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Evaluation of Persistence of Viruses in Landfill Leachate

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Evaluation of Persistence of Viruses in Landfill Leachate

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Abbreviations/Acronyms

AHA	activity hazard analysis
APHIS	U.S. Department of Agriculture Animal and Plant Health Inspection Service
ATCC	American Type Culture Collection
BOD	biological oxygen demand
BSL	Biosafety Level
BW	biological warfare
°C	degree(s) Celsius
CAA	Clean Air Act
CFU	colony forming unit(s)
CFU/mL	colony forming unit(s) per milliliter
cm	centimeter(s)
CPE	cytopathic effect
CoA	Certificate of Analysis
COC	chain of custody
COD	chemical oxygen demand
DAL	double agar layer
DHL	DHL Analytical, Inc.
EDTA	Ethylenediaminetetraacetic acid
EMEM	Eagle's Minimum Essential Medium
E. coli	*Escherichia coli*
EPA	U.S. Environmental Protection Agency
°F	degree(s) Fahrenheit
FBS	Fetal Bovine Serum
FSMA	Food Safety Modernization Act
g	gram(s)
HDPE	high density polyethylene
L	liter(s)
LBA	Luria Bertani Agar
LBB	Luria Bertani Broth
LBTA	Luria Bertani Top Agar
ln	natural logarithm
LOD	limit of detection
MERS	Middle East Respiratory Syndrome

MHV	mouse hepatitis virus
µm	micrometer(s)
mg	milligram(s)
µL	microliter(s)
mL	milliliter(s)
min	minute(s)
MOI	multiplicity of infection
MOP	miscellaneous operating procedure
MS2	MS2 bacteriophage
MSW	Municipal Solid Waste
NHSRC	EPA National Homeland Security Research Center
ORP	oxidation reduction potential
Phi6	Phi6 bacteriophage
PBS	Phosphate Buffered Saline
PD	proportional distance
PDA	potato dextrose agar
PE	performance evaluation
PFU	plaque forming unit(s)
PPE	personal protective equipment
ppm	parts per million
P. syringiae	*Pseudomonas syringiae*
QA	quality assurance
QAPP	quality assurance project plan
QC	quality control
QMP	Quality Management Plan
rcf	relative centrifugal force
RCRA	Resource Conservation and Recovery Act
RNA	ribonucleic acid
SARS	Severe Acute Respiratory Syndrome
ST	swine testicular
T&E II	Testing and Evaluation II Contract
$TCID_{50}$	50 % Tissue Culture Infectious Dose
TDS	total dissolved solids
TGEV	Transmissible Gastroenteritis Virus
TNTC	too numerous to count
TO	task order
TOC	total organic carbon
TOCOR	Task Order Contracting Officer's Representative
TOL	Task Order Leader
TSA	Tryptic Soy Agar

TSB	Tryptic Soy Broth
TSS	total suspended solids
TSTA	Tryptic Soy Top Agar
USDA	U.S. Department of Agriculture
VEEV	Venezuelan Equine Encephalitis Virus

Executive Summary

The purpose of this effort was to assess the persistence of viruses in landfill leachate. Due to the limited capacity of incinerators and hazardous waste sites, municipal solid waste (MSW) landfills, or landfills of a similar design, may receive decontaminated building materials following a terrorist attack with biological warfare (BW) agents, a natural outbreak of a highly infectious viral pathogen (i.e., Ebola virus) and/or an unintentional release. The ultimate fate of those materials is of great concern, particularly if the materials were incompletely decontaminated and contain residual amounts of BW agents. To determine whether active viruses could pose a threat to human and environmental health once introduced into a landfill, laboratory testing was performed to measure the decay rate of viral agents in landfill leachate. This effort was performed using surrogate test agents similar to BW agents following the well-established hypothesis that, though the diversity of viral contaminants may be quite large, a limited list of viral surrogates can be chosen that qualitatively represent the likely BW threat agents of interest.

This effort evaluated the persistence of Transmissible Gastroenteritis Virus (TGEV), MS2 bacteriophage (MS2), and Phi-6 bacteriophage (Phi6) in landfill leachates collected from three landfills. Each of the three landfill leachates was individually spiked with a known quantity of virus, dispensed into replicate screw-top vials, and statically incubated. One set of all three virus-spiked samples was incubated at 12°C, and one set of MS2 and Phi6-spiked samples was incubated at 37°C; TGEV was not analyzed at 37°C. Throughout the course of the study, triplicate samples were assayed for infectious viruses (via infectious viral titer) at up to seven time points post T_0. TGEV-spiked samples were assayed using a 50% tissue culture infectious dose assay ($TCID_{50}$), in which samples were serially diluted, and a range of dilutions was assessed for infectivity on Swine Testicular (ST) cells. For MS2 and Phi6-spiked samples, triplicate samples were assayed using the standard Double Agar Layer (DAL) method, in which serial dilutions of samples were analyzed on *Escherichia coli* (MS2-specific) and *Pseudomonas syringae* (Phi6-specific) host strains. Data were graphed as concentration versus time, and best fit regression curves were used to calculate persistence (the time where the measured linear rate of decay intersects with the assay limit of detection) and decay rate (D-value, time required for the viral titer to reduce by 90%, or to 10% of the starting titer).

Data generated from this study included viral persistence and decay rates in landfill leachates for three surrogate viral test agents. Viral persistence and decay rates in three unique landfill leachates were determined under two temperature conditions (illustrated in Executive Summary Tables 1 and 2, respectively). Data indicated viral surrogate agents (TGEV, MS2 and Phi6) can persist for weeks to months in landfill leachates, and persistence varies by environmental condition; viruses persisted longer at mild temperatures (12 degrees Celsius [°C]) and decayed far more rapidly at warmer temperatures (37°C). The study results suggest that viruses may persist in landfill leachates for a lengthy period of time (weeks to months) under the mild conditions present in the majority of the US. Should waste from an attack with viral agents still containing residual agent be disposed of in a landfill, knowledge of the persistence of the virus in the leachate will allow landfill operations to be adapted to minimize potential exposures to waste management workers and the public.

Executive Summary Table 1. Persistence of Various Viruses in Three Landfill Leachates

Virus	Temperature Test Condition	Calculated Days Until No Longer Detected[a]			
		Leachate A	Leachate B	Leachate C	Control Matrix[b]
Transmissible Gastroenteritis Virus (enveloped RNA virus)	12°C	5	17	7	43
MS2 Bacteriophage (non-enveloped phage)	12°C	113	75	87	NR[c]
	37°C	3	2	2	NR[c]
Phi6 Bacteriophage (enveloped phage)	12°C	55	66	81	122
	37°C	0.3	0.2	0.2	2

[a]Calculated time (days) when measured linear decay rate intersects with assay limit of detection.
[b]TGEV in sterile incomplete Eagle's Minimum Essential Medium (EMEM) medium; bacteriophage in sterile phosphate buffered saline.
[c]No decay, or minimal, observed within incubation period tested.

Executive Summary Table 2. Decay Rates of Viral Agents in Three Landfill Leachates

Virus	Temperature Test Condition	Measured D-value in Days			
		Leachate A	Leachate B	Leachate C	Control Matrix[a]
Transmissible Gastroenteritis Virus (enveloped RNA virus)	12°C	1	4	2	7
MS2 Bacteriophage (non-enveloped phage)	12°C	10	7	8	189
	37°C	0.3	0.2	0.2	NR[b]
Phi6 Bacteriophage (enveloped phage)	12°C	6	10	12	17
	37°C	< 0.1	< 0.1	<0.1	0.15

[a]TGEV in sterile incomplete EMEM; bacteriophage in sterile phosphate buffered saline (PBS).
[b]No decay observed within incubation period tested.

1.0 Introduction

U.S. Environmental Protection Agency (EPA) is designated as a coordinating Agency under the National Response Framework to prepare for, respond to, and recover from threats to public health, welfare, or the environment caused by actual or potential oil and hazardous materials incidents. Hazardous materials include accidentally or intentionally released chemical, biological, and radiological substances. The EPA is also designated as a support Agency, to support the U.S. Department of Agriculture's (USDA's) Animal and Plant Health Inspection Service (APHIS) activities in agricultural emergency response. In addition, the EPA is a lead agency under Section 208 of the Food Safety Modernization Act (FSMA), tasked with developing model plans for protecting the nation's food and agricultural infrastructure to safeguard human health and the environment. Management of waste resulting from cleanup after incidents involving contamination with biological agents typically involves some sort of treatment process (e.g., decontamination, incineration, autoclaving) followed by disposal of the treatment residues in a secure landfill. Secure landfills include Resource Conservation and Recovery Act (RCRA) Subtitle C (hazardous waste) or RCRA Subtitle D (municipal waste) landfills, depending on the decisions that are made, typically at the state level.

This study assessed the persistence and decay rate of viral surrogates in landfill leachate. Waste generated during natural outbreaks (i.e., Ebola virus waste), clean-up of unintentional releases, or following a terrorist attack involving biological agents may be placed in MSW landfills. The ultimate fate of the BW agent(s), in this case viral agents, is of concern. Although these materials will be decontaminated, large quantities, heterogeneous materials, and laboratory limitations may lead to residual biological contamination. To evaluate whether infectious viruses in landfill leachate could survive to be a risk to human and environmental health, laboratory testing was performed to measure the decay rate of viral surrogates in landfill leachates.

A scientific basis to assess this concern was developed to assess the potential for residual agent to: 1) persist in the landfill environment; and 2) be transported within the landfill environment to different media (e.g., waste, leachate, gas). This study aimed to evaluate survivability and/or persistence of viral agents in landfill leachate. Data from this study provide a good framework for

estimating and determining the fate of residual viral agents that may be placed into a landfill. This study provides confidence in the ability to effectively predict the fate of infectious viral BW agents in these types of waste and reduce the need for characterization of highly infectious BW viral agents represented by these surrogates.

This study used three viral agents as surrogates for highly pathogenic BW viral agents. Three ribonucleic acid (RNA) viruses were selected: one enveloped mammalian virus (TGEV), one enveloped bacteriophage (Phi6) and one non-enveloped bacteriophage (MS2). These surrogates were selected because they represent three common classes of viruses, all which can easily be manipulated in biosafety level (BSL)-2 facilities, and do not include human infectious agents. TGEV is an *Alphacoronavirus* that causes severe disease in young swine (mortality close to 100% in piglets) and is related to several human coronaviruses. TGEV was used here as a model for Risk Group 3 Coronaviruses, including Severe Acute Respiratory Syndrome (SARS) and Middle East Respiratory Syndrome (MERS) coronaviruses, as well as other emerging human enveloped RNA viruses (e.g., influenza). Risk Group 3 agents are agents associated with serious or lethal human disease, for which preventative or therapeutic interventions may not be available. These agents represent high individual risk, and low community risk if released from a laboratory, and often (as is the case of MERS and SARS) are manipulated in BSL-3 facilities. Phi6 is an enveloped RNA bacteriophage that infects *Pseudomonas syringae*. This phage contains a tripartite double-stranded RNA genome and was used in this study as an intermediate stability enveloped RNA virus. MS2 is a non-enveloped single-stranded RNA virus that infects *Escherichia coli*. This phage was used as a surrogate for non-enveloped human infectious viral agents, including poliovirus, norovirus, parvovirus, rotavirus, hepatitis A and E viruses, and Coxsackievirus.

Study deliverables included time-course survivability data and specific persistence and D-values (i.e., decay rates) for the three surrogate viruses in three unique landfill leachates under two temperature conditions. These deliverables are included as appendices to this report.

2.0 Approach

The study was divided into the following three tasks consecutively performed.

Task 1: Landfill Leachate Acquisition and Characterization.

Task 2: Method Development

Task 3: Virus Persistence Testing

2.1 Task 1 - Landfill Leachate Acquisition and Characterization

Three landfill facilities were used to support this evaluation. These facilities were selected based on meeting the following acceptance criteria:

- "Large" in size (capacity of ≥ 2.5 million tons MSW) and subject to Clean Air Act (CAA) requirements;
- RCRA Subtitle D-type waste (with RCRA Subtitle C-type construction if possible);
- Operational for at least five years;
- All three with similar design and operating characteristics such as waste composition and gas extraction and capture, including no active leachate recirculation;
- Steady leachate composition and quantity (demonstrated by available historical monitoring data);
- Not under any enforcement action (for any local, state, or federal regulations); and
- Willing to allow access to research staff to collect leachate from an accessible leachate collection point representative of leachate across the landfill.

Approximately 10 liters (L) of landfill leachate was collected and returned the laboratory where a portion of each was sent to an analytical laboratory for characterization analysis. The remaining portion was stored under refrigeration and used as needed for virus persistence testing. The details of the procedures used for landfill leachate facility selection, landfill leachate acquisition processes, and characterization testing are described in Section 3.0, and the results are presented in Section 5.1 . The three landfills selected have been kept anonymous in this report and are referred to herein as Landfills A, B, and C. Each of these landfills met the primary selection criteria, including Landfill B which accepts animal carcasses.

2.2 Method Development

This task involved acquiring and preparing three viral agents (Table 1), establishing virus quantitation assays (mammalian-based $TCID_{50}$ and double agar layer [DAL] plaque assays), assessing each landfill leachate for the presence of indigenous viral agents that could cause false positive results, identifying whether each landfill leachate causes assay inhibition, and developing sample analysis procedures (extraction and quantitation) so that assay inhibition is minimized or eliminated while viral concentration is accurately measured. This task was not intended to be an exhaustive method optimization activity but was intended to develop virus recovery methods for generating accurate, defensible virus persistence data from landfill leachates using existing standard methods.

Methods to recover live viral agent from each of the three leachates, while minimizing mammalian cell cytotoxicity were evaluated. Landfill leachate is a very complex matrix, consisting of many chemical and biological constituents. Therefore, tests were performed to determine if leachates induced cytotoxicity on mammalian cell monolayers used for the TGEV quantification assay, or adversely effected bacterial cells used in the DAL assay for the bacteriophage quantification assays. The most robust method for minimizing assay inhibition/interference and cytotoxic effects was dilution. Further testing of sample processing procedures were proposed (including filtration and precipitation) if dilution was not successful, but fortunately dilution adequately eliminated the majority of the effects of the leachate on the TGEV $TCID_{50}$ assay. The leachate also interfered with the Phi6 DAL assay, and a short slow-speed centrifugation step applied to the leachate sample prior to initiating the DAL assay was effective in eliminating assay inhibition. No leachate-induced adverse effects were observed on the MS2 DAL assay. In addition to inducing cytotoxicity or other adverse effects, leachate may have harbored indigenous viruses that could interfere with detecting surrogate agents, thus generating false positive results. Each leachate was screened for indigenous viruses using the appropriate assay system; no indigenous viruses were identified using either assay system.

Upon completion of preliminary evaluation, analytical methods for each viral agent were identified and shown to be effective in efficiently and accurately determining target viral agent concentrations from each landfill leachate. A detailed summary of the method development

activities performed and results are provided in Appendix A. The final methods that were utilized for the persistence study were written into a Miscellaneous Operating Procedure (MOP). The MOP (provided in Appendix B) included sample preparation, incubation, and processing procedures and is discussed in detail in Section 3.0.

2.3 Virus Persistence Testing

Persistence of three viral surrogates (Table 1) was evaluated in the three landfill leachates over time (12°C or 37°C) using methods described in Section 2.2 and procedures described in Section 3.0 (also refer to MOP in Appendix B). To accurately measure the persistence and decay rate of the surrogate agents in the landfill leachate, a decay (kill) curve was generated. To generate this curve, samples of each agent in each leachate were prepared (4 milliliter [mL] aliquots in 5 mL screw-top tubes spiked with virus) and incubated at the test temperature. Triplicate test samples, triplicate positive controls (TGEV spiked into Eagle's Minimum Essential Medium [EMEM] without fetal bovine serum [FBS] or MS2/Phi6 spiked into phosphate buffered saline [PBS]) and negative controls per leachate (leachate without virus) were removed and analyzed for viral titer at each time point. Samples were analyzed over eight weeks or until the samples were below the limit of detection for two sequential time points. The analysis included up to seven time points after T_0.

Persistence testing was designed to capture decreasing agent viability at each incubation temperature, and time points were selected to capture decay across at least three sequential time points. Initially, time points were chosen to capture viable agent over one to three days, and time between sample time points was adjusted based on immediately preceding results. MS2 and Phi6 assay results were obtained 24 hours after samples were analyzed, and the data were used to make informed decisions regarding subsequent time points. The TGEV assay has a longer read-out time, and assay results were thus acquired three to four days after the samples were processed, preventing informed day-to-day decision making early in the persistence study. Time points for TGEV analysis were selected using an assumption of rapid viral decay for the first three time points, and then refined using these data thereafter. Details of sample preparation, incubation, quantitation assay and data analysis are described in Section 3.0.

Table 1. Test Matrix for Virus Persistence Evaluation in Landfill Leachates.

Parameter	Description
Virus surrogates (3)	TGEV coronavirus (enveloped) Phi 6 (enveloped bacteriophage) MS2 (non-enveloped bacteriophage)
Landfill Leachate	Three; each from different landfill facilities
Incubation Temperature	12˚C for TGEV[a]; 12˚C and 37˚C for MS2 and Phi6
Time Points[b]	0 (baseline), 1, 3, 7, 14, 21, 28, 35, 42, 49, 56 days

[a] TGEV persistence was not measured at 37°C, as its survival at 12°C suggested that it would not survive for more than several hours at 37°C.

[b]Subject to change throughout test and amended as deemed necessary based on results of previous time points. Actual time points tested are shown in Table 3.

2.4 Microbial Activity

Microbial activity intrinsic to each leachate was analyzed using standard plate count methods. Heterotrophic bacterial and fungal concentrations in each leachate were characterized approximately 2½ months apart, once at the onset of each test. Microbial diversity was qualitatively assessed by analyzing bacterial and fungal colonies on non-selective media for heterotrophic bacteria (Tryptic Soy Agar, a non-selective medium) and fungi (Potato Dextrose Agar, a reduced pH medium to support fungal growth and minimize bacterial growth). Colonies were enumerated on spread plates to calculate colony forming units per milliliter (CFU/mL), and colony morphology was observed at the same time. Colony morphology was recorded during both heterotrophic plate counts; however, recovered colonies were not identified or characterized. Results are described in section 5.5 and Table 8.

