SERRI Report 09-81200-01

Phase I Final Report

SERRI Project: Aerobic Decomposition-Alternative Method for Managing Large Scale Animal Fatalities

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This material is based upon work supported by the U.S. Department of Homeland Security under U.S. Department of Energy Interagency Agreement 43WT10301. The views and conclusions contained in this document are those of the authors and should not be interpreted as necessarily representing the official policies, either expressed or implied, of the U.S. Department of Homeland Security.

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SERRI Project: Aerobic Decomposition - Alternative Method for
Managing Large Scale Animal Fatalities (81200)

PHASE I FINAL REPORT

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Date Published:

January 2011

Prepared for
U.S. Department of Homeland Security
under U.S. Department of Energy Interagency Agreement 43WT10301

Prepared by
OAK RIDGE NATIONAL LABORATORY
Oak Ridge, Tennessee 37831-6283
managed by
UT-BATTELLE, LLC
for the
U.S. DEPARTMENT OF ENERGY
under contract DE-AC05-00OR22725

ACKNOWLEDGEMENTS

The authors wish to express their gratitude to Mr. Nick Fielder, Director of Homeland Security for the Tennessee Department of Environment and Conservation; Mr. John Sanford, Food Manufacturing Administrator for the Tennessee Department of Agriculture; and Dr. Lee Meadows Jantz, Coordinator for the Forensic Anthropology Center at the University of Tennessee – Knoxville. Their review and input on this paper is greatly appreciated.

The authors also wish to think Mr. Ben Thomas of the Southeast Region Research Imitative of Oak Ridge National Laboratory. His knowledge and patience have proven invaluable throughout this research.

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ACRONYMS

CFU	colony forming unit
ClO_2	chlorine dioxide
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immunosorbent assay
GC	Agilent Technologies 6890 GC Series II
GC-MS	gas chromatography-mass spectrometry
mM	millimolar
MPN	most probable number
MSD	Agilent Technologies 5973 Mass Selective Detector
MSRV	modified semisolid Rapport-Vassiliadis medium
MTSU	Middle Tennessee State University
PCR	polymerase chain reaction
PMI	postmortem interval
ppb	parts per billion
ppbv	parts per billion volume
ppm	parts per million
ppmv	parts per million volume
ppt	parts per trillion
rDNA	ribosomal deoxyribonucleic acid
SPME	solid phase microextraction
TDEC	Tennessee Department of Environment and Conservation
TSA	Trypticase™ Soy Agar
U.S. EPA	United States Environmental Protection Agency
VOC	volatile organic compound

SOUTHEAST REGION RESEARCH INITIATIVE

In 2006, the U.S. Department of Homeland Security commissioned UT-Battelle at the Oak Ridge National Laboratory (ORNL) to establish and manage a program to develop regional systems and solutions to address homeland security issues that can have national implications. The project, called the Southeast Region Research Initiative (SERRI), is intended to combine science and technology with validated operational approaches to address regionally unique requirements and suggest regional solutions with potential national implications. As a principal activity, SERRI will sponsor university research directed toward important homeland security problems of regional and national interest.

SERRI's regional approach capitalizes on the inherent power resident in the southeastern United States. The project partners, ORNL, the Y-12 National Security Complex, the Savannah River National Laboratory, and a host of regional research universities and industrial partners, are all tightly linked to the full spectrum of regional and national research universities and organizations, thus providing a gateway to cutting-edge science and technology unmatched by any other homeland security organization.

As part of its mission, SERRI supports technology transfer and implementation of innovations based upon SERRI-sponsored research to ensure research results are transitioned to useful products and services available to homeland security responders and practitioners.

For more information on SERRI, go to the SERRI Web site: www.serri.org.

1. INTRODUCTION

In late August 2005, Hurricane Katrina made landfall and became the most costly hurricane in American history, with the greatest death toll and property damage occurring in New Orleans, Louisiana. Initial estimates for the number of human fatalities were exceedingly large. However, in disasters such as this, it is not only the human loss that is of concern, but depending upon the nature of the incident, it could involve the loss of large numbers of animals. Mass numbers of decaying animals may present a health hazard for the general population, especially if a biological agent is involved. Dead animals also represent a risk, especially if an infectious agent, such as anthrax or hoof and mouth disease, is responsible for the deaths.

Each year, thousands of animals of all sizes reach their end of life. While burial, rendering, cremation, and placement in landfills have been acceptable disposal alternatives in the past, current environmental and water quality regulations limit their use in most states. Recent legislation has limited the processing of horses for human consumption and their export to Mexico and Canada for processing will soon be illegal (Congressional Bills H.R.503 and S.311). Thus in the near future, the United States will need to dispose of 100,000 horses each year if the current rate of breeding and use of horses continue. The United States annually disposes of millions of cats and dogs mostly by incineration, but the size of horses and other large animals make this method impractical, especially with the rising cost of fuel. Rendering is an acceptable method but has met resistance if the animal has been euthanized with drugs that may taint the eventual product. Tennessee has one of the nation's largest populations of horses, and an alternative method of disposal is needed. Aerobic decomposition through composting has been used in the last decade for disposal of poultry and swine (Fulhage 1994; Glanville and Trampel 1997), but no recommendations are in place for the composting of large animals.

The equine industry, along with other large animal industries, needs research to develop alternatives of disposal not only for the normal end of life occurrences but also for mass disasters in which large numbers of animals die or have to be put down. This project examines aerobic decomposition as a method that could be developed to accommodate disposal of animals in mass disaster situations or on small farms or commercial operations. Factors involving the best aerobic media for decomposition, the proper temperatures, rate of decomposition, and effects on pathogens need to be identified. Additionally, attention must be paid to means of neutralizing the environmental impact of pentobarbital used to euthanize animals, the volatile compounds responsible for the odors of decomposition, and the use of chlorine dioxide as a disinfectant when agents such as anthrax or other pathogens are involved.

Many of the findings of this study are valuable in establishing protocol for high-magnitude mass disasters involving humans. Original estimates of the number of dead in New Orleans from Hurricane Katrina were thought to be as high as 10,000, a number that would easily outstrip local, state, and federal resources dedicated to the care of fatalities. In 2004, the Southeast Louisiana Catastrophic Hurricane Functional Plan stated, "Identifiable bodies will be placed in refrigerated trucks and stored until they can be processed and claimed" (Innovative Emergency Management, 2004). The National Association of Medical Examiners Mass Fatality Plan (2007) calls for "Refrigerated Trucks with metal floors which allow decontamination: 20 bodies per 40 foot trailer at 35 - 38ºF." Guidelines for Reporting and Managing Mass Fatality Events

with the Virginia Medical Examiner System further clarifies this by noting that "Forty foot refrigerated trucks will hold 22 remains without shelving. Shelving should be considered for a large number of remains to increase capacity. Shelving should not be above the waist to prevent back injuries to morgue staff" (The Virginia Medical Examiner's Disaster Casualty Guidelines 2005). Protocol for handling casualties in mass disasters usually involves refrigeration trucks, and if low shelving is used, it is reasonable to conclude that each truck can accommodate a maximum of 40 bodies. At this rate, 250 refrigeration trucks would have been required if the number of fatalities in New Orleans had reached 10,000. The space required to operate using this large number of trucks as well as the generators and other support equipment would have been a logistical nightmare. Immediate processing of the unidentified to document identifiers and burial in an appropriate burial media could replace refrigeration trucks. The animal model used in this study provides the foundation for additional research needed to develop protocol for managing large scale large animal and human fatalities.

1.1 Objective

The general goal for this research is to create an end product that will provide a scientifically proven method for the appropriate disposal of deceased animals in high-magnitude mass fatalities that is acceptable and cost efficient. Additionally, some of the findings can be applied to high-magnitude human fatalities.

The proposed research was originally planned for three phases; unfortunately, only Phase 1 was funded. In Phase 1, the basic infrastructure necessary for the experiments would be constructed in a secluded place with appropriate security, and initial data would be collected using 200-pound pigs and three burial media—sand, sawdust, and soil—to evaluate the advantages and disadvantages of each medium. The findings from Phase 1 were to provide the basis for the large animal study in Phase 2 in which horses were to be used. Phase 3, the concluding phase, was to address remaining questions from Phases 1 and 2 and allow the development of protocol for managing high-magnitude, large animal fatalities.

Phase 1 tasks involved the building of a containment facility to evaluate the effects of three different burial media (i.e., sand, sawdust, and soil) on the decomposition rate of 200-pound pigs as the initial animal model. Runoff was tested for contaminants, and volatile chemical compounds responsible for the odor produced by decomposition were examined. In addition, the utility of chlorine dioxide as an external decontaminate of corpses was researched, and the potential for developing a microorganism that could breakdown pentobarbital—an environmentally harmful compound used to euthanize large animals—was investigated.

1.2 Significance

The findings of this study are based in several disciplines and can be used in the development of mass disaster protocol. Additionally, some of the findings have application in numerous other areas. An understanding of the volatile chemical compounds associated with decomposition can provide the basis for developing a chemical means of handling offensive decomposition odors. Current research (Vass 2008) has investigated the odor of decomposition to identify "odor signatures" specific to humans. A better understanding of

odor signatures can be used to more effectively train cadaver dogs and develop field portable analytical instruments.

Pentobarbital is commonly used to euthanize large animals and may be required in mass disaster settings. However, its presence in the environment represents a hazard that would be amplified if large numbers of euthanized animals were buried in a mass grave. The development of a microorganism that breaks down pentobarbital would be of value not only in mass disasters but also on farms and ranches where individual animals must be euthanized.

Chlorine dioxide was used (Gugliotta and Warrick 2001) to fumigate anthrax after the terrorist attack on 9/11. At that time, technology required the mixture of the chlorine dioxide on site—a logistically cumbersome approach. Subsequently, technical advances have made the use of chlorine dioxide for decontamination more feasible for mass disaster situations. Findings from this study illuminate the utility of chlorine dioxide as a disinfectant for corpses, both animal and human.

1.3 Relevance to the Department of Homeland Security (DHS)

Currently, there exists no protocol to handle high-magnitude mass disasters involving the losses of large numbers of humans and large animals. This project was designed to address questions needed to design such a protocol. Unfortunately, only the initial phase was funded, and this report contains only the baseline findings.

2. PROJECT TEAM AND WORKFORCE DEVELOPMENT

2.1 Personnel

The project was carried out by an interdisciplinary team consisting of eight faculty members, six graduate students, and four undergraduate students from the Departments of Chemistry and Biology at Middle Tennessee State University (MTSU), MTSU's School of Agribusiness and Agriscience, and MTSU's Forensic Institute for Research and Education. The project team consisted of the following members:

Hugh E. Berryman, PhD, D-ABFA, Director, Forensic Institute for Research and Education, and Research Professor, Department of Sociology and Anthropology (Co-Principal Investigator)

John C. Haffner, DVM, Clinical Specialist in Equine Reproduction and Wellness (Co-Principal Investigator)

Anthony L. Farone, PhD, Professor, Department of Biology (Co-Investigator for runoff of contaminants and microorganisms that break down pentobarbital research)

Mary B. Farone, PhD, Associate Professor, Department of Biology (Co-Investigator for runoff of contaminants and microorganisms that break down pentobarbital research)

Ngee Sing Chong, PhD, Professor, Department of Chemistry (Co-Investigator for volatile organic compounds released during decomposition research)

Anthony L. Newsome, PhD, Professor, Department of Biology (Co-Investigator for chlorine dioxide as an external decontaminate of corpses research)

Warren Gill, PhD, Professor of Animal Science (Co-Investigator)

David Whitaker, PhD, Director, Horse Science (Co-Investigator)

Corbett Oullette (Bachelor Degree student, Biology, May 2011)

Luke Bolin (Bachelor Degree student, Chemistry, May 2011)

Dustin Hills (Bachelor Degree student, Biology, May 2011)

Jeannie Stubblefield (Bachelor Degree student, Biology, May 2011)

Michael Chad Cozart (M.S. in Professional Science, August 2010)

Samantha Keene (Masters Degree student, Chemistry, August 2011)

Lydia Rickman (Masters Degree student, Chemistry, August 2011)

Justin Lee (Masters Degree student, Biology, August 2011)

Alison Jordan (Masters Degree student, Biology, May 2011)

Tiffany Saul (Masters Degree student, Biology, May 2011)

The project team operated as four subgroups, each headed by a faculty Co-Investigator or one of the Co-Principal Investigators. The Co-Investigators contributed in areas of planning, experimental design, data collection and analysis, and report preparation. Students participated in all areas and gained knowledge and experience not only in chemistry and biology, but also in research design, application, and the mechanics of externally funded projects.

2.2 Presentations

Hugh E. Berryman and John C. Haffner. "Aerobic Decomposition for Large Scale Animal Fatalities." Presented at the Southeast Regional Research Initiative (SERRI) Semi-Annual Projects Review Meeting, Washington D.C., October 6, 2010.

Lydia Rickman. "Isolating Odor Signatures in Decomposing Animal Remains." Presented at the 95th International Association for Identification's (IAI) Annual International Educational Conference, Spokane, Washington, July 11-17, 2011.

Jeannie M. Stubblefield and Anthony L. Newsome. "Potential Use of Chlorine Dioxide to Decontaminate Skin Surfaces in an Animal Mass Casualty Response." Presented at the DHS University Network Summit, Washington, D.C., March 28-April 1, 2011.

Jeannie M. Stubblefield and Anthony L. Newsome. "Potential Use of Chlorine Dioxide to Decontaminate Skin Surfaces in an Animal Mass Casualty Response." Presented at Middle Tennessee State University Scholars Week, Murfreesboro, TN, April 4-8, 2011.

Jeannie M. Stubblefield and Anthony L. Newsome. "Potential Use of Chlorine Dioxide to Decontaminate Skin Surfaces in an Animal Mass Casualty Response." Presented at Posters on the Hill, Washington, D.C., April 13, 2011.

3. ACCOMPLISHMENTS

3.1 Construction of Facility

A concrete-barrier containment facility was constructed for the purpose of examining the effects of different burial media (i.e., sand, sawdust, and soil) on the decomposition process. The facility was located approximately six miles from campus on a farm owned by the Department of Agribusiness and Agriscience at MTSU. A remote site in a wooded area approximately 250 yards from a paved county road was chosen as the site. A gravel-based road was built to the site from the paved road, where the entrance was gated.

The base for the proposed research facility (Figure 1) is a concrete pad built 32 feet by 50 feet. The pad was formed by pouring four inches of concrete on a four inch deep bed of crusher run rock. It was configured with drainage grooves appropriate for the slope to handle any rain-based runoff. Wood dividers define eight separate stalls into distinct research compartments. The concrete pad is surrounded by a 72 feet by 102 feet chain link fence that is eight feet tall to give the pad security from animal and human intruders. The fence is 20 feet removed on all sides of the concrete pad. A small 12 by 12 feet storage building is placed within the fence line to secure tools and equipment needed for the project.

Figure 1. Photograph of the decomposition facility.

