BONE MICROARCHITECTURE, KIDNEY HEAVY METAL AND METALLOTHIONEIN CONCENTRATIONS IN *PEROMYSCUS LEUCOPUS* FROM TAR CREEK SUPERFUND SITE

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Abstract: Tar Creek Superfund Site (TCSFS) is highly contaminated by toxic heavy metals. Cd, Pb, and Zn concentrations in soil and kidney specimens of *P. leucopus* were analyzed using inductively coupled plasma-mass spectroscopy (ICP-MS). The fourth lumbar vertebra (L_4) of *P. leucopus* was used to assess heavy metal effects on bone density and fragility using micro-computed tomography (µCT). Metallothionein (MT) was measured by using an Enzyme Linked Immune Sorbent Assay (ELISA). The results showed significantly higher concentrations of Pb, Cd, and Zn in TCSFS soil than in the reference sites (Oologah Wildlife Management Area [OWMA] and Sequoyah National Wildlife Refuge [SNWR]). The soil Pb concentrations at the three sites were 1132±278, 6.4±1.1, and 2.3 ±0.3 mg/kg, respectively. Concentrations of Cd were 48±4, 0.15 ±0.03, and 0.06±0.01 mg/kg. A similar pattern was seen for Pb and Cd concentrations in kidney specimens with TCSFS being higher than the reference sites. The kidney Pb concentrations were 0.57±0.10, 0.04±0.01, 0.05±0.01 mg/g, and Cd concentrations were 4.62±0.71, 0.53±0.08, and 0.53±0.06 mg/g for TCSFS and two reference sites. In addition, micro CT analysis of L_4 from TCSFS showed significant Pearson's correlation coefficients between Cd concentrations and trabecular bone number (-0.67, $p \leq 0.05$) and trabecular separation (0.72, $p \leq 0.05$).The results showed no correlation between bone parameters and mineral concentrations at reference sites. MT-1 concentrations in *P. leucopus* did not show statistically significant differences between TCSFS and reference sites ($P \geq 0.09$). In conclusion, this study showed significant heavy metal contamination in kidney of small mammals from TCSFS and confirmed previous studies. However, the study added new physiological endpoints for assessment of the effects of environmental contaminants on small wild mammals used as indicators for human health.

TABLE OF CONTENTS

Chapter Page

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

Environmental science as a multidisciplinary field integrates physical, biological and information sciences that address environmental problems and find solutions to these problems. This project studied the toxicological effects of heavy metals and physiological alterations in a wild animal species (*Peromyscous leucopus)* captured from the contaminated Tar Creek Superfund Site (TCSFS) and other uncontaminated sites. The study combined several scientific disciplines that are concerned with toxic effects of heavy metals on cellular and bone physiological responses in humans. A wild small mammal species was an appropriate model to determine the effects of heavy metals as an environmental problem.

TCSFS is a part of the Tri-state Mining District which includes Kansas, Missouri, and Oklahoma (Drake, 1999; ODEQ, 2003 ; United States Environmental Protection Agency USEPA, 2005). Environmental health issues are documented at TCSFS in Ottawa County more than at other superfund sites in the Tri-state mining district due to the large area and large human population, the large volume of mine tailings, and high levels of cadmium (Cd), lead (Pb), and zinc (Zn) (Health & Human Services, 1993; Neuberger, Hu, Drake, & Jim, 2009; Ostrowski et al., 1999).

There are many reasons to choose TCSFS for this study. Firstly, according to the National Priorities List of the U.S. Environmental Protection Agency (EPA), TCSFS was heavily mined for lead and zinc from the 1890s to 1970. This site is highly contaminated with lead, cadmium and zinc (United States Environmental Protection Agency USEPA, 2005). Secondly, there is public and scientific concern regarding the presence of these environmental contaminants in soils, plants, and water. Thirdly, TCSFS provides a good field location to study how toxicity of heavy metal contaminants affects human health and biological systems and how physiological processes can tolerate these contaminations.

