Leachate Analysis from an Organic Burial Pit Containing Euthanized Large Animals

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Abstract

The disposal of animal mortalities creates surface and ground water pollution concerns. In the case of euthanized animals, there is particular concern for drugs residuals that may be mobilized in leachate as carcasses decompose. In this study, we describe an experimental organic burial pit containing mature dairy cattle euthanized after participation in a clinical vaccination trial. The animals were buried in wood chips to promote composting and rapid decomposition of the carcasses. The pit included a plastic liner that allowed rainfall derived leachate to be collected from the pit. New GC/MS detection methods were developed to analyze the leachate for the two barbiturates used to euthanize the animals, namely xylazine and pentobarbital. Our findings indicate that the xylazine degrades quickly, but that the pentobarbital was persistent over a period of several weeks and easily detectable in the leachate. We describe the burial experiment, the new GC/MS detection techniques for xylazine and pentobarbital, and leachate concentrations of xylazine, pentobarbital, E. coli, and oxygen demand.

Introduction

Tennessee's livestock owners have limited disposal options for large animal mortalities:

- Downer cows can no longer be processed for consumption.
- Cattle carcass material is now prohibited in animal feed (brain, spinal cord, certain lymphatic tissue).
- In 2011, the last two rendering services ended in south central and north western Tennessee.
- There are no remaining horse slaughter facilities in the United States.
- Only 35 Class I landfills in Tennessee can legally accept dead animals, but only a third readily accept them.
- Burial is not practical in many areas because the topsoil is shallow.

One disposal option that has been proposed is large scale composting or organic burial using utility wood chips. Such a facility would serve perhaps a one or two county area. One of the concerns would be management of rainfall leachate from the compost/burial area. For example, the animal carcasses could be the source of veterinary pharmaceuticals or pathogenic microorganisms that could contaminate surface and ground water. In particular, euthanasia medications like pentobarbital (PB) are a concern because they are commonly used to euthanize horses (as well as small animals housed at county animal pounds). **The objective of this study was to determine if the leachate from a large animal composting operation contains veterinary pharmaceuticals and/or potentially pathogenic microorganisms.**

Organic Burial Design

Twenty euthanized mature Holstein dairy cows were placed in a compost/organic burial pit (euthanasia was mandated because the cattle received an experimental vaccination) (**Figure 1**). The pit was excavated at the site of a former silage trench and was underlain by a 160 foot long, 10 foot wide concrete pad. A 60 mil HDPE liner was placed in the trench. Xylazine and pentobarbital (CAS numbers 7361-61-7 and 76-74-4, respectively) were administered in 5 ml and 100 ml doses for sedation and euthanasia, respectively, and the animals were promptly placed in the trench (within two hours). Each carcass was underlain and covered with at least two feet of utility wood chips; carcass torsos were separated by at least two feet. Leachate was collected from a sump at the end of the organic burial trench and archived at –20°C. A field negative control was composed of a wood chip leachate collection barrel without carcass addition.

Quantification of Pharmaceuticals

A GC/MS method was developed to detect the xylazine and the pentobarbital (PB) in the burial pit leachate. The samples were thawed and centrifuged at 10k rpm for 30 minutes to remove leachate solids. The supernatant was applied to Atlantic™ Hydrophilic-Lipophylic-Balanced solid phase extraction columns and eluted serially using 10 ml of n-hexane and 20 ml of a 1:1 mixture of methanol and acetone. Both eluents were concentrated to 1 ml using N_2 purging gas. Detection required derivitization using Supelco Trimethylphenylanilinium hydrolxide (2 μl of eluent, 98 μl TMAH), which likely stabilized the compounds via methylation. Xylazine and PB were subsequently quantified in the derivitization solutions using a Hewlett Packard 6890/5973 GC/Inert Mass Selective Detector outfitted with a J&W Scientific DB-5MS column (5% phenyl reactive matrix; 30 m long, 0.25 mm diameter, 0.25 μm film thickness). Euthanasia solution dilutions were used as standards.

Microbial Characterization

Quantitative PCR assays targeting total 16S ribosomal RNA and bovine-associated *Bacteroides* (BoBac) 16S rRNA genes were used to quantify the total bacterial and BoBac populations using universal primers 1055F/1392R and BoBac367F/467R primers respectively. Two burial pit leachate samples (from 4/21/11 and 5/12/11, designated "C") and their respective control samples (designated as "W") were selected for 16S rRNA gene amplicon pyrosequencing on a ROCHE 454 FLX Genome Sequencer at the UT-ORNL Joint Institute for Biological Sciences. After initial processing of the sequences on Roche software, the software package MOTHUR was used to filter low quality sequences and compare the samples.

