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USDA

Large-Scale Shallow Burial with Carbon Field Study in Oklahoma

Contact

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Executive Summary

This report presents the findings of a field study to evaluate the practicality, safety, and effectiveness of shallow burial with carbon (SBC) for disposal of swine, in case of an African swine fever (ASF) outbreak in the United States. The work was funded by the U.S. Department of Agriculture (USDA) and included partners from USDA's Animal and Plant Health Inspection Service (APHIS); Oklahoma Department of Agriculture, Food, and Forestry (ODAFF); Oklahoma State University Department of Veterinary Pathobiology; Oklahoma State University Department of Entomology and Plant Pathology; and Virginia Department of Environmental Quality.

The study focused on virus survival in bone marrow, construction logistical requirements, environmental impacts, scavenger intrusion, and insect activity to 1) identify the insects associated with SBC and 2) determine the virus viability in house flies (*Musca domestica*), which might serve as vectors to spread pathogens.

This study produced extensive data related to the functioning of SBC for managing mass animal mortalities resulting from an animal disease outbreak. However, the data suggests there are still unanswered questions and the study team recommended several areas for possible continued work.

Researchers began the field study in September 2019 by burying 100 sow carcasses in two SBC trenches near Stillwater, OK, to determine if the SBC process could inactivate pathogens and decompose carcasses without significant environmental impact, scavenger activity, or insect activity. The sows weighed an average of 500 pounds each. The study team excavated the trenches an average of 21.5 inches deep, 6.5-7 feet wide, and 113 to 117 feet long. A foot of wood shavings was placed in the bottom of each trench. To prepare the sow carcasses, the researchers inoculated $10^{6.8}$ $TCID_{50}$/ml swine pox virus (SwPV) into the bone marrow of each femur and then placed the carcasses in a single layer on the wood shavings. The carcasses were covered with the excavated soil and seeded with a regionally and seasonally appropriate grass seed.

The study team collected background samples (10 femurs from each trench) on the day of inoculation. They collected 20 femurs from 10 carcasses, half from each trench, at around days 7, 14, 21, 28, 60, 90, 180, and 360 after inoculation, then analyzed the bone marrow by qPCR and virus isolation. To further support the findings from the SBC project, the team performed a bench scale study to explore the effect of the bone marrow environment on SwPV inactivation. The viability of SwPV, bovine viral diarrhea virus (BVDV), porcine parvovirus (PPV), and Senecavirus (SVA) was assessed using an in-vitro system.

Background soil samples were collected during trench construction and again at 2, 4, 6, and 12 months after construction and analyzed for pH, nitrates, ammonium, percent organic matter, total nitrogen, magnesium, calcium, sulfate, and salinity management parameters including total dissolved solids. The team analyzed leachate encountered after heavy rain soon after trench construction for total coliforms and SwPV. They analyzed forage for moisture, protein, and other factors. Wildlife attraction was monitored through the use of motion-activated trail cameras.

The team monitored insect activity with malaise and pitfall traps to compare background insect type and abundance with insect type and abundance associated with the SBC plots. They collected insects encountered in the trenches during soil sampling events for virus testing. They classified all insect samples down to insect order and forensically important species of beetles and flies using appropriate diagnostic keys. The researchers developed a predictive degree-hour model to estimate the expected

forensically important insect activity over time, then compared the model results with actual observations of forensically important flies collected. In addition to the field study, the team performed an in-vitro study to evaluate the ability of house flies to transport pathogens away from an infected source.

The study found:

1. Site Restoration

- No bones or tissue were visible on surface of plots after 1 year, although buried tissue was forced from a soft spot due to the weight of final grading equipment and had to be covered manually.

2. Virus Survival (Field)

- Nucleic acid detection by qPCR: SwPv Viral DNA was detectable by qPCR assay throughout the 12 months of the study in the majority of the samples.
- Swinepox virus survival: Viable virus was only detected in samples collected at Day 7 post-burial in 30 percent (3/10) of femurs from the west trench and 80 percent (8/10) of femurs from the east trench.

3. Statistical Analysis of Field Virus Survival

- Theanalysis found very high probability that no viable virus existed by the end of the study consistent with the rapid inactivation of the virus shown by viral titers.
- Virus concentration reaches near-zero at an estimated 10.7 days under the specific conditions of this study.

4. Virus Survival (In-Vitro)

- Virus viability in bone marrow samples - Bone marrow material had no direct negative impact on the titer of SwPV, supporting the conclusion that the SBC process was responsible for virus inactivation, not the presence of bone marrow tissue.
- Virus viability in house flies - Results demonstrated the ability of five flies to carry viruses when feeding in a solution containing the virus.

5. Environmental Monitoring

- Soil ammonium - Ammonium reached background levels within 2 feet below the trench bottom (BTB) during all time periods; SBC may release slightly more ammonium than composting, but significantly less than deep burial.
- Soil potassium - Potassium reached background within about a foot or less BTB during all time periods; it is generally not considered a pollutant.
- Soil calcium - Calcium was below background for the first 6-12 inches BTB in both trenches, then somewhat above background levels for the remaining depths at most time periods; if excess levels of calcium from soil reach groundwater used for drinking, it is not listed as a primary or secondary contaminant under the Safe Drinking Water Act.

- Soil pH - pH below both trenches is more acidic than background for the first foot or two BTB before returning to near-background for the next several feet of depth, then rising above background below both trenches for most time periods.
- Soil total dissolved solids (TDS) - TDS neared background within 3-5 feet BTB during all time periods in both trenches.
- Soil sulfate ions (SO4) - Levels neared background at 3-5 feet BTB; if excess sulfate from soil reaches groundwater used for drinking, it is a secondary drinking water contaminant for taste.
- Soil magnesium (Mg) - Levels were still elevated above background at 1 year for all depths in the west trench, but had returned to background levels at 1 year in the east trench by 7 feet BTB; it is not a known pollutant.
- Soil percent organic matter (%OM) - %OM levels returned to background levels at all time points by about 4 ft BTB.
- Soil total nitrogen (TN) - TN levels returned to background levels at all time points by about 2 feet BTB.
- Soil nitrates/nitrites - Nitrate/nitrite levels were at or below background levels under the west trench for all but one time point; the east trench exceeded background levels for nitrates/nitrites at 6 months and 1 year at 10-13 ft BTB. It is unlikely nitrates/nitrites exceeded safe levels at any time or depth.
- Leachate coliforms - Elevated total coliforms were measured in leachate produced near the east and west trenches after heavy rain soon after trench construction.

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1 Introduction

1.1 Purpose

The purpose of this study was to evaluate the practicality, safety, and effectiveness of shallow burial with carbon (SBC) for disposal of 100 sows, in case of an African swine fever outbreak in the United States. The study focused on virus survival in bone marrow, construction logistical requirements, environmental impacts, scavenger intrusion, and insect activity to 1) identify the insects associated with SBC and 2) determine the virus viability in house flies (*Musca domestica*), which might serve as vectors to spread pathogens.

1.2 Background

USDA-APHIS Veterinary Services (VS) is the lead U.S. Federal agency in charge of responding to foreign animal disease (FAD) outbreaks in farmed food animals. As part of that responsibility, VS manages national response efforts, supports States in their response activities, and supports producers by providing resources for depopulation, disposal, and decontamination of infected farms to protect American agriculture. During the 2014-2015 highly pathogenic avian influenza (HPAI) response, disposal activities were the second-most significant cost, following indemnity, based on actual 2015 response cost data from the USDA Center for Epidemiology and Animal Health.

There are limited disposal options available for large numbers of livestock or poultry carcasses, including on-site deep burial, burning, or composting, as well as off-site landfill, rendering, or incineration. Each option has advantages and disadvantages. Although on-site deep burial is the most familiar method for animal disposal to many people, it can have significant drawbacks depending on where and how it is implemented. The Virginia Department of Environmental Quality in collaboration with Virginia Polytechnic Institute and State University Extension Service and livestock industry officials recognized that responders might choose to bury livestock and poultry carcasses despite the potential drawbacks. They developed the shallow burial with carbon (SBC) concept to mitigate some of the deep burial drawbacks. Once the concept was formulated, the team conducted several relatively small-scale field trials to test and refine the method.

SBC involves excavating an approximately 2-foot deep trench, placing a 1-foot layer of wood chips or similar material in the bottom of the trench, then placing a layer of carcasses on the wood chips. The carcasses are covered with the excavated soil, and the plots are seeded with seasonally and regionally appropriate grass seed (Figure 1- Shallow Burial with Carbon Diagram).

In 2019, the Oklahoma State Veterinarian, in collaboration with the Oklahoma Pork Council, set up a 2-day meeting at the request of the Oklahoma pork industry to discuss issues related to planning for ASF outbreak response, should it be needed. During discussions regarding stop movement, euthanasia, and disposal, industry officials asked about deep burial options. An official from the Oklahoma Department of Agriculture, Food, and Forestry (ODAFF) Agricultural Environmental Management Services (AEMS) Division explained the potential human health and environmental risks of deep burial and introduced the potentially safer alternative of SBC. Officials from industry, Oklahoma State University, ODAFF, and USDA expressed interest in doing a field trial of the method, which led to this project.

Figure 1. Shallow burial with carbon diagram

The work was funded by USDA and included partners from USDA APHIS, ODAFF, Oklahoma State University Department of Veterinary Pathobiology, Oklahoma State University Department of Entomology and Plant Pathology, and Virginia Department of Environmental Quality.

1.3   Report Organization

This report is organized into five sections, including this introduction, and sections on methodology, findings, conclusions, and recommendations, as well as references.

2   Methodology

2.1   Construction Logistics and Implementation

2.1.1   Site Selection

To select a suitable testing site, the team evaluated several locations in Payne County, OK, managed by Oklahoma State University. Initially, the researchers screened the locations using published data including:

- USDA National Resource Conservation Service (NRCS) Web Soil Survey carcass burial suitability ratings;
- Flood maps;
- Data regarding depth to groundwater, bedrock, or other restricting layers; and
- Distance to wells/springs, surface water, property lines, sink holes, rock outcrops, physical structures, and drain tiles.

If a candidate site was found to have suitable soil textures and properties, to meet state regulatory setback requirements, and to have enough soil depth to fully evaluate any changes to the soil chemistry under the carcasses (minimum depth of 15 feet, or 180 inches), then the site was considered for further field evaluation. Four candidate locations were selected for field evaluation

where the soil was sampled using a manual 4-inch diameter stainless steel bucket auger with extension tubes and a tee handle. Soil samples were collected and evaluated in 6-inch increments.

Site 1 appeared to be suitable based on the desktop review, but field evaluation showed that the area had limited soil depth (Figure 2) and was not a suitable site due to a limiting (dense compacted) layer at approximately 30 inches below ground surface.
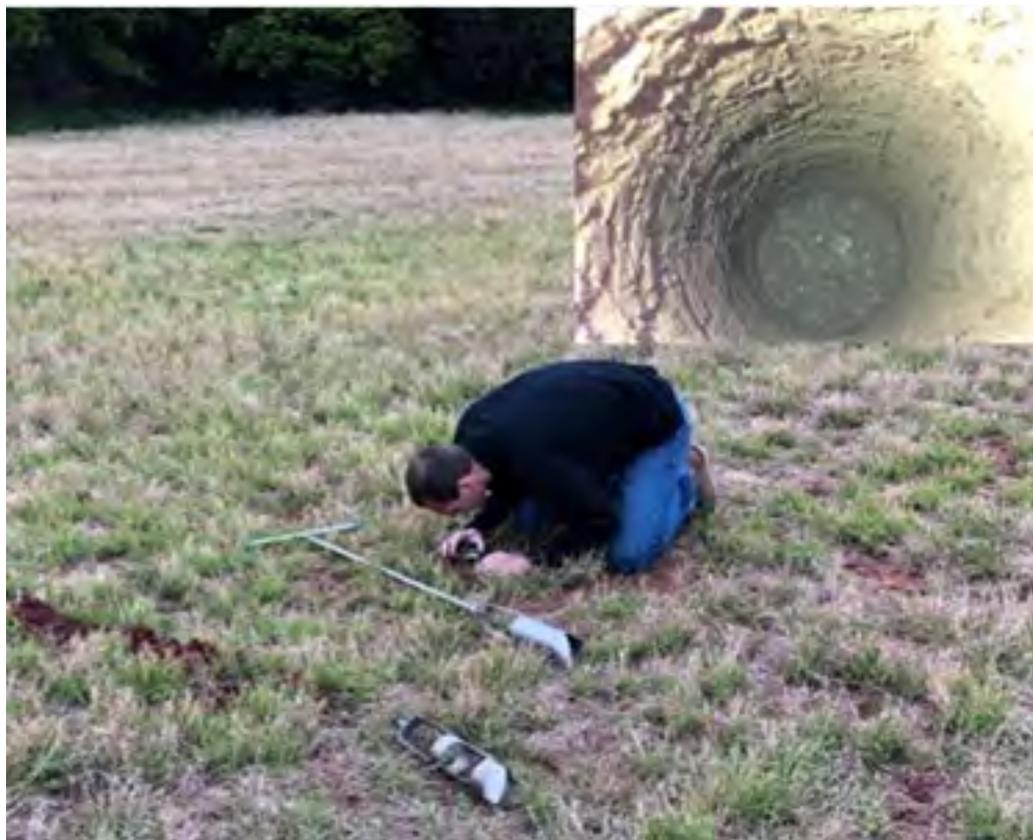
Figure 2. Photo of limiting layer at Site 1

Site 2 also appeared to be suitable based on the desktop review, but field evaluation showed that the area would not be suitable because strong redoximorphic depletions (color patterns in the soil formed by the oxidation and reduction of iron and/or manganese caused by saturated conditions within the soil) were encountered suggesting periodic soil wetness, and water began seeping into the test holes at a depth of approximately 132 inches (Figure 3).

Site 3 was determined not suitable because it was difficult to access and the team encountered water in the test holes at a depth of approximately 64 inches below the ground surface.

At Site 4, the team was able to hand-auger to a depth of 16 feet below the ground surface. They installed four test holes, and root channels with dark organic matter (Figure 4) from old decaying roots were observed at depths of 9 feet below the ground surface. Some redoximorphic features were observed in all four test holes, but no free water was observed. Soil was moist throughout the profile in all four test holes.

Figure 16. Photo of discolored, wet soil sample from Site 2

Site 4 offered easy access, was in a discreet area, and had suitable soil properties to the required depth. The site did need some clearing and management to properly lay out the trenches for data collection and monitoring, but overall, it had the most favorable characteristics of the four candidate sites and was selected as the test site.

Figure 4. Organic matter from old roots at Site 4

2.1.2 Site Description

2.1.2.1   *Soil*

A USDA-NRCS Major Land Resource Area (MLRA) soil scientist classified soil at the selected site. The site was identified as a Pond Creek-like soil. The Pond Creek soil is described as a very deep, well-drained soil that formed in calcareous loamy material high in silt and very fine sand, which was derived from a combination of loess, alluvium, and residuum from red beds. These soils occur on paleoterraces on broad uplands in the Central Rolling Red Prairies (MLRA 80A). Slopes range from 0 to 8 percent. The taxonomic class is a fine, mixed, superactive, thermic Oxyaquic Argiustolls and is typical of a geomorphic setting of a backslope of tread of paleoterrace on an alluvial plain remnant.

2.1.2.2   *Groundwater*

The site's ground surface has an elevation of 294 meters above mean sea level (AMSL) and Stillwater Creek, which is located approximately three-tenths of a mile south, has an elevation of 287 meters AMSL. Figure 5 depicts a cross-sectional view of the research site elevation relative to the creek.
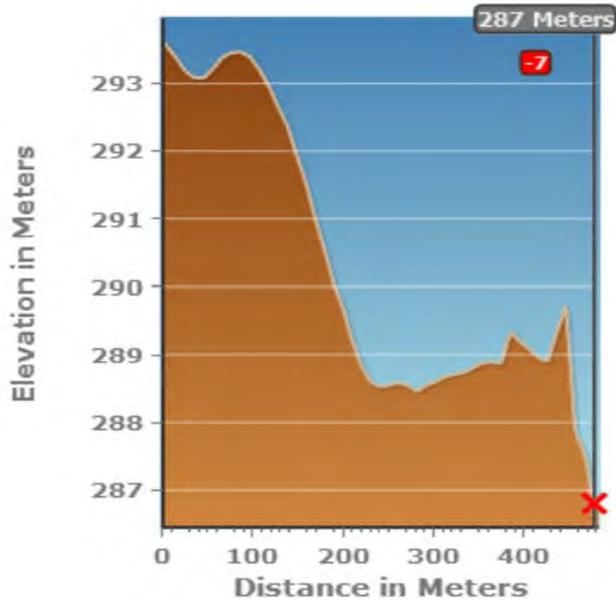
Figure 5. Horizontal profile from project to Stillwater Creek

2.1.2.3   *Weather*

Payne County typically gets 71 cumulative days of precipitation per year, totaling 38 inches on average annually. The average annual temperature is 60°F (Figure 6). The average maximum temperature is 72°F and the average minimum temperature is 48°F. The record high temperature is 117°F and the record low is -19°F. The area typically gets 74 days greater than 90°F and 25 days less than 32°F. There are generally 207 days in the growing season.

2.1.2.4   *Vegetation*

Existing vegetation prior to plot construction included multiple species of trees and unmanaged regrowth following previous clearing. Trees included sumac *(Rhus spp.)*, honey locust *(Gleditsia*
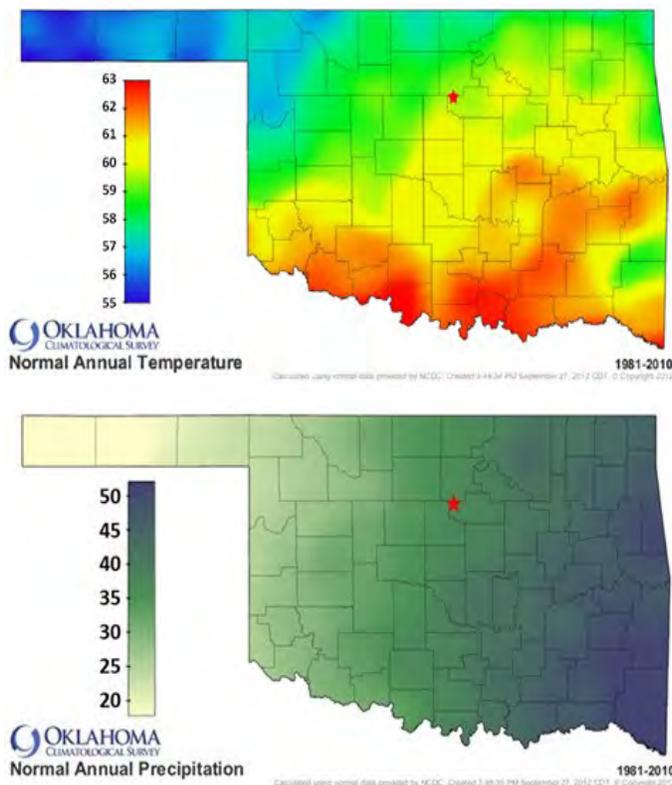
Figure 6. Normal annual temperature and precipitation

triacanthos), and Eastern red cedar *(Juniperus virginiana).* Other vegetation included ragweed *(Ambrosia spp.)*, skunkbrush *(Rhus trilobata)*, poison ivy *(Toxicodendron radicans)*, green briar *(Smilax glauca)*, sand plum *(Prunus angustifolia)*, honeysuckle *(Lonicera sp.)*, sericea lespedeza *(Lespedeza cuneata)*, passion flower *(Passiflora incarnata)*, goldenrod *(Solidago spp.)*, marestail *(Conyza Canadensis)*, Illinois bundle flower *(Desmanthus illinoensis)*, doveweed *(Croton sp.)*, ironweed *(Vernonia sp.)*, wood sorrel *(Oxalis stricta)* and other miscellaneous varieties.

2.1.3   Plot Construction

2.1.3.1   *Site Preparation*

The site had been previously cleared using two dozers that pulled a large chain between them, leaving the area scattered with dead logs and brush *(Figure 7)*. The site had to be recleared with a tractor using a brush hog mower. Dead logs and brush were pushed aside, clearing an area approximately 150 feet square *(Figure 8).* The two trenches, east trench and west trench, were marked in a north-to-south orientation. Both trenches were measured, outlined with string, and the ground was spray-painted with a dashed line. Trench dimensions were approximately 6½ to 7 feet wide by 100 feet

Figure 18. Condition before mowing and clearing

long (*Figure 9*). Wooden stakes were driven into the ground to mark the four corners on both trenches. Care was taken to ensure ample distance between the trenches to install fencing while maintaining a comfortable work area.

2.1.3.2   Swine Euthanasia

One hundred cull sows weighing an average of 300 pounds each were separated into two groups of 50 each and euthanized with penetrating captive bolt 1 week apart, in accordance with the Institutional Animal Care and Use Committee Protocol VM1919. Death was confirmed by the absence of a heartbeat, absence of a corneal reflex, and cyanosis of mucous membranes. After animal death was confirmed, the carcasses were taken to the study site.

Figure 19. Condition after mowing and clearing

2.1.3.3   Carcass Staging Area

To prevent carcass residue from accumulating on the ground and attracting potential scavengers and insects, the team constructed an 8-foot wide by 16-foot long staging area with ¾-inch CDX plywood and 2x6 boards placed on edge creating a containment area (Figure 10*). String-reinforced (6-mil) polyethylene construction fabric was placed inside the structure covering the bottom and draping over the sides to secure it in place (Figure 11). Twenty-four cubic feet of pine shavings were scattered inside the staging area. Shavings were used to absorb fluids from the carcasses. Batches of five pigs each were unloaded to prevent overwhelming other field activities and to minimize attracting insects, which were part of the project study.
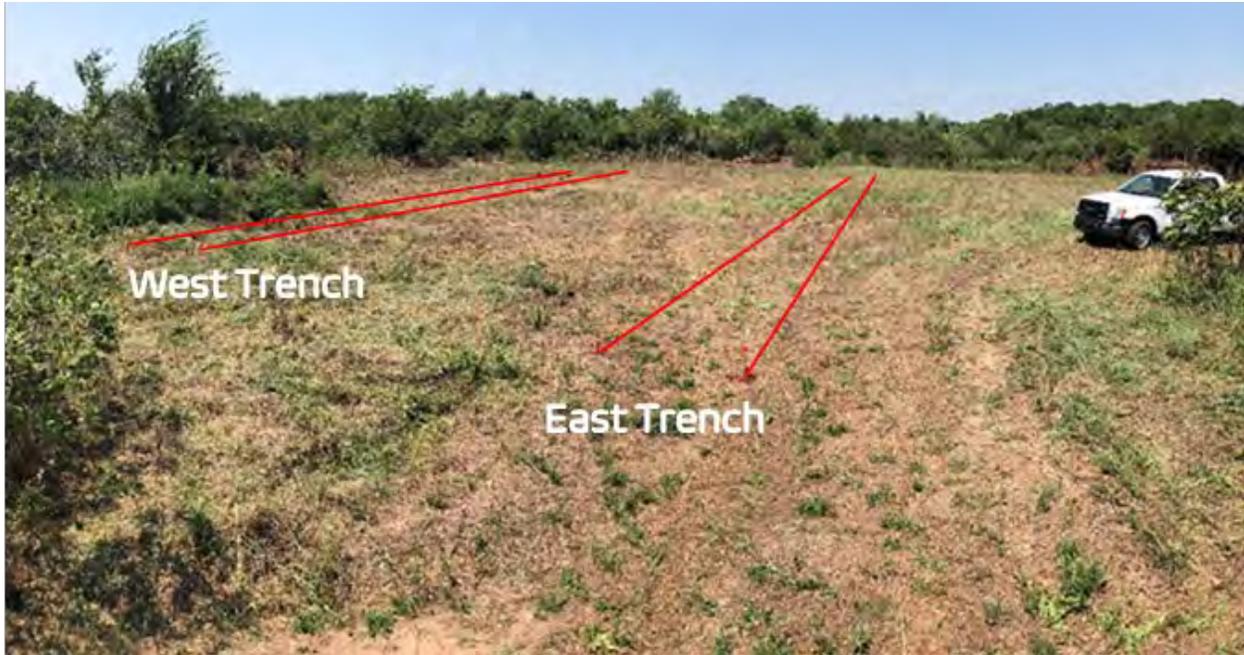
Figure 20. West and east trenches with corner stakes depicting trench lines

2.1.3.4 *Trench Excavation and Construction*

On September 3, 2019, the researchers excavated the west trench with a John Deere 310C backhoe. Recent heavy rainfall had softened the ground, causing the backhoe to sink into the moist soil, slowing

operations. The 12-inch trenching bucket was replaced with a wider general-purpose bucket as soils dried out to increase excavation speed and compensate for the delay. The trench was excavated to the dimensions described in the previous section along the spray-painted lines. The width of the trench was determined based on the length of the hogs plus 6 inches on either end of the animals. The west trench averaged 6 feet 4 inches wide (Figure 12).

Figures 20 and 20. Carcass staging area

Figure 21. Trench excavation and preparation

The trench was designed to be 20 to 24 inches deep, so a fencing stake was marked to 24 inches to serve as a depth gauge. The final average depth of the trench was 21.5 inches (Figure 13).

Excavated soil was piled along one edge of the trench, leaving space to maneuver equipment for back filling. The length of the trench was estimated based on 2.5-3 feet for each of the 50 mature sows, resulting in a total estimated trench length of 125-150 feet. The actual length of the west trench was 113 feet or 2.26 feet per sow. Once the trenches were excavated, pine shavings were placed in the bottom of the trench to a depth of approximately 12 inches. The shavings were scattered and roughly leveled with garden rakes *(Figures 14-15),* again using marked wooden stakes as a depth gauge. The team estimated the total volume of shavings procured by multiplying the trench width by the trench length by 1 foot of depth. Because wood shavings tend to compress, the procured volume was increased by approximately 25 percent, making the base layer slightly deeper than 12 inches. Pine shavings were selected as the carbon source for several reasons. First, they were readily available locally. Second, they provide a porous structure below the carcasses to promote aerobic decomposition. Third, they absorb leachate.

On September 10, 2019, the team constructed the east trench in the same manner as the west trench. The average depth of the east trench was 21.5 inches, and the average width was approximately 7 feet. The total length of the trench was 117 feet long or 2.34 feet per sow. The final average depth of the trench was 21.5 inches with an approximately 12-inch depth of shavings.

Figure 22. Researchers measuring trench depth

Figure 23. Trench with pine shavings

Figure 23. Researchers placing carbon in the trench

2.1.3.5 *Carcass Placement*

Initially, the carcasses were moved from the staging area to each trench with a backhoe. However, the backhoe had a mechanical failure mid-way through the effort, so a skid loader was used to finish the

placement of the carcasses. The carcasses were lifted with shackles and a chain from the staging area and transported to the trench. Field operators guided each carcass into the trench by grasping the front legs while the skid loader operator lowered the bucket. The carcass was then unshackled, with the operators making final adjustments to ensure the head or other extremities were not above ground level. A hay hook was used for final positioning. This process was repeated until 50 sows were placed in

each trench. Wooden stakes were placed between every group of five pigs to easily distinguish between a sample group for future data collection. The stakes also served as an indicator of the depth of cap material.

Because the average carcass weight was greater than 300 pounds, the abdomen of each carcass was opened from belly to sternum to aid in the venting of gasses and to insert temperature sensors.

After the carcasses were placed in the trench, each femur was accessed by making a medial incision in the upper leg and drilling into the bone to create a passage to the bone marrow. A portion of bone marrow was removed to facilitate the inoculation of 10 mL of SwPV with a tissue culture infectious dose (TCID$_{50}$) of 10$^{6.8}$ per mL. The passage was sealed with a ½-inch threaded stainless-steel pipe plug fitting (Figure 16).
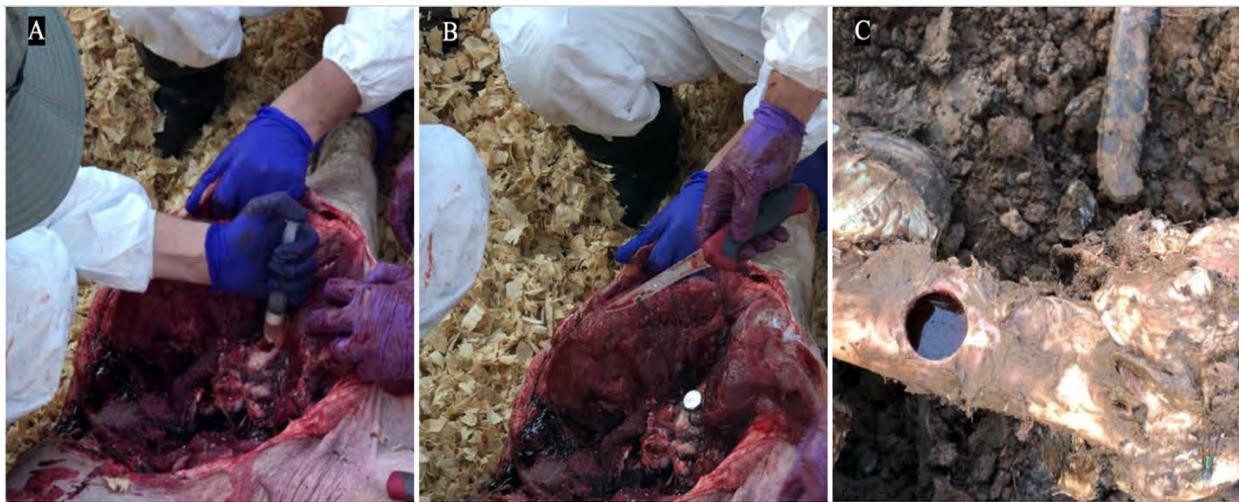
Figure 16. Virus inoculation and sampling. Femur was exposed, drilled and the SwPV inoculated (A). A metal plug was used to seal the inoculation access (B). For sampling, the metal plug was removed, and a syringe was used to collect the sample. Image of virus-bone marrow material 14 days post-inoculation (C).

Figure 17. Onset HOBO U12 Data Logger

2.1.3.6 Temperature Sensor Installation

Two Onset HOBO U12 outdoor/industrial use temperature data loggers with four channels (Figure 17) were used to measure temperatures in each trench. Two data-monitoring probe leads were placed on the 12th, 24th, 36th, and 48th carcass on top of the chest cavity *(Figure 18)* and inside the abdomen (Figure 19). To ensure the probe on top of the chest cavity did not move while backfilling the trench, the team either placed a stick parallel to the trench length or cut a small tab in the hide to stabilize the probe. Field technicians monitored the backfilling process and used hand tools to stabilize the probes if needed.

Figure 18. HOBO Sensor on carcasses
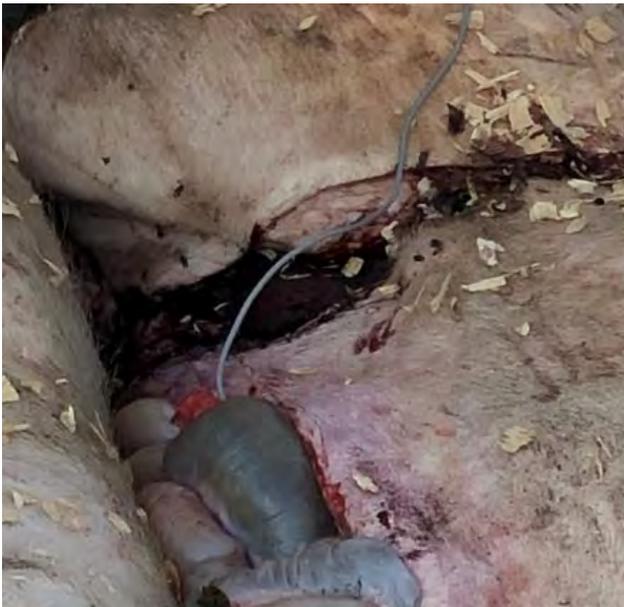
Figure 19. HOBO Sensor in the abdomen

2.1.3.7 *Soil Cover*

After femur inoculation and sensor placement, the excavated soil was gently pushed back on top of the trench (Figure 20) with the front-end loader, forming a dome shape while ensuring no femurs or sensors were damaged. The team used shovels and rakes to fine-tune the configuration of the trench dome geometry to facilitate precipitation run-off. Equipment operators avoided compacting the plots with heavy equipment.

Figure20. Finished trench with wooden stakes

2.1.3.8 *Trench Revegetation*

Annual ryegrass *(Lolium multiflorum)* was applied to the cover soil with a hand-held broadcast applicator to establish cool-season vegetative cover.

2.1.3.9   *Fencing and Camera Installation*

2.1.3.9.1   Fencing

Upon completion of each trench, 5-foot tall by 16-foot long fence panels were installed around each trench on all four sides (Figure 21). The purpose of the fence was to prevent scavengers from accessing the plots.

Figure 21. Fence panels around the east trench

2.1.3.9.2   Cameras

The team installed four BlazeVideo No Glow Infrared Trail Hunting Game Cameras to monitor scavenger activity at the site. These cameras were installed on the inside of the fence panels at the north or south ends of each trench. Two of these cameras were set up to take motion-activated photos 24 hours a day. The other two cameras were set up to take a daily photo at approximately the same time each day to gather time-lapse images.

Additionally, two Spartan 4G LTE GoCam Wireless Trail Cameras were installed to provide real-time photos via email based on motion-detected activity. One Spartan camera was installed on an 8-foot T-post located on the north end of the west trench and the second Spartan camera was installed on an 8-foot T-post on the south end of the east trench.

2.2   Virus Testing

2.2.1   Surrogate Selection

Because the ASF virus is a severe threat to pork production, it is considered a select agent and requires strict containment practices. Therefore, the actual virus could not be used in these field experiments,

and a surrogate was required. Although there are no other viruses in the same family as ASF, the swinepox virus is similar in structure and was once classified in the same family as ASF, based on a personal conversation with Dr. Manuel Borca in 2019. Based on that similarity, and the fact that swinepox is endemic in the United States and can be controlled with good sanitation, it was used as a substitute for ASF in these experiments.

2.2.2   Virus amplification

Porcine kidney cells (cell line PK15) were cultured at 37°C with 5 percent CO2 in MEM medium (Corning) supplemented with 10 to 20 percent fetal bovine serum (FBS; Seradigm), 2 mM l-glutamine (Corning), 1 percent Antibiotic-Antimycotic 100X (Gibco), and gentamicin (50 μg/ml; Corning). The swinepox virus (SwPV; NVSL catalog number 002-PDV) stock was prepared in 70 percent confluent PK15 cells in T175 flasks using a total volume of 30ml of MEM per flask. Two sets of the virus were prepared—each set consisted of 70 flasks. The two sets were titrated using a method previously described (Finney 1952), and the lot with the highest viral titer was used in the study.

2.2.3   Virus characterization

2.2.3.1   *Inoculum metagenomics and quality control*

To perform quality control of the SwPV inoculum, the researchers conducted metagenomics sequencing. For this, 50ml of the SwPV stock was ultracentrifuged on a 25 percent sucrose cushion at 100,000 × g for 3 h at 4°C in a Sorvall AH629 rotor. The pellet containing the viral particles was incubated for 90 minutes with DNase and RNase (Thermo Fisher Scientific, Waltham, MA, USA) following a protocol previously described (Thurber et al. 2009). DNA extraction used a phenol-chloroform protocol (Sambrook and Russel 2001).

Viral DNA was enriched through multiple displacement amplification (MDA), using GenomePlex® Complete Whole Genome Amplification Kit (Sigma-Aldrich, St. Louis, MO, USA). Viral RNA was reverse-transcribed and further enriched using the SeqPlex RNA Amplification Kit (Sigma-Aldrich, St. Louis, MO, USA) following the manufacturer's recommendations. The enriched DNA products were purified using the PureLink™ Quick Gel Extraction and PCR Purification Combo Kit (Thermo Fisher Scientific). The quality and quantity of the DNA were assessed through spectrophotometry and fluorometry performed with NanoDrop™ (Thermo Fisher Scientific) and Qubit™ 4 (Thermo Fisher Scientific), respectively. The viral libraries were prepared with 1 ng of purified DNA using the Nextera XT DNA Library Preparation Kit and sequenced using the Illumina iSeq 100 System conducted with the Illumina i1 reagent kit (2 × 150 paired-end reads).

Additionally, technicians at the Oklahoma Animal Disease Diagnostic Laboratory tested the viral stock for bovine respiratory syndrome virus (BRSV), porcine reproductive and respiratory syndrome virus (PRRSV), Senecavirus A (SVA), and swine influenza virus (SIV) using qPCR, following standard diagnostic protocols.

2.2.3.2   *Metagenome analysis*

The quality of the generated sequences was evaluated using the FastQC tool. Only high-quality sequences were selected for the analysis. The data was de novo assembled on BaseSpace Cloud (Illumina) with the metaSPAdes genome assembler (Version 3.0). Sequences were mapped to a

reference sequence 17077-99 (Genbank accession number AF410153), and gene annotations transferred using the Geneious Prime software (2020.2.1) as previously described (Paim et al., 2021).

2.2.4   Bone Marrow Sample Collection

Background samples (10 femurs from each trench) were collected on the day of inoculation after spiking the femurs. The samples were stored at -70°C as a positive control for the study. Twenty femurs were collected from 10 carcasses, half from each trench, at around days 7, 14, 21, 28, 60, 90, 180, and 360 after inoculation. Bone marrow samples were collected by locating the marked carcasses, removing the plug that had been placed in the femur during construction, and aspirating the bone marrow material with a syringe (Figure 17c). For samples without liquid material remaining (most of the femurs after Week 4), 10ml of phosphate-buffered saline was added to the femur, and the solution was gently moved within the femur cavity to aid recovery of viral material.

2.2.5   DNA extraction and qPCR

Viral DNA was extracted from collected femur samples using the MagMAX viral RNA/DNA isolation kit (Life Technologies), according to the manufacturer's instructions, in an automated nucleic acid extractor (KingFisher Purification System; Thermo Fisher Scientific).

The following primers and probe sequences were used:

Primer Pox3 (forward) 5'-TCAGTACATCCAATTGTCAAGGA-3'

Primer Pox3 (reverse) 5'-CTGGCTAAATAGAATGAGTGAAACG-3'

Probe Pox3 5'-[6FAM]ACTTCCAGAAACGAGTAATCCTTACAAGAC[BHQ-2]-3'

Amplification and detection were performed with an Applied Biosystems 7500 real-time PCR system. The qPCR conditions employed were 40 cycles of 50ºC for 30 sec followed by 95 ºC for 10 sec and 62.5°C for 30 sec.

2.2.6   Virus isolation from bone marrow samples

Virus isolation was performed on the mixture of viral and bone marrow material in PK15 cells. Due to the microbial contamination of the sample, an initial dilution of 1:20 was conducted in the material. About 150 µl of the sample was inoculated in 70 percent confluent PK15 cell layer. The first passage was inoculated and incubated for 48 hours. Four additional serial passages were conducted with an incubation period of 96 to 120 hours. Samples were monitored daily for the presence of cytopathic effect characteristic of SwPV. The virus isolation procedure was conducted in 24-well plates using cell culture reagents and conditions described earlier for the virus amplification.

2.2.7   Statistical analysis of femur data

Statistical analysis of field femur data was performed using the statistical software R. Team members used a Bayesian analysis of the virus isolation data to estimate the lower bound on the proportion of nonviable virus in the population at the end of the study. In general, a population is the broadest group that we want to make inference on, and a sample from that population is used to make this connection. For this experiment, the sample is the group of 100 pigs and their spiked femurs physically buried using

the shallow burial with carbon technique, as previously described. The population is the theoretically infinite group of pigs buried in the same manner as in this experiment. The sample size limited the precision of the estimated proportion of nonviable virus in the population; increasing the number of pigs in this study would have led to a more precise or higher value for the lower bound on nonviable virus, presuming their virus viability outcomes mimicked the results found in this study. However, increasing the number of pigs used in this study was not logistically or monetarily feasible.

A linear calibration regression model was fit to the log10 concentrations from the four Day 0 samples evaluated (two syringes of inoculum and two of the extra femurs taken to the field but not used) and the detection limit of $10^{1.8}$ TCID$_{50}$/ml on Day 7. Due to limited data above the detection threshold from the virus titration, we could not evaluate goodness of fit.

2.2.8   In-Vitro Study of Virus Survival in Bone Marrow Cells

To further support the findings from the SBC Project, the team performed a bench scale study to explore the effect of the bone marrow environment on SwPV inactivation since this effect was not directly evaluated in the SBC Project. The viability of SwPV and additional viruses were assessed in bone marrow samples with the limited introduction of other contaminants using an in-vitro system (in a test tube, culture dish, or elsewhere outside a living organism). The tested viruses included the SwPV, bovine viral diarrhea virus (BVDV), porcine parvovirus (PPV), and SVA. The BVDV (from the same viral family as the classical swine fever virus) is a virus that under typical pathogenesis replicates in cells present in the bone marrow, and there are reports of pigs infected with BVDV. Therefore, the swine bone marrow could be considered a friendly environment for BVDV. The PPV and SVA are known to be viruses with increased resistance to various environmental conditions including heat and, because of that, were included in the study as positive controls.

For the amplification of the four viruses, three cell lines were used: PK15 for SwPV; ST for SVA and PPV; and MDBK for BVDV amplification. Cells were cultured using protocols described in previous sections of this report. Media used for viral stock preparation was then restricted of the antibiotics and antimycotic to reduce interference on the tissue microbial. The titration methodology and virus titer calculations were conducted using the method previously described (Finney 1952). The initial titers for SVA were about 10^9 TCID50/ml while initial titers for SwPV, PPV, and BVDV were around 10^7 TCID$_{50}$/ml.

Bone marrow was collected from the femurs of pigs over 4 months of age in a biosafety cabinet. Bone marrow samples were mixed 1:1 with the virus (500mg of tissue with 500ul of virus solution). As a control, tubes containing only the virus were prepared. The samples were kept at room temperature. The sampling points were at days 0, 2, 5, 7, 10, 15, 20, and 30 post-inoculation. For each of the time points, the team collected three samples and three controls and tested for virus viability by viral titration. Viral titrations used serial log10 dilutions of the samples to assess the level of infectivity remaining in the samples and controls. The use of indirect immune fluorescence assay using conjugate antibodies (VMRD) was used for BVDV and PPV to validate the results.

In addition to the bone marrow testing, house flies were also exposed to SwPV, SVA, PPV, and BVDV in a controlled system to explore the fly's possible role in transporting viruses from the depopulation site. See Section 2.4 for more details on this aspect.

Statistical analysis of *in-vitro* data

The study team completed the statistical analysis of in-vitro data using the statistical software R. A regression model with unequal intercepts and slopes for the control and bone marrow samples was fit to the SwPV TCID$_{50}$ values across time. A Student's t-test was used to test equality of intercept and slopes..

2.3 Environmental Sampling and Analysis

2.3.1 Soil

2.3.1.1 *Background and 12-Month Soil Sampling*

After trench excavation but prior to placement of shavings in both trenches, the team collected background soil samples from two separate locations in each trench (Figure 22) approximately 50 ft. from the ends. For each sample location, a 4-in bucket auger was used to collect soil samples in 6-in intervals to 180 inches deep. Each interval was composited, mixed, bagged, and labeled for delivery to the Soil, Water, and Forage Analytical Laboratory (SWFAL) to be analyzed. The results from the two sample locations for each trench were averaged to establish background samples for each 6-in. horizon.

2.3.1.2 *Two-, Four- and Six-Month Soil Sampling*

At 2, 4, and 6 months after trench construction, one location in each trench was sampled at 6-inch intervals to 180 inches of depth and prepared as described in the previous section.

2.3.1.3 *Analytical Parameters*

SWFAL analyzed each soil sample for the following parameters:

- pH (1:1), Lime Requirement (Sikora, 2006) method
- Nitrate-nitrogen (NO3-N), Soil Test P&K (Mehlich, 1984) method
- Ammonium nitrogen (NH4-N) (1M KCl) method (LACHAT, 1994)
- Textural Class (Hydrometer); Percent sand, silt and clay of each 6-inch interval
- Percent Organic Matter (%OM)
- Percent Total Nitrogen (%TN) (LECO) method (Nelson and Sommers, 1996)
- Secondary Nutrients (magnesium (Mg), calcium (Ca) (Mehlich, 1984), (Soltanpour et al., 1996), and sulfate ion (SO4) (0.008M Calcium Phosphate)
- Salinity Management Parameters (1:1 Soil to Water Extraction) including Sodium (Na), Calcium (Ca), Magnesium (Mg), Potassium (K), Boron (B), (Soltanpour et al., 1996), Electrical Conductivity (EC), Total Dissolved Solids (TDS), Sodium Adsorption Ratio (SAR), Exchangeable sodium percentage, (USDA, 1954) and pH.

Figure 22. Researchers collecting background soil samples

2.3.2   Groundwater

Groundwater was absent from all 180-inch deep soil borings. The Oklahoma Water Resources Board online portal indicates the nearest groundwater well is 1.4 miles southwest of the test site, where the water table is 49 feet below the ground surface. Other wells within a 2- to 3-mile radius are also approximately 45-50 feet deep, which validates that groundwater is not expected at 180 inches below ground surface at the test site. Groundwater will not be discussed further in this report.

2.3.3   Leachate

After a heavy rain event on September 25, 2019 (approximately 2 weeks after plot construction), a red liquid was observed pooled over an estimated 60 by 30 inch area adjacent to the east trench. The team collected a grab sample of the red liquid in a plastic wide-mouthed sample bottle. Another sample of clear liquid pooled adjacent to the west trench was collected in a similar manner. The team sent both samples to OSU, where they were analyzed for total coliforms. The leachate was also analyzed for swine pox virus using qPCR as described in Section 2.2.5.

2.3.4   Forage

Six months after plot construction, the researchers collected vegetation growing over the trenches and in nearby undisturbed areas to determine if there was a significant difference in quality based on growth location. They collected annual ryegrass forage (*Festuca perennis*) by clipping the top 4 to 5 inches of green growth from multiple locations above each trench and replicated the same procedure from multiple locations in the undisturbed area around the plots. The vegetation was placed in paper bags, one for each source, and sent to the SWFAL as two sets of composite samples (see Figures 23-26). Samples were analyzed for the parameters listed below in accordance with National Forage Testing Association guidelines.

Moisture (%) = amount of moisture
Protein (%) = amino acids essential for reproduction, lactation, growth, and maintenance of the body
ADF (%) = acid detergent fiber which is a measure of cellulose and lignin. Higher percent means lower digestibility. Ideal is about 25-50 percent.
TDN (%)= total digestible nutrients as a sum of digestible protein, digestible carbohydrates, and 2.25 times the digestible fat.
Maint (%) = net energy for maintenance; an estimate of the energy value of a feed to maintain animal tissue with gain or loss of weight.
Lact (%) = net energy for lactation which estimates the energy available from the feed to support an animal's requirements for maintenance plus milk production.
Gain (%) = net energy for gain; an estimate of a feed energy value for body weight gain above the energy required for maintenance.

**Figure 353. Forage quality on plots
compared to surrounding area – east trench**

Figure 364. East trench

Figure 365. West trench

Figure 376. West trench

2.3.5 Olfactory Qualitative Assessment

At 2, 4, 6, and 12 months after plot construction, while soil samples were being collected, various odor levels were observed and documented. The odors were characterized according to the scale shown below.

Scale	Categorical Descriptions
1	Little to no smell
2	Disturbed soil
3	Earthy soil
4	Sweet soil
5	Mild earthy and sweet
6	Slight hog non-decomposing smell
7	Hog non-decomposing smell
8	Slight decomposing smell (no ammonia)
9	Moderate decomposing smell (slight detectable hints of ammonia and sulfur compounds present)
10	Active decomposing smell (ammonia and sulfur type smell easily detected).

2.3.6 Weather

The Lake Carl Blackwell Mesonet Station, located approximately 360 feet southwest of the study site (Figure 27), is located 293 meters above mean sea level (961 feet). The station contains a set of

environmental measuring instruments located on or near a 10-meter-tall tower. The measurements are packaged into "observations," then transmitted to a central facility 24 hours per day year-round. The

Figure 387. Lake Carl Blackwell Mesonet Station

station collects a variety of data at 5, 10, or 30-minute intervals. The measurements of interest for this project included daily rainfall, air temperature at 1.5 m above the ground surface, soil temperature at 10 cm below the ground surface, and soil temperature at 60 cm below the ground surface.

2.3.7 Wildlife

Wildlife visits were measured by reviewing trail-cam photos from the cameras described in Section 2.1.3.10.2. Approximately 200,000 instances of motion were captured during the year-long study. The team reviewed images captured for the first 2 months and the last month to identify any wildlife approaching the plots or entering the plot areas. The numbers and types of wildlife were recorded and analyzed.

2.4 Insect Monitoring

2.4.1 Sample Collection, Classification, and Analysis

2.4.1.1 Collected Insect Virus Testing

During femur collection at days 7, 14, and 21, when the soil cap was removed, the researchers collected insects encountered in the soil for virus testing.

2.4.1.2 Vicinity Insect Traps

Two trap types were used for this study: malaise and pitfall. The malaise traps were H-shaped traps from Bio Quip and were established according to the manufacturer's directions (Figure 28). The preliminary/control malaise trap was set with openings running north and south while the animal mortality trench malaise traps were oriented east and west.

Figure 39. Malaise trap set up for the preliminary site

The pitfall traps were constructed by using 1,000 mL paint cups with lids, garden edging, and garden stakes (Figure 29). The garden edging was cut into 60 cm lengths and staked into the ground with two garden stakes. Each trap set had one central cup and three garden-edging rays with another cup at the end of each ray. The lids of the paint cups were suspended over the cups with two garden stakes to help prevent rain from entering and to reduce evaporation. The lip of each cup was placed at ground level. Prestone® lowtox antifreeze was added to the pitfall traps as a killing and preserving agent.

Figure 40. Pitfall trap set. A total of 23 arrays were established for this study.

Preliminary traps were set out 2 weeks before the animal mortality trenches were excavated. These traps consisted of one malaise and three sets of pitfall traps. The purpose of these traps was to set a baseline to compare to the traps placed near the trenches since space was limited. The traps were collected every 2 to 3 days and then became the control traps once the first set of pigs were placed in the animal mortality trenches. The team set the traps over the trenches the day after animal placement. The pigs were placed in two sets, one week apart. Ten sets of pitfall traps were placed around each trench, five on each side, and one malaise trap was placed per trench. The final trap layout is shown in Figure 30. The team collected insects from the traps every 2 to 3 days for the first month and then increased the time between trap collection with a decrease in specimens collected. The contents of the traps were placed in 70 percent ethanol for later identification. Traps were repaired and reset as needed. Figure 31 shows one of the trenches after the insect traps were installed.
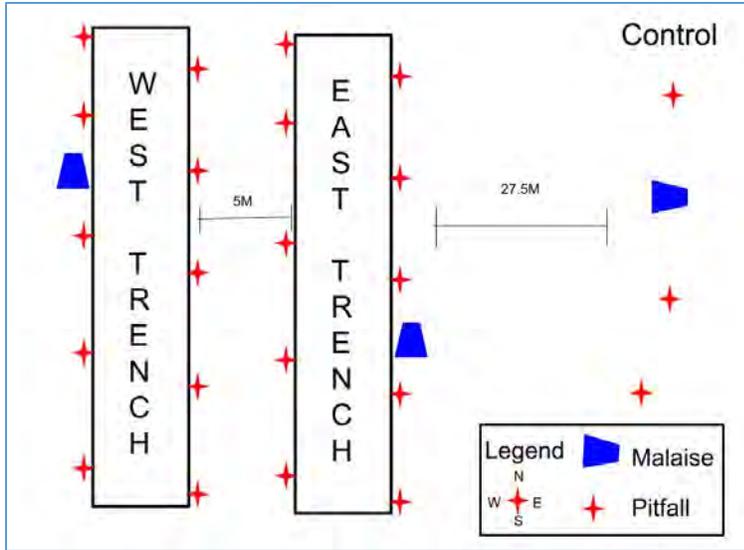
Figure 30. Map of the field site and trap layout

Figure 411. Animal mortality trench after animal and trap placement

2.4.1.3   Sample Classification

All insect samples were classified down to insect order using common diagnostic keys. Specialized diagnostic keys were used to further classify down to forensically important species of beetles and flies.

2.4.1.4   Data Analysis

All insect data analyses were conducted within the first month of animal placement in the animal mortality trenches due to a lack of specimens after this period. Using the response variable "abundance," the total number of forensically important species within Coleoptera (beetles) and Diptera (flies) were compared by trap type between the animal mortality trenches and control site and between the preliminary site and animal mortality trenches. The association between different sampling areas (animal mortality trenches, control, and preliminary sites) and abundance of forensically important insects was tested using a Chi-Square test with a p-value less than 0.05 to denote significant differences. Further diversity and abundance indices (such as Simpson's and Shannon-Wiener) were used for insect orders, Coleopteran families (beetles), and Dipteran families (flies) to compare the different sampling areas. A predictive degree-hour model was developed based on previous work done for specific species for common calliphorid flies (blow flies) and the two proven vectors of ASF virus, *Musca domestica* (house fly), and *Stomoxys calcitrans* (stable fly) (Olesen et al. 2018, Herm et al. 2020).

2.4.2   In-Vitro Virus Viability in house flies (Musca domestica)

2.4.2.1   Viruses and cells

In the present study, swinepox virus was selected as a surrogate for ASFvirus, along with SVA, and BVD virus as surrogates for foot-and-mouth disease (FMD) virus and classical swine fever (CSF) virus. Surrogate viruses for high impact animal diseases have been used to evaluate potential viral transport by house flies. SVA belongs to the family *Picornaviridae*, the same family as FMD virus, while CSF virus and BVD virus both belong to the family *Flaviviridae* (ICTV, https://ictv.global/taxonomy/).

The SwPV virus NVSL (catalog number 002-PDV) and SVA strain Hawaii were amplified in ST (Swine testis) cells, while the BVDV1 strain Singer was amplified in Madin-Darby bovine kidney (MDBK) cells. Cells were cultured at 37°C with 5 percent CO_2 in MEM medium (Corning) supplemented with 10 to 20 percent fetal bovine serum (FBS; Seradigm), 2 mM l-glutamine (Corning), 1 percent Antibiotic-Antimycotic 100X (Gibco), and gentamicin (50 μg/ml; Corning). Virus titration was conducted using the Reed–Muench method (Reed and Muench, 1938). The titer for SVA was about 10^9 tissue culture infectious dose ($TCID_{50}$/ml) while the titers for SwPV and BVDV were around 10^7 $TCID_{50}$/ml.

2.4.2.2   House Flies

The flies used in this study were house flies (*Musca domestica*) in the family Muscidae, from a lab colony at Oklahoma State University. The flies used for the trials were at least 10 generations removed from the initial population. They were housed at 80 percent humidity and 26.7^0C. The larvae were raised on a mixture of wheat bran, calf manna, and vermiculite.

2.4.2.3   Study Design

For the exposure of each virus, 120 newly emerged and unfed house flies were divided into five cages. Each cage measured 30.5 cm^3, had aluminum screen sides and a mesh stockinette sleeve, and held 24 flies (12 males and 12 females). Two milliliters of virus solution were placed in a 25 mm petri dish and set inside each cage. A dish with 10ml of the viral solution was added to the cage. The titer was about 10^9 $TCID_{50}$/ml for SVA, and around 10^7 $TCID_{50}$/ml for SwPV, PPV, and BVDV. The virus solution was

removed after 1 hour and water and sugar cubes were placed in the cages through the sleeve. Flies from one cage were collected in each of the sampling points: 0, 6, 12, 24, and 48 hours post-exposure. To collect the flies, the cage was placed in a -20 °C freezer for at least 10 minutes at each time point. The flies were then moved from the cage to microtubes and placed in a -80 freezer for later processing.

For each time point, five male and five female flies were tested. Viral nucleic acid was extracted from the flies using a commercial kit (Zymo Research). The viral nucleic acid detection was conducted by real-time PCR using primers and probes specifically designed for each viral strain used in the studies. PCR conditions were the same as described for SwPV. The research team evaluated infectious particles present in the tested samples as described earlier, using the appropriate cell line for each virus.

2.4.2.4 *Virus Isolation in Exposed Flies*
Five hundred microliters of PBS (Corning) were added to the flies for virus isolation processing. Microtubes containing the flies were crushed with a pestle and bead beat twice at 2,000 rpm for 2 minutes. Samples were then centrifuged at 5,000 x g for 10min. The supernatant was used as the inoculum. Virus isolations (VI) were conducted in 24 well plates with 70 percent confluent cells. For each virus, the VI procedure was conducted in the appropriate cell line as described above. A total of 100 µL of the supernatant was incubated and adsorbed for 1 h. The inoculum was then removed from each well and washed three times with 500 µL of MEM. After washing, 500 µL of complete MEM was added to each well and the plate was incubated for 48 to 96 h. A total of three passages was conducted. Cells were examined daily for cytopathic effect. At the end of the third passage, plates were frozen and thawed and the supernatant was used to extract nucleic acid using the Quick-DNA/RNA Viral kit (Zymo® Research) following the manufacturer's protocol. The nucleic acid was then submitted to qPCR (SwPV samples) or RT-qPCR (SVA and BVDV samples).

2.4.2.5 *Nucleic acid amplification - qPCR and RT-qPCR*
After the extraction, nucleic acid amplification was conducted using Luna® universal probe qPCR master mix (New England Biolabs) for SwPV and Luna® universal probe one-step RT-qPCR kit for SVA and BVDV. The reactions were prepared following the manufacturer's recommendations. Two microliters of extracted viral nucleic acid were added to the master mix, containing specific primers and probes as described in Table 1.

All samples were run in an Applied Biosystems™ 7500 Real-Time PCR (Thermo Fisher Scientific). DNA samples were run for 1 cycle at 95°C for 60 seconds, followed by 40 cycles at 95°C for 15 seconds, and 60°C for 30 seconds. The RNA samples had an initial hold phase at 55°C for 10 minutes, followed by 95°C for 60 seconds, and then 40 cycles at 95°C for 10 seconds, and 60°C for 30 seconds. As a positive control, specific synthetic DNA fragments with 500 base pairs were used (gblocks® gene fragment - Integrated DNA Technologies).

Gblocks were diluted in TE in a log10 series. Dilutions were run as described above and enabled the estimation of the nucleic acid copy numbers. Ultra-pure water was used as a negative control. Both samples and controls were run on the same plate and in duplicates.

Table 1. Primer and probe sequences used for each virus

Virus	Primer Probe	Primers
SVA	5'-/56-FAM/CGGATTAGCGGGTCTCCTCACAAA/36-TAMSp/-3'	5'-GTAGCCAAGAGGTTCAAGATT-3' 5'- CAGTAGACTTCTCGACCTCCT-3'
BVDV	5'-/56-FAM/ACAGGGTAGTCGTCAGTGGTTCGA/36-TAMSp/-3'	5'-TGGCATCTCGAGACCTTTATTC-3' 5'-AGTGGTGAGTTCGTTGGATG-3'
SwPV	5'-/56-FAM/AACATCGAGGACTTTGCTCCGGAC/36-TAMSp/-3'	5'-GTCGTCGGTCGCTGTTAAAT-3' 5'-TGGTTCACCCGGTAGATAGT-3'

2.4.3   Data Analysis

Insects collected from the trenches during the first 4 weeks of the study were tested by qPCR as described in Section 2.2.5, above. The only modification was that the DNA was extracted using Quick-DNA Tissue/Insect Kit following the manufacturer's instructions (Zymo Research).

3   Findings

3.1   Construction Logistics and Implementability

3.1.1   Implementability

The team used a John Deere 310C backhoe with a front end loader to construct both trenches. The backhoe was delivered with a narrow trenching bucket (~ 10 inches wide), which resulted in slow construction progress. A wider trenching bucket (~ 24 inches wide) was delivered to the site and installed on the backhoe. The wider bucket improved overall efficiency and time of construction. Additionally, the site had soft working conditions during construction of the west trench, which caused minor challenges in the operation of heavy equipment.

3.1.2   Site Restoration

At completion of the study after 12 months, the plots were regraded to original topography with a John Deere tractor with a standard 6-ft wide box blade. No bones or tissue were visible. One area on the east trench was still soft where samples had recently been collected and the tractor tire sank into the pit, forcing some buried tissue to the surface. The soft area was manually backfilled and regraded with extra soil. Figure 32 shows the final site condition.

Figure 452. Final site condition and vegetative cover

3.2   Virus Survival

3.2.1   Virus amplification and characterization

The viral titer for the two lots of SwPV was $10^{6.0}$ TCID$_{50}$/ml for Lot 1 and $10^{6.8}$ TCID$_{50}$/ml for Lot 2. Lot 2 was selected for sequencing procedures and femur inoculation. About 99.5 percent of the genome was retrieved using the Illumina sequencing platform. The average coverage for the genome retrieved was 194× (Figure 33). The genome has over 149K base pairs in length and is about 99.93 percent similar to the isolate 17077-99. The team identified a total of 150 open reading frames (ORFs) (Figure 34). The SwPV inoculum was free of BRSV, SVA, SIV, and PRRSV by specific qPCR. And SwPV was the only pathogen identified in the inoculum by the analyses of the metagenomics results.
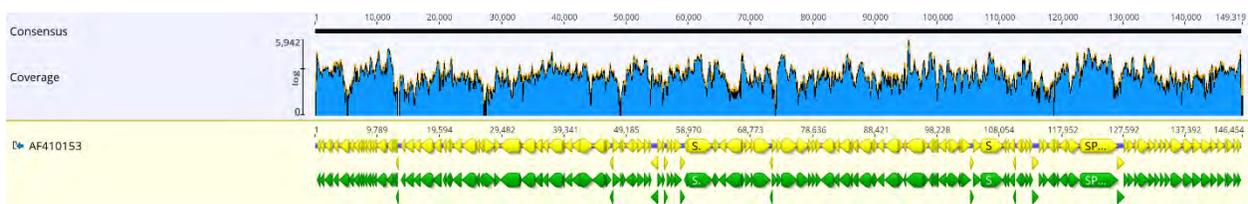
Figure 453. Schematic representation of the sequence reads mapped over the full-length genome of SwPV (blue bars represent the coverage)
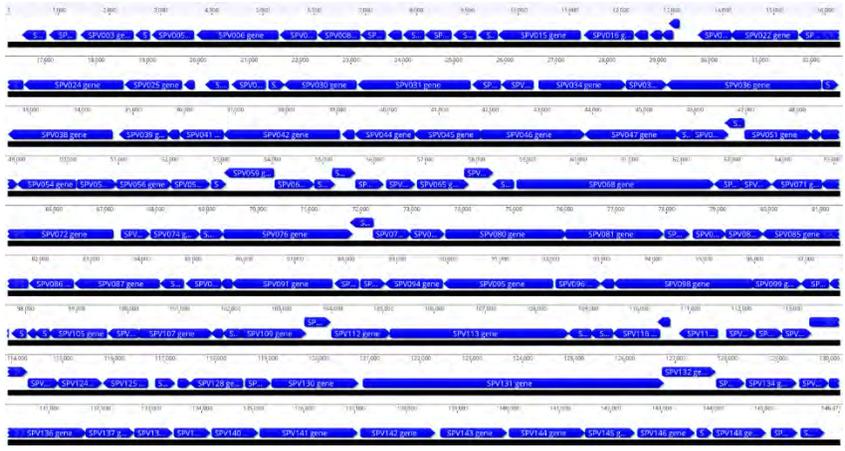
Figure 464. Schematic representation of the full genome of the SwPV used in the study. Blue arrows represent the 150 ORFs identified by similarity with the strain 17077-99

3.2.2 Nucleic acid detection by qPCR

Viral DNA was detectable by qPCR assay throughout the 12 months of the study in the majority of samples (Figure 35). The overall Cq values slightly varied over the tested period with a trend of increasing Cq values over time.
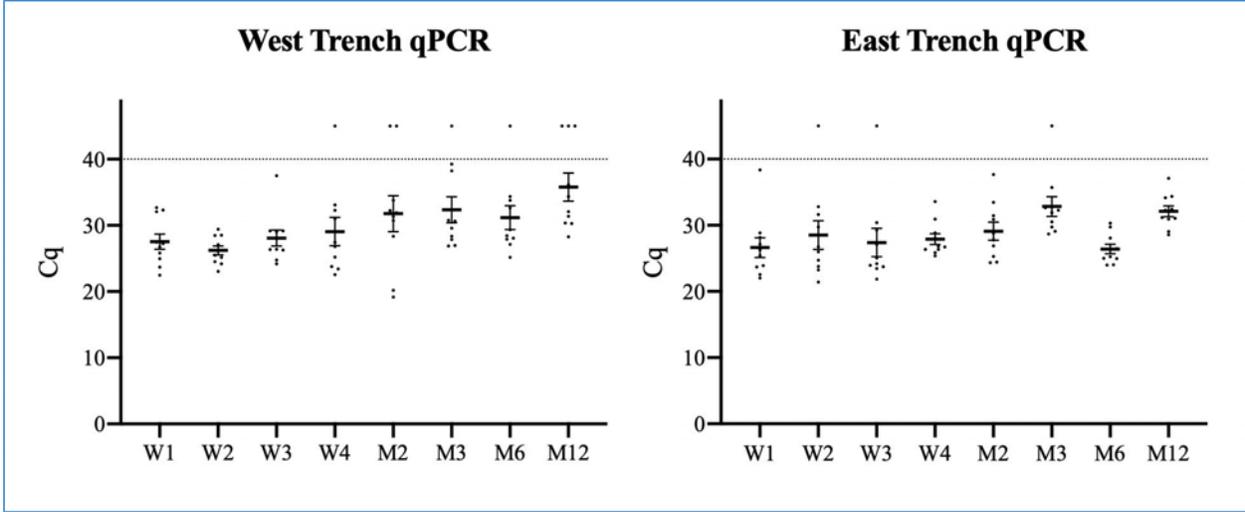
Figure 465. qPCR results for bone marrow samples collected during the study times points from week 1 (W1) to month 12 (M12). Samples represented over the dotted line are negative samples. Bars represent the mean of the Cq values for the positive samples with the standard error of the mean.

3.2.3 Swinepox virus viability in bone marrow

Viable virus was only detected in samples collected at Day 7 post-burial in 30 percent (3/10) of femurs from the west trench and 80 percent (8/10) of femurs from east trench (Table 2). All samples with infectious virus had a titer lower than $10^{1.8}$ TCID$_{50}$/ml, demonstrating rapid inactivation of the virus within a 1-week period. The differences in the results comparing trenches might be attributed to the decreased time of carcass exposure to direct sunlight, suggested by lower carcass temperatures

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recorded on day 0 for the east trench (Figure 38). Although they noted differences, the researchers did not observe a statistical significance between trenches.

Table 2. Virus isolation (VI) results for samples collected 7 days after inoculation

Sampling point*	Pig ID	Leg position (Top or Bottom)	Positive VI - West Trench	Positive VI - East Trench
Day 7	Pig 1 R	Top	X	X
	Pig 1 L	Bottom		X
	Pig 2 R	Top	X	X
	Pig 2 L	Bottom		X
	Pig 3 R	Top		
	Pig 3 L	Bottom		
	Pig 4 R	Top	X	X
	Pig 4 L	Bottom		X
	Pig 5 R	Top		X
	Pig 5 L	Bottom		X

* The samples from the remaining sampling points after Day 7 were all negative for viable virus

Numerous studies have shown that increasing temperature decreases virus viability. This study was initiated during late summer, with warm ambient temperatures serving to promote virus inactivation. Figure 36 shows the carcass temperatures recorded by the probes for the west trench (36A) and east trench (36B) during the first 14 days post-burial. For the first 10 days, the average of the internal and external temperatures of the monitored pigs in both trenches was above 90°F. It is important to note that the results found here may not apply to other situations in which the SBC method is used. Many environmental conditions affect viral inactivation, with temperature likely paying a major role.
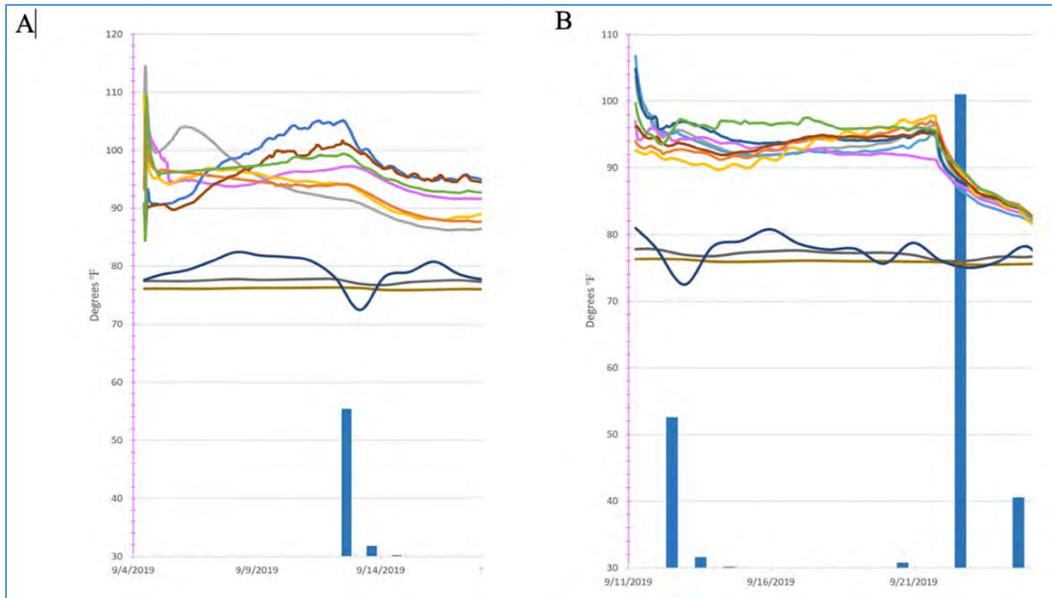
Figure 36. Temperature recorded during the first 2-weeks of the study for each trench. The group of lines around or above 90°F on day 0 are the temperature in the carcasses or between carcasses. The group of lines around 80°F represent the air temperature (blue), soil at a depth of 25 cm (grey) and 60 cm (brown).

3.2.4   Statistical analysis of femur data

No viable virus was detected in samples collected at day 14 or later post-burial from either trench. Assuming 99.9 percent sensitivity and specificity for the virus isolation process, a Bayesian analysis on the cumulative data from Day 14 to Day 360 estimates that the proportion of nonviable virus in the population after 52 weeks is greater than 98.4 percent with 95 percent confidence. This reflects a very high probability that no viable virus existed by the end of the study consistent with the rapid inactivation of the virus shown by viral titers. The analysis assumes that if no viable virus was detected on Day 14, Day 21, or any time point up to Day 360, no viable virus would have been detected on Day 360 had the sample remained buried. This estimate did not include Day 7 data because viable virus was detected at this time and disease freedom or zero detection analyses only apply when all samples are negative. The precision of the lower bound on the percent nonviable virus is limited by the number of samples contributing to the cumulative negative evidence available and is likely an underestimate of true proportion of nonviable virus in the theoretical population of pigs inoculated in the manner of this study.

Based on the linear calibration regression fit to log10 concentration, viral concentration is estimated to be at 1 $TCID_{50}$ in an average of 10.7 days. A confidence interval could not be provided because no estimate of variability was available. This is a conservative estimate because the model used the detection limit as the mean concentration on Day 7 even though concentration in all samples was below the detection limit. Nonetheless, the results demonstrated significant virus inactivation in a short period of time (less than 2 weeks). The viral concentration results are consistent with the lower bound of 98.4

percent nonviable virus from the viral viability results, supporting that the lower bound of nonviable virus would likely be much higher if a larger sample size was used in this study.

3.2.5 In-Vitro Study of Virus Survival in Bone Marrow Cells

3.2.5.1 *Virus viability in bone marrow samples*

Because the SwPV was inactivated in less than 2 weeks and the virus does not normally replicate in bone marrow cells, it was necessary to verify that the bone marrow environment was not the cause of the rapid inactivation, as discussed in Section 2. The viability results for the four tested viruses over a period of 30 days are presented in Figure 37A-D. Both SwPV and SVA remained positive over the 30 days in bone marrow samples. The remaining titer of both SwPV and SVA at the end of the study was about 70 percent of the initial concentration. Similar results were noted for the SwPV control.
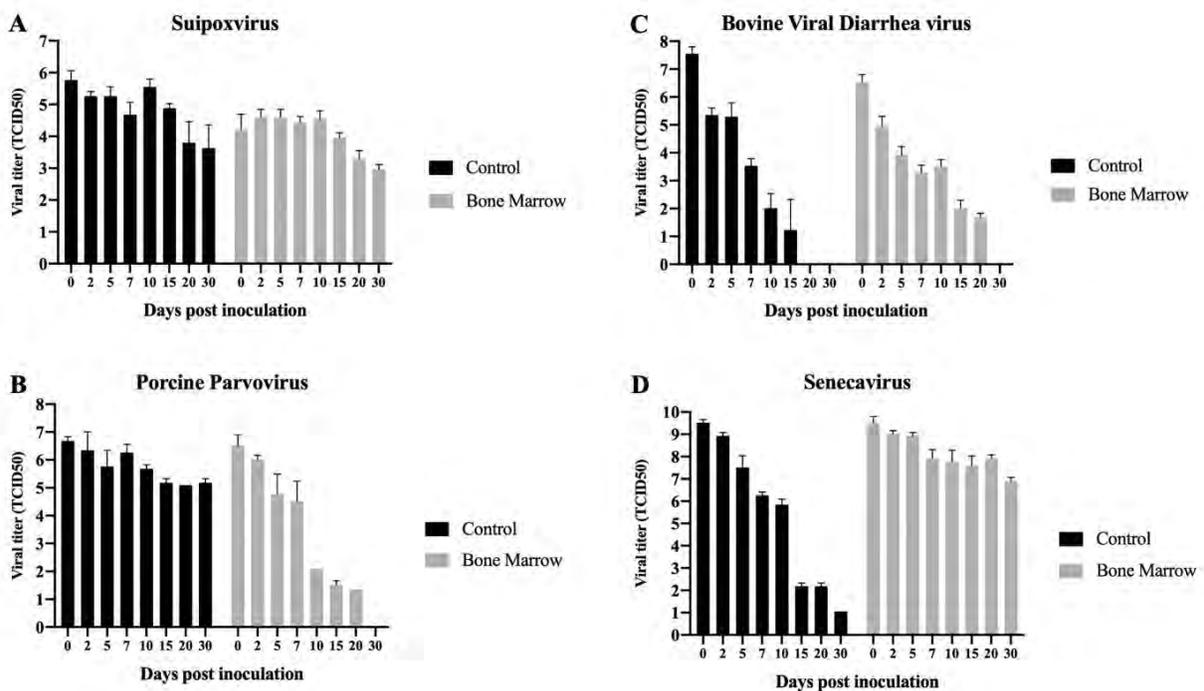
Figure 497. Viability results for SwPV (A), PPV (B), BVDV (C) and SVA (D). Bars represent the mean of the titers with the standard error of the mean.

For SwPV, there was no significant difference (p=0.344) in the estimated slopes of -0.068 and -0.056 $TCID_{50}$per day for controls and bone marrow samples, respectively (Figure 40). Viral concentration decreased by 2.0 and 1.5 $TCID_{50}$ over the 30 days of the study for controls and bone marrow samples, respectively, with final viral concentration estimated at 3.6 and 3.0 $TCID_{50}$ at the end of the study.

However, the decrease of the titer in the SVA control was about 90 percent, indicating that the bone marrow tissue provided a friendly environment for SVA survival. The SVA result contrasts with PPV, in which the bone marrow samples yielded negative results at day 30 whereas the remaining viral concentration for controls was about 70 percent of the original titer. Among all tested viruses, BVDV was demonstrated to be the most sensitive to the conditions used. Both BVDV control and bone marrow

samples were negative at day 30. However, there is evidence that the bone marrow material provided some protective influence to BVDV, considering the viral titer at day 20 in bone marrow was still about 25 percent of the initial concentration, yet negative for the control samples.
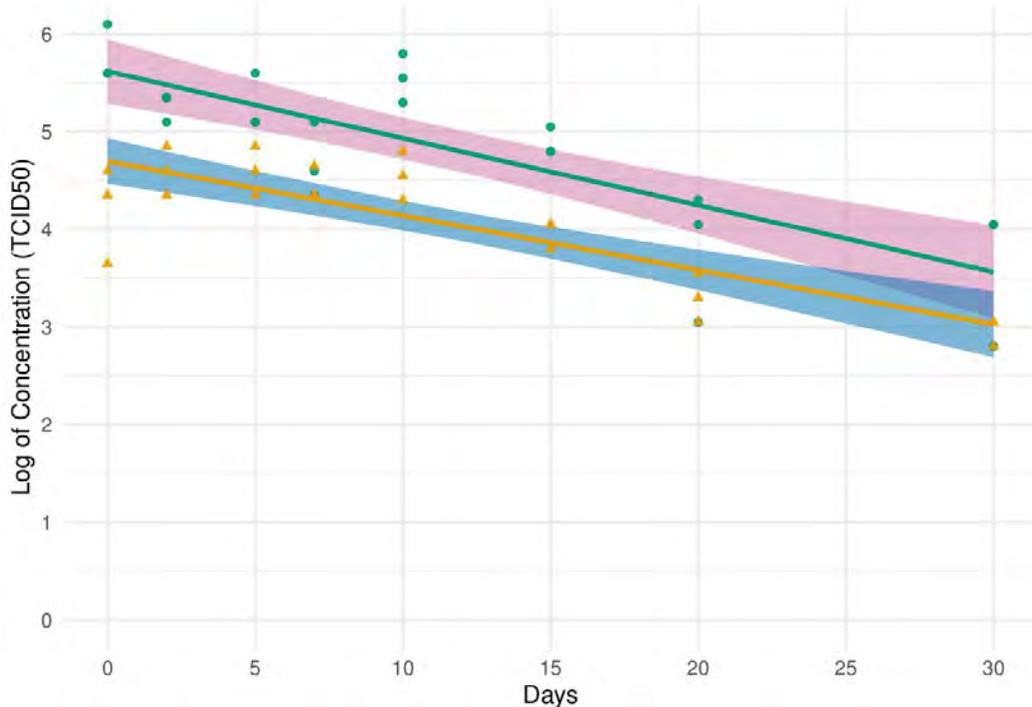
Figure 38. Decrease in viral titer at room temperature for bone marrow samples (orange triangle) and control samples (green circle) with accompanying best fit regression lines and their 95% confidence bands.

Please note that it is impossible to replicate all the conditions that the virus undergoes in the SBC process using an in-vitro system. However, the tests showed the bone marrow material had no direct negative impact on the titer of SwPV, supporting the conclusion that the SBC process was responsible for virus inactivation, not the presence of bone marrow tissue. The in-vitro viability of the virus in the lab was longer than in the SBC plots. The temperature in the lab was lower than the temperature in the plots, emphasizing the potential temperature effect over virus viability. This study also provided evidence that not every virus will be inactivated at the same rate and that different matrices can drastically affect the virus inactivation rate.

3.2.5.2   *Virus viability in house flies*
The methodology employed to expose the flies to the viruses was adequate based on the high number of groups that tested positive for the viruses by the real-time PCR assay (Table 3). Even though each group included only five flies and they were were tested per time point, the researchers did retrieve positive samples with the infectious virus. All samples tested for SVA were positive (Table 3), whereas infectious SwPV was detected in only one group at the 6h time point. Infectious BVDV was only identified at the 0h in one group and no infectious PPV was detected. The virus isolation positive samples are highlighted in yellow in Table 3.

These results demonstrated the ability of five flies to carry viruses when feeding in a solution containing the virus. Under natural exposure, with an increased number of flies, the likelihood for carrying infectious particles could be more probable. The high positivity level of SVA samples is not surprising because SVA is known to be very resistant in variable conditions. In the study, it is not possible to establish whether the infectious viral particles were in the digestive tract of the fly or on the body surface. It's possible that the digestive enzymes did not inactivate SVA during the fly feeding, although we note that the SVA solution had the highest viral concentration. This likely favored the transport of higher concentrations of virus. These results suggest that flies may carry significant viral concentrations. However, further studies are required to address transmissibility to naïve animals by flies exposed to these viruses.

Table 3. Real-time PCR results for flies exposed to SVA, SwPV, PPV, and BVDV. Highlighted in yellow are the samples positive in the virus isolation assay

Groups	Sampling point	Viruses			
		SVA	SwPV	PPV	BVDV
Male	0	21.496	21.994	34.916	30.311
	6	23.519	27.524	23.926	33.969
	12	27.865	25.709	34.89	34.023
	24	30.311	33.673	Neg	34.337
	48	30.537	32.605	28.138	33.332
Female	0	20.606	25.111	Neg	29.7825
	6	23.537	25.792	Neg	31.824
	12	27.923	27.527	Neg	33.583
	24	21.954	32.339	30.585	31.428
	48	27.479	29.521	34.784	33.0175
Positive control		15.798	18.107	17.63	15.198
Negative control		Neg	Neg	Neg	Neg

3.3   Environmental Monitoring

3.3.1   Soil

As discussed in Section 2, soil samples were analyzed for 19 parameters representing the compounds most mobile in soil and those related to salinity. Of the 19, only 10 varied noticeably from background levels. Those 10 parameters are discussed below.

3.3.1.1   *Ammonium*

Figures 39-40 show the concentration of ammonium ions (NH4) in the west and east trenches, respectively, over time at various depths below the trench bottom. Two months after installation, the

ammonium levels reached a peak of about 1,900 ppm at the bottom of the west trench, while the ammonium levels at the bottom of the east trench reached a peak of approximately 2,200 ppm at 1 year. Background levels were approximately 2-4 ppm. In general, ammonium reached background levels within 2 feet BTB during all time periods and the lowest ammonium levels were observed in the winter.
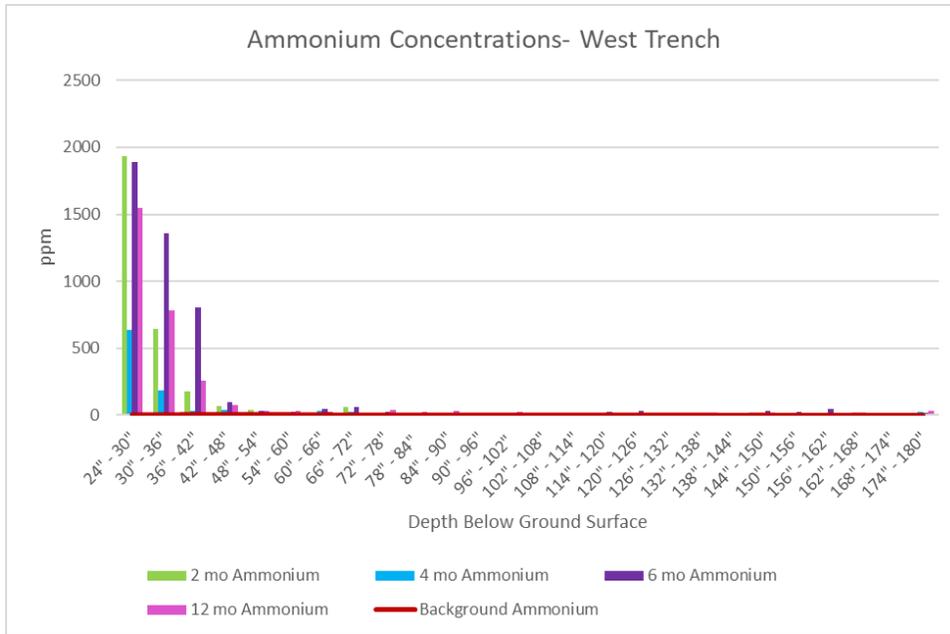
Figure 529. Ammonium concentration beneath West Trench at 0, 2, 4, 6, and 12 months
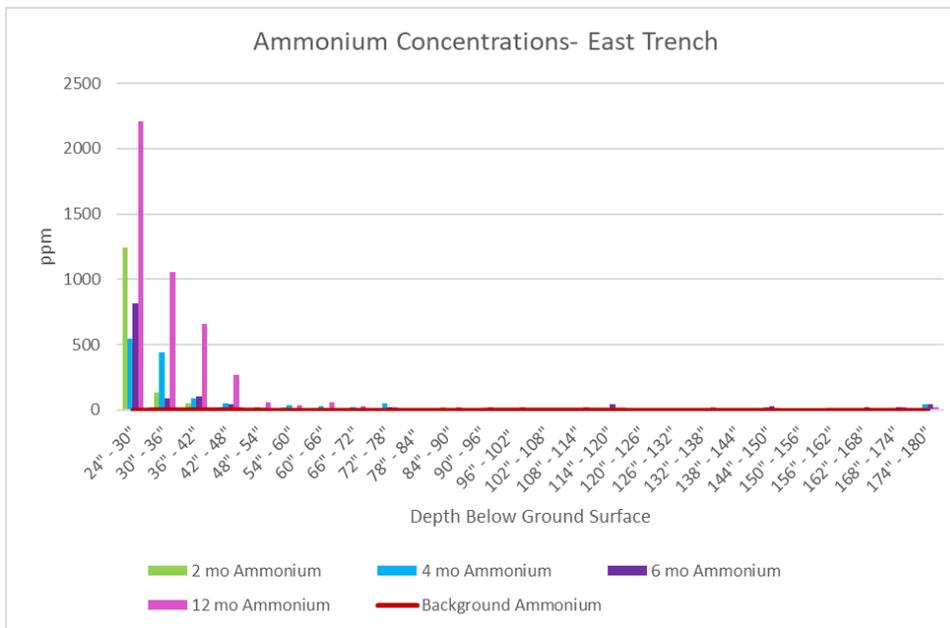
Figure 520. Ammonium concentration beneath East Trench at 0, 2, 4, 6, and 12 months

Ammonium ions can convert to nitrates and nitrites depending on soil type and pose a risk to human and environmental health. This conversion (nitrification) is often a cause of soil acidity. Acidity is produced when ammonium containing materials are transformed to nitrate in the soil. The more ammoniacal nitrogen fertilizer is applied, the more acidic the soil gets. As a point of reference, one State (Kansas, 2010) allows a maximum level of 40 ppm ammonium at 24 inches below the ground surface; this is two orders of magnitude less than the observed levels under the trenches. Levels of ammonium have been measured at 1,400 ppm beneath compost piles (Glanville, 2006) and ranging from 3,500-12,000 ppm beneath deep burial sites (Pratt, 2009). This suggests SBC may release slightly more ammonium than composting, but significantly less than deep burial.

3.3.1.2   Potassium

Figures 41-42 show the concentration of potassium in the west and east trenches, respectively, over time at various depths BTB. Two months after installing the plots (November), the potassium levels at the bottom of the west trench peaked at 600 ppm, while the east trench peak of about 440 ppm occurred at one year. Background levels ranged from 75-220 ppm. In general, potassium reached background within about a foot or less BTB during all time periods, and no excess potassium was measured in the winter.
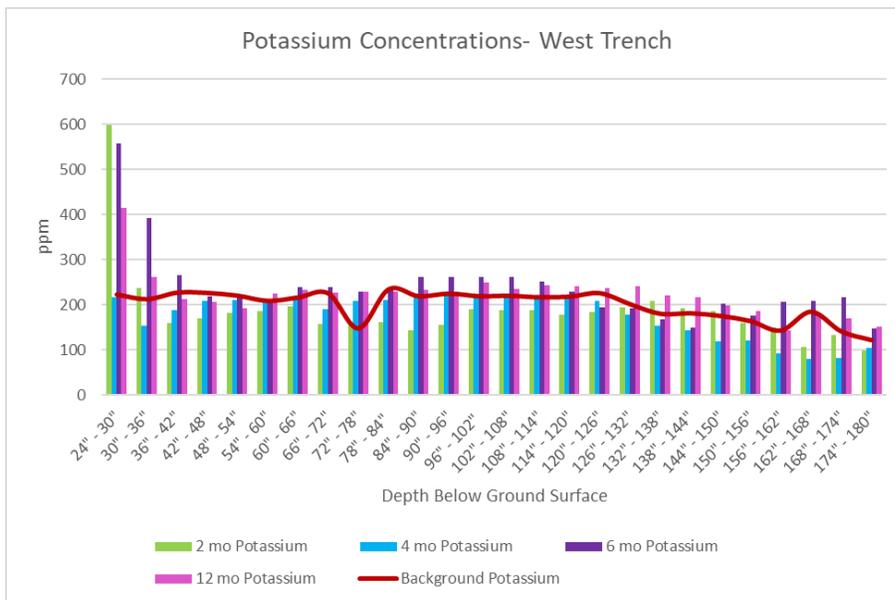
**Figure 531. Potassium concentration beneath west trench at 0, 2, 4, 6, and 12 months**
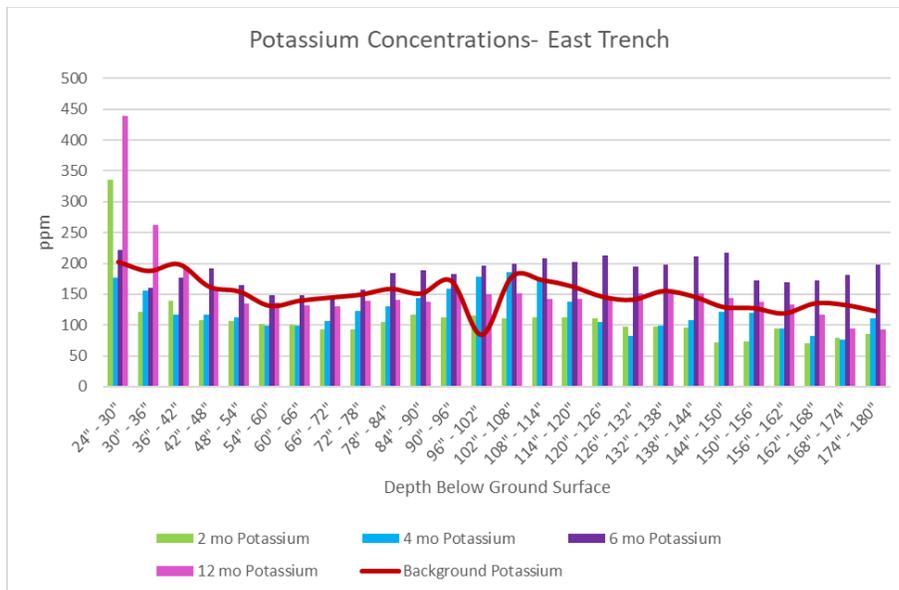
Figure 542. Potassium concentration beneath east trench at 0, 2, 4, 6, and 12 months

In most soils in Oklahoma (except extremely sandy soils in high rainfall regions), total potassium contents are high. Like nitrogen and phosphorus, not all of the total potassium is available for plant growth. Only 1 to 2 percent of the total potassium in soils is readily available. Of this, approximately 90 percent is exchangeable or attached to the outside edge of clays, and the remaining 10 percent is in the soil solution (Oklahoma Soil Fertility Handbook 2017). A maximum of 125 ppm in soil is recommended for optimal crop production in Oklahoma. If excess potassium from the soil reaches groundwater used for drinking, it is generally not considered a pollutant and is listed neither as a primary nor a secondary drinking water contaminant under the Safe Drinking Water Act (EPA, 2021).

3.3.1.3   *Calcium*

Figures 43-44 show the concentration of calcium in the west and east trenches, respectively, over time at various depths BTB. Background levels of calcium were measured at a peak of 5,500 ppm at about 12 inches BTB in both trenches. At months 4, 6, and 12 the peak calcium levels were about 5,100 ppm at 12-18 inches BTB in the west trench and the peak was 6,000 ppm in the east trench at 12 inches BTB. In general, calcium was below background for the first 6-12 inches BTB in both trenches, then somewhat above background levels for the remaining depths at most time periods. Calcium levels were aligned with background levels in January in the west trench.

Calcium is a soil nutrient and is taken up by plants as the cation, Ca2+. Calcium functions in the plant in cell wall development and formation. Calcium is not translocated in plants and consequently, the deficiency of calcium will be observed first in the new, developing plant tissue. The calcium ion is referred to as a basic ion because the element reacts with water to form the strong base calcium hydroxide, Ca(OH)2. Calcium is held tightly on the negatively charged clay and organic particles in soils and is abundant in soils that have developed in arid and semi-arid climates. Because of this, it is primarily responsible for maintaining these soils at or near a neutral pH (Oklahoma Soil Fertility Handbook 2017). Clay soils often have 2,500 ppm calcium or more (FSA2118). If excess levels of calcium from soil reach groundwater used for drinking, it is not listed as a primary or secondary contaminant under the Safe Drinking Water Act (EPA, 2021).
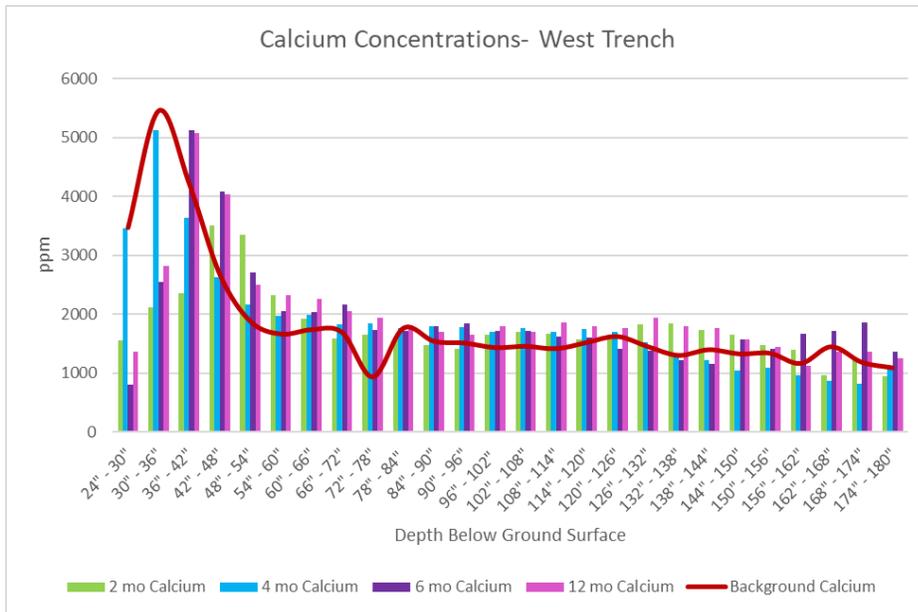
Figure 553. Calcium concentration beneath west trench at 0, 2, 4, 6, and 12 months
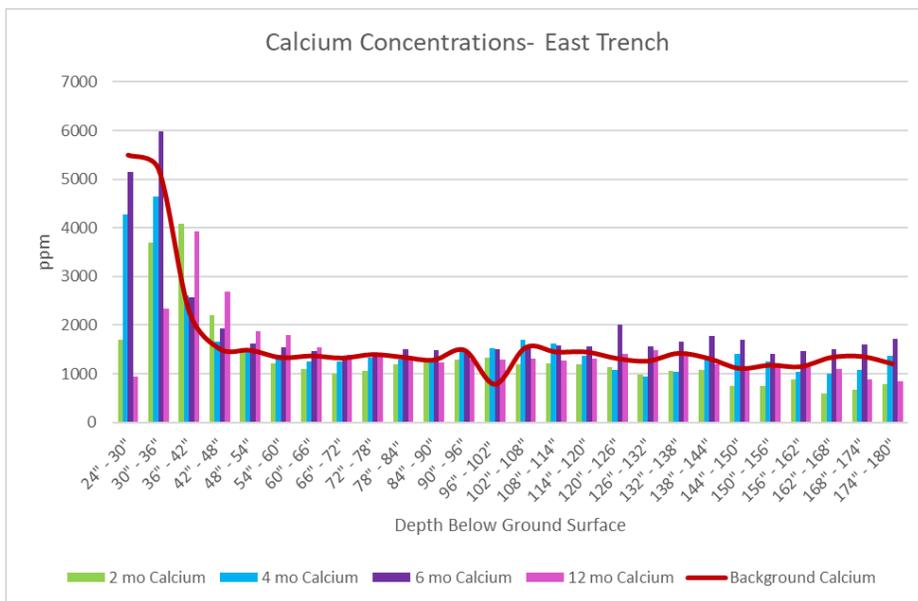
Figure 554. Calcium concentration beneath east trench at 0, 2, 4, 6, and 12 months

3.3.1.4   *pH*

Figures 45-46 show the pH in the west and east trenches, respectively, over time at various depths BTB. Background pH levels below both trenches were above 8 at trench bottom, then dipping below pH 6 between 6.5 ft - 9 ft BTB under the west trench and between 4.5 ft - 7 ft under the east trench. For all treatment time periods, the pH below both trenches is more acidic than background for the first foot or two BTB before returning to near-background for the next several feet of depth, then rising above background below both trenches for most time periods. It is possible that a localized soil pH decrease may result from nitrification of ammonium ions to nitrates and nitrates, as well as mineralization of

organic S to SO4, and other organic compounds breaking down in the carcasses. In addition, as the organics and minerals (having these characteristics) migrate in the soil, the background soil may serve as a buffer to stabilize pH levels. The high concentration of calcium may add to the increases in soil pH with depth.
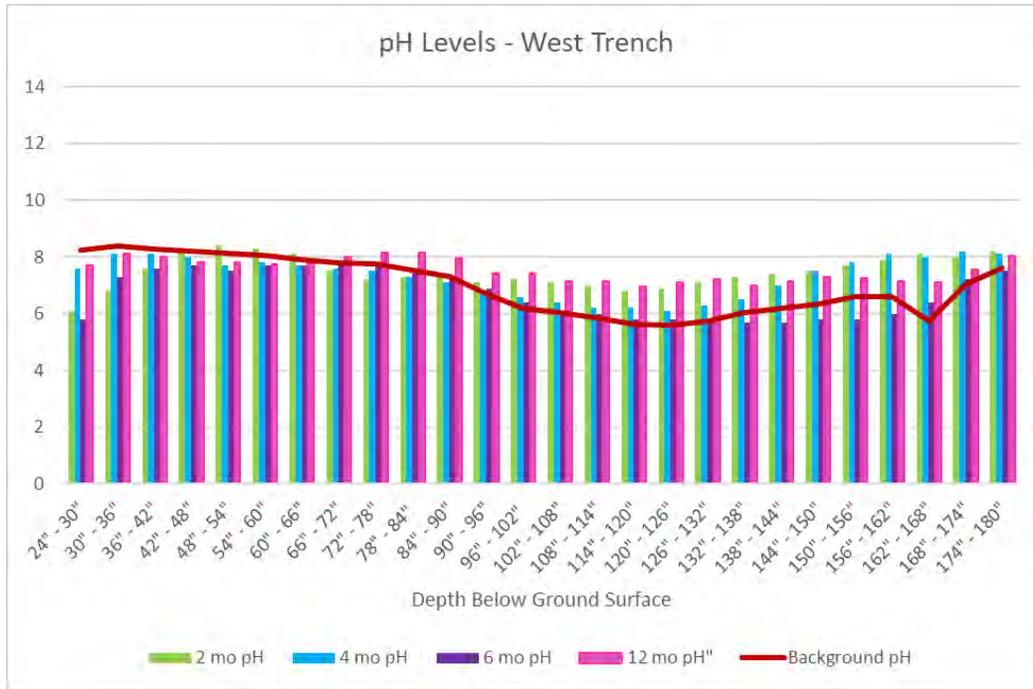
Figure 565. pH levels beneath west trench at 0, 2, 4, 6, and 12 months
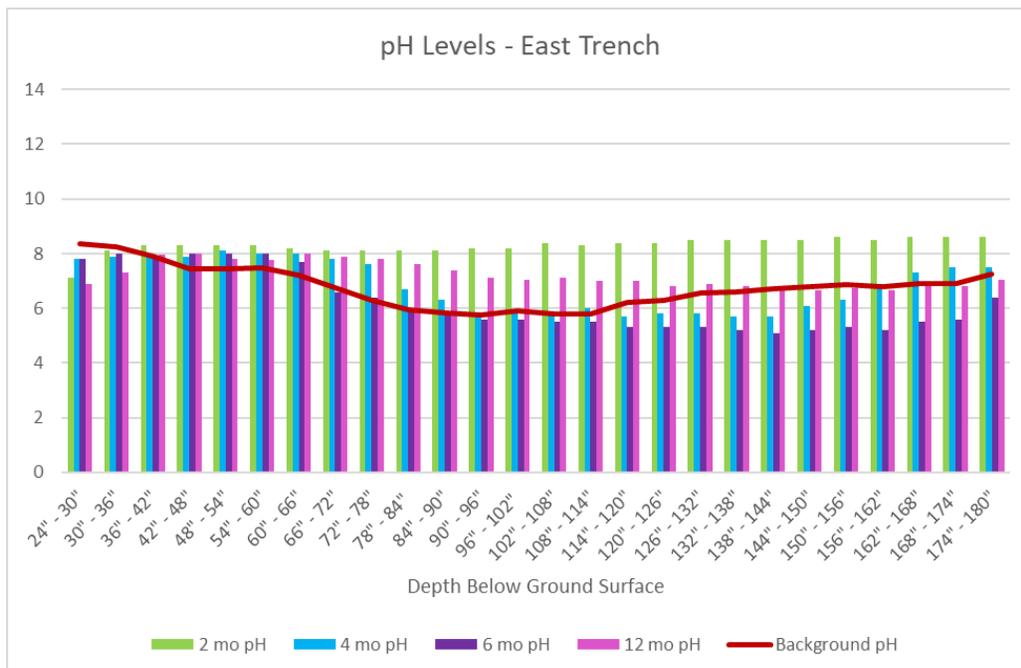
Figure 566. pH levels beneath east trench at 0, 2, 4, 6, and 12 months

3.3.1.5   Total Dissolved Solids (TDS)

Figures 47-49 show the concentration of total dissolved solids in the west and east trenches, respectively, over time at various depths BTB. Background levels were measured between 823 ppm and 2,535 ppm in the two trenches at various depths. At two months, the TDS level peaked at 20,000 ppm and almost 12,000 ppm at the bottom of the west and east trenches, respectively. In general, TDS neared background within 3-5 feet BTB during all time periods in both trenches.

TDS indicates the presence of dissolved nutrients. If excess levels of dissolved nutrients from soil reach groundwater used for drinking, the secondary drinking water standard for TDS is 500 ppm (EPA, 2021) because it causes hardness, deposits, colored water, staining, and salty taste at that level.
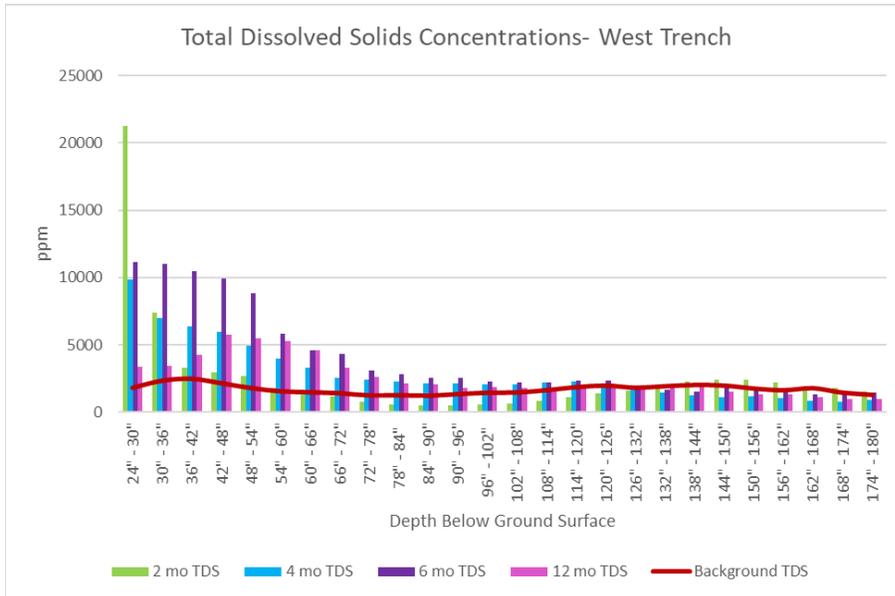
Figure 577. Total dissolved solids concentration beneath west trench at 0, 2, 4, 6, and 12 months
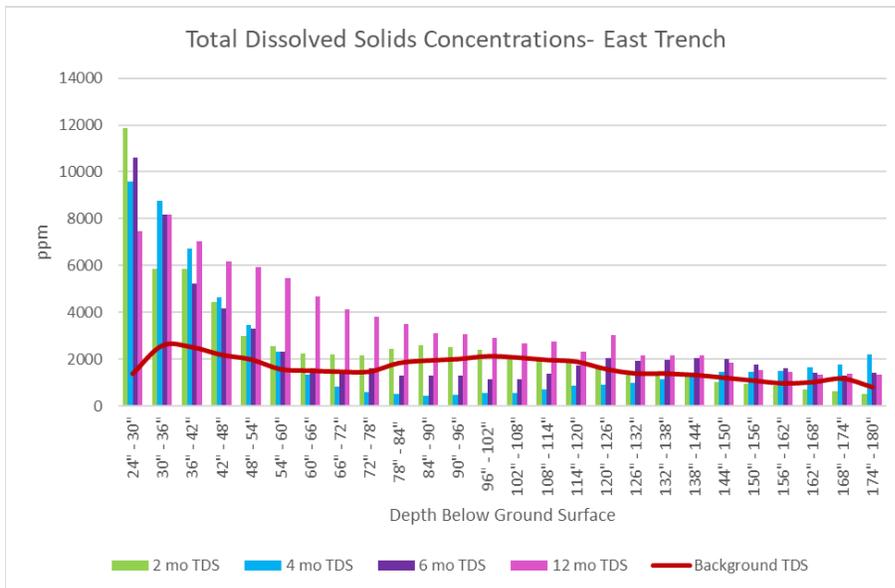
Figure 57. Total dissolved solids concentration beneath east trench at 0, 2, 4, 6, and 12 months

3.3.1.6   Sulfate Ions (SO4)

Figures 49-50 show the concentration of sulfate ions in the west and east trenches, respectively, over time at various depths BTB. Background levels of sulfate ions ranged from 9 ppm to 64 ppm at various depths below both trenches. The peak sulfate ion levels were 145 ppm at 4 months a foot BTB in the west trench and 180 ppm at the same depth at 2 months in the east trench. In general, sulfate ions were highest in November and January about 1-2 feet BTB. Levels neared background at 3-5 feet BTB.

SO4 is the only form of sulfur that is absorbed by plant roots. Sulfur is removed from the soil system by two major mechanisms, crop uptake and removal and leaching. Excessive rainfall or irrigation water can move SO4-S below the root zone, especially on well or excessively drained (coarse textured) soils. Leaching of SO4-S from the root zone of poorly drained (medium and fine-textured) soils is unlikely. Plant roots were observed at the study site to a depth of 7 feet BTB.

Sulfates are found in fertilizer at concentrations up to 15 ppm. Natural soil with gypsum contains 1,400 ppm or more. If excess sulfates from soil reach groundwater used for drinking, the secondary drinking water standard for sulfate is 250 ppm because it causes a salty taste at that level (EPA, 2021).
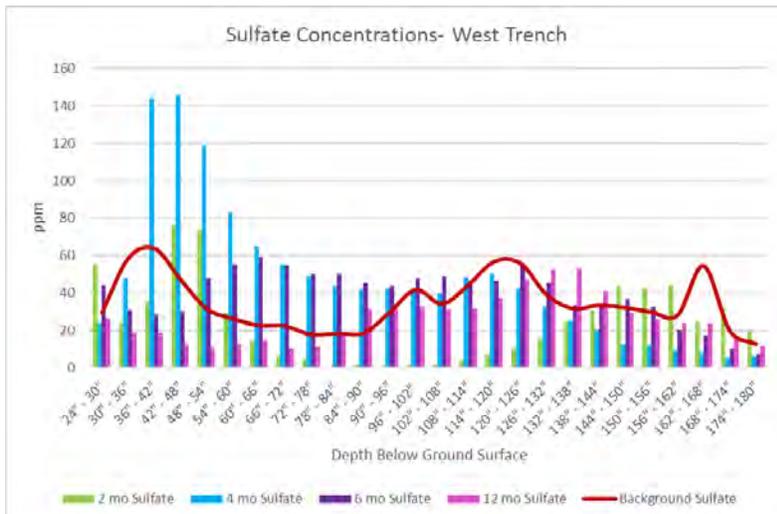
Figure 49. Sulfate concentration beneath west trench at 0, 2, 4, 6, and 12 months
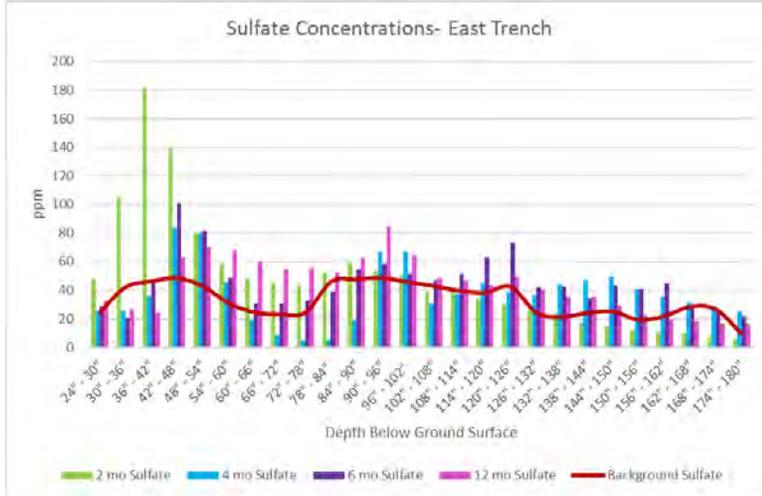
Figure 580. Sulfate concentration beneath east trench at 0, 2, 4, 6, and 12 months

3.3.1.7 Magnesium (Mg)

Figures 51-53 show the concentrations of magnesium in the west and east trenches, respectively, over time at various depths BTB. Background levels of magnesium ranged from 234 ppm to 1034 ppm at various depths below both trenches. The peak magnesium levels were 892 ppm at 4 months per foot BTB in the west trench and 1,054 ppm at the same depth at 2 months in the east trench. In general, magnesium levels were highest in November and January about 1-2 feet BTB. Levels were still elevated above background at 1 year for all depths in the west trench, but had returned to background levels at 1 year in the east trench by 7 feet BTB.

Magnesium reactions in soils are similar to calcium in many respects. Magnesium, like calcium, is a basic ion that generally is abundant in arid and semi-arid soils with near-neutral pH (Oklahoma Soil Fertility Handbook 2017). Magnesium is an essential element for plant growth and photosynthesis. If excess magnesium from soil reaches groundwater used for drinking, there are no maximum contaminant levels for magnesium in drinking water because it has no known health or aesthetic effects (EPA, 2021).
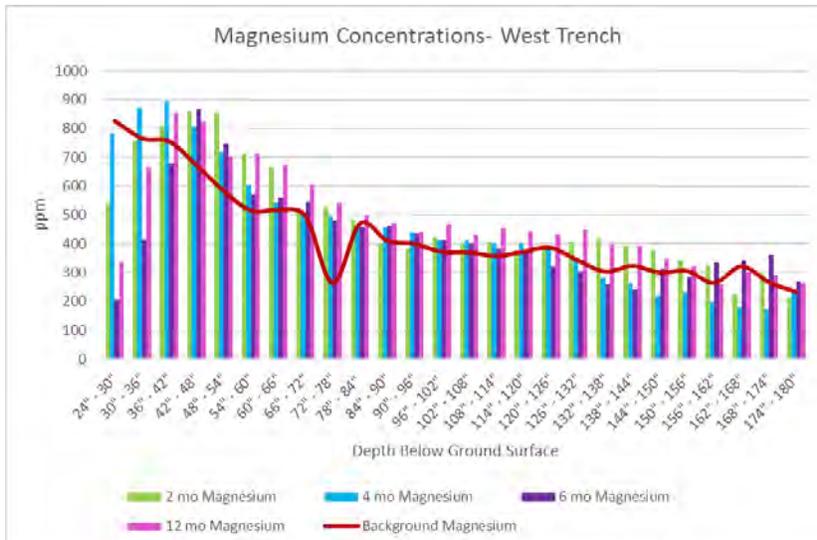
**Figure 591. Magnesium concentration beneath west trench at 0, 2, 4, 6, and 12 months**
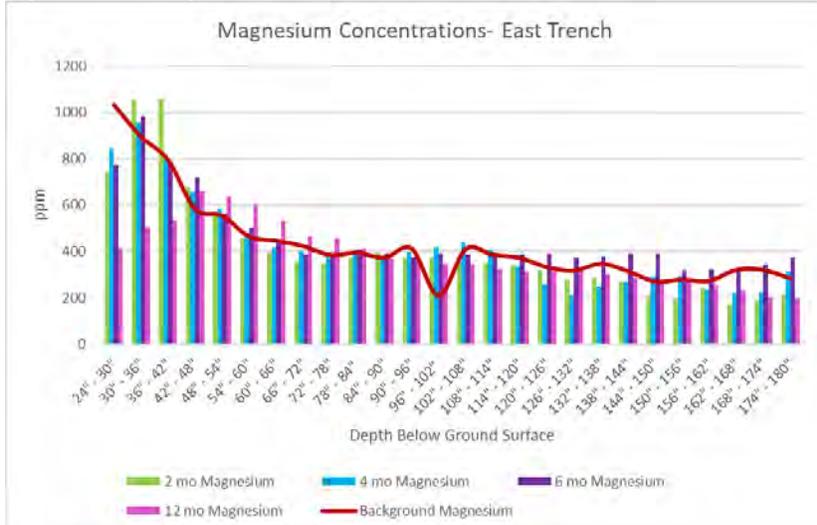
Figure 592. Magnesium concentration beneath east trench at 0, 2, 4, 6, and 12 months

3.3.1.8 *Percent Organic Matter (%OM)*

Figures 53-54 show the percent organic matter in the west and east trenches, respectively, over time at various depths BTB. Background levels of %OM ranged from 0.06 percent to 1.26 percent various depths below both trenches, with the highest levels near the trench bottom. The peak %OM levels were observed at 2 months for both trenches immediately below the trench bottom. The west trench peak was 3.32 percent, and the east trench peak was 1.79 percent. In general, %OM levels returned to background levels at all time points by about 4 ft BTB. Decaying organic matter produces H+ which can be responsible for acidity. The carbon dioxide (CO_2) produced by decaying organic matter reacts with water in the soil to form the weak acid called carbonic acid. This is the same acid that develops when CO_2 in the atmosphere reacts with rain to form acid rain. Several organic acids are also produced by decaying organic matter, but they are also weak acids. (Oklahoma Soil Fertility Handbook 2017)

According to the OSU Cooperative Extension Service publication "Understanding your Soil Test Report," %OM measures the humic substances in the soil; soils with higher OM are generally more productive than soils with lower OM. A medium OM range is classified as 1.5 percent - 3.0 percent OM; more than 3.0 percent is considered high, but no upper limit is listed (OSU, 2002).
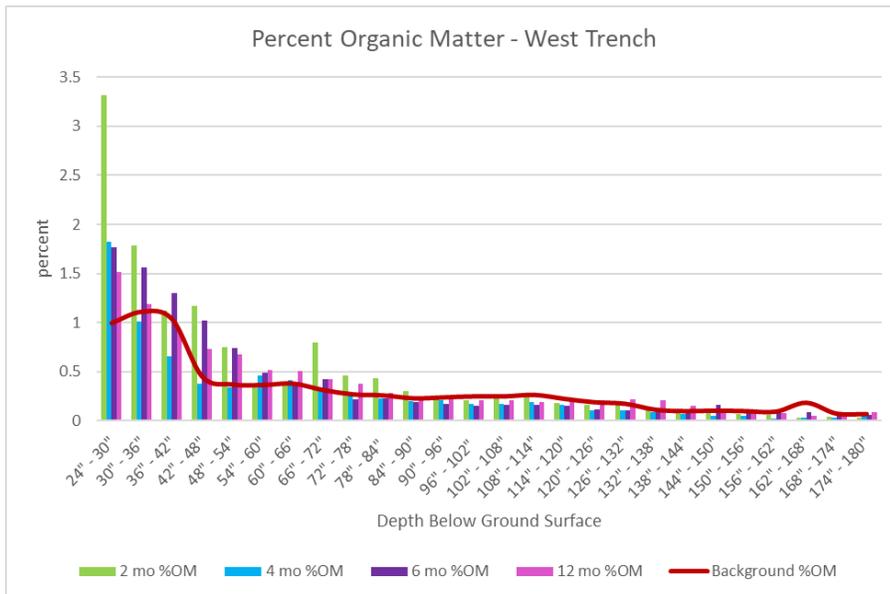
Figure 603. Percent organic matter concentration beneath west trench at 0, 2, 4, 6, and 12 months

60
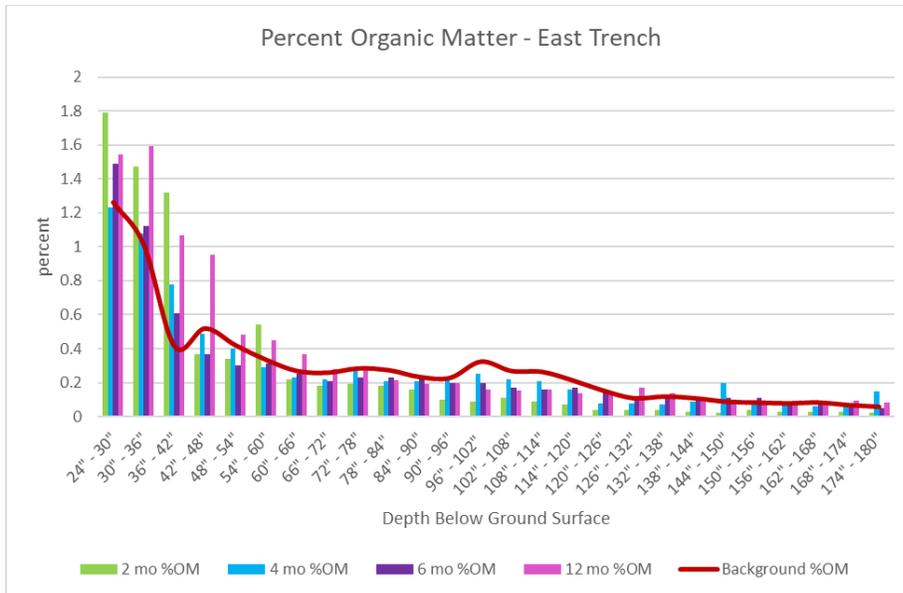
Figure 614. Percent organic matter concentration beneath east trench at 0, 2, 4, 6, and 12 months

3.3.1.9   *Total Nitrogen (TN)*

Figures 57-56 show the total nitrogen concentrations in the west and east trenches, respectively, over time at various depths BTB. Background levels of TN ranged from 250 ppm to 750 ppm at various depths below both trenches, with the highest levels near the trench bottom. The peak TN level for the west trench was 4,800 ppm at 2 months immediately below the trench bottom. The east trench peak was 3,550 ppm at 1 year at the trench bottom. In general, TN levels returned to background levels at all time points by about 2 ft BTB.

TN is the sum of ammonium, organic nitrogen, and nitrates/nitrites. Ammonium was previously discussed above, it represents the majority of TN, and the ammonium data correlates closely with the TN data presented here.
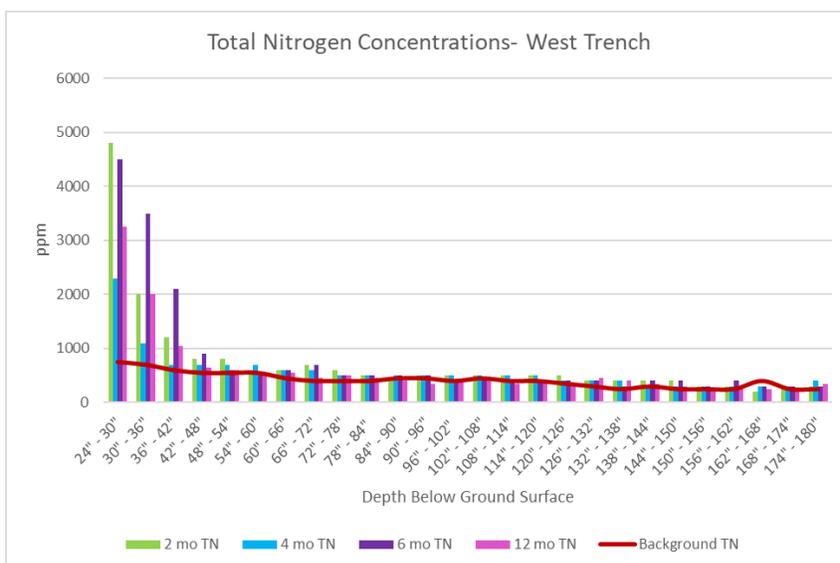
Figure 615. Total nitrogen concentration beneath west trench at 0, 2, 4, 6, and 12 months
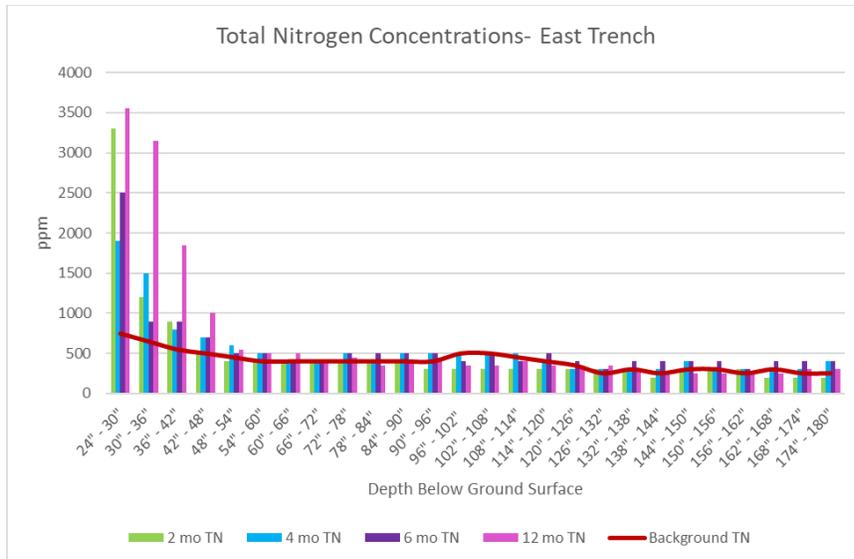
Figure 626. Total nitrogen concentration beneath east trench

3.3.1.10 *Nitrates/Nitrites*

Figures 57-59 show the nitrate/nitrite concentrations in the west and east trenches, respectively, over time at various depths BTB. Background levels of nitrates/nitrites ranged from 0.25 ppm to 1.5 ppm at various depths below both trenches, with the highest levels near the trench bottom under the west trench, but more evenly distributed with depth under the east trench. The peak nitrate/nitrite level for the west trench was 1.75 ppm at 1 year immediately below the trench bottom. The east trench peak was 1.0 ppm at 6 months at 9-10 ft BTB. In general, nitrate/nitrite levels were at or below background levels under the west trench for all but one time point. The east trench exceeded background levels for nitrates/nitrites at 6 months and 1 year at 10-13 ft BTB.

Nitrates/nitrites can be toxic to infants and young animals. The U.S. Environmental Protection Agency (EPA) sets drinking water limits at 10 ppm for nitrates and 1 ppm for nitrites. The soil test data from this project did not differentiate between nitrates and nitrates, so it is not known if nitrites exceeded 1 ppm. The total concentrations of nitrates and nitrates only exceeded 1 ppm at one data point, so it seems unlikely nitrites exceeded 1 ppm. Also, the EPA standards are for drinking water; it is likely the levels of nitrates/nitrites measured under the trenches would decrease further before reaching groundwater.
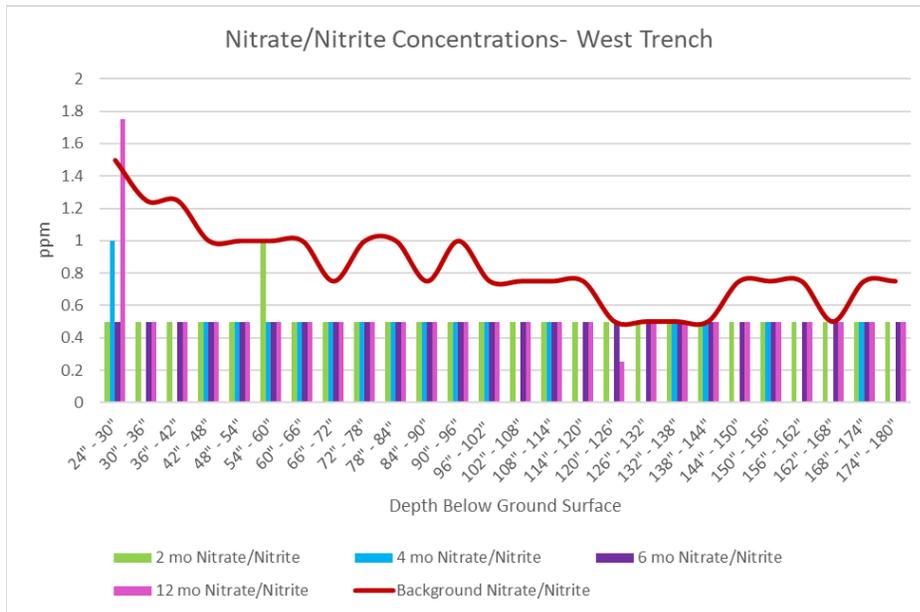
Figure 637. Nitrate/nitrite concentration beneath west trench
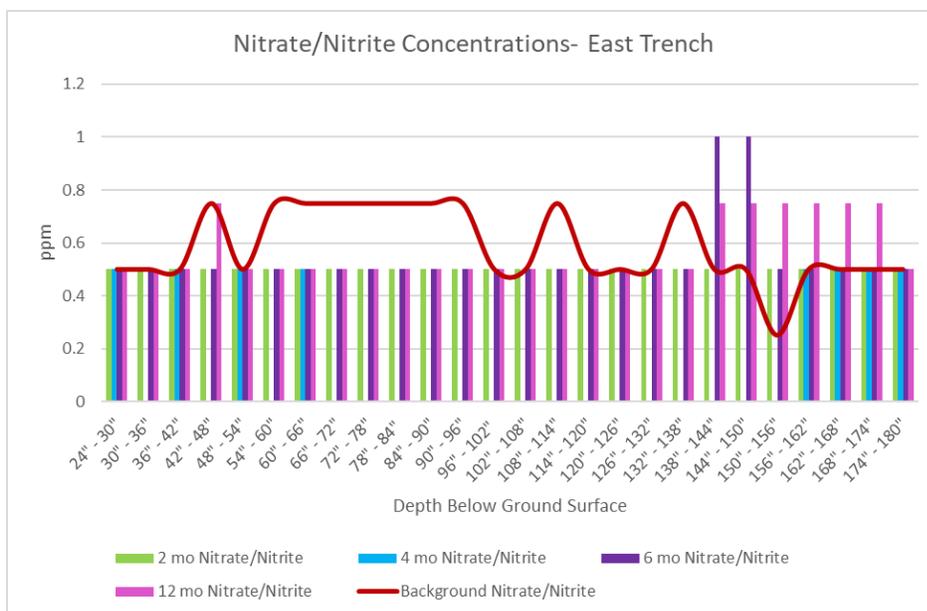
Figure 63. Nitrate/nitrite concentration beneath east trench

3.3.2   Leachate

Figure 59 shows an image of leachate ponded near the east trench after a heavy rain about 2 weeks after the plot was constructed. Figure 60 shows an image of the samples collected from ponded liquid adjacent to the east and west trenches, respectively. Total coliforms in leachate were measured by soil coliform culture at SWFAL at 4,500 colony forming units (CFU)/mL adjacent to the east trench and 510 CFU/mL adjacent to the west trench.

The World Health Organization (1993) reported 133,000 CFU/mL thermotolerant coliforms in primary sewage effluent in Sydney, Australia. Southwestern Oklahoma State University (O'Neal, 2007) measured

a mean of 237 CFU/mL total coliforms in surface water (Little Deep Creek Lake) after a heavy rain. The U.S. EPA maximum contaminant level for total coliforms in drinking water is zero.

Figure 59. Leachate ponded near east trench

Figure 60. Leachate samples

Forage

Table 4 and Figures 61-62 show forage results for the west and east trenches, respectively. For both trenches, the percent moisture and percent protein were significantly higher in the forage growing above the trenches than the forage in the undisturbed surrounding area. The ADF (%) (acid detergent fiber, a measure of cellulose and lignin; higher percent means lower digestibility) showed slightly better digestibility in the forage over the west trench than surrounding forage, while the digestability was slightly less for the forage over the east trench compared to the surrounding forage. The TDN (%) (total digestible nutrients as a sum of digestible protein, digestible carbohydrates, and 2.25 times the digestible fat) were slightly higher for west trench forage than the surrounding area, while the forage over the east trench had slightly less TDN (%) than surrounding forage.

Table 4. Forage sampling results

Sampling Event	Sample Location	Moist (%)	Protein (%)	ADF (%)	TDN (%)	Maint (%)	Lact (%)	Gain (%)
6 Month	ET Top Forage	74.5	31.7	22.7	71.2	0.76	0.74	0.48
	ET Side Edge Forage	57.5	11.8	21.6	72.1	0.77	0.75	0.49
	WT Top Forage	69.6	29.8	21.8	71.9	0.77	0.75	0.49
	WT Side Edge Forage	46.6	9.2	24	70.2	0.75	0.73	0.47

Moisture = percent water
Protein = amino acids essential for reproduction, lactation, growth and maintenance of the body
ADF = acid detergent fiber which is a measure of cellulose and lignin. Higher percent means lower digestibility. Ideal is about 25-50%.
TDN = total digestible nutrients as a sum of digestible protein, digestible carbohydrates, and 2.25 times the digestible fat.
Maint = net energy for maintenance; an estimate of the energy value of a feed to maintain animal tissue with gain or loss of weight.
Lact = net energy for lactation with estimates the energy available from the feed to support an animals' requirements for maintenance plus milk production.
Gain = net energy for gain; an estimate of a feed energy value for body weight gain above the energy required for maintenance.

Maint (%) (net energy for weight maintenance), Lact (%) (net energy for lactation), and Gain (%) (net energy for weight gain) were similar for forage growing over the trenches compared to surrounding forage for both trenches. In general, forage growing over the trenches had more moisture and protein than forage growing on undisturbed soil around the trenches.
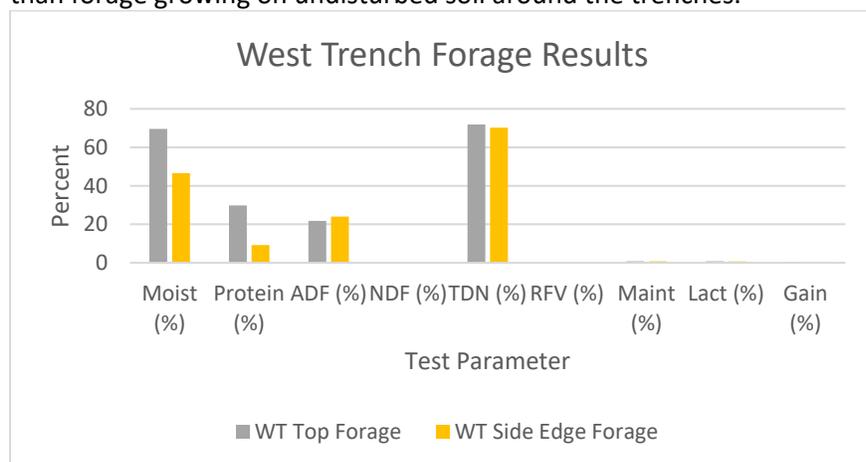
Figure 651. West trench forage results
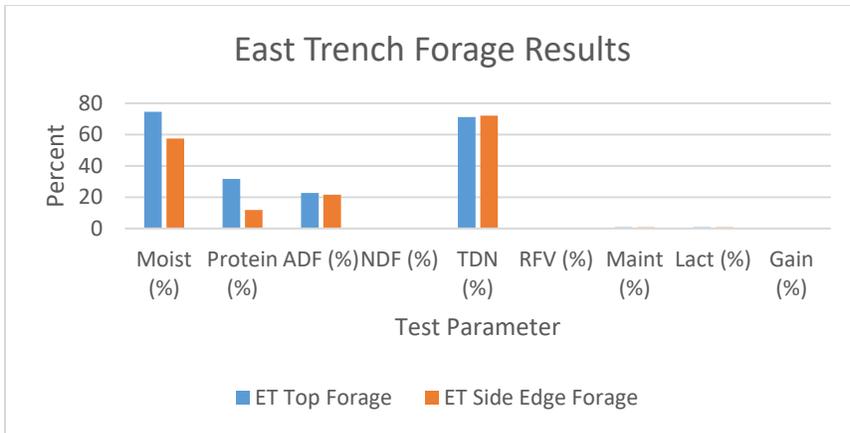
Figure 662. East trench forage results

3.3.3 Olfactory Qualitative Assessment

Figures 63-64 show the level of odors noticed at various depths during the 2, 4, 6, and 12-month sampling events for the west and east trenches, respectively. The odor level was highest immediately below the west trench at the 2-month event; with levels generally dropping with time and depth. Odor levels in the east trench were significantly higher for the first two sampling events (Months 2 and 4), then dropping off with time and depth.
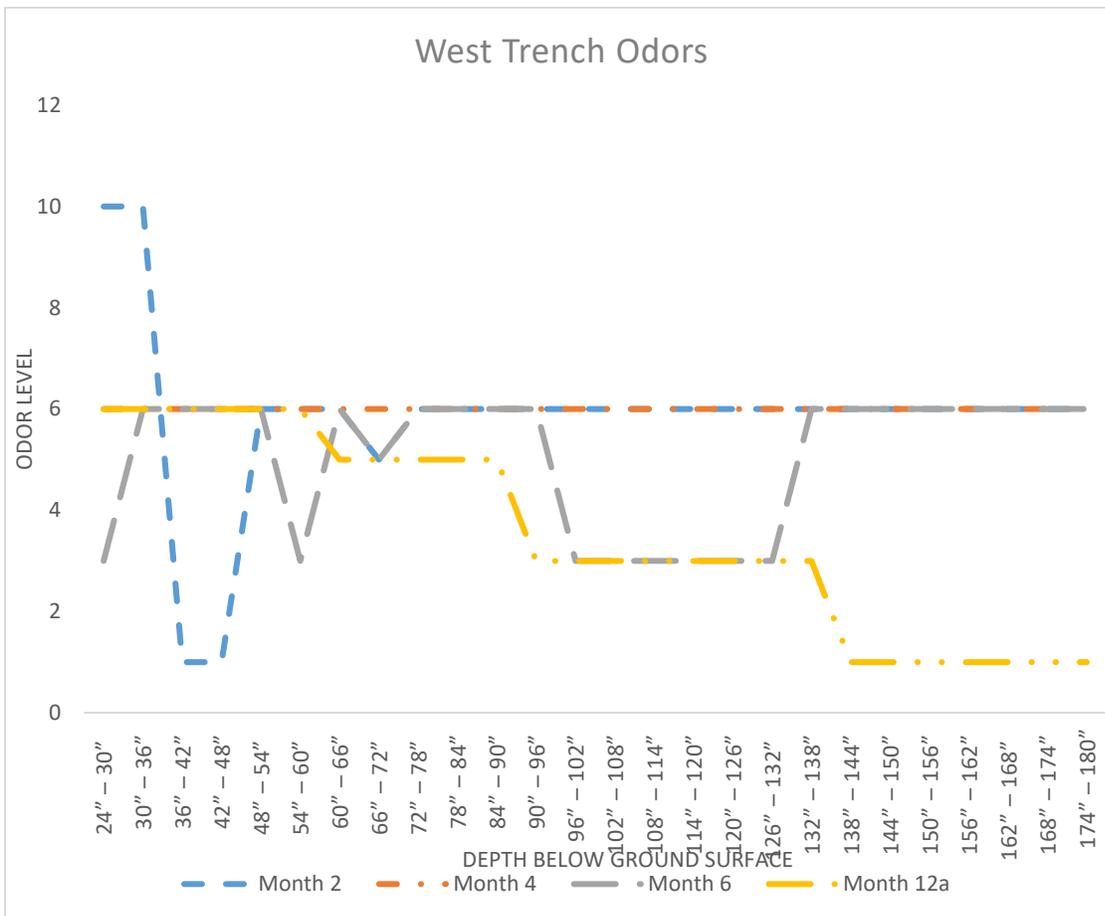
Figure 663. West trench odors
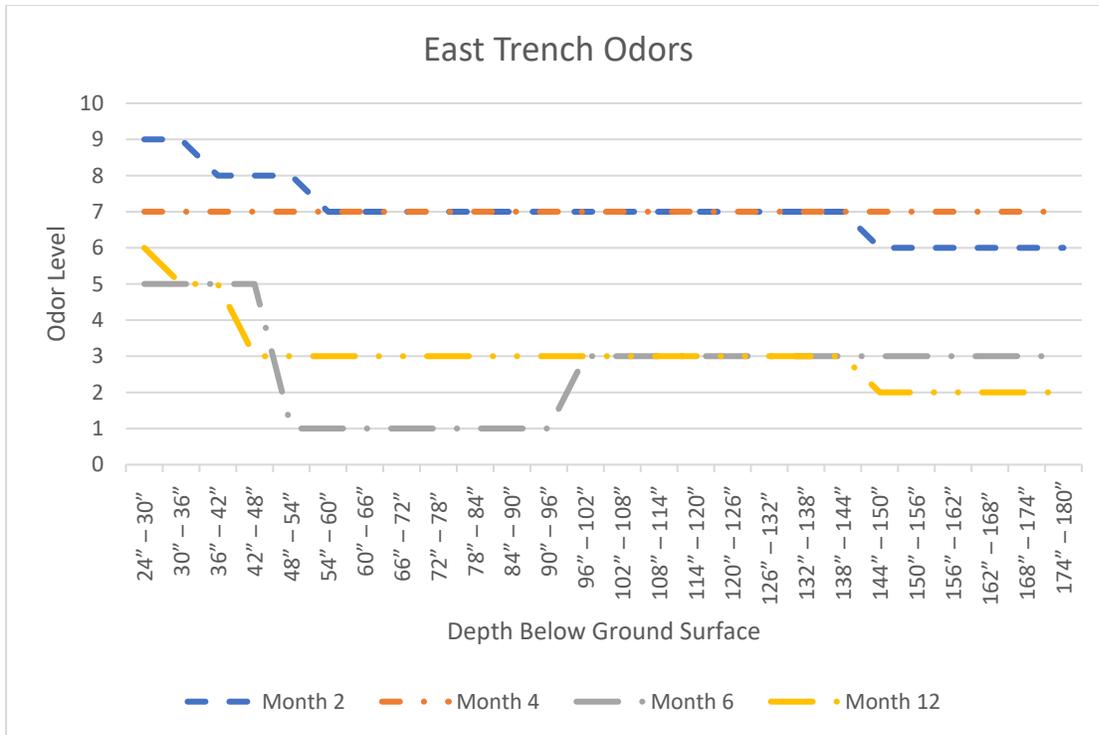
Figure 674. East trench odors

3.3.4 Soil and Trench Temperatures

Figure 65 shows the average soil temperature about 10 inches (25 cm) below ground surface at the weather station approximately 100 m from the trenches to vary from about 25.7°C in late August to about 23.8°C in late September of 2019, around the time the plots were constructed. West trench temperatures for a similar time period were measured to range from about 39°C - 27°C inside the pigs and to range from 37°- 27°C at the pig/soil interface (Figure 66). East trench temperatures ranged from 39°C-27°C inside the pigs and from 35°-27° at the pig soil interface (Figure 67). Temperatures in both trenches were about 10 degrees higher than ambient for the first week after plot construction, dropping to within 3°C of ambient after 2-3 weeks. This data representing the first few months after trench construction was useful for the insect modeling.
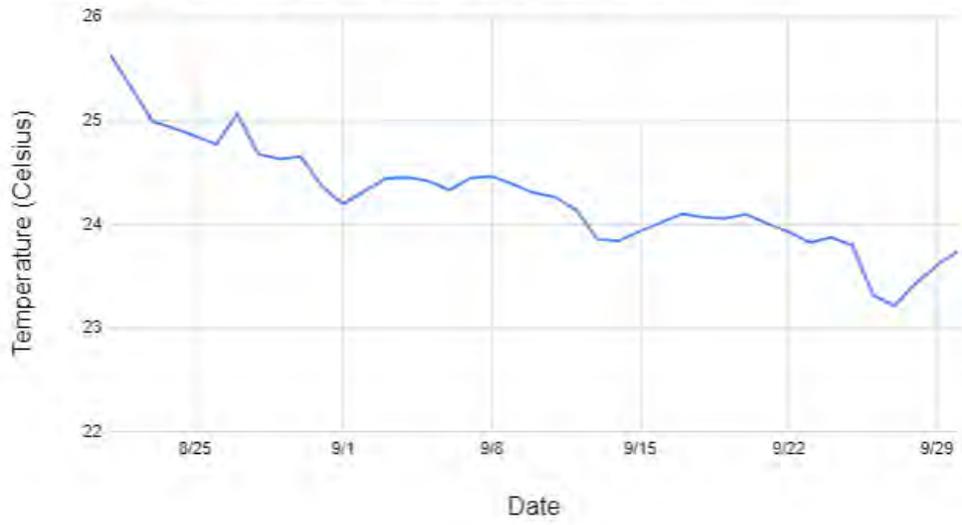
Figure 685. Mesonet temperature (soil) collected 25cm underground from Lake Carl Blackwell station approximately 100m from field site. Collected from 8/24/2019 to 9/30/2019.
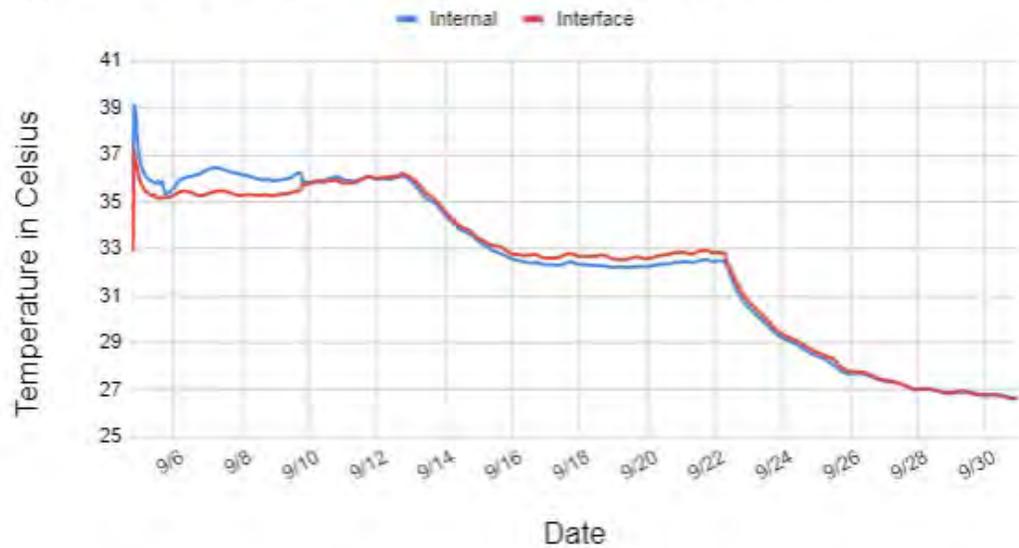
Figure 686. Temperature of west trench from 9/4/2019 to 9/30/2019

Figure 697. Temperature of the east trench from 9/12/2019 to 9/30/2019

Figures 68 and 69 show temperature data for the entire study year for west and east trenches, respectively. Each figure shows ambient air temperature as a light blue line, the ambient soil temperature at 9.84 inches below the ground surface as a red line, the internal temperature of Pig 36 as a green line, and the external temperature (on the hide of the pig) as a dark blue line.

The ambient soil temperature closely follows the ambient air temperature for both trenches throughout the study period. However, the trench temperatures only tracked closely with ambient when air temperatures were below the upper 50s. When air temperatures exceeded about 60°F, the temperatures in the carcasses were elevated up to 20 degrees higher than ambient soil temperatures.

The trench temperatures were noticeably higher than ambient between September to late October 2019 and between May and September 2020. This suggests that the warmer temperatures promoted microbial activity in the carcasses, which would facilitate decomposition during the warmer months of the year.
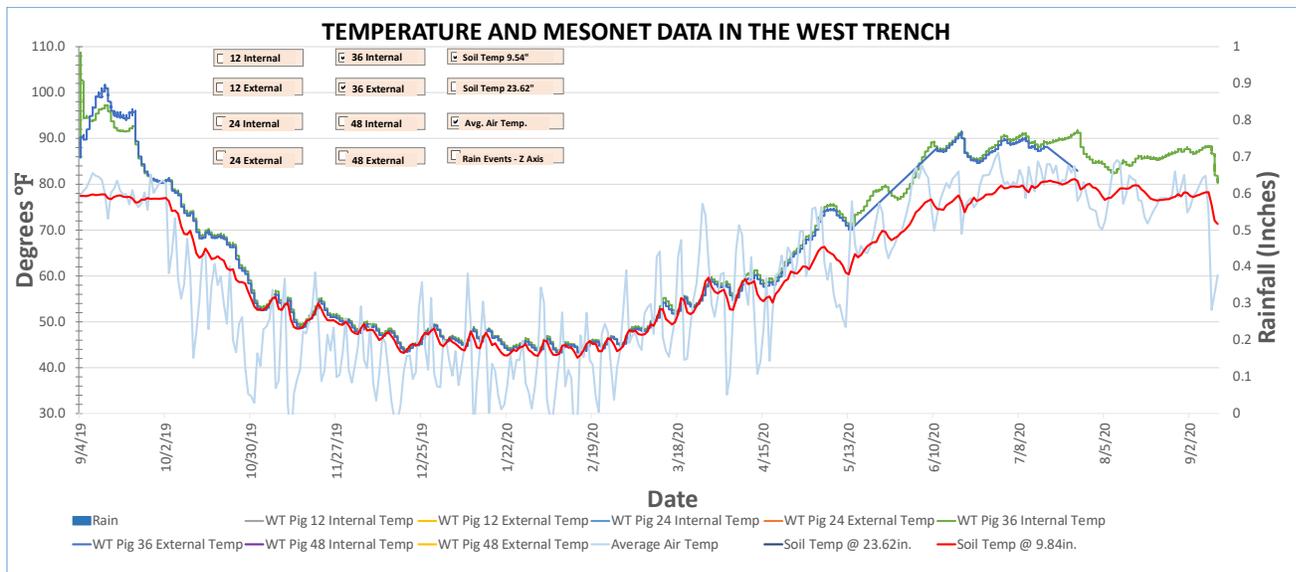
Figure 70. Ambient air, ambient soil, and trench temperatures in the west trench
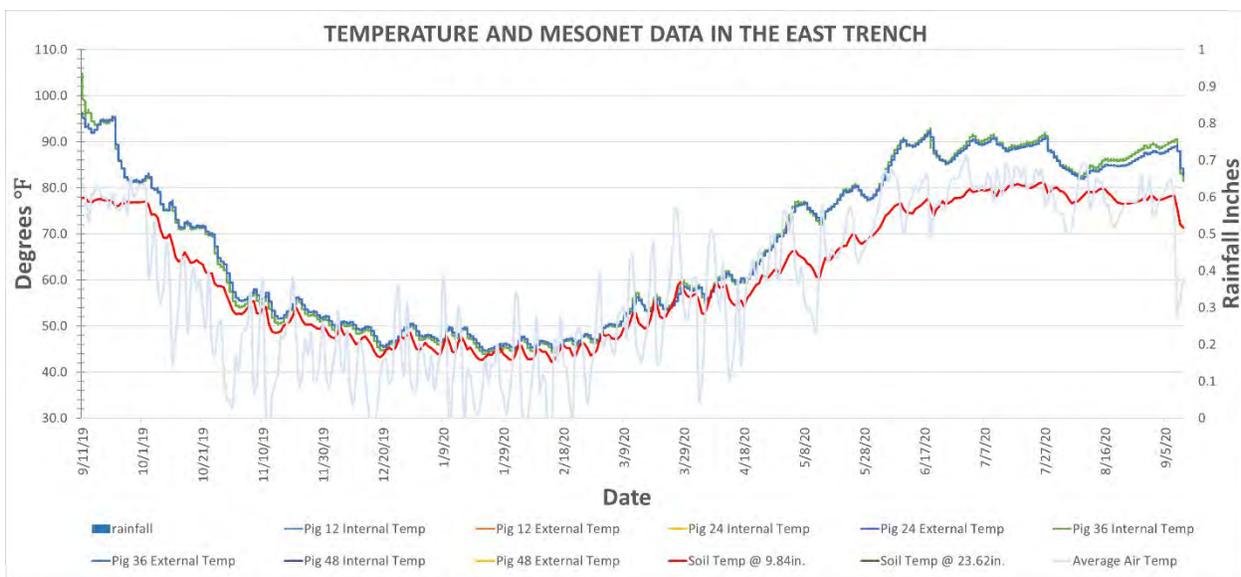
Figure 70. Ambient air, ambient soil, and trench temperatures in the east trench

3.3.5   Wildlife

Over the course of the study, the motion-activated cameras captured approximately 200,000 images. The team reviewed all photos collected in September, October, November, and December 2019, as well as those collected in June and August of 2020. Birds observed sitting on the fence or in flight were not counted as sightings. Some sighting images are shown in Figure 70.

Of the 108,925 images reviewed, 53 contained discrete animal visits to the plots. Often, there were multiple photos of the same animal or herd at a single time, but those images were only counted as one sighting. Of the 53 sightings, 75 percent were non-scavengers (30 deer and 9 rabbit). The deer and rabbits were observed walking by or through the plots.

70

The remaining 25 percent of the sightings were scavengers, including nine opossum, two racoons, and one each armadillo, bobcat, and turkey vulture. All the scavengers observed were walking by or through the plots, except for the turkey vulture, which was seen resting on the east trench. None of the scavengers were observed digging into or disturbing the plots, and there was no evidence of animal disturbance on the plots or at the fence line.

Figure 70. Game cameras capturing wildlife images

3.4   Insect Monitoring

3.4.1   Diversity and Abundance

A total of 28,575 specimens were identified from the field site. The total specimens in pitfall traps total 5,845 specimens, with the primary orders being Coleoptera (beetles), Hymenoptera (ants and wasps), and Orthoptera (grasshoppers). The preliminary, control, and animal mortality trenches had 665; 1,843; and 3,337 total specimens, respectively. Hymenoptera (ants and wasps) and Orthoptera (grasshoppers) specimens were the dominant taxa collected in the preliminary (Figure 71) and control (Figure 72) but in the animal mortality trench pitfall traps the majority of all specimens collected were in the order Coleoptera (beetles)(Figure 73). There were significantly more Coleoptera (beetles) and Orthoptera (grasshoppers) in the animal mortality trenches than the control site pitfall traps.

A total of 22,730 specimens were identified from the malaise traps with the dominant specimens representing the orders Diptera (flies) 16,148; Hymenoptera (ants and wasps) 3,496; and Hemiptera (true bugs associated with plants) 1,977. Dipteran (flies) specimens remained the large group across the malaise trap accounting for 71 percent of the total specimens caught in the malaise traps. The percentage of Hymenoptera (ants and wasps) specimens increased from the preliminary site (Figure 76) to the control site (Figure 75) with the animal mortality trenches exhibiting the highest percentage of Hymenopteran (ants and wasps)(Figure 76) while Diptera (flies) decreased in total percentage from the malaise traps from 82 percent to 71 percent of total specimens. Even though the total percentage collected decreased the number of dipteran (fly) specimens collected rose from 427 in the preliminary malaise traps to 16,148 in the animal mortality trenches. There were significantly more Diptera (flies), Hemiptera (true bugs associated with plants) and Hymenoptera (ants and wasps) in the animal mortality trenches than the control site pitfall traps.

A total of 1,893 beetles (Coleoptera) (Table 5) and 672 flies (Diptera) (Table 6) were collected in pitfall traps. Forensically important beetle families totaled 1,084 specimens. A total of 480 Scarabaeidae (scarab beetles including dung beetles), 320 Silphidae (carrion beetles), 185 Staphylinidae (rove beetles),

and 7 Trogidae (hide beetles) were collected in the pitfall traps located near the animal mortality trenches. Thirty-one Scarabaeidae (scarab beetles including dung beetles) and 17 Staphylinidae (rove beetles) were collected in the control traps. Thirty-nine Scarabaeidae (scarab beetles including dung beetles) and four Staphylinidae (rove beetles) were caught in the preliminary traps. Scarabaeidae (scarab beetles including dung beetles) and Staphylinidae (rove beetles) were not significantly different between animal mortality trenches and preliminary (Scarabaeidae; Staphylinidae) or control (Scarabaeidae; Staphylinidae) pitfall traps. Animal mortality trench pitfall traps collected significantly more Silphidae (carrion beetles) than the control and preliminary pitfall traps.
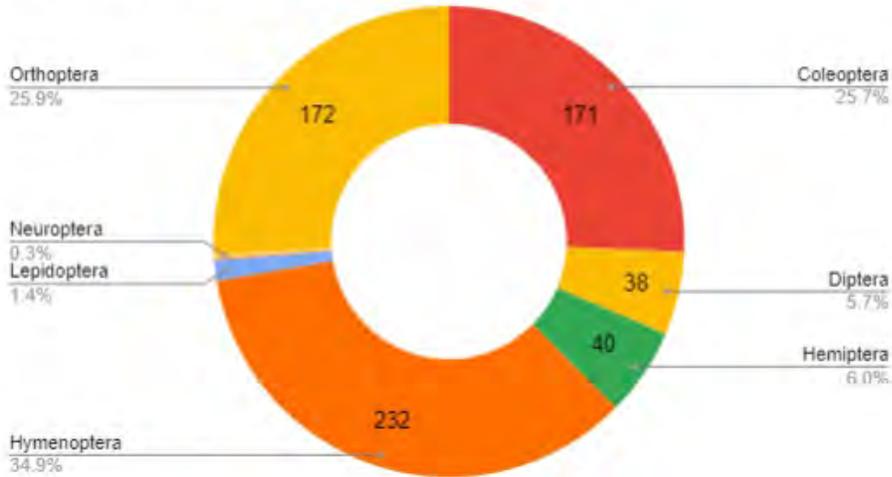
Figure 721. Percentage and number of specimens associated with certain insect orders collected in preliminary pitfall traps
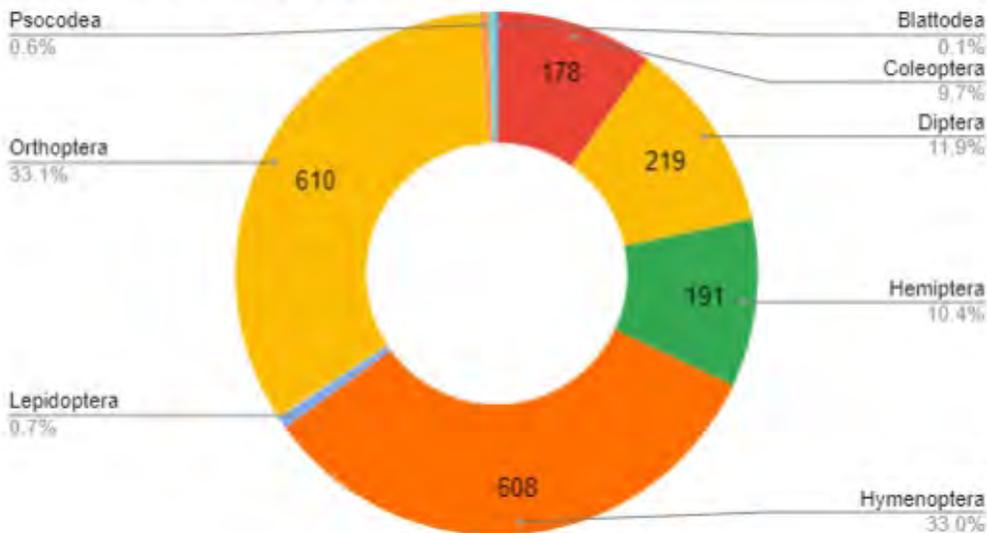
Figure 722. Percentage and number of specimens associated with certain insect orders collected in control pitfall traps
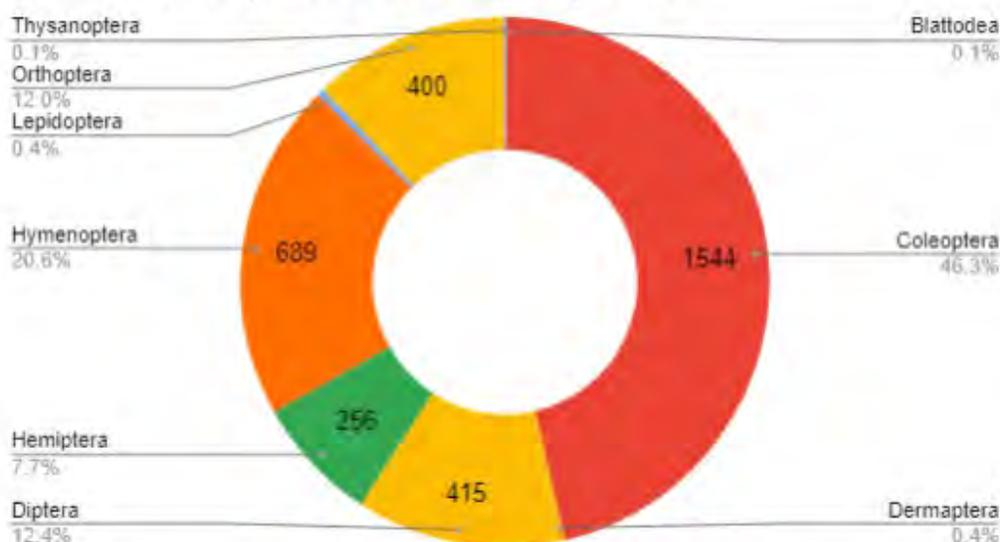
Figure 733. Percentage and number of specimens associated with certain insect orders collected in pitfall traps around the animal mortality trenches

Table 5. Total number of Coleopteran (beetle) specimens collected per family from each sampling area (trench = animal mortality trenches; control = located away from the trenches; prelim = preliminary site before disturbance and animal placement)

Family	Trench Pitfall	Trench Malaise	Control Pitfall	Control Malaise	Prelim Pitfall	Prelim Malaise
Annobidae	1	0	15	0	0	0
Anthibidae	11	0	1	0	0	0
Bostrichidae	34	0	1	0	2	0
Buprestidae	6	1	1	0	0	0
Cantharidae	3	5	2	0	0	0
Carabidae	129	2	12	4	68*	0
Cerambycidae	212	7	41	4	0*	18*
Chrysomelidae	45	10	7	2	17	1
Coccinellidae	0	0	0	0	0	1
Curculionidae	59	8	17	0	10	0
Dermestidae	0	1	0	0	0	0
Elateridae	1	0	7	0	5	0
Erotylidae	2	1	1	0	0	0
Eucinetidae	0	0	1	0	0	0
Histeridae	3	0	2	0	0	0
Lampyridae	1	2	0	1	0	0
Meloidae	18	2	7	0	25	0

Mordellidae	4	2	8	0	0	0
Phalacridae	0	7	0	0	0	0
Ptilodactylidae	0	0	2	0	0	0
Scarabaeidae	480	0	31	0	39	0
Silphidae	320	0	0*	0	0*	0
Staphylinidae	185	0	17	0	4	0
Tenebrionidae	1	0	0	0	0	0
Troogidae	5	0	1	0	2	0
Unknown	24	0	3	0	0	0

* $p<0.05$, chi squared 3.841, df=1. Significance was compared between animal mortality trenches and control or animal mortality trenches and preliminary for families with over 200 specimens collected or forensically important.

Preliminary Malaise Trap Orders

Orthoptera 2.1%
Hymenoptera 4.0%
Hemiptera 7.9%
Coleoptera 3.8%
Diptera 82.1%

21
20
41
427
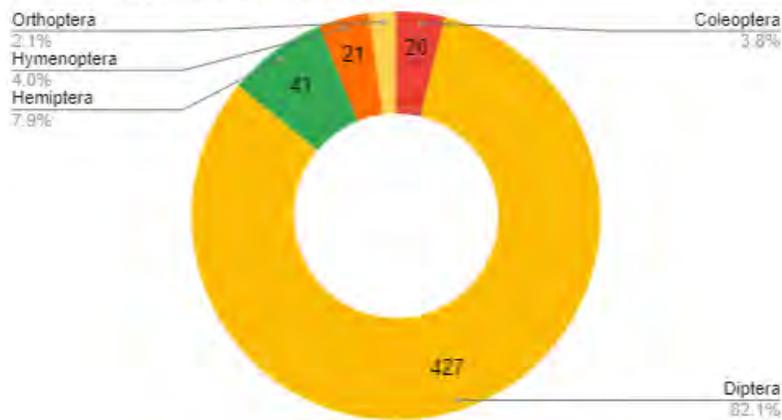
Figure 744. Percentage and number of specimens associated with certain insect orders collected in preliminary Malaise traps

Control Malaise Trap Orders

Lepidoptera 5.3%
Hymenoptera 9.8%
Hemiptera 5.7%
Coleoptera 0.2%
Diptera 78.5%

303
565
330
4510
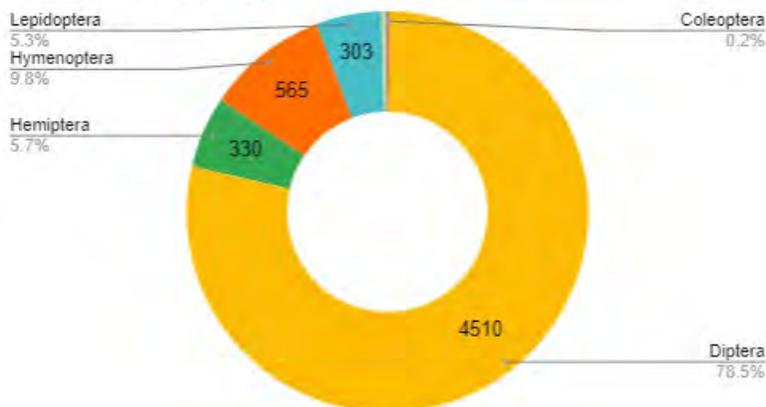
Figure 745. Percentage and number of specimens associated with certain insect orders collected in control Malaise traps
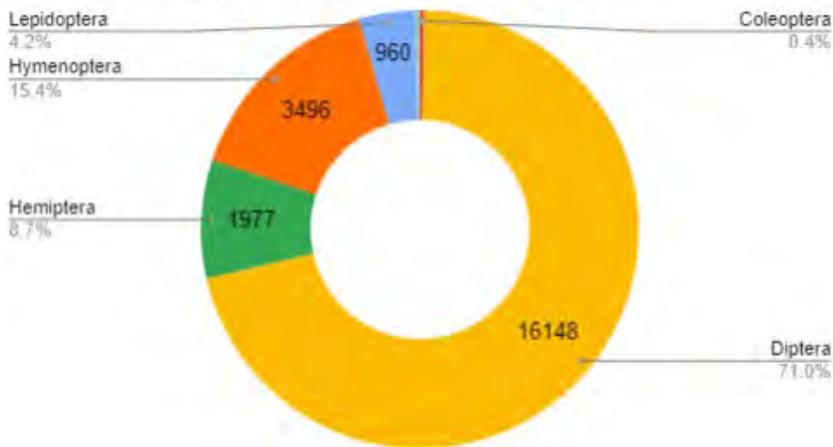
Figure 756. Percentage and number of specimens associated with certain insect orders collected in Malaise traps around the animal mortality trenches

A total of 285 fly specimens that are considered forensically important were caught during the preliminary sampling periods. A total of 39, 7, and 0 fly Calliphoridae (blow flies) specimen(s) were caught in pitfall traps near the animal mortality trenches, control, and preliminary pitfall traps, respectively. The Calliphoridae (blow flies) specimens collected in the pitfall traps were not significantly different when comparing the animal mortality trenches to the control or the preliminary site. A total of 13, 12, and 1 fly specimen(s) in the family Sarcophagidae (flesh flies) and a total of 16, 14, and 0 fly specimen(s) in the family Muscidae (house flies and kin) caught in the animal mortality trenches, control and preliminary pitfall traps, respectively. The Sarcophagidae (flesh flies) specimens collected in the pitfall traps were not significantly different when comparing the animal mortality trenches to the control or the preliminary. The Muscidae (house flies and kin) specimens collected in the pitfall traps were not significantly different when comparing the animal mortality trenches to the control or the preliminary. A total of 148, 28, and 7 fly specimens within the Phoridae (humpbacked flies) family were caught in the animal mortality trenches, control, and preliminary, respectively. The Phoridae (humpbacked flies) specimens collected in the pitfall traps were not significantly different when comparing the animal mortality trenches to the control or the preliminary.

Table 6. Dipeteran (fly) family totals from each sampling area (trench = animal mortality trenches; control = located away from the trenches; prelim = preliminary site before disturbance and animal placement)

Family	Trench Pitfall	Trench Malaise	Control Pitfall	Control Malaise	Prelim Pitfall	Prelim Malaise
Anthomyiidae	0	805	8	50*	1	0*
Asilidae	0	2	0	4	0	0
Bombilidae	0	2	0		0	0
Calliphoridae	39	2568	7	322*	0	4*
Chironomidae	0	1	0	0	0	0
Choloropidae	0	1	0	0	0	0
Culicidae	87	1836	86*	1195*	21	1*

Dolichopodidae	3	1054	43*	319*	4	105*
Drosophilidae	0	0	4	1	2	0
Empididae	0	20	0	0	0	7
Ephydridae	0	20	3	22	0	0
Lauxaniidae	0	0	0	3	0	3
Muscidae	16	2169	14	657*	0	36*
Phoridae	148	202	28	31*	7	0*
Sarcophagidae	13	1542	12	1452*	1	172*
Scathophagidae	0	23	1	0	0	0
Simullidae	0	5	2	10	0	0
Stratiomyidae	0	2	0	0	0	0
Syrphidae	0	129	3	48	0	2
Tabanidae	0	16	1	32	0	6
Tachinidae	0	40	0	43	0	2
Tephritidae	0	12	0	17	0	48
Tipulidae	0	89	1	42	0	5
Ulidiidae	1	387	2	98*	2	0*
Unknown	108	286	4	164	0	36

* p<0.05, chi squared 3.841, df=1. Significance was compared between animal mortality trenches and control or animal mortality trenches and preliminary for families with over 200 specimens collected or forensically important.

A total of 16,148 flies (Diptera) (Table 6) and 79 beetles (Coleoptera) (Table 5) were caught in both pitfall and malaise traps. Of the 16,148 flies, 9,155 were from forensically important families with the animal mortality trench malaise traps containing 2,568 Calliphoridae (blow flies), 1,542 Sarcophagidae (flesh flies), 2,169 Muscidae (house flies and kin), and 202 Phoridae (humpbacked flies). The control traps were significantly different than the animal mortality trenches containing 322 Calliphoridae (blow flies), 1,452 Sarcophagidae (flesh flies), 657 Muscidae (house flies and kin), and 31 Phoridae (humpbacked flies). The preliminary traps were also significantly lower than the animal mortality trenches containing 4 Calliphoridae (blow flies), 172 Sarcophagidae (flesh flies), 36 Muscidae (house flies and kin), and zero Phoridae (humpbacked flies). No beetles from forensically important families were found in the malaise traps during the preliminary sites.

Silphidae (carrion beetles) were the only coleopteran family found in traps around the animal mortality trenches and not at any other sampling area. This is to be expected since these beetles are commonly associated with carrion (Dubie and Talley 2017, Rosa et al. 2011). There was an increase in specimens in the dipteran families of Calliphoridae (blow flies), Muscidae (house flies and kin), and Sarcophagidae (flesh flies), which are all commonly associated with decomposing animals. Calliphorid flies (blow flies) were the most abundant flies collected in the animal mortality trenches.

Forensically important insects were identified to lowest possible taxa listed on Table 7. The majority were only collected after placement of animals. There was a significant increase in the beetle genera and species identified around the animal mortality trenches compared to the control site, while the flies are not significantly different between the trenches and the control site. *Musca domestica*, (house fly) a proven vector of ASF virus, was found in both the trenches and control sites. Although this common fly

was caught in both the animal mortality trenches and control site, this fly was observed in such low numbers there is no trend to determine if this was statistically different or was associated with the animal mortality trenches. *Necrodes surinamensis* (red-linded carrion beetle) and *Cochliomyia macellaria* (secondary screwworm) were both collected around the animal mortality trenches but not the other sites.

Table 7. List of genera and species taxa identified. (+) = is at least one specimens of that species was found in either a pitfall or a malaise trap for that site.

Coleoptera	Preliminary	Control	Mortality Trenches
Staphylinidae			
Aleocharinae			
Homalota spp.			+
Phanerota spp.		+	
Dasycerinae			+
Leptotyphlinae			+
Habrocerinae			+
Habrocerus spp.			
Micropepinae			+
Oxyporinae		+	+
Oxypus spp.			
Oxytelinae			+
Paederinae			+
Homaeotarus spp.			
Scaphidiinae			+
Cyparium spp.			
Staphylininae			
Ocypus spp.			+
Platydracus spp.		+	+
Scarabaeidae			
Aphodiinae		+	+
Ataenius			
A. confertus			
Cetoniinae	+	+	+
Euphoria			
E. sepulcralis			
Malancanthon			+
Aphodiinae	+	+	+
Aphodius spp.			
Melolontine		+	+
Hoplia spp.			
Omalinae			
Occiephilinus spp.			+
Haida spp.			+
Vellica spp.			+
Scarabaeinae			
Ateuchus			+
A. hiseroides			
Phanaeus spp.			+
Onthophagus			+
O. pennsylvanicus			
Silphidae			+

Taxon			
Necrodes			
N. surinamensis			
Trogidae	+	+	+
Omorgus spp.			
Diptera			
Muscidae			
Atherigona	+	+	+
A. reversura			
Caricea spp.			+
Eudasyphora		+	+
E. cyanella			
Fannia spp.		+	
Haematobia irritans			+
Musca		+	+
M. domestica			
Neodexiopsis	+	+	+
N. ovata			
Phaonia spp.		+	+
Stomoxys		+	
S. calcitrans			
Sarcophagidae			
Boettcheria spp.		+	+
Ravinia spp.	+	+	+
R. stimulans		+	
Sarcophaga spp.		+	+
Calliphoridae			
Cochliomyia		+	+
C. macellaria			+
Cynomya spp.			+
Lucilia			
L. silvarum		+	
L. coeruleiviridia		+	
L. elongata			+
L. eximia			+
L. sericata	+	+	+
Pollenia			
P. griseotomentosa			+
P. pediculata			+
P. rudis			+
Phoridae			
Dohriniphora spp.		+	
Stichilus spp.		+	+

The percent change in certain forensically important fly families such as Muscidae (house flies and kin), Calliphoridae (blow flies), and Phoridae (humpbacked flies) was larger than the percent change observed in Sarcophagidae (flesh flies) between the control malaise trap compared to the animal mortality trench malaise traps (Table 8). The percent change in Calliphorid (blow fly) specimens between the animal mortality trenches and the preliminary malaise traps was the largest at 32,000 percent. The percent change in important dipteran families suggests that animal placement into a mortality management

area will increase fly abundance especially when comparing the preliminary and control sites to the animal mortality trench sites.

Table 8. Average number of Calliphorids (blow flies), Sarcophagids (flesh flies), Muscids (house flies and kin) and Phorids (humpbacked flies) per malaise trap and percent change compared either from the preliminary sites or control sites to the animal mortality trenches

	Calliphoridae	Sarcophagidae	Muscidae	Phoridae
Trench Malaise	1284	771	1084.5	101
Control Malaise	332	1452	657	31
Prelim Malaise	4	172	36	0
%change Malaise Control/Trench	299%	*46%	65%	225%
%change Malaise Prelim/Trench	32,000%	348%	291%	N/A

The study team calculated Simpson's index (S), Shannon-Wiener index (SW), evenness (E), and richness (R) for each of the sites. Order diversity was the highest in the animal mortality trenches (0.395 S and 1.256 SW), followed by the control site (0.426 S and 1.232 SW) and then the preliminary site (0.618 S and 0.743 SW) (Table 9).

Table 9. Diversity and abundance indexes for orders, Coleoptera (beetle) families, and Diptera (fly) families from each collection area

	Preliminary	Control	Animal Mortality Trenches
Orders			
Simpson's	0.681	0.426	0.395
Shannon-Wiener	0.743	1.232	1.256
Evenness	0.414	0.514	0.524
Richness	6	11	11
Coleoptera Families			
Simpson's	0.204	0.122	0.175
Shannon-Wiener	1.857	2.416	2.064
Evenness	0.774	0.794	0.657
Richness	11	21	23
Diptera Families			
Simpson's	0.219	0.202	0.147
Shannon-Wiener	1.894	1.937	2.119
Evenness	0.669	0.646	0.676
Richness	17	20	23

The dipteran (fly) families followed the same pattern with animal mortality trenches (0.147 S and 2.119 SW), control (0.202 S and 1.937 SW), and preliminary (0.219 S and 1.857 SW). The control site was the most diverse in coleopteran (beetle) families (0.122 S and 2.416 SW) then animal mortality trenches (0.175 S and 2.064 SW) and preliminary (0.204 S and 1.857 SW). The animal mortality trenches had the lowest evenness (.657 E) for coleopteran (beetle) families indicating higher presence of coleopteran

(beetle) taxa. The animal mortality trenches had the highest number of taxa across all three categories shown by the richness index (11 R in orders, 23R for both family groups). The animal mortality trenches had five additional orders, 12 additional coleopteran (beetle) families and five additional dipteran (flies) families than the preliminary site. The animal mortality trenches also had two additional coleopteran (beetle) families and three additional dipteran (fly) families when compared to the control site.

3.4.2   Data Analysis

3.4.2.1   *Fly Degree Hour Model*

Mesonet data collected from the Lake Carl Blackwell station shows a steady decline in the temperature after animal placement (Figure 67). The temperature of the pigs in the trenches remained above 30°C then started to decrease at a faster rate after September 22, 2019 (Figures 66 and 67).

This data was removed to better show the trend in the temperature. The graphs focus on 1 month post-burial for the degree-hour model. This timeframe includes all expected emergence dates (Table 10), the virus viability time (7 days post burial), and the peaks in forensically important flies.

Table 10. Predictive emergence of adult *Muscidae* (House flies and kin) and *Calliphoridae* (blow flies) fly species based on degree hour models

Species	Lower Developmental Threshold	Accumulated Degree Hours Needed (ADH)	Date ADH was Achieved Per Trench	
			West	East
Stomoxys calcitrans[A]	10°C	5832	9/13/2019	9/20/2019
Musca domestica[A]	10°C	5328	9/13/2019	9/20/2019
Phormia regina[B]	10°C	4976	9/12/2019	9/19/2019
Lucilia sericata[C]	10°C	5069	9/12/2019	9/19/2019
Cynomya cadaverina[C]	6°C	5511	9/13/2019	9/20/2019
Cochliomyia macellaria[D]	10°C	4698	9/12/2019	9/19/2019

[A]= Lysyk 1993; [B]= Nabity et al. 2006; [C]= Wang et al. 2016; [D]= Boatright and Tomberlin 2010

The predictive model (Table 10) demonstrated peaks of forensically important files on September 12-13 and September 19-20. Specimens within the Calliphoridae (blow flies) family had the highest abundance of forensically important fly specimens with peaks in adults collected on September 16, 22, and 27, 2019 (Figures 77 and 78). Specimens in the families Muscidae (house flies and kin), Sarcophagidae (flesh flies), and Phoridae (humpbacked flies) also demonstrated an increase in abundance on September 16, 2019. The highest peak for Muscidae (house flies and kin), Calliphoridae (blow flies), and Phoridae (humpbacked flies) was September 27, 2019. For Sarcophagidae (flesh flies), the highest peak was on September 25, 2019. The predictive model shows an expected increase in fly activity 8-9 days post burial, which is supported by the timeline of forensically important flies collected. Burial dates were September 4, 2019, and September 11, 2019, respectively, for the west and east trenches.

3.4.2.2   *Insect Prevalence Associated with Trenches*

This study has shown that shallow trench composting attracts similar entomological fauna as other methods of mass animal mortality management (Rosa et al., 2011). Both this study and Rosa et al. (2011) found Diptera (flies) to be the most dominant order at 56 percent and 80 percent, respectively, and Sarcophagidae (flesh flies), Muscidae (house flies and kin), and Calliphoridae (blow flies) to be the most dominant fly families. The number of individuals within the Calliphoridae (blow flies) family was
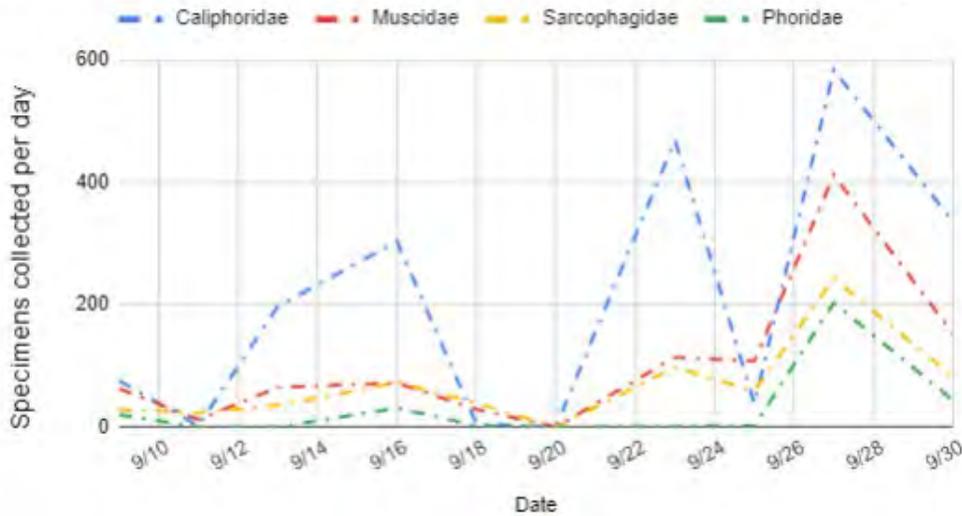
Figure 817. Number of forensically important flies collected per day from the west animal mortality trench near Stillwater, OK in 2019. Calliphoridae = blow flies, Muscidae = house flies and kin, Sarcophagidae = flesh flies, and Phoridae = humpbacked flies.
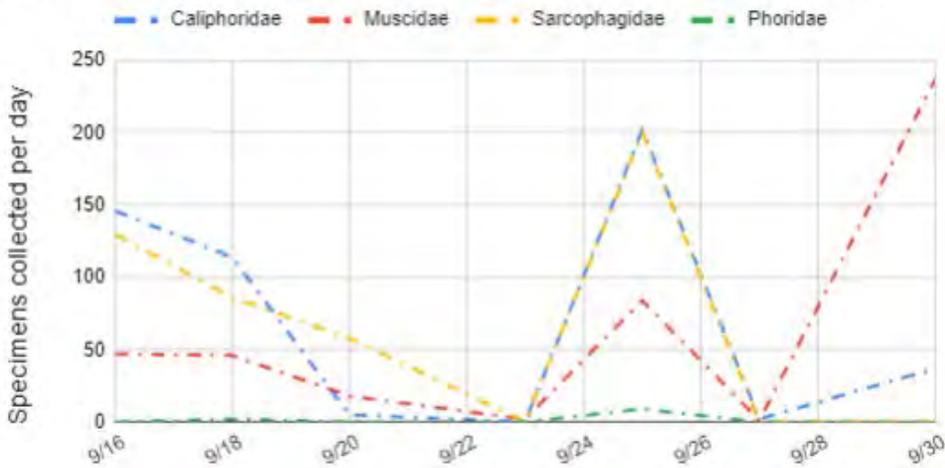
Figure 81. Number of forensically important flies collected per day from the east animal mortality trench near Stillwater, OK, in 2019. Calliphoridae = blow flies, Muscidae = house flies and kin, Sarcophagidae = flesh flies, and Phoridae = humpbacked flies

not as large of a portion as in some previous studies, 18 percent compared to 49 percent of total flies collected (Dubie and Talley 2017). This agrees with previous studies that stated Calliphoridae (blow flies) are not found past 10cm deep unless eggs are deposited on the carcasses pre-burial (Amendt et al. 2010, Gunn and Bird 2011).

Calliphoridae (blow flies), Muscidae (house flies and kin), and Culicidae (mosquitoes) were the three dipeteran (fly) families with the largest representation in the traps that were around the animal

mortality trenches. Dubie et al. (2017) also found Calliphorid (blow fly) and Muscid (house fly and kin) flies as two of the most common families in terms of specimens collected but did not report any specimens in the family of Culicidae (mosquitoes).

Standing water was present at the trap sites, which likely explains the large number of mosquitoes (Figure 79). Another possibility for the presence of mosquitoes is that the decomposition generates CO_2 that keeps them in the same area. The species found by Dubie and Talley (2017) were similar with a few noticeable differences. Dubie and Talley (2017) identified more taxa in the families Sarcophagidae (flesh flies) and Calliphoridae (blow flies) but less in the Muscidae (house fly and kin) family. Both studies commonly found *Musca domestica* (house fly) a proven vector of ASF virus (Dubie and Talley 2017). The only species of Silphidae (carrion beetle) found in our study was *Necrodes surinamensis* (red-lined carrion beetle) while the other study found *N. surinamensis, Necrophilia Americana* (American Carrion Beetle), and other unidentified species (Dubie and Talley 2017).

Figure 82. Water standing between the two animal mortality trenches

Silphidae (carrion beetles) were only caught in the traps around the animal mortality trenches and were observed crawling in and out of the dirt that covered the animal mortality trenches. This study was conducted in a field setting while Dubie and Talley (2017) conducted their study close to a feedlot. This

change in habitat could account for the diversity difference. *Philonthus spp.* (rove beetles), a member of the subfamily Staphylininae (rove beetles), was mainly found by Gaudry et al. (2006) at 10cm and 30 cm deep. Similarly, this study found more beetles in this family and subfamily in the animal mortality trenches compared to the preliminary and control site, though not significantly different. Beetles in the family Histeridae (clown beetles) were not collected in this study. Previous studies have collected these beetles (Amendt et al. 2010, Pastula and Merritt 2013, Dubie and Talley 2017). These beetles are usually collected ~7 days after burial (Pastula and Merritt 2013). One thing to note is that most other studies did not mention the method of collection. The two studies that did list collection method were Dubie and Talley (2017) and Pastula and Merritt (2013) and they used pitfall and hand collection, respectively. Difference in collection type could explain some of the difference in insect fauna. Parasitic Hymenoptera (wasps) have been found in burial sites up to 1 meter deep (Amendt et al. 2010). Parasitic hymenoptera (wasps) could explain the significant increase between animal mortality trenches and the control site.

Due to the time it took to inoculate the femurs of the pigs, the pigs were exposed to flies between 9 to 11 hours before burial. This allowed for flies to oviposit their eggs on the carcasses, which could have resulted in the mass emergence observed (Figure 80.) It is unclear if that was the sole reason for the mass emergence. Muscid (house flies and kin) flies have been shown to travel 10cm down in compacted soil and 40cm in loose soil (Gunn and Bird 2011). Other possible explanations are the wooden stakes used to mark the location for certain groups of pigs, which created an opening for developing flies (Figure 83) to emerge from the trenches. Another route for flies to emerge or gain access to the buried pigs were the zip ties used to locate the femurs of the swine, which were exposed and could allow flies to oviposit eggs onto the pig carcasses. Calliphorid flies (blow flies) have been known to oviposit on buried carcasses 105 days after they have been exposed by scavengers (Turner and Wiltshire 1999). When collecting femurs, we exposed the pigs for approximately 1 hour. This could have attracted more flies and allowed them to oviposit more eggs on the carcasses. This could also explain the additional peak in the collected flies' timeline. However, combined with the degree-hour model and the collection timeline, it is more probable to conclude that the eggs were laid on the day of burial.

The research team recovered viable virus inoculated into the femurs of the pigs 7 days post-burial. The six species of flies in the degree-hour model demonstrated an expected adult emergence after 8 or 9 days. This is enough time to reach third instar larvae for the flies with viable virus in the animal mortality trenches. Pastula and Merritt (2013) found that burying the carcasses at 30cm or 60cm does not significantly affect the accumulated degree days when compared to the ambient temperature. This study showed a ~6-day increase in the expected emergence date when comparing the Mesonet data, which represents the normal soil temperature nearby, to the Hobo® data loggers' data, which represents the temperatures in or around the buried swine. A point to consider is that Pastula and Merritt (2013) were examining burials and this study was examining a shallow trench composting system that incorporates a composting cycle that could retain or produce higher temperatures. Another potential issue that highlights the importance of completely covering buried carcasses is that some areas of the pigs were exposed after the burial process (Figure 82), which allowed certain Calliphorid flies (blow flies) to colonize those exposed areas. These areas were completely covered after a thorough inspection of both animal mortality trenches. Both Muscid (house flies and kin) and Calliphorid flies (blow flies) have been shown to lay eggs on the surface of the soil so the larvae can crawl down to the carcasses (Amendt et al. 2010, Gunn and Bird 2011).

Figure 840. Dipteran larvae emerging from one of animal mortality trenches

Figure 884. Wooden stake used to mark were certain pig groups were located within one of the animal mortality trenches with flies resting on it

Figure 852. Exposed pig snout with Calliphoridae flies crawling over it from an area of the animal mortality trenches that were not completely covered

3.4.3   Collected Insect Virus Viability

The team extracted the DNA of 16 insects collected from the trenches and conducted qPCR testing. One sample was considered inconclusive, and due to the limited amount of available material, we did not achieve a confirmatory result. All the remaining 15 samples were considered negative by qPCR for SwPV.

3.4.4   In-Vitro Virus Viability in house flies (*Musca domestica*)

In the event of animal disease outbreaks, mass animal mortality generally cannot be avoided. Carcass management systems all have one issue in common: for a period of time, the carcasses are exposed to insects; specifically flies. The flies that are attracted to the carcasses are not the common blood-feeding vectors that are normally involved in disease transmission cycles (Dubie and Talley 2017). Filth flies like house flies are in animal facilities and areas where carcasses are found (Watson and Carlton 2005). Previous studies have shown that house flies can carry pathogens from one area to another (Khamesipour et al. 2018; Graczyk et al. 1999; Fasanella et al. 2010). Joshi (2016) detected SVA from house flies on a farm that had no history of vesicular disease.

The importance of house flies as vectors for ASFV has been shown (Herm et al. 2020). Notably, pigs were successfully infected with flies exposed to ASFV (Herm et al. 2020). However, that study did not evaluate the time length that viable virus was present in the flies. Since house flies are common in mortality management systems and can carry pathogens, we evaluated the virus viability in exposed flies under an in vitro setting. In this study, house flies were successfully exposed to BVDV, SVA, and SwPV as described in Section 2.4.2. The SwPV DNA was detected at every time point except in males 24 hours post-exposure (Table 11).

There was a 75 percent and 65 percent decrease in number of viral copies between zero hours post exposure and 6 hours for males and females, respectively. One sample, female flies 6 hours post exposure, was positive in the virus isolation (VI) assay. The BVDV RNA was recovered from the females at each time point and males at zero hours after exposure (Table 11). The only viable virus was detected

from male house flies at hour 0 post exposure through virus isolation. The viable virus was confirmed by RT-qPCR after the third passage during virus isolation. The viral RNA was recovered from all the samples for SVA and the virus isolation showed an increase in viral load for each time point (Table 11). Notably, viable virus was obtained from all SVA-tested samples. Cytopathic effect and RT-qPCR demonstrated virus replication.

Infectious viruses were successfully recovered up to 48h for SVA and less frequently from SwPV and BVDV. Infectious virus was recovered from the BVDV-exposed flies immediately following exposure and from SwPV-exposed flies 6 hours post exposure. The SVA is known to be a more resistant virus under different abiotic situations (Dee et al. 2018) and it is demonstrated to have a long viability in flies. Lysyk and Axtell (1986)

Virus	Sample	Hour	CT pre VI	Estimated number of copies pre VI	Virus isolation	CT post VI	Estimated number of copies post VI
BVDV	Male	0	30.008	968,989	Pos	20.2865	477,492,250
		6	36.0385	20,701	Neg	*	*
		12	UD	N/A	Neg	*	*
		24	UD	N/A	Neg	*	*
		48	UD	N/A	Neg	*	*
	Female	0	25.9215	13,127,802	Neg	*	*
		6	33.393	111878	Neg	*	*
		12	34.805	45,462	Neg	*	*
		24	35.56	28,088	Neg	*	*
		48	32.7585	167,683	Neg	*	*
SVA	Male	0	22.486	46,633	Pos	13.1645	21,822,536
		6	23.688	21,104	Pos	13.188	21,486,886
		12	26.608	3,075	Pos	13.2185	21,058,941
		24	23.7115	20,779	Pos	13.4615	17,940,207
		48	28.325	990	Pos	14.606	8,432,906
	Female	0	19.474	340,019	Pos	13.31	19,825,571
		6	22.687	40,842	Pos	13.5115	17,358,196
		12	21.785	74,046	Pos	14.1065	11,723,644
		24	22.784	38,311	Pos	14.3085	10,261,203
		48	33.3255	36	Pos	14.2185	10,888,785
SwPV	Male	0	28.3115	13,218	Neg	*	*
		6	30.382	3,081	Neg	*	*
		12	30.8925	2,152	Neg	*	*
		24	UD	N/A	Neg	*	*
		48	33.44	358	Neg	*	*
	Female	0	28.679	10,207	Neg	*	*
		6	30.1835	3,543	Pos	*	*
		12	30.814	2,274	Neg	*	*
		24	33.418	364	Neg	*	*
		48	33.7225	294	Neg	*	*

collected flies over a 3-day period and captured flies released 20 km away. However, most flies associated with animal facilities or animal burial sites will stay within a shorter distance if resources are readily available. Long distance movement of flies that are potentially carrying viruses is caused by extreme weather events or unintended transport on animal transport vehicles.

Table 11. Cycle threshold (CT) to estimated number of virus copies conversion for each virus from flies collected and virus isolation (VI) plates if needed for confirmation. * Samples without evidence of virus replication were not resubmitted to qPCR or RT-qPCR.

The BVDV-CSFV surrogate was only viable from male flies immediately after exposure (hour 0). Stewart et al. (1975) showed mechanical transmission of CSFV via *Ades aegypti*. Additionally, CSFV has been shown to be mechanically transmitted by fomites and biting insects (Blome et al. 2017, Penrith et al. 2011).

Our findings suggest that flies can potentially carry viable BVDV virus for a shorter period of time compared to SVA or SwPV, still contributing to the movement of this pathogen into livestock operations.

This study provides evidence that house flies could be a potential vector for any of the viral pathogens tested. It should be noted that only five flies from each time point compose each tested sample. Nevertheless, viable virus was recovered from at least one sample from all tested viruses. These results clearly indicate that flies exposed to solutions containing infectious virus are able to transport viable virus for a variable period of time. In a mass mortality event, hundreds or thousands of flies will be exposed to infected body fluids, increasing the probability for virus transport and transmission. Additional studies are required to demonstrate viral transmission from house flies exposed to viral pathogens. A future project could also include other species of flies in the filth fly groups, including *Phormia regina* or *Cochliomyia macellaria,* to determine their role in virus transmission and retention.

The first study showed that the insects attracted to the animal carcass burial in a shallow trench composting system do not differ from the ones in traditional mortality management system (Rosa et al. 2011, Dubie and Talley 2017). The most common dipteran (fly) families are Calliphoridae (blow flies), Muscidae (house flies and kin), and Sarcophagidae (flesh flies) and the most common coleopteran (beetle) families are Silphidae (carrion beetles) and Staphylinidae (rove beetles). These studies have also shown that certain viruses can serve as surrogates to test the potential of house flies to mechanically transmit highly pathogenic viral strains. Based on these two factors and previous studies, we developed a table showing the potential risk of fly and beetle families for transmitting ASFV (Table 12). The low risk are insects that were captured at the field site but are not known to be associated with carcasses of dead animals. The medium risk are insects associated with carrion but not thought of as common pathogen vectors. This includes insects that could potentially transmit the pathogen mechanically if the virus can survive. The high risk are common vectors of pathogens that feed on blood. These insects have been proven in studies to transmit other pathogens. The final category are proven vectors of ASFV studies demonstrated by Herm et al. (2020) and Olesen et al. (2020) that have established house flies and stable flies as vectors of ASFV.

Table 12. Potential risk of beetle and fly families found during this study for the transmission of ASF

No Risk	Low Risk	Medium Risk	High Risk	Proven
Because we are just starting to understand insect transmission of ASF, all arthropods attracted to a euthanasia site are considered at this point.	Not known to feed on carcasses	Not yet proven to be common vectors of viruses associated with carcasses	Known vectors of other viral pathogens	Proven to vector ASF
	Dolichopodidae long-legged fly	Sarcophagidae Flesh fly	Muscidae Horn fly	Muscidae Stable fly House fly
	Ulidiidae Picture-winged fly	Anthomyiidae root-maggot fly		Argasidae Soft ticks
	Chrysomelidae Leaf beetles	Calliphoridae Blow fly		
	Cerambycidae Longhorn beetles	Culicidae mosquitoes		
		Tabanidae Horse fly		
		Staphylinidae Rove beetles		
		Silphidae Carrion beetles		

4   Conclusions and Recommendations

4.1   Conclusions

Based on the data collected during this study, we can draw a number of conclusions from the findings as summarized below and in Table 13.

4.1.1   Construction Logistics and Implementability

4.1.1.1   *Implementability*

- A 24-inch wide backhoe bucket worked better than a 10-inch bucket for excavating the trenches;
- Thick stakes should be used to prevent breakage or movement during back filling operations;
- A necropsy knife was the most effective knife for opening carcasses; frequent sharpening is needed;
- Field staff should use gloves and a face shield or goggles.

4.1.1.2   *Site Restoration*

- No bones or tissue were visible on the plot surfaces after 1 year;
- Buried tissue was forced out of a soft spot on the east trench from the weight of final grading equipment and had to be covered manually.

4.1.2   Virus Survival (Field)

4.1.2.1   *Nucleic acid detection by qPCR*

Viral DNA was detectable by qPCR assay throughout the 12 months of the study in the majority of samples.

4.1.2.2 *Swinepox virus survival*

Viable virus was only detected in samples collected at Day 7 post-burial in 30 percent (3/10) of femurs from the west trench and 80 percent (8/10) of femurs from east trench. No viable virus was detected after Day 7 in any samples.

4.1.3 Statistical analysis of field virus survival

4.1.3.1 *Probability of virus survival at one year*

There is a very high probability that no viable virus existed by the end of the study consistent with the rapid inactivation of the virus shown by viral titers.

4.1.3.2 *Time for viable virus to reach near-zero*

Virus concentration reaches near-zero at an estimated 10.7 days under the specific conditions of this study.

4.1.4 Virus Survival (In-Vitro)

4.1.4.1 *Virus viability in bone marrow samples*

Bone marrow material had no direct negative impact on the titer of SwPV, supporting the conclusion that the SBC process was responsible for virus inactivation, not the presence of bone marrow tissue.

4.1.4.2 *Virus viability in house flies*

In-vitro results demonstrated the ability of five flies to carry viruses when feeding in a solution containing the virus.

4.1.5 Environmental Monitoring

4.1.5.1 *Soil Ammonium*

- Ammonium reached background levels within 2 feet BTB during all time periods;
- SBC may release slightly more ammonium than composting, but significantly less than deep burial.

4.1.5.2 *Soil Potassium*

Potassium reached background within about 1 foot or less BTB during all time periods; it is generally not considered a pollutant.

4.1.5.3 *Soil Calcium*

- Calcium was below background for the first 6-12 inches BTB in both trenches, then somewhat above background levels for the remaining depths at most time periods;
- If excess levels of calcium from soil reach groundwater used for drinking, it is not listed as a primary or secondary contaminant under the Safe Drinking Water Act.

4.1.5.4 *Soil pH*

pH below both trenches is more acidic than background for the first foot or two BTB before returning to near-background for the next several feet of depth, then rising above background below both trenches for most time periods.

4.1.5.5 *Soil Total Dissolved Solids (TDS)*

TDS neared background within 3-5 feet BTB during all time periods in both trenches.

4.1.5.6   *Soil Sulfate Ions (SO4)*
- Levels neared background at 3-5 feet BTB;
- If excess sulfate from soil reaches groundwater used for drinking, it may affect taste.

4.1.5.7   *Soil Magnesium (Mg)*
- Levels were elevated above background at 1 year for all depths in the west trench, but had returned to background levels at 1 year in the east trench by 7 feet BTB;
- Mg is not a known pollutant

4.1.5.8   *Soil Percent Organic Matter (%OM)*
%OM levels returned to background levels at all time points in both trenches by about 4 feet BTB.

4.1.5.9   *Soil Total Nitrogen (TN)*
TN levels returned to background levels at all time points by about 2 feet BTB.

4.1.5.10   *Soil Nitrates/Nitrites*
- Nitrate/nitrite levels were at or below background levels under the west trench for all but one time point;
- The east trench exceeded background levels for nitrates/nitrites at 6 months and 1 year at 10-13 feet BTB.
- If high levels of nitrate/nitrite from soil reaches groundwater used for drinking, it can be harmful or fatal to infants and young animals, but it is unlikely nitrates/nitrites exceeded safe levels at any time or depth.

4.1.5.11   *Leachate Coliforms*
Elevated total coliforms were measured in leachate produced near the east and west trenches after heavy rain soon after trench construction.

4.1.5.12   *Forage Characteristics*
Forage growing over the trenches had more moisture and protein than forage growing on undisturbed soil around the trenches.

4.1.5.13   *Trench Olfactory Qualitative Assessment*
Odors in soil beneath the trenches were highest at the 2-4 month sampling events, decreasing with time and depth.

4.1.5.14   *Soil and Trench Temperatures*
- Temperatures in both trenches were up to 20 degrees higher than ambient for the first week after plot construction, dropping to near ambient after 2-3 weeks, then rising again from May to September 2020.
- Trench temperatures were similar to ambient from November through April, suggesting little microbial activity during the colder months.

4.1.5.15   *Wildlife Intrusion*
There were no signs of animal disturbance on the plots or at the fence line at any time during the study period.

4.1.6   Insect Monitoring

4.1.6.1   *Diversity and Abundance*
- There were significantly more beetles, especially carrion beetles, and grasshoppers in the animal mortality trenches than in the control site pitfall traps.
- There were significantly more flies, true bugs associated with plants, and ants and wasps in the animal mortality trenches than in the control site malaise traps.

4.1.6.2   *Fly Degree Hour Model*
The Fly Degree Hour predictive model shows an expected increase in fly activity 8-9 days post-burial, which is supported by the timeline of forensically important flies collected.

4.1.6.3   *Insect Prevalence Associated with Trenches*
- SBC attracts similar entomological fauna as other methods of mass animal mortality management;
- Flies were the most dominant order with flesh flies, house flies and kin, and blow flies the most dominant fly families.

4.1.6.4   *Mosquitoes*
Standing water near the trap sites likely explains the large number of mosquitoes; the decomposition-generated CO_2 may also have attracted them.

4.1.6.5   *Mass emergence of flies*
- Mass emergence of flies was most likely due to the time it took to inoculate the femurs of the pigs, allowing exposure to flies for 9-11 hours before burial;
- The time required to collect femoral samples may have contributed to mass fly emergence.
- The wooden stakes and zip ties marking sampling points may have contributed to mass fly emergence.
- Some tissue remained exposed after the burial process, which allowed blow flies to colonize those exposed areas.

4.1.6.6   *Collected Insect Virus Viability*
Of the 16 insects collected from the trenches, one sample was considered inconclusive, and all the remaining 15 samples were considered negative by qPCR for SwPV.

4.1.6.7   *In-Vitro Virus Viability in house flies*
House flies could be a potential vector for any of the viral pathogens tested.

4.2   Recommendations
This study produced extensive data related to the functioning of SBC for managing mass animal mortalities resulting from an animal disease outbreak. However, the data suggests there are still some unanswered questions. We provide the following recommendations for future consideration and ongoing work.

4.2.1   Monitor Plots for More Than a Year
Further work is needed to document continuing decomposition of carcasses in a shallow burial with carbon system beyond 1 year. The plots used for the subject study could be monitored at 3 years and beyond for this purpose.

4.2.2   Evaluate Change in Carcass Mass over Time

To document the stages of decomposition occurring during SBC, it is necessary to develop and validate a process to evaluate the change in carcass mass over time. This could be accomplished by weighing carcasses at the time of burial, then exhuming them at 6 months and 1 year post-burial and weighing the remains again.

4.2.3   Measure Virus Inactivation Rate in Winter

The subject plots were constructed in late summer during relatively warm weather, inactivating the surrogate virus in less than 2 weeks. The data showed that plot temperatures fell during winter, suggesting reduced decomposition during that time. To better understand the effectiveness of SBC when plots are constructed in the winter, another study could be conducted to measure the virus inactivation rate for winter plots.

4.2.4   Repeat Study with ASFv

It would be beneficial to study ASFv inactivation directly in swine carcasses that were actively infected with ASFv while still alive. ASFv inactivation may behave differently than SwPV. The viral load of a fully infected carcass may take longer to degrade than the method of inoculating virus into a femur bone as done in this field study. In addition, the statistical analysis can be improved by increasing sample size (number of carcasses). Further, choosing earlier time points to excavate samples would capture more of the ASFv decay curve before the concentration is below the detection threshold of the laboratory test.

4.2.5   Virus Transmission by Insects in Various Stages

Future studies should examine filth flies and their role in disseminating viral pathogens away from animal mortality management areas. The European Food Safety Authority et al. (2021) lists future projects to improve the understanding of mechanical vector transmission of ASFV. There are current gaps in knowledge related to filth flies like *Musca domestica* (house flies) and their role in ASFV transmission, especially from animal mortality management areas. Our current study is a proof of concept and more work is needed to detail the role house flies play in transmitting viruses. This study also shows the importance of timely management of animal carcasses with the mass emergence of fly larvae. Animal carcasses should be buried or placed in areas that limit the access of insects if timely management is not available. Also, future research should include the role of fly and beetle larvae in passing certain viral pathogens to subsequent life stages to gain a better understanding of immature stage in vector competence.

4.2.6   Impacts of Insecticide Treatments on Mass Fly Emergence

Data from the insect virus work and the degree-day model suggest that even after a certain time with certain temperatures, flies could become an issue. Specifically, more work is needed to determine if additional management such as applying insecticides to the carcasses as they are placed into the trenches will influence fly development or the decomposition process of the pigs. The time of exposure of animals before they are placed into an animal mortality management system is very important especially during the active fly season. In scenarios where animals cannot be immediately placed into a compost pile, burial pit, or a shallow-trench composting area, then additional steps will be needed to reduce the risk of certain flies from spreading infectious agents into the surrounding environment or other animal production areas.

Table 13. Conclusions

Construction Logistics and Implementability	
Implementability	- 24-inch wide backhoe bucket worked better than 10-inch bucket
Site Restoration	- No bones or tissue were visible on surface after 1 year - Buried tissue was forced out of soft spot from weight of final grading equipment and had to be covered manually
Virus Survival (Field)	
Virus amplification and characterization	- Nothing to note.
Nucleic acid detection by qPCR	- Viral DNA was detectable by qPCR assay throughout the 12 months of the study in the majority of the samples
Swinepox virus survival in field study	- Viable virus was only detected in samples collected at Day 7 post-burial in 30% (3/10) of femurs from the West Trench and 80% (8/10) of femurs from East Trench
Statistical analysis of femur data	- very high probability that no viable virus existed by the end of the study consistent with the rapid inactivation of the virus shown by viral titers. Virus concentration reaches near-zero at an estimated 10.7 days under the specific conditions of this study.
Virus Survival (In-Vitro)	
- Virus viability in bone marrow samples	- bone marrow material had no direct negative impact on the titer of SwPV, supporting the conclusion that the shallow burial with carbon (SBC) process was responsible for virus inactivation, not the presence of bone marrow tissue
- Virus viability in house flies	- results demonstrated the ability of 5 flies to carry viruses when feeding in a solution containing the virus
Environmental Monitoring	
Soil	
- Ammonium	- ammonium reached background levels within two feet BTB during all time periods - SBC may release slightly more ammonium than composting, but significantly less than deep burial
- Potassium	- potassium reached background within about a foot or less BTB during all time periods - it is generally not considered a pollutant
- Calcium	- calcium was below background for the first 6-12 inches BTB in both trenches, then somewhat above background levels for the remaining depths at most time periods

	- If excess levels of calcium from soil reach groundwater used for drinking, it is not listed as a primary or secondary contaminant under the Safe Drinking Water Act
- pH	- pH below both trenches is more acidic than background for the first foot or two BTB before returning to near-background for the next several feet of depth, then rising above background below both trenches for most time periods
- Total Dissolved Solids (TDS)	- TDS neared background within 3-5 feet BTB during all time periods in both trenches
- Sulfate Ions (SO4)	- Levels neared background at 3-5 feet BTB - Secondary drinking water contaminant for taste
- Magnesium (Mg)	- Levels were still elevated above background at 1 yr for all depths in the West Trench, but had returned to background levels at 1 year in the East Trench by 7 feet BTB. - Not a known pollutant
- Percent Organic Matter (%OM)	- %OM levels returned to background levels at all time points by about 4 ft BTB
- Total Nitrogen (TN)	- TN levels returned to background levels at all time points by about 2 ft BTB.
- Nitrates/Nitrites	- Nitrates/nitrites can be toxic to infants and young animals. - nitrate/nitrite levels were at or below background levels under the West Trench for all but one time point. - The East Trench exceeded background levels for nitrates/nitrites at 6 months and 1 year at 10-13 ft BTB. - It is unlikely nitrates/nitrites exceeded safe levels at any time or depth.
Leachate	Elevated total coliforms were measured in leachate produced near the East and West Trenches respectively after heavy rain soon after trench construction.
Forage	forage growing over the trenches had more moisture and protein than forage growing on undisturbed soil around the trenches.
Olfactory Qualitative Assessment	Odors in soil beneath the trenches were highest at the 2-4 month sampling events, decreasing with time and depth.
Soil and Trench Temperatures	Temperatures in both trenches were up to 20 degrees higher than ambient for the first week after plot construction, dropping to near ambient after 2-3 weeks, then rising again in the spring, suggesting most microbial activity occurs in spring, summer and fall.
Wildlife	There were no signs of animal disturbance on the plots or at the fence line.
Insect Monitoring	

Diversity and Abundance	- There were significantly more beetles, especially carrion beetles, and grasshoppers in the animal mortality trenches than the control site pitfall traps. - There were significantly more flies, true bugs associated with plants, and ants and wasps in the animal mortality trenches than the control site malaise traps.
Data Analysis	
- Fly Degree Hour Model	The predictive model shows an expected increase in fly activity 8-9 days post burial, which is supported by the timeline of forensically important flies collected.
- Insect Prevalence Associated with Trenches	- SBC attracts similar entomological fauna as other methods of mass animal mortality management - Flies were the most dominant order with flesh flies, house flies and kin, and blow flies the most dominant fly families - Standing water near the trap sites likely explains the large number of mosquitoes or the decomposition-generated CO_2 may have attracted them. - Mass emergence of flies was most likely due to the time it took to inoculate the femurs of the pigs, allowing exposure to flies for 9-11 hours before burial; also contributing may have been the time required to collect femoral samples, or due to the wooden stakes and zip ties marking sampling points. - Some tissue remained exposed after the burial process which allowed blow flies to colonize those exposed areas.
Collected Insect Virus Viability	Of the 16 insects collected from the trenches, one sample was considered inconclusive, and all the remaining 15 samples were considered negative by qPCR for SwPV.
In-Vitro Virus Viability in house flies (*Musca domestica*)	House flies could be a potential vector for any of the viral pathogens tested.

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