The main goal of this study was to determine the concentrations of cadmium, lead and zinc in soil samples and kidney specimens of *Peromyscous leucopus* collected from Beaver Creek, TCSFS and two uncontaminated sites - Sequoyah National Wildlife Refuge (SNWR) and Oologah Wildlife Management Area (OWMA) (Hays, 2010; K. L. Phelps, 2006). The Cd, Pb, and Zn soil concentration in the contaminated site and in two reference (uncontaminated) sites are presented in chapter IV of this dissertation. Another goal presented in chapter IV was to determine relationships between tissue heavy metal concentrations and density and fragility of lumbar vertebra in *P. leucopus.* Correlations between mineral concentrations in kidney specimens with bone parameters are presented in chapter IV.

In chapter V, heavy metal concentrations in kidney specimens were related to differences in metallothionein concentrations in kidney specimens of *P. leucopus* from contaminated and uncontaminated sites. The relationship between observed metallothionein induction and metal toxicity showed how *P. leucopus* responded to

environmental contamination. Their response can be compared to metallothionein induction caused by environmental contamination in the Old World species, *Apodemus sylvaticus* (Fritsch et al., 2010). This study compared the relationships among soil metal concentrations, tissue metal concentrations, bone fragility, and metallothionein-1 induction of *P. leucopus* in contaminated and uncontaminated sites. This project aimed to understand the environmental contamination effects on the detoxification function in wildlife populations and extrapolate to humans. The project produced new information about how the population of *P. leucopus* at TCSFS responds to chronic exposure to heavy metals in soil. Metallothionein induction and bone microarchitectural features are the biomarkers that were used to identify the physiological functions in this species.

From previous studies at TCSFS, we know that *P. leucopus* is able to maintain higher population levels at this highly contaminated site compared to other common rodent species (Phelps & McBee, 2009). We also know that *P. leucopus* collected from TCSFS showed no significant differences in mean minimum longevity or reproductive success compared to *P. leucopus* at the uncontaminated sites SNWR and OWMA (Phelps & McBee, 2010). This suggests that *P. leucopus* might demonstrate more resilience to heavy metal contaminants or may have enhanced detoxification capabilities compared to other species. This study provides evidence of tissue mineral residue levels of the primary contaminants at TCSFS and allowed me to determine if animals from TCSFS had increased MT-1 in comparison with animals from uncontaminated sites. My research has broad implications because responses to chronic exposure to heavy metals in *P. leucopus* may help predict impacts of such exposure on physiological processes of humans and other species. Although MT analysis has been conducted on numerous vertebrate and

invertebrate aquatic species, there has been little work done on metallothioneins in small mammals from contaminated sites in North America.

This study opened more questions about metallothionein functions in humans and small mammals in contaminated sites. It may provide a basis for another project to resolve and reduce Pb and Cd exposure at TCSFS and other contaminated sites in the United States. Moreover, this study will add information to investigate the effects of Pb and Cd on different functions in humans and other mammalian species.

CHAPTER II

REVIEW OF LITERATURE

We are regularly exposed to metals through food, air and skin contact from many environmental sources. Heavy metals have toxic effects on biological cycles (Glanze, 1996). Although we need a small amount of several metals for biological functions, large amounts or long exposure to any metal may be detrimental for health. In general, there are 35 metals, called heavy metals, that are of concern for human health. Common health problems that arise from exposure to heavy metals can be chronic or acute depending on the length of exposure and the dose.

Health problems that can result from heavy metal toxicity include reduced function of the nervous system, alteration of blood cell counts, circulatory and respiratory problems, liver dysfunction, and reproductive and renal problems. Some kinds of chronic diseases such as Alzheimer's, Parkinson's, muscular dystrophy, multiple sclerosis, osteoporosis, and some kinds of cancers may be related to long-term exposure (Casarett, Doull, & Klaassen, 2008).

Heavy metal in biological systems affects humans as well as domestic and wild animals (Mollazadeh, Esmaili, & Ghasempouri, 2011; Rajaganapathy, Xavier, Sreekumar, & Mandal, 2011). Studying bioindicator animals, such as the wild mice observed in this study, helps us to understand the effects of environmental pollution on human health.

Description of Tar Creek Superfund Site (TCSFS)

According to the National Priorities List of the U.S. Environmental Protection Agency, the TCSFS was heavily mined for lead and zinc from the 1890s to 1970. As a result, this site is highly contaminated with lead, zinc, and cadmium (United States Environmental Protection Agency USEPA, 2005). Consequently, there is public and scientific concern about the presence of these environmental contaminants in soil, plants, and water. TCSFS provides an ideal field location to study how toxicity of heavy metal contaminants affects biological systems. TCSFS, located around the towns of Picher, Quapaw, and Miami in Ottawa County, Oklahoma, covers 104 km^2 and is part of the historical Tri-State Mining District. The Tri-State Mining District (northeastern Oklahoma, southeastern Kansas and southwestern Missouri) covers 3,000 km^2 and includes parts of Ottawa County, OK, Cherokee County, KS and Jasper and Newton counties, MO (Gibson, 1972). The area was mined for sulfide forms of Pb (galena), Pb carbonate, Pb phosphate (pyromorphite), Zn carbonate (smithsonite) and other plentiful minerals for more than 80 years.

This mining and smelting produced several sources of contamination (Beyer et al., 2005). Both waste metals and rocky waste called chat were produced from milled ore (Oklahoma Department of Environmental Quality, 2003).

A study by the Environmental Protection Agency (EPA) found that chert is the main component of chat, and the siliceous rock mined with the ore chat samples from TCSFS contained high concentrations of Pb, 270-732 mg, Cd, 41-57 mg and Zn, 8,266-11,086 mg per kg of chat (United States Environmental Protection Agency USEPA, 2005). Another study found that Tar Creek itself is contaminated with water from mines; samples of surface sediments in Tar Creek contained 8,700 mg of Pb, 130 mg of Cd and 35,000 mg of Zn per kg (Parkhurst, 1988). Soil samples are good indicators to determine levels of lead, cadmium, and zinc contamination at TCSFS.

Toxic effects of Cd, Pb, and Zn

Cadmium toxicity

One of the common metals at TCSFS is cadmium. Small amounts of free cadmium ions are more toxic than bound cadmium ions, and cadmium can cause toxicity in different organs including the pancreas, testis, and nervous system. Elimination of cadmium by the kidneys is slow. Chronic exposures to cadmium ions can result in proteinuria and tubular dysfunction in the proximal tubules (Chang, Magos, & Suzuki, 1996; Godt et al., 2006). Renal toxicity from cadmium exposure is correlated with the amount of cadmium ions in kidney tubule cells, reabsorption, degradation of cadmium metallothionein complexes, and excess production of metallothionein by renal tubules (Chang et al., 1996). Cadmium toxicity observed in the renal cortex of laboratory rats (*Rattus rattus*) resulted in cytosolic damage and renal malfunction after oral administration of cadmium chloride ($CdCl_2$) (Siddiqui, 2010). Cadmium ions can bind to mitochondria, endoplasmic reticulum, and nuclei of the liver, causing hepatic toxicity (Chang et al., 1996). Cadmium ions have limited capacity to pass the blood-brain barrier,

but exposure to cadmium can cause central nervous system (CNS) lesions in neonates and peripheral nervous system (PNS) lesions in adults (Chang et al., 1996). In blood, cadmium is bound with albumin and other plasma proteins (Chang et al., 1996).

The main effects of cadmium on the liver include hepatic failure, increased levels of plasma enzymes (asparatate amino transferase and sorbitol dehydrogenase), cytoplasmic eosinophilia, necrosis, and histological changes (Goering & Klaassen, 1984). Several studies demonstrated that cadmium exposure causes carcinogenic effects in humans including lung cancer, prostatic cancer, renal and hepatic cancer, and pancreatic tumors (Chang et al., 1996; Godt et al., 2006). Low doses of cadmium can stimulate DNA replication (Lohmann & Beyersmann, 1994) and may cause genotoxicity with spontaneously occurring DNA damage; however, high doses of cadmium cause direct damage to DNA and disrupt genetic material (Chang et al., 1996; Godt et al., 2006; Usuda et al., 2010). The cadmium chloride ion has been shown to induce sub-chronic effects on guinea pigs that result in immune toxicity expressed as T- and B-cell suppression in the lymphoid organ (Boroskova & Dvoroznakova, 1997). Overall, these studies indicate that Cd causes different effects at different levels such as cellular and tissue levels.

Lead toxicity

TCSFS is highly contaminated by lead. Lead has no known role in the normal physiological function of animals and humans, and it causes toxic effects on many different systems and processes (Chang et al., 1996; Goyer, 1990). Lead impacts development, IQ, and behavior in children at low levels of exposure (Chang et al., 1996; Schwartz, 1994).

Lead also may cause hypertension and neurotoxic effects in adult humans by influencing neurotransmitters and ion channels through calcium-sodium ATP pump processes that affect cellular energy production (Chang et al., 1996). Chang et al. (1996) found that lead damages myelin sheaths of nerves and disrupts electrochemical impulse transmission. Furthermore, it causes necrosis of small capillaries in the brain that can lead to hemorrhage, vaculation, necrotic foci, and edema; it damages the prefrontal cortex, cerebellum, and hippocampus on molecular, cellular, and intracellular levels in the human nervous system. Biomolecular studies have shown that lead has toxic effects on gene expression, signal transduction, and calcium's function as a messenger system (Chang et al., 1996; Goyer, 1990). In two studies on lead ion, one focused on exposure to fumes during metal utilization by battery and pigment plant workers (Fu & Boffetta, 1995) and another focused on plumbers (Chang et al., 1996). Stomach, lung, bladder and kidney cancer are recorded in workers who are heavily exposed to lead from tobacco smoking and occupational carcinogens (Fu & Boffetta, 1995). Lead toxicity causes carcinogenic and genotoxic effects in lung, kidney, and stomach tissues (Chang et al. (1996).

Similar damaging effects were found in small mammals. For example, lead-induced kidney neoplasia was observed in mice after cutaneous exposure to lead naphthenate even though there were no skin tumors in the mice (Baldwin, Cunningham, & Pratt, 1964). Although some studies did not show significant mutagenic effects of lead, Amacher and co-workers determined a weak induction of mutation in mouse lymphoma cells caused by lead ions (Amacher, Paillet, & Zelljadt, 1982). Also, Razani-Boroujerdi, Edwards, and Sopori (1999) showed that only high-dose exposure to lead acetate caused

immunotoxic effects and induced lymphocyte proliferation in cultured rat spleen cells. Multiple effects of lead were observed in laboratory animals including biochemical, structural, and molecular changes in hepatic cells, liver hyperplasia (cell proliferation), and oxidative stress (Mudipalli, 2007).

Zinc toxicity

Zinc is another metal that is common in TCSFS. Zinc is an essential metal for humans and animals. Zinc toxicity is uncommon, but there is some experimental evidence that zinc overexposure has carcinogenic effects (Walsh, Sandstead, Prasad, Newberne, & Fraker, 1994).

High zinc and copper concentrations in soil and vegetables were recorded in 12 districts of England and Wales, and these records correlated with lethal gastric cancer in residents who lived in that area (Stocks & Davies, 1964). Zinc salt exposure can induce mutagenic effects in humans (Chang et al., 1996; Stocks & Davies, 1964; Walsh et al., 1994). The zinc ion may cause lymphocyte proliferation (Chang et al., 1996; Walsh et al., 1994). Even with short exposure, zinc can cause a disease called metal fume fever through inhalation of zinc fumes (Roney, Smith, Williams, Osier, & Paikoff, 2005). Ingestion of high amounts of zinc may inhibit copper absorption by the intestine through competition for metallothionein binding in the mucosal cells. This inhibition can cause copper excretion, and decreases copper in plasma (Roney et al., 2005).

Toxic effects on humans at Tar Creek Superfund Site

Increased rates of human mortality and health problems, such as stroke and heart disease, have been reported at TCSFS associated with the exposure to heavy metals in the area (Neuberger et al., 2009). The Center for Disease Control and Prevention's (CDC)

advisory committee on childhood lead poisoning prevention detected blood lead levels (BLLs) >10 µg/dL in children at TCSFS. As a result of lead contamination in Ottawa County, 40% of children in this area had high blood lead levels. The CDC reported that children's mental and physical ability may be affected even by BLLs less than 10µg/dL. The Indian Health Service reported that 34% of 192 Native American children from TCSFS had BLLs in excess of 10 µg/dL, and 15% had higher than 20 µg/dL BLL (United States Environmental Protection Agency USEPA, 1994). Polluted water and soil were identified as sources of the high Pb and Zn. The exposure to Pb and Zn affected blood parameters as well as the nervous system and emotional of pregnant women, and intellectual development of infants and children in the TCSFS area (Hu, Shine, & Wright, 2007).

According to the Occupational Safety and Health Administration (OSHA), short term (acute) overexposure to lead can affect the brain and can cause seizures, coma and death (Occupational Safety & Health Administration, 1991). The acute exposure to lead can cause acute encephalopathy and several other health problems in a short time of exposure (Occupational Safety & Health Administration, 1991). OSHA is concerned with workers who are more exposed to lead, and it aims to protect people and remove those who had more than 50 µg/dL of blood lead from the workplace. Long term (chronic) exposure to lead causes severe health problems such as anxiety, nausea, insomnia, weakness, headache, nerve irritation, numbness, and muscle pain (Occupational Safety & Health Administration, 1991). Chronic exposure leads to kidney disease, reproductive system diseases and disrupted blood cell synthesis causing anemia and weakness (Occupational Safety & Health Administration, 1991).

Industrial activities are the main sources of lead exposure. OSHA (2010) recorded elevation of blood lead in 804,000 workers in general industries and 838,000 workers in construction. Workers were exposed to lead through inhalation and ingestion during such work activities as transportation, maintenance, construction, painting and recycling materials. OSHA has developed a safety program to protect workers from lead exposure in accordance with Code 29 of Federal Regulations (CFR). Industrial employers are required to follow OSHA lead standards in general industry (29 CFR 1910.1025), construction (29 CFR 1926.62), and shipyards (29 CFR 1915.1025) to protect workers (OSHA, 2010).

Industrial workers also are potentially exposed to cadmium through inhalation. The Agency of Toxic Substances and Diseases Registry (ATSDR) recorded that 500,000 workers are exposed to cadmium annually in the United States (OSHA, 2010). Long term exposure to cadmium can cause severe health problems such as lung and prostate cancer, as well as kidney disease and dysfunction. Employers and workers who may be exposed to cadmium are required to receive training before being involved in the industrial works to understand safety and protection limits against cadmium exposure (OSHA, 2010).

In addition to the EPA, the Agency for Toxic Substances, Disease Registry and OSHA expressed their concerns about the presence of zinc in the environment and its effects on human health (Roney et al., 2005). Food and water that contain amounts of zinc higher than the Recommended Dietary Allowances (RDAs), tolerable upper intake level (UL) of 40 mg/day for adults can cause severe health problems such as vomiting, nausea, anemia, pancreatic damage, and decreased high density lipoprotein levels (HDL) (Roney et al., 2005). OSHA states permissible exposure level limit (PEL) for airborne

zinc oxide fumes as 5 mg/m^3, 15 mg/m^3 for total dust, and 5mg/m^3 for respirable dust in eight hours of exposure (OSHA, 2012).

Heavy metal effects on wildlife at TCSFS

Studies at TCSFS have also reported the toxic effects of heavy metals on wildlife species. Human health and environmental risks have been estimated in the Tri-State Mining District by measuring concentrations of Pb, Cd, and Zn in crayfish (*Orconectes spp*.) and six species of fish frequently consumed by Native Americans living in the Tri-State Mining District - common carp *(Cyprinus carpio)*, channel catfish *(Ictalurus punctatus*), flathead catfish *(Pylodictis olivaris)*, largemouth bass (*Micropterus salmoides)*, spotted bass, (*M. punctulatus*) and white crappie (*Pomoxis annularis)* (Schmitt, Brumbaugh, Linder, & Hinck, 2006). Fish and crayfish samples were collected from the Spring River and Neosho River downstream from the Tar Creek drainage in northeastern Oklahoma and from a contaminated site in eastern Missouri. The samples were compared with reference fish including pond-raised largemouth bass and commercially raised channel catfish. The results showed high concentrations of Pb, Cd, and Zn in fish and crayfish from the Neosho and Spring rivers that were assumed to be from historical mining in the Tri-State Mining District (Schmitt et al., 2006)

Roark and Brown (1996) analyzed the genetic effects of Zn and Pb on three species of fish. The mosquito fish *(Gambusia affinis*), Bluntnose minnow (*Pimephales notatus)*, and blackstripe topminnow (*Fundulus notatus)* were compared between Willow Creek (contaminated) and Brush Creek (reference) in the Tri-State Mining District in Kansas. The genetic structure and levels of variation within species were not different between the two communities of small fish sampled, showing no evidence of a selective

effect of zinc or lead on allozymic distributions. Additionally, experiments showed a difference in allozymic sensitivity to zinc for glucose phosphate isomerase (Gpi) and phosphoglucose dehydrogenase (Pgdh) loci but not for maltase dehydrogenase (Mdh). There was no apparent allozymic sensitivity to lead concentration.

In contrast, Hays and McBee (2007) found significant differences in blood cadmium and lead levels in red-eared sliders (*Trachemys scripta*) collected at TCSFS as compared to turtles sampled at Sequoyah National Wildlife Refuge (SNWR). They found no statistical difference in coefficients of variation for nuclear DNA content between turtles from TCSFS and SNWR, but did find a significantly higher frequency of aneuploidy in turtles from TCSFS compared to reference sites. Pb concentrations in tissues of American robins (*Turdus migratorius*), northern cardinals (*Cardinalis cardinalis*), and several species of waterfowl from TCSFS were higher in comparison with Pb concentrations in reference birds (Beyer et al., 2005). Also delta-aminolevulinic dehydrogenase (ALAD) activity in red blood cells of birds from TCSFS was less than 50% of levels in reference birds (Beyer et al., 2005).

Hays (2010) used amplified fragment length polymorphism (AFLP) analysis to determine differences in heterozygosity and population genetic structure of *P. leucopus* collected from two contaminated sites within TCSFS and six reference sites in Oklahoma. Although animals from TCSFS clustered separately from some reference sites, geographic separation has explained more of the observed variation than whether animals were from contaminated or uncontaminated sites. Metaphase chromosome analysis in *P. leucopus* from TCSFS and reference sites showed no significant elevation in frequency of chromosomal aberrations in animals from Beaver Creek, OK at TCSFS

(Hays, 2010). Coolon, Jones, Narayanan, and Wisely (2010) used sequence data to compare microbial communities in soil and in feces collected from deer mice (*Peromyscus maniculatus*) and *P. leucopus* trapped within the Tri-State Mining District. They found no difference in community diversity indices for feces or soil microbial communities, but samples showed altered abundances of intestinal microbes in relation to soil contamination. This finding indicates that polluted soil increases microbial infection in these two species. Also, body mass and the amount of fