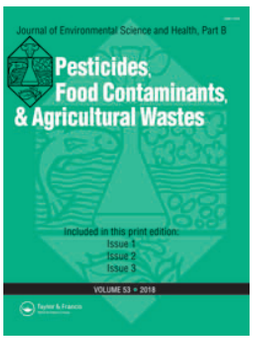
# EXHIBIT 8

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# Stability of pentobarbital in soil

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**ABSTRACT**

Intravenous injection of barbiturates, particularly pentobarbital (5-ethyl-5-pentan-2-yl-1,3-diazinane-2,4,5-trione), is a widely used method to euthanize large animals such as horses. However, one concern with this method is the fate of pentobarbital after the disposal of the carcass. As tissues decompose, pentobarbital may leach into the soil and from there migrate to groundwater. A method using methanol extraction, solid phase concentration, and liquid chromatography (LC/MS) has been developed to measure pentobarbital in soils. Recovery of pentobarbital from soil averaged approximately 85% from different soil types including topsoil, potting soil, sand, stall sweepings, and loam. The method was capable of detecting pentobarbital levels of 0.1 ppm. A calibration curve was constructed with a linear range of 1 ppm to 100 ppm. The limit of quantification was 0.5 ppm. The rate of degradation of pentobarbital in sand, topsoil, and potting soil was measured over a 17-week period. At the end of week 17, approximately 17% of the pentobarbital remained in the sand, 19% remained in the topsoil, and 10% remained in the potting soil. While there was a significant decrease in the pentobarbital recovered from the soil, there were still detectable amounts of pentobarbital present in the soil after 17 weeks. To determine the importance of bacterial degradation, the three soil types were autoclaved before addition of pentobarbital. After autoclaving, no degradation of pentobarbital was observed in sand and one topsoil sample, while there was no difference in the degradation of pentobarbital in autoclaved potting soil versus potting soil that had not undergone autoclaving.

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Pentobarbital; degradation; horse disposal; veterinary drugs; soil

## Introduction

While a large number of studies have examined pharmaceuticals in the environment, the bulk of these studies have concentrated on drugs used by humans. Drugs used in veterinary medicine have received less attention, although that is changing.[1] Even among those studies, most of the published studies have examined antibiotics and hormones used in livestock production.[2] An often-overlooked class of veterinary drugs are those used to euthanize animals, such as pentobarbital (5-ethyl-5-pentan-2-yl-1,3-diazinane-2,4,6-trione). In addition to their behavior in the animal carcass, once the tissues have decomposed and the drug is released to the environment, interactions with a complex matrix such as soil could significantly change the fate and impact of the drug on the environment.[3]

Methods of euthanasia approved by the American Veterinary Medical Association for large animals such as horses include pentobarbital overdose, and captive bolt to the temporal lobe.[4] With a lethal dose of 2–10 g for humans, the approximately 30–40 g of pentobarbital typically used to euthanize a large animal represents a significant reservoir of the drug.[5] For smaller animals such as dogs, the lethal dose is approximately 390 mg per 4.5 kg of body weight.[6] After euthanasia by lethal injection, disposal of the carcass represents a problem. Disposal methods include burying, composting, cremation, and rendering.[7] Pentobarbital is known to survive the rendering process.[8] Meat containing pentobarbital from euthanized animals has found its way into pet food.[9] As recently as February 2017, Evanger brand dog food was recalled after reports of animals dying or becoming sick due to pentobarbital ingestion.[10]

Burying the carcass can pose two major threats. The carcass itself represents a source of pentobarbital and there are several reports of secondary poisoning of wild animals such as birds of prey and pets by the carcasses of euthanized animals.[11,12,13] In the tissues of euthanized animals, pentobarbital has been shown to survive for long periods of time.[14,15] One of the reported cases of secondary poisoning involved two dogs that disturbed an unburied horse carcass that had been euthanized 2 years previously.[12] In 2003, the FDA issued a warning stating "euthanized animals must be properly disposed by deep burial, incineration, or other method in compliance with the state and local laws to prevent consumption of carcass material by scavenging wildlife."[16]

The second source of contamination involves the release of pentobarbital into the environment as the carcass decomposes. While the study of pentobarbital in the environment has been limited, there are a few reports. Pentobarbital was detected in ground water 300 m from a landfill that had been closed 21 years earlier.[17] Also, the presence of the barbiturate,

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5,5-diallyl barbituric acid, was detected in leachates near a landfill.[18] It was the purpose of this study to determine the rate of decomposition of pentobarbital after sorption to various types of soil.

