Persistence of Poliovirus Types 2 and 3 in Waste-Impacted Sediment and Water

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A thesis

submitted in partial fulfillment of the

requirements for the degree of

Master of Science

University of Washington

2019

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Program Authorized to Offer Degree:

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Abstract

Persistence of Poliovirus types 2 and 3 in waste-impacted sediment and water

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The objective of this study was to evaluate the persistence of poliovirus type 2 (PV2) and type 3 (PV3) in domestic wastewater and sediment. Microcosms containing *(a)* domestic influent wastewater and *(b)* wastewater with a sediment matrix [1:1 waste biosolids and loamy sand] were seeded with between 10^5 and 10^6 plaque-forming units either PV2 or PV3, and stored for 126 days at three temperatures (4°C, room temperature (RT), and 30°C). Viable PV in the liquid of (*a)*, and the sediment and liquid portions of (*b)* were sampled and plaques counted ten separate time points [0, 1, 3, 7, 14, 21, 28, 42, 84 and 126 days] over 18 weeks using Buffalo-Green Monkey Kidney [BGMK] plaque assay. The time points at which >99% reduction was reached were determined. Both viable PV in the sediment portion of (*b)* reached >99% reduction at 30°C, RT, and 4 °C at 28, 84, and >126 days respectively. At 126 days in sediment at 4°C, the percent reduction of viable PV was 78.3% and 85.6% respectively. In the liquid portion of *(b)*, >99% reduction of both viable PV was reached for 30°C, RT, and 4°C at 14, 42 and >126 days respectively. At 126 days in the liquid of *(b)* at 4 °C, the percent reduction of viable PV was 95.9% and 91.5% respectively. This study demonstrates that liquid vs sediment sample, and temperature both play roles in PV viability.

Table of Contents

Abstract..2

List of Appendices...4

List of Tables 4

List of Figures..5

Acronyms..6

Acknowledgements...7

Literature Review..8

Study Aims...14

Methods...15

Results..20

Discussion...28

Conclusion..31

References..32

List of Appendices

Appendix A: Extraction Method....40

Appendix B: Supplementary data....42

List of Tables

Table 1: Plaque assay results at Number of days at which >99% PFU reduction was reached....21

Table 2: Plaque assay results at 126 days for 4 °C....21

Table 3: Slope values for associated mixed linear model....25

Table 4: P-values for model differences between sample type within a temperature....26

Table 5: P-values for model differences between temperatures within a sample type....26

Table 6: Estimated time to 99% PFU reduction of PV....27

Table 7: R^2 values for models....27

Table 8: Table 8: Eluents tested on sediment recovery....41

Table 9: pHs tested for $NaNO_3$ + 3% beef extract sediment recovery 41

Table 10: Average recoveries for the first run of PV2 and PV3....42

Table 11: Composition of biosolids....47

List of Figures

Figure 1: Run 1 of PV2 at 4°C....22

Figure 2: Run 1 of PV2 at RT....22

Figure 3: Run 1 of PV2 at 30°C....23

Figure 4: Run 1 of PV3 at 4°C....23

Figure 5: Run 1 of PV3 at RT....24

Figure 6: Run 1 of PV3 at 30°C....24

Figure 7: Run 2 of PV2 at 4°C....43

Figure 8: Run 2 of PV2 at RT....44

Figure 9: Run 2 of PV2 at 30°C....44

Figure 10: Run 2 of PV3 at 4°C....45

Figure 11: Run 2 of PV3 at RT....45

Figure 12: Run 2 of PV3 at 30°C....46

Acronyms

AFP: Acute Flaccid Paralysis

BGMK: Buffalo-Green Monkey Kidney

CDC: Centers for Disease Control

GPEI: Global Polio Eradication Initiative

IPV: Inactivated Polio Vaccine

MM: Mixed Microcosm

mOPV: Monovalent Oral Polio Vaccine

OPV: Oral Polio Vaccine

PFU: Plaque Forming Unit

PV: Poliovirus

PV1: Poliovirus type 1

PV2: Poliovirus type 2

PV3: Poliovirus type 3

RT: Room Temperature

tOPV: Trivalent Oral Polio Vaccine

VDPV: Vaccine Derived Poliovirus

WPV: wild poliovirus

WWO: Wastewater Only

Acknowledgements

This research project could not have been completed without the support of my thesis committee Marilyn Roberts and Scott Meschke, who provided advice and support through the entirety of the project and ensured its success. Thanks also goes to the students and staff of the Environmental and Occupational Microbiology Laboratory at the University of Washington, Nicola Beck, Alexandra Kossik, Christine Fagnant-Speralti, Nicolette Zhou, Jeff Shirai, Joanna Harrison, James Januch, and Erika Keim for their project support, which included training in laboratory techniques, performing lab analyses, assisting with writing, and providing project advice. Thanks to Lianne Shepherd and Pauline Trinh for biostatistics advising for data analysis.

Thanks also goes to those that provided samples: Phuong Truong at West Point Wastewater Treatment Plant in Seattle for providing biosolids and wastewater, Rob Harrison at University of Washington for providing the sandy-loamy soil sample, and the Centers for Disease Control for providing the PV2 and PV3 stocks.

Finally, I want to thank my cohort in the Department of Environmental and Occupational Health Sciences and my friends and family for their continued support and encouragement throughout the past two years, which kept me going.

Funding for this research project was provided by PATH (GAT.2186-01558814-SUB) with key project oversight from the Gates Foundation.

Literature Review

This study aimed to characterize persistence of poliovirus types 2 and 3 in waste-impacted sediment and water in order to apply results to environmental surveillance of poliovirus in Pakistan, a country still endemic to poliovirus. There are many factors that affect how a virus persists, such as temperature, sample type, and pH, and there are also factors in Pakistan that affect how the virus can circulate. Consistent migration between Afghanistan and Pakistan and poor sanitation are two things discussed that may affect increased input of the virus into the environment, which may affect whether environmental surveillance positives are from persistence or increased input into the environment. Previous studies have focused on poliovirus type 1 (PV1), and in sterile environments, demonstrating a need for PV2 and PV3 research that focuses on waste-impacted environments, similar to what is found in Pakistan. With a high asymptomatic rate of poliomyelitis and increased chance of silent transmission of PV, environmental surveillance and the role of persistence of PV within it, are important to fully understand to achieve eradication.

Vaccination

Vaccination for poliovirus was introduced in 1955 with the creation of the Inactivated Polio Vaccine (IPV) by Jonas Salk, and 6 years later the live attenuated Oral Polio Vaccine was introduced in 1961 by Albert Sabin (Baicus 2012). The introduction of the vaccine greatly decreased incidence of poliovirus infection, for instance in the United States from 1955 – 1961 case incidence of poliovirus went down 94.2% (Baicus 2012). Once eradication seemed feasible, the Global Polio Eradication Initiative (GPEI) was created in 1988 in order to achieve global eradiation. Vaccination approaches have varied in different countries. In endemic areas, the GPEI works partially through vaccination campaigns with trivalent OPV due to its effectiveness,

less expensive price compared to IPV, and ease of administration (Mast and Cochi 2017). With OPV, there is a risk of mutation of the vaccine strain resulting in vaccine-derived poliovirus (VDPV), which can cause PV infection (Minor 2009). After WPV2 was last seen in 1999 and the risk of circulating VDPV type 2 causing acute flaccid paralysis, in April 2016, there was a global switch from trivalent OPV to bivalent OPV (types 1 and 3), with targeted monovalent OPV (mOPV) type 2 vaccination campaigns during VDPV type 2 outbreaks (Mast and Cochi 2017). PV2 and PV3 are still a concern in countries affected by PV due to VDPV, and vaccination campaigns affect input of PV into the environment.

Pakistan and Afghanistan

Pakistan is one of the three countries that is still considered endemic for wild poliovirus (WPV) and subsequently a focus of eradication efforts (GPEI 2017). There has been a 96% polio case reduction in Pakistan since 2014, with consistent positive environmental samples indicating continued circulation in the environment. In 2019, there have been 17 cases of polio to date, indicating the effects of continued circulation of PV (WHO 2017). Reasons for this continued barrier to eradication of poliovirus in Pakistan include transmission from Afghanistan (also considered endemic for WPV), incomplete vaccine dosage, particularly in rural areas with low healthcare access, and poor sanitation, which could potentially lead to increased circulation (Shah et al 2011).

Afghanistan has a similar case load to Pakistan, with 21 cases of WPV in 2018 and 3 in 2019 (WHO 2017). Cases from 2006-2010 showed clustering at the border between Afghanistan and Pakistan and they are considered to be in the same epidemiological block, showing the importance of migration transmission in eradication efforts (Shah et al 2011). In Pakistan, areas with lowest vaccination rates, mainly rural, have been shown to have higher case loads, which

also corresponded with poor infrastructure and inaccessibility (Noori et al 2017, Shah et al 2011).

Poor sanitation may be leading to the increased circulation and persistence of poliovirus in the environment (Rasheed et al 2009). High contamination in drinking water in Pakistan has been associated with effects from natural disasters (Rasheed et al 2009). Poor living conditions along with mismanagement of waste facilities, which are uncommon and/or do not function properly (Rasheed et al 2009, Hisam et al 2014). Safe drinking water in Pakistan is available to 56% of the population, with even poorer coverage in rural areas (45%) (Farooq et al 2008). In a review of Pakistani total coliform contamination in drinking water, it was found that 20-100% of samples were contaminated with total coliforms, though level of contamination was not provided. The number of samples contaminated depended on the region, but all regions sampled were contaminated, demonstrating the wide-spread nature of fecal contamination in Pakistan (Azizullah et al 2011). High fecal waste contamination in the environment in Pakistan suggests increased levels of poliovirus occurring in the environment because it is fecal shed. Therefore, environmental surveillance in these areas is important due to the high fecal contamination, and persistence of poliovirus may impact this circulation due to continued input from wastewater.

Environmental Surveillance

Environmental surveillance of infectious poliovirus, as a supplement to the gold standard surveillance of Acute Flaccid Paralysis (AFP) cases, has been set up as a system to monitor poliovirus in the community (GPEI 2017). Up to 90% of people infected with polio are asymptomatic (Racaniello 2006). This can potentially lead to undetected PV circulation within a population. One model predicted that it takes an estimated 326 - 512 days from transmission within the community until the first symptomatic case is detected by AFP surveillance systems

(Bencsko and Ferenci 2016). This was also shown in a study of poliovirus in Hispaniola waters, where it was determined that poor vaccination rates and poor surveillance of poliovirus cases and environmental samples could result in undetected circulation of poliovirus in the environment for up to 2 years before cases arose (Vinje et al 2004). Environmental surveillance is beneficial in areas that have poor sanitation and subsequently high sewage contamination in water sources (Ndiaye et al 2014). A study focused in Senegal from 2007-2013 found poliovirus in 34.3% of sewage samples demonstrating that poliovirus presence can be significant in sewage impacted waters and that environmental surveillance helps to understand circulation and distribution of poliovirus in impacted areas (Ndiaye et al 2014). Other studies have established the importance of source appropriation using molecular techniques on environmental surveillance positives to determine source and geographic clustering (Vinje et al 2004, Manor et al 2007). One study in Gaza was able to determine that it was re-importation not silent endemic circulation that allowed for renewed transmission of WPV (Manor et al 2007). With up to 90% of cases being asymptomatic and cases decreasing as eradication nears, molecular characterization of environmental surveillance isolates will play an important role in informing eradication efforts whether the source of environmental surveillance positives is from re-importation, silent circulation or persistence.

The source of positive poliovirus environmental surveillance samples can be attributed to a multitude of factors including re-importation as mentioned previously, but additionally persistent shedding of infected individuals and environmental persistence can both contributed to environmental surveillance positives. Previous studies have not agreed on the level of shedding rates and duration of poliovirus in fecal samples from people vaccinated with live virus. Alexander et al 1997 showed that concentration of the virus shed in feces immediately following

vaccination rapidly drops in the first week (71-100% drop), and fewer than 20% of patients were still shedding after 5 weeks (Alexander et al 1997). This differs with other studies that have shown shedding of poliovirus lasting up to 6 months post-vaccination (Ferreyra-Reyes 2017) or in another study even up to 22 months from a vaccine-derived strain (Hovi et al 2013). The latter study demonstrates that long shedding or infection of vaccine derived asymptomatic poliovirus may not be as rare as previously determined (Hovi et al 2013). Differences in type of vaccination have been shown to affect shedding periods as well (Saleem et al 2018). In a study comparing Inactivated Polio Vaccine (IPV), Trivalent Oral Polio Vaccine (tOPV) and monovalent Oral Polio Vaccine (mOPV), it was found that after one week post-vaccination mOPV had a higher proportion of continued shedding than tOPV at a p-value of <0.001 (Saleem et al 2018). Understanding the shedding rates are important in understanding the contribution of shedding to persistence and contribution to environmental surveillance samples positive for PV.

Persistence Factors

Temperature

Temperature has been found to be of great importance in predicting viral PV1 persistence in the environment (Hurst et al 1980). Environmental temperatures above 55 C^{o} rapidly inactivate poliovirus in < 30 min versus die-off at 90 days at 10 ºC, 20 ºC, and 30 ºC (Clarke et al 1961). Another study found that both in ground water and wastewater that die off was the slowest define what slowest means at 10 ºC, and that wastewater was protective of viability over time defined when compared with groundwater (Nasser et al 1993). When comparing PV1 reduction in surface water, one study found average reduction rates of 0.14 log10 PFU/day in 4ºC and 0.19 log10 PFU/day 25 ºC (Bae & Schwab 2008). Another study characterizing WPV1 persistence in soil and on vegetables were still presence in soil > 96 d in the winter (-13.9 °C –

26.7 °C), 11 days in the summer (15 °C - 34.4 °C) (Tierney et al 1977). Previous studies have thus concluded that low temperatures are protective of PV viability.

Sample type

The persistence of PV in sediment and soil is largely dependent on soil properties as demonstrated below. Sand has been shown to have a lower affinity for poliovirus with lower adsorption percentages of PV to the soil in multiple studies and clay, high affinity with higher adsorption percentages of PV to the soil (>99%) in multiple studies, and virus survival was correlated significantly ($P < 0.05$) with adsorption to soil and saturation pH (Gerba et al 1975, Hurst et al 1980). One study characterized reduction of WPV type 1 in different soil types and found persistence in both clay and sand >24 weeks. However, PV1 was reduced more in sand vs clay soil at 50 weeks, starting with 6 logs at week 1, 2.5 log reductions in sand vs 1.5 log reductions in clay (Meschke & Sobsey 2003). Other studies suggest that there is longer persistence, yet, when compared with water, sediment and different soils allow for longer persistence of PV (Sobsey 1980, Gerba et al 1975, Labelle & Gerba 1980). In contrast the saturation of water in sediment contributes to decreased survival over time more information here (Hurst et al 1980). One study comparing PV1 in sediment and water showed persistence in water lasting up to 9 days and persistence in sediment lasting >19 days (Rao et al 1984).

When comparing between surface water and groundwater, PV1 persists longer in groundwater (Bae & Schwab 2008, Nasser et al 1993). Previous research demonstrates decreased loss of PV1 with increased filtration or sterilization of water (Gerba et al 1975, Labelle & Gerba 1980, Sobsey et al 1980). These papers suggest that with PV1, sediment type may be explain the increased persistence in groundwater and demonstrates the importance of microbiological activity in persistence of PV1.

pH

Viruses are greatly influenced by pH due to their isoelectric points, the pH value at which the net zero surface charge of the virus changes. This will greatly impact the movement of the virus in environmental media (Michen and Graule 2010). One isoelectric point of poliovirus has been determined to be near neutral pH; one study characterizing Sabin types 1, 2, and 3 found isoelectric points at pH of 7.4, 7.2, and 6.3 respectively (Thomassen et al 2013). The other isoelectric point of PV has been found to be around pH 4.5 (Vrijsen et al. 1984). This can influence virus adsorption to surfaces including sediment particles depending on pH, which may influence the extent of persistence (Bitton 1975). In one study testing effects of pH on virus adsorption to soil materials, it was found that poliovirus adsorption to sand when compared with adsorption to clay was more impacted by pH. When comparing pH 7.5 to pH 3.5 poliovirus adsorbed to sandy soil at 59% (7.5) and 97% (3.5) compared with clay soils with adsorption at 98% (7.5) and >99% (3.5) (Sobsey et al 1980). Poliovirus itself is stable at a wide range of pH from 3.8 to 8.5 for 25 weeks, which may help increase its persistence in environmental media (Pollard 1949).

Study Aims

Aim 1: Determine the Persistence of viable poliovirus types 2 and 3 in wastewater and waste-impacted sediment

Aim 2: Compare persistence between temperatures and sample type

Materials and Methods

Wastewater and Sediment sources

Approximately 6 L of wastewater was collected at the wastewater influent division channel at West Point Treatment Plant [Seattle, WA, USA] on June 14 [PV3], and June 28, 2018 [PV2], and was kept in a 4 °C fridge between 0-4 d until use. Sediment was collected from Alderwood, WA, USA where the soil is a gravelly-sandy-loam and was used for all samples. Gravelly-sandy-loam was chosen due to its similarity to the textural composition of soil found in Pakistan where poliovirus outbreaks still occur (Khan et al 2018). The sediment was mixed in a 1:1 ratio with waste solids obtained from West Point Treatment Plant in order to simulate sediments in waste-impacted waters in Pakistan (Azizullah et al 2011, Nabeela et al 2014).

Microcosms

The relationship between plaque forming units [PFU] of PV2 and PV3, time [0 -126 d], temperature [4, RT, 30 ºC], type of sample (liquid or sediment) was tested using a longitudinal experimental study. Controlled microcosms were set up at the three different temperatures. The room temperature was measured using a temperature probe that measured temperature every 10 min and was varied between 19.6°C and 23°C. Microcosms were set up in sterile conical 50 mL centrifuge tubes [VWR; Radnor, PA, USA] with each tube a time point and temperature.

Two different types of microcosms were prepared: tubes with domestic influent wastewater only to test for PV persistence in the absence of sediment, and tubes with both domestic wastewater and a sediment matrix [1:1] ratio of waste biosolids and gravelly-loamy-sand]. Microcosms were prepared in plastic centrifuge tubes so that liquid and sediment could be separated easily, as previous described (Höglund et al 2002, Hurst et al 1980). Both waste solids and soil were used as wet weight to emulate environmental conditions. Tubes with wastewater and sediment that were referred to as mixed microcosm [MM] had 3 g of sediment to 10 mL of

wastewater. The second microcosm type prepared contained 10 mL of wastewater and were referred to as wastewater only [WWO]. The MM tubes were prepared in duplicate for each time point and temperature and the WWO tubes were prepared singularly for each time point and temperature. This resulted in 20 MM tubes per temperature for each of three temperatures for a total of 60 tubes of MM microcosm tubes per run. Ten WWO tubes were prepared per temperature for each of three temperatures for a total of 30 WWO microcosm tubes per run. A full run of the experiment was performed separately each for PV2 and PV3. A second, smaller scale duplicate run with both MM and WWO microcosms was performed with three time points (0, 21, 84 days) each for PV2 and PV3 to confirm results.

