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MERCURY AND OTHER TRACE METAL ANALYSIS IN BAT GUANO

A Thesis
Presented to
The Faculty in the Department of Chemistry
Western Kentucky University
Bowling Green, Kentucky

In Partial Fulfillment
Of the Requirements for the Degree
Master of Science

By
Amanda Lee Houchens

May 2020

MERCURY AND OTHER TRACE METAL ANALYSIS IN BAT GUANO

Date Recommended March 27, 2020

Cathleen J. Webb

Cathleen Webb, Director of Thesis

Bangbo Yan

Christopher Groves

Cheryl D Davis Digitally signed by Cheryl D Davis
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Dean, Graduate School Date

ACKNOWLEDGEMENTS

To begin, I would like to thank Dr. Cathleen Webb, my research advisor, for all of the support that she has provided during my experience at Western Kentucky University. I first met Dr. Webb as an undergraduate student when she recruited me as a chemistry major and again when she forced me out of my comfort zone as a TA in a summer PChem lab. She definitely saw something in me that I hadn’t yet discovered for myself. Thank you again for helping me grow my confidence as a scientist.

I would also like to thank Pauline Norris at the Advanced Materials Institute at the WKU Center for Research and Development, part of the WKU Applied Research and Technology Program. Pauline provided excellent instruction and support as I learned how to use the various analytical instruments in the lab. And thank you to Alicia Pesterfield for always making sure I knew how to be safe in the lab. From the first time I met you as an undergraduate, you have always been a sound provider of advice.

Thank you to the rest of the faculty of the Chemistry Department in the Ogden College of Science and Engineering for enriching my graduate experience over the past five years. I would like to recognize the WKU Graduate School for providing me with a Graduate Student Research Grant to fund this research. A special thank you to Dr. Bangbo Yan and Dr. Christopher Groves for serving on my thesis committee. And thank you, Dr. Groves, for assisting me in the collection of the guano samples for my research.

Last, but certainly not least, I would like to thank my family and friends for providing the love, support, and encouragement I needed to keep going when I couldn’t see the light at the end of the tunnel. I owe a deep debt of gratitude to my Mom for shaping me into the strong-willed woman I am today. And to my partner, Travis, thank you for always supporting my strong-willed pursuits.

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MERCURY AND OTHER TRACE METAL ANALYSIS IN BAT GUANO

Amanda Lee Houchens May 2020 52 Pages

Directed by: Cathleen Webb, Bangbo Yan, and Christopher Groves

Department of Chemistry Western Kentucky University

Heavy metal pollution in the environment pose risks to ecosystems and the populations that reside in them. Mercury, lead, and cadmium negatively impact humans by way of neurological disorders, various cancers, and damage the reproductive organs, kidneys, and lungs. Bats have been studied as a bioindicator species to identify possibly elevated levels of these metals in the environment. Previous studies have identified correlation between metal concentrations within bat tissues and fur. Many bat species are endangered or at risk due to white-nose syndrome so collection of tissues and fur for analysis can impose stress on bat colonies. This study investigates the presence of a correlation between mercury concentrations and lead and cadmium concentrations in guano. Thirty-seven guano samples from a breeding colony of federally endangered gray bats were analyzed for mercury using a mercury analyzer. Lead and cadmium concentrations were determined using acid digestion and ICP-OES. Analysis indicates a positive correlation between mercury, lead, and cadmium. Guano samples from the same cores consistently mirror these findings. In conclusion, when the concentration of mercury, lead, or cadmium is determined for a bat guano sample the remaining two concentrations can be predicted.

1. GENERAL INTRODUCTION AND LITERATURE REVIEW

Mercury and other heavy metals are a growing environmental and health concern in the United States and across the globe. Graening and Brown detected toxic concentrations of metals in water, sediment, and animal tissues (2003). Many of these metals are biomagnified as they contaminate air, water, and land. While they may be synthetic or natural in origin, these trace metals are toxic and bioaccumulative (Tiefenbacher, 2000) making them of special importance.

Karst ecosystems have several underground features of note: caves, pits, sinkholes, rivers, etc. Special attention is being paid to karst ecosystems because they play a vital role in providing a habitat for several endangered species as well as contributing to the hydrogeology and hydrology of water storage and circulation (Bonacci, Pipan, and Culver, 2008).

1.1 Sources of Mercury and Trace Metals in the Environment

Previous research has identified synthetic and natural sources of mercury and trace metal contamination in the environment. Phosphate rocks are commonly used to manufacture phosphate fertilizers for the soil, providing a significant source of cadmium metal in the soil and food chain (Aydin, Aydin, Saydut, Bakirdere, and Hamamci, 2010; Cheraghi, Lorestani, and Merrikhpour, 2011; Gupta, Chatterjee, Datta, Veer, and Walther, 2014; Mar and Okazaki, 2010). Cuculic, Cuckrov, Zeljko, and Mlakar determined that carbonate rocks also provide a source of minor cadmium, copper, lead, zinc contamination in the water column (2011).

Agriculture is a contributor to heavy metal contamination in the environment. The use and overuse of phosphate rocks as a source of phosphorus in fertilizers adds heavy metals to the environment like cadmium, arsenic, lead, and mercury (Aydin, Aydin, Saydut, Bakirdere, and Hamamci, 2010). The use of biogenic sources of phosphorus fertilizers, such as bird and bat guano, also contribute to heavy metal pollution in the environment (Cheraghi, Lorestani, and Merrikhpour, 2011), although the amount of bird and bat guano used for fertilizers is small relative to other sources of phosphorus. Fertilizers create a cycle of heavy metals moving through the environment (Liu, Nie, Sun, and Emslie, 2013).

Roadside ecosystems encounter higher than normal levels of metals such as lead, cadmium, nickel, and zinc. Cadmium enters the environment as a result of tire wear while nickel originates from fuels and lubricating oils. Despite lead being removed from most gasoline in 1996, lead still contaminates roadside environments. Once contaminants are on the road surface, surface and groundwater, consumption of plants and animals by animals from outside the ecosystem, and migrating animals are the primary means for spreading contamination throughout the environment (Scanlon, 1991).

In addition to highway contamination, industrial process also contribute to heavy metal pollution in the environment. Tetra ethyl lead (TEL) and tetra methyl lead (TML) are still used in aviation gasoline (Kraus, 2011). Additionally, lead is released into the environment by industrial manufacturing processes. Cadmium is released via industrial processes like metal plating and alloy production and is also a byproduct of phosphate fertilizers and coal combustion (Gupta, Chatterjee, Datta, Veer, and Walther, 2014; Thies and Gregory, 1994).

Mercury can easily travel through terrestrial and aquatic environments, passing through natural filtration systems in the form of methylmercury (MeHg), the most deleterious form of mercury to living organisms due to its organometallic character (Gilmour, Henry, and Mitchell, 1992; Grasman, 2002; Lison, Espin, Aroca, Calvo, and Garcia-Fernandez, 2016; Morel, Kraepiel, and Amyot, 1998). Methylmercury can easily be absorbed, stored, and accumulated in various tissues of aquatic animals and fish (Gilmour, Henry, and Mitchell, 1992; Graening and Brown, 2003; Mansour, Soliman, and Soliman, 2016; Milan, 2009; Zukal, Pikula, and Bandouchova, 2015).

Once this happens, MeHg passes into the food web where it bioaccumulates and becomes biomagnified (Lison, Espin, Aroca, Calvo, and Garcia-Fernandez, 2016). Bat guano has also been identified as a source of trace metal contamination in aquatic environments, specifically cadmium (Cd), lead (Pb), copper (Cu), zinc (Zn), and mercury (Hg). In anchialine objects like caves, sinkholes, and caverns this can be especially problematic as they serve as important aquatic environments and aquifers containing reserves of potable water (Cuculic, Cukrov, Kwokal, and Mlakar, 2011). Studies have established that concentrations of these trace metals increase following heavy periods of rain (Bonacci, Pipan, and Culver, 2008; Cuculic, Cukrov, Kwokal, and Mlakar, 2011; Dodge-Wan, Prasanna, Nagarajan, and Anandkumar, 2017).

As populations increase in karst terrains, surface water and groundwater sources are becoming increasingly threatened. Bonacci, Pipan, and Culver indicate several factors negatively impacting the karst ecosystem, including human influence, water pollution, unsustainable agriculture and forestry, and deficiencies in the legal framework that should protect these areas (2008). Whether organisms in the ecosystem are aquatic or terrestrial, water sources in the karst system are important for the survival of species, thus warranting a study of contamination.

According to a publication by the United States Geological Survey, every state in the United States has karst features in the landscape or has soluble rocks with the potential to develop into karst features like caves and sinkholes. This area accounts for 18% of the land area in the United States (Weary and Doctor, 2014) and approximately 20% of the groundwater used by the population (Maupin and Barber, 2005). Additionally, more than 25% of the world's population lives in karst regions or obtains their water from a karst aquifer with karst terrain accounting for 10% of the Earth's surface (Maupin and Barber, 2005)

Dodge-Wan, Prasanna, Nagarajan, and Anandkumar detected correlations between several trace metals in a epiphreatic cave. A correlation between iron (Fe) and manganese (Mn) as well as cobalt (Co), copper (Cu), and cadmium (Cd) suggest that some trace metal contamination is originating from soil and rock leaching into the environment (2017). Open pit mines provide a source of heavy metal contamination when they are exposed to large amounts of water in the instance of becoming filled with water and forming a lake (Zocche, Leffa, Damiana, Carvalhalo, Mendonca, Santos, Boufleur, Dias, and Andrade, 2010). Heavy metals pass through the food chain as a result.

1.2 Effects of Mercury and Trace Metals on Health and the Environment

Methylmercury is recognized as an agent that can cause neurodegenerative effects and also affects the reproductive system in mammals. MeHg also crosses the placental barrier and can concentrate in the fetal brain causing developmental problems in the fetus, potentially resulting in fetal death. Furthermore, MeHg can also be transferred to offspring via lactational transfer (Lison, Espin, Aroca, Calvo, and Garcia-Fernandez, 2016).

The first well-documented case of wide-spread acute methylmercury poisoning occurred in 1953 in Minamata, Japan. The Chisso Corporation’s chemical factory released industrial wastewater containing methylmercury into Minamata Bay which bioaccumulated and biomagnified in the fish and shellfish which were consumed by the city’s population. As a result, individuals who ate contaminated fish and shellfish developed symptoms including ataxia, numbness in the extremities, muscle weakness, vision and hearing loss, insanity, paralysis, and coma (Ekino, Susa, Ninomiya, et al, 2007; Nabi, 2014). More than 1,700 people died as a result of this severe mercury poisoning, referred to as Chisso-Minamata Disease (Takaoka, 2011). There is also a congenital form of Chisso-Minamata Disease that can pass to fetuses in utero (Ekino, Susa, Ninomiya, et al, 2007).

Other trace metals present serious considerations for the health of mammals in the environment. Cadmium can retard growth, cause anemia, damage kidney and testicular tissue, interfere with the metabolism of copper and zinc, and increases hypertension in animals. Excess nickel negatively impacts growth rate, reproduction, disrupts liver metabolism, and muscle glycogen metabolism. An overabundance of zinc in an organism impedes copper metabolism, causes anemia, and interferes with the function of the gastrointestinal tract, liver enzymes, and skeletal formation (Scanlon, 1991; Thies, Gregory, 1994).

Arsenic and arsenic compounds cause abnormal development in mammalian embryos, degenerative tissue changes, cancer, damage to chromosomes, and death (Thies, Gregory, 1994). The largest poisoning of a population in the world is due to arsenic contamination. Between 35 and 75 million of the 125 million people in Bangladesh receive drinking water from groundwater sources that have been contaminated with arsenic naturally occurring in the ground (Hossain, 2006; Sahu, Saha, 2019).

A summary of biological effects of mercury, lead, and cadmium is summarized in Table 1.

Table 1: *Biological Effects of Heavy Metal Toxicities*

Metal	Biological Impacts
Mercury	Bioaccumulates in fat due to methylation, elemental form readily methylates into methylmercury, which: ● Causes neurodegenerative effects ● Affects the reproductive system in mammals ● Crosses the placental barrier and can concentrate in the fetal brain causing developmental problems in the fetus and potential fetal death ● Can also be transferred to offspring via lactational transfer (Lison, Espin, Aroca, Calvo, and Garcia-Fernandez, 2016)
Lead	Mimics the function of calcium in mammals and can: ● Retard growth ● Cause anemia ● Damage kidney and testicular tissue ● Interfere with the metabolism of copper and zinc ● Increases hypertension in animals (Scanlon, 1991; Thies and Gregory, 1994)
Cadmium	Mimics the function of zinc in mammals and can: ● Result in flu-like symptoms (chills, fever, and muscle pain) ● Damage the lungs ● Cause kidney, bone and lung disease ● Cause various cancers (United States Department of Labor, 2019)

A study of heavy metal contamination of bats in Britain concluded that lead and cadmium have a direct relationship in renal concentrations in multiple species of bats. The study did not yield the same correlation between lead or cadmium and mercury. Lead and cadmium appear to be introduced into the environment from similar contamination sources, while mercury comes from different sources (Walker, Simpson, Rockett, Wienburg, and Shore, 2007).

Some research suggests bats exposed to heavy metals and other anthropogenic stressors simultaneously may have antagonistic or synergistic effects. For example, 13% of bats with potentially toxic lead levels and 4% of bats with potentially toxic levels of arsenic also presented with white-nose syndrome (Courtin, Stone, Risatti, Gilbert, and VanKruiningen, 2010; Jones, Jacobs, Kunz, Willig, and Racey, 2009). In addition, Grasman determined that areas contaminated by environmental pollutants endured more severe epizootic infectious diseases (2002), suggesting a level of immunosuppression within populations due to contamination in the environment.

White-nose syndrome arrived in Mammoth Cave National Park in 2016. Since the arrival, there has been an 18.5% decline in the population of the most abundant bat species in the cave system, *Myotis septentrionalis* (Northern Long-eared Myotis) (Thalken, Lacki, Johnson, 2018). Mercury and methylmercury have long been documented in the Mammoth Cave National Park System (Helf, 2003). It is possible that the bioaccumulation of methylmercury in the bats made them more susceptible to contracting white-nose syndrome.

Increased concentrations of mercury in bat tissues leads to a decrease in neutrophil counts, the white blood cells responsible for fighting bacterial and fungal infections (Beldomenico, Telfer, Gebert, et al, 2008). This can make bats more susceptible to contracting white-nose Syndrome. Over time, chronic heavy metal exposure can increase glucocorticoid hormones, which are associated with chronic stress. This causes an increase in inflammatory response in bats, making them more prone to contract white-nose syndrome (Becker, Chumchal, Bentz, et al, 2017).

1.3 Bats as Bioindicators

Because of their wide range of geographical distribution, relatively long life span, high metabolic rate and food intake, and high trophic position, bats are considered an excellent bioindicator for mercury (Hg) contamination in the environment. Consumption of water and insects that may be contaminated with Hg and other trace metals can be detected in bats due to the bioaccumulative nature of these metals. Due to the length of the life-span, insectivorous bats are at an increased risk of obtaining toxic concentrations of a variety of trace metals (Jones, Jacobs, Kunz, Willig, and Racey, 2009; Lison, Espin, Aroca, Calvo, Garcia-Fernandez, 2016; Walker, Simpson, Rockett, Wienburg, and Shore, 2007). The collection of bat guano for analysis is also useful for heavy metal analysis due to containing undigested portions of food which can help expose the source of contamination (Mansour, Soliman, and Soliman, 2016).

The majority of studies cited in the literature use destructive samples such as kidney, liver, muscle, and brain which limits sample size. In addition, while carcasses obtained in the field are occasionally used, the samples available are limited and must be handled with extreme caution. Authors seek validation of existing research findings by means of non-destructive samples such as guano and fur (Bird, Boobyer, Bryant, Lewis, Paz, and Stephens, 2001; Graening and Brown, 2003; Lison, et al., 2016; Milan, 1990; Scanlon, 1991; Walker, Simpson, Rockett, Wienberg, and Shore, 2006; Wurster, Munksgaard, Zwart, and Bird, 2015; Zocche, Leffa, Damiana, Carvalhalo, Mendonca, Santos, Boufleur, Dias, and Andrade, 2010; Zukal, Pikula, and Bandouchova, 2015). Validation of existing results is especially important as many bat species are rare, threatened, or endangered making specimen collection difficult (Lison, Espin, Aroca, Calvo, and Garcia-Fernandez, 2016).

Concentrations of metal pollution in small mammals, including bats, is determined to increase with increasing traffic flow in and near the environment. Insectivores are known to have higher metal concentrations than herbivores in the same environments. Scanlon also notes that as organisms die, the heavy metal contaminants contained in tissues returns to circulate through the ecosystem (1991). Studies suggest that current levels of heavy metal concentrations in a variety of bats is not decreasing in relation to historical levels. Continued monitoring of heavy metal contamination in bats is imperative to monitoring the health of ecosystems (Walker, Simpson, Rockett, Wienburg, and Shore, 2007.

There are a limited number of studies on the exposure and potential impacts of heavy metal exposure to bats and even fewer studies on bats from habitats near areas near coal sources. Studies are also limited on human impacts of heavy metal exposure in and around coal areas, especially downstream from coal mining areas. As humans and bats share a trophic level, bats seem especially useful in serving as a bioindicator for potential human exposure to these toxic metals as well. There are limitations to this comparison however, as humans are more complex due to mobility and diets not necessarily being locally derived (Zocche, Leffa, Damiana, Carvalhalo, Mendonca, Santos, Boufleur, Dias, and Andrade, 2010).

1.4 Current Analysis Methods of Mercury and Trace Metals in Bats

A review by Zukal, Pikula, and Bandouchova selected fifty-two studies and revealed heavy metal exposure, including arsenic, cadmium, cobalt, chromium, copper, mercury, manganese, nickel, lead, tin, and thallium, is continual and even increasing in parts of the world (2015). Despite wide recognition of bats as a bioindicator species, there is limited documentation in the literature on the negative effects of heavy metals on wild bat populations. One study documented the presence of heavy metals in wild bat populations (Jones, Jacobs, Kunz, Willig, and Racey, 2009).

Existing literature utilizes a variety of methods for heavy metal analysis in tissue, hair, and guano samples. However, consistent units of concentration are utilized throughout the literature so direct comparisons of data sets can be evaluated. Some studies use wet weight while others use dry weight of tissues (Zukal, Pikula, and Bandouchova, 2015) while relying on a conversion factor to draw comparisons between the two, impacting the results (Mochizuki, Mori, Hondo, and Ueda, 2008). Due to the processes involved in heavy metal accumulation in various organs and tissues, it is difficult to make a correlation between concentrations of different metals in different tissue types within a single organism. It is easier to expose a relationship between various metals in a single tissue type, but it must be carefully assessed (Hariono, Ng, and Sutton, 1993).

Little is known about potential toxicity levels of heavy metals in bats. While no studies have been done to quantify toxic thresholds of heavy metals in bats, studies of other insectivorous mammals have shown that a higher tolerance for heavy metals exists when compared to rodents (Ma and Talmage, 2001).

Milan concluded, through analysis of mercury concentrations in insect prey and mercury concentrations in the Big Brown Bat and Little Brown Bat that there was not a statistically significant relationship between the two. However, the concentrations of mercury in the surrounding natural environment and in the bats were proportional suggesting that bioaccumulation through the food chain and environmental factors are responsible for mercury accumulation in bats (1990). This relationship further supports the use of bats as a bioindicator of mercury contamination in the environment.

Hariono, Ng, and Sutton ascertained lead concentrations in bat fur was positively correlated to lead concentrations in the kidneys and liver (correlation coefficients of 0.55 and 0.51), respectively. A greater correlation existed for lead concentrations in fur washings and lead concentrations in the kidneys and liver (correlation coefficients of 0.73 and 0.94, respectively) (1993). Another study by Mansour, Soliman, and Soliman, indicated strong correlation between heavy metal concentrations in bat guano and heavy metal concentrations in liver and kidney samples for insectivorous bats (2016). Mulec, Covington, and Walochnik suggest that analysis of guano is an excellent alternative means when direct sampling of bats is impossible (2013) whether collection is limited, the species being studied is protected, or the researcher wants to limit stressors to the bat colony.

The purpose of this research is to determine if a correlation between the concentrations of mercury, lead, and cadmium in bat guano. By using mercury concentration to predict concentrations of lead and cadmium, analysis of environmental conditions can be done in a more rapid, economical manner. By conducting the analysis using bat guano in lieu of bat tissues or hair, minimal disruption is required to the bat colony under investigation. This is important as many species, like the Gray Bat, are threatened or endangered.

To accomplish these goals, bat guano was collected from a colony of Gray Bats and prepared for analysis by drying. Direct analysis of mercury concentration was completed using an AMA-254 instrument while analysis of lead and cadmium was completed with inductively coupled plasma- optical emission spectroscopy (ICP-OES) after the samples were prepared by acid digestion. Data for the concentrations of lead and cadmium were compared to the data for the concentrations of mercury to determine if mercury concentrations can be used to predict other metal concentrations in guano.

2. METHODOLOGY

Safety Considerations

When working with bat guano in the laboratory setting certain safety considerations must be observed. Bat guano may contain Histoplasmosis or Cryptococcosis spores. While not all samples may be contaminated, all samples should be treated as if they may be contaminated. Because of this, goggles, gloves, and a N-95 (or better) respirator should be worn when handling and conducting research with dried guano samples in the lab. Work areas should be cleaned with a disinfectant (Texas A&M University Biosafety Occupational Health Program, 2015).

Additional caution must be exercised in the collection of guano samples when bats are present. Since samples were collected from an active maternity roost of gray bats, care must be taken not to disturb the bats to reduce the chance for rabies exposure from a bite. In Kentucky, it is more common for skunks to be carriers of rabies than bats. However, bats account for the majority of rabies infections in humans in the United States. Should a direct exposure or bite occur, one should seek medical attention and begin a post-exposure prophylaxis treatment regimen (Kentucky Cabinet for Health and Family Services, 2019) .

Sample Collection and Preparation

Guano samples were collected with the assistance of Dr. Christopher Groves at Crumps Cave, a 1.5 mile long cave located in the Smiths Grove community of Warren County, Kentucky. The gated cave is owned and managed by Western Kentucky University and there is limited access to the cave as it is the summer home of a breeding colony of the transient, federally endangered gray bat as seen in Figure 1. The guano collected for this investigation was a mound 24 inches in height from this colony.

Figure 1: The gray bat, *Myotis grisescens* (US Fish and Wildlife Service, 2019).

Core samples were collected by driving a series of 1 inch PVC pipes through the deepest section of the guano mound (Figure 2a). The ends of the pipe were sealed for removal from the cave and were then transported to a freezer for storage until the cores could be sectioned for analysis (Figure 2b).

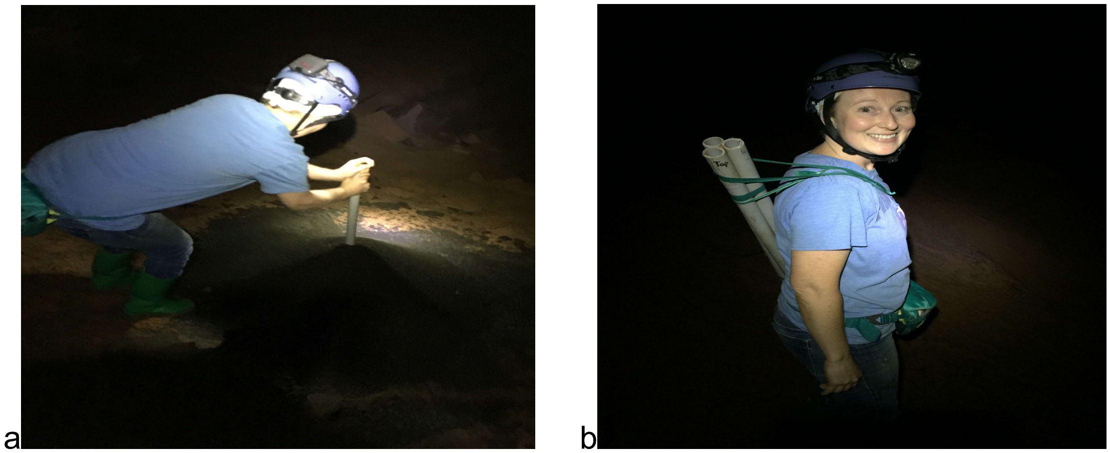

Figure 2: a) Collecting guano samples and b) transporting the collected samples from Crumps Cave, Smiths Grove, KY.

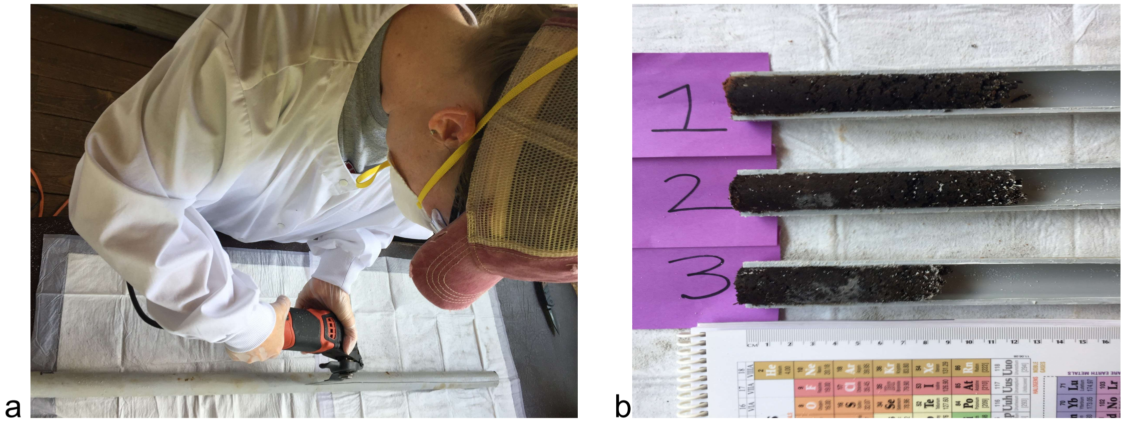

Figure 3: a) Cutting open the PVC pipes used to collect the guano samples to reveal b) the cores were compacted during collection.

To prepare the samples for analysis, the PVC pipes were cut open using an oscillating tool fitted with an oscillating saw blade (Figure 3a). Due to the freshness of the guano collected, the cores underwent some compaction during the collection process (Figure 3b). Once the sample cores were removed, they were measured, divided into samples, numbered (Figure 4), and transferred to paper envelopes to dry for 1 week. The samples had an average of 41.2% moisture content.

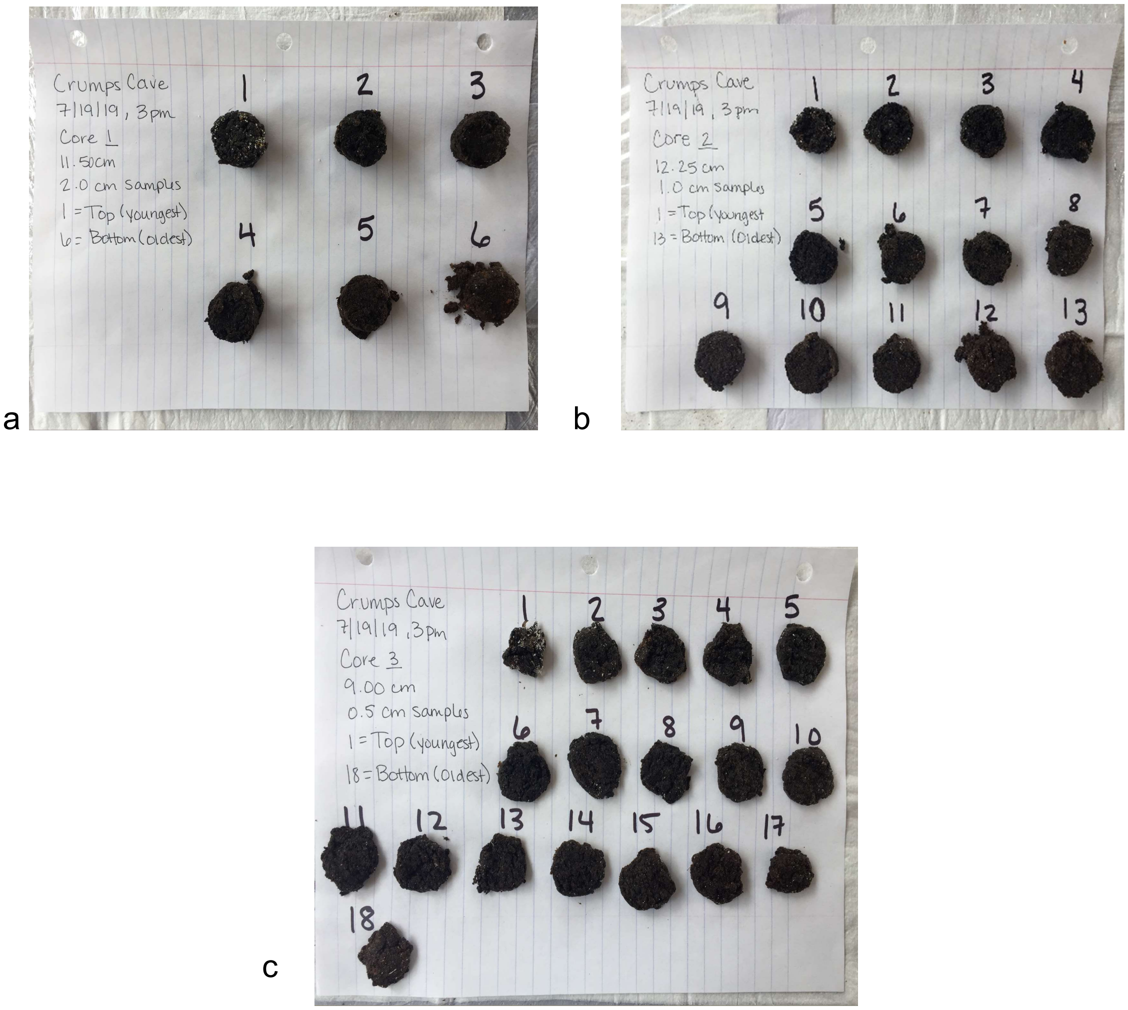

Figure 4: Cores a) one, b) two, and c) three were divided into smaller sections for drying and analysis.

Table 2: Division of Core Samples

Core	Total Length (cm)	Length of Sample (cm)	Number of Samples
1	11.5 cm	2.0 cm	6
2	12.25 cm	1.0 cm	13
3	9.0 cm	0.5 cm	18

Once dried, samples were evaluated for mercury, lead, and cadmium content at the Advanced Materials Institute at the Center for Research and Development on the campus of Western Kentucky University, a unit of the WKU Applied Research and Technology Program.

Mercury Analysis

AMA254

Figure 5: The AMA-254 Advanced Mercury Analyzer (LECO Corporation, US, 2008).

The bat guano samples are analyzed for mercury concentration using the AMA254 Mercury Analyzer by LECO Corporation, US and QuickSilver software

(Figure 5). The AMA254 can quantify mercury content in samples in about 5 minutes with detection levels from 5ppb to 5ppm and precision of 2.5ppb or ≥5% RSD, whichever is greater. The instrument operates in three phases: decomposition, collection, and detection (LECO Corporation, US, 2008). This process is outlined in Figure 6.

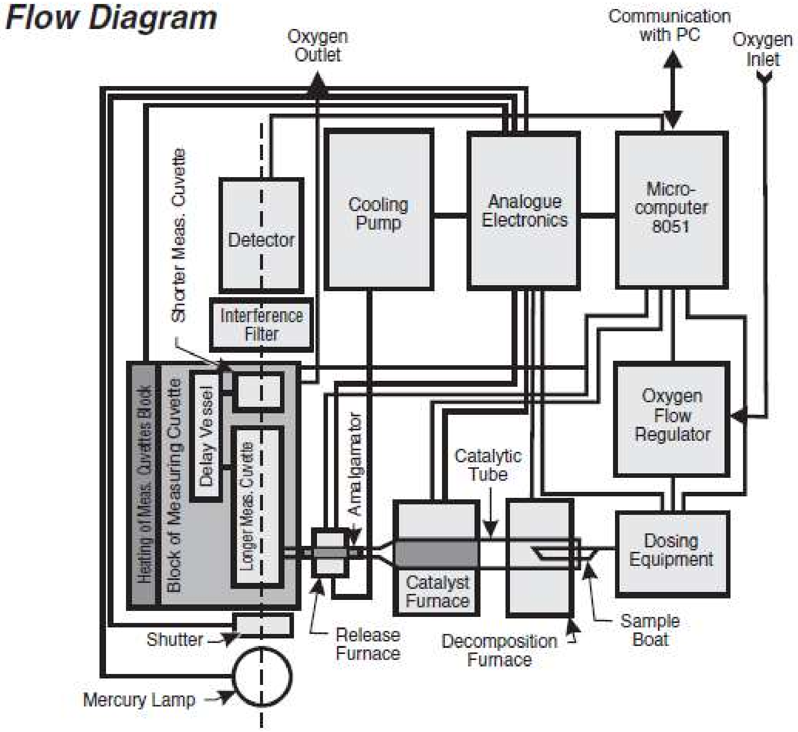

Figure 6. Flow Diagram for AMA-254 (LECO Corporation, US, 2008).

After solid contaminants, such as small rocks, were removed from the guano sample, 0.100 to 0.200 grams of guano was added to the nickel sample boat (Figure 7). The exact mass was entered into the QuickSilver software and the analysis process began. A total of 37 samples were divided out of 3 cores to be examined to ensure measurements were repeatable and consistent throughout the cores.

During decomposition, the sample is inserted into the instrument and it is heated to approximately 750°C to thermally decompose the sample and release gaseous components, including $Hg_{(g)}$. Oxygen carrier gas moves the gaseous portion of the sample to the catalyst furnace where impurities are removed (LECO Corporation, US, 2008).

Figure 7. The Nickel Sample Boats for AMA-254; (L to R) empty, guano sample, remaining ash.

The cleaned gas is carried to the mercury amalgamator to begin the collection phase. The amalgamator is composed of gold plated ceramic beads. Gold has a high affinity for mercury at a significantly lower temperature than required for the decomposition phase. Once all mercury has been collected in the amalgamator, the beads are quickly heated to 900° Celsius to release all of the mercury vapor, trapping the mercury vapor for detection phase (LECO Corporation, US, 2008).

In the detection phase, the mercury gas passes through a cuvette in the path of an Atomic Absorption Spectrophotometer with a lamp set to 253.7 nm, which is a wavelength specific to mercury absorption. The amount of mercury is then quantified by a UV diode detector for mercury (LECO Corporation, US, 2008).

Calibration of AMA254

The AMA-254 is unique in its analysis and calibration as the instrument measures total mercury. The mass of the standard sample is entered into the QuickSilver software and once the total mercury in the sample is measured, the concentration is calculated in parts per million. Concentration of the standard sample remains the same regardless of the amount used, however the intensity is directly related to the mass of the sample. A sample with a larger mass will have more mercury in it and therefore a higher intensity when compared to a sample with a smaller mass. The concentration of the mercury will be the same. Conversely, if there are two samples of the same mass with two different concentrations of mercury, the samples with the highest total mercury concentration will display the highest intensity.

The standard used in this analysis is fly ash (NIST 1633b.) This standard is from the National Institute of Standards and Technology and is known to have a mercury concentration of 0.141 ± 0.019 ppm (National Institute of Standards and Technology, 1993).

Other Trace Metal Analysis

Acid Digestion

In order to examine the guano samples for the concentration of lead and cadmium by ICP-OES, the samples were prepared using acid digestion. A 10 mL volume of nitric acid, trace metal analysis concentration, was added to approximately 0.500 g of guano in a 50 mL polypropylene digestion vessel (Figure 8a). The digestion vessels containing the samples were heated at 85°C for one hour in a HotBlock (Figure 8b). Once cooled, the samples were diluted to a total volume of 25 mL using deionized water (Figure 8c).

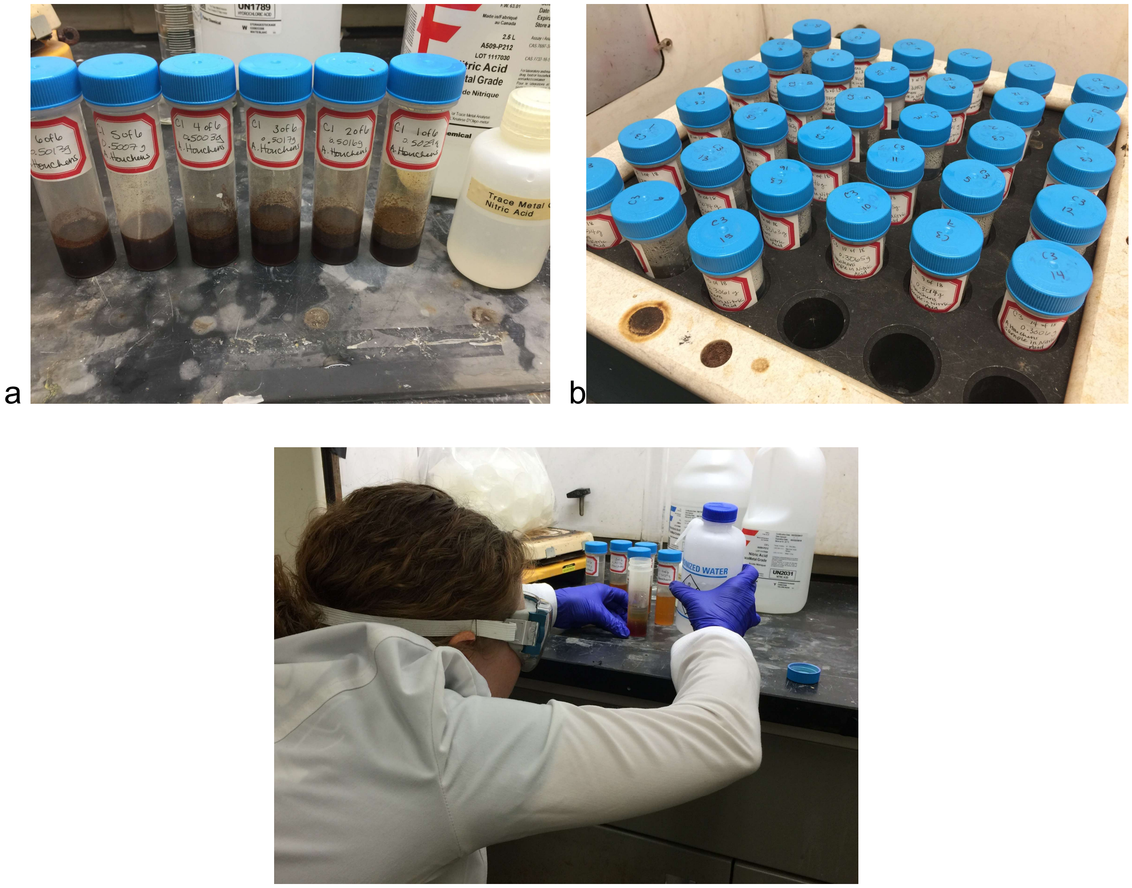

Figure 8: Preparation of guano samples for analysis: a) mixing with nitric acid, b) digesting in the HotBlock, and c) diluting with deionized water.

Domestic sludge (NIST 2781) was used as a standard for comparison for lead and cadmium concentrations using ICP-OES and was prepared for analysis by acid digestion as well. This standard is from the National Institute of Standards and Technology and is known to have a lead concentration of 200.8 ± 4.2 ppm and cadmium concentration of 12.78 ± 0.63 ppm (National Institute of Standards and Technology, 2018).

A 5 mL volume of hydrochloric acid, trace metal analysis concentrated, and 10 mL of nitric acid, trace metal analysis concentration, was added to approximately 0.500 g of standard in a 50 mL polypropylene digestion vessel. It was also heated at 85°C in a HotBlock but required 90 minutes of heating and an additional treatment of hydrogen peroxide to complete the digestion. Once cooled, the standard sample was diluted to a total volume of 50 mL using deionized water.

ICP-OES

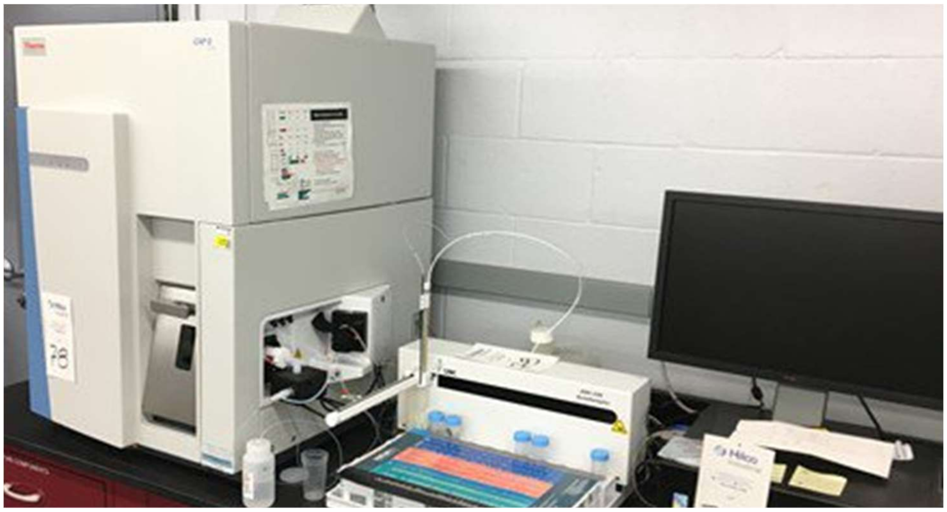

Figure 9: The iCAP 6000 series ICP spectrometer by Thermo Scientific, with ASX-520 Autosampler.

The bat guano samples were analyzed for cadmium and lead using the iCAP 6000 series ICP spectrometer, by Thermo Scientific. Additionally, the ASX-520 Autosampler was used throughout the analysis (Figure 9). The iTEVA software was used to control the unit during analysis and the method used was developed to utilize a sample flush time of 80 seconds with three repeats and the plasma viewer was set to axial. Three measurements were made for each metal concentration for each sample assessed and an average was recorded.

Lead and cadmium are not commonly analyzed in samples at the Advanced Materials Institute therefore the standard typically used to calibrate the ICP spectrometer did not contain lead or cadmium. To ensure quality control, lead and cadmium standards, ranging from 0.001 ppm to 1.000 ppm were made and used to calibrate the instrument before analysis.

To prepare samples for analysis, all samples and domestic sludge standards were centrifuged to remove any small particulate matter that might clog the tubing of the instrument. A 15 mL volume was then loaded into the autosampler for analysis for each sample.

3. RESULTS

The raw data collected for mercury, cadmium, and lead concentrations in the bat guano samples was scrutinized and graphed to demonstrate relationships between the metal concentrations. A positive correlation between each of the metals exists (cadmium and mercury, lead and mercury, and lead and cadmium) suggesting that determining the concentration of one metal in a guano sample would allow for the prediction of another metal in the guano sample.

The concentrations of mercury, cadmium, and lead were also investigated to see how each changed with varying depth of the guano sample. There were fluctuations in the concentrations of each metal of the three metals throughout the depth of the cores. These fluctuations varied similarly throughout the depth of each core.

Correlation of One Metal Concentration to Another Metal Concentration

The graphs in Figures 10, 11, and 12 illustrate the relationship between the three metal concentrations. The mound sampled was from an entire breeding colony comprised of bats of varying ages. Due to the biological nature of bat guano, a 15% error bar is included for all data points.

Cadmium Concentration versus Mercury Concentration

Figure 10 demonstrates that as the concentration of mercury increases in the guano, the concentration of cadmium also increases. Mercury concentrations range from 0.0755 ppm to 0.2572 ppm while cadmium concentrations range from 0.3617 ppm to 1.4354 ppm. The data points represent a linear relationship with most data points close to the trendline. The data does not contain clusters of points.

Statistical calculations from the graph in Figure 10 indicate there is a positive correlation value of 0.4896 between the concentrations of cadmium and mercury. Furthermore, the data collected allows for a high confidence in the prediction of cadmium concentration based on mercury concentration in a guano sample with a 0.0462 standard error of prediction from the data.

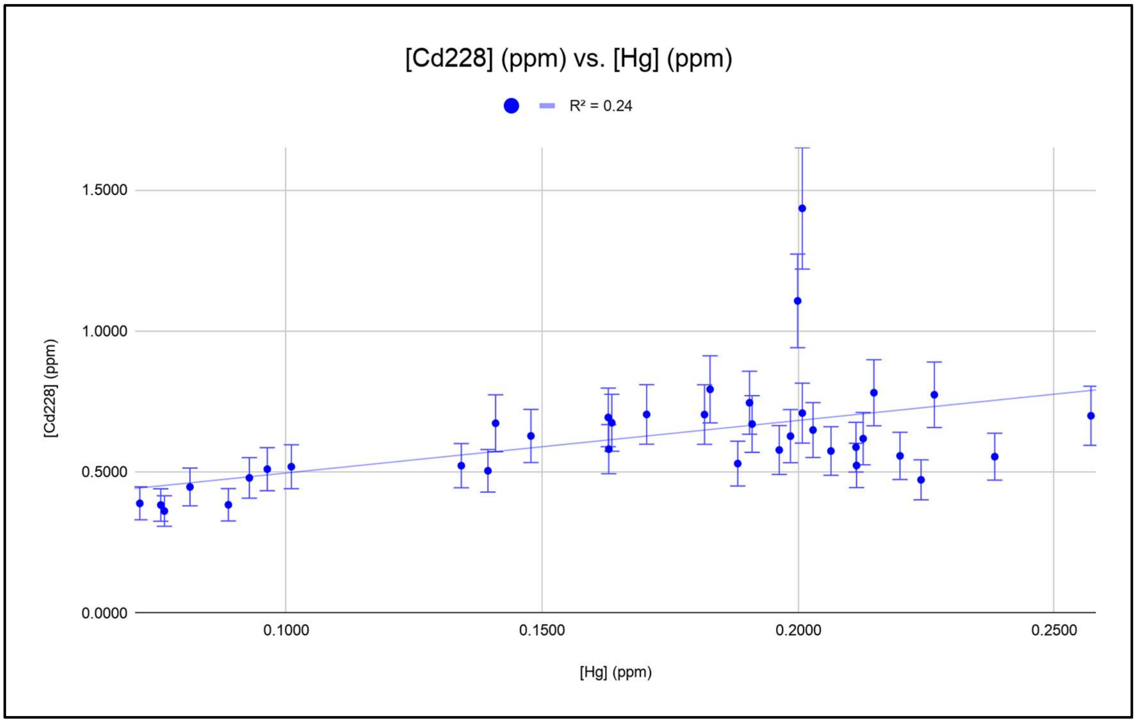

Figure 10: Concentration of cadmium vs. concentration of mercury in bat guano cores.

Lead Concentration versus Mercury Concentration

Figure 11 illustrates the concentration of mercury rises in a sample of guano in trend with the concentration of lead. Mercury concentrations range from 0.0755 ppm to 0.2572 ppm while lead concentrations range from 0.3880 ppm to 1.5260 ppm. A linear relationship exists between the variables and most data points are found close to the trendline. The data also appears to be evenly dispersed, forming no clusters.

Statistical calculations on the graph in Figure 11 determined a positive correlation value of 0.2552 between the concentrations of lead and mercury. Furthermore, the data collected results in a high confidence in the prediction of lead concentration based on mercury concentration in a guano sample due to a standard error of prediction of 0.0512.

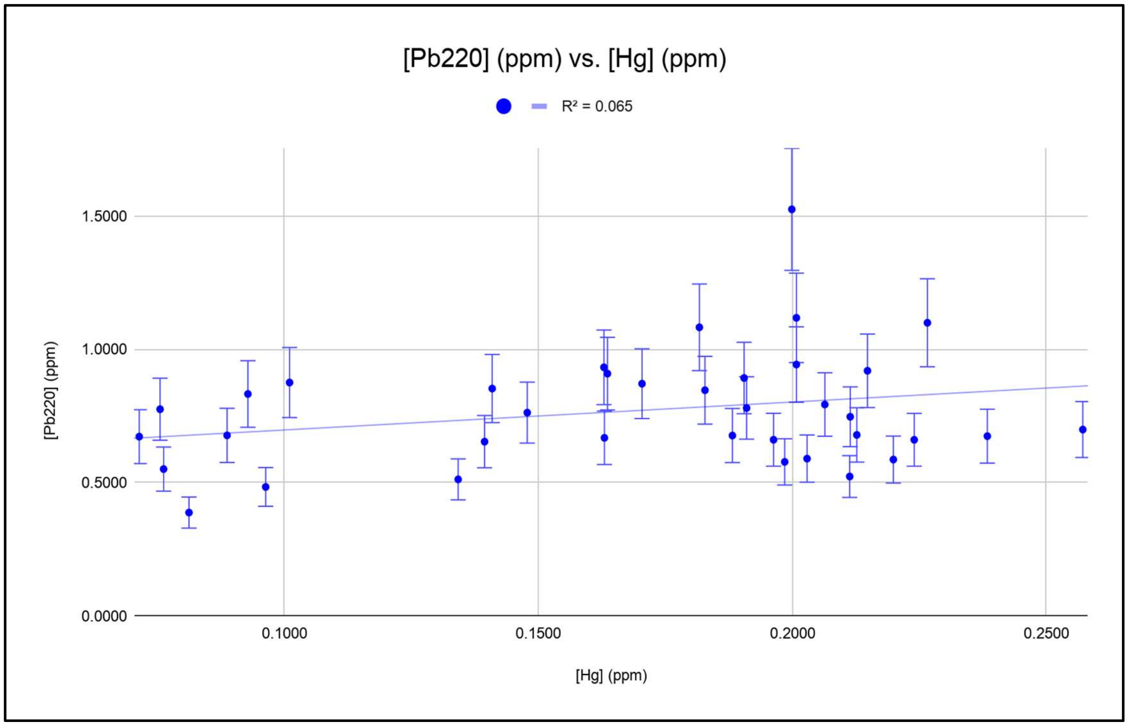

Figure 11: Concentration of lead vs. concentration of mercury in bat guano cores.

Lead Concentration versus Cadmium Concentration

Figure 12 indicates the concentration of cadmium in a sample of guano increases in trend with the concentration of lead. Cadmium concentrations range from 0.3617 ppm to 1.4354 ppm while the concentrations of lead range from 0.3880 ppm to 1.5260 ppm. Similar to the trends for cadmium and lead concentrations in trend with mercury concentrations, the data points revealed a linear relationship. Most data points lie close to the trendline illustrated on the graph and almost all data also appears to be evenly dispersed across the graph.

Statistical calculations show there is a positive correlation value of 0.7138 between the concentrations of lead and cadmium. Furthermore, the data collected allows for a high confidence in the prediction of lead concentration based on cadmium concentration in a guano sample with a 0.1523 standard error of prediction from the data.

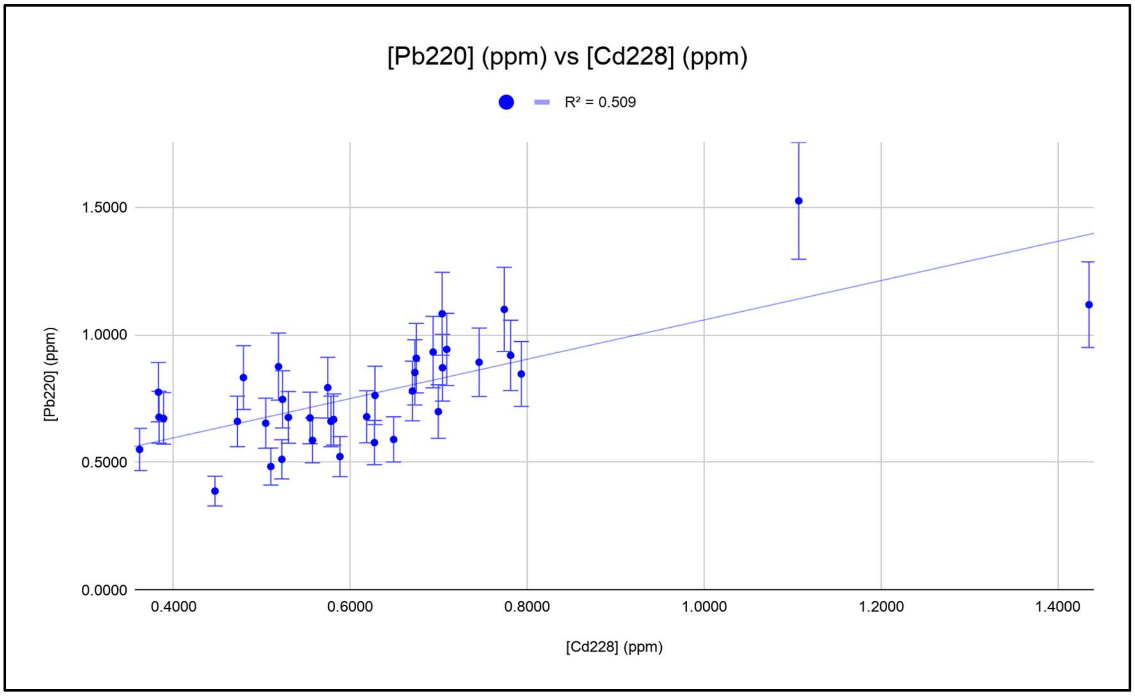

Figure 12: Concentration of lead vs. concentration of cadmium in bat guano cores.

Table 3 provides statistical validation for the prediction of one metal concentration from the concentration of another metal. A positive covariance value indicates that metal concentrations tend to change together in the same direction while the Pearson correlation, r, provides data on the linear relationship between the two variables being examined. The standard error of prediction is between 5% and 15% for all three relationships.

Also included in Table 3 is covariance data, which was previously unmentioned. This is due to the fact that a positive covariance only indicates that two variables are changing together, in the same direction. Due to the data having a positive covariance value, it validates the need for additional calculations to expose the correlation of the concentrations along with the standard error of prediction for the data.

Table 3: Correlation, standard error, and covariance data for the relationship of mercury, lead, and cadmium concentrations in the bat guano cores.

Metals Compared	Pearson Correlation (r)	Standard Error of Prediction (dependent from independent)	Covariance
Cadmium vs. Mercury	0.4896	0.0462	0.0049
Lead vs. Mercury	0.2552	0.0512	0.0028
Lead vs Cadmium	0.7138	0.1523	0.0296

The high correlation between lead and cadmium concentrations was expected, as lead and cadmium tend to have similar sources of contamination in the environment. Mercury is not typically observed as sharing a source of contamination (Walker, Simpson, Rockett, Weinburg, and Shore, 2007). Additionally, lead and cadmium tend to accumulate from local sources of contamination whereas mercury tends to accumulate from regional and global sources and become persistent in the environment.

General Comparison and Trends in Metal Concentration Correlation

Generally, observations and data support an increase in concentration of any of the three metals in the guano indicates that there is a high probability of a higher concentration of the other two metals. While the previous graphs appear to have outliers, when the data points are removed and statistical calculations are repeated, there were no statistically significant changes in the results. That is to say, there were no changes in the outcomes of the calculations outside of the anticipated range of error. Additionally, the points that appear to be outliers on two of the graphs originate from the same sample within the same core. Therefore, all data collected from the guano samples are included on the graphs and in the statistical calculations.

Table 4 provides the correlation values for each individual core and correlation data for all three cores combined. The only consistent correlation across all three cores is between lead and mercury concentrations. The correlation between cadmium and mercury and between lead and cadmium is not as close between all three cores. Despite the variance in the correlations between the three core samples, all indicate a positive correlation in each of the metal concentration relationships. This underscores the importance of using a large number of samples for data, especially when analyzing biological samples.

Table 4: Comparison of the correlation data for the relationship of mercury, lead, and cadmium concentrations in each of the bat guano cores.

Metals Compared	Core 1 (6 samples)	Core 2 (13 samples)	Core 3 (18 samples)	All Cores
Cadmium vs. Mercury	0.3514	0.4305	0.7032	0.4896
Lead vs. Mercury	0.2256	0.2769	0.2456	0.2552
Lead vs Cadmium	0.9855	0.8001	0.5988	0.7138

Correlation of Metal Concentration with Depth of Guano Mound

The graphs in Figures 13, 14, and 15 demonstrate how the concentration of each metal changes with depth along the three core samples. To better understand the graphs, a depth of 0 cm indicates top of the guano mound, or the most recent sample. An increasing depth indicates a sample deeper within the mound, or an older sample. A 15% error bar is also included on these graphs because of the expected natural variance of bat guano samples.

Mercury Concentration versus Depth of Guano Mound

Figure 13 depicts how the concentration of mercury changes along the depth of the guano cores. In this graph, data is included from all three cores. It is observed that the concentration of mercury increases with increasing depth of the guano mound for all three core samples taken. This indicates that the oldest guano samples, located at the bottom of the mound, would be expected to have the highest concentrations of mercury, while the most recently deposited guano has the lowest concentration.

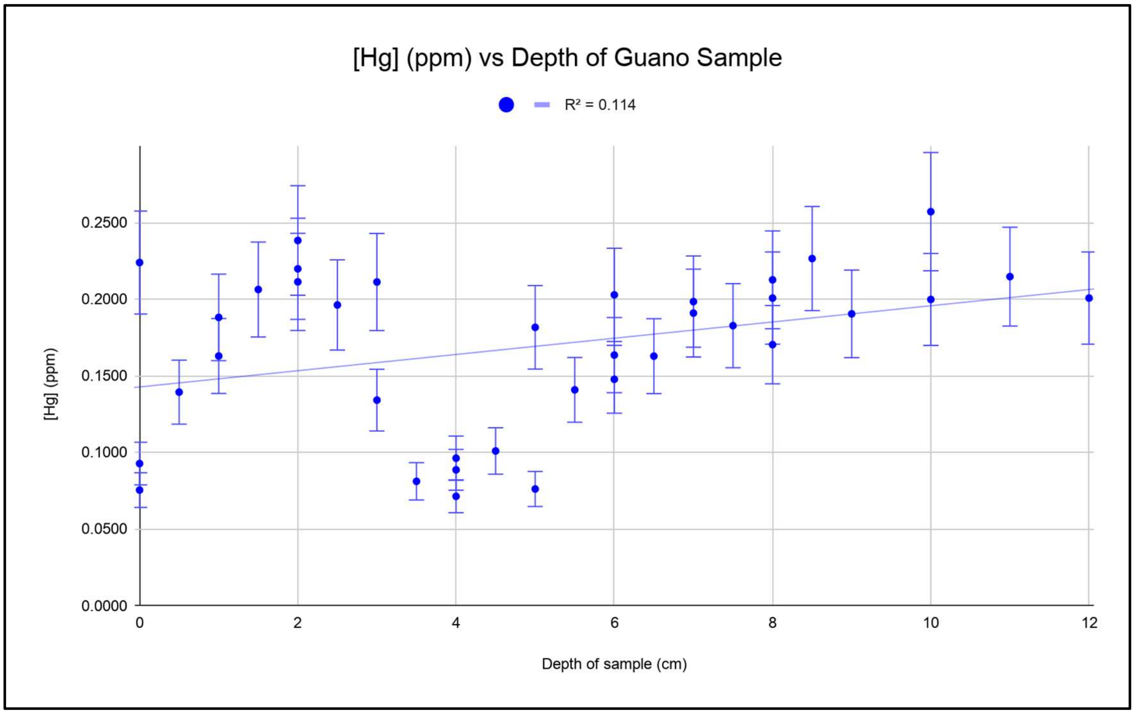

Figure 13: A line graph depicting the concentration of mercury vs. depth of the bat guano cores.

Cadmium Concentration versus Depth of Guano Mound

Figure 14 depicts how the concentration of cadmium changes along the depth of the guano cores. In this graph, data from all three cores is represented. It is observed that the concentration of cadmium increases with increasing depth of the guano mound for all three core samples taken. This implies that the oldest guano samples, located at the bottom of the mound, would be expected to have the highest concentrations of cadmium, while the most recently deposited guano has the lowest concentration.

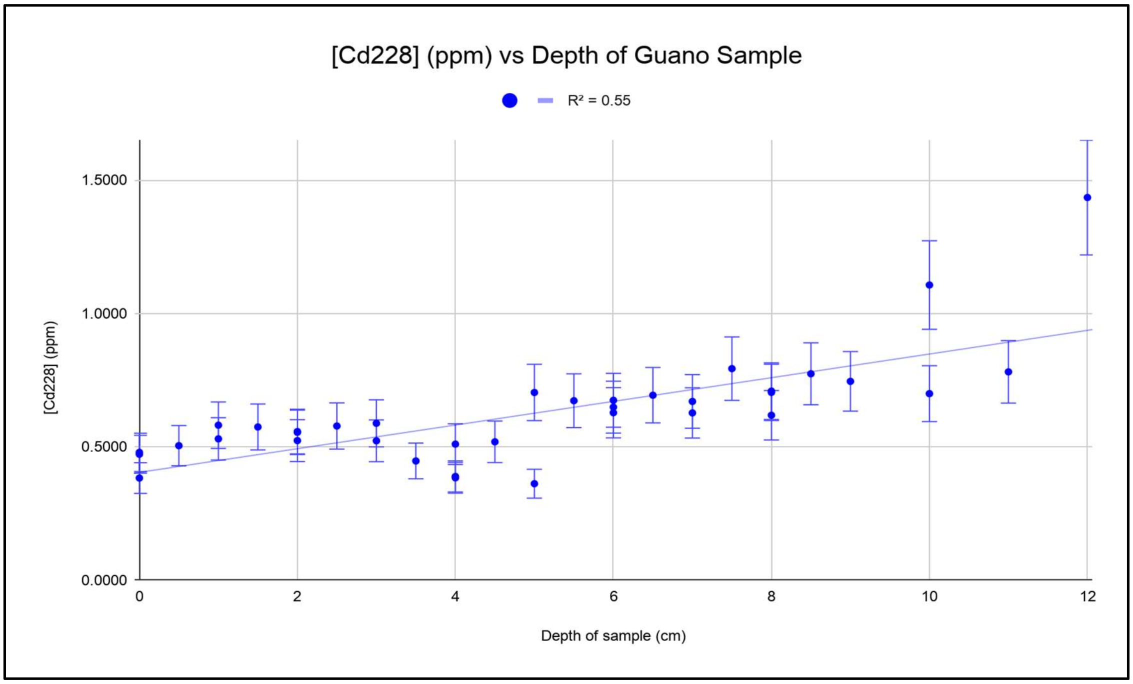

Figure 14: A line graph depicting the concentration of cadmium vs. depth of the bat guano cores.

Lead Concentration versus Depth of Guano Mound

Figure 15 represents how the concentration of lead changes along the depth of the guano cores. In this graph, data is visualized from all three cores. The graph reveals that the concentration of lead generally increases with increasing depth of the guano mound for all three core samples taken. This indicates that the oldest guano samples, located at the bottom of the mound, would be expected to have the highest concentrations of lead, while the most recently deposited guano has the lowest concentration.

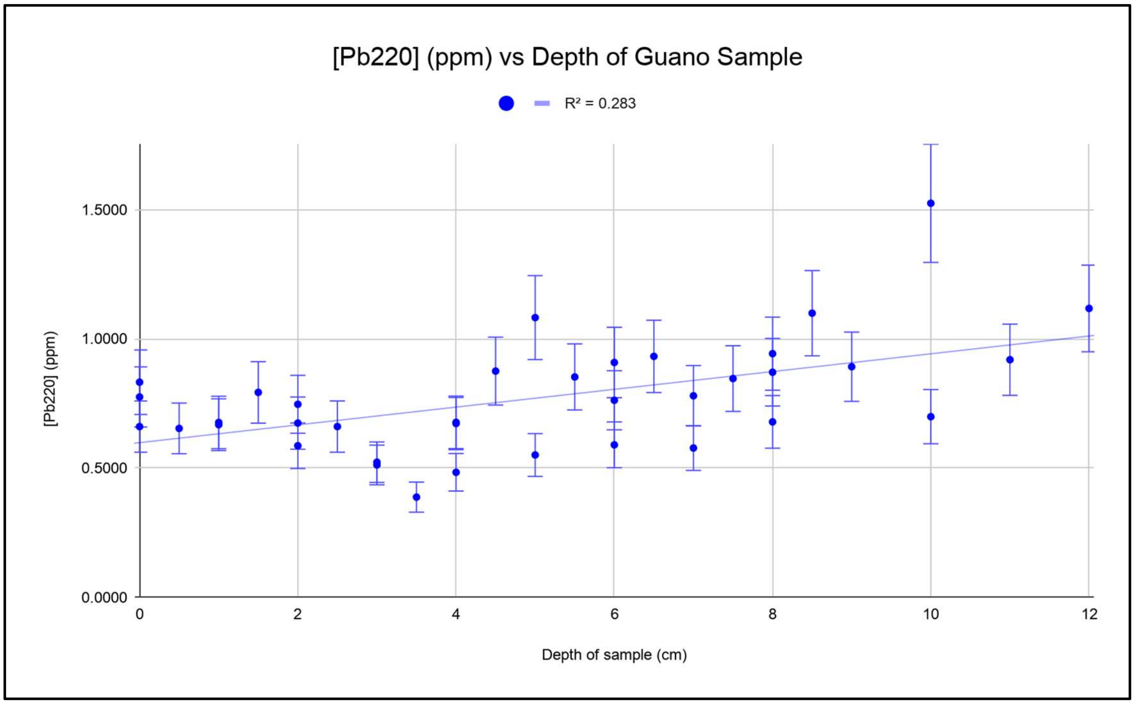

Figure 15: A line graph depicting the concentration of lead vs. depth of the bat guano cores.

General Comparison and Trends in Metal Concentration versus Depth of Guano Mound

When the three graphs represented in Figures 13, 14, and 15 are compared, it is evident that all three metals have some fluctuations. The graphs also seem to reveal some outliers. However, excluding these data points did not significantly change the outcomes of the correlation calculations within the anticipated range of error so they have not been excluded.

Table 5 gives Pearson correlation, standard error of prediction, and covariance data for the previous data sets, for the concentration of metal versus the depth of the guano cores. All three data sets yield a positive covariance, warranting a correlation calculation to judge the strength of the relationship between the variables.

Additionally, the standard error of prediction has been calculated. Mercury presents the lowest correlation value, but allows for a prediction with a fairly low error expected from predictions made from the data. Cadmium provides the highest correlation of concentration prediction from depth and also yields a relatively low error from the prediction, given the variance of a biological sample.

Table 5: Correlation, standard error, and covariance data for the relationship of mercury, lead, and cadmium concentrations with depth in the bat guano cores.

Metal	Pearson Correlation (r)	Standard Error of Prediction	Covariance with Depth
Mercury	0.3369	0.0499	0.0568
Cadmium	0.7416	0.1352	0.4755
Lead	0.5324	0.1841	0.3685

The metal concentrations all generally increased with increasing depth. Mercury had the weakest correlation with depth suggesting that most of the mercury in the guano was in its methylated form and bound in the guano matrix. It is possible, since lead and cadmium have a greater increase in concentration with depth in the guano mound, that the moisture in the sample allowed for a "trickle down" effect of those unbound metals.

To see if this trend was consistent for all three metals in each of the cores sampled, the raw data for each core was evaluated. Pearson correlation values were calculated for each metal as depth increased in each core. This data is located in Table 6 for comparison.

Table 6: Comparison of the correlation data for the relationship of depth and mercury, lead, and cadmium concentrations in each of the bat guano cores.

Metals	Core 1 (6 samples)	Core 2 (13 samples)	Core 3 (18 samples)	All Cores (37 samples)
Mercury	0.0833	0.4707	0.3044	0.3369
Cadmium	0.8350	0.6918	0.7743	0.7416
Lead	0.8503	0.5827	0.4402	0.5324

Cadmium is most consistent when the correlation values for each core are compared to the data for all three cores together. Lead was the next most consistent metal and mercury was the least consistent across all three cores. It is also notable that correlation values for Core 3 were close to the overall correlation values for the metal concentration and depth relationship. This is most likely due to the fact that Core 3 was divided into smaller samples, therefore providing more data points for the calculation. However, as guano is expected to have natural variance, this data also highlights the importance of a larger data set in order to draw conclusions.

Average Total Metal Concentrations in Guano Samples

Table 7 gives the average concentration for each metal in each core. Additionally, an average metal concentration for all three cores was calculated and the standard deviation was computed. The standard deviations range from 6.16% to 12.18% of the mean values. With such a small standard deviation for each of the three metal concentrations in the cores, it can be reasoned that the three core samples are representative of the whole guano mound. Furthermore, these small standard deviation values support the use of 15% error bars on the data in the line graphs used to represent the data.

Table 7: Average metal concentration in each bat guano core.

Core Sample	Mercury (ppm)	Cadmium (ppm)	Lead (ppm)
1	0.1814	0.6450	0.9109
2	0.1764	0.6656	0.7377
3	0.1600	0.5901	0.7476
Average of all cores	0.1726	0.6336	0.7987
Standard Deviation	0.0112	0.0390	0.0973

The persistence of these metals in bat guano is reason for concern as Milan determined that the presence of heavy metals in bats is proportional to concentrations of the metals in the environment (1990). Mansour, Soliman, and Soliman confirmed this again when they determined a correlation exists in heavy metal concentrations found in bat guano and liver and kidney tissues (2016). It is biologically important to make sure that a single source is analyzed for heavy metals, especially when studying endangered species like the gray bat.

Analysis of guano limits the stress placed on the bats and is the most minimally invasive of sample collection methods (Mulec, Covington, and

Walochnik, 2013). By collecting guano from a mound, it is possible to study a population of bats collectively. While there may be advantages to being able to link concentrations to an individual bat, it does present more stress on the bat.

Heavy metals like mercury, cadmium, and lead introduce stress on bat populations, especially endangered ones like the gray bats considered in this project. Since white-nose syndrome is present in bats in the general area of the cave where the guano samples were collected, care was taken to minimize stress on the bat colony. Care was taken to minimize noise in the cave, lighting was kept to a minimum, and lights were not aimed at the colony. Collection of guano also allows the researcher to get a wide snap-shot of a population while also decreasing risks to the individual person, like rabies or histoplasmosis.

4. CONCLUSIONS

This investigation set out to uncover if the concentration of heavy metals were related to one another in a biological sample. Specifically, core samples of bat guano from a guano mound belonging to a transient, breeding colony of migratory gray bats was experimentally investigated for mercury, cadmium, and lead concentrations. Data was reviewed to determine possible correlation between the concentrations of cadmium and mercury, lead and mercury, and lead and cadmium.

Concentrations of cadmium and lead can be forecast from the concentration of mercury due to a 0.4896 and 0.2552 correlation and 0.0462 and 0.0512 standard error of prediction, respectively.

The lower correlation for lead prediction can possibly be attributed to the fact that it can be more difficult to get a reading of lead concentration using inductively coupled plasma- optical emission spectroscopy (ICP-OES), when compared to determining the concentration of cadmium using the same instrumentation and methods. All standards and blanks analyzed alongside that samples fell within quality control ranges.

Furthermore, when concentrations of cadmium are known, concentrations of lead can also be predicted. The data yields a 0.7138 correlation with a 0.1523 standard error of prediction, supporting that there is a close relationship between the data.

While there are few sources of heavy metal pollution that all three metals have in common, for example batteries, all three metals are commonly used in industrial processes. This leads to the suggestion that areas of the world that have experienced significant levels of industrialization could expect to find the three metals in measurable concentrations in the environment. While the three are not released together in most industrial processes, it is common to find industries co-existing near each other that together, have the potential to release all three.

Mercury can be released by burning fossil fuels, through the electrical industry, and old paints. Cadmium can be introduced through the production of alloys and pigments, while lead is released via mining processes, the burning of fossil fuels, and through leaching into water and the ground via old pipes. Interstate 65 is a major north/ south highway that runs through Smiths Grove, Kentucky, where Crumps Cave is located. It is possible that the fossil fuels being burned as vehicles have passed and currently pass through the area have contributed to the significant relationship between the mercury and cadmium concentrations.

A shared source of cadmium and lead is the earth's crust, especially sedimentary rocks, which are present in karst areas like the one being evaluated in this investigation. Due to the karst environment and habitat of the gray bats being studied, it is likely that the environment is contributing to the lead and cadmium concentrations since the correlation between the two is high.

These findings are useful because a laboratory procedure to determine mercury content in a sample of guano can be completed in a matter of minutes using the Advanced Mercury Analyzer instrument. This time requirement is significantly shorter compared to the time needed to process and quantify a guano sample for cadmium or lead, since an acid digestion is also required in order to run a biological sample using the ICP-OES instrumentation.

One possible way to account for the fluctuation and variance of heavy metal concentration in the bat guano is related to the reproductive cycle of the gray bat. The gray bat is transient throughout the year depending on the season. Females store sperm over the winter and when they emerge from hibernation in the spring, they become pregnant. This occurs during late March and early April. Females then form maternity colonies and their pups are born in late May or early June.

The oldest guano (greater depth) comes from adult females who bring in metal contaminants from their previous location. Additionally, they are newly pregnant or become pregnant shortly after arrival. Prior research has established that heavy metals can cross the placental barrier so the guano from the females would be expected to experience a drop in heavy metal concentration in their guano. Once the pups are born, the female bats up their food intake to produce milk for their young, as the pups will not fly for approximately 20 days. Increasing food intake would account for an influx of heavy metal concentration.

Eventually, the pups emerge and will consume insects at rates similar to the adult females. This would lead one to expect an increase in concentration of heavy metals near the top of the mound. However, the weeks leading up to the collection of guano samples were especially wet and rainy, possibly diluting the metal concentrations in the environment and therefore the bat food sources.

This interpretation is based on the labeling of the Kentucky Department of Fish and Wildlife and USDA at the entrance of Crumps Cave that the cave is home to a breeding colony of Gray Bats. Future work could include sexing the bats to determine with certainty that this is a maternal roosting colony and not a bachelor colony during the summertime.

Heavy metal concentrations were also graphed and compared to each other compared to the depth from which each sample was taken in the cores. The concentrations not only displayed a uniform trend of increasing metal concentration with depth of the mound, the concentrations also followed a similar pattern of fluctuation.

One other possible interpretation and explanation for this data is changes in atmospheric chemistry due to changes in environmental regulations regarding disposal of certain wastes and emissions of industrial gases. Future research could include Cesium-154 dating to determine a more exact age of the bat guano from the mound and compare it to existing environmental regulations at the time.

5. BROADER IMPACT

The research presented here is important because three toxic metals that build up in the environment are being studied- mercury, lead, and cadmium. When these metals are present, they can work their way through the food chain which means humans are also at risk of their toxic effects. Mercury primarily affects the nervous system and can cause damage to the reproductive system. Lead exposure can cause anemia, weakness, kidney and brain damage, fertility issues in men and women, and could even lead to death. Cadmium can cause slowed growth, cause anemia, damage the kidneys and testicular tissue, and also causes high blood pressure. Mercury and lead can freely cross the placental barrier and cause miscarriages, stillbirths, and developmental delays, while cadmium is very limited in its ability to do so.

Bats, like humans, are at the top of their food chain. These metals bioaccumulate, which means they get more and more concentrated as they go up the chain, so the closer you are to the top the more concentrated the metals tend to get. By researching bats, one can get a good estimate of these toxic metals in the environment. The lab test that checks for the amount of mercury is quick and easy compared to the test for lead and cadmium. If a relationship can be established between the amount of mercury and the amounts of lead and cadmium, then one can predict the amounts of the other two based on the amount of mercury.

These findings could also be used by scientists researching birth defects in areas with pollution, behavioral scientists studying neurological incidents in areas polluted with metals, or incidents of organ failure in areas with high concentrations of these metals.

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