Figure 1. A. Finished burial trench. B. Close-up of leachate collection sump. C. Placing the carcasses in the trench (April 11, 2011).

Quantification of Pentobarbitol (PB)

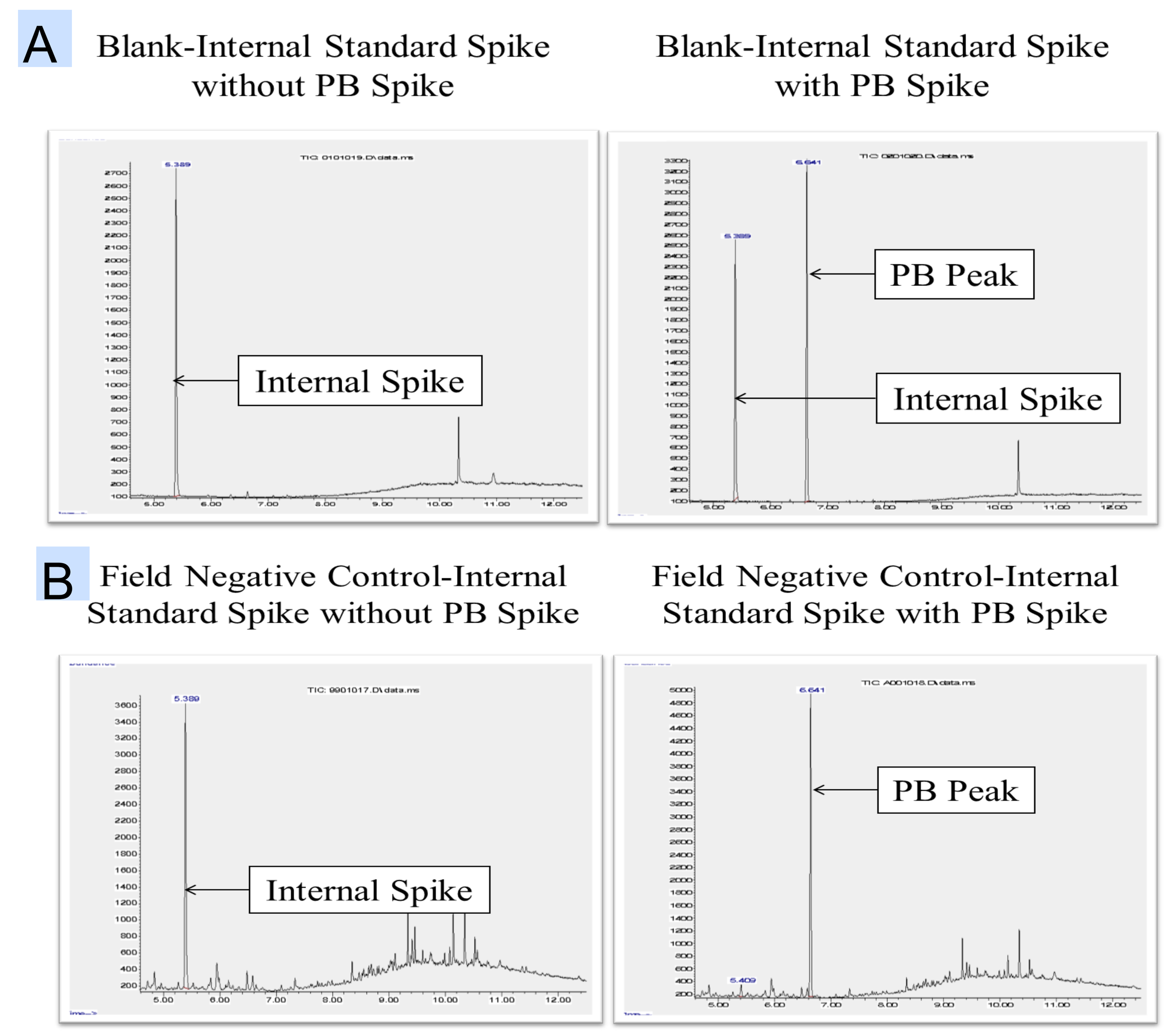

Figure 2. A. Successful detection of PB in a water blank. B. Absence of PB in the field negative control; detection of PB in the spiked field negative control.