3.0 Procedures

3.1 Landfill Leachate Acquisition and Characterization

3.1.1 Landfill Selection

Potentially suitable landfills were identified using the EPA's Incident Waste Decision Support Tool (I-WASTE) tool [1] (V6.4; http://www2.ergweb.com/bdrtool/login.asp), selecting "MSW Landfills" and "Large Landfills" as search terms, and collating results for facilities meeting the capacity criterion for the States of Indiana, Michigan, Pennsylvania, and Ohio. These states were selected to achieve operational efficiency for project personnel located in Newtown, Pennsylvania, and Columbus, Ohio. After multiple potentially suitable landfills were identified, the short-listed facilities were contacted to obtain commitment from the owners/operators to participate in the study. Three landfills were selected that met the primary selection criteria, including Landfill B which accepts animal carcasses. Basic characteristics about each landfill are provided in Table 2.

Table 2. Landfill Characteristics

Characteristic	Landfill A	Landfill B	Landfill C
Waste Acceptance Rate	In 2014, accepted approximately 3,200 tons per day	3,500 to 5,000 tons per day, Approximately 1,000,000 tons of waste received in 2014	Average 1,400 tons/day
Footprint	100 acres permitted to accept waste	283 acres permitted to accept waste	168 acres permitted to accept waste
Year Opened	1997	1995	1995
Expected Closure Date	2023 or 2024 (pursuing an expansion which could extend life by 25 years)	2030 to 2045	Information not provided
Gas collection system	Yes	Yes (approximately 190 gas collection wells or points)	No

3.1.2 Logistics

For the leachate sampling, a field sampling technician traveled to the sampling sites with coolers, sampling equipment, and supplies. The coolers contained unpreserved 1.89 L (1/2 U.S. –gallon) and 3.78 L (one U.S. gallon) high density polyethylene (HDPE) containers, laboratory sample

containers provided by the selected study laboratory, DHL Analytical, Inc. (DHL) of Round Rock, Texas, and frozen blue-ice packs to keep the samples cold after collection. Equipment included a multi-parameter water quality instrument with pH, oxidation reduction potential (ORP), and temperature probes, disposable Teflon bailers, a peristaltic pump, and a sample collection rod. Supplies included chain of custody (COC) forms, sample bottle labels, deionized water, nylon rope, tubing, disposable beakers, labels, a spray bottle with bleach solution, a 9.47 L (2.5 U.S. gallon) bucket, Ziploc® bags, nitrile gloves, Tyvek coats, hard hat, face shield, paper towels, and trash bags.

Project health and safety was governed by a project-specific activity hazard analysis (AHA). The AHA was strictly adhered to during the course of performing landfill leachate sampling activities, including the use of appropriate personal protective equipment (PPE).

3.1.3 Leachate Collection

All three leachates were collected within a two-day window, October 7 - 8, 2015. Leachate was collected directly from an accessible leachate collection point into two 3.78-L and two 1.89-L HDPE containers. The containers were completely filled and sealed with minimal headspace to avoid oxygenation of the leachate.

At Landfill A, leachate was collected from a leachate accumulation area, consisting of three above-ground 8,976 L (34,000 U.S. gallon) storage tanks situated in a cement containment area (Figure 1). A three-inch discharge line connected to the tanks is routinely used by Landfill A personnel to collect leachate samples for analysis. The leachate flow from the tanks is controlled by a ball valve. Sample collection began at approximately 9:00 AM. The discharge line was purged at a high flow rate for approximately five minutes. After purging, the flow rate was reduced using the ball valve to a controlled laminar flow for sample collection. The leachate appeared yellow and without significant particulate matter. The collection time was written on the labels, and the labels were placed on the appropriate sample containers.

Figure 1. Landfill A Leachate Accumulation Area

At Landfill B, leachate was collected from a leachate sump area (Figure 2). Sample collection began at approximately 12:00 PM. The leachate level was approximately three meters below the surface of the sump. Nylon rope was tied to the 9.47 L bucket and dropped into the sump. The leachate appeared brown with significant particulate matter. The collection time was written on the labels, and the labels were placed on the appropriate sample containers.

Figure 2. Landfill B leachate sump area.

At Landfill C, leachate was collected from a leachate accumulation area in which the leachate is stored in a 22,176 L (84,000 U.S. gallon) tank surrounded by a containment area (Figure 3). A three-inch discharge line was connected to the tank routinely used by Landfill C personnel to

collect leachate samples for analysis. The leachate flow from the tank was controlled by a ball valve. Sample collection began at approximately 11:00 AM. The leachate line was purged at a high flow rate for five minutes before sample collection. After purging the line, the flow rate was reduced using the ball valve to a controlled laminar flow for sample collection. The leachate appeared dark brown and contained significant particulate matter. The collection time was written on the labels, and labels were placed on the appropriate sample containers.

Figure 3. Landfill C leachate accumulation area.

At each site, a disposable beaker was filled with leachate, and the pH, oxidation reduction potential (ORP), and temperature were measured using the multi-parameter water quality instrument immediately after leachate collection. Following the measurements, the leachate was immediately placed in a cooler packed with ice packs and delivered to the contractor laboratory. Upon receipt, leachate samples were inspected, photographed, assigned a unique lot number, inventoried, and stored at 4^{o}C. Figure 4 illustrates the visual appearance of the leachates in 3.78 L bottles.

Figure 4. Landfill Leachates A, B, and C in 3.78 L containers.

For each landfill leachate, one 1.89 L bottle of leachate was shipped to a commercial analytical laboratory (DHL) in coolers loaded with wet ice on the same day as sample collection (expedited overnight shipping) for characterization testing. The remaining 1.89 L and two 3.78 L HDPE containers were relinquished to the custody of virology laboratory staff for method development activities and the persistence study.

Leachate from each of the three landfill facilities selected for the study was submitted to DHL for analysis of the following parameters (unless otherwise noted):

- Alkalinity (total)
- Ammonia
- Anions
    - Chloride
    - Nitrate
    - Sulfate
- Biological oxygen demand (BOD)
- Chemical oxygen demand (COD)
- Metals
    - Calcium
    - Iron
    - Magnesium
    - Manganese
    - Potassium
    - Sodium
    - Zinc
- pH (performed in the field by Battelle)

- ORP; performed in the field by Battelle Technical Staff
- Total dissolved solids (TDS)
- Total organic carbon (TOC)
- Total suspended solids (TSS)

The analytical data received from DHL are summarized in Table 4 in Section 5.1, with data derived in the field at the time of sample collection from a multi-parameter water quality instrument (ORP, pH, and temperature) and visual characteristics.

3.1.4 Microbial Activity

Heterotrophic microbial activity in each leachate sample was measured at two points during this study: (1) start of the initial persistence testing and (2) start of second persistence testing. Microbial activity was determined using a standard plate count assay. Leachate samples were serially diluted in PBS out to 10^{-4} dilution and 100 microliter (µL) samples of each dilution were plated in triplicate on Tryptic Soy Agar (TSA) and Potato Dextrose Agar (PDA). Bacterial activity was quantitated using TSA and fungal activity using PDA. Samples were incubated at 25°C for 60-72 hours, and colonies were enumerated. Colony morphology on each media was noted, however colonies were not identified or characterized. Microbial activity was determined as colony-forming units per mL (CFU/mL) using the equation:

$$\frac{CFU}{mL} = \frac{Average\ number\ of\ colonies\ per\ dilution}{(dilution)\ \times (volume\ added\ to\ plate)}$$

3.2 Virus Propagation

3.2.1 TGEV Propagation

TGEV Purdue strain (ATCC, VR-763 lot 4) was acquired from the American Type Culture Collection (ATCC). The ATCC stock is the only known commercially available source of TGEV.. As the ATCC TGEV stock is contaminated with bacteria [2], filtration was used to generate a bacteria-free sample prior to seed stock generation. TGEV was propagated on swine testicular (ST) cells (ATCC CRL1746). The virus was thawed on ice, diluted 1:2 in incomplete growth medium (Modified Eagle's Medium supplemented with Earle's buffered salt solution, L-glutamine and 1 % antibiotic/antimycotic) and sterile-filtered using a 0.22 micrometer (µm) low-protein binding

filter. An additional 1 mL of sterile incomplete growth medium was passed through the filter to ensure the virus was completely removed from the filter. Filtered virus was centrifuged (12,000 relative centrifugal force [rcf], four minutes [min]) to remove any residual cells and debris, and inoculated onto a healthy confluent monolayer of ST cells in a 25 cm^2 tissue culture flask (T25) and incubated for one hour (37°C, 5% CO_2); the flask was rocked every 15 minutes. Additional incomplete growth medium (5 mL) was added, and the flask was returned to the incubator (37°C, 5% CO_2). Inoculated cells were observed for cytopathic effect (CPE), and virus was harvested when the cells exhibited 70-90% CPE (approximately 24 hours post-inoculation). Virus was harvested in a two-step process: the flask was frozen at -15 to -30°C for at least one hour, then thawed at room temperature, and virus seed stock was prepared by centrifugation (4°C, 400 rcf for 20 min), and the supernatant was aliquoted and frozen at -75 to -80°C.

TGEV seed stock was used to prepare a second pass stock (TGEV p2). Undiluted seed stock was directly inoculated onto a healthy confluent monolayer of ST cells in a 75 cm^2 tissue culture flask (T75) and incubated for one hour (37°C, 5% CO_2); the flask was rocked every 15 minutes. Additional incomplete growth medium (8 mL) was added, and the flask was returned to the incubator (37°C, 5% CO_2). Virus was identically harvested to the seed stock at 21 hours post-inoculation. Virus supernatant was aliquoted and stored at -75 to -80°C. TGEV p2 virus titer was 4.6×10^6 $TCID_{50}$/mL via $TCID_{50}$ assay on ST cells (see section 3.3.3).

A working stock (third pass) of TGEV was prepared from TGEV p2 (TGEV p3). TGEV p2 was diluted in incomplete medium to a multiplicity of infection (MOI) of 0.1 and inoculated onto a healthy 70 % confluent monolayer of ST cells in 150 cm^2 tissue culture flasks (T150). Flasks were incubated for one hour (37°C, 5% CO_2) and rocked every 15 minutes. Additional incomplete growth medium (25 mL) was added, and the flask was returned to the incubator (37°C, 5% CO_2). Virus was identically harvested to the seed stock at 21 hours post-inoculation. Virus supernatant was frozen in 1 mL aliquots at -65 to -80°C. TGEV p3 virus titer was 4.1×10^6 $TCID_{50}$/mL via $TCID_{50}$ assay on ST cells (see Section 3.3.3). TGEV p3 was used for all persistence studies.

3.2.2 Bacteriophage Propagation

Master stocks of MS2 (ATCC 155597-B1) and Phi6 (EPA-provided) bacteriophages were propagated from infected agar cultures of *Escherichia coli (E. coli)* (ATCC 700891) and *Pseudomonas syringae* (*P. syringae*) LM2489 (EPA-provided), respectively.

To propagate MS2, a log phase broth *E. coli* culture was grown in Luria Bertani Broth (LBB) for approximately 3.5 hours. This culture was added to molten Luria Burtani Top Agar (LBTA) and overlaid onto Luria Bertani agar (LBA). A lyophilized pellet of MS2 (ATCC) was rehydrated in 0.5 mL LBB and overlaid onto *E. coli*-inoculated LBTA and incubated for approximately 24 hours at 35-37°C. The soft top agar, consisting of MS2-infected *E. coli* cells, was scraped off, transferred to a tube containing 15 mL SM buffer, and centrifuged at 7000 rcf for 15 minutes. Virus supernatant was filtered through a 0.2 µm syringe filter to remove residual bacterial cells. Filtered supernatant was designated the MS2 master stock (assigned lot number MS2091515) and was stored as replicate 0.5 mL aliquots at < -70°C. MS2 master stock titer was measured using a standard DAL method (see Section 3.3.3) and determined to be 4.0×10^{9} plaque forming units per milliliter (PFU/mL).

To propagate Phi6, a 100 µL aliquot of the EPA Phi6 stock was suspended in 30 mL Tryptic Soy Broth (TSB) supplemented with magnesium (TSB-Mg) and combined with 6 mL of a *P. syringae* culture grown for approximately 24 hours in TSB-Mg stock and 90 mL of molten Tryptic Soy Top Agar (TSTA) supplemented with magnesium (TSTA-Mg). Phi6 suspension was gently mixed, overlaid as 4 mL aliquots onto each of approximately 30 TSTA-Mg plates, and incubated for approximately 20 hours at 25-27°C. Phi6 was harvested by adding 5 mL TSB-Mg to each plate, incubating at 25-27°C for two hours, and resuspended by gently swirling the plates. The Phi6 stock was filtered through a 0.2 µm syringe filter to remove residual bacterial cells. Filtered supernatant was designated the Phi6 master stock (assigned lot number PHI6092516) and was stored as replicate 1 mL and 5 mL aliquots at < -70°C. Phi6 master stock titer was measured using a standard DAL method (see Section 3.3.3) and determined to be 1.8×10^{10} PFU/mL.

3.3 Persistence Testing

The persistence of TGEV, MS2, and Phi6 viruses in landfill leachates were evaluated as follows: leachates were individually spiked with a known quantity of virus, dispensed into replicate screw-top vials, and statically incubated for up to 56 days or until the virus was no longer detected. One set of TGEV spiked samples was incubated at 12°C, and two sets of each bacteriophage samples (MS2 and Phi6) were incubated (one at 12°C and the second set at 37°C). TGEV persistence was not measured at 37°C, as its survival at 12°C suggested it would not survive for more than several hours at 37°C. Throughout the incubation period, triplicate samples were assayed at the initiation of testing (T_0) and up to seven following time points.

TGEV spiked samples were assayed using an end-point dilution $TCID_{50}$ assay on ST cells. Briefly, each replicate sample was serially diluted and plated on ST cells. $TCID_{50}$ assays measure infectious virus and identified the dilution of the virus at which 50 % of cell cultures were infected. MS2- and Phi6-spiked samples were assayed using a standard DAL method. Triplicate samples were serially diluted in a buffer, and dilutions were used to infect either *E. col*i (MS2) or *P. syringae* (Phi6), followed by the DAL assay.

Results from each time point were assessed as concentration versus time, and these data were subsequently used to determine the time at which the viral agent was no longer detectable. Decay rates were expressed as D-values: the time required for the reduction of the infectious virus titer by 90 %. The key activities associated with persistence testing included sample preparation, incubation, analysis, and data analysis; each is discussed in detail in the following sections.

3.3.1 Sample Preparation

Aliquots of Leachates A, B, and C were dispensed into replicate tubes approximately 24 hours prior to the initiation of persistence testing. Each leachate was mixed well by manually and vigorously swirling a 3.78 L container of leachate and immediately dispensing a 500 mL aliquot into a sterile 1 L flask. Leachate was then continuously mixed on medium-high speed for five minutes, and 4 mL aliquots were dispensed into replicate 5 mL cryotubes. All sample tubes were labeled and stored overnight at 2-8°C.

3.3.1.1 Test Samples

TGEV, MS2 and Phi6 samples were prepared in pre-dispensed leachate samples (Section 3.3.1). Persistence test samples were separately prepared for each virus in triplicate for each leachate and each incubation test condition.

TGEV Samples: TGEV virus was rapidly thawed and spiked into each 4-mL leachate sample at a final concentration of 5×10^4 $TCID_{50}$/mL (80 µL of pooled TGEV p3). Each sample was mixed by swirling and inverting three times. Samples were incubated upright at 12 ± 1°C with no mixing. Samples were incubated without mixing to ensure that all effects on viral infectivity were due to the matrix, and not mechanical stress from periodic mixing.

Bacteriophage Samples: Bacteriophage (MS2 and Phi6) master stocks were thawed and diluted in PBS to generate 1×10^8 PFU/mL working stocks. Each bacteriophage was separately spiked into each 4-mL leachate sample at a final concentration of 1×10^6 PFU/mL (40 µL of the working stock). Samples were incubated upright as described in Section 3.3.2. Three test samples per virus were assessed per persistence time point and incubation temperature.

3.3.1.2 Negative Controls

Negative controls were generated from pre-dispensed leachate samples (Section 3.3.1). Leachate samples were removed from refrigerated storage and incubated with the respective test and positive control samples. One negative sample was assessed per persistence time point.

3.3.1.3 Positive Controls

Positive samples were generated in sterile media. TGEV positive samples were generated in sterile incomplete medium (EMEM supplemented with 1 % antibiotic/antimycotic). MS2 and Phi6 samples were prepared in the PBS in the same manner as the test samples (Section 3.3.1.1). TGEV positive samples were generated at the same time and with the same pooled virus as the test samples (Section 3.3.1.1). Each sample was mixed by swirling and inverting three times. These controls were incubated with their respective test and negative control samples.

3.3.1.4 Evaporation Controls

To assess the role of evaporation, pre-dispensed leachate samples (Section 3.3.1) were spiked with 40 µL of incomplete medium (EMEM supplemented with 1 % antibiotic/antimycotic) or 40 µL of PBS and incubated with each set of test samples. Each sample was weighed on an analytical balance to the nearest 0.0001 g at each time point and returned to the incubators.

3.3.2 Incubation

Test samples were statically incubated upright with negative (one per time point) and positive (three per time point) samples and evaporation controls in cryoboxes (Figure 5). Samples were incubated within incubators (Figure 6) set to operate at 10 - 14°C or 35 - 39°C with desired set points of 12°C and 37°C, respectively. Incubator temperatures were monitored throughout the incubation period using calibrated thermometers or via a calibrated electronic temperature monitoring system.