## Materials and methods

Pentobarbital sodium salt was purchased from Sigma-Aldrich. Strata-X C19 solid phase extraction units and HyperClone ODS C18 (150 × 4.6 5 $\mu$m) HPLC columns were purchased from Phenomenex. Millex-GV PVDF 0.22 $\mu$m syringe filters were obtained from Millipore. All other chemicals were reagent grade. Expert Gardener All Purpose Potting Soil Mix was purchased from Lowe's Home Improvement Center. Topsoil A (0–10 cm) and topsoil B (11–20 cm) were obtained from the Department of Agriculture and Environmental Science at Tennessee State University. The other soil samples, sand, horse stall sweepings, and loam were obtained from the University of Tennessee Extension site in Lewisburg, TN.

### Standards

Stock solutions of pentobarbital (10,000 ppm) were prepared by dissolving 10 mg of pentobarbital sodium salt in 1 mL of methanol. Any solution not used immediately was stored at 4°C. Working standards were prepared by serial dilution with methanol of the stock solution.

### Characterization of soil samples

To determine the pH of the soil, 20 mL of reagent grade water were added to 20 g of soil sample. The sample was covered and stirred for 5 min. The suspension was allowed to stand for 1 h to allow the soil to settle. The pH of the supernatant was measured using an Accumet AB150 pH meter equipped with an Accumet combination electrode.

The moisture content of the soil was determined by drying 20 g of soil sample at 100°C to a constant weight. The percent moisture was calculated by dividing the difference between the original weight and the weight of the dried sample by the original weight of the sample and multiplying by 100.

The organic content of the sample was determined by ashing 5 g of the dried sample used to determine the moisture content at 440°C overnight in a muffle furnace. The organic content was calculated by dividing the mass of the ashed soil by the original mass of the dried soil and multiplying by 100.

### Extraction of pentobarbital from soil

Soil samples (5 g) were weighed and ground by mortar and pestle to break up large clumps to produce a more uniform mixture. The soil sample was spiked with varying amounts of the stock pentobarbital solution to produce the desired concentration of pentobarbital in the soil. The sample was allowed to air dry for 2 h. Methanol (25 mL) was added to the soil sample to extract the pentobarbital. The sample was thoroughly mixed on an automated rocker overnight and the solids allowed to settle. The supernatant was decanted into a 50 mL centrifuge tube. Another 10 mL of methanol was added to the soil sample, and again mixed thoroughly by shaking. The sample was allowed to settle for 1 h. The liquid was decanted into the same 50 mL centrifuge tube. The combined methanol solution was centrifuged at 1,900 rpm for 10 min. The supernatant was collected into a fresh centrifuge tube. The process was repeated an additional two times and solution evaporated to near dryness on a rotary evaporator at 37°C. An additional 5 mL of water was added to the residue and the solution filtered through a Millex-GV PVDF 0.22 $\mu$m syringe filter. The SPE cartridge was activated and conditioned with 2 mL of 5% methanol, then equilibrated with 2 mL of 1 M sodium acetate buffer (pH 7.0). The pentobarbital sample was passed through the cartridge at a rate of 5–8 drops per 10 sec. The cartridge was washed with 1 mL of 1 M sodium acetate buffer and allowed to dry under vacuum for a minimum of 5 min. The pentobarbital was eluted into a fresh flask with 1.1 mL of 50:50 20% methanol/acetonitrile and the solution transferred to an HPLC vial for LC/MS analysis.

### Liquid chromatography/mass spectrometry (LC/MS)

Samples were chromatographed on a Phenomenex HyperClone ODS C18 (150 × 4.6 mm) 5 $\mu$m column on an Agilent 1100 LC/MS. The column was eluted with an isocratic mobile phase of 60/40 acetonitrile/water with a flow rate of 0.4 mL min$^{-1}$. The column temperature was 40°C and 20 $\mu$L of sample were injected. The mass detector ionization mode was electrospray atmospheric pressure ionization (ES-API) in negative polarity with a fragmentation voltage of 90 V.

### Calibration curve

A calibration curve was constructed based on multiple injections of stock pentobarbital solutions in methanol ranging in concentration from 1 to 100 ppm. Multiple injections of pentobarbital in methanol using known low concentrations were used to determine the limit of detection (LOD). The limit of detection was the concentration of pentobarbital that yielded a signal-to-noise of 3 compared to a blank sample. The limit of quantification was determined in a similar manner to that used to determine the limit of detection using a signal-to-noise of 7 compared to a blank sample.

### Stability studies

Samples of sand, potting soil, and topsoil (35 g each) were placed in 50 mL disposable centrifuge tubes and spiked with 0.07 mg of pentobarbital. The samples were mixed thoroughly and divided into 5 g portions and stored at 37°C. The amount of pentobarbital remaining in the soil was determined at intervals using the previously described method.

### Bacterial degradation of pentobarbital

Soil samples were autoclaved and spiked with pentobarbital as described above. The amount of pentobarbital remaining in the soil was determined by LC/MS as described above.

## Results and discussion

Because of the expected complex nature of the soil sample extracts, LC/MS was chosen as the method to identify and quantify pentobarbital recovered from various soil samples. A standard sample of pentobarbital was used to determine the retention time of pentobarbital and its mass spectra (Fig. 1). Pentobarbital had a retention time of 3.8 min and the extracted ion spectra of the peak at 3.8 min gave a major peak with an *m/z* value of 225 (Fig. 1 inset) in the negative ion mode which is consistent with the [M-H] spectra of pentobarbital. The polarity of the mass analyzer was the most significant instrument parameter in determining the signal intensity. In the negative ion mode pentobarbital could be detected at the part per million level, while in the positive ion mode no signal for pentobarbital was observed.

A calibration curve was constructed for pentobarbital to determine the range of linearity, limit of detection (LOD), and limit of quantitation (LOQ) for the method. The range of linearity was determined using multiple concentrations ranging from 0.1 ppm to 10,000 ppm and a calibration curve constructed (data not shown). Above a pentobarbital concentration of 100 ppm, deviation from linearity was observed. Using concentrations ranging from 1 ppm to 100 ppm, a linear regression of the data resulted in a correlation coefficient of 0.9951 between peak area of pentobarbital and concentration of pentobarbital. The limit of detection was determined by injecting increasingly dilute solutions of pentobarbital until the signal to noise (S/N) was less than 3. This occurred at 0.1 ppm. This is consistent with previously reported detection limits for pentobarbital of 0.1 ppm by gas chromatography-mass spectrometry coupled to a limit of quantitation, signal-to-noise ratio of at least 7, and range of linearity, of 0.5–100 ppm.[19]

A number of methods have been developed to measure pentobarbital concentrations in different types of matrices. Most of these methods involve the analysis of pentobarbital levels in various tissues and biological samples.[20–22] A second group of analyses involves the determination of pentobarbital levels in pet food.[23–25] Finally, a limited number of studies have determined the levels of pharmaceuticals in soils or other materials.[26,27]

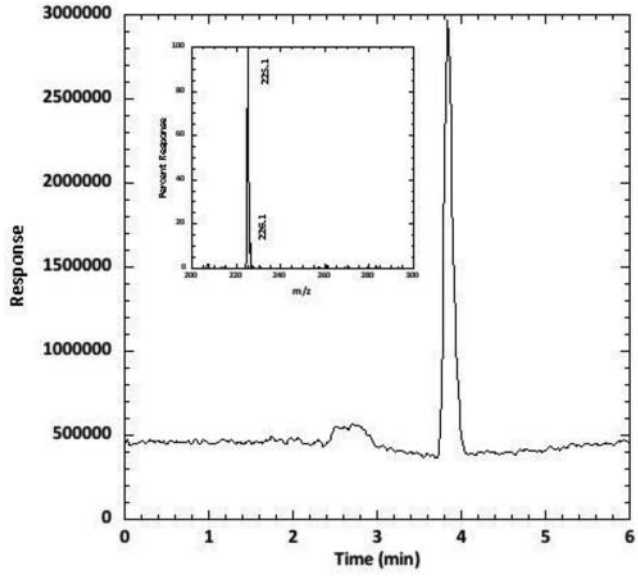


**Figure 1.** Total ion chromatogram of pentobarbital with a retention time of 3.84 min. Inset: Extracted ion chromatogram of peak at 3.84 min. The peak at *m/z* of 225 is consistent with the [M-H] peak of pentobarbital with a molecular weight of 226.