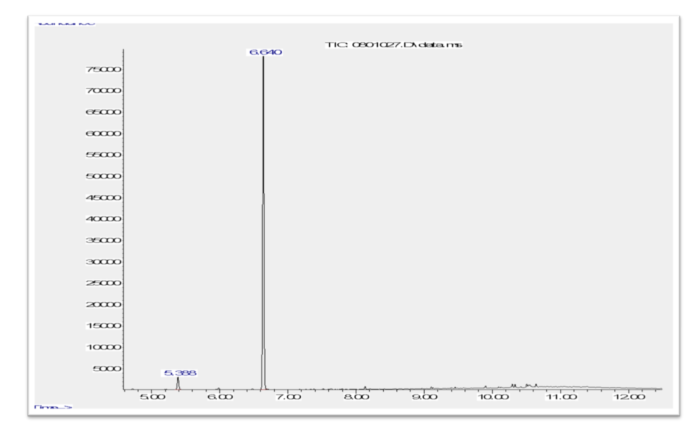

Figure 3. Mass chromatogram illustrating detection of PB in organic burial pit leachate.

Table 1. Quantification of PB in leachate from the organic burial pit.

Sample Type	Date	Volume	n-Hex	Metha-nol:Acetone	TOTAL - ppb	Average - ppb
Leachate	4/19/2011	1 L	4.14	20.18	**24.3**	**24 ± 1**
		1 L	5.41	18.32	**23.7**	
Field Negative Control	5/3/2011	1 L	13.30	39.77	**53.1*spike**	**BDL**
		1 L	0	0.08	**0.1**	
		0.72 L	0	0.98	**1.0**	
Leachate	5/3/2011	1 L	8.22	156.59	**164.8**	**149 ± 18**
		1 L	16.90	135.66	**152.6**	
		0.54 L	12.50	117.82	**130.3**	
Leachate	5/12/2011	1.58 L	38.08	261.54	**299.6**	**267 ± 47**
		2 L	38.60	194.82	**233.4**	
Leachate	5/25/2011	1 L	26.50	131.26	**157.8**	**194 ± 39**
		1 L	41.50	193.37	**234.9**	
		1 L	38.73	151.49	**190.2**	

Microbial Characterization

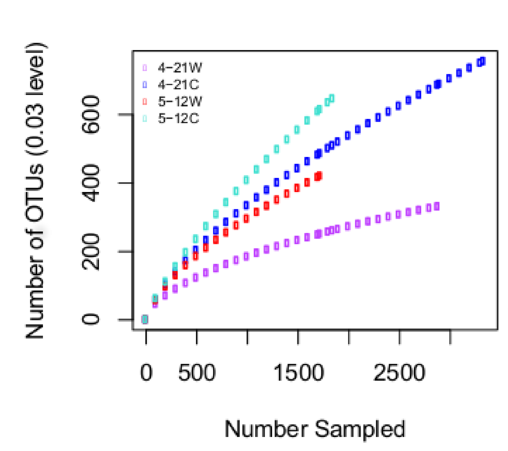

Figure 4. Number of OTUs as a result of the sampling effort in the 4 pyrosequencing libraries. "C" is from leachate; "W" are the field negative controls.

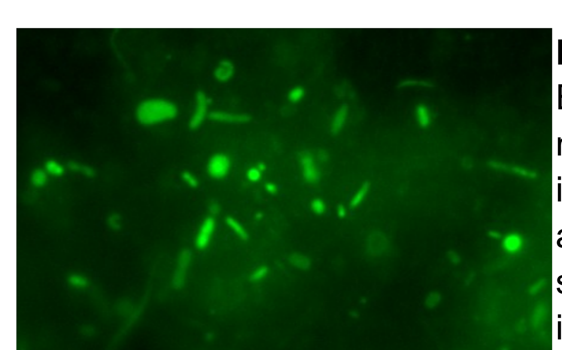

Figure 5. Epifluorescent microscopy images of acridine orange-stained bacteria in leachate.

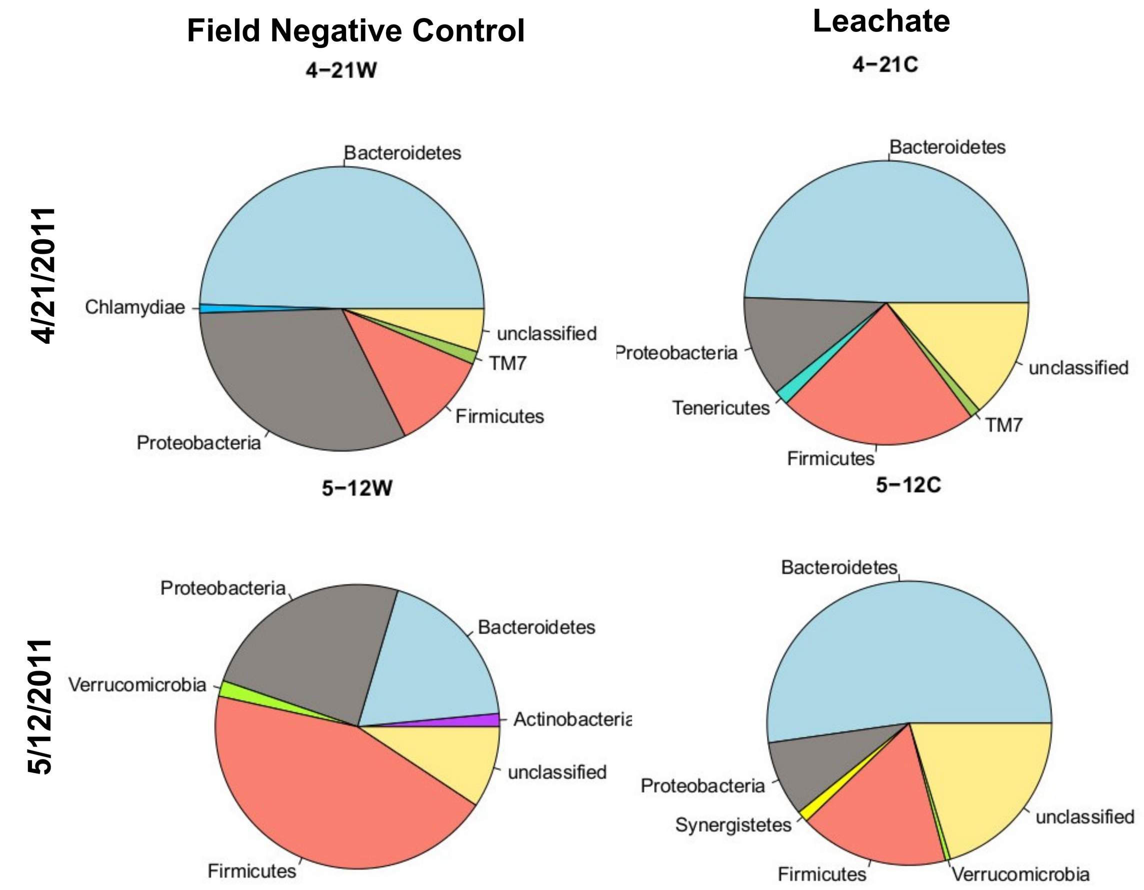

Figure 6. Dominant bacterial OTUs by phyla in each sample

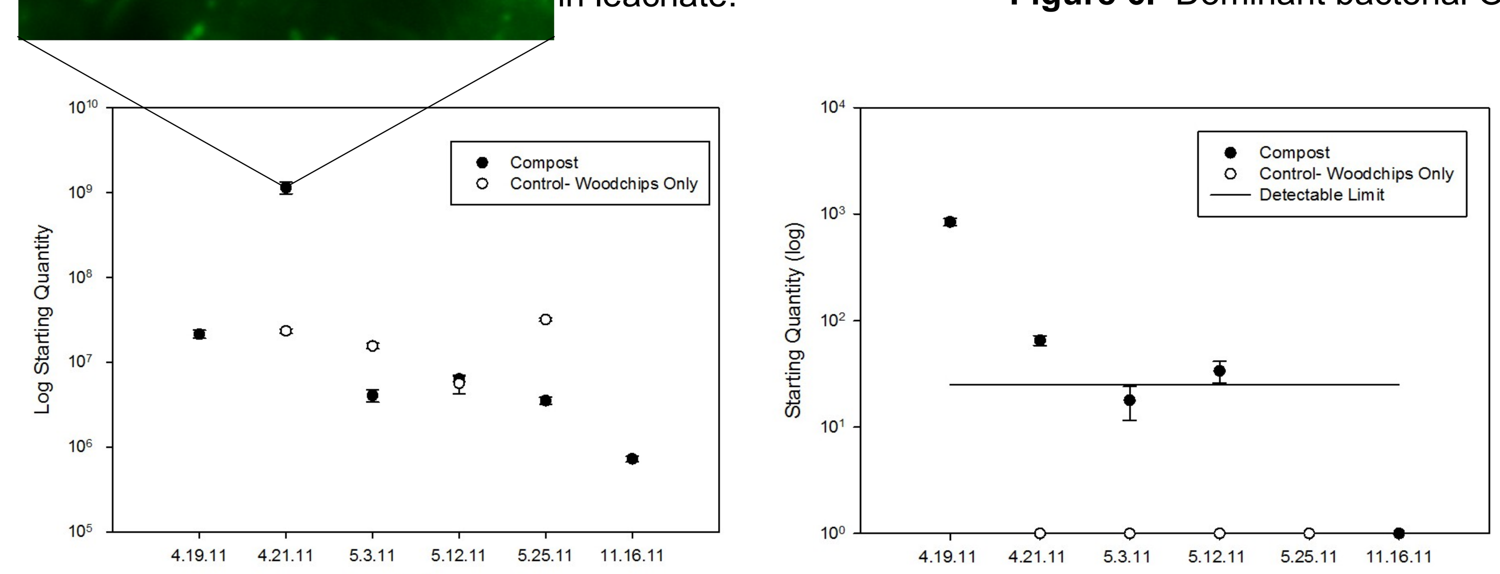

Figure 7. Quantitative PCR abundance of total 16S rRNA genes (left) and Bovine-associated Bacteroides 16S rRNA genes (right) in DNA extracts from organic burial pit leachate and field negative controls.

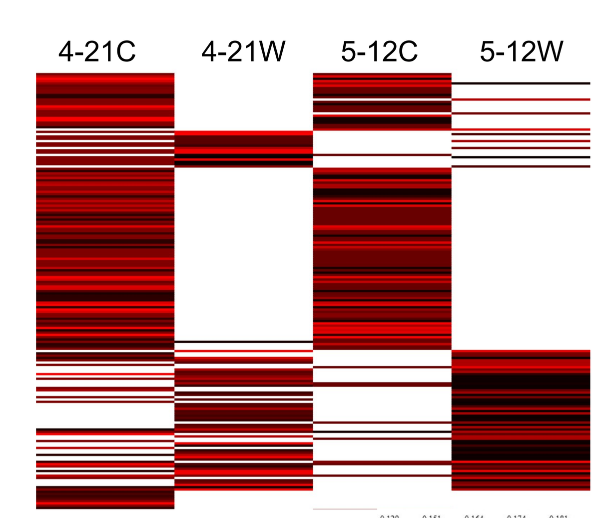

Figure 8. Heatmap of the relative abundance of each dominant OTU across the 4 samples.

Results & Discussion

PB was detected in the compost/organic burial pit leachate (**Table 1**); xylazine was not detected in any sample. A series of controls including both lab and field positive and negative controls were successful and demonstrated that PB could be detected as low as 2 ppb (**Figure 2**). PB was detected in each leachate sample collected (**Figure 3**) and ranged from a low of 24±1 ppb 8 days following burial, to a high of 267 ± 47 ppb one month after burial (**Table 1**). Six weeks following burial, the concentrated declined to 194±39 ppb.

16S rRNA gene sequencing revealed a diverse bacterial community in the leachate (**Figure 4 and 5**) compared to the field negative control. The community structure also differed between the leachate samples and their respective negative controls (**Figure 6 and 8**): In particular, leachate samples contained *Tenerictues* and *Synergistetes* phyla, which include common GI tract anaerobes. Bovine-associated *Bacteroides* (indicator organisms for fecal contamination) were only detected on the first four dates sampled, indicating that they persisted in the burial pit for at least 30 days and then dropped below detectable limits (**Figure 7**).

The findings indicate that if large scale composting or organic burial is offered as a county wide service to livestock owners, the leachate should be collected and properly treated to remove euthanasia drug residues and potentially pathogenic bacteria.

Acknowledgements

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