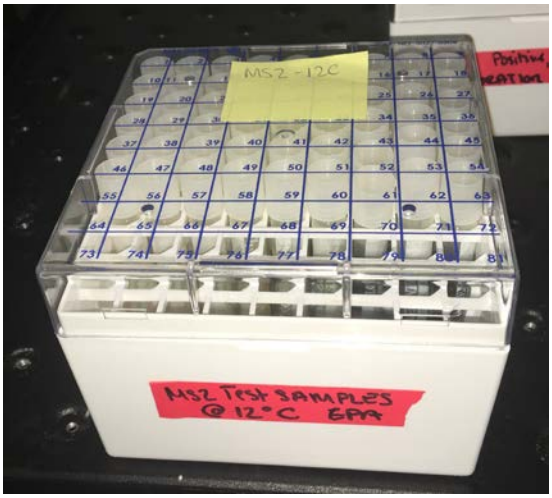


Figure 5. Persistence samples in 5-mL cryovials placed within cryobox.

Figure 6. Sample incubators.

3.3.3 Sample Analysis

Persistence testing on all three landfill leachates was simultaneously conducted . Initial persistence testing evaluated TGEV persistence at 12°C, and MS2 and Phi6 at 12°C and 37°C. Initial tests successfully evaluated TGEV, MS2 and Phi6 at 12°C; however, the decay rate at 37°C was too rapid to be captured within the initial tested timeframe (3 days). In fact, no MS2 or Phi6 virus was detected after T_0. A second MS2 and Phi6 persistence test was performed at 37°C using a shorter series of time points to capture the linear decay rate (Table 3); however, TGEV persistence was not measured at 37°C, as its survival at 12°C suggested it would not survive for more than several hours at 37°C.

Table 3. Sample Analysis Time Points

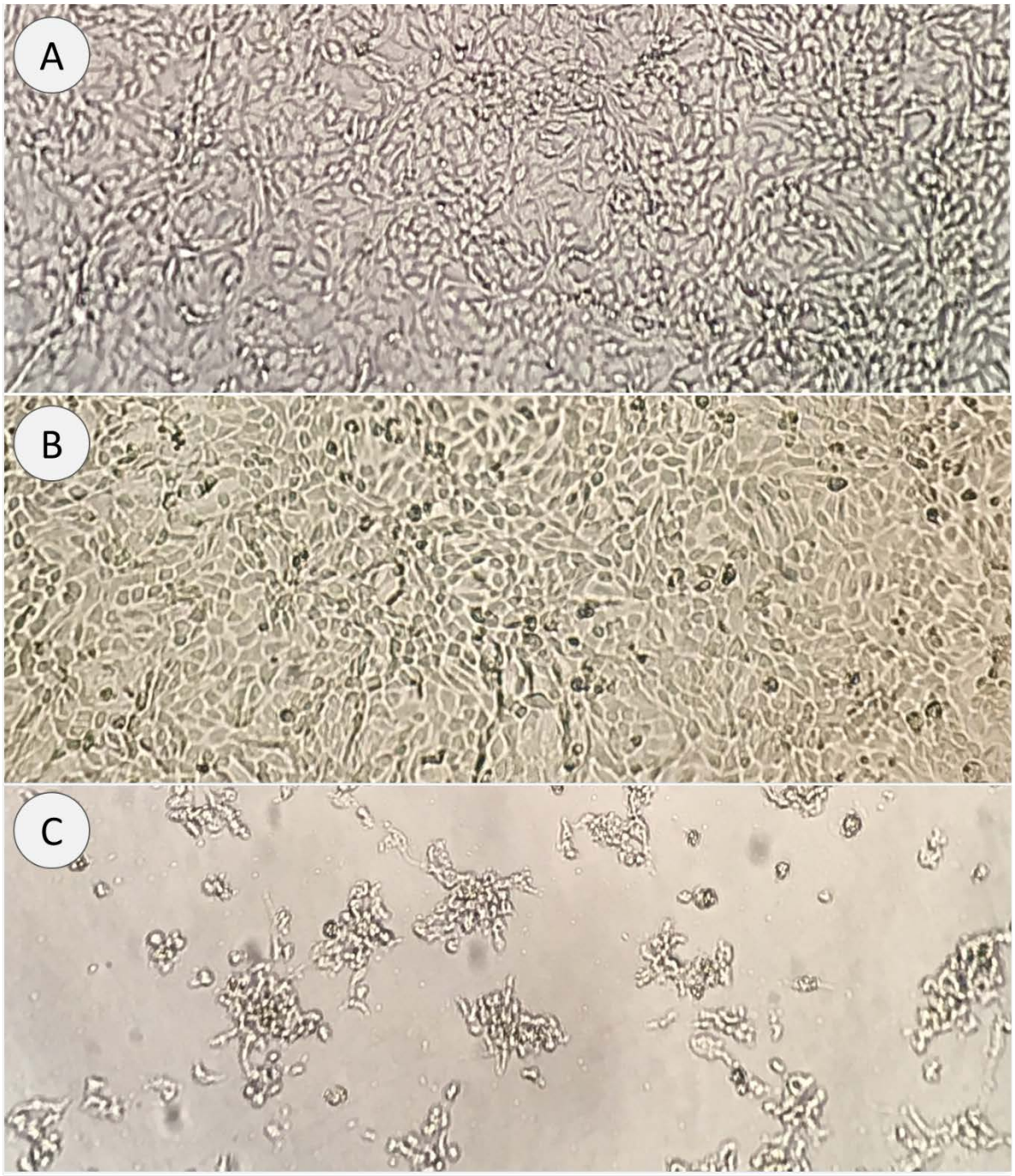
Viral Agent	Temperature	Sample Analysis Time Points
TGEV	12°C	0, 3, 7, 10, 14, 21, 28, 42 days
MS2determined fo	12°C	0, 3, 7, 14, 21, 35, 56 days
	37°C	0, 3, and 7 days
	37°C (2nd test)	0, 6, 12, 24, 34 hours
Phi6	12°C	0, 3, 7, 14, 21, 35, 42, 56 days
	37°C	0, 3, and 7 days
	37°C (2nd test)	0, 6, 12, 24, 30 hours

The baseline (starting concentration) of each analysis set was determined by immediately analyzing one set of samples (the T_0 set). Per leachate type (A, B, and C), three test samples, three positive samples and one negative sample were analyzed within two hours of dosing using the MOPs generated in method development. All remaining test, positive control, and negative control samples were incubated and analyzed over time (Table 3). At each test point, evaporation samples were weighed to the nearest 0.0001 g to assess evaporation during testing.

3.3.3.1 TGEV Sample Analysis

Per leachate, triplicate test samples and single negative control samples, along with triplicate positive control samples, were analyzed using a $TCID_{50}$ assay. The TGEV $TCID_{50}$ assay was adapted from a standard mouse hepatitis virus (MHV) $TCID_{50}$ assay. All samples were serially diluted using a twofold and/or tenfold dilution in complete medium (EMEM, supplemented with 10% FBS and 1% antibiotic/antimycotic). Each dilution was added to a healthy confluent monolayer of ST-cells across one row (12 wells) of a 96-well plate at 0.1 mL diluted sample/well. The lowest dilution able to be used without showing leachate-induced cytotoxicity was 10^{-1}. The calculated assay limit of detection was determined for both natural logarithm (ln) and base-10 logarithm (log) to be: 230 $TCID_{50}$/mL (2.3 log ($TCID_{50}$) or 5.4 ln ($TCID_{50}$)). The 96-well plates were incubated for two days (37°C, 5% CO_2) and manually scored by experienced virologists for CPE using a phase-contract microscope. All bacterial or fungal contaminations were noted, and those wells were not included in titer calculations. TGEV titer (concentration) calculated via $TCID_{50}$ reflects the concentration of virus in a sample in which 50 % of the sample wells were infected. Positive wells were scored and documented for all dilutions plated, and viral titer was

determined as $TCID_{50}$ using the Reed-Muench method [3] in Excel 2013. Examples of viral CPE are shown in Figure 7, and $TCID_{50}$ calculations are shown in Figure 8.



ST-cell morphology and TGEV infection CPE. (A) Control ST-cells at 100 % confluence, (B) ST-cells infected with low levels of TGEV and (C) ST-cells demonstrating CPE due to TGEV infection.

Figure 7. TGEV CPE on ST Cells

1) Calculate the percentage of wells infected for each dilution.

For example, if 4 of 12 wells are scored infected

$$\frac{4\ infected\ wells}{12\ total\ wells} * 100\ \% = 33.3\ \%\ wells\ infected$$

If there is contamination, calculate the total of infected wells without those wells. *For example, if there are four infected wells and one with fungal contamination.*

$$\frac{4\ infected\ wells}{11\ total\ wells\ counted} * 100\ \% = 36.4\ \%\ wells\ infected$$

2) Calculate the proportional distance (PD).

i. Choose two dilutions

The dilution that has just above 50 % of wells infected: _____ (a)

The next highest dilution (more dilute): _____ (b)

ii. Calculate proportional distance.

$$PD = \frac{(\%\ of\ wells\ infected\ at\ (a)) - 50\ \%}{(\%\ of\ wells\ infected\ at\ (a)) - (\%\ of\ wells\ infected\ at\ (b))}$$

iii. Calculate $TCID_{50}$.

$$Log(TCID50) = \log(b) + \log(dilution\ series) * PD$$

- *(b) refers to (b) identified in step 2i.*
- *PD is the proportional distance calculated in step 2ii.*
- *Dilution series. This will determine the correction factor to accurately calculate titer. Use 10 for a tenfold series and 2 for a two-fold series. The correction factor for a ten-fold dilution series is 1.0, for a two-fold dilution series is 0.3.*

Figure 8. $TCID_{50}$ Titer Calculation adapted from [3]

3.3.3.2 MS2 and Phi6 Sample Analysis

Per leachate, triplicate test samples and single negative control samples, along with triplicate positive control samples, were analyzed using the DAL assay. Samples were each vortexed at moderate speed for 30 seconds and serially diluted using a tenfold dilution series in PBS no further than the 10^{-5} dilution. MS2 samples were diluted neat, and Phi6 samples were vortexed briefly and centrifuged at 12,000 rcf for two min prior to dilution. Appropriate serial dilutions were selected based on initial viral titer and previously-analyzed time points to select dilutions that would most likely provide plaque counts within the desired countable range of 25-250 plaques per

plate. In general, positive control samples were plated with $10^{-3, -4, -5}$ dilutions, and negative controls were plated without dilution.

Per dilution, triplicate suspensions of the following mixture were prepared in individual 50 mL conical tubes, as follows:

1) MS2 analysis: 0.1 mL of a log-phase *E. coli* culture (approximately three-six hours old) grown in LBB, 0.1 mL of sample (undiluted or diluted), and 5 mL of molten (50°C) LBTA supplemented with 15 mg/mL streptomycin and 15 mg/mL ampicillin (LBTA+S+A).
2) Phi6 analysis: triplicate suspensions of the following mixture were prepared in individual 50 mL conical tubes: 0.1 mL of an overnight *P. syringae* culture grown in TSB-Mg, 0.1 mL of sample (undiluted or diluted), and 5 mL of molten TSTA supplemented with 20 mg/mL ampicillin (TSTA+A20).

Conical tubes containing virus, agar and bacteria mixtures were promptly swirled and overlaid onto 10-cm Petri dishes. MS2/*E. coli* was overlaid onto LBA-S+A, and Phi6/*P. syringae* was overlaid onto Tryptic Soy Agar (TSA) supplemented with magnesium and 20 mg/mL ampicillin (TSA-Mg+A20). Plates were incubated overnight (MS2 at 37±2°C; Phi6 at 25±2°C). Viral titers were determined using the standard PFU calculation (see below). Plates having 0-250 plaques were counted and recorded. Plaque counts were used to calculate decay rates and persistence values as described later in this section. The appearance of typical MS2 plaques are shown in Figure 9, and typical Phi6 plaques are shown in Figure 10.

Calculation of viral titer:

Plates having 25-250 plaques were used to calculate the viral titer in PFU/mL. The PFU/mL was calculated by multiplying the mean PFU/plate by the dilution factor. Total PFU recovered per sample were calculated by multiplying the PFU/mL recovered by the total sample volume (4 mL). These values were converted to log PFU and natural log (ln) PFU and plotted versus time. Log graphs are shown in Section 5.0 and represent virus persistence in each leachate and positive control matrix. Graphs of ln PFU versus time were used to calculate decay rates. Viral persistence was calculated to be the time at which the linear decay rate intersects the assay theoretical limit of

detection (LOD). The calculated LOD for the DAL assay is 40 PFU, equivalent to 1.6 log PFU or 3.7 ln PFU. This value was calculated using a detection limit of 1 PFU per 0.1 mL plated, equivalent to 10 PFU/mL and 40 PFU/sample in a 4-mL leachate test sample. Decay rate and persistence calculations are detailed in Section 3.3.4.

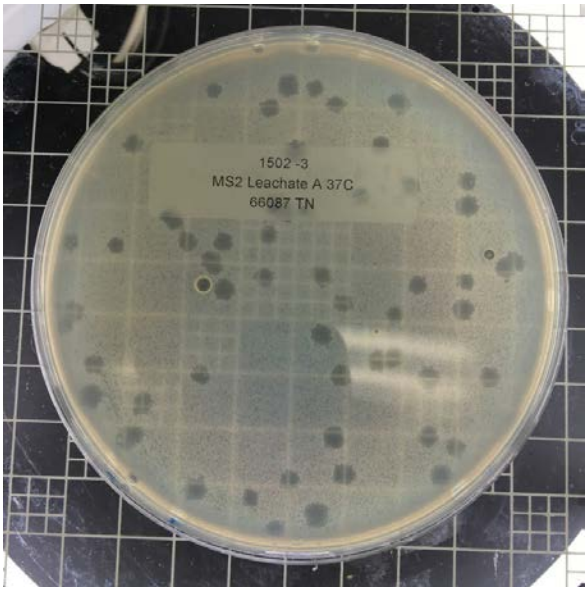


Figure 9. MS2 plaques on *E. coli*.

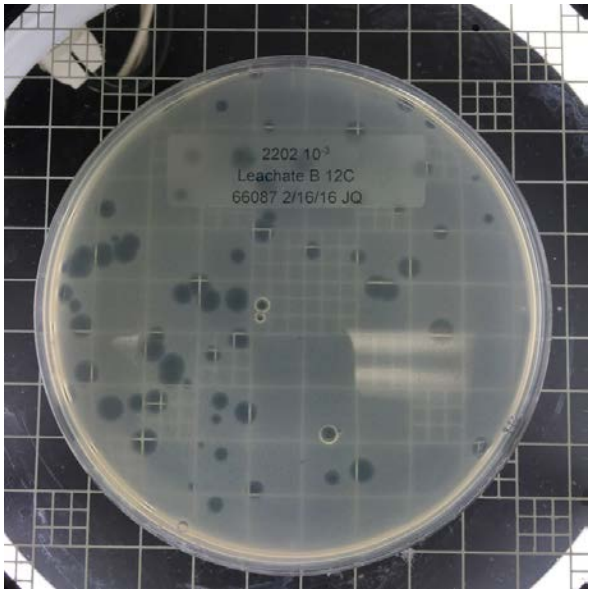


Figure 10. Phi6 plaques on *P. syringae*.

3.3.4 Data Analysis and Interpretation

Viral decay rate was determined by measuring the decrease of infectivity and was measured using a first order decay equation and calculating decimal reduction times (D-values, the time required for the viable concentration to be reduced to 10 % of the starting concentration). These decay kinetics are commonly used to measure biological agent decay, making this a practical and appropriate approach to measure viral persistence. D-values were calculated using persistence data plotted as ln PFU recovered (for MS2 and Phi6) or ln $TCID_{50}$/mL (for TGEV) versus time. A linear decay curve was fitted to data points that included data points in which viral recovery was detected in at least two or three replicates, including the initial T_0 time point.

A linear regression of the data was generated using the following formula:

$$y = mx + b$$

where:

y = concentration (ln PFU recovered for MS2 and Phi6, or ln $TCID_{50}$/mL for TGEV)

m = slope

x = time (days)

b = y-intercept

The slope (m) from the linear regression was used to calculate D-values using the following formula:

$$D - value = \frac{1}{m}X - 1$$

where:

m= slope

Viral persistence time (x) was calculated as the time (in days) required for the rate of linear decay to intercept the assay LOD using the following formula:

$$x = \frac{(y - b)}{m}$$

where

y = ln (assay LOD)

b = y-intercept of linear decay rate

m = slope of linear decay rate

in which Y equals:

5.4 ln per mL for TGEV (eq. to 230 $TCID_{50}$/mL)

3.7 ln (eq. to 40 PFU) for MS2 and Phi6

Persistence (log (viral titer) versus time) and decay curves (linear regression plots used to calculate D-value and persistence) are shown and discussed in Section 5.0.

4.0 Quality Assurance/Quality Control

QA/QC procedures were performed in accordance with the previously approved quality documents for this evaluation.

4.1 Performance Evaluation Audit

A Performance Evaluation (PE) audit was inadvertently not performed for temperature, volume, and PFU calculation measurements in project documentation. However, assurance that these measurements were accurate throughout this study can be demonstrated based on the following:

- All analysts were technically competent, several with many years of experience with these assays. All equipment used was calibrated.
- Calibrated micropipettes were used to perform all sample enumerations as documented in the daily worksheets used to perform the study. All micropipettes were used within their calibration due date.
- There is sufficient evidence in the raw data that volume, temperature and PFU calculations were accurately recorded. All PFU calculations were verified on the completed worksheets as indicated by the reviewer signatures on these worksheets. The Quality Assurance (QA) representative verified that the raw data and calculations transcribed into the Excel spreadsheets were accurate.

A deviation report was written and included in the study file. The impact was deemed as "minimal" as there was sufficient documented evidence that these measurements were accurate.

4.2 Technical System Audit

The QA Manager performed a technical systems audit on February 23 through 25, 2016, to confirm compliance with both TO and program level quality documents. The audit focused on both virus and bacteriophage sample preparation, plating, and reading of results. Procedures followed requirements in the MOP developed under this task order (see Appendix B).

4.3 Data Quality Audit

At least 10 % of the data acquired during the evaluation were audited. The QA Officer traced the data from the initial acquisition, through reduction and statistical analysis, to final reporting, to

ensure the integrity of the reported results. All calculations performed on the audited data were checked for accuracy. The audit revealed three activity measurement transcription errors that were corrected in the report and data spreadsheets.

4.4 QA/QC Reporting

Each assessment and audit was documented in accordance with the quality system developed for the testing and evaluation program.

5.0 Results

5.1 Landfill Leachate Characterization

The landfill leachate characterization data are summarized in Table 4. Although Leachate A was chemically markedly different from Leachates B and C (Figure 4), analytical data illustrated that all three leachates showed numerous similarities and distinct differences in composition and characteristics. Similarities between all three leachates included pH (varying only between 7.1 to 7.9) and temperature (ranging between 20 to 25°C). Chemical characteristics that did not vary by >1 order of magnitude included iron, magnesium, potassium, sodium, chloride, ammonia concentrations, alkalinity, COD, TDS, TOC, and TSS. Distinct differences (varying by >1 order of magnitude) in one leachate versus the other two included calcium, zinc, nitrate, sulfate concentrations, BOD, and ORP.

Viral persistence data and calculated decay rates in leachate (discussed in Sections 5.2 to 5.4), identified that MS2 and Phi6 decay rates were only slightly different at each temperature. Interestingly, leachate effects on viruses were not consistent between viruses. Decay rates for MS2 (at 12°C and 37°C) and Phi6 (at 37°C) were slowest for Leachate A, followed by Leachate C, and most rapid in Leachate B. However, decay rates of Phi6 at 12°C showed the opposite trend: slowest in Leachate C, followed by Leachate B, and most rapid in Leachate A. The decay rates for TGEV (at 12°C) showed a trend different from both MS2 and Phi6, decaying slowest in Leachate B (four days), followed by Leachate C (two days) and decaying fastest in Leachate A. Results from all tests are summarized in Executive Summary Tables 1 and 2. Unsurprisingly, all viruses persisted significantly longer in control matrices (incomplete EMEM for TGEV, PBS for MS2 and Phi6) than leachate matrices.

Leachate constituents clearly contributed to viral inactivation; however, this study did not assess the contribution of individual constituents of the leachate to viral inactivation. Further persistence testing with viral agents in a much larger sample of different leachates from different landfills, or artificial leachate substitutes varying in constituents, would be required to gain further insight into the root cause(s) of viral inactivation and chemical markers that could indicate the rate of viral inactivation in landfill leachate.

Table 4. Landfill Leachate Characterization Data

Analyte	Leachate A	Leachate B	Leachate C
Metals (mg/L)			
Calcium	11.6	200	312
Iron	6.36	17.4	31.5
Magnesium	130	84.3	297
Manganese	0.0468	0.152	2.26
Potassium	468	260	937
Sodium	1,880	1,500	2,360
Zinc	0.140	0.0199	0.0711
Biological Oxygen Demand (mg/L)			
BOD	187	2,020	2,350
Anions (mg/L)			
Chloride	2,070	1,980	2,810
Nitrate-N	4.00	3.08	<1.00
Sulfate	3.19	10.1	33.0
Total Alkalinity as $CaCO_3$ (mg/L)			
Total Alkalinity	6,100	2,600	8,040
Ammonia as Nitrogen (mg/L)			
Ammonia	1,050	386	1,370
Chemical Oxygen Demand (mg/L)			
COD	1,500	2,470	9,060
pH (Standard Units)			
pH	7.76	7.06	7.55
pH (field)	7.88	7.14	7.36
Oxidation Reduction Potential (millivolts)			
ORP (field)	47.4	-60.7	-96.8
Temperature (ºC)			
Temperature (field)	21.8	25.0	20.0
Total Dissolved Solids (mg/L)			
TDS	6,680	5,980	13,500
Total Organic Carbon (mg/L)			
TOC	448	796	2,960
Total Suspended Solids (mg/L)			
TSS	12.3	82.0	72.0
Visual Observations			
Color	yellow	brown	dark brown
Particulates	not significant	significant	significant

5.2 TGEV Persistence

TGEV persistence was analyzed in all three different leachates (A, B and C) at intervals over 42 days at 12 ± 1°C. Samples were removed from incubation and quantified via $TCID_{50}$ assay at 0, 3, 7, 10, 14, 21, 28 and 42 days post inoculation. Viral titer in $TCID_{50}$ was used to generate decay curves. Viral titer, expressed as mean log ($TCID_{50}$/mL) or ln ($TCID_{50}$/mL) versus time (Figure 11 and Figure 12 respectively), and a linear regression of the data were used to calculate D-values and persistence time (Table 5).

Results indicated that TGEV persisted for 4.6 to 16.6 days at 12°C in leachate (Figure 11). While viral concentrations close to the limit of detection can result in varying results (e.g., concentration reporting at 0 or close to the limit of detection) at low dilutions (e.g., 10^{-1}), viral CPE was distinguishable from leachate-induced toxicity. TGEV was observed to be inactivated fastest in Leachate A (4.6 days), followed by Leachate C (6.7 days), and slowest in Leachate B (16.6 days). Leachates A and C initially showed similar decay rates; however, virus incubated in Leachate C did result in a measurable titer at 14 days after dropping below the limit of detection on day 10. This rebound in infectious virus may have been due to difficulties in separating leachate-induced cytotoxicity from viral CPE. Data from Day 14 for Leachate C were obtained from the 10^{-1} dilution and were only observed in the 2^{-5} and 2^{-6} dilution (1:32 and 1:64 dilutions from neat sample), with results very close to the limit of detection. It is likely the Day 10 data in Leachate C were occluded by leachate cytotoxicity and were at a similar titer.

TGEV also degraded in the control matrix (EMEM supplemented with 1 % antibiotic/ antimycotic), reducing the viral titer to the neat assay detection limit on Day 42 (the final time point). Viral inactivation in the control matrix was expected; however, viral survival greater than one-two days in the leachate was unexpected.

Table 5. TGEV Measured D-values at 12°C

Matrix	Slope	Measured D-Value (days)	Persistence[a] (days)
Leachate A	-1.2003	0.8	4.6
Leachate B	-0.2654	3.8	16.6
Leachate C	-0.4580	2.2	6.7
Incomplete EMEM Medium (Positive Control)	-0.1428	7.0	43.1

[a]Calculated time in days at which measured linear decay rate intersects with assay limit of detection.

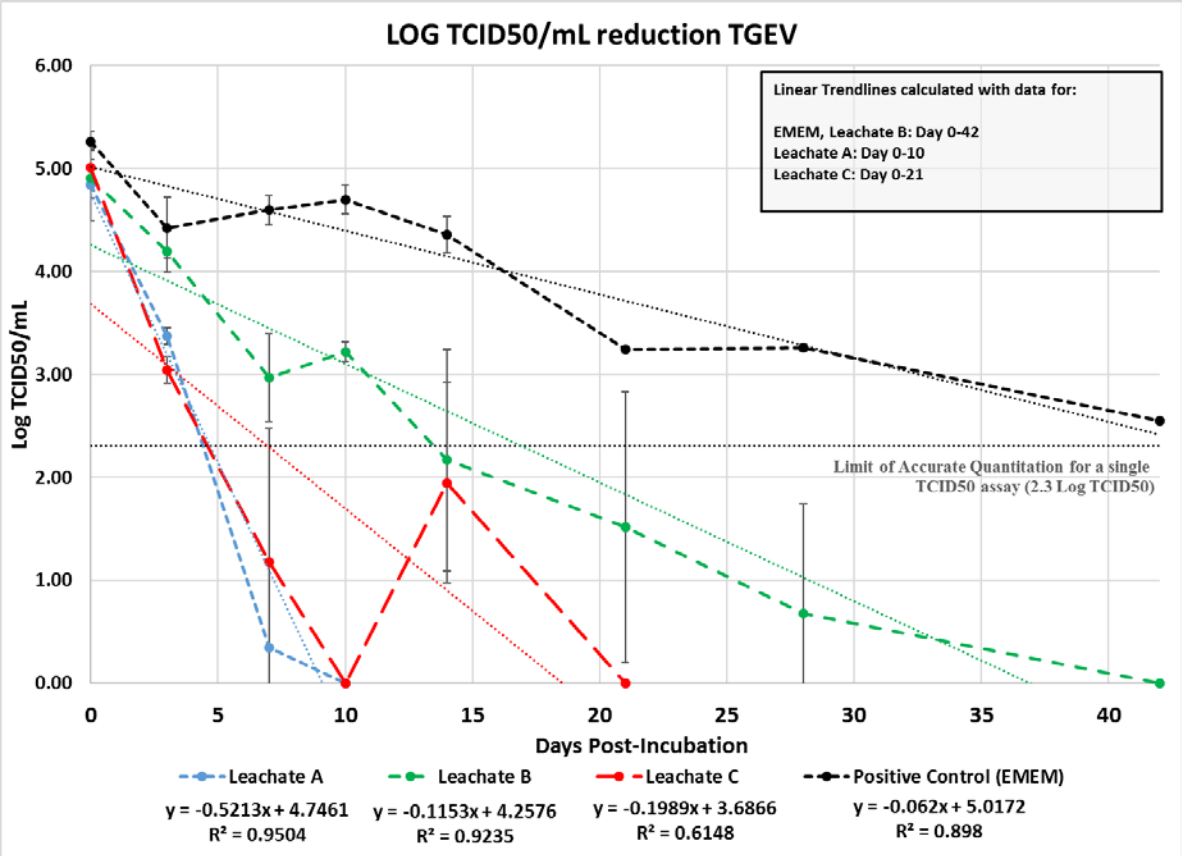


Figure 11. TGEV Persistence at 12°C

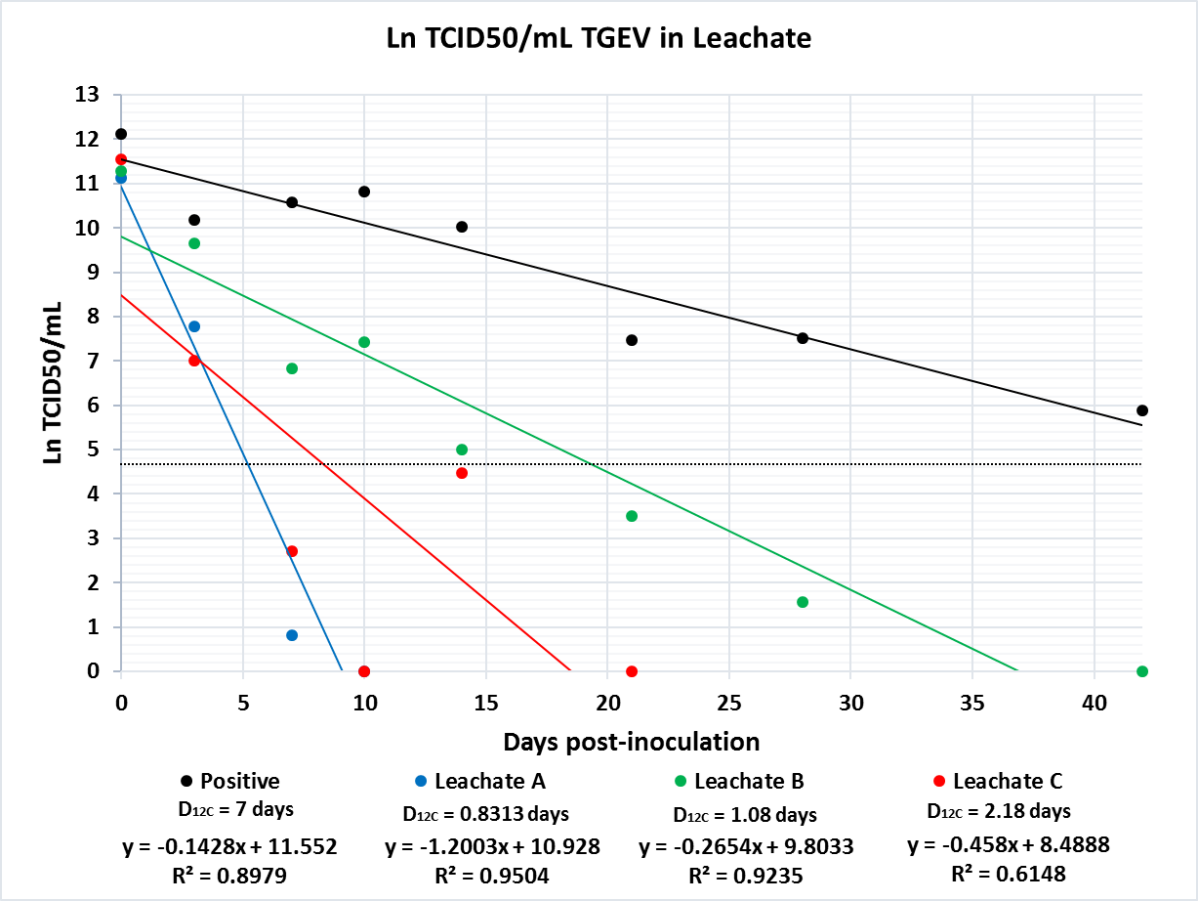


Figure 12. TGEV Decay Curves at 12°C

5.3 MS2 Persistence

MS2 persistence in leachate was expressed as mean log (PFU recovered) versus time at 12°C and 37°C (Figure 13 and Figure 14, respectively). Mean ln (PFU recovered) was calculated and expressed versus time to determine linear decay rates (Figure 15 and Figure 16, respectively). Linear regression of the data and the slope of the decay curves was used to calculate D-values and persistence time (Table 6).

MS2 was deactivated very rapidly in leachate at 37°C, therefore data from the initial MS2 37°C persistence test are not presented, as no virus was recovered after the initial time point (T_0; the first subsequent time point was on Day 3). Data presented for MS2 persistence at 37°C are from the second test performed several months later, using the same landfill leachates (stored refrigerated).

As shown in Figure 13 and Figure 14, respectively, results indicated that MS2 persisted much longer at 12°C (approximately 2.5 to four months) than at 37°C (two to three days). While temperature did affect the decay rate, decay was slowest in Leachate A, followed by Leachate C, and fastest in Leachate B at both 12°C and 37°C. In the control matrix (PBS), MS2 was very stable at both temperatures during test duration (35-52 days) with no discernible decay observed.

Table 6. MS2 D-values and Persistence

Matrix	12°C			37°C		
	Slope	D-Value (days)	Persistence[a] (days)	Slope	D-Value (days)	Persistence[a] (days)
Leachate A	-0.1002	10.0	113	-0.1643	0.3	3
Leachate B	-0.1377	7.3	75	-0.2384	0.2	2
Leachate C	-0.1216	8.2	87	-0.1989	0.2	2
PBS	-0.0053	188.7	NR[b]	0.0147	NR[b]	NR[b]

[a]Calculated time in days at which measured linear decay rate intersects with assay limit of detection.
[b]No decay observed.

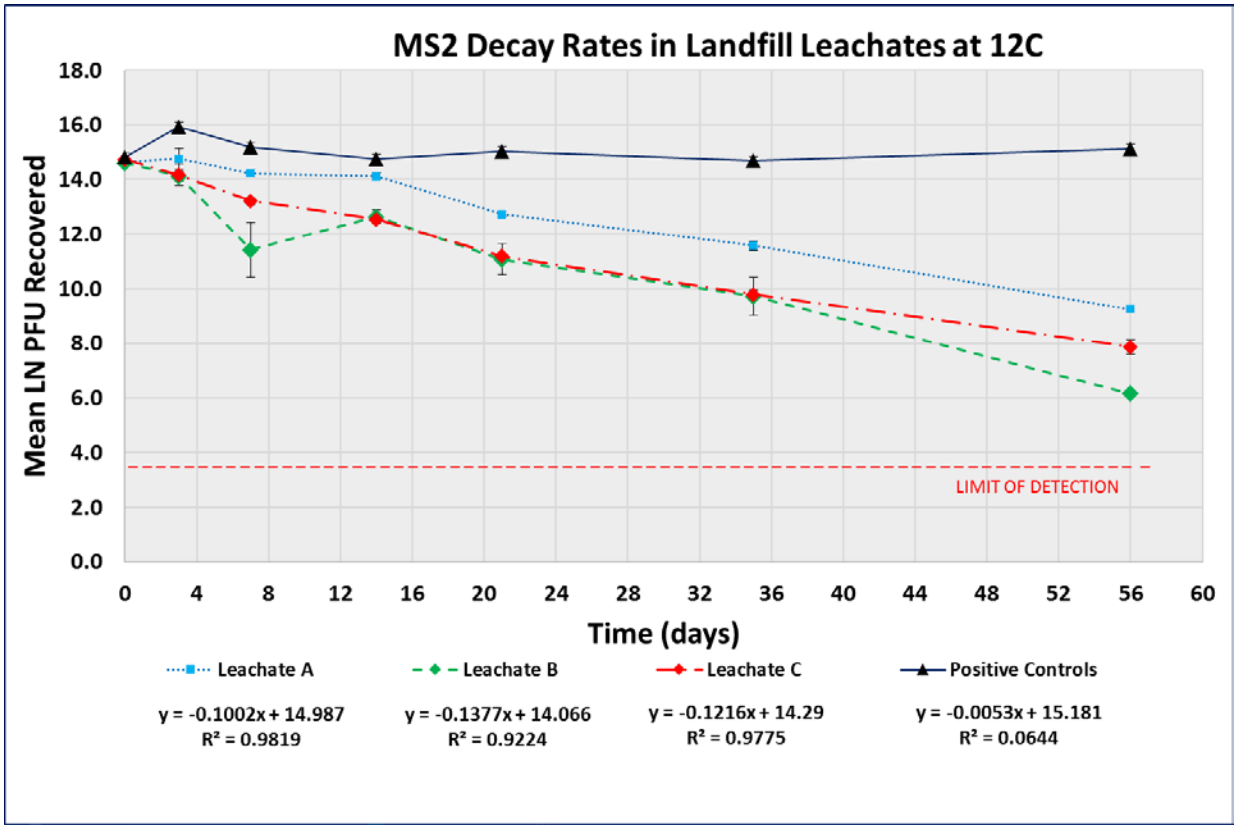


Figure 13. MS2 Persistence at 12°C

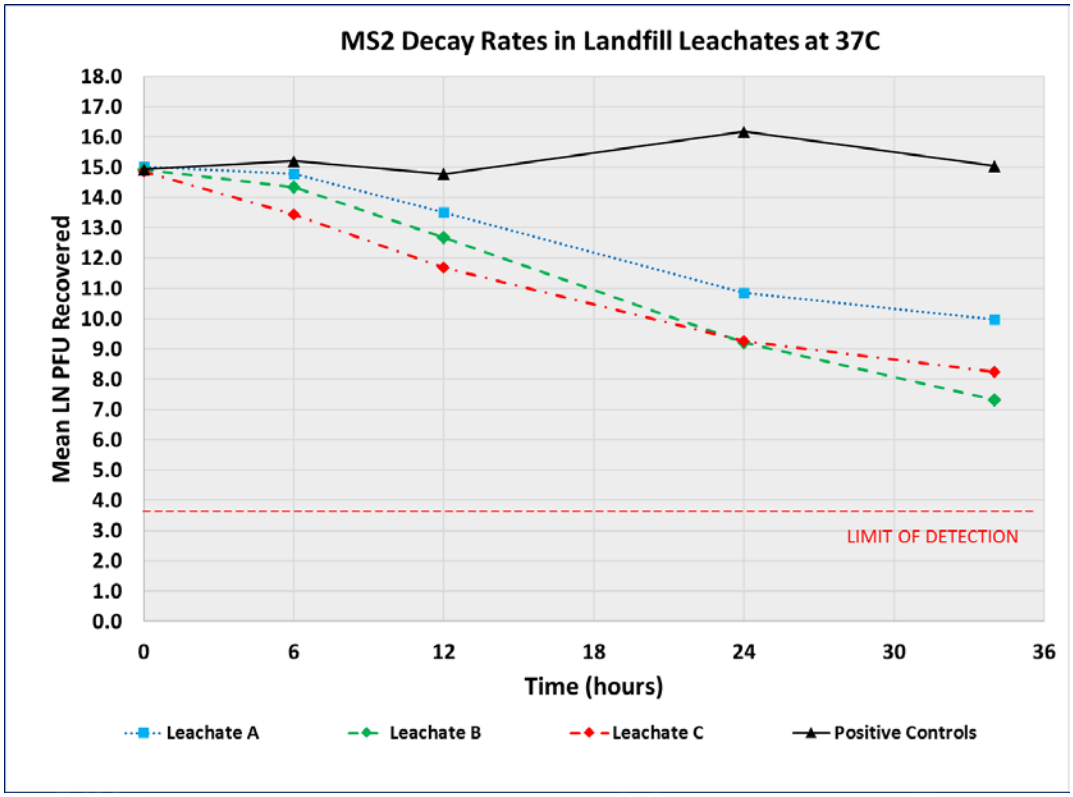


Figure 14. MS2 Persistence at 37°C

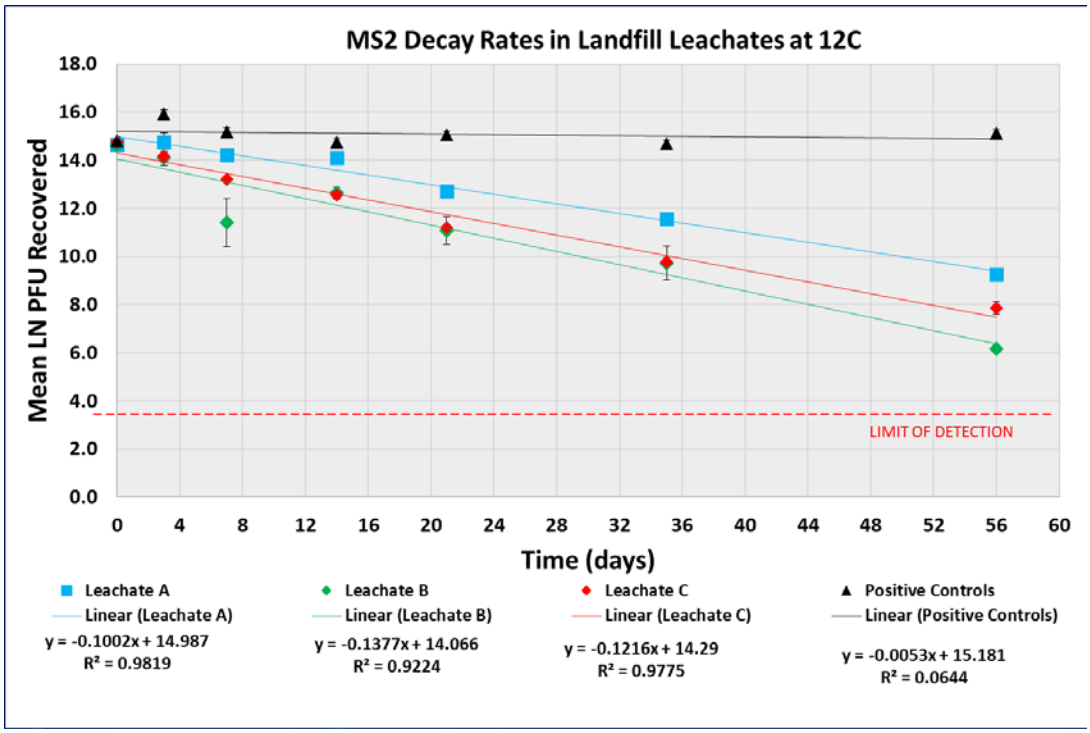


Figure 15. MS2 Decay Rate at 12°C

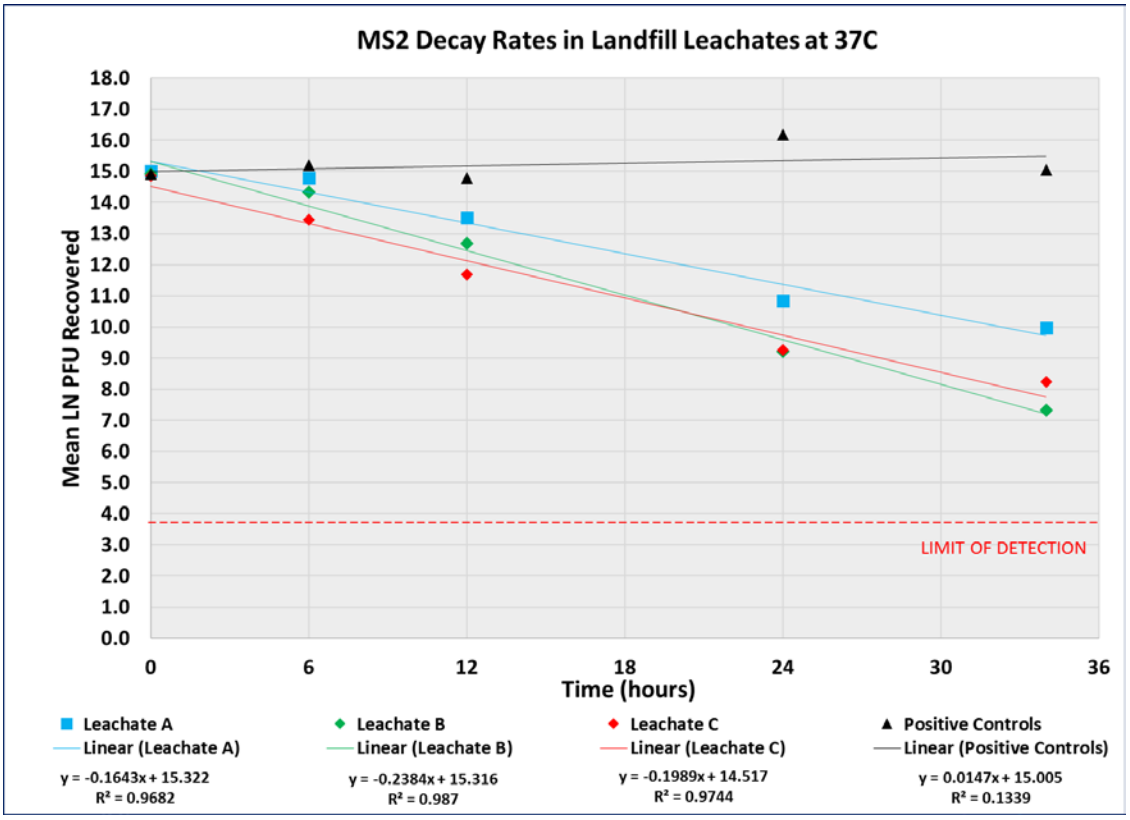


Figure 16. MS2 Decay Rate at 37°C

5.4 Phi6 Persistence

Phi6 persistence in leachate was expressed as mean log (PFU recovered) versus time at 12°C and 37°C (Figure 17 and Figure 18, respectively). Mean ln (PFU recovered) was calculated and expressed versus time in each leachate at 12°C and 37°C to determine linear decay rate (Figure 19 and Figure 20, respectively). Linear regression of the data and the measured slope of the decay curves were used to calculate D-values and persistence (results summarized in Table 7).

Like with the MS2 virus, Phi6 virus was deactivated very rapidly at 37°C, therefore data from the initial Phi6 37°C persistence test are not presented, as no virus was recovered after the initial time point (T_0; the first subsequent time point was on day 3). Data presented for Phi6 persistence at 37°C are from the second test performed several months later using the same landfill leachates (stored refrigerated).

Results demonstrated that Phi6 persisted much longer at 12°C (approximately two to three months) than at 37°C with rapid decay to the assay detection limit within six hours. While temperature also

affected the decay rate, decay was noticeably fastest in Leachate A, with decay in Leachates B and C occurring slower. Unlike MS2, Phi6 did decay in the control matrix (PBS) at both temperatures, significantly more so at 37°C.

Table 7. Phi6 D-Values and Persistence

Matrix	12°C			37°C		
	Slope	Measured D-Value (days)	Persistence[a] (days)	Slope	Measured D-Value (days)	Persistence[a] (days)
Leachate A	-0.1584	6.3	55	-1.5867	0.03	0.3
Leachate B	-0.0981	10.2	66	-1.7500	0.02	0.2
Leachate C	-0.0819	12.2	81	-2.0968	0.02	0.2
PBS	-0.0604	16.6	122	-0.2701	0.15	1.8

[a]Calculated time in days at which measured linear decay rate intersects with assay limit of detection

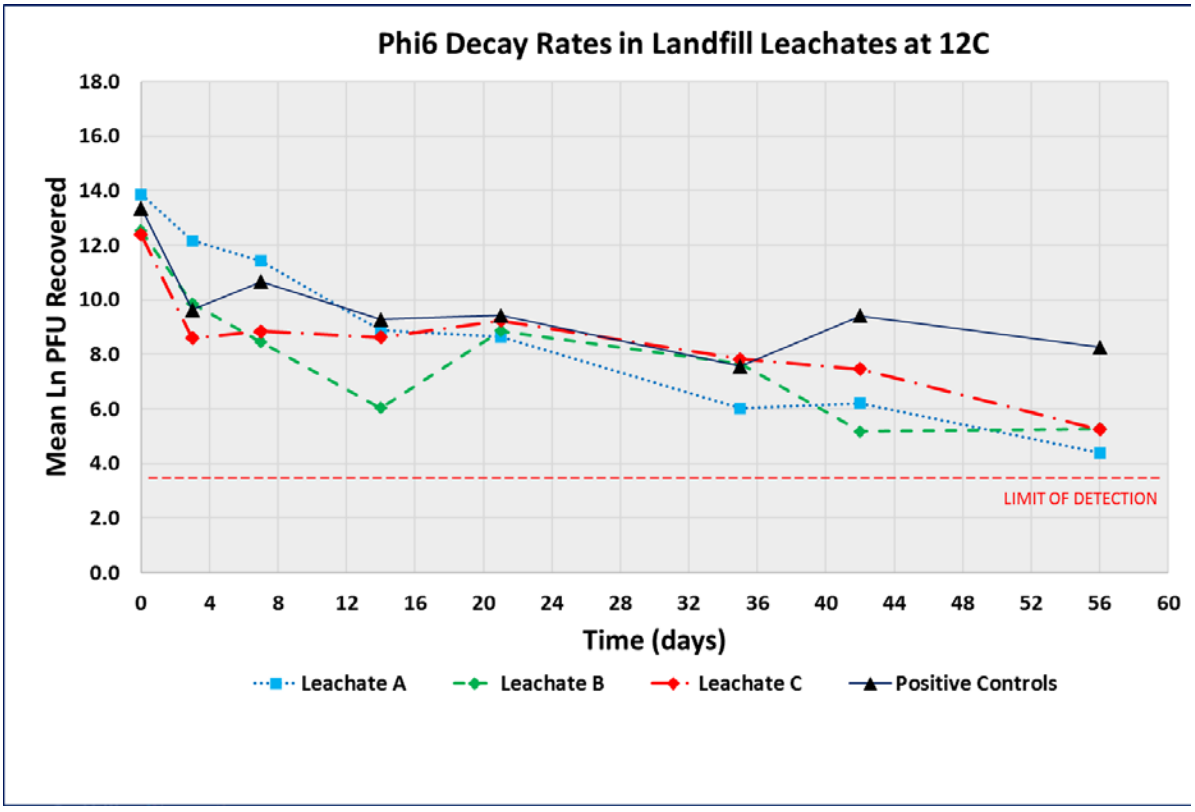


Figure 17. Phi6 Persistence at 12°C

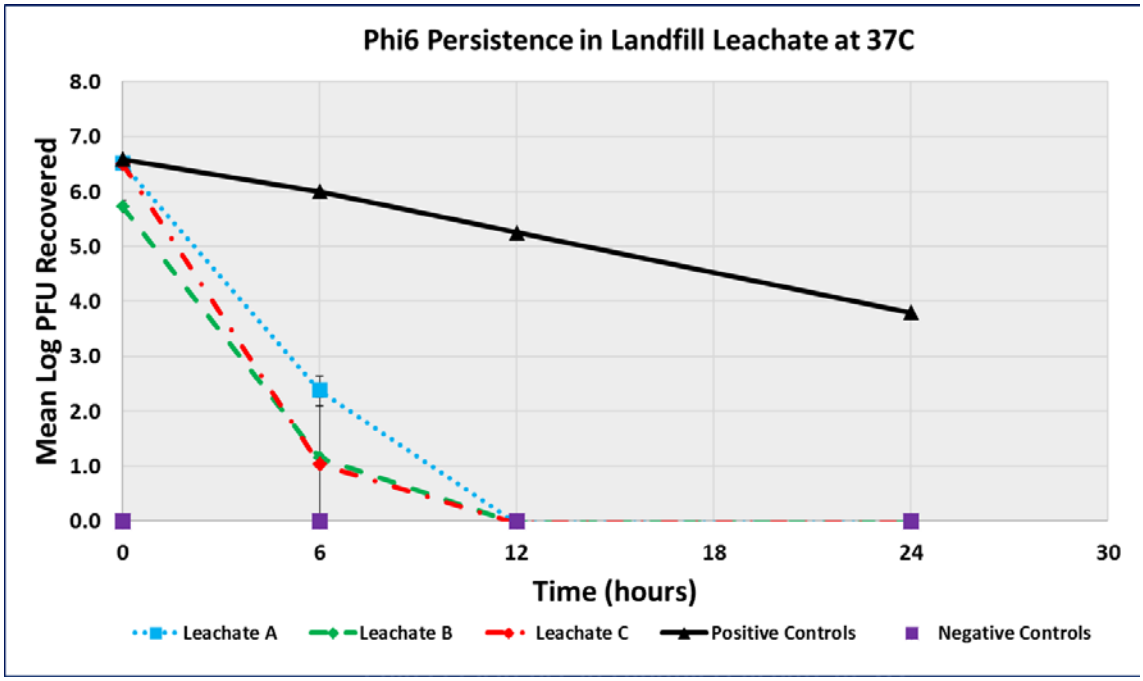


Figure 18. Phi6 Persistence at 37°C

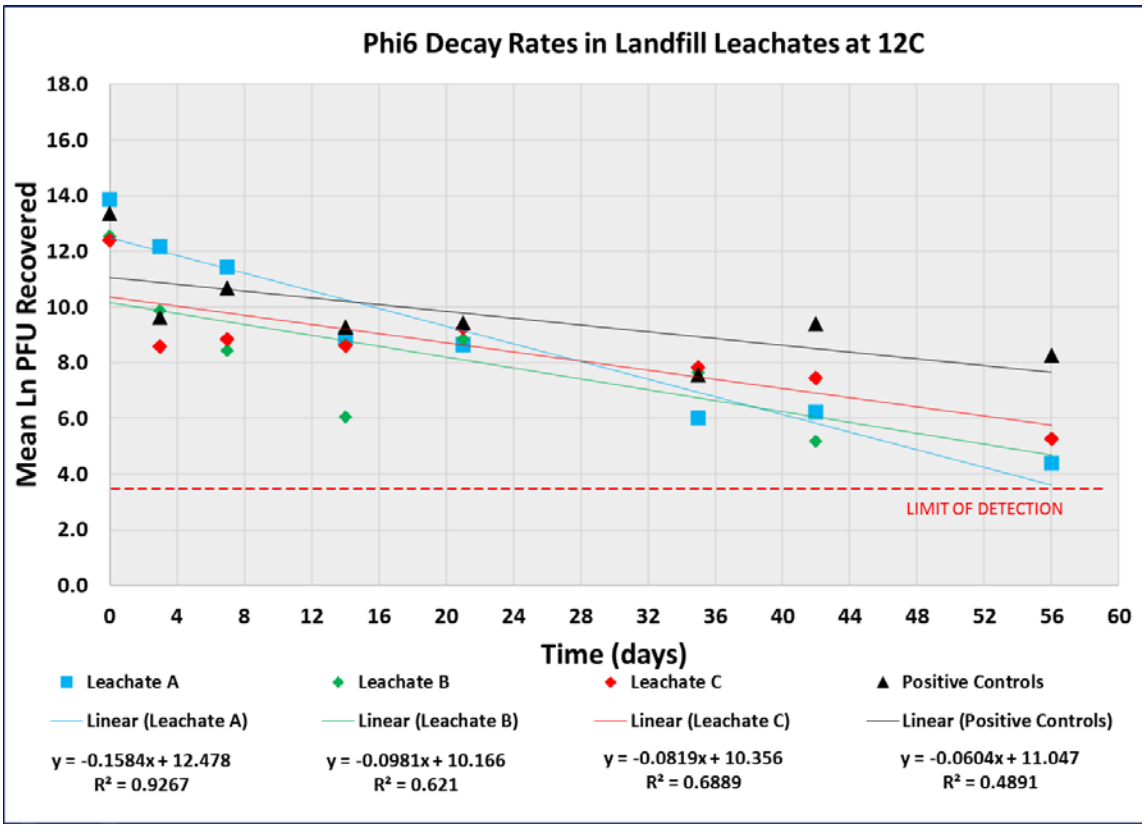


Figure 19. Phi Decay Rates at 12°C

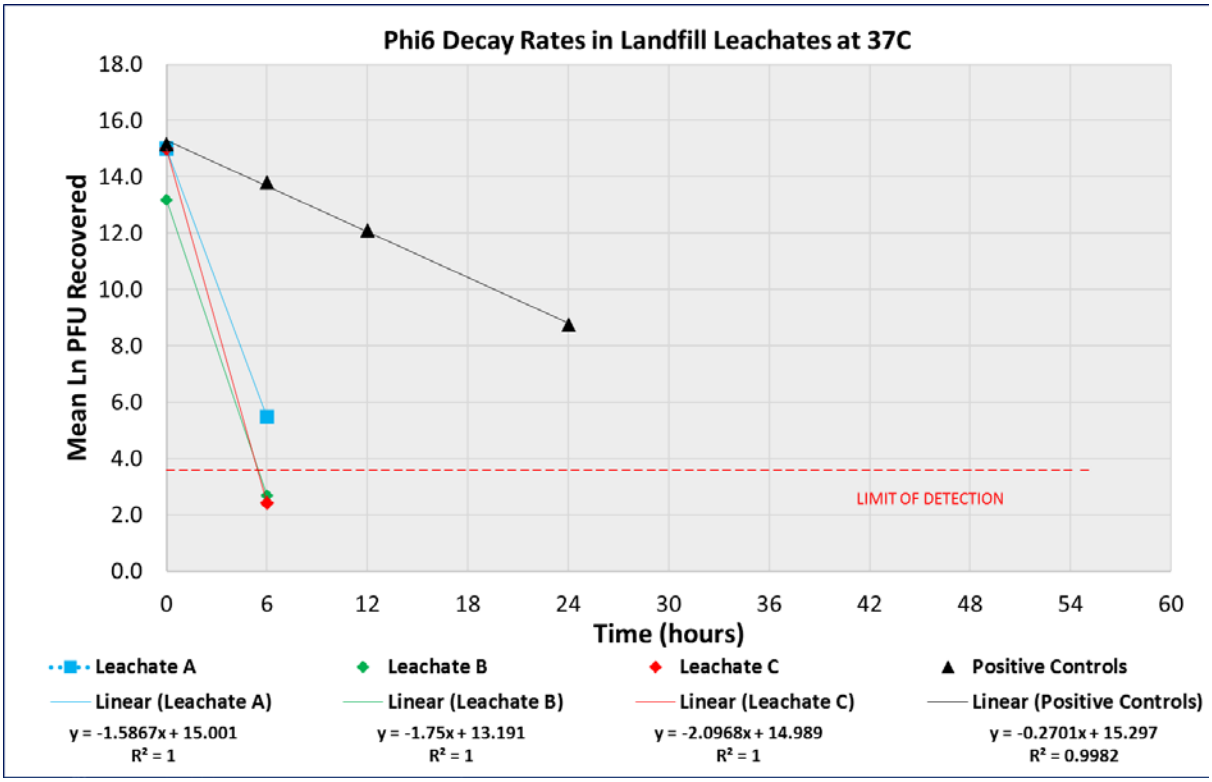


Figure 20. Phi6 Decay Rates at 37°C

5.5 Microbial Activity

Heterotrophic bacterial and fungal concentrations in each leachate were characterized approximately 2½ months apart, once at the onset of each test (Table 8). Results indicated that landfill leachates were biologically active, with Leachate A showing less fungal activity than Leachates B and C. Based on these limited data, leachates generally remain biologically active between the first and second persistence tests, a period of approximately 2½ months. Heterotrophic bacterial concentration remained approximately the same (10^5 to 10^7 CFU/mL) throughout the incubation. Heterotrophic fungal concentration did appear to increase slightly from 10^2-10^4 to 10^5 CFU/mL over the 2½ month incubation.

Fungal and bacterial colony types observed on plates differed between leachates, and over time. Although recovered colonies from heterotrophic plate counts (representative images in Figure 21 and Figure 22) were notes, they were not identified or characterized, so this analysis was purely qualitative and was not intended to measure population or diversity shifts.

Another indication that microbial activity altered between the first and second persistence tests was identified during method development. During initial testing, Leachate B interfered with the Phi6 DAL assay, preventing plaques from forming. However, Phi6 could be recovered from autoclaved Leachate B, suggesting that the assay inhibition was caused by a biological constituent. This issue was resolved by adding a centrifugation step post-spiking, prior to the DAL assay, that allowed a high percentage of Phi6 to be recovered from the supernatant using TSA supplemented with magnesium and 20 ppm ampicillin (Appendix A, Table 3). Interestingly, interference of Leachate B with Phi6 was not evident in a follow-on experiment, suggesting that microbial activity, and/or the population of specific microbes, changed during storage from the time the leachate was collected.

Table 8. Microbial Activity in Landfill Leachates

Test	Start Date	Leachate	pH	CFU/mL	
				Bacteria	Fungi
Initial Test with all three viruses	2/15/16	A	8.02	$3x10^6$ (3-5 colony types)	$3x10^2$ (3 colony types)
		B	7.37	$9x10^5$ (3-4 colony types)	$8x10^4$ (10-20 colony types)
		C	7.73	$8x10^5$ (8-10 colony types)	$9x10^3$ (10-20 colony types)
MS2 2nd Iteration at 37°C	4/27/16	A	ND	$>3x10^7$ [a] (~5 colony types)	$6x10^5$ (3 colony types)
		B	ND	$1x10^6$ (4 colony types)	$6x10^5$ (5-10 colony types)
		C	ND	$5x10^6$ (~5 colony types)	$3x10^3$ (~10 colony types)

ND= not determined (note: pH of the leachates was measured in July, 2016, and did not shown discernible changes, having pH readings of 8.09, 7.36, and 7.55 for Leachates A, B, and C, respectively.)
a: Colonies on all plates of all dilutions returned were too numerous to count. As the maximum number of countable colonies per plate is 300, the CFU/mL of the sample is greater than this quantity.

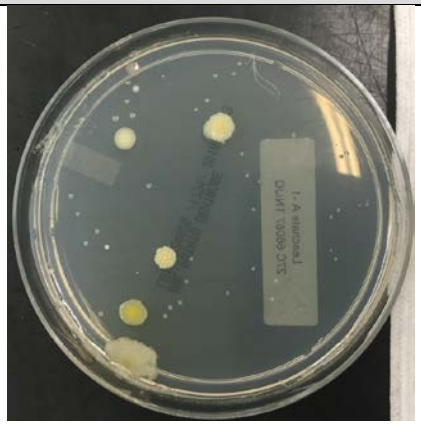
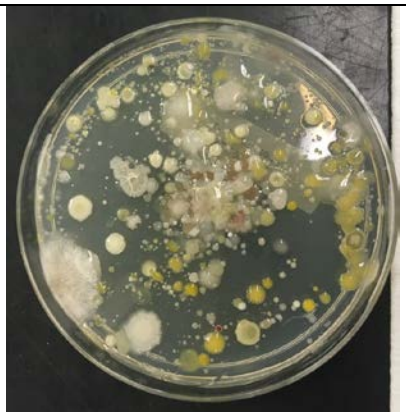
Leachate	Colonies on Tryptic Soy Agar (TSA)	Colonies on Potato Dextrose Agar (PDA)
A		
B		
C		

Figure 21. Bacterial and Fungal Growth on TSA and PDA growth media (initial analysis on 2/15/2016).

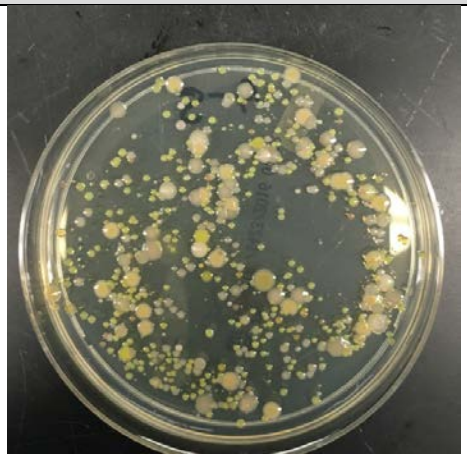
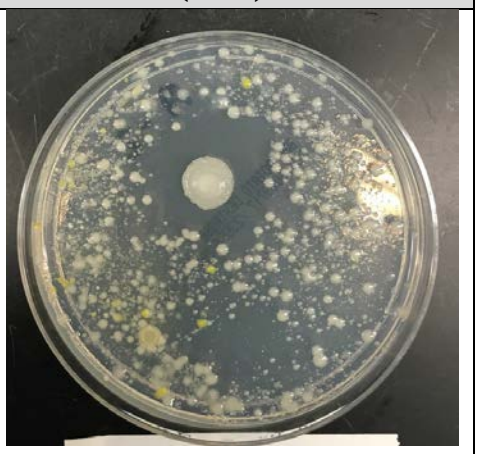
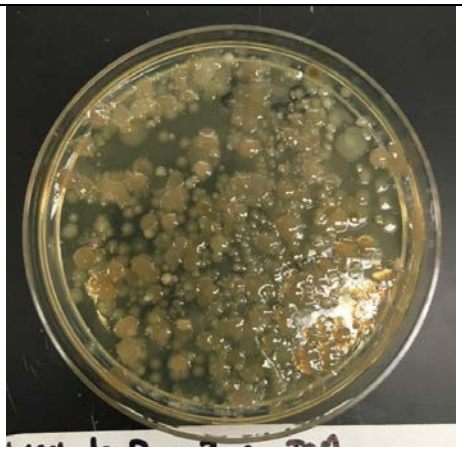
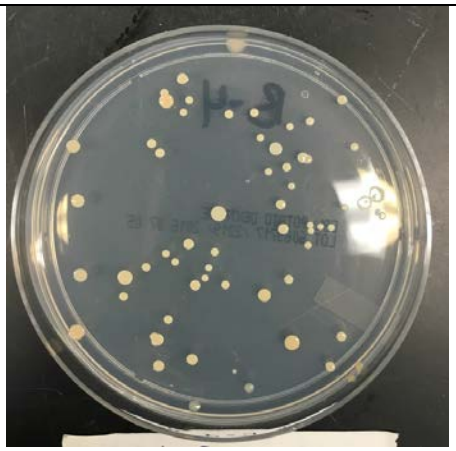
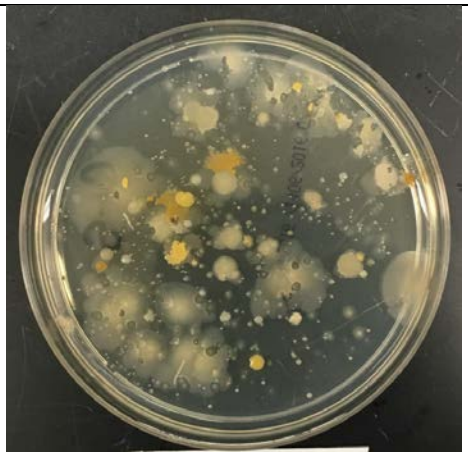
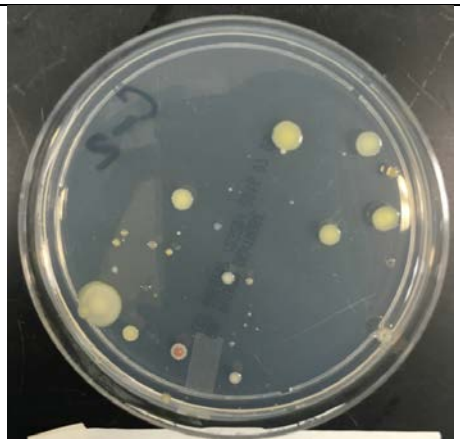
Leachate	Colonies on Tryptic Soy Agar (TSA)	Colonies on Potato Dextrose Agar (PDA)
A		
B		
C		

Figure 22. Bacterial and Fungal Growth on TSA and PDA growth media (second analysis on 4/27/2016).

5.6 Evaporation

The weights of “evaporation control” samples were measured at each time point and showed no change, demonstrating that evaporation of the leachate within the vials did not occur. Therefore, observed changes in virus concentration over time could not be attributed to evaporation as the total volume in the sample tubes had not changed.

6.0 Discussion

Viral persistence varied by agent and temperature. At 12°C, MS2 persisted the longest in the leachate (three-four months), Phi6 persisted for a slightly shorter period (two-three months) and TGEV persisted the shortest period (5-16 days). At 37°C, MS2 survived only a few days, and Phi6 decayed very rapidly, within the first six hours. TGEV persistence was not measured at 37°C, as its survival at 12°C suggested it would not survive for more than several hours at 37°C.

Variation in agent survival time was expected as each virus has a distinctive structure. MS2 and Phi6 are bacteriophages (viruses that infect bacteria). MS2 is a non-enveloped single-stranded RNA virus with a very small 3.5 kilobase genome, while Phi6 is an enveloped double-stranded RNA virus with a 13.5 kilobase genome. TGEV is an enveloped single-stranded positive sense RNA mammalian virus (a *Coronavirus*) with a 28.6 kilobase RNA genome. Non-enveloped viruses are generally more environmentally stable than enveloped viruses. Enveloped viruses are thought to be less stable due in part to the outer lipid layer, which is susceptible to dehydration and disruption by a variety of environmental and chemical factors (e.g., pH, humidity or water activity, heavy metals). Data from this study were in accordance with this general trend, showing the most stable virus (MS2) to be the non-enveloped virus, while the enveloped viruses (TGEV and Phi6) were less stable. In addition, Phi6 was substantially more stable than TGEV, even though both are enveloped viruses, suggesting that viral characteristics beyond basic structure can influence viral stability.

Interestingly, leachate effects on viruses were not consistent between agents: for example, TGEV persisted the longest in Leachate B, MS2 persisted the longest in Leachate A, and Phi6 persisted the longest in Leachate C (see Executive Summary Tables 1 and 2), although both enveloped viruses were most affected by Leachate A. This observation suggests that each class of virus is affected by leachate constituents in unique ways, and that non-enveloped and enveloped viruses may maintain stability in different types of leachate. While pH can inactivate viruses, primarily by altering surface protein structure and/or the lipid envelope, all three leachates displayed pH readings similar to environments that do not affect viral infectivity (within physiological levels

[pH 7.1-7.9]). The data suggests that viral agents were affected primarily by the varying chemical constituents in the leachate.

Statistical analysis was attempted to try to distinguish any trends with respect to the chemical analysis of the leachate samples and the respective persistence of viruses in those leachate, but no statistically significant trends were identified.

Differences in decay rates between leachates was not surprising, as each landfill used in this study varied in intake and management, so each leachate showed its own biological and chemical fingerprint, sometimes substantially varying in constituent concentration. In fact, Leachate B interfered with the Phi6 plaque assay but not the MS2 plaque assay, and Leachate C interfered with the TGEV $TCID_{50}$ assay but not the MS2 or Phi6 plaque assays.

7.0 Conclusions

This controlled laboratory study evaluated the persistence of three uniquely different viral agents: one enveloped single-stranded RNA virus (*Coronavirus*, TGEV), one enveloped bacteriophage (Phi6), and one non-enveloped bacteriophage (MS2) in three unique landfill leachates. Viral inactivation in each landfill leachate was measured at 12°C, and bacteriophage inactivation was also evaluated at 37°C. These data were used to establish decay rates and estimate viral persistence in leachate (persistence: time at which a viral agent reached the assay limit of detection). This study showed that, in general, viral agents can persist for weeks or months in landfill leachate if the leachate remains at a mild temperature, and that viral decay rates increase rapidly as leachate warms. These results also suggest that the chemical characteristics of a specific landfill can affect viral decay rates although we were not able to statistically identify any relevant trends with respect to chemical composition of leachate and the persistence of viruses.

This study showed that live infectious viral agents can persist for days, weeks, even months in the landfill leachate under certain environmental conditions.

Key findings and observations from this study are the following:

- Some viral biological agents likely can persist in landfill leachate for several months; especially at lower temperatures.
- Moderately elevated temperatures, such as 37°C (99°F), can drastically reduce viral persistence and infectivity and can decrease D-values to < 1 day.
- Leachate composition likely dramatically effect viral decay rates and persistence. This study did not identify how leachate composition affects viral inactivation, however differences in viral persistence between leachates is likely due to chemical constituents and concentrations. As this study investigated leachates from only three landfills, it is unknown how these data correspond to leachate from other landfills across the U.S. Further persistence analysis of these viral agents in a much larger number of landfill leachates would be needed to gain insight into the key characteristics that effect viral decay rates, and generate actionable data for use in waste management.

- Non-enveloped viruses were found to persist longer than enveloped viruses. This result was expected based on the persistence of both of these viral types in other environments.

8.0 References

1. Thornelow, S.A., et al., *EPA's Suite of Homeland Security Decision Support Tools For Managing Disaster-Generated Waste and Debris,* in *Proceedings Global Waste Management Symposium, Promoting Technology and Scientific Innovation*2008: Copper Mountain Conference Center, Colorado USA.
2. (ATCC), A.T.C.C. *Transmissible gastroenteritis virus (ATCC VR-763)*. 2016 [cited 2016 10/05/2016]; ATCC Catalogue].
3. Reed, L.J. and H. Muench, *A Simple Method of Estimating Fifty Per Cent Endpoints.* American Journal of Epidemiology, 1938. **27**(3): p. 493-497.

Appendix A:

Method Development Summary Report

Landfill Leachate Virus Persistence Study under Contract No. EP-C-15-002

Task 3 (Method Development) Summary

Described below is summary of the key results of laboratory activities performed for developing recovery assays of TGEV, MS2, and Phi6 bacteriophage from three landfill leachates designated A, B, and C. Based on these results, the recommended persistence study design and methods is summarized in Table 6.

TGEV

Assay Inhibition/Interference

Prior to testing the recovery of TGEV from leachate, a cytotoxicity assay was performed to assess if there was a toxic effect on the ST-cells due to the unique matrix of Leachate A, B and C. Additionally, a comparison of autoclaved versus untreated leachates allowed for a screening for virus within the leachate matrix which could potentially interfere with the assay. Three treatments of leachate (untreated, autoclaved and EDTA at 0.1M) were inoculated onto a monolayer ST-cells at six dilutions (neat, 1:5, 1:10, 1:20, 1:50 and 1:100). Cells were observed at 18, 24, 48, 72 and 96 hours post inoculation for cytopathic effect (CPE).

No evidence of mammalian virus (viral-induced CPE) was observed on ST-1 cells in untreated leachate matrix A, B or C. No evidence of cytotoxcity was observed on ST-1 cells when cellular monolayers were exposed to a 1:0 dilution of untreated leachate, however concentrated leachate did cause cytotoxic effects. The 1:10 dilution of leachate was determined sufficient to prevent cell death due to leachate toxicity. Previous studies used 0.1M EDTA treated Leachate to reduce mammalian cytotoxcity, however this treatment resulted in increased cytotoxcity over untreated leachate matrix, and thus was not explored further.

Recovery Efficiency

To assess the recovery of TGEV from Leachates A, B and C, three TCID50 assays were performed per leachate. Leachate aliquots were spiked at a 1:100 ratio with stock TGEV (lot#: TGEV-P3-121515) for an estimated titer of 4e+4 TCID50/mL (i.e., 4.6 log TCID50/mL). Leachate aliquots were then immediately serially diluted in complete growth media (5% FBS in Eagles Minimum Essential Media (EMEM)) and plated on ST-cells for TCID50 assays. Assays were scored for CPE at 48 hours post-infection. Positive, leachate and negative controls were ran with the experiment for comparison. As shown in table 1, TGEV was recovered at >90% from all three leachates. The recovery % is calculated using empirical data from the positive control results (an average of 4.68 log TCID50 inoculated per sample).

Table 1. Recovery of TGEV

Leachate	Spiked (log TCID50)	Recovered	Coefficient of Variance	Recovery with 95% CI
A	4.68	95.19%	6.03%	95% +/- 44%
B	4.68	96%	2.05%	96% +/- 15%
C	4.68	98.62%	4.17%	99% +/- 31%

Limit of Detection (LOD) Assay and Inter-Analyst Observations
The LOD of the TGEV TCID50 assay, TGEV (lot# TGEV-P3-121515) was determined in two independent experiments, each of which was read by three Battelle analysts. Results from replicate 1 and 2 were used to evaluate the limit of detection and results from each analyst were used to determine inter-analyst variation. The LOD was determined in each assay by diluting stock TGEV to extinction in Complete Growth Medium (5% FBS in EMEM), followed by triplicate TCID50 assays on low concentrations of TGEV (see Table 2). All TCID50 assays were read separately by three analysts at 48 hours post-infection. Results are shown in Table 2.

The limit of detection was estimated by comparing the concentration of virus in each high dilution to the calculated titer present in each sample. The coefficient of variance (variability) was used to assess the reliability of each result. Calculated titers (shown in Table 2) were determined using positive control samples run with each replicate for accuracy. Conservatively, the LOD was estimated to be approximately 200 TCID50/mL.

As the TCID50 assay is dependent on analyst ability to differentiate cytopathic effect (CPE) of TGEV from any other cellular damage/death on ST-cell monolayers (including leachate-induced cytotoxcity), and results are often dependent on sequential reading of many assay plates (up to 27 plates per assay), analyst calls for positive CPE wells and overall results from analyst results were compared to determine if there was significant inter-analyst variation in the results.

Analyst observations were compared using a Paired Two Sample for Means t-Test (Microsoft Excel 2007). No significant differences between the mean numbers of TICD50/ml observations was perceived between three analysts (significant differences defined as $p<0.01$).

Table 2: Limit of Detection results for replicate TCID50 assays using both 2-fold (Table 2A) and 10-fold (Table 2B) dilution schemes. The 2-fold dilution scheme was able to identify lower concentrations of virus, however the 10-fold dilution scheme resulted in less variable results. Legend: * Dilution was not repeated during the second run of the assay; - N/A; [1] High CV reflects substantial increase in variation in TCID50 assays when using low concentration of virus. TCID50 calculations are dependent on at least 50% + CPE in at least one dilution of starting material (e.g. one row on one plate). At a concentration of 40 TCID50/mL, it is likely that only a small number of wells were inoculated with enough virus to cause + CPE.

Table 2A: LOD results using 2-fold dilution series

Limit of Detection Results for TGEV TCID50 assay using 2-fold dilution series											
		Replicate Experiment 1					Replicate Experiment 2				
Dilution Series used for TCID50 assay	Dilution from Stock for TCID50	Calculated titer TCID50/mL	Empirically determined TCID50 of stock solution	Standard Deviation	Coefficient of Variation	Empirical Titer / Stock Titer	Calculated titer TCID50/mL	Empirically determined TCID50 of stock solution	Standard Deviation	Coefficient of Variation	Empirical Titer / Stock Titer
2-fold	1.00E-07	0.28	0.00	0	-	-	0.21	*	*	*	-
2-fold	1.00E-06	2.82	0.00	0	-	-	2.07	0.00	0	-	-
2-fold	1.00E-05	28.20	37.36	9.46	25.32%	132.48%	20.70	5.85	10.13	173.21%[1]	28.25%
2-fold	1.00E-04	282.00	339.66	56.58	16.66%	120.45%	207.00	91.60	21.71	23.71%	44.25%
10-fold	Stock	2.82E+06	2.82E+06	2.94E+05	10.46%	100.00%	2.07E+06	2.07E+06	1.77E+06	85.77%	100.00%

Table 2B: LOD results using 10-fold dilution series

Limit of Detection Results for TGEV TCID50 assay using 10-fold dilution series											
		Replicate Experiment 1					Replicate Experiment 2				
Dilution Series used for TCID50 assay	Dilution from Stock for TCID50	Calculated titer TCID50/mL	Empirically determined TCID50 of stock solution	Standard Deviation	Coefficient of Variation	Empirical Titer / Stock Titer	Calculated titer TCID50/mL	Empirically determined TCID50 of stock solution	Standard Deviation	Coefficient of Variation	Empirical Titer / Stock Titer
10-Fold	1.00E-07	0.28	0.00	0	-	-	0.21	*	*	*	
10-Fold	1.00E-06	2.82	0.00	0	-	-	2.07	*	*	*	-
10-Fold	1.00E-05	28.20	32.35	0.67	7.77%	114.72%	20.70	*	*	*	-
10-Fold	1.00E-04	282.00	321.24	24.95	6.27%	113.91%	207.00	147.83	39.18	26.50%	71.42%
10-fold	Stock	2.82E+06	2.82E+06	2.95E+05	10.46%	100.00%	2.07E+06	2.07E+06	1.77E+06	85.77%	100.00%

Proposed Recovery Method
For the persistence study, the following test methods are proposed:

- Spike 3 mL leachate samples at a ratio of 1:50 with TGEV stock to achieve a starting concentration of 5 log10 TCID50/mL. Spike EMEM without FBS, with antibiotics (Incomplete growth medium) as Positive Controls. Include non-spiked leachate as Negative Controls.
- Incubate samples at 12C in screw-top, polypropylene tubes.
- Assay triplicate test samples per time point along with triplicate Positive Control sample (stock virus of the same lot as spiked sample), and a single Negative Control per leachate.

BACTERIOPHAGE

The Double Agar Overlay (DAL) method was used to measure the concentration of MS2 (*E. coli* ATCC 700891 as host) and Phi6 (*Pseudomonas syringae* ATCC LM2489 as host). Luria Bertani Agar (LBA) was used for MS2 testing and Tryptic Soy Agar supplemented with MgCl2 (TSA+Mg) was used for Phi6 testing. Working stocks of MS2 and Phi6 were prepared at 3.6e+9 and 1.0E+10 pfu/mL respectively. These stocks were stored frozen as 1 ml aliquots and used as needed.

Assay Inhibition/Interference
Leachate samples that were non-diluted and diluted 1:5 and 1:10 were plated using the DAL method and showed that that none of the leachates caused assay inhibition for MS2 nor did leachates A and C for Phi6. Leachate B did however did contain constituents that prevented Phi6 plaques from forming. This same inhibition effect of Leachate B with Phi6 was observed when all three leachates were spike with MS2 and Phi6 at 10^4 pfu/mL as there was virtually no reduction with MS2 and Phi6 with leachates A and C, and no Phi6 was recovered from Leachate B. A subsequent test with autoclaved Leachate B showed that assay inhibition was caused by a biological constituent as Phi6 was recovered from the autoclaved material.

The use of antibiotics in the bottom and top agar was also evaluated. For MS2, Luria Bertani agar supplemented at 15 ppm streptomycin and 15 ppm ampicillin (LA+S+A) was evaluated. For Phi, TSA+Mg supplemented with ampicillin at 20, 50, 100, 150, and 200 ppm was evaluated. In short, the addition of antibiotics in the media for MS2 neither aided in recovery nor adversely affected recovery. The addition of ampicillin didn't eliminate Leachate B inhibition. The Phi6 plaques are also less distinct and more difficult to count using media containing ≥ 50 ppm ampicillin.

A follow-on experiment showed that if Leachate B was centrifuged post-spiking to pellet the microbes and particulates from the bacteriophage, a high percentage of Phi6 (Table 3) was recovered in the supernatant using TSA+Mg+A20 medium. Interestingly, Leachate B no longer showed assay inhibition. One explanation may be that the concentration of microbe(s) that caused the inhibition may have significantly declined during storage from the time the leachate was collected.

Table 3. Recovery of Phi6 from Leachate B post-centrifugation

Matrix	PFU/mL Recovered	
	Pre-Centrifugation	Post-Centrifugation
PBS	2.7e+3	n/d
Leachate B	1.9E+3	1.6E+3

Recovery

Leachates A, B, and C were spiked at 1e+10^4 pfu/mL (i.e., 4 $\log_{10}$/mL), serial diluted with PBS, and the assayed using the DAL method. The results (Table 4) illustrate that the phage can be recovered very well in leachates A, B, and C as the log reduction observed was insignificant with the exception of Phi6 from Leachate B.

Table 4. Phage Recovery

Leachate	Reduction of Recovered Phage Observed ($\log_{10}$ PFU/mL)	
	MS2	Phi6
A	0	No data (assay inhibition)
B	0.2	-0.3
C	0.2	-0.2

LOD and Intra-assay Precision

To LOD of the DAL method was determined assaying five replicate 0.1 aliquots of MS2 and Phi6 prepared in PBS at 10, 50, 100, 500, and 1000 PFU/mL. LA+S+A medium was used for MS2 quantitation and LA+Mg+A20 was used for Phi 6 quantitation. Two analyst assayed each suspension.

In short, the results suggest the LOD for the bacteriophage is approximately 500 pfu/mL (i.e., 2.7 log pfu/mL) based on the phage was detected in all replicate sample from both analysts at this concentration. The results (Table 5) also illustrate the assay has a high precision as the results between the two analyst were very comparable. The recovery efficiency was lower than desired 50% but since the initial spike concentration for the persistence study will be 6 $\log_{10}$ pfu/mL, a range of 4 logs of decay will still be able to be measured.

Proposed Bacteriophage Recovery Methods

Based on these results, the proposed recovery assay for MS2 is to serial dilute the test samples with phosphate buffered saline, and assay triplicate aliquots of various dilutions with the DAL method using bottom and top Luria Bertani Agar supplemented with 15 ppm streptomycin and 15 ppm ampicillin. These antibiotic concentrations are often used with the E. coli host strain and did not adversely affect recovery of MS2.

The proposed method for recovering Phi6 is centrifuge two 1 mL aliquots at 12,000 xg for 2 minutes, combine the supernatants, serial dilute with PBS, then using DAL method, assay triplicate 0.1 ml aliquots per dilution using TSA+Mg+A20 medium.

Table 5. LOD of DAL Method

Phage	Analyst	Theoretical Concentration (PFU/mL)	Measured Conc. (PFU/mL)	% Recovery
MS2	1	10	2	20%
		50	4	8%
		100	10	10%
		500	64	13%
		1000	158	16%
	2	10	0	0%
		50	6	12%
		100	8	8%
		500	70	14%
		1000	138	14%
Phage	Analyst	Theoretical Concentration (PFU/mL)	Measured Conc. (PFU/mL)	% Recovery
Phi6	1	10	2	20%
		50	4	8%
		100	14	14%
		500	100	20%
		1000	252	25%
	2	10	6	60%
		50	10	20%
		100	20	20%
		500	84	17%
		1000	176	18%

PROPOSED DESIGN OF PERSISTENCE STUDY

Table 6. Recommended Test Design for Persistence Study

Test Parameter	TGEV	MS2 and Phi6
Spike Concentration and Sample Volume	Spike at a ratio of 1:50 • 60 µL virus stock into 2.96 mL leachate to achieve starting conc. of 4.9 $\log_{10}$ TCID50/mL • Sample volume= 3 mL Sample Tube: 5 mL, screw-top polypropylene	Spike at ratio of 1:100 • Spike with 1E+8 pfu/mL suspension prepare in PBS to achieve starting conc. of 1E+6 pfu/mL (or 6 $\log_{10}$ pfu/mL) • Spike 0.3 ml phage into 2.7 mL leachate • Sample volume= 3 mL
Spiking	Option A: Add replicate 3 ml aliquots of leachate per 5 mL screw-top polypropylene tubes, then spike each tube with virus or phage. Prepare three replicates per each time point listed below. Incubate tubes in test tube racks with caps tightly fastened in incubators set at temperatures indicated below. Option B: Spike leachate with virus or phage, mix well, and dispense into individual tubes. Then incubate as described in Option A.	
Controls	Per Time Point: 1 Neg. Control Per Leachate (non-spiked leachate) 3 Positive Controls* (spiked EMEM without FBS, with Antibiotics Complete Growth)	Per Time Point: 1 Neg. Control Per Leachate (non-spiked leachate) 3 Positive Controls (spiked PBS)
Incubation Temperature	12ºC	12ºC and 37ºC
Time Points (subject to change throughout testing based on real-time results)	0, 3, 7, 14, 21, 28, 35, and 42 days	0, 3, 7, 14, 21, 28, 35, and 42 days
Recovery Assay Assay triplicate samples per time point	Mix sample well via repeated pipeting then dilute sample 1:10 in Complete Growth Medium. Continue with serial dilutions (1:2 and/or 1:10 as deemed appropriate) using 96-well plate. Assay triplicate wells per dilution using 2-day TCID50 assay. Persistence assays will be performed in triplicate. A second analyst will score results for 50% of the experiments. This will give second observations for 50% of the persistence experiments. Data will be reported as an average of both analysts for those experiments. Recommend spiking virus into leachate at a minimum of 1:50.	Mix sample well via moderate vortexing. • MS2: serial dilute with PBS to 10-4, then using DAL method, assay triplicate 0.1 ml aliquots per dilution using LA+15S/A medium. • Phi6: centrifuge two 1 mL aliquots at 12,000 xg for 2 minutes, combine supernatants, serial dilute to 10-4, then using DAL method, assay triplicate 0.1 ml aliquots per dilution using TSA+Mg+A20 medium. Data will reported as mean pfu/sample

*3 reps at T_0 to determine baseline spiking concentration, but may be reduced to one replicate in subsequent time points.

Appendix B:

Miscellaneous Operating Procedure

Miscellaneous Operating Procedure (MOP) to Evaluate the Persistence of Transmissible Gastroenteritis Virus (TGEV), MS2 bacteriophage, and Phi6 bacteriophage in Landfill Leachate

1.0 Purpose and Scope

To evaluate the persistence of Transmissible Gastroenteritis Virus (TGEV), MS2 bacteriophage, and Phi6 bacteriophage in landfill leachate collected from three landfills. Each of the three landfill leachates will be spiked individually with a known quantity of virus or bacteriophage, dispensed into replicate screw-top vials, and incubated statically over the course of approximately 8 weeks. All TGEV-spiked samples will be incubated at 12 degrees Celsius (°C), and one set of the bacteriophage-spiked samples will be incubated a 12 °C and another set at 37 °C. Per time point, triplicate samples will be assayed periodically over time for up to eight time points including T_0. TGEV-spiked samples will be assayed for infective ("viable") virus by serially diluting the sample in tissue culture medium and then assaying a range of dilutions using a $TCID_{50}$ assay on Swine Testicular (ST) cells. For bacteriophage-spiked samples, triplicate samples will be serially diluted in a buffer and assayed for infective bacteriophage using a standard Double Top Agar Overlay (DAL) method using *Escherichia coli* and *Pseudomonas syringae* as the host strains for MS2 and Phi6, respectively. Samples will be assayed over time until either the virus/bacteriophage is no longer detected or until the samples for all eight time points have been assayed.

2.0 Materials and Reagents

A. Tryptic Soy Bottom Agar supplemented with Magnesium Chloride and Ampicillin at 20 ppm (TSA+Mg+A20)
B. Tryptic Soy Top Agar supplemented with Magnesium Chloride and Ampicillin at 20 ppm (TSTA+Mg+A20)
C. Luria Bertani Bottom Agar supplemented Streptomycin and Ampicillin at 15 ppm (LBA+S+A)
D. Luria Bertani Top Agar supplemented Streptomycin and Ampicillin at 15 ppm (LBTA+S+A)
E. Tryptic Soy Broth (TSB)
F. Tryptic Soy Agar (TSA)
G. Luria Bertani Broth (LBB)
H. Potato Dextrose Agar (PDA)
I. Phosphate Buffered Saline, pH 7.0 to 7.2, sterile (PBS)
J. 70% Isopropyl Alcohol Spray (IPA)
K. IPA Wipes, Sterile
L. Bleach, 10% (or 0.5% sodium hypochlorite solution)
M. Bleach, household (5.25 to 6.5% sodium hypochlorite)
N. *Escherichia coli* American Type Culture Collection (ATCC) 700891
O. MS2 bacteriophage ATCC 155597-B1
P. *Pseudomonas syringae* LM2489

Q. Phi6 bacteriophage (EPA provided)
R. Leachate A, lot number A100715, collected 10/7/2015
S. Leachate B, lot number B100715, collected 10/7/2015
T. Leachate C, lot number C100815, collected 10/8/2015
U. Complete Growth Medium: 1X Eagle's Minimum Essential Medium (EMEM) containing 5% v/v fetal bovine serum (FBS) and 1% v/v antibiotic-antimycotic solution (Anti-Anti)
V. Incomplete Growth Medium: 1X EMEM supplemented with 1% v/v antibiotic-antimycotic solution (Anti-Anti)
W. Transmissible Gastroenteritis Virus (TGEV); ATCC VR-763
X. Swine Testicular (ST) cells (ATCC CRL-1746) maintained in Complete Growth Medium

3.0 Equipment and Supplies

A. Incubator, 36-38 °C
B. Refrigerator Incubator, 11-13 °C
C. Water bath, 45-50 °C
D. Class II Biological Safety Cabinet (BSC)
E. Vortex
F. Lock n Lock Boxes or equivalent plasticware container
G. Biohazard bin
H. Necessary personal protective equipment (PPE) for general lab use
I. Wypalls or paper towels
J. Liquid waste container, 500 milliliter (mL) to 1 liter (L)
K. Conical tubes, 50 mL, sterile
L. Cyrovials, 5 mL polypropylene, screw-top tubes, sterile
M. Test tube racks
N. Flask, glass, 1L with stir bar, sterilized by autoclaving
O. Flasks, glass, 250 mL with stir bar, sterilize by autoclaving
P. 96 well tissue culture plates with monolayers of swine testis (ST)-cells
Q. Tissue culture reservoirs
R. 96 deep well plates
S. Micropipettes, P-20, P-100, P-200, P-1000
T. 200 microliter Multichannel pipette, L-200
U. Assorted sterile filtered pipette tips
V. Aspirator
W. Non-filtered pipette tips
X. Pipet aid
Y. Assorted column sterile disposable pipettes, graduated
Z. Analytical Balance
AA. Calibrated weight set

4.0 Acronyms

A. ATCC - American Type Culture Collection
B. BSC – Biological Safety Cabinet

C. BSL – Biological Safety Level
D. CFU – Colony Forming Unit
E. CGM – Complete Growth Medium
F. CPE- Cytopathic Effect
G. DAL – Double Top Agar Overly
H. EMEM – Eagle's Minimum Essential Medium
I. FBS – Fetal Bovine Serum
J. IPA – 70% Isopropyl Alcohol
K. LBA – Luria Bertani Bottom Agar
L. LBB – Luria Bertani Broth
M. LBTA – Luria Bertani Top Agar
N. NC – Negative Control
O. PBS – Phosphate Buffered Saline
P. PC – Positive Control
Q. PFU – Plaque Forming Units
R. PPE – personal protective equipment
S. ppm – parts per million
T. PDA – Potato Dextrose Agar
U. $TCID_{50}$ –Median Tissue Culture Infective Dose.
V. TGEV – Transmissible Gastroenteritis Virus
W. TNTC – Too numerous to count
X. TSA – tryptic soy agar
Y. TSB – tryptic soy broth
Z. TSTA – tryptic soy top agar

5.0 Methods

A. Documentation

1. Record work performed using the worksheets listed below:

Bacteriophage Worksheets	Worksheet for Preparing Samples for MS2 Bacteriophage Persistence Study
	Worksheet for Preparing Samples for Phi 6 Bacteriophage Persistence Study
	Worksheet for Recovering MS2 from Landfill Leachate
	Worksheet for Recovering Phi6 from Landfill Leachate
	Worksheet to Prepare Tryptic Soy Bottom Agar supplemented with Magnesium Chloride and Ampicillin at 20 ppm (TSA-Mg+A20)
	Worksheet to Prepare Tryptic Soy Agar supplemented with Magnesium Chloride and Ampicillin at 20 ppm (TSTA-Mg+A20)
	Worksheet to Prepare Luria Bertani Bottom Agar supplemented Streptomycin and Ampicillin at 15 ppm (LBA+S+A)

	Worksheet to Prepare Luria Bertani Top Agar supplemented Streptomycin and Ampicillin at 15 ppm (LBTA+S+A)
	Worksheet to Prepare Tryptic Soy Broth (TSB)
	Worksheet to Prepare Luria Bertani Broth Broth (LBB)
	Worksheet to Prepare Potato Dextrose Agar (PDA)
	Worksheet to Prepare Tryptic Soy Agar (TSA)
TGEV Worksheets	Worksheet for Preparing Samples for TGEV Persistence Study
	Worksheet for Propagation of TGEV
	Worksheet for Recovery of TGEV from Leachate with ST cells
	Worksheet for Seeding 96 Well Plates with ST-Cells
	Preparation of Complete Growth Medium (CGM) for ST Cells
	Preparation of Incomplete Growth Medium for ST Cells
	Cell Passage Form

B. Sample Preparation – MS2 and Phi6

Note 1: Perform all manipulations with virus and bacteriophage in a certified, class II BSC.
Note 2: Perform all laboratory work following biosafety level (BSL) II guidelines.
Note 3: Confirm concentration of bacteriophage stocks within 1 week of starting persistence study using the DAL method.
Note 4: Pre-label all 5 mL sample tubes prior to preparing the samples. Labels shall include a unique sample ID number, and identify the viral agent, type of leachate (A, B, or C), storage temperature (12 °C or 37 °C), and the study number. .

1. Test Samples (per bacteriophage):
   a) Per leachate (this step can be performed up to 1 day prior to spiking with virus):
      i. Remove one of the large bottles of leachate from refrigerated storage and mix very well by swirling vigorously.
      ii. Immediately dispense approximately 500 mL into a sterile 1L Erlenmeyer flask containing a stir bar.
      iii. Mix leachate on a stir plate set at medium-high speed for at least 5 minutes.
      iv. While leachate continues to mix a stir plate, use a 5 ml graduated pipet to dispense 4 ml of leachate into pre-labeled tubes.
      v. Store samples refrigerated if they are to be spiked the following day. If spiked the same day, proceed to next step.
   b) Remove bacteriophage stock from cold storage.
   c) Dilute bacteriophage stocks to $1x10^8$ plaque forming units (PFU)/mL using PBS, representing the spiking suspensions. Mix suspensions well by vortexing at low-moderate speed.
   d) Using a calibrated P-200, spike each 4 mL leachate sample with 40 µL of the appropriate spiking suspension to achieve a target starting concentration of $1x10^6$ PFU/mL. *Important: Mix spiking suspension frequently to maintain a homogenous suspension while spiking the samples.*

e) Ensure screw-caps of each sample are tightly fastened, then mix each sample by swirling and inverting the sample 3 time times.
f) Place tubes in a rack, and then immediately place in an incubator set to operate at either 12 °C or 37 °C.
g) Promptly enumerate the 10^8 PFU/mL spiking stock and triplicate spike leachate samples as described in step E.

2. Positive Controls (per bacteriophage)
   a) Using a graduated 5 mL pipet, accurately dispense 4 mL of sterile PBS into pre-labeled tubes.
   b) Using the same spiking suspension prepare in step B.1.c and using a calibrated P-200 micropipette, spike each 4 mL PBS sample with 40 µL of the appropriate spiking suspension to achieve a target starting concentration of $1x10^6$ PFU/mL. *Important: Mix spiking suspension frequently to maintain a homogenous suspension while spiking the samples.*
   c) Ensure screw-caps of each sample are tightly fastened, then mix each sample by swirling and inverting the sample 3 times.
   d) Place tubes in a rack, and then immediately place in an incubator set to operate at either 12 °C or 37 °C.
   e) Promptly assay three positive controls spiking stocks using the DAL method as described in section E.

3. Negative Controls (per bacteriophage)
   a) Using the leachates that are prepared in step B.1.a and that are mixing on a stir plate, dispense 4 mL aliquots of the appropriate leachate into pre-labeled, replicate 5 mL screw-top tubes.
   b) Ensure screw-caps of each sample are tightly fastened.
   c) Place tubes in a rack, and then immediately place in an incubator set to operate at either 12 °C or 37 °C.

4. Evaporation Samples
   a) Dispense 4 mL of Leachate A, B, and C, and 4 ml of PBS into individual, pre-labeled 5 mL tubes. Prepare one set per bacteriophage and per incubation temperature.
   b) Ensure caps are fastened securely.
   c) Weigh each sample on an analytical balance. Record weight out to 0.0001 grams.
   d) Place tubes in a rack, and then immediately place in an incubator set to operate at either 12 °C or 37 °C.
   Note: These samples will be weighed at each time point throughout the persistence study, weighed, and then returned to the appropriate incubator.

C. Sample Preparation - TGEV
   1. Test Samples
      a) Per leachate (this step can be performed up to 1 day prior to spiking with virus):
         i. Remove one of the large bottles of leachate from refrigerated storage and mix very well by swirling vigorously.
         ii. Immediately dispense approximately 500 mL into a sterile 1L Erlenmeyer flask containing a stir bar.
         iii. Mix leachate on a stir plate set at medium-high speed for at least 5 minutes.
         iv. While leachate continues to mix a stir plate, use a graduated 5 ml pipet to dispense 4 ml of leachate into pre-labeled tubes.
         v. Store samples refrigerated if they are to be spiked the following day. If spiked the same day, proceed to next step.
      b) Thaw TGEV aliquots and pool into one 15 mL conical tube. Thaw enough TGEV to spike all leachate and positive control samples.
      c) Invert vial 5 times and thoroughly triturate without introducing bubbles into the media for 20s.
      d) Spike each 4 mL leachate sample, aliquoted in step C.1.a, with 80 µL TGEV to achieve a target starting concentration of at least $5x10^4$ $TCID_{50}$/mL.
      e) Ensure screw-caps of each sample are tightly fastened, then mix each sample by swirling and inverting the samples 3 times.
      f) Place tubes in a rack and incubate at 12 °C.

   2. Positive Controls
      a) Using a graduated 5 mL pipet, accurately dispense 4 mL of sterile incomplete EMEM into the appropriate pre-labeled tubes.
      b) Using the same spiking suspension prepared in step C.1.c, and using a calibrated P-200 micropipette, spike each 4 mL incomplete EMEM sample with 80 µL of the spiking suspension to achieve a target starting concentration a target starting concentration of at least $5x10^4$ $TCID_{50}$/mL.
      c) Ensure screw-caps of each sample are tightly fastened, then mix each sample by swirling and inverting the samples 3 times without introducing bubbles into the sample.
      d) Place tubes in a rack, and then immediately place in an incubator set to operate at 12 °C.
      e) Promptly assay three positive controls spiking stocks using the TCID50 method as described in section F.

   3. Negative Controls
      a) Using the leachates that are prepared in step C.1.a and that are mixing on a stir plate, dispense 4 mL aliquots of the appropriate leachate into pre-labeled, replicate 5 mL screw-top tubes.
      b) Ensure screw-caps of each sample are tightly fastened.
      c) Place tubes in a rack, and then immediately place in an incubator set to operate at either 12 °C.

4. Evaporation Samples
   a) Dispense of 4 mL of Leachate A, B, and C, and 4 ml of Incomplete Growth Medium into individual, pre-labeled 5 mL tubes.
   b) Ensure caps are fastened securely.
   c) Weigh each sample on an analytical balance. Record weight out to 0.0001 grams.
   d) Place tubes in a rack, and then immediately place in an incubator set to operate at either 12 °C.
   Note: These samples will be weighed at each time point throughout the persistence study, weighed, and then returned to the incubator.

D. Incubation

1. Place test samples, positive controls and negative controls in an appropriate incubator. Record start time, date, and temperature.
2. Monitor temperature of incubator at least 3x per week or monitor continuously if a temperature data logger is available.
3. Remove triplicate test samples, triplicate positive controls, and one negative control per the following time points: 3, 7, 14, 21, 28, 35, 42 days. *Note: Time points are subject to change based on real-time results. The goal is to acquire data points during the linear range of decay.*
4. Record date, time of day, and temperature when samples are removed per time point.
5. At each time point, remove Evaporation Samples from incubator, measure sample weights, and return them to the incubator.

E. Sample Analysis – MS2 and Phi6

Note: Per day of sample analysis, process then negative controls first followed by the test samples, and then finish with the positive controls.

1. Culture Preparation
   a) **For MS2, on the day of sample analysis**, inoculate 10 mL LBB with 1-2 colonies of a ≤ 7 day old *E. coli* culture grown on LBA. Incubate broth culture in an incubator set at 35-37 °C and 200 rpm. Use broth culture ideally when it has been incubating 3 to 6 hours).
   b) **For Phi6, one day prior to sample analysis,** inoculate 10 mL TSB with 1-2 colonies of a ≤ 7 day old *P. syringae* culture grown on TSA. Incubate broth culture overnight in an incubator set at 23-25 °C and 200 rpm. Use broth culture ideally when it has been incubating for 14 to 24 hours.
2. Removed bottom agar plates from refrigerated storage and allow to come to room temperature.
3. Prepare molten top agar and stored in a water bath set at 45-50 °C.

4. Prepare sample:
   a) For MS2 Samples (test and controls):
      i. Vortex sample at moderate speed for 30 seconds.
      ii. Serially dilute to 10^{-4} using PBS (per dilution, add 0.5 mL phage-laden sample to 4.5 mL PBS).
   b) For Phi6 Samples (test and controls):
      i. Vortex sample at moderate speed for 30 seconds.
      ii. Dispense two 1 mL aliquots into microcentrifuge tubes.
      iii. Centrifuge at 12,000 xg for 2 minutes.
      iv. Pool 0.8 mL of supernatant from both tubes in a new tube.
      v. Serially dilute to 10^{-4} using PBS (per dilution, add 0.5 mL phage-laden sample to 4.5 mL PBS).

   Note 1: As bacteriophage begins to decay throughout the incubation period, fewer and fewer dilutions will be required. The Principal Investigator will determine which dilutions are needed per time point.
   Note 2: Negative controls, only assay neat (non-diluted sample).
   Note 3: For Positive Controls, serially dilute to 10^{-4}, and only plate dilutions $10^{-2, -3, \text{and } -4}$ (final dilution of $10^{-3, -4, -5}$ on plates.).

5. Sample Analysis using the DAL Method (per sample)
   Note 1: For MS2, use E. coli as the host and LBA+S+A as the bottom agar and LBTA+S+A as the top agar.
   Note 2: For Phi6, use P. syringae as the host and TSA-Mg+A20 as the bottom agar and TSTA-Mg+A20 as the top agar.

   a) Dispense 0.1 mL broth culture prepared in step E.1 into triplicate 50 mL conical tubes.
   b) Dispense 0.1 ml of bacteriophage sample (undiluted or diluted) into each tube with broth culture.
   c) Within 10 minutes, add 5 mL of molten top agar to each tube, promptly mix tube by swirling (avoid creating bubbles) and pour entire contents onto the bottom agar plate.
   d) Allow top agar to solidify (generally just a few minutes needed), and then incubate MS2 plates at 35-37 °C and Phi6 plates at 25-27 °C until plaques reach a countable size (generally 14 to 30 hours).
   e) Store remaining sample and dilution tubes at 2-8 °C until the sample results are tabulated in the event the Principal Investigator elects to have one or more sample re-enumerated.
   f) Count plates that have 0 to 250 PFU/plate. Record results and calculated PFU/mL using the mean of plates ranging from 25-250 PFU/plate. Record plates having >250 PFU per plate as Too Numerous to Count (TNTC).
   g) Per triplicates for each time point, calculate mean PFU recovered and the log PFU recovered. Plot mean log PFU recovered versus time.

F. Sample Analysis – TGEV
   1. Cell Culture Preparation
      a) ST-Cells harvested one day prior to time point testing.
      b) Cells counted and seeded at appropriate concentration in 96-well tissue culture plate to ensure 100% confluent monolayer of ST-cells the following day. NOTE: Seed between 15,000-20,000 cells per well of a 96-well plate.
      c) Incubate at 37 °C and 5% CO_2 overnight.
      d) Prior to inoculation for $TCID_{50}$ removed media and wash cells with 200 µL of Complete Growth medium.

   2. Sample Preparation
      a) Remove sample, positive and negative control vials from appropriate incubator.
      b) Work Steps F.2.c – F.3.c with negative controls first, then test samples and then positive controls to limit cross contamination between samples.
      c) Serially dilute sample/positive control/negative control to extinction using a 10-fold and/or 2-fold dilution scheme. Dilution end point will be based on the stock concentration of TGEV and the prior recovery during the previous time point. Use 1X Complete Growth Medium as the diluent and perform dilutions in a 96 deep well plate on ice.

   3. $TCID_{50}$
      a) Remove wash from ST-cells plated in 96 well plates.
      b) Inoculate with dilutions prepared in step F.2.b.
      c) Record inoculation time for each plate and incubate a 37 °C and 5% CO_2 for 48 hours.
      d) Observe plates for cytopathic effect (CPE) 48 hour post-inoculation.
      e) Calculate mean $TCID_{50}$ recovered and log $TCID_{50}$ recovered per time point. Plot mean log $TCID_{50}$ recovered versus time.

6.0 References

A. BIO IV-018 SOP for the Operation and Maintenance of a Biological Safety Cabinet
B. BIO I-001 SOP for Receipt, Storage, Transport, and Shipment of Biological Materials
C. BIO IV-027 SOP for the Use and Maintenance of Incubators

7.0 Revision History

None. This is original version.

Miscellaneous Operating Procedure (MOP) to Evaluate the Persistence of TGEV, MS2 bacteriophage, and Phi6 bacteriophage in Landfill Leachate

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Agency

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