Because of the low pentobarbital concentration, these methods often involve an extraction and concentration step prior to chromatography. The most widely used methods of detecting pentobarbital and other veterinary pharmaceuticals are gas chromatography/mass spectrometry and liquid chromatography/mass spectrometry after extraction and concentration using a solid phase cartridge.

The recovery of pentobarbital from various types of soils was measured to determine the suitability of the method. Five different soil types (5 g) were spiked with 0.001 mg pentobarbital resulting in a concentration of 0.2 ppm. The types of soil analyzed were Expert Gardener All Purpose Potting Soil Mix from Lowe's Home Improvement Center, sand, horse stall sweepings, and loam obtained from the University of Tennessee Extension site in Lewisburg, TN, and topsoil sample A (1–10 cm) and topsoil sample B (11–20 cm) obtained from the Department of Agriculture and Environmental Science at Tennessee State University. The method described earlier was used to extract pentobarbital from each soil type and the percent recovery of pentobarbital determined (Table 1). The percent recovery was defined as the percentage of pentobarbital recovered from the soil as measured by mass spectrometry divided by the amount of pentobarbital added to the soil sample. Methanol was used to extract pentobarbital from the soil sample and the pentobarbital was concentrated by solid phase extraction. Blank soil samples in which no pentobarbital was added showed no pentobarbital present (Fig. 2). The percent recovery of pentobarbital varied from a low of 70% for topsoil A to a high of 111% for topsoil B, while the remaining recoveries were approximately 85%. The lower limit of detection was 0.0005 mg pentobarbital added to 5 g of soil corresponding to 100 ppb. This is slightly higher than the levels of pentobarbital Adam and Reeves[23] were able to detect in dog food. Using a combination of methanol extraction, solid-phase extraction, and gas chromatography/mass spectrometry, they were able to determine pentobarbital at 5–20 ppb.

To determine the effect of pentobarbital concentration on recovery, the percent recovery was determined using potting soil and varying the amount of pentobarbital added to the 5 g soil sample from 0.001 mg (0.2 ppm) to 0.1 mg (20 ppm). The average recovery for a total of 18 trials was 86.4 ± 11.8%. In general as the concentration of pentobarbital increased the percent recovery increased. When the amount of pentobarbital dropped to 0.0005 mg in 5 g soil, the percent recovery was significantly lower at 59% compared to the other concentrations.

**Table 1.** Recovery of pentobarbital from various soil types.

| Soil | Recovery (%)* |
|---|---|
| Topsoil A (0–10 cm) | 70 ± 3.5 |
| Topsoil B (11–20 cm) | 111 ± 4.0 |
| Sand | 87 ± 3.0 |
| Stall sweepings | 85 ± 4.5 |
| Loam | 88 ± 3.8 |
| Potting soil | 86 ± 3.5 |

*Values are the average of three trials.

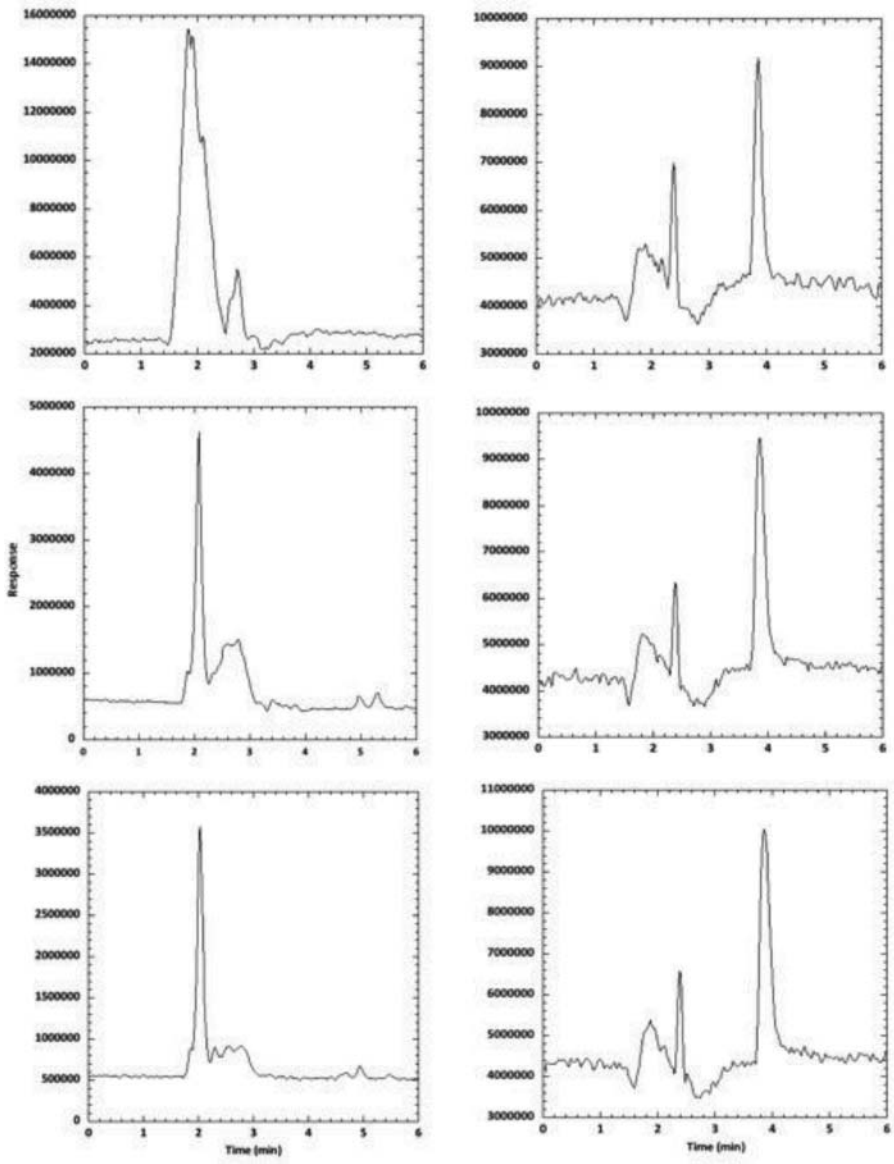


**Figure 2.** Chromatograms of soil samples after extraction and LC/MS analysis: chromatograms on the left are before addition of pentobarbital and those on the right are samples during the course of the stability study. (A) Potting soil; (B) topsoil; (C) sand. The pentobarbital peak at 3.84 min has a retention time and extracted ion consistent with pentobarbital standard. The peaks at the earlier retention times do not have an extracted ion consistent with pentobarbital.

The solid phase extraction step was critical to the concentration and cleanup of the samples extracted from the soil. While methanol was necessary to the extraction of pentobarbital, its inclusion in the sample loaded onto the SPE cartridge prevented the binding of pentobarbital. Therefore while the soil was extracted with methanol, a step to remove the methanol by evaporation was included. The use of the sodium acetate buffer was also necessary to the analysis of pentobarbital. Without the sodium acetate buffer, the recovery of pentobarbital from the SPE cartridge was significantly lower; moreover, numerous other compounds were present in the chromatogram resulting in difficulty in quantifying the pentobarbital recovered.

An analysis of the decay of pentobarbital adsorbed to three different soil types was conducted over a 17-week period. The moisture content, organic content, and pH of the three types of soil samples were determined (Table 2). Potting soil had the highest pH, while topsoil and potting soil had similar pHs of approximately 6. Potting soil had a significantly higher moisture and organic content compared to topsoil and sand.

Three 35 g samples of potting soil, sand, and topsoil B (11–20 cm) were spiked with 70 $\mu$g of pentobarbital and mixed thoroughly. The initial concentration of pentobarbital in the soil samples was 2 ppm, a low but detectable concentration of pentobarbital. Each sample was divided into seven 5 g samples, stored in 50 mL centrifuge tubes, and incubated at 37°C. There was a steady decrease in the amount of pentobarbital recovered in each soil type as time passed,

**Table 2.** Properties of soils.

| Soil | Moisture content* *n*(%) | pH* | Organic content* (%) |
|---|---|---|---|
| Topsoil B | 1.73 | 5.88 | 6.3 |
| Sand | 0.007 | 7.30 | 0.2 |
| Potting soil | 47.221 | 5.98 | 43.5 |

*Values are the average of two trials.

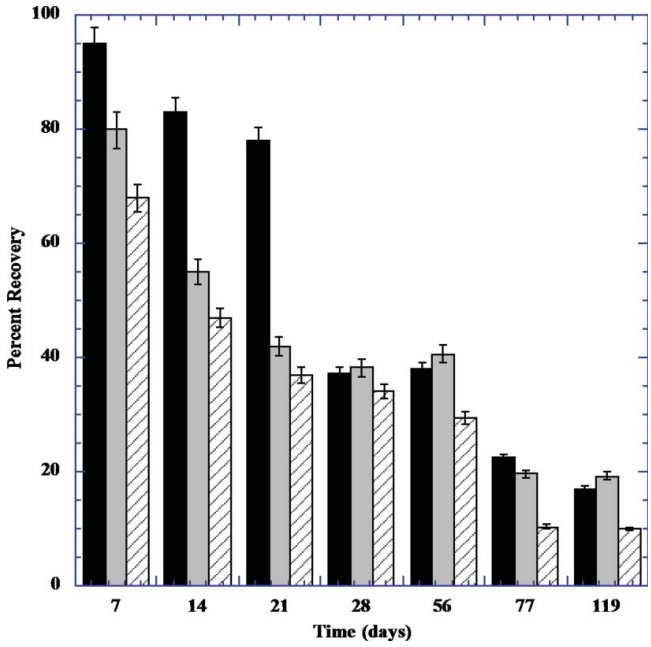


Figure 3. Time course of recovery of pentobarbital from three soil types, sand, topsoil B, and potting soil. The percentages are the average of two trials. Black bars, sand; gray bars, topsoil B; diagonal lines potting soil.

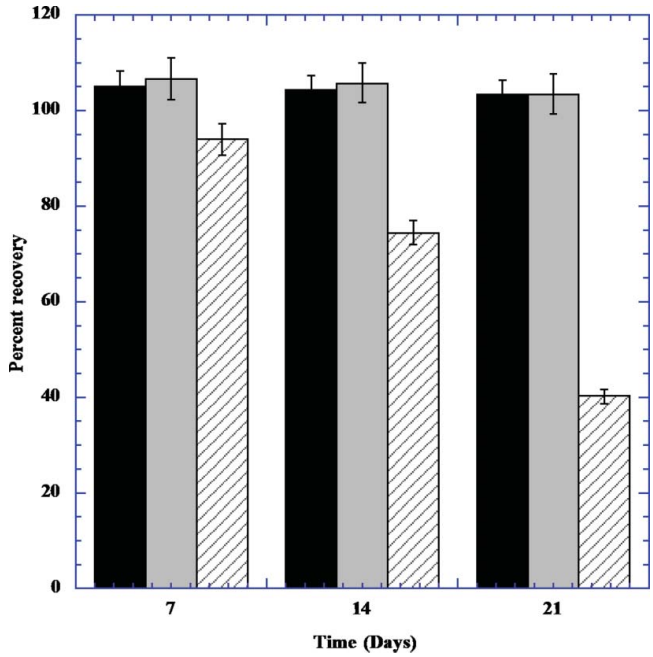


Figure 4. Time course of recovery of pentobarbital from autoclaved soils, sand, topsoil B, and potting soil. The percentages are the average of two trials. Black bars, sand; gray bars, topsoil B, diagonal lines, potting soil.

although there are differences in the rates of degradation between the soil types (Fig. 3). The degradation of pentobarbital in potting soil had the fastest initial rate, followed by degradation in topsoil B. Sand had the slowest initial rate of degradation. However, after 28 days the amount of pentobarbital in all three soil types was approximately equal at 3.8 $\mu$g (0.8 ppm). Between 77 days and 119 days the amount of pentobarbital remained relatively constant at approximately 2.0 $\mu$g in topsoil B and sand and 1.0 $\mu$g in potting soil corresponding to 0.4 ppm and 0.2 ppm in each 5 g soil sample. While a significant amount of pentobarbital had undergone degradation, at 119 days measurable amounts of pentobarbital persisted in all three types of soil.

These results are consistent with studies measuring the persistence of pentobarbital in compost piles containing equine carcasses.[28–31] While pentobarbital decreased during the time period, it persisted in a compost pile 180 days after introduction of a euthanized horse carcass. In another study, sodium pentobarbital was still detectable at 367 days.

The breakdown of compounds such as antibiotics in soils is mainly due to the action of microorganisms.[32] The mechanism of pentobarbital degradation in soil is unclear. The breakdown of barbiturates in the tissues of animal and human organisms has been widely studied.[33] However, their degradation in the environment is not well studied. One mechanism by which pentobarbital can be degraded is through the action of the bacterium *Rhodococcus erythropolis*. Barbiturase, an enzyme of the oxidative pyrimidine pathway of *R. erythropolis*, catalyzes the conversion of barbituric acid to ureidomalonic acid.[34] During a related project four potential pentobarbital-degrading bacteria were isolated from horse stall litter.[35] Work is continuing on the characterization of these bacteria.

To determine if the degradation of pentobarbital was due to bacterial action, samples of potting soil, topsoil B, and sand were autoclaved. The soil samples were spiked with pentobarbital as described above and the amount of pentobarbital determined as a function of time (Fig. 4). In autoclaved sand and topsoil B, degradation of pentobarbital was not observed. Over a three-week period, the average recovery of pentobarbital from sand was 104.3 $\pm$ 3.9%, while it was 104.5 $\pm$ 2.8% from topsoil B. In contrast there was significant degradation of pentobarbital added to autoclaved potting soil.

At the end of the first week, the amount of pentobarbital recovered was 93.2%, at the end of the second week the percent recovery was 72.6%, and at the end of the third week the pentobarbital recovery was 45.1%. This was comparable to the recovery of pentobarbital from potting soil that was not autoclaved. The results indicate that several mechanisms may be at work in the degradation of pentobarbital. The most important mechanism in topsoil and sand appears to be bacterial. In potting soil, in addition to bacterial degradation there appears to be another mechanism. There was no significant difference in the degradation of pentobarbital between potting soil and autoclaved potting soil. The potting soil used in this study contained peat moss, composted bark, pasteurized poultry litter, and an organic wetting agent. Because of its complex nature there appear to be substances in the potting soil that are capable of degrading pentobarbital. Finally, potting soil contains a wide diversity of bacterial populations, including thermophilic bacteria.[36] While the temperatures reached in the autoclave would normally be sufficient to kill any bacteria present, the heterogenous nature of the potting soil may have resulted in uneven heating of sections of the sample. Within these sections, thermophilic bacteria may have survived. Further work is being conducted to characterize the composition of the potting soil in relation to the observed degradation of pentobarbital.

## Conclusions

A method utilizing methanol extraction, solid phase extraction, and liquid chromatography was developed to measure pentobarbital in various soil types. The method was capable of detecting pentobarbital levels down to 100 ppb in soil. The stability of pentobarbital in three types of soils was determined over a 17 week period. Three types of soil, sand, topsoil, and potting soil, were spiked with pentobarbital equal to a concentration of 2 ppm. While there was significant degradation of pentobarbital, even after 4 months detectable amounts of pentobarbital remained. The concentration of pentobarbital in sand and topsoil after 17 weeks was 0.4 ppm and in potting soil it was 0.2 ppm. These results reinforce the need for the proper disposal of euthanized animals to avoid contamination of the environment with barbiturates.

In sand and topsoil, bacterial degradation appears to be the chief mechanism by which pentobarbital degrades. Autoclaving the soil before spiking with pentobarbital resulted in no degradation of pentobarbital. However, autoclaving potting soil did not result in a significant change in the rate of pentobarbital degradation, indicating a different mechanism of degradation may be at work. Further work will be necessary to elucidate the mechanisms of pentobarbital degradation to evaluate the risk associated with the pentobarbital introduced into the environment through euthanized animals.

## Funding

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**Exhibit 8**