Poliovirus

Sabin type 2 and Sabin type 3 vaccine strain poliovirus stocks were provided by Centers for Disease Control (CDC) and were grown separately on Buffalo Green Monkey Kidney (BGMK) cells through confluent lysis of cell monolayers (Melnick et al 1954). Grown stocks were frozen in cell culture cryogenic plastic tubes [Midland Scientific, Denver, CO, USA] at -80 °C until use. PV2 and PV3 stocks were diluted 5-fold into a total of 10 mL of 1X Phosphate-buffered saline (PBS) at pH 7.0 in order to achieve a concentration~ 1 x 10^5 PFU per 200 uL. All 90 tubes per run were seeded individually in order to ensure even distribution between samples with 500 uL of the stock dilution and vortexed for 5 min in order to evenly distribute the virus within the sample. All samples were then left to settle for 24 h at RT. After 24 h, 30 tubes were incubated at room temperature, 30 tubes were refrigerated at 4°C, and 30 tubes incubated at 30 °C for each run. At day 0, tubes for day 0 (2 MM samples and 1 WWO sample) from each temperature and sample type was incubated for 1 h at their respective temperatures either 4 °C,

RT or 30 °C and then the day 0 time point was processed for quantification of PFU. Additional samples were processed at days 1, 3, 7, 14, 21, 28, 42, 84, and 126.

Processing Sediment from MM microcosms

For detection and quantification of PV2 and PV3 in sediment, a method was adapted from previously described methods used to isolate poliovirus from oyster tissue (Mullendore 2001, Sobsey 1978). The MM tubes were taken from their respective temperatures on sample dates and were centrifuged for 20 min at 5,000 x G to separate liquid and sediment and 10 mL of the liquid was pipetted off and placed in a new centrifuge tube for separate processing. The sediments were processed after the liquid portion was removed using a 2M $NaNO_3$ in 3% beef extract eluent at a 3:1 (W/V) ratio, vortexed for 5 min, and set to shake for 15 min to separate the virus from the sediment. Samples were centrifuged at 5,000 x G for 20 min and the top liquid was pulled off. Then a 3% sterile skim milk solution was added to the supernatant from the eluent step at a 0.1% volume concentration, the pH was modified with 5M HCL to a pH of between 3-4 using pH strips and set to shake for 2 h as a flocculation step. After shaking, samples were then centrifuged for 30 min at 3500 x G. The supernatant was poured off leaving the floc, containing the virus, at the bottom and 5 mL of PBS was added to re-concentrate the virus (Falman et. al 2019). A Vertrel XF [Miller-Stephenson, Danbury, CT, USA] extraction was then done which is described as follows: 0.5X volume of Vertrel XF was added to samples, samples were vortexed for 2 minutes, and centrifuged at 3,000 x G for 15 minutes, and then the top supernatant was pipetted off carefully. A Vertrel XF extraction was done as a replacement for chloroform extraction in order to remove organics from the sample (Mendez 2000).

Processing Liquid from MM and WWO samples

After the MM tubes were taken from their respective temperatures, and were centrifuged for 20 min at 5,000 x G [Beckman Coulter Avanti Centrifuge J-20 XPI] to separate liquid and sediment portions and then the 10 mL of the liquid portion for both MM and WWO tubes was pipetted off and placed in a new centrifuge tube for separate processing. A Vertrel XF extraction was done on the liquid portions of both MM and WWO tubes to remove organics as previously described (Falman et al 2019, Mendez 2000). Then a skim milk secondary concentration step was done as described in the sediment processing above.

Plaque Assay

BGMK cells were grown for exactly 4 days to 95% confluent monolayers in 25 cm^2 polystyrene flasks with vented caps [Corning; Corning, New York, USA], and then transferred into 9.5 cm^2, 6-well tissue culture plates [Corning; Corning, New York, USA] before inoculation with the sample (Dahling and Wright 1986). The MM and WWO samples were taken from the 4°C refrigerator and several ten-fold dilution series were made ranging from 10^0 to 10^{-4}, with time points farther out requiring less dilution. Each sample had three dilutions and time point used in order to ensure it was within the limit of detection. For each dilution, in replicates of three, a volume of 200 uL of sample was put on each well, and left to incubate for 1 h, swirling every 15 min to ensure virus absorption and even distribution of virus onto the cells. Cells were overlaid with a 3% Avicel overlay [FMC Health and Nutrition, Philadelphia, PA, USA] (Matrosovich, et al 2006), which was made with 3% sterile Avicel in de-ionized water, 1% filter-sterilized 200 mM L-glutamine [Corning, Corning, NY, USA], 1% filter-sterilized 35.6% HEPES [Fisher Bioreagents; Pittsburgh, PA, USA], 0.5% filter-sterilized gentamicin sulfate [Sigma-Aldrich; St. Louis, MO, USA], 0.5% filter-sterilized kanamycin sulfate [Sigma-Aldrich;

St. Louis, MO, USA], 3% filter-sterilized 7.5% sodium bicarbonate [Sigma-Aldrich; St. Louis, MO, USA], 1.2% filter-sterilized Minimum Essential Medium Eagle (MEM) [Sigma-Aldrich; St. Louis, MO, USA], 1.2% filter-sterilized nystatin [Sigma-Aldrich; St. Louis, MO, USA], and 2% Fetal Bovine Serum (FBS) [ATCC; Manassas, VA, USA] (Matrosovich, et al 2006). The infected BGMK cells were incubated for 40-44 hours at 37°C and 5% CO_2, and then stained with 2% crystal violet in 20% methanol and the Plaques were counted and remaining PFU determined for both PV2 and PV3 samples of the experiment (Falman et al 2019).

Analysis

Data from plaque assay results were analyzed through creation of a series of mixed linear models in the nlme package in R (Pinheiro et al 2013) characterized as follows:

1. Six mixed linear models were run to evaluate the role of sample type in the persistence of poliovirus within a temperature. Data was separated by PV type and then further separated by temperature, and for each PV type and temperature within PV type the following model was run:

$$E\,[\log(PFUs)] = \beta_0 + \beta_1 X_{Time} + \beta_2 X_{sediment} + \beta_3 X_{wastewater\text{-}only} + \beta_4 X_{Time*sediment} + \beta_5 X_{time*wastewater\text{-}only} + \rho_{i\ time} + \rho_{i\ Run\ ID} + \varepsilon_{ij}$$

2. Six mixed linear models were run to evaluate the role of temperature in the persistence of poliovirus within a sample type. Data was separated by PV type and then further separated by sample type, and for each PV type and sample type within PV type the following model was run:

$$E\,[\log(PFUs)] = \beta_0 + \beta_1 X_{Time} + \beta_2 X_{room\ temperature} + \beta_3 X_{30\ degree} + \beta_4 X_{Time*room\ temperature} + \beta_5 X_{time*30\ degree} + \rho_{i\ time} + \rho_{i\ Run\ ID} + \varepsilon_{ij}$$

Slope values were obtained for each PV type, temperature and sample type and p-values and 95% confidence intervals were determined for differences between temperatures within a sample type and PV type, and differences between sample types within a temperature and PV type. Using the models, a time until 99% reduction in PFU was determined.

Results

Plaque assay results

Table 1 summarizes results from the plaque assay with the time point tested (in days) at which >99% reduction was observed for each serotype, temperature and environmental media tested. At 4 °C >99% reduction of both the PV2 and PV3 was not reached within the 126-day time period with the exception of the WWO sample in the PV3 (Table 1**).** The percent reductions at 126 days and 4 °C for PV2 were 78.3% (sediment) (95% CI: 75.6 – 79.8%), 95.7% (wastewater) (95% CI: 95.6 - 95.7%), and 98.6% (wastewater only) (95% CI: 98.5 - 98.7%). For PV3 the reductions were comparable at 85.6% (95% CI: 84.1 - 86.7%)(sediment), 91.5 (wastewater) (95% CI: 91.46 - 91.58%), and 99.8% (wastewater only) (95% CI: 99.83 - 99.84%) (Table 2) PV2 and PV3 persisted longer in the MM liquid samples than in the WWO liquid only. Graphs of die-off over time are displayed in Figures 1 – 6.

Table 1: Plaque assay results at Number of days at which >99% PFU reduction was reached

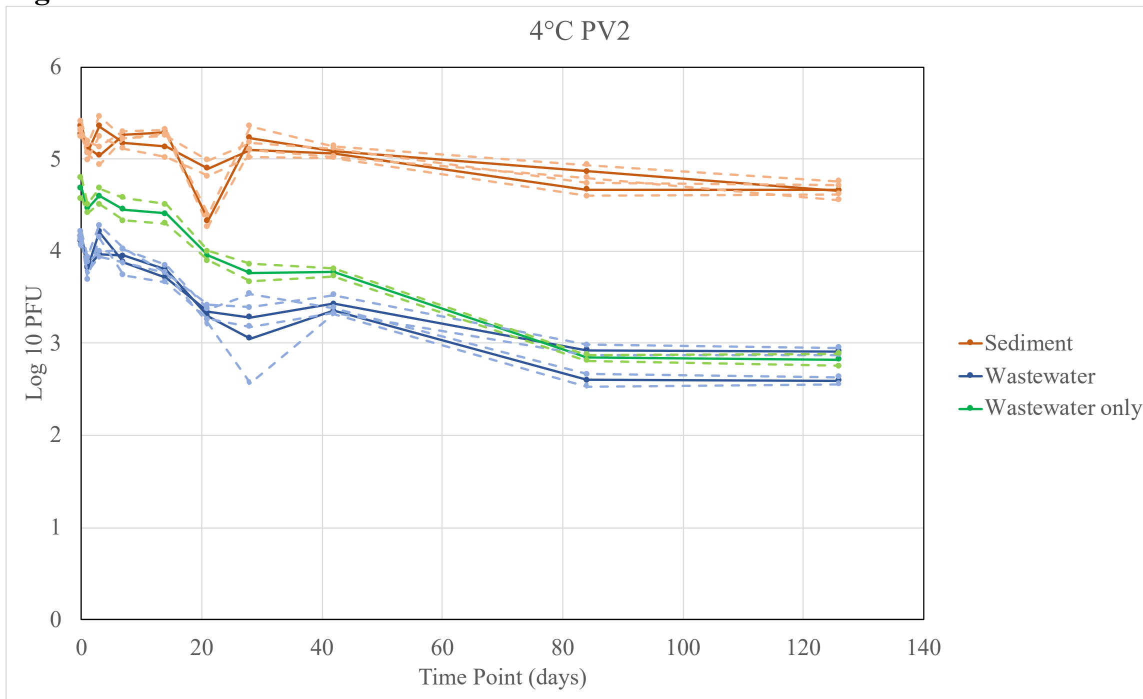
Time points tested: 0, 1, 3, 7, 14, 21, 28, 42, 84, 126	Number of days at which >99% PFU reduction was reached	
Sample	**PV2**	**PV3**
4 °C		
Sediment	>126	>126
Liquid	>126	>126
Liquid only	126	>126
Room Temperature		
Sediment	84	84
Liquid	42	42
Liquid only	21	14
30 °C		
Sediment	28	28
Liquid	14	14
Liquid only	14	*NA

*PV3 30 °C Wastewater only sample was inhibited by Cytotoxic effects (CTE) and reached 90.1% PFU reduction in 3 days

Table 2: Plaque assay results at 126 days for 4 °C

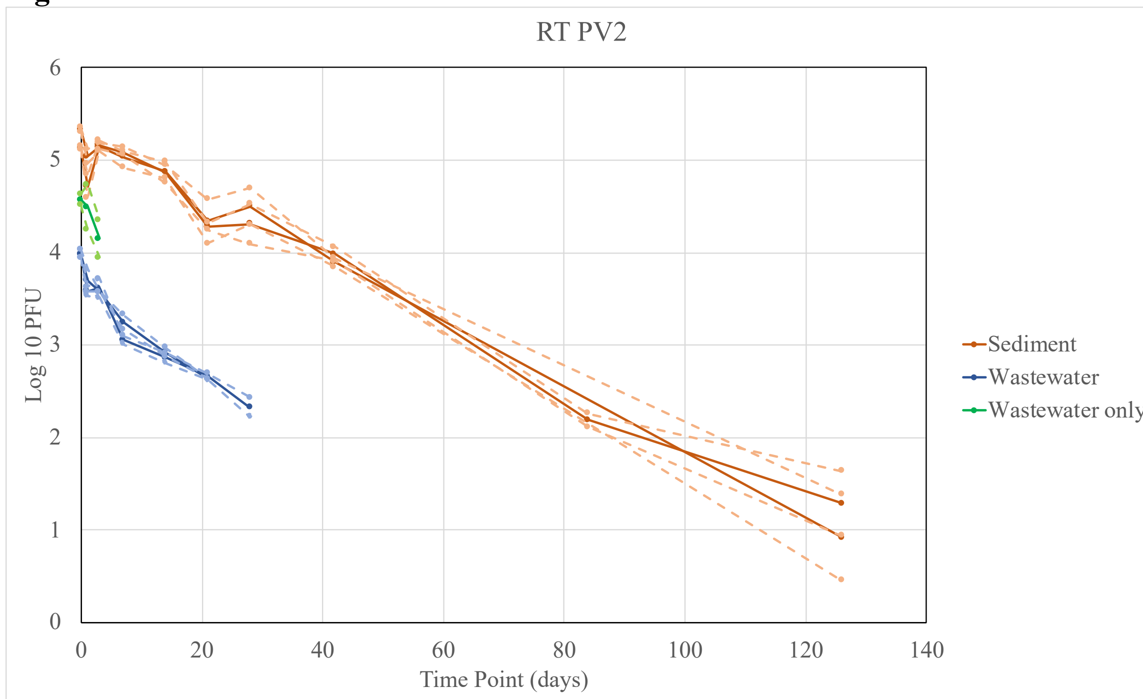
Sample type	4 °C % reduction at 126 days	
	PV2	**PV3**
Sediment	78.3%	85.6%
Liquid	95.7%	91.5%
Liquid-only	98.6%	99.8%

Figure 1: Run 1 of PV2 at 4°C



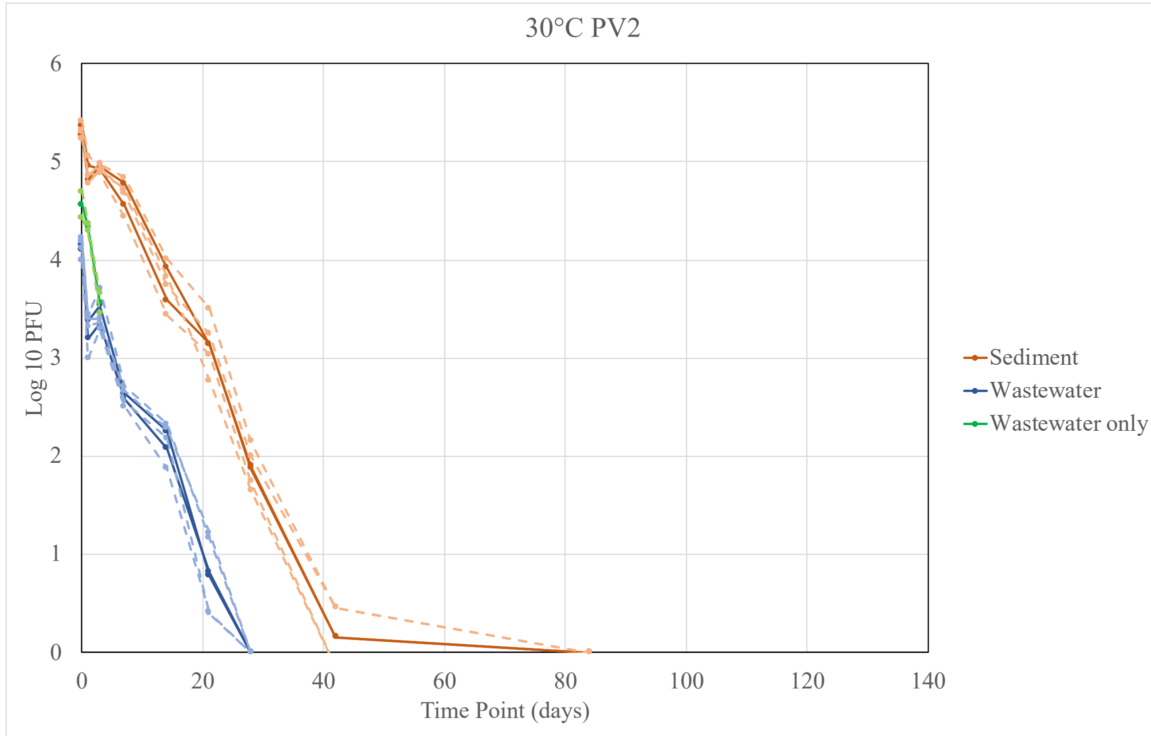
Dashed lines are 95% confidence intervals based on multiple wells plated during plaque assay

Figure 2: Run 1 of PV2 at RT



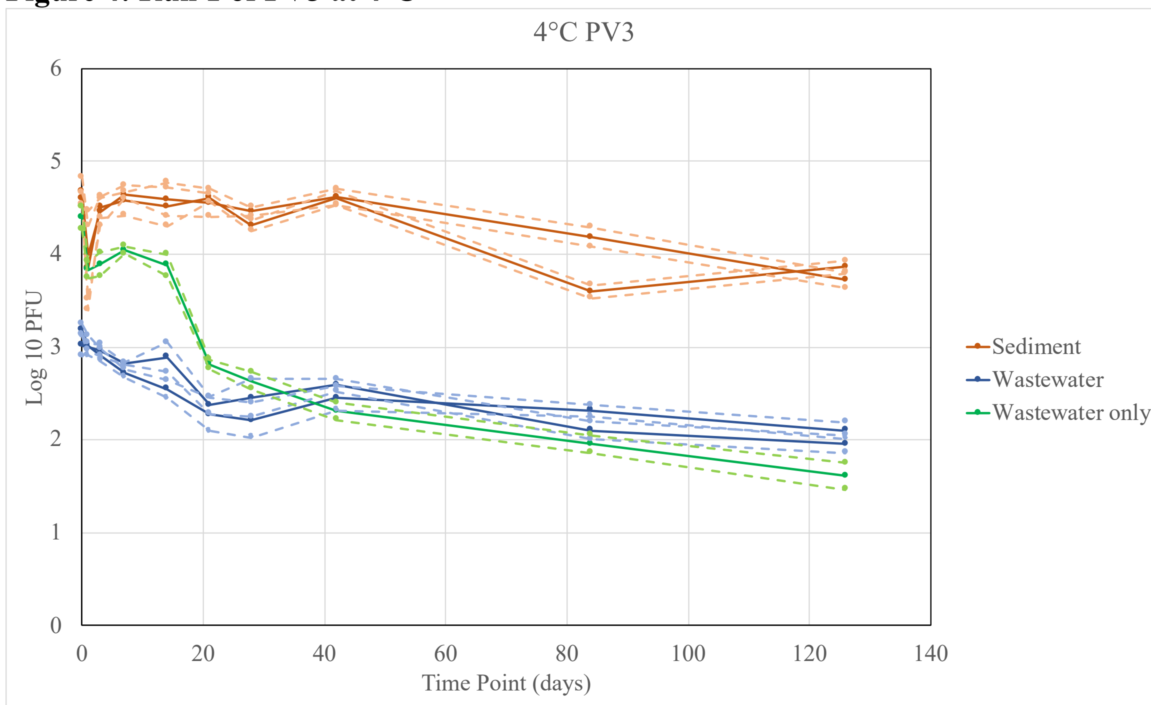
Dashed lines are 95% confidence intervals based on multiple wells plated during plaque assay.

Figure 3: Run 1 of PV2 at 30°C



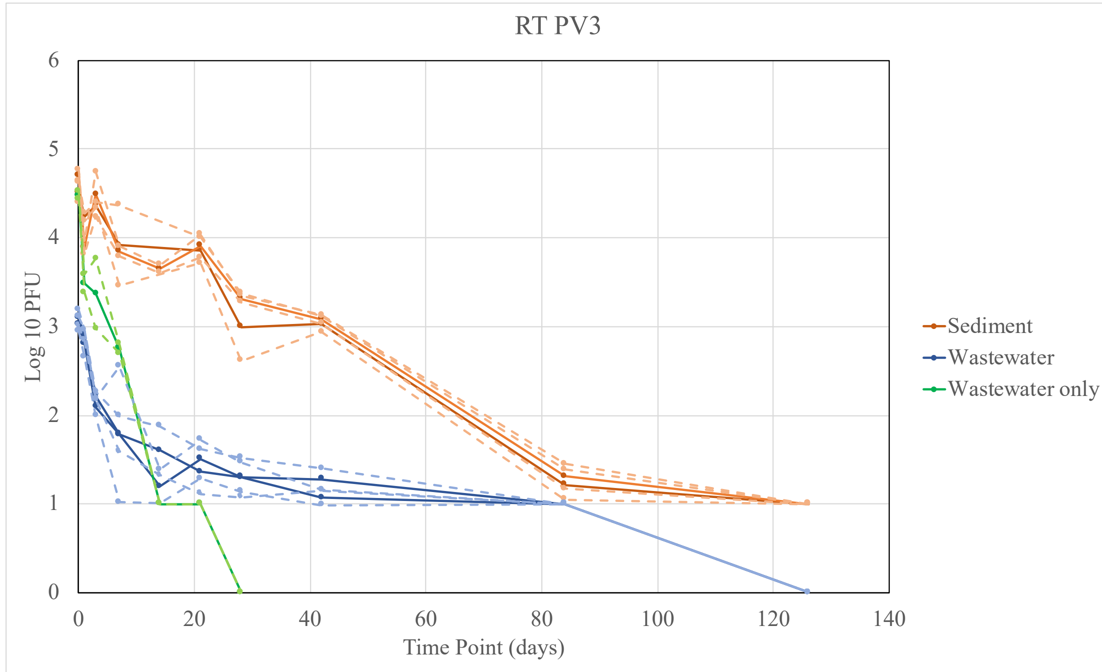
Dashed lines are 95% confidence intervals based on multiple wells plated during plaque assay.

Figure 4: Run 1 of PV3 at 4°C



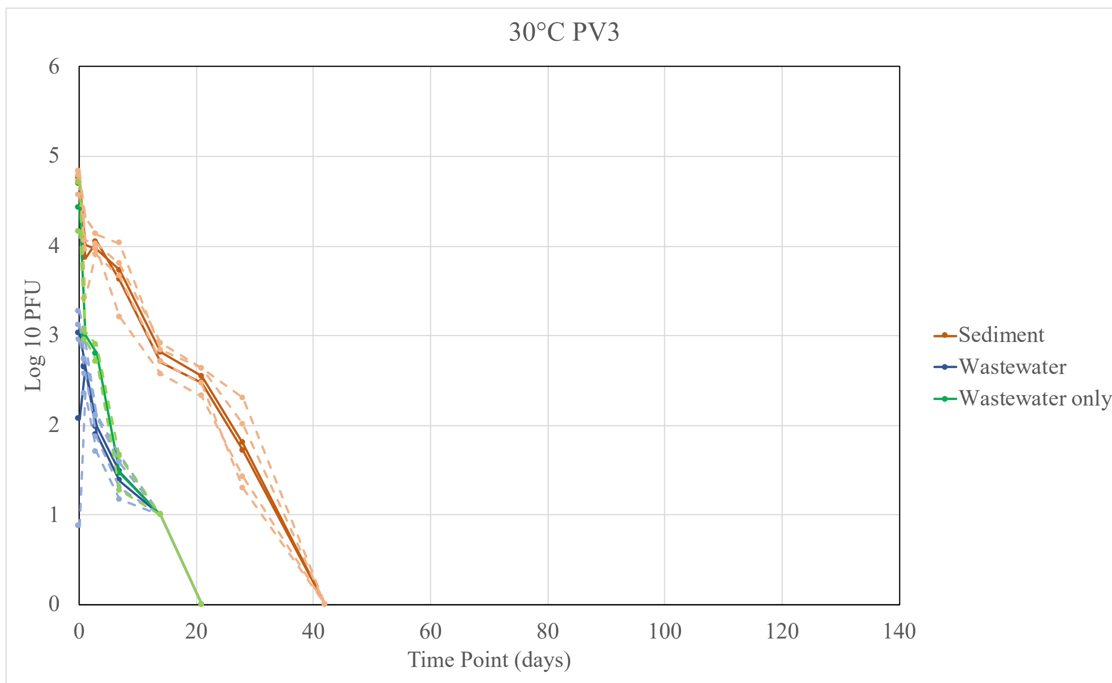
Dashed lines are 95% confidence intervals based on multiple wells plated during plaque assay.

Figure 5: Run 1 of PV3 at RT



Dashed lines are 95% confidence intervals based on multiple wells plated during plaque assay.

Figure 6: Run 1 of PV3 at 30°C



Dashed lines are 95% confidence intervals based on multiple wells plated during plaque assay.

Plaque assay analysis

Slope values and associated p-values for differences based on slope determined from the models are available in Tables 5, 6, and 7. Slopes become steeper with increasing temperature and sediment slopes are steeper than liquid slopes (Table 3). P-values in Tables 4-5 show this to be significant in that there are significant differences at a significance level set at <0.05 between the die-off over time of PFU at almost every single interaction. The only exceptions were at 4°C for PV3 when comparing sediment and wastewater there was no significant difference. Additionally, when comparing between RT and 30°C in WWO samples for both PV2 and PV3, no significant differences were seen. Models were then used to predict a time point at which 99% reduction of PV would be reached and results are seen in Table 6. Table 7 displays the R^2 values for each model.

Table 3: Slope values for associated mixed linear model

$ß_4$ or $ß_5$ Term interpretation (Time*Sample type)		
	PV2	**PV3**
4 °C		
Sediment	-0.004	-0.006
Wastewater	-0.011	-0.007
Wastewater only	-0.016	-0.021
RT		
Sediment	-0.031	-0.033
Wastewater	-0.091	-0.032
Wastewater only	-0.202	-0.290
30 °C		
Sediment	-0.113	-0.099
Wastewater	-0.133	-0.206
Wastewater only	-0.334	-0.285

Table 4: P-values for model differences between sample type within a temperature

P-values for ß$_4$ or ß$_5$ Term (Time*Sample type)		
	PV2	**PV3**
4 °C		
Sediment : Wastewater	0.0012	0.4863
Wastewater : Wastewater only	0.0394	<0.0001
Wastewater only : Sediment	<0.0001	<0.0001
RT		
Sediment : Wastewater	<0.0001	0.8869
Wastewater : Wastewater only	0.0036	0.0002
Wastewater only : Sediment	<0.0001	0.0002
30 °C		
Sediment : Wastewater	0.0123	<0.0001
Wastewater : Wastewater only	<0.0001	0.0177
Wastewater only : Sediment	<0.0001	<0.0001

Table 5: P-values for model differences between temperatures within a sample type

P-values for ß$_4$ or ß$_5$ Term (Time*Temperature)		
	PV2	**PV3**
Sediment		
4 °C: 30 °C	<0.0001	<0.0001
RT : 4 °C	<0.0001	<0.0001
30 °C : RT	<0.0001	<0.0001
Wastewater		
4 °C : 30 °C	<0.0001	<0.0001
RT : 4 °C	<0.0001	<0.0001
30 °C: RT	0.0006	<0.0001
Wastewater only		
4 °C : 30 °C	0.0008	<0.0001
RT : 4 °C	0.0005	<0.0001
30 °C : RT	0.1408	0.9276

Table 6. Estimated time to 99% PFU reduction of PV

	Time (days)	
	PV2	**PV3**
4°C		
Sediment	428	334
Wastewater	160	237
Wastewater only	115	69
Room Temperature		
Sediment	63	91
Wastewater	22	42
Wastewater only	8	13
30°C		
Sediment	17	20
Wastewater	13	2
Wastewater only	6	5

Table 7: R^2 values for models

R^2 Values for Linear Regression						
Sample Type	**4**		**RT**		**30**	
	PV2	**PV3**	**PV2**	**PV3**	**PV2**	**PV3**
Sediment	0.613	0.5316	0.981	0.9431	0.8593	0.9166
Wastewater	0.7813	0.6937	0.998	0.8068	0.9453	0.9822
Wastewater only	0.8936	0.6178	0.9338	0.8785	0.9933	0.9198

Discussion

Previous studies

The results from this study are similar to previous research studying poliovirus persistence using PV1. In a persistence study with PV1 using sandy-loamy soil in anaerobic, non-sterile conditions, it was found that one log10 reduction of PV1 was not reached at >75 days at 1°C, with an estimated time at which one log10 reduction was reached at 323 days. One log10 reduction was reached 33 days at 23°C and 3.5 days at 37°C (Hurst 1988). In the same study in sandy-loamy soil in anaerobic sterile conditions it was found that one log10 reduction of PV1 was not reached at 75 days at 1°C with an estimated time at which 1 log10 reduction was reached at 688 days. One log10 reduction was reached at 30 days at 23°C and 1.7 days at 37°C (Hurst 1988). As discussed previously, soil type, pH and temperature have significant effects on persistence and therefore direct comparisons of PV1 persistence studies to this study are difficult to interpret. However, Hurst 1988 is the most similar to the sediment conditions of this study and therefore may be comparable. An estimated time to 99% reduction in the 4°C sediment in this study for PV2 and PV3 were 428 and 334 days respectively. This indicates that previous studies with PV1 are comparable to what is seen with PV2 and PV3, however, the addition of the impact of waste is unclear. Yet, the addition of microbial activity when comparing the sterile vs. non-sterile conditions demonstrated that microbial antagonism can contribute to decreased persistence, indicating a lower persistence due to the input of sewage and increased microbial activity (Hurst 1988). In Finland in 1988 after an outbreak and vaccination campaign, PV2 and PV3 were detected in sewage after 3.5 months, while PV1 was no longer detected immediately after the vaccination campaign ended, indicating the possibility that waste addition may have an unknown impact on PV1 when compared with PV2 and PV3 (Pöyry et al 1988). No study to date

has compared persistence of PV1, PV2, and PV3 at one time. The creation of this study with a soil type and environmental conditions similar to what is seen in Pakistan is important for being able to apply it to environmental surveillance in Pakistan.

Temperature was found to be of significance in this study in determining length of persistence and rate of die-off over time. This is consistent with previous literature, as discussed previously, not only with PV1, but with other viruses. All are consistent with this study in that low temperatures are protective of virus viability in the environment, and that increased temperatures increase die-off (Pirtle et al 1991, Hurst et al 1980, Hurst 1988, Clarke et al, Nasser et al 1993, Tierney et al 1977).

Environmental Surveillance implications

There were significant differences when comparing sediment and wastewater samples in the MM and sediment and WWO samples, and there were significant differences when comparing wastewater samples from the MM to the WWO samples (Table 4). This indicates that sediment is protective of both PV2 and PV3 viability and that sediment resuspension in the wastewater portion of the MM may be impacting increased persistence over time. Others have hypothesized that the sediment protects the viral capsid when attached to sediment particles (Rao et al 1984, Tierney et al 1977, Meschke and Sobsey 2003). This is important in the context of environmental surveillance in that sediment resuspension could contribute to water samples being positive for PV.

Linearity

A linear mixed model was used to interpret the data and used the entirety of the data for both association and prediction. One assumption of a linear mixed model is that the data is linear, which this study is limited by complete linearity of the data, which is shown when looking at

Figures 1 – 6 and the R^2 values in Table 7. The 4°C models were slightly less correlated and less linear when compared to other temperatures (Table 7). When using linear models, correlation is more important for prediction than for association analyses (Shmueli 2010). Therefore, when comparing the sample types and temperatures for significant differences in die-off of PV over time, the differences in linearity would not affect the interpretation of the results. However, when using the models to predict a time point at which 99% reduction was reached (Table 6), the linearity becomes more important. In this case, however, when looking at Figures 1-6, lower correlation values may have to do with quicker die-off in the beginning followed by slower die-off towards the end of the 126 days. This would cause an underestimate of days at which 99% reduction was reached, and the data should then be interpreted as such. Additionally, the low sample number and only 10 time points taken limit the interpretation of the data, especially towards 126 days. Future research can address more full characterization of the tail end of persistence of poliovirus so interpretation of the time point at which complete die-off is reached is more accurate.

Conclusion

This study's characterization of PV2 and PV3 persistence displays the importance of sediment and temperature in preserving the viability of poliovirus in the environment, which will be able to inform environmental surveillance measures. Future work should evaluate persistence of PV2 and PV3 for longer periods of time for cooler temperatures and focus more on characterizing persistence as PFU nears zero in order to fully characterize die-off of poliovirus in the environment. With the importance of environmental surveillance in eradication efforts, and the importance of understanding persistence of poliovirus in environmental surveillance, as discussed previously, the results from this study can help interpret environmental surveillance positives.

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Appendix A: **Extraction Method**

Method

Multiple eluents were tested to determine the eluent which gave the best viral recovery in sediment. Four separate eluents were examined for viral recovery and included a.) sterile Soy milk b.) 2M $NaNO_3$ [Fisher Bioreagents; Pittsburgh, PA, USA] diluted in sterile 3% beef extract [BD Biosciences; San Jose, CA, USA] c.) 2M Glycine [TCI American, Portland, OR, USA] diluted in sterile 3% beef extract d.) sterile 2M Glycine diluted in 2M NaCl [Fisher Bioreagents; Pittsburgh, PA, USA]. After it was determined that 2M $NaNO_3$ in 3% beef extract had the highest recovery of 25.75% (Table 8). The pHs of 5.5, 7.5 and 9.5 were tested, and the highest recovery at pH 7.5 and was subsequently used in the experiment (Table 9).

Determination of best eluent for the recovery of virus samples

The 2M $NaNO_3$ in 3% beef extract eluent had the highest recovery tested at 25.7% recovery (Table 8). This recovery is comparable or higher than the current method used in poliovirus environmental surveillance, which is two-phase separation, which has been shown to have low recoveries in sewage samples averaging 5% in one study (Fattal et al 1977). Of the three pHs tested, 5.5, 7.5 and 9.5, the 2M $NaNO_3$ eluent with pH 7.5 had the highest recovery of 19.5% compared to recovery rates of other tested pHs of 5.5 (3.8%) and 9.5 (15.2%) (Table 9). A 2M $NaNO_3$ in 3% beef extract eluent at pH 7.5 was then used in the experiments.

Table 8: Eluents tested on sediment recovery

Eluent	Recovery percentage
$NaNO_3$ + 3% beef extract	25.75%
Glycine + 3% beef extract	23.91%
Glycine + NaCl	2.45%
Soy Milk	13.28%

Table 9: pHs tested for $NaNO_3$ + 3% beef extract sediment recovery

pH $NaNO_3$	Recovery percentage
5.5	3.77%
7.5	19.53%
9.5	15.20%

For the determination of best eluent for virus recovery, it was determined that a sodium nitrate with 3% beef extract eluent at pH 7.5 had the highest recovery tested, which is seen in other PV recovery methods from sediment (Wait and Sobsey 1983). In addition, PV recovery methods have been shown to have consistent recovery with beef extract-containing eluents (Monpoeho et al. 2001). It has been the hypothesis that is due to the inclusion of aromatic amino acid content in beef extract and the neutral pH isoelectric point of poliovirus (Farrah and Bitton 1979, Thomassen et al 2013). The method used in this study is comparable to two-phase recovery, which is the approved method of the WHO for poliovirus environmental surveillance (GPEI 2019). Two-phase recovery in waste-impacted water samples has had low recoveries averaging at 5% (Fattal et al 1977).

Appendix B: Supplementary Data

Recovery & Compartmentalization in the experiment

Compartmentalization of poliovirus in the MM is valuable to know when determining the persistence in the wastewater portion of the MM when comparing it to persistence in the wastewater of the WWO. Compartmentalization was determined using average recoveries for each separate run of PV2 and PV3. Average recovery for each run was determined using the formula below

$$\% \, Recovery = \frac{Experimental \; PV \; PFU - Stock \; PV \; PFU}{Stock \; PV \; PFU} \times 100$$

Average recoveries for PV2 and PV3 for each run are displayed in Table 10. Using the average recoveries, compartmentalization was determined by dividing the sediment portion by the sediment average recovery and the liquid portion by the average WWO recovery and then dividing the MM liquid PFU by the total PFU sum in the microcosm. The average compartmentalization of PV2 in liquid was 30.4 ± 6.3%, and the average compartmentalization of PV3 in liquid was 3.66 ± 1.9%.

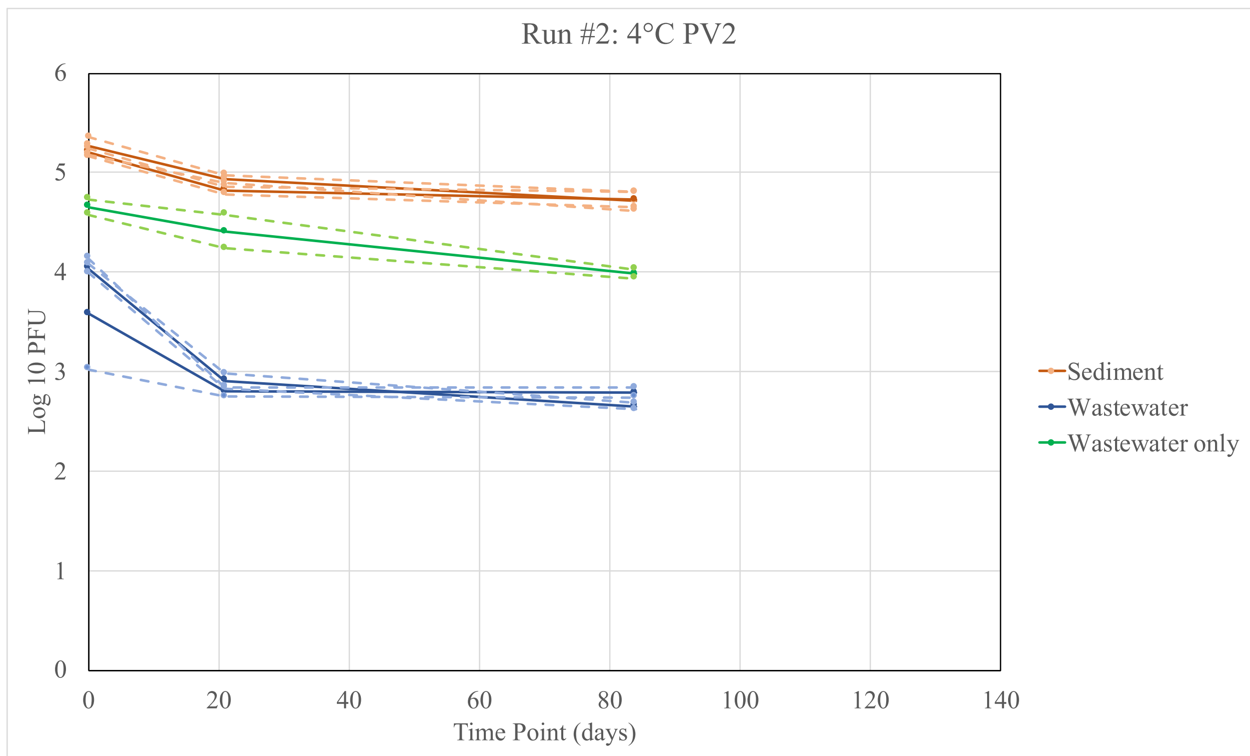
Table 10: **Average recoveries for the first run of PV2 and PV3**

Average Recovery in % recovered					
PV2			PV3		
MM: Sediment	MM: Wastewater	WWO: Wastewater	MM: Sediment	MM: Wastewater	WWO: Wastewater
22.8 ± 4.2	1.4 ± 0.2	4.8 ± 0.8	20.8 ± 3.8	0.5 ± 0.09	12.6 ± 1.3

Second Run

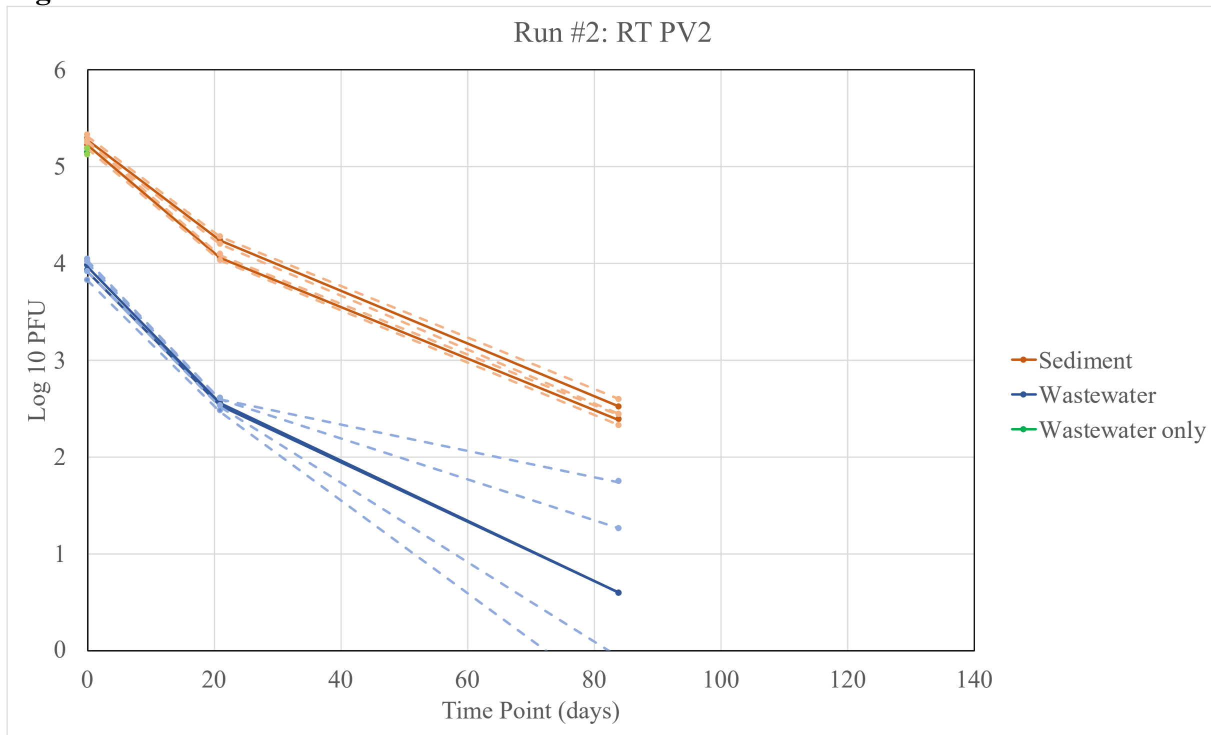
A second run of the experiment was conducted as described previously. Results from the second run were not included in the analysis due interpretation inaccuracies from truncation of the time points. Figures showing results from the second run are shown below in Figures 7-12. General patterns of decrease in PFU over time are similar to what is seen in the original experimental runs of PV2 and PV3 in that die off is slower at 4 °C, and in the sediment portions of the MM microcosm.

Figure 7: Run 2 of PV2 at 4°C



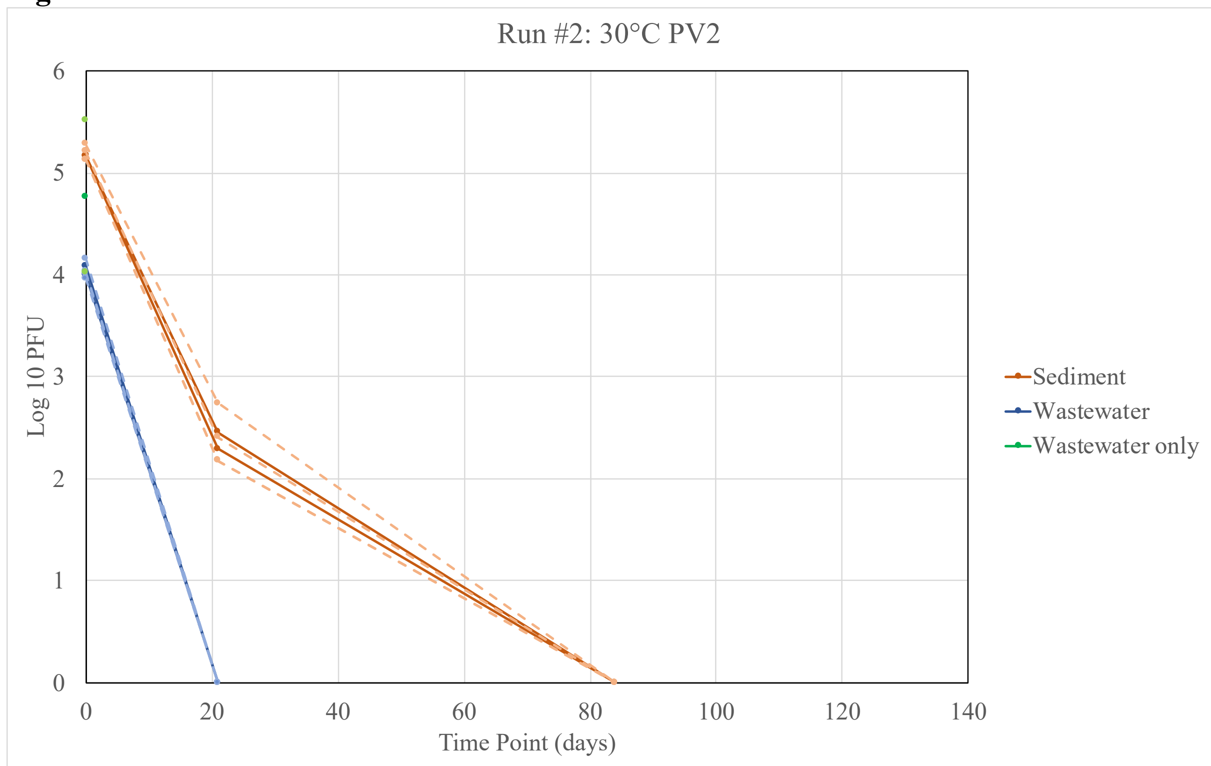
Dashed lines are 95% confidence intervals based on multiple wells plated during plaque assay.

Figure 8: Run 2 of PV2 at RT



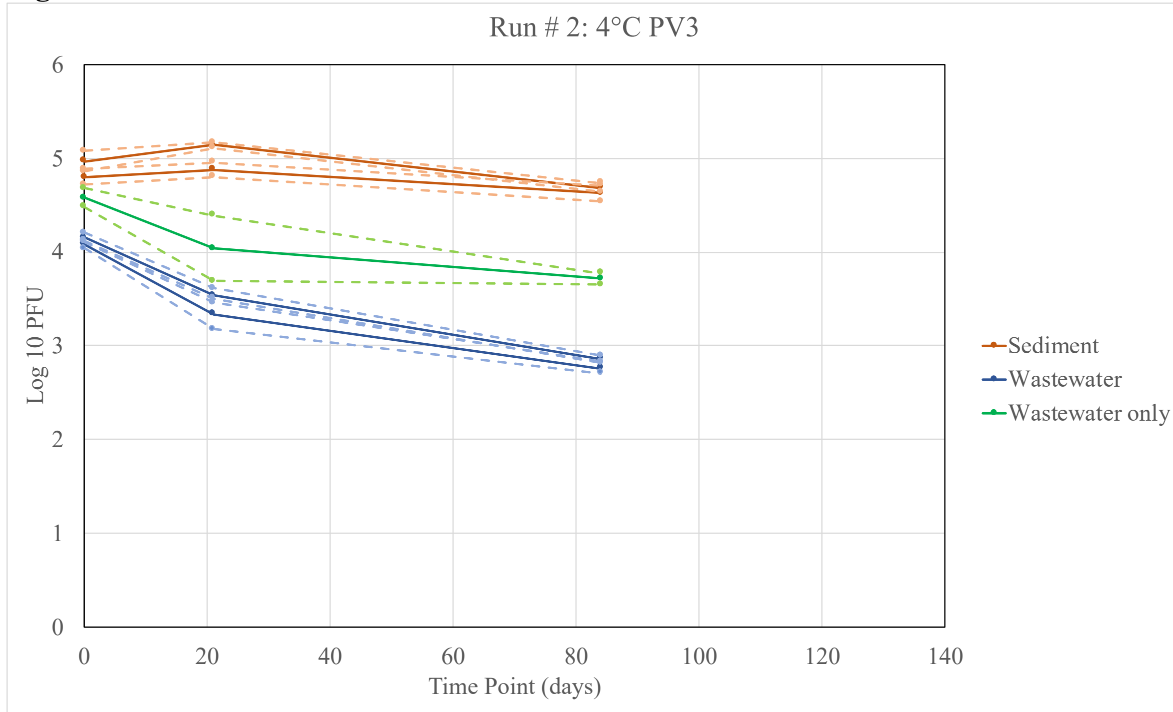
Dashed lines are 95% confidence intervals based on multiple wells plated during plaque assay.

Figure 9: Run 2 of PV2 at 30°C



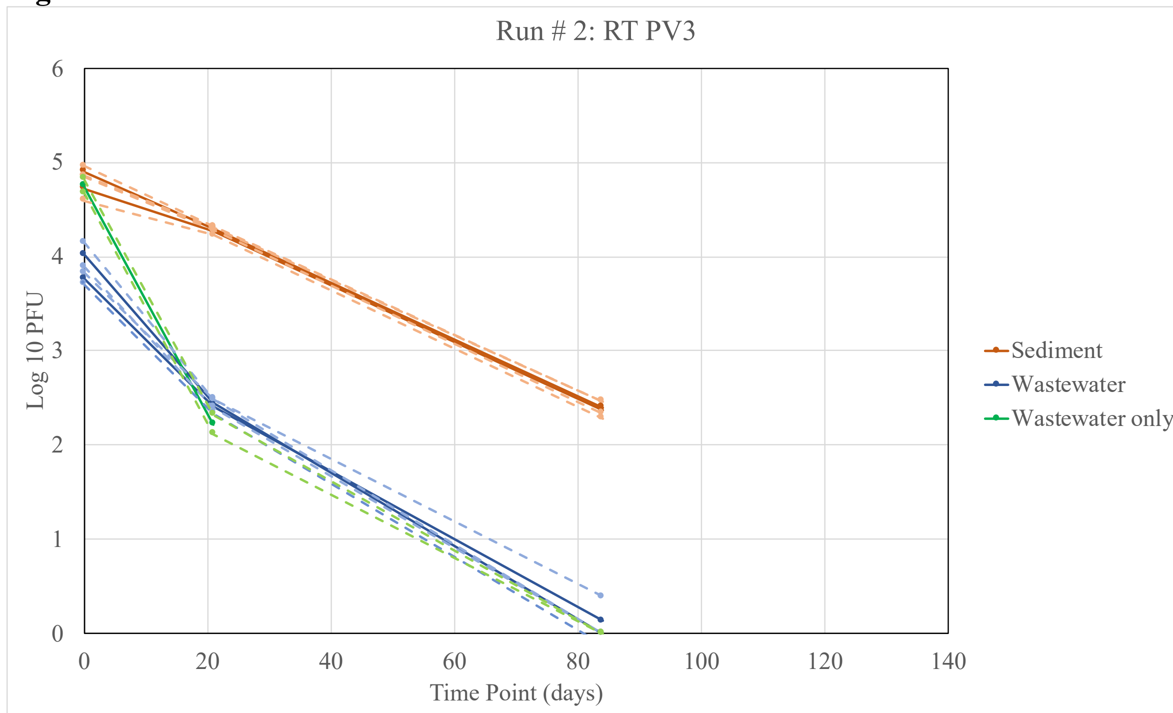
Dashed lines are 95% confidence intervals based on multiple wells plated during plaque assay.

Figure 10: Run 2 of PV3 at 4°C



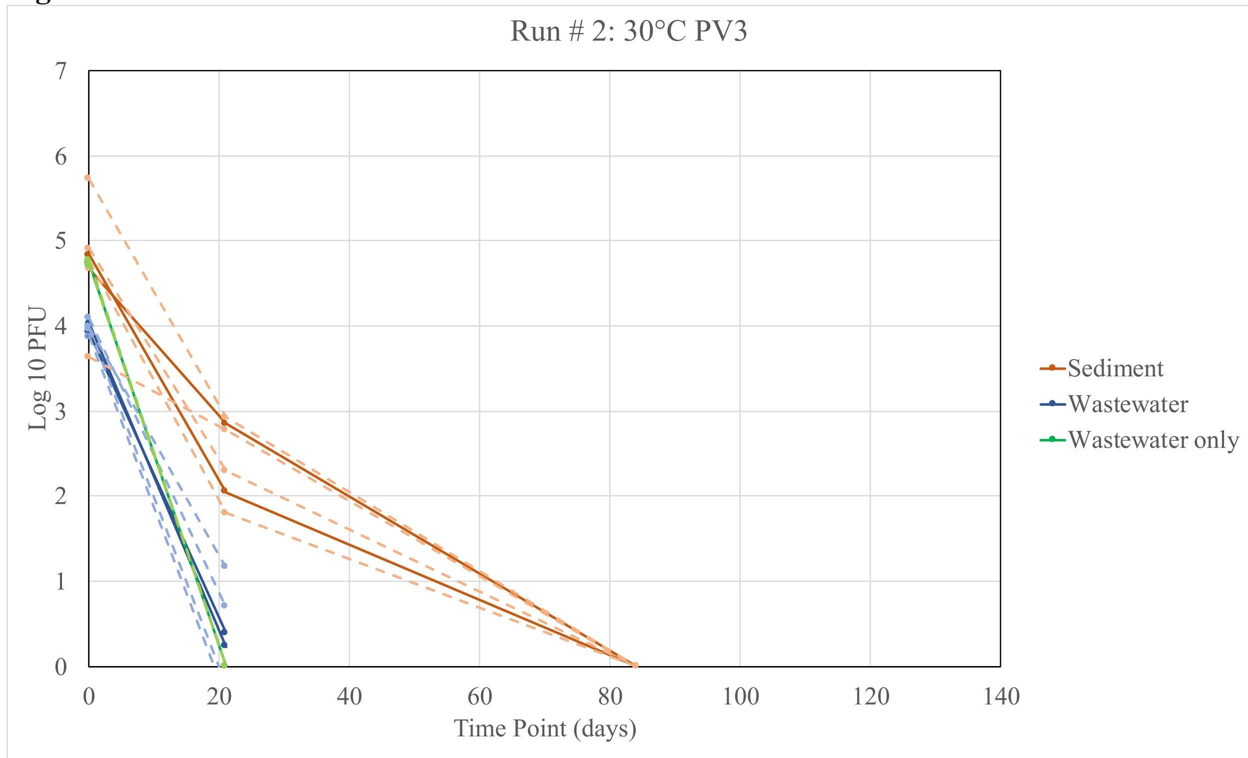
Dashed lines are 95% confidence intervals based on multiple wells plated during plaque assay.

Figure 11: Run 2 of PV3 at RT



Dashed lines are 95% confidence intervals based on multiple wells plated during plaque assay.

Figure 12: Run 2 of PV3 at 30°C



Dashed lines are 95% confidence intervals based on multiple wells plated during plaque assay.

Composition of the Biosolids

Composition of the biosolids was received from West Point Treatment plant and is seen in Table 11 below. Composition includes metals, pH, % Solids TS and % Solids VS.

Table 11: Composition of biosolids

WEST POINT BIOSOLIDS COMPOSITION

Ag	*As*	*Ba*	*Be*	*Cd*	*Cr*	*Cu*	*Hg*	*K*	*Mg*	*Mn*
mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg
3.2	4.63	160	0.12	1.8	29.4	373	0.68	1180	6010	578

Mo	*Ni*	*Pb*	*Se*	*Zn*	NH3	TKN	TP	BOD	pH
mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/l	mg/l	mg/l	mg/l	su
7.8	20.4	57.8	6.2	787	2490	18293	4793	23225	8.91

% Solids TS	% Solids VS
26.8	69.87