3.2 Effects of Different Media on the Decomposition Process

3.2.1 Introduction

The rationale for selecting the appropriate medium for the burial of animals and humans may be distinct. With animals, the complete digestion of soft and hard tissue and the elimination of pathogens are most desirable. With humans, extended preservation of hard tissue—allowing retrieval and repatriation after identification—is most desirable. Each burial medium provides its own advantages and disadvantages. Temperatures in sawdust increase during decomposition, speeding the process and therefore more effectively eliminating pathogens. In composting settings, this approach will break down bone, representing the optimum medium for large animals, but is problematic for humans where bone preservation is desired. Sand may be better for preserving bone but may tend to desiccate soft tissue, hampering complete decomposition. In a mass disaster, both sand and soil are more easily obtainable than composting material and probably less expensive. Soil may be more substantial and resistant to erosion than sand, but the advantages and disadvantages of soil over sand or sawdust for decomposition are not as well understood.

3.2.2 Materials and Methods

On May 13, 2010, five pig carcasses weighing 200 pounds each were placed in three burial media—one in sand, one in soil, two in sawdust, and one was placed on the surface as a control. Each carcass was placed on its left side on top of 30 cm of media base and then covered by media to a total depth of approximately one meter. Temperature sensors were placed in each animal and in the media. The control was placed on its left side directly on the concrete floor of the facility. These five pig carcasses were used in Trial 1.

In addition, on July 28, 2010, a second set of six pigs weighing between 30 and 40 pounds each were buried. One animal each was buried in two separate mounds of each media type (sawdust, sand, and soil), for a total of six animals in six mounds. Two temperature sensors (one inside the animal, one inside the mound) were placed in one mound of each media. The other three mounds and animals (one of each media) were without a temperature sensor. These six pigs were used in Trial 2.

3.2.3 Results

The carcasses from Trial 1 were excavated on September 15, 2010—four months after they were buried—and compared to the control (the remains exposed on the surface). Overall, the control carcass was in the dry stage of decomposition (Figure 2). The remains were irregularly covered by dry skin and soft tissue with windows exposing the cranium, mandible, cervical vertebrae, and portions of ribs and bones from the legs. The bones of the upper legs tended to be exposed while the lower leg bones were covered by desiccated soft tissue. Hair was present on portions of the pelvis, back, abdomen, shoulder, skull, and mandible. There was continued insect activity, mostly beetle, but relatively little odor.

Figure 2. Control pig placed exposed on concrete.

The sawdust carcasses exhibited (Figure 3) with much more soft tissue than the control. Approximately half of the bones were exposed on the right side of the body while the majority of soft tissue remained on the left or lower side of the body. Bone was partially exposed in many areas including the cranium, pelvis, and distal legs and feet. A considerable amount of skin and hair was present, as were many soft tissue body parts, such as the nose and ears. Exposed bones were oily, and there was a slight odor. No insect or maggot activity was noted.

The carcass buried in soil (Figure 4) had more advanced decomposition than the two buried in sawdust. Most of the bones on the right or upper side of the body were exposed and adipocere—a white, soap-like substance produced by the anaerobic bacterial hydrolysis of body fat—was abundant. The soil seemed to hold the body fluids, speeding decomposition. There was an abundance of large maggots in the abdomen, mouth, and other areas, and odor was prominent. The soil cover also provided a cool, dark, moist environment, facilitating maggot activity.

The sand showed the least amount of decomposition with few bones exposed and more soft tissue than carcasses in either the sawdust or soil (Figure 5). Muscle, pink in color, and fat encased the bones. The only exposed bones were portions of the mandible and bones of the lower legs and feet. Much of the soft tissue was desiccated, and there was little or no odor. Assessment was difficult due to the tendency of sand to adhere to the soft tissue.

Figure 3. Pig carcass buried in sawdust.

Figure 4. Pig carcass buried in soil.

Figure 5. Pig carcass buried in sand.

3.2.4 Conclusion

The carcass buried in the soil seemed to show the most decomposition as well as the most intense odor and presence of active groups of large maggots. The sawdust burials showed less decomposition than surface remains and less than the carcass buried in soil. The sawdust burials also exhibited less intense odor and no obvious insect or maggot activity. The sand showed the least amount of decomposition with considerable amounts of muscle and fat remaining and few bones exposed. The muscle, though it appeared to be somewhat desiccated, was pink in color. The sand burial showed surprisingly little odor for the amount of soft tissue remaining. There was no insect activity associated with the sand burial.

If the goal of a project is to reduce the remains to bone, burial in soil seems to be the best approach. However, if the goal is to retard decomposition and reduce odor, this initial test suggests that burial in sand is best. It is important to remember that these findings represent the initial elements of the Phase 1 baseline. More research is needed to draw meaningful conclusions.

3.3 Runoff of Contaminants

3.3.1 Introduction

Large scale mortalities of animal populations due to natural disasters or a biological attack would require management of the animal carcasses and their associated potentially pathogenic bacteria. Although surface decontamination of carcasses could reduce pathogens, the leaching of internal, intestinal bacteria into the environment could pose a

potential risk. This mortality composting generally involves the burial of animals in a pile or mound composed of media such as sawdust, straw, litter (containing manure), or wood shavings (Kalbasi et al. 2005). The temperatures of 55-60°C achieved in the mounds can inactivate most enteric pathogens (Wilkinson 2006). However, data on composting is generally limited to studies with manure or sewage biosolids (Berge et al. 2009). Information on initial pathogen containment or eventual pathogen elimination during animal composting using local materials easily accessible during emergency situations must still be addressed. In the instance of open bin systems, as would occur with natural disasters, ensuring that environmental conditions do not contribute to pathogen re-population is another important consideration. For example, mass mortalities following flooding might be covered with excessively moist material, or the re-wetting of compost piles can occur with rainfall events. The increased moisture can provide environments for pathogens to repopulate (Soares et al. 1995). For these studies, the destruction of the naturally occurring intestinal bacteria of the composted pig carcasses was compared for the three different burial media.

3.3.2 Materials and Methods

3.3.2.1 Collection of samples

For these studies, two trials were performed. In the first trial, one pig carcass was buried in sand and soil medium, two pig carcasses were buried in sawdust medium, and one was placed on the concrete surface as a control. For the second trial, two pig carcasses were buried in separate mounds for each burial medium. Prior to burial of the pig carcasses, samples were taken of all of the composting media to assess the initial presence of bacteria. For coliform and *Escherichia coli* sampling, samples were collected at regular intervals from the base of each mound (Figure 6) by inserting a 20 inch soil probe into the base approximately 2-3 inches above the concrete pad to remove a 6 x ¾ inch core (Figure 7). Two base samples from opposite sides of the mound were collected. Base samples were collected to assess organisms that would most likely be present in runoff. Base and core samples were used for *Salmonella* detection. The core sampler was chemically disinfected between samples. Samples were refrigerated up to 72 hours before testing for intestinal bacteria.

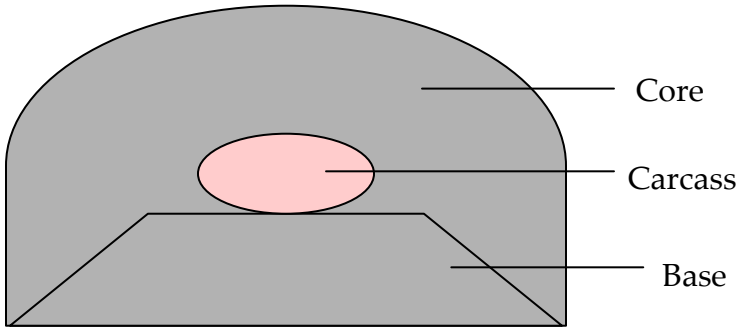

Figure 6. Representation of burial mound.

Figure 7. Core sampler used for collection.

3.3.2.2 Microbial testing of samples

Assessment of total coliform and *Escherichia coli* present in the samples was determined using IDEXX Colilert® and Quanti-Tray/2000®. For these studies, 10 g of the sample was added to 90 mL of sterile distilled water and disrupted using a Seward® stomacher. The disrupted mixture was serially diluted to 1 x 10-2 and 1 x 10-4 in 99 mL volumes of sterile distilled water. Following dilution, a Colisure® packet was added to each mixture. The bottles were incubated at 35°C for 24 hours and the presence of coliforms was confirmed by the appearance of a yellow color. The presence of *E. coli* was determined by looking for fluorescence after shining a 6-watt, 365 nanometer ultraviolet light 3-5 inches from the bottle. If samples were positive for coliforms or *E. coli*, the samples were further examined by pouring the bottle contents into a Quanti-Tray/2000®. The tray was sealed and incubated as above. After incubation, the numbers of yellow and fluorescent wells were determined and the most probable numbers (MPN) of coliforms and *E. coli* calculated using the accompanying MPN table. All samples were also tested for the presence of *Salmonella* species using the United States Environmental Protection Agency (U.S. EPA) Method 1682 (2006) for the detection of *Salmonella* in biosolids by modified semisolid Rappaport-Vassiliadis (MSRV) medium. Briefly, 25 g of the sample was homogenized using a stomacher in 225 mL of sterile distilled water. Samples were inoculated into 1x and 3x tryptic soy broth and incubated at 36.5°C for 24 hours. Turbid tubes were recorded and 30 L samples from turbid tubes were spotted onto plates of MSRV medium and incubated at 42°C for 18 hours. After incubation, plates were examined for the presence of a whitish halo and positive samples were streaked onto Xylose Lysine Dextrose and Hektoen Enteric media. Positive colonies appeared black on the media and were inoculated into tubes of

triple sugar iron agar, lysine iron agar, and urea broth for presumptive confirmation as *Salmonella*.

3.3.3 Results

Total coliforms and *E. coli* were measured for all media types and plotted along with precipitation data. In Trial 1, the sand burial medium had no detectable coliforms or *E. coli* on the day the medium was used for burial. No organisms were detectable 1 week following the burial (Figure 8A); however, by week 2, the detection of coliforms coincided with significant amounts of precipitation. By week 3, coliforms were not detected and remained undetectable throughout the sampling period. No *E. coli* was detected in the sand throughout the sampling period.

When soil was used for the burial medium, coliforms but not *E. coli* were detected in the soil used for burial. The coliform amount increased by week 2 (Figure 8B) and remained elevated throughout week 5, after which it decreased to initial concentrations. Rainfall also coincided with the initial increase in the coliform number. No *E. coli* was detected in these mounds.

The sawdust had high concentrations of *E. coli* (and identical concentrations of coliforms) in the initial burial material. Concentrations of *E. coli* increased by week 2 (Figure 8C), again coinciding with precipitation. The amount of *E. coli* remained elevated throughout the sampling period. Sampling of the mound at weeks 17 and 21 also resulted in concentrations of *E. coli* and coliforms greater than 1×10^6 colony forming unit (CFU)/mL, despite the fact that there were no detectable amounts of coliforms or *E. coli* in either the sand or soil mounds.

It is interesting that *Salmonella* was only detected during week 2 of sampling in the soil mound. This detection also coincided with significant rainfall and it is unclear whether this could have represented contamination from an outside source such as bird droppings.

For Trial 2, there were duplicate mounds for each burial medium. Initial sampling of the burial media showed that both the soil and sawdust were positive for coliforms but not *E. coli*. The sand contained no detectable coliforms or *E. coli*. However, following burial of the pig carcass (Figure 8D), coliforms were detected in one of the sand mounds during weeks 1 and 2 (data not shown on graph). *E. coli*, and hence coliforms, were detected at concentrations of greater than 1 x 106 CFU/mL in sand mound 1 during week 5. As noted for Trial 1, this increase in bacterial detection followed a precipitation event of more than one inch of rainfall. By week 6, no coliforms, *E. coli*, or *Salmonella* were detected in either of the sand mounds.

The soil mounds in Trial 2 were positive for coliforms throughout the sampling period including the initial sampling of the burial medium (data not shown on graph). However, *E. coli* was detected in only one of the mounds during week 6 (Figure 8E), which coincided with a significant rainfall of over two inches. This suggests that viable *E. coli* were still present in the mound and that rainfall facilitated the movement of these bacteria to the base. Interestingly, *Salmonella* was detected in core samples from one of the mounds during weeks 9 and 10. This indicates that the bacteria had survived the decomposition temperatures of the mound and may be leaching from the interior of the pig carcass. No further *Salmonella* was detected in the core or base samples of this mound.

The sawdust used as burial medium was positive for coliforms at concentrations of greater than 1×10^6 CFU/mL but was negative for *E. coli* before it was used for carcasses.

Coliforms were detected in both sawdust mounds at these same concentrations throughout the sampling period (data not shown on graph). *E. coli* concentrations, however, were not consistently elevated as in Trial 1. Both mounds had detectable *E. coli* between weeks 1 and 3, but the bacterium was not detected again until rainfall events occurred during weeks 6 and 8. During week 6, both mounds were positive for *E. coli*, but only one of the mounds was positive for week 8. *Salmonella* was detected on day 1 from the sawdust base of mound 1 and most likely represented contamination from the sawdust material itself since no significant decomposition had occurred. *Salmonella* was detected again following rainfall events at weeks 5 and 9, which again indicated the potential survival of this bacterium within the mounds.

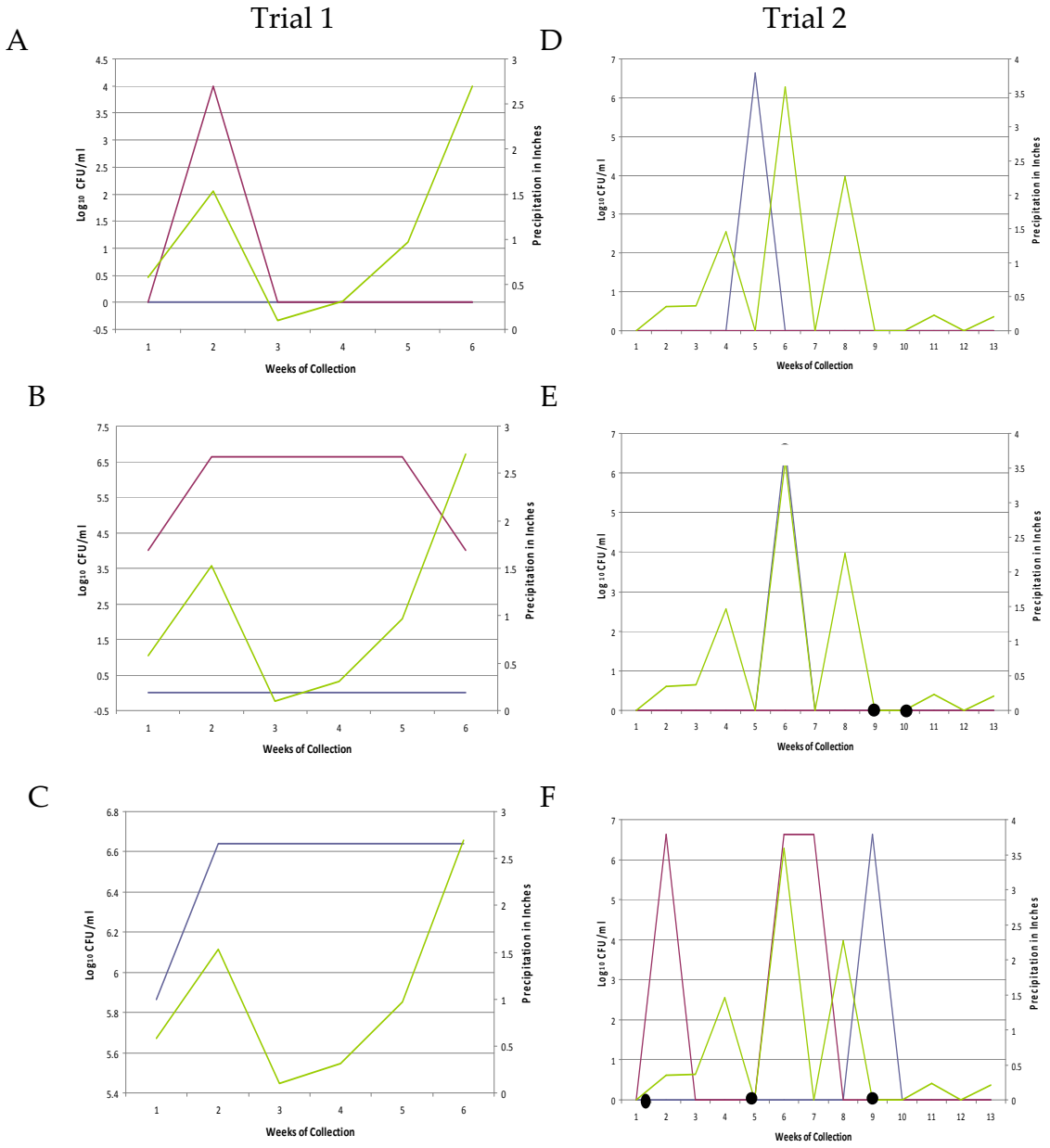

Figure 8. Trial 1 sampling of coliforms (—) and *E. coli* (—) from (A) sand, (B) soil, and (C) sawdust. For the sawdust sample, concentrations of *E. coli* and coliforms were identical. Trial 2 sampling of *E. coli* from mound 1 (—) and mound 2 (—) of (A) sand, (B) soil, and (C) sawdust. Concentrations of bacteria are plotted vs. precipitation (—) on the right axis. The circle (●) indicates the detection of *Salmonella* from the mound.

3.3.4 Conclusion

Of the three burial media, sand was the most effective at eliminating potentially pathogenic bacteria. Although sand is often recommended as a base material for mortality composting, very little data exists as to its effectiveness in composting or for pathogen destruction. In Trial 1, coliforms but not *E. coli* were detected following rainfall and in Trial 2, viable *E. coli* and coliforms could be detected during week 5 from one of the mounds after a rainfall event. Although none of these intestinal bacteria were detected after week 5, precipitation events could contribute to the movement of any viable bacteria from these mounds into the surrounding environment. The soil material used for the burials was contaminated with coliforms. These levels of coliforms remained elevated throughout the experiment, so it was difficult to assess whether coliforms from the carcasses were effectively eliminated. *E. coli* and *Salmonella* were not detected in the soil mound from Trial 1 but were detected during week 6 from one of the mounds but not in following weeks. S*almonella* was detected during weeks 9 and 10. This may indicate that the bacteria survived the internal composting temperatures and that rainfall facilitated the movement of these bacteria to the outer, cooler portions of the mound. This may also represent a regrowth of *Salmonella* following depletion of competing microorganisms in the mound as nutrients became limited (Wilkinson 2007). The sawdust was the least dense of the all the burial media and could more easily facilitate the movement of internal microorganisms to the outer portions of the mound. The sawdust contained coliforms that remained elevated throughout the sampling period. *E. coli* and *Salmonella* could also be detected in the mounds throughout weeks 8 and 9 respectively indicating long-term survival of these potential pathogens in this material. However, because of contamination of the initial medium, it is difficult to determine whether these bacteria represent the indigenous organisms or intestinal bacterial from the carcasses. Nonetheless, this medium supported the continuing survival of these bacteria. Consultation with Gregory Denton, manager of the Planning and Standards Section of the Tennessee Department of Environment and Conservation (TDEC) Division of Water Pollution Control confirmed the importance of reducing coliform runoff into water systems. Of the burial materials used, sand was the least likely to have indigenous coliforms, and it also eliminated coliforms and pathogens by week 5.

3.4 Microorganisms that Break Down Pentobarbital

3.4.1 Introduction

Barbiturates, which include the euthanasia drug sodium pentobarbital, have been shown to stably persist in the environment over long periods of time (Peschka et al. 2006). Large animals such as horses euthanized with barbiturates are considered environmental hazards and disposal options are limited (Lenz 2009). Large scale euthanasia of animal populations could potentially result in the release of active barbiturate compounds into the environment that could be harmful to humans and wildlife. Uncovered carcasses disposed of in landfills or used as donated carcass meat for carnivores in zoological institutions have resulted in barbiturate toxicity (Harrison et al. 2006; National Wildlife Health Center 1999). Large animals could have 30-40 g of barbiturates in the carcass following euthanasia (Wolfgang 2009). Further study is needed on the stability of this drug in the environment and the potential for bioremediation by barbiturate degrading microorganisms.

3.4.2 Materials and Methods

3.4.2.1 Barbital enrichment

Barbiturate-degrading microorganisms were isolated from samples taken from horse stall litter that had been left to dry at the edge of a field before spreading. It was rationalized that naturally occurring, thermophilic microorganisms could be present in stall litter with the potential to degrade barbiturates. Barbiturate-degrading bacteria were isolated from the material by enrichment in sodium pentobarbital. For these studies, 1 g of the stall litter was diluted into 99 mL of sterile distilled water and shaken vigorously. A 1 mL volume of this mixture was added to minimal broth (Davis formulation; Becton-Dickinson) to which 0.25% glucose and 0.002 mg/mL sodium pentobarbital (Sigma-Aldrich Chemical Company) were added. Cultures were incubated at 37°C until turbid. A 0.1 mL volume of each turbid culture was transferred to minimal broth containing 0.125% glucose and 0.004 mg/mL sodium pentobarbital. These cultures were incubated at 37°C until turbid. The process was repeated, incrementally decreasing the concentration of glucose and increasing the concentration of sodium pentobarbital to 0.04 mg/mL. This concentration is equivalent to 40 parts per million (ppm) and represents dilution of the compound in the burial medium. Concentrations as high as 250-400 ppm have been reported in the livers of euthanized animals (Wolfgang 2009).

3.4.2.2 Temperature enrichment

Once cultures containing barbiturate-degrading microorganisms were obtained, 0.1 mL volumes of the cultures were transferred to the 0.4 millimolar (mM) sodium pentobarbital broths and incubated at increasing increments of 2°C. Temperatures were increased until broths remained clear for at least five days.

3.4.2.3 Identification of bacteria

Broths that were turbid at elevated temperatures were streaked for isolation onto tryptic soy agar plates. Isolated colonies were inoculated into 0.4 mM sodium pentobarbital broth and incubated at the temperature from which they were isolated. Bacteria that were re-isolated from these broths were identified by sequencing of the small subunit ribosomal deoxyribonucleic acid (rDNA) gene as described in Berk et al. (2005). Briefly, deoxyribonucleic acid (DNA) was extracted from bacterial colonies and amplified by the polymerase chain reaction (PCR) using universal primers for the small subunit rDNA gene. The PCR product was sequenced on an Applied Biosystems™ 3130xl Genetic Analyzer using universal bacterial primers and BigDye® terminator version 3.1 (Applied Biosystems™) chemistry. To determine the identity of the bacteria, the resulting sequences were compared to known sequences in the National Center for Biotechnology Information database using the Basic Local Alignment Search Tool (BLAST).

3.4.2.4 Barbiturate enzyme-linked immunosorbent assay

Supernatants from potential barbiturate-degrading bacteria were collected by removal of the bacteria by centrifugation at 8000 centrifugal force relative to gravity (xg) for 10 minutes. The resulting supernatants were diluted 2000-fold in phosphate buffered saline. The 0.4 mM

sodium pentobarbital broth was also diluted 2000-fold for use in the enzyme-linked immunosorbent assay (ELISA) protocol. Supernatants were tested for the presence of barbiturates by the BIOQUANT Barbiturate Direct ELISA Kit, which can detect between 0-52 nanograms of pentobarbital.

3.4.3 Results

Following barbital and temperature enrichment, four bacteria were identified as potential barbiturate-degrading microorganisms. The designations of these bacteria are reported in Table 1, along with their maximum temperature limit for growth in 0.4 mM sodium pentobarbital. The two organisms that grew at 47 and 49°C were identified by 16S rDNA sequencing as members of the genus Pseudomonas.

Table 1. Characteristics of Sodium Pentobarbital-degrading Bacteria

Organism designation	Maximum growth temperature	Fold decrease in pentobarbital	Identification
W3-A	45°C	2.5	ND*
HSC-A2-A	45°C	2.9	ND*
HSC-A2-D	47°C	2.5	*Pseudomonas fluorescens*
HSC-B-C	49°C	2.6	*Pseudomonas aeruginosa*

* ND = not determined

3.4.4 Conclusion

Using enrichment methods, barbiturate degrading bacteria were isolated. Because the bacteria were isolated from composting manure, these bacteria represent organisms which may not only be thermophilic but also represent indigenous organisms from either the environment or intestinal tracts of the animals. Thus introduction of these bacteria into media for mortality composting does not represent a potential health hazard. The bacteria were able to degrade the sodium pentobarbital at temperatures up to 49°C. Although internal temperatures of decomposition mounds may reach temperatures of greater than 60°C, the presence of these bacteria in the cooler, outer portions of the mounds may prevent the movement of barbiturates into the environment. Wolfgang (2009) has reported the persistence of barbiturates in mortality compost mounds for 80-90 days with potential detection for one year. In instances of a mass disaster necessitating euthanasia, 30-40 g of barbiturates per carcass could be present. These concentrations could pose a threat to ground water and watersheds. TDEC regulations prohibit the release of pollutants into the water supply that may be detrimental to water as a source of domestic water supply (TDEC 2008). Although naturally-occurring degraders in compost materials may facilitate the degradation of barbiturates, the addition of known degraders to composting material could enhance the process and reduce environmental contamination. Further studies will be needed to determine the efficacy of adding barbiturate-degrading bacteria to compost material.

3.5 Volatile Organic Compounds Released During Decomposition

3.5.1 Introduction

The release of volatile organic compounds (VOCs) from decomposing animal carcasses or human remains is inherently important to the forensic community. Law enforcement officers often use cadaver dogs to aid them in the search for the bodies of homicide victims. Canines are able to differentiate between living and non-living humans, which suggests there is a distinguishable scent emitted by cadavers which the dogs can detect long after humans can. Often the canines pick up on scents that are not specific to humans, or they identify ancient remains. By fully characterizing the odors being emitted during decomposition of pig carcasses and comparing their chemical identities to odors or VOCs released from human remains as reported in a previous study (Vass et al. 2002; Vass et al. 2008), the distinguishing characteristics of chemical vapors unique to human remains may serve as training aids for the cadaver canines to achieve a higher accuracy of detecting human remains. Furthermore, the chemical profile obtained from this study may be used to help select candidate compounds for training cadaver dogs used in search and recovery operations or developing powerful and yet portable sensors for finding human remains.

This research involved monitoring the volatile organic compounds (VOCs) emitted during the decomposition of pig carcasses, which were buried in soil, sand, and sawdust. Pigs were used since their anatomy resembles that of a human, and they are readily available as research specimens (Lorenzo et al. 2003). This research will also aid in understanding the process of animal decay under varying environmental conditions that can be used to facilitate the management of mass casualties after catastrophic events. Another advantage of analyzing the VOCs released during decomposition is the development of a method to more accurately determine postmortem interval (PMI). Currently, however, there are few scientific methods based on chemical measurements that can be used to provide the PMI estimate. Typically, the PMI estimate is obtained by visual inspection of the body for signs of rigor and livor mortis and determining the core body temperature and gastric contents. In this project, the dependence of the chemical profile of gaseous emission on the decomposition period is examined and useful biomarkers are identified and rationalized based on catabolic reactions in the decomposition of biomolecules such as proteins, lipids, and carbohydrates present in the body mass of pig carcasses. Weather conditions such as the temperature, rainfall, and relative humidity, which were capable of influencing decomposition rates, were recorded over the course of the decomposition of animal carcasses.

This project also provided opportunities for the assessment of analytical techniques for measuring trace levels of VOCs released by the pig carcasses. In order to analyze the volatile organic compounds in the range of parts per trillion (ppt) to parts per billion (ppb) levels, three analytical sampling approaches were implemented. The first one involves sampling using Tedlar™ bags that were filled by using a pump to create a partial vacuum in an airtight suitcase in which clean Tedlar™ bags were placed and connected to the sources of VOCs from pig carcasses via Teflon® tubing. The second method involved connecting an evacuated bottle to a metal probe inserted into the media for burying the carcasses. Both the bags and bottles were subsequently analyzed by gas chromatography-mass spectrometry (GC-MS) analysis via a pre-concentrator equipped with three traps for maximal transfer of analytes with minimal interference from the carbon dioxide and moisture released during

the decomposition process. Figure 9 shows the photo of the preconcentrator coupled to a 16-positon autosampler that can handle the automated and unattended sequence analysis of up to 16 gas samples in bags, bottles, or canisters. It also includes a schematic diagram of the glass bead trap, the Tenax trap, and the cryofocuser being connected via the two 6-port valves to the inlet of the GC-MS so that various heating, cooling, flow, and timing parameters can be adjusted to improve the preconcentration efficiency to achieve the detection limits down to 0.1 parts per billion volume (ppbv) or 100 ppt level. The third sampling approach involves the use of a solid phase microextraction (SPME) sampler as depicted in Figure 10. The sampling device consists of a retractable silica fiber coated with sorbent materials that was exposed to the gas samples collected from the decomposing carcass or tissues to allow the extraction of the trace VOCs onto the sorbent coating. The sorbent-coated silica fiber was subsequently injected into the heated injector of a gas chromatograph to desorb the compounds for GC-MS analysis. The SPME process is not automated and is fairly tedious.

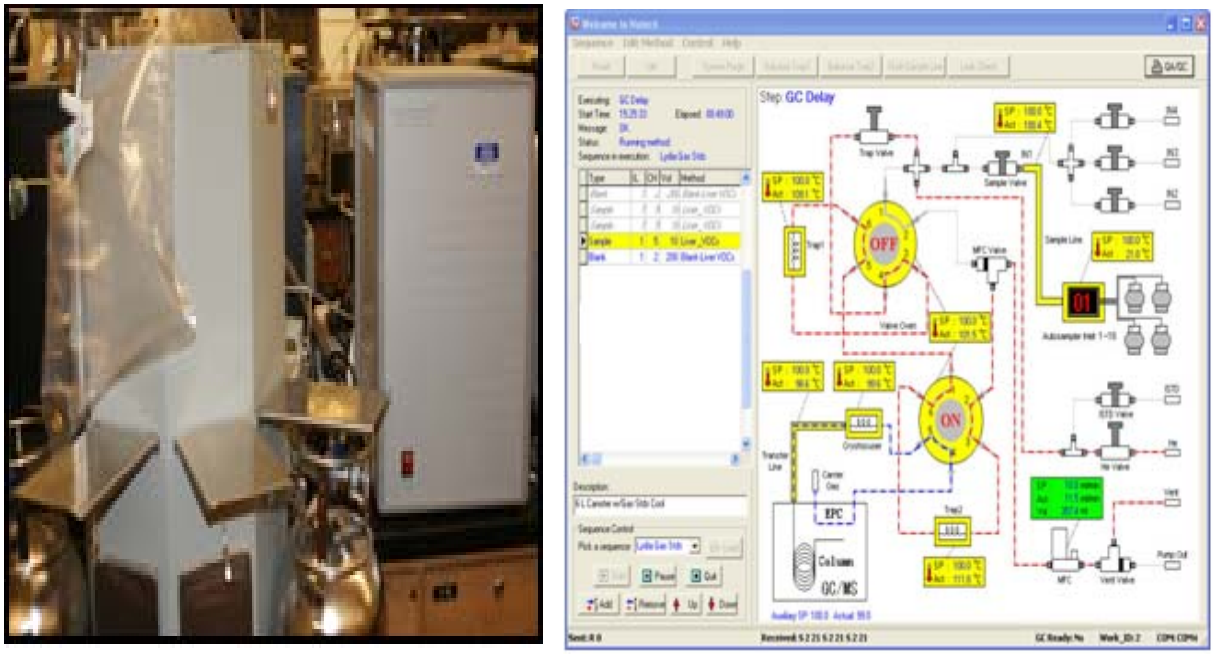

Figure 9. Photo of the Nutech Preconcentrator and Autosampler as well as the schematic of the flow paths for the preconcentrator.

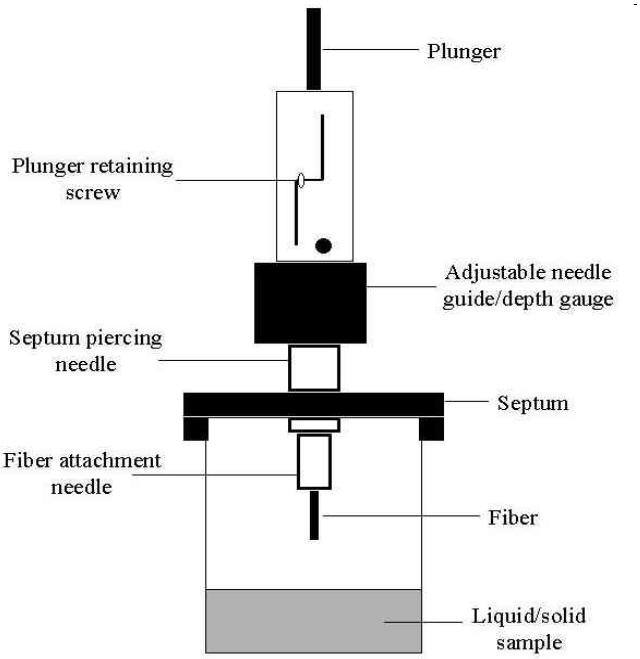

Figure 10. Schematic diagram of a solid phase micro-extraction setup.

3.5.2 Materials and Methods

For this part of the study, the pig carcasses from Trial 2 were utilized. These pig carcasses were buried in sand, sawdust, and soil along with the deployment of temperature, rainfall, and dew point sensors on July 28, 2010, and gaseous samples from the emission of VOCs during the decomposition of carcasses were collected for almost two months until September 21, 2010. The VOCs released from pig carcasses were sampled at the frequency of two to three times per week for the first month, and the sampling frequency decreased to once or twice per week for the second month.

The sampling containers were predominantly evacuated bottles along with some Tedlar™ bags and stainless steel canisters. The Tedlar™ bags were filled with ultra-high purity nitrogen and emptied three times prior to being used for sampling. Likewise, the bottles and canisters were also filled with nitrogen and evacuated at least two times before being used to collect samples. Teflon® tubing of 0.25 inch o.d. was buried in proximity to the pigs and the tubing outlet was covered to prevent rainwater from getting into the gas sampling tubing. Tedlar™ bags were used to collect samples with a pump according to differential pressure conditions; whereas, the pre-evacuated bottles or canisters were used for instantaneous sampling by attaching it to a soil vapor sampling probe.

Analysis of emission samples was conducted using the Agilent Technologies 6890 GC Series II (GC) coupled with the 5973 Mass Selective Detector (MSD). The GC was coupled to a Nutech 8900 Preconcentrator with a Nutech 3602 Autosampler to which Tedlar™ bags, glass bottles, and canisters containing the samples or standards were attached. The conditions of the preconcentrator and GC-MS are shown in Table 2. A sample volume of 100 mL was transferred to the Nutech 8900 Cryogenic Preconcentrator. The GC capillary

column used for VOC analysis was a Restex MXT®-1 (100% polydimethysiloxane) column of 60 meters in length, 0.25 mm i.d., and a film thickness of 0.50 mm. The helium carrier gas had a linear flow velocity of 32 cm/sec and the MSD was set to scan mode with a mass range of 33.0-455.0 amu.

Table 2. Operating Conditions of Preconcentrator and GC-MS Instrument

Preconcentrator Conditions	
Glass Bead Trap	Cools to -150°C Desorbs at 80°C
Tenax Multimedia Trap	Cools to -20°C Desorbs at 200°C
Cryofocuser	Cools to -150°C Desorbs at 200°C
GC Method Conditions	
Initial Oven Temp and Time	32°C for 8 min
Ramp 1 Rate	5°C/min to 150°C, 1 min hold
Ramp 2 Rate	20°C/min to 300°C, 1 min hold
Column Type	Restek MXT®-1
Column Length	60.0 m
Column Diameter	250 µm
Column Film Thickness	0.50 µm
Helium Carrier Flow	1.5 mL/min
Column Pressure	22.66 psi
Linear Flow Velocity	32 cm/sec

3.5.3 Results

Among the VOCs detected from the pig carcasses, the classes of compounds found in the greatest abundance were esters, aldehydes, and sulfur-containing compounds. Some of these compounds were found at levels as high as 1-10 parts ppm. Furans and aromatic compounds were measured at intermediate levels of 2-750 ppbv and 5-200 ppbv, respectively. Amines and chlorinated compounds were detected not only at lower concentrations of <100 ppbv, generally, there were also substantially less chemical species in these two categories relative to the other compound classes.

For the aldehydes, hexanal generally was found at the highest concentrations in most instances for all three carcasses, but petanal occasionally was measured at higher concentrations than hexanal during the first three weeks for the pig carcass buried in soil (Figure 11). Alkanes found in the emission samples have carbon numbers ranging from C4 to C13 based on the cryofocusing GC-MS technique used (Figure 12). However, C1-C3 alkanes were likely to be present but could not be measured using the current method. Future analysis will need to use extractive infrared spectrometry with a long pathlength gas cell. Among the aromatic compounds detected, hexylbenzene, 1,2,4-trimethylbenzene, and methoxybenzene were present at higher levels than others (Figure 13). Naphthalene was found at significantly higher levels in the soil than in the other two media. It was unclear if the higher levels were related to a higher level of microbial activity in the soil. Alcohols were the most polar compounds observed during the pig decomposition process (Figure 14) and were represented by 1-petanol, 1-hexanol, 1-heptanol, and 1-octanol in roughly the descending order of concentrations. After each episode of rain and especially pronounced during the August 17-20 period, the alcohols were completely soluble in the rainwater and were effectively removed from the gas phase. The most common esters found in the gas samples were amyl butyrate and ethyl butyrate (Figure 14).

The presence of furans like 2-pentylfuran and 2-heptylfuran were likely formed from the catabolic reactions of fatty acids from the breakdown of animal fat (Figure 15). The levels of furans in the soil were significantly higher than those in the sawdust and the sand. Three reasons could have contributed to this disparity. The first is that the degree of mixing and hence dilution is larger in the sawdust and sand media relative to the soil media. Secondly, the soil condition was more conducive toward the production of singlet oxygen that was cited as being responsible for the metabolic transformation of linolenic acid from fatty tissues into 2-pentylfuran (Min, Callison, and Lee 2003). Thirdly, since it has been reported that a fungal strain named *Aspergillus fumigatus* may be responsible for the production of 2-pentylfuran (Syhre, Scotter, and Chambers 2008), it was possible that the *Aspergillus* fungus may be more prevalent in the soil than the other two media. The metabolic by-products in the form of ketones were unique compared to other VOCs detected because they were usually found at higher concentrations in the later stage of decomposition or more than a month since the carcasses were buried (Figure 16). This trend was particularly obvious for the pig carcass buried in soil. Acetone was the most abundant ketone found, but other ketones of lower molecular weight were also found in lower concentrations. The presence of sulfur-containing metabolites such as methanethiol, dimethylsulfide, dimethyldisulfide, and dimethyltrisulfide was linked to the biochemical degradation of sulfur-containing amino acids like methionine, cystine, and cysteine (Figure 17). The presence of dimethyldisulfide and dimethyltrisulfide may be linked to cystine, which is due to the dimerization of two cysteine units and is commonly found in collagen (i.e. connective tissue) and keratin

(hooves and hair). The production of sulfur compounds was persistent throughout the project period. On the contrary, 1,1-difluoroethane was typically found after at least 7-10 days since the inception of decomposition. It was suspected then as the decomposition progressed to the point that the microbes metabolized the bone tissues, the fluoride migrated from the bones and reacted with hydrocarbons to form fluorinated compounds.

The data plots from the environmental sensors for temperature, dew point, and rainfall are shown in Figures 18-20. It was apparent that the decomposition of pig carcasses released heat to the surrounding soil where the probe was buried. The heat raised the temperature in the soil by about 6-10 °C for the initial decomposition period of about the first three weeks before leveling off. The extent of temperature increase was less apparent in sand and was most likely less apparent in sawdust also, but a defective probe provided inaccurate data. The higher temperature during the initial period of decomposition was due to the concomitant factors of higher ambient air temperature in early August and the aerobic decomposition process that is exothermic. The porosity of the media also affected the VOCs concentrations significantly because of the varying extent of mixing with ambient air. In general, the VOC concentrations of the pig carcasses decrease in the order of soil, sand, and sawdust as the decomposition media.

The most salient observation of this study of porcine decomposition is that the identities and concentrations of VOCs released yield important telltale clues of forensic significance regarding the decomposition process. Although one would intuitively expect the VOC concentrations to reflect the rate of decomposition that increases initially as the insects and microorganisms accelerate the breakdown of the organic matter and eventually decreases to a minimal level, the prevailing environmental conditions surrounding the decomposing carcass is of paramount importance in affecting the actual gas phase concentrations measured. The most notable environmental condition in influencing VOC levels is the amount of rainfall, which plays a role in reducing the level of the more polar compounds such as alcohols, ketones, and aldehydes. These compounds have high water solubility and tend to partition in the aqueous phase when the media of sand, sawdust, and soil are soaked with rainwater. This phenomenon of "washing out" the polar VOCs was observed for all compounds for pig carcasses buried in all three media after each episode of rainfall, especially after the rain that lasted from August 17 till August 20 that totaled 63 mm of rainwater. The VOC levels gradually returned to the expected higher values when the rainwater in the media evaporated and the VOCs were released in gas phase.

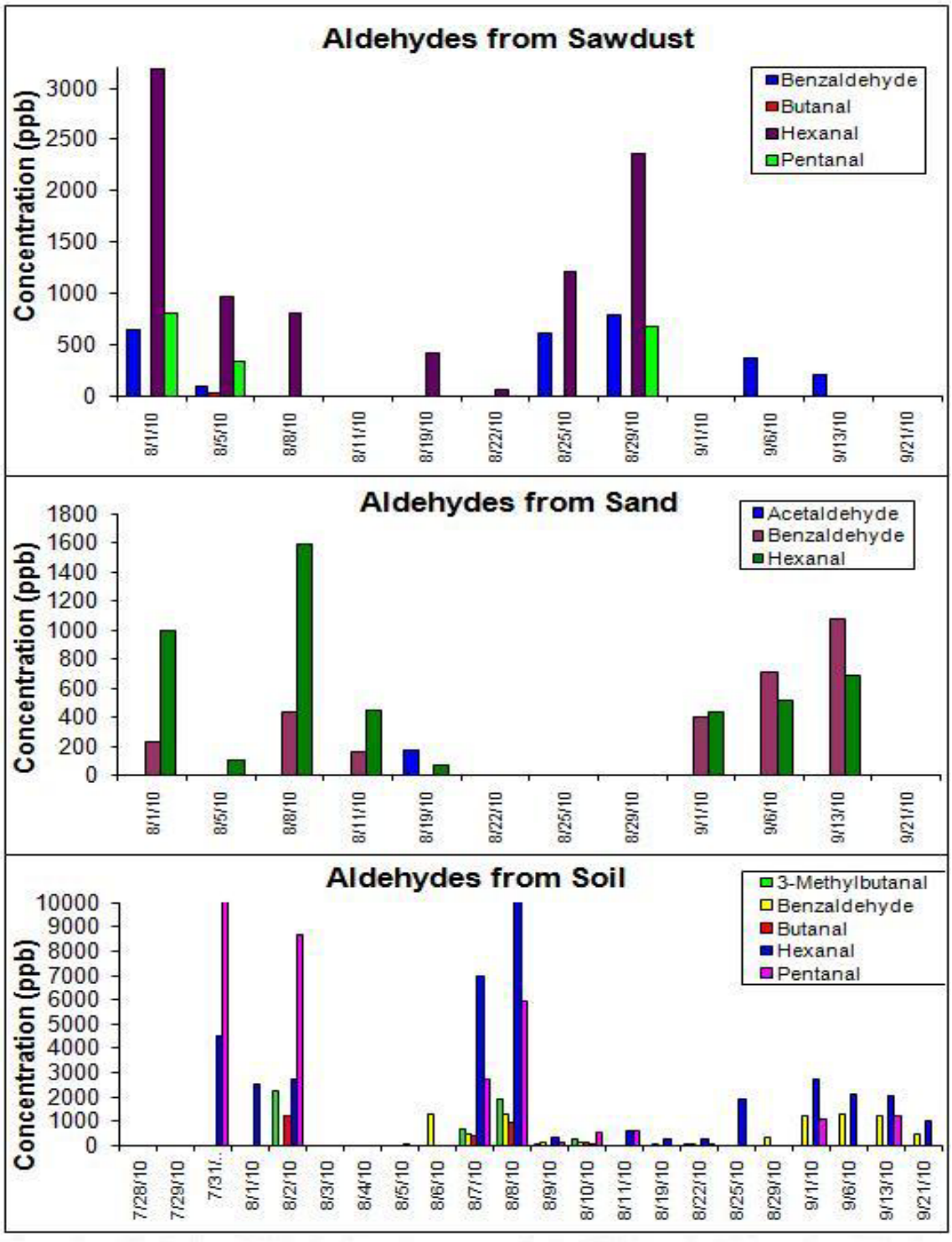

Figure 11. Emission of aldehydes from pig carcasses buried in soil, sand, sawdust.

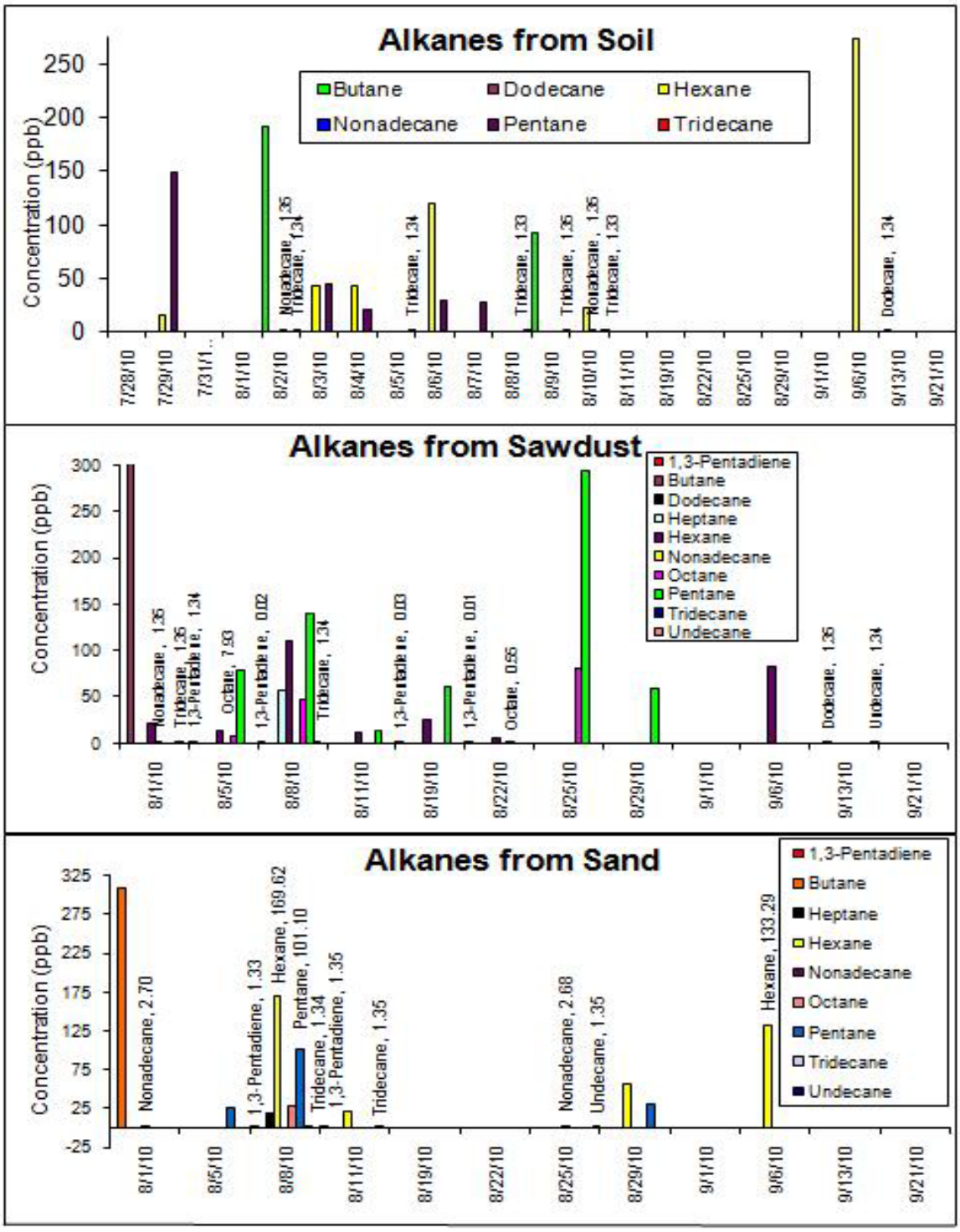

Figure 12. Emission of alkanes from pig carcasses buried in soil, sand, sawdust.

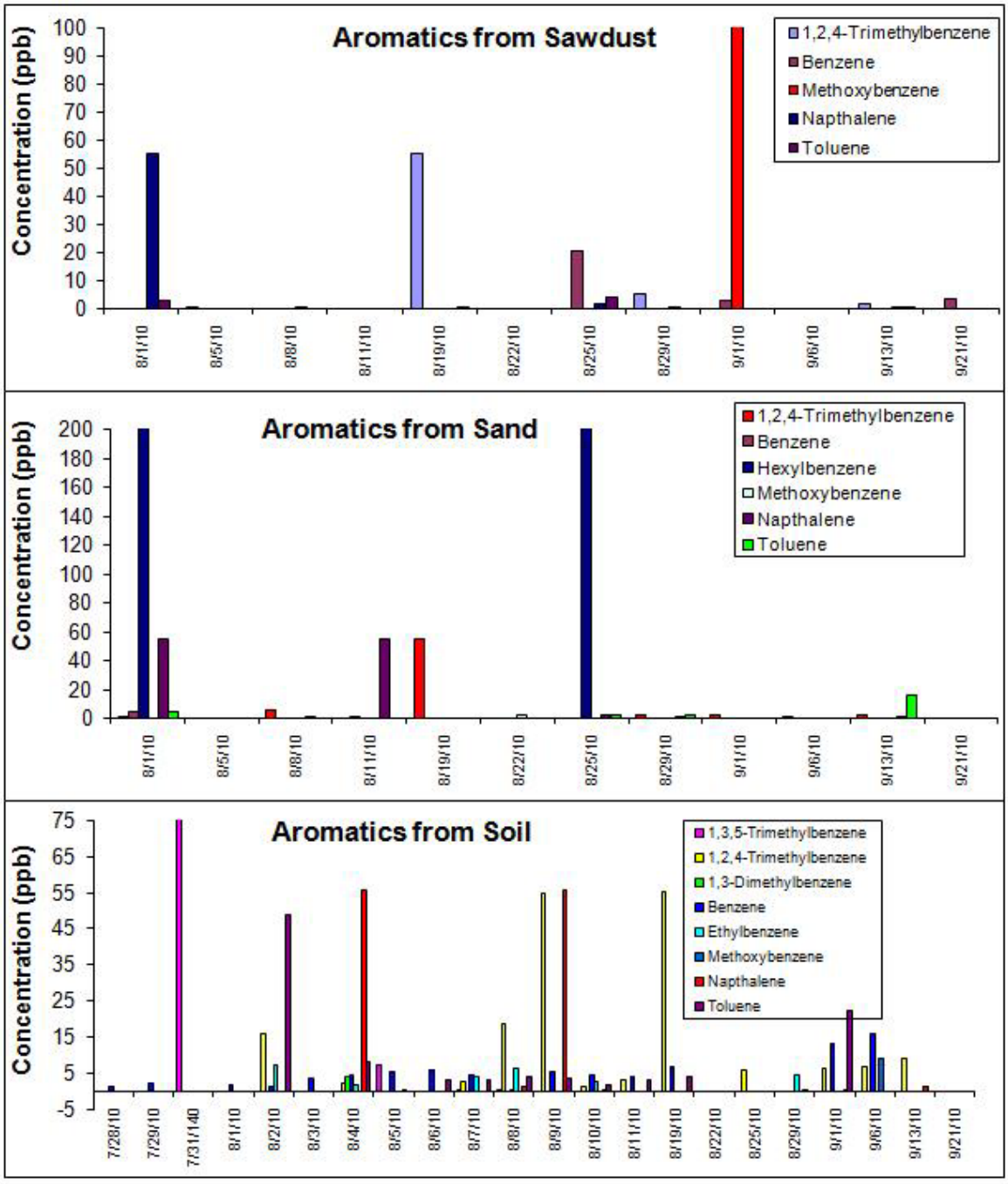

Figure 13. Emission of aromatics from pig carcasses buried in soil, sand, sawdust.

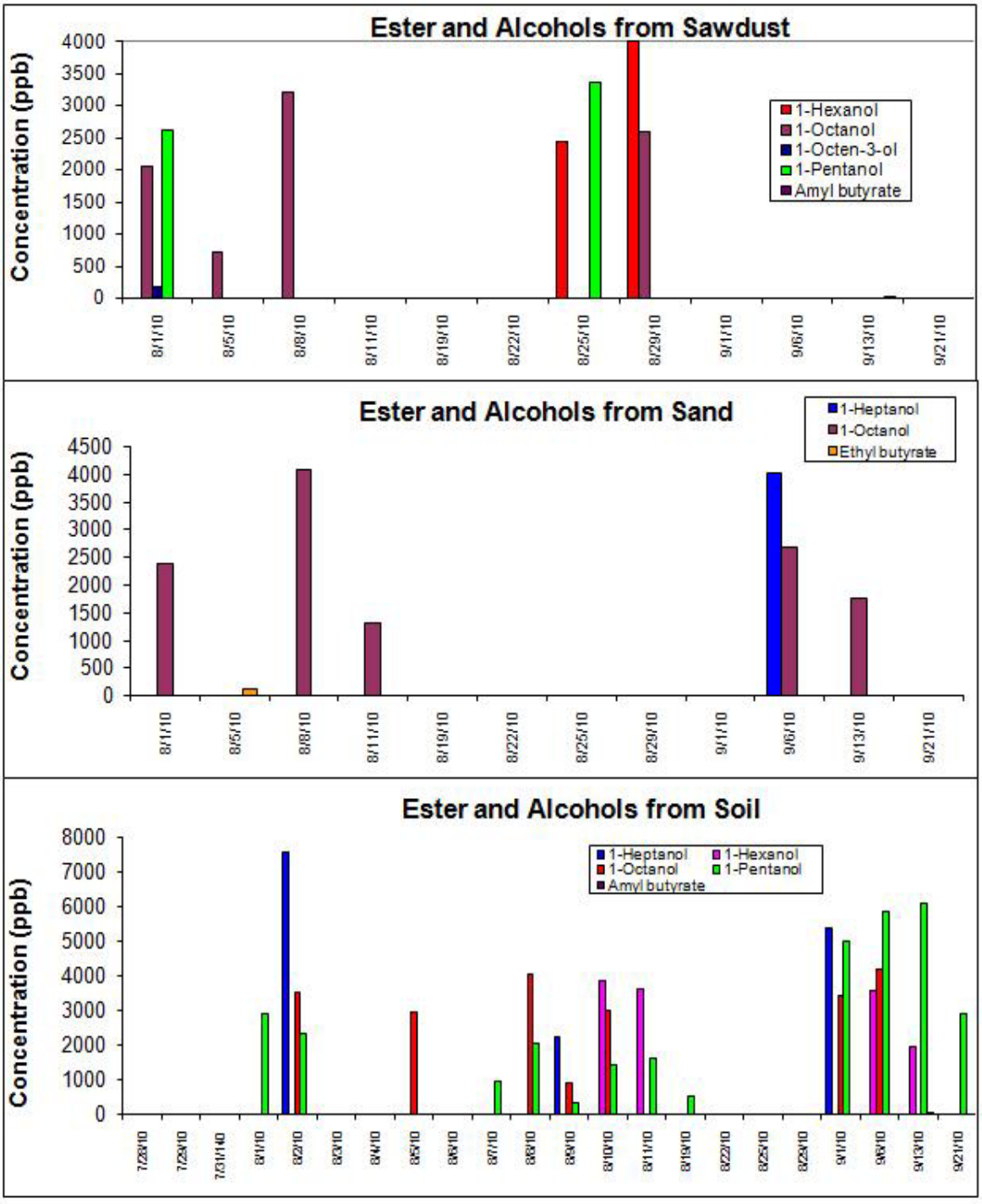

Figure 14. Emission of esters and alcohols from pig carcasses buried in soil, sand, sawdust.

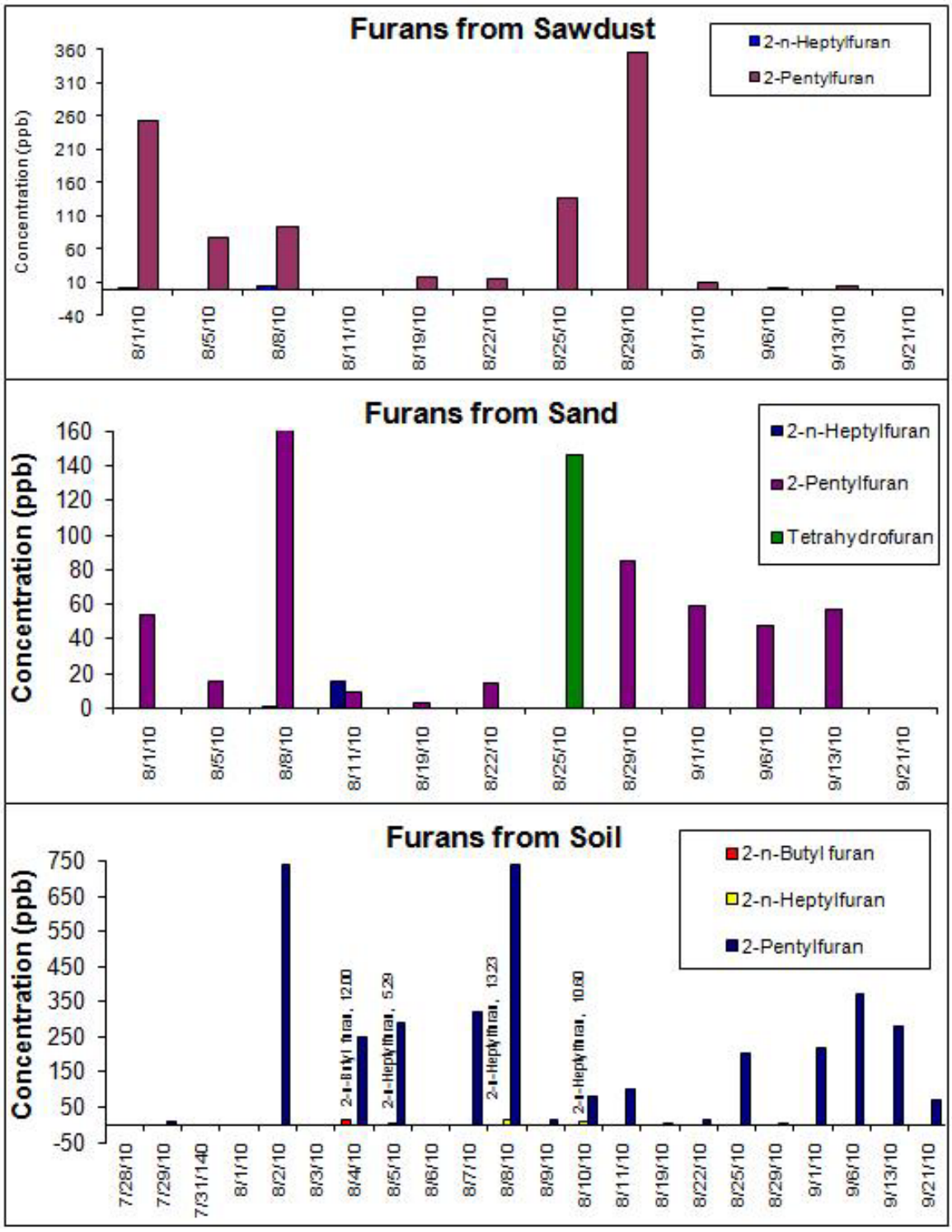

Figure 15. Emission of furans from pig carcasses buried in soil, sand, sawdust.

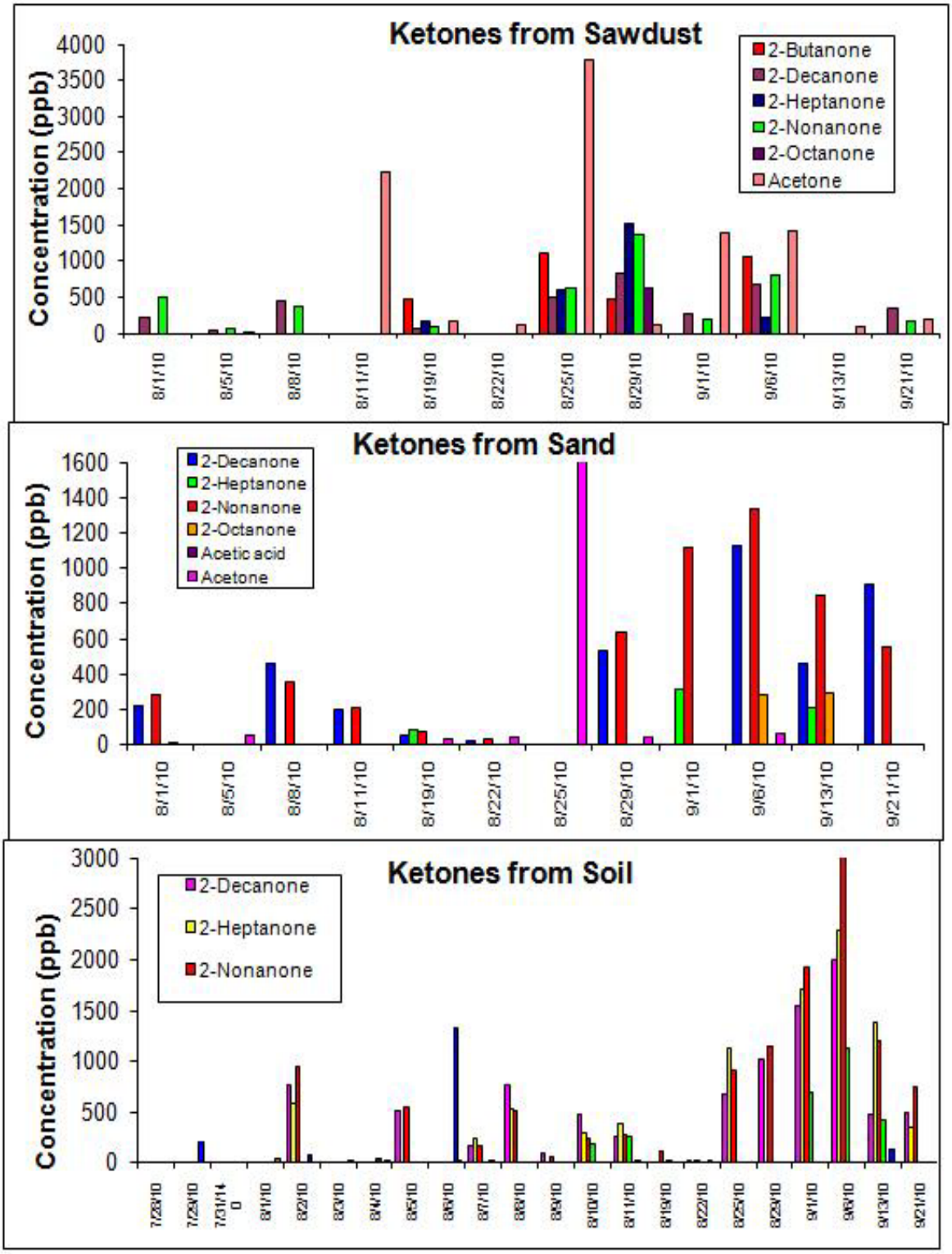

Figure 16. Emission of ketones from pig carcasses buried in soil, sand, sawdust.

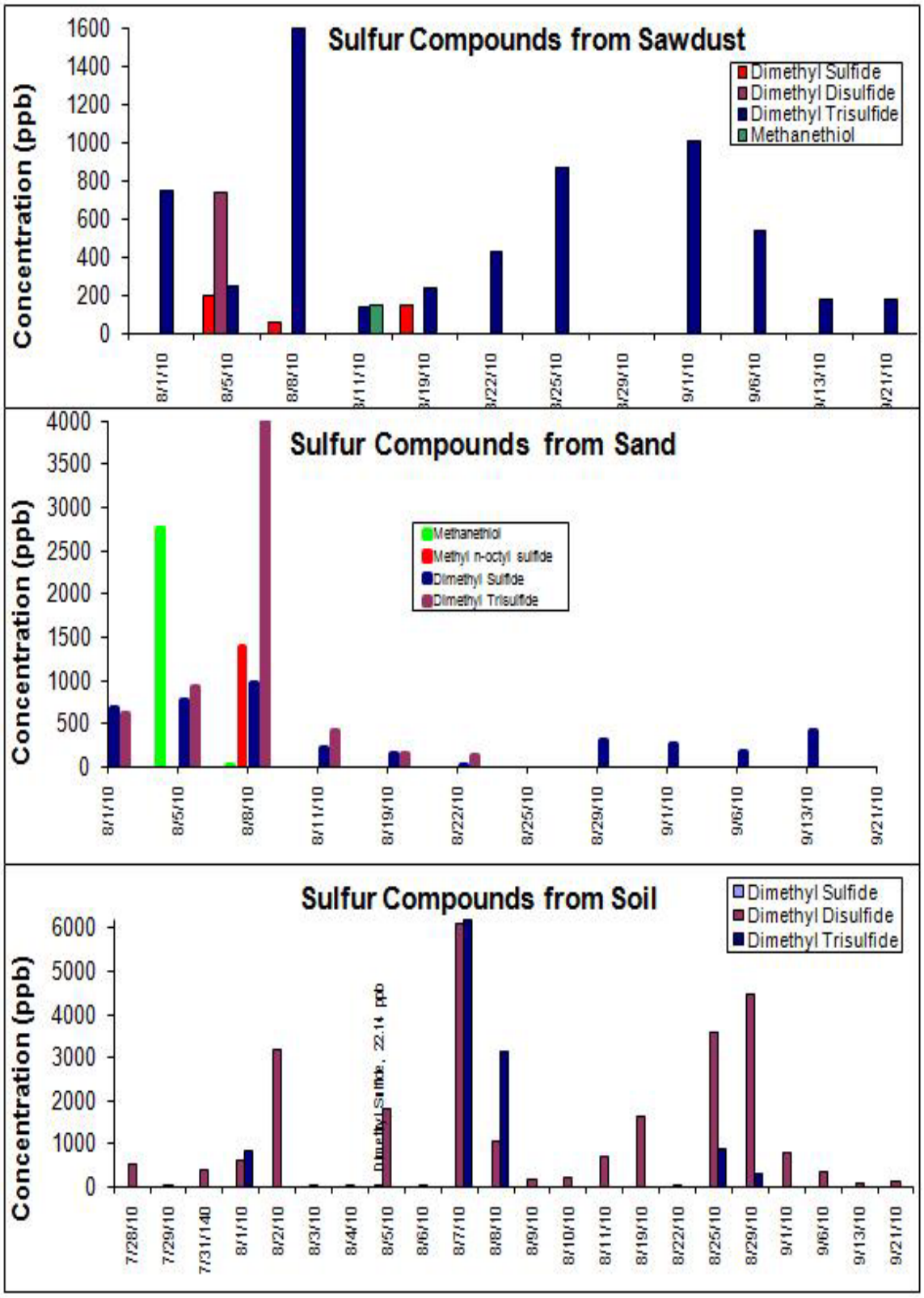

Figure 17. Emission of sulfur compounds from pig carcasses buried in soil, sand, sawdust.

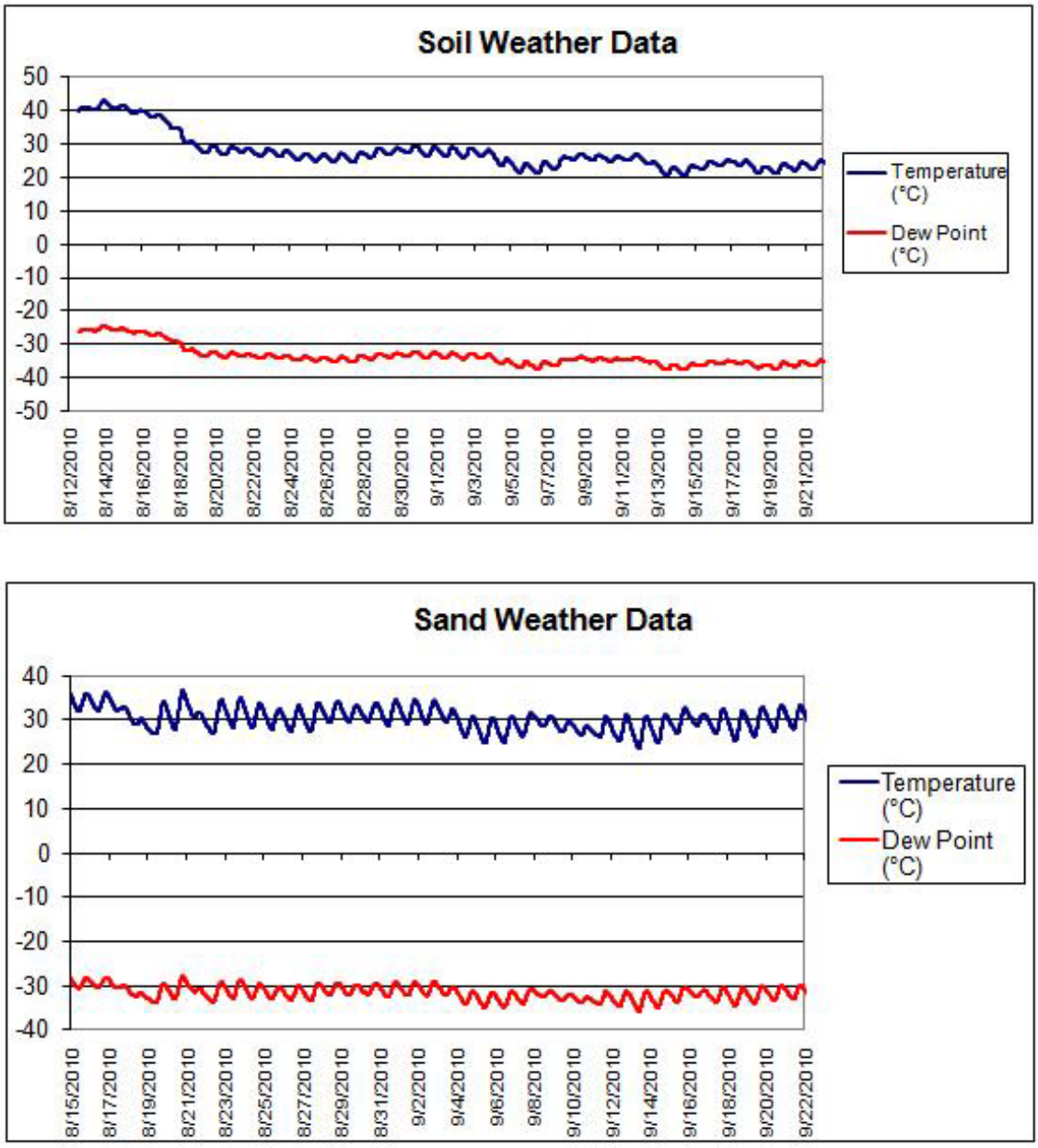

Figure 18. Plots of probe temperature and dew point in soil and sand as a function of decomposition time.

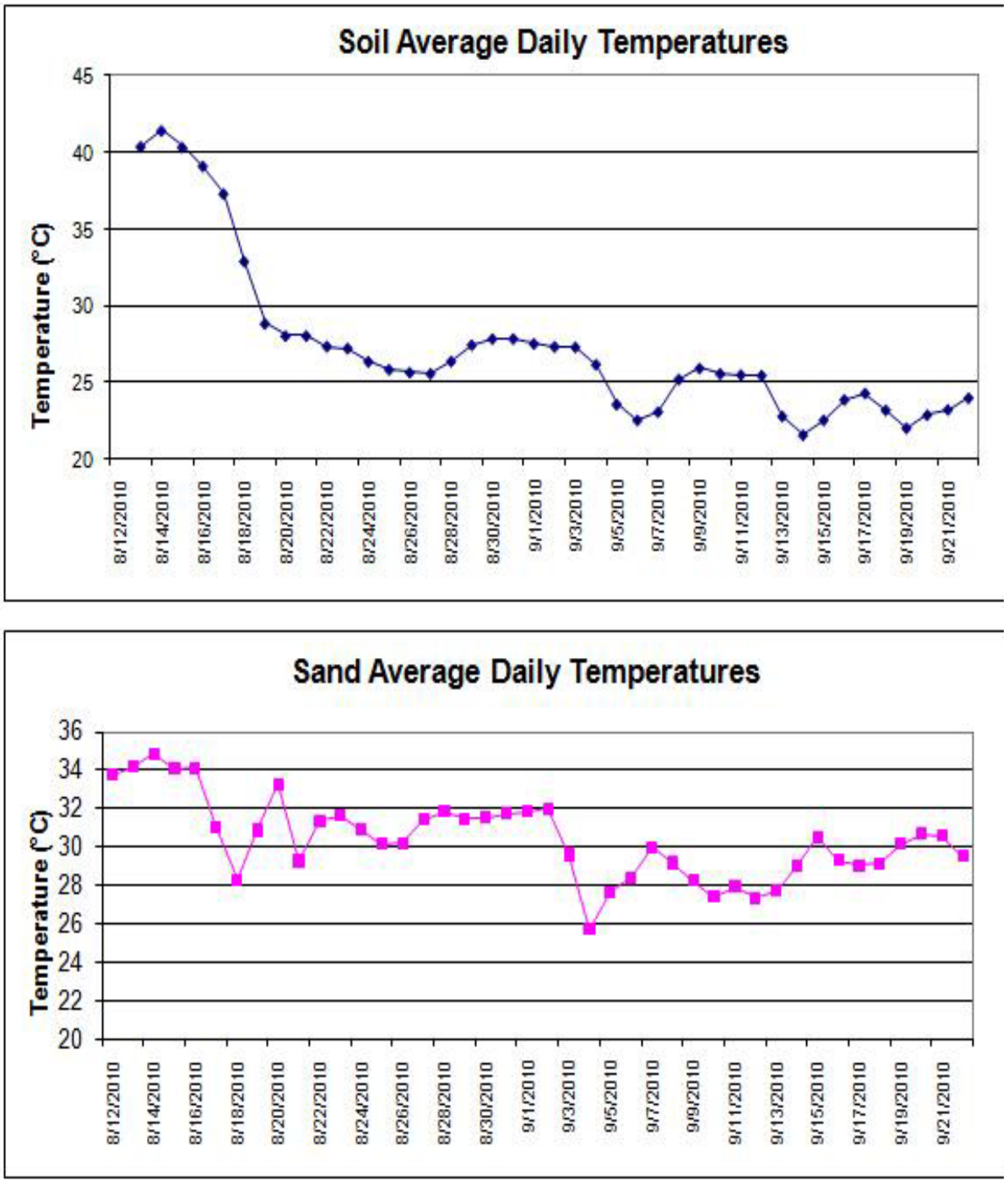

Figure 19. Plots of daily probe temperature in soil, and sand as a function of decomposition time.

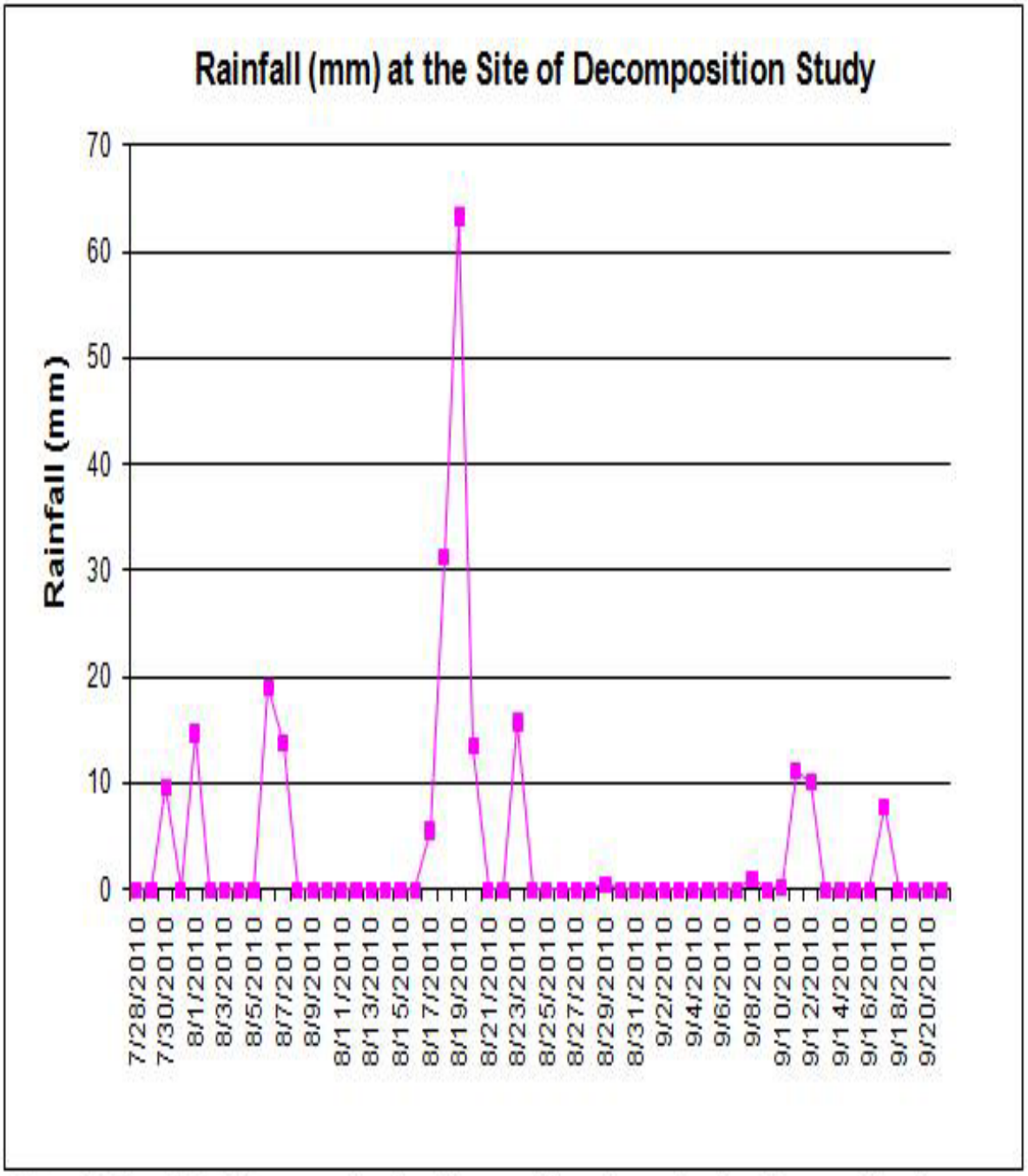

Figure 20. Plot of rainfall amount at the site of decomposition study as a function of decomposition time.

3.5.4 Conclusion

This study shows that the analytical method of GC-MS with prior preconcentration of analytes by a three-trap system of a glass bead trap, Tenax trap, and a cryofocuser is suitable for analyzing a wide range of VOCs including alkanes, aromatics, aldehydes, ketones, alcohols, esters, halogenated compounds, and sulfur compounds. The GC stationary phase of 100% dimethylsiloxane as present in the MXT®-1 column gives satisfactory resolution of the majority of compounds released during porcine decomposition. The collection of samples using evacuated bottles seems to be reliable, compact, and is certainly more convenient than Tedlar™ bags, which are difficult to clean. The bottles are also more economical than the stainless steel canisters, which are about 7-9 times more expensive. The 32-ounce bottles have a volume of 460 mL each, which will easily allows 3 injections of 100-mL sample volumes. The analytical method provides acceptable calibration range for all the compounds measured in the study that span the range of 1 ppbv to 20 ppmv. The SPME method gave excellent sensitivity for selected classes of compounds including non-polar to moderately polar oxygenated VOCs. However, the fact that the calibration procedure is too laborious and the implementation of analytical setup is too cumbersome caused a decision to be made to abandon the SPME approach in favor of the preconcentrator configuration with the pre-evacuated sampling bottles.

The concentrations of the VOCs detected are highly dependent on the media used to bury the pig carcasses and the environmental conditions, especially the rainfall. It was observed that after each rainy period, the concentrations of VOCs decreased drastically and the degree of reduction follows the order of alcohols>esters, ketones, aldehydes>sulfides, thiols, and aromatic compounds>alkanes. The levels of VOCs are also generally higher in the soil compared to the sand and sawdust samples. This may be due to the lowest porosity of soil that prevents the VOCs from permeating to the ambient air. Sawdust, on the other hand, allowed VOCs to permeate and mix freely with ambient air and resulted in the lowest VOC concentrations in general. Another reason that may be responsible for the higher levels of VOCs in soil is because there was a higher level of microbial activity in soil that expedited the decomposition process and enhanced the degradation kinetics. This explanation was partly supported by the fact that the temperature of the soil became elevated by 6-10 °C during the initial decomposition period as a result of the exothermic aerobic degradation process.

From the standpoint of evaluating management practice for disposal of animal carcasses, the porosity of the sawdust is a drawback because it allows VOCs to readily contaminate the ambient air and is more likely to give rise to odor complaint problems. The lack of intimate contact and the limited microbial population in sawdust also lead to the incomplete degradation of organic matter and the emission of potentially harmful VOCs to the ambient atmosphere. Sand is an interesting candidate for the disposal of animal remains because its solid matrix allows more effective subsequent disinfection treatment by heat. However, the decomposition may still be somewhat slower than that of soil because it depends on the indigenous microbes in the bodies of animal carcasses for biodegradation.

This project identified several important compounds that may be of forensic significance in determining the extent of decomposition or post-mortem interval as well as whether a site was used for the burial or concealment of dead animal or human remains. The sulfur compounds, 1,1-difluoroethane, 2-pentylfuran, and low molecular weight aldehydes and ketones can be found readily in soil samples during the first three months after the inception

of decomposition when the animal remains were buried. Larger molecular aldehydes and ketones such as nonanal, nonanone, decanal, decanone, benzaldehyde, as well as dimethyltrisulfide and 1,1-difluoroethane may be useful for determining the duration of animal or human remains that had been buried for more than three months since the GC-MS detection limits is generally at the levels of 0.1-10 ppbv for most compounds and that soil samples with these biomarkers can be heated to detect ultratrace levels present at the concentrations of 1-100 ppt. The quantitative determination of post-mortem interval will be extremely challenging because the weather conditions, especially rainfall, have to be accounted for, and a simulated decomposition using the site-specific conditions will be necessary.

3.6 Chlorine Dioxide as an External Decontaminant of Corpses

3.6.1 Introduction

The objective of this research component was to document the potential of newer chlorine dioxide (ClO_2) disinfection technology to eliminate bacteria (including spore forming bacteria) that might be associated with the body surface of animal carcasses. In the event of large scale animal fatalities (whether naturally occurring or the result of biological terror activity), there exists great potential for microbes associated with deceased animals to pose an infectious threat to humans associated with animal disposal, other animals, and the environment. The goal was to document efficacy of the disinfectant application methods and outline physical conditions (method of application, concentration of disinfectant, temperature, time, etc.) necessary to obtain meaningful reduction of bacteria associated with the carcass skin surface.

Chlorine dioxide has a long history of use as a disinfectant. It is used worldwide for treatment of potable water and in food preparation (Ieta and Berg 1986; Simpson, Laxton, and Clements 1993). Proper use of ClO_2 gas and solutions typically do not produce cumulative adverse health effects, is not carcinogenic, and is not a reproductive hazard. In recent years, the chemotherapeutic potential of ClO_2 has been realized through the use of products for human use such as antibacterial gels (Babad 1999), skin treatments, and a mouthwash. It is also apparent the gas can inactivate viruses (such as the influenza virus) at levels that are safe for human respiration (Otaga and Shibata 2008). Chlorine dioxide gas is too unstable to be shipped and must be prepared on-site. Historically, this has often required the use of dedicated equipment and the training of personnel. Advances in ClO_2 generation technology have resulted in the ability to produce small amounts of gas and solutions to meet a variety of specific needs. One system recently developed by ICA TriNova is composed of a two part impregnate enclosed within a sachet that is gas permeable but water impermeable. When submerged into water, ClO_2 gas is released, creating a bactericidal solution. The ppm of ClO_2 can be varied to meet specific needs. Previous in-vitro tests from this laboratory and others have demonstrated that this system effectively generated small levels of ClO_2 gas, and solutions locally produced had a rapid bactericidal effect against vegetative bacteria cells, spores (at higher ClO_2 dosage), and protozoa (Wu and Kim 2007; Ernst, Issac, and Newsome 2002; Newsome, Ernst, and Issac 2002). Most recently the elimination of naturally occurring heterogeneous bacteria populations on sports equipment has been demonstrated as well as the effective elimination of *Staphylococcus aureus* (Newsome, DuBois, and Tenney 2009).

Since the intentional release of *Bacillus anthracis* spores in the United States in 2001 (and the potential use of this bacteria as a biological agent capable of causing mass fatalities), much interest has centered on means of inactivation of these spores following their intentional release into the environment. Interest is especially centered around animals that might become contaminated with the spores on skin surfaces. Bacteria spores can survive in the environment for an extended period of time and are resistant to a wide variety of treatments to inactivate spores. Gaseous forms of ClO_2 have been used for the inactivation of *Bacillus* spores including federal office buildings (Hass 2001). This fumigating agent could be advantageous for both large scale and small scale decontamination needs.

Needs exist to develop easily used surface decontamination technology for biological agents such as *Bacillus anthracis*. Chlorine dioxide has the potential to make a significant contribution to these needs. This research documents its efficacy using a gas phase, a spray, and a whole tissue dip. Similar methods are currently used in the food industry (both meat and vegetable) to control bacteria pathogens. For this, however, lower levels of ClO_2 are used and its use is directed primarily at non-spore forming bacteria such as *Escherichia*, *Salmonella*, or fungi. This research outlines necessary parameters and shows that newer chlorine dioxide generating technology can also be used to achieve a state of decontamination, which includes spore-forming bacteria. In addition, it demonstrates that the technology is portable, easily used, and requires little training.

The proposed newer, unique technology is easy and safe to use. A precursor chemical (sodium chlorite) and an activator chemical are transported and stored in separate packages thus ensuring shelf stability. To generate ClO_2, both chemicals are combined in a "generator sachet" that is gas permeable but water impermeable. The sachet is placed in a mixing pail filled with water. The chemical reaction inside the sachet disperses the ClO_2 gas out through the porous membrane of the sachet and into the water. When the reaction is completed, the spent sachet with all materials inside is removed and disposed of as common, environmentally safe trash. Chlorine dioxide has a long been acknowledged by the U.S. EPA and scientific bodies as an effective disinfectant, with no adverse health or environmental implications.

There is a clear need to have technology on hand to inactivate potential pathogens in the event of mass animal causalities. It is also important that the technology is user friendly, requires no dedicated equipment (except for ClO_2 generating precursors) or material, and requires no extensive training or preparation for its use. Developments to meet these needs also have direct application for use in food preparation and safety, preparation of potable water, and most recently, as an effective disinfectant for sewage waste and reuse water.

3.6.2 Materials and Methods

3.6.2.1 Test surfaces

Disinfection testing was performed on pig skin taken from carcasses from a local pork production facility. Skin was cut into squares measuring approximately 8 x 8 cm. Tests were performed to assess killing of naturally occurring bacteria on the skin surface and also on pig skin that was inoculated with 10^7 spores of *Bacillus atrophaeus,* which served as a surrogate for *B. anthracis*. For this, 10 ul of a 10^9 spore suspension were pipetted onto pig skin surface in small, drop wise fashion.

3.6.2.2 Assessment of bacteria killing on pig skin surface

At its inception, it was proposed to use cotton swabs to recover bacteria from both untreated and chlorine dioxide treated pig skin surfaces. In this process, the cotton swab is rubbed onto the surface of Trypticase™ Soy Agar (TSA) plates. Plates are then incubated at 37°C and then assessed for outgrowth of bacteria. Although this method was used in a previous study of disinfection of sports equipment (Newsome, DuBois, and Tenney 2009), it is subject to some variability because cotton swab application to surfaces is variable and an undetermined number of bacteria could remain attached to the swab when it is rubbed onto the surface of a TSA plate. A search to identify methodology that might prove more useful for this study identified one method that seemed to be especially relevant.

Tests were conducted using agar plates to remove bacteria from both untreated and chlorine dioxide treated pig skin surfaces. With these agar plates, the agar medium extends beyond the lip of the Petri dish. To sample a surface for the presence of bacteria, simply press the agar plate onto the surface to be tested and bacteria adhere directly to the agar surface, thus bypassing the need of using a cotton swab to transfer bacteria. In addition, an area of specific size is tested. Since the plates are designed to be used in the detection and enumeration of microorganisms present on surfaces of "sanitary importance", these plates are the choice for sampling surfaces in "clean rooms" used for the manufacturing of drugs for human use.

After testing, it became apparent that this is a better method for sampling surfaces such as skin; therefore, 65mm x 15 mm RODAC™ plates were utilized by adding TSA. The plates were pressed against the skin and incubated for 48 hours (24 hours at 37°C and 24 hours at room temperature). Recovery of bacteria was based on appearance of colonies on the plates (Figure 21).

Fig. 21. Recovery of bacteria from pig skin using TSA RODAC™ plates. Plates were incubated 24 hours at 37 °C + 24 hours at room temperature.

3.6.2.3 Preparation of chlorine dioxide gas and solution

Chlorine gas studies were conducted in common use containers such as plastic bags and plastic cans. For gas studies, the reaction must occur within an enclosed container to prevent dispersal of the gas. Although this provides ease of use, it does not allow a precise measurement of the amount of gas in ppm. One way to accurately measure the ppm of ClO_2 during the treatment process was incorporation and use of a novel device termed a "ClO_2Clave." In this device, test materials such as pig skin are placed within an enclosed container, and ClO_2 generating sachets are placed inside. The amount of gas generated is measured in ppm (Figure 22).

Figure 22. ClO_2 Clave for chlorine dioxide treatments and measurement of chlorine dioxide gas in ppm.

Pig skin was treated at various time intervals and the level of ClO_2 gas was recorded. Before and after treatment, pig skin was assessed for the presence of recoverable bacteria by use of RODAC™ plates prepared with TSA.

Chlorine dioxide solutions were prepared by immersing a sachet containing the two gas generating components in a container of water overnight. For the studies involving solutions (dip test and spray test), solutions were prepared using deionized water and water from a local stream. Spray tests were conducted by administering 10 mL of solution using spray bottles obtained from a local retailer.

3.6.3 Results

3.6.3.1 Representative spray treatment results

Chlorine dioxide solutions with concentrations from 100 to 1,000 ppm were tested using a spray treatment of pig skin that had naturally-occurring bacteria as well as spores of *B. atrophaeus* that were added to the sample. Skin was sampled using RODAC™ plates prepared with TSA at various time intervals following the spray treatment. Although there was a marked reduction in naturally-occurring bacteria one hour after spray treatments, it was not effective in elimination of spores. Representative results with 500 ppm solution are provided in Figure 23.

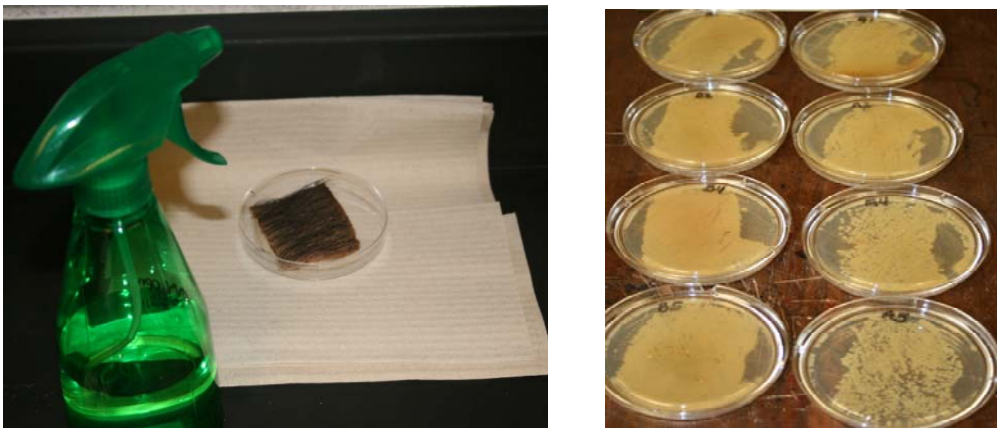

Figure 23. Reduction of bacteria using spray treatment on pig skin surfaces inoculated with 10^7 spores of *B. atrophaeus*.

3.6.3.2 Representative dip treatment results

Chlorine dioxide solutions prepared using deionized water and water from a local stream were prepared with concentrations from 100 to 1,000 ppm and tested by immersing skin samples in solution for various time intervals. The dip treatments showed a marked reduction in growth of naturally-occurring bacteria on the skin surface but were not effective in eliminating spores. Results showed that there was no apparent difference in effectiveness between solutions prepared from different water sources. Results also indicated that the dip treatment did not appear to be any more effective than the spray treatment. Representative results using 1,000 ppm chlorine dioxide solutions prepared with deionized water and water from a local stream are provided in Figure 24.

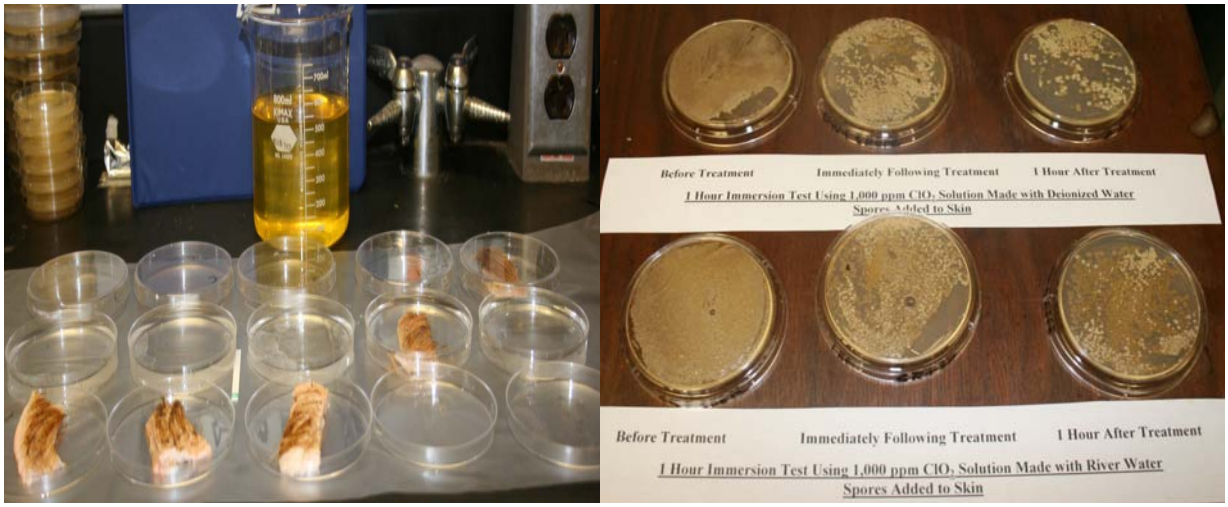

Figure 24. Reduction of bacteria using dip treatment on pig skin surfaces inoculated with 10^7 spores of *B. atrophaeus*.

3.6.3.3 Representative gas treatment results

Studies using the ClO_2Clave demonstrated that elimination of naturally-occurring bacteria and spores inoculated onto the skin surface could be achieved. Elimination of bacteria was dependent on time and concentration of chlorine dioxide gas used in the treatment. Chlorine dioxide gas concentrations between 1,000 and 2,000 ppm (temperatures ranging from 4°C to 32°C) with exposure times greater than six hours were particularly effective in eliminating naturally-occurring skin bacteria as well as spores that were added to the skin surface. Representative results are provided in Figure 25. Containers that would be readily available in a local response, such as plastic garbage bags and buckets, were also tested with no observed difference in the effectiveness as compared to treatments in the ClO_2Clave. In these containers, results indicated that the elimination of bacteria was concentration and time dependent.

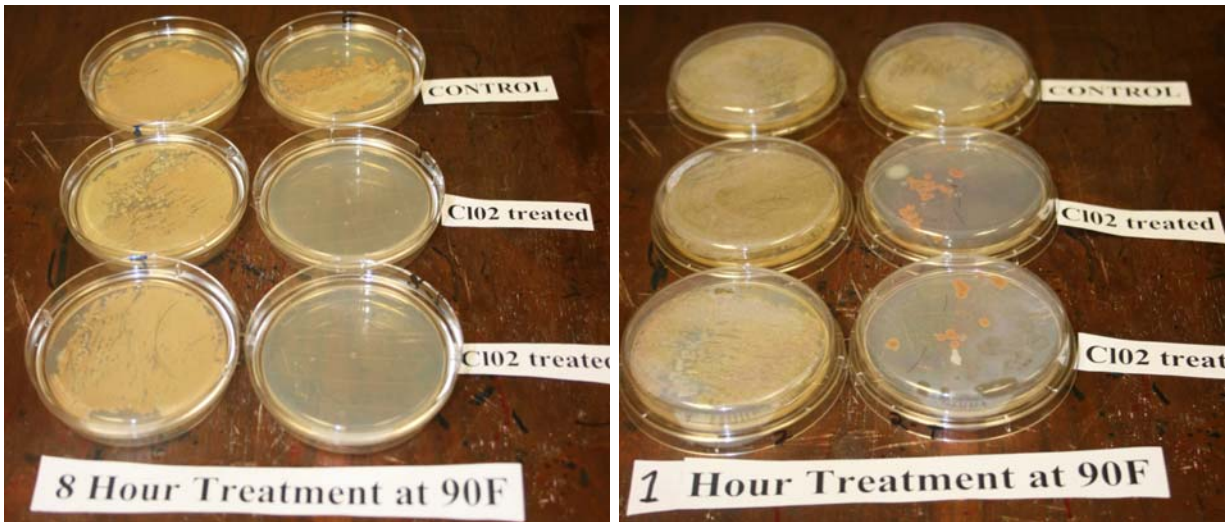

Figure 25. Elimination of bacteria using gas treatment of pig skin surfaces inoculated with 10^7 spores of *B. atrophaeus*.

3.6.4 Conclusion

Results of this study have demonstrated that ClO_2 can effectively eliminate naturally occurring bacteria associated with pig skin surfaces. In addition, when spores of *B. atrophaeus* were added, inactivation of spores was apparent based on the lack of outgrowth of spores on RODAC™ plates prepared using TSA. The degree to which bacteria were inactivated was both time and ClO_2 concentration dependent. Of particular interest was that the use of ClO_2 gas was superior to a ClO_2 based liquid spray or a whole tissue dip. This has important implications in future efforts to design and potentially implement disinfection technology. Results from this study support the concept that gas is superior to liquid in the disinfection of pig (and likely other animal surface tissue) skin surfaces. This is perhaps a reflection of the ability of gas to penetrate small pores associated with skin surfaces. In addition, the gas has an affinity for moisture, which may be present on pig skin surfaces. It is noteworthy that in a previous study, the ability of ClO_2 gas to inactivate spores of *B. atrophaeus* when they were inoculated onto surfaces such as wood, wallboard, Formica, concrete, metal, and carpet was documented (Salehzadeh 2009).

4. SUMMARY

The genesis of this study came from the realization that no protocol exists to deal with the dead in high-magnitude mass disasters involving humans and large animals. Although only the first of three phases was funded in this study, the baseline information gathered in Phase 1 in the areas of burial media characteristics, runoff, pentobarbital, decomposition gases, and chlorine dioxide offers potential for future research efforts.

Although more experimentation is needed before the true nature of decomposition in sand, soil, and sawdust can be determined, the initial findings indicate that bodies buried in sand tend to decompose more slowly than bodies buried in sawdust or soil. Results of the runoff study point to sand as the most effective at eliminating pathogenic bacteria. The levels of coliforms in soil remained elevated throughout the study, making it difficult to determine whether coliforms from the carcass were eliminated. Sawdust was contaminated from its previous use as stall litter, making it difficult to determine if the bacteria present was indigenous or originated from the intestines. Unlike the other media, sand eliminated all coliforms and pathogens by the fifth week.

The burial media used and environmental conditions (e.g., rainfall) affected the concentrations of volatile organic compounds, which were detected with levels generally higher in soil than in sand or sawdust. This is probably due to higher microbial activity in soil and its low porosity restricting the movement of decomposition gases. Lowest concentrations were found in sawdust, making it disadvantageous as a burial media. Its increased porosity may result in an increase in decomposition gases being released into the ambient air, causing odor complaints. Sand compacts around the carcass becoming somewhat of a solid matrix that allows a more effective disinfection treatment by heat. Unfortunately, decomposition with sand must rely upon the indigenous microbes within the carcasses, slowing the process.

To address the environmental risk of pentobarbital leaching from decomposing euthanized animals, barbiturate-degrading bacteria were isolated from composting manure using enrichment methods. The bacteria were able to degrade sodium pentobarbital in temperatures as high as 49°C. Since composting temperatures can reach 60°C, which is too high for the survival of the barbiturate-degrading bacteria, one strategy would be to apply it to the perimeter of composting piles where the temperatures are lower. Further studies are needed to evaluate the effectiveness of these barbiturate-degrading bacteria.

Mass disasters in which biological weapons are used represent their own unique problems, with the primary concern being worker safety and the containment of the infectious agent. Chlorine dioxide gas exhibits potential as a disinfectant for contaminated skin surfaces, even when tenacious agents such as anthrax are involved. This study suggests that the use of chlorine dioxide in a gas form was superior as a disinfectant to sprays or dips, which has implications for the planning of future mass disaster protocols.

It must be stressed that this was a Phase 1 study. The objective of this study was to obtain baseline information that with further study could be used to develop a cost efficient and acceptable protocol for handling high-magnitude mass disasters involving humans and animals. These initial findings were to serve as direction for future research design involving larger animals.

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APPENDIX A. RESEARCH REVIEW LETTER

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STATE OF TENNESSEE
DEPARTMENT OF ENVIRONMENT AND CONSERVATION
NASHVILLE, TENNESSEE 37243-1535

HOULEE J. MARTINEAU, JR.
COMMISSIONER

BILL HASLAM
GOVERNOR

February 24, 2011

Dr. Hugh Berryman, Director
Forensic Institute for Research and Education (FIRE)
MTSO Todd Hall, Room 307
Box 89
Murfreesboro, TN 37132

RE: Phase I report on SERRI Project: Aerobic Decomposition Alternative Method for Managing Large Scale Animal Fatalities,
Project Principal Co-Investigators: Dr. Hugh E. Berryman and Dr. John C. Haffner

Dear Dr. Berryman:

I have reviewed the above referenced report from the standpoint of an emergency manager faced with making decisions in a disaster that resulted in large numbers of human and/or large animal fatalities. My professional background is in the field of forensic archaeology as well as an emergency manager with the Department of Environment and Conservation and Tennessee Emergency Management Agency. I also helped write the Tennessee Mass Fatality Plan currently on file with TEMA.

Like you, I watched closely the treatment of human remains that followed Hurricane Katrina and the Haiti earthquake. By reference, our current estimate for the number fatalities that would result from a 7.7 magnitude earthquake on the New Madrid Earthquake Zone falls between those two disasters. We think that Tennessee alone would have several thousand corpses to deal with under extreme conditions, high media visibility and widespread destruction of supporting infrastructural systems.

I am pleased that you and your colleagues have begun to develop empirical data on what kinds of methods could be used for such a mass fatality event. The process you have begun is systematic, scientifically based and testable. I think your research design is sound and expandable to other lines of inquiry.

I would like to stress that the end result of the decomposition process for the large animals is different for human remains. The human remains decomposition needs to

Dr. Hugh Berryman, Director
February 14, 2011
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stabilize or be stopped at the point of skeletalization so that the remains can be returned to their families post identification if possible.

I think your research is an excellent first step in understanding the science of the process of handling human remains. The more we can work out the procedures in advance of when they are needed, the better the decisions will be at the time of a crisis. The decomposition studies you have done indicate that aerobic methods hold promise for finding a workable solution to the problems we will face.

I urge you and your funding agencies to continue the research to look at the logistics aspects of transport and temporary storage of human remains prior to identification. A pilot study might be a suitable next step.

Thank you again for asking me to review this important work.

Sincerely,

Nick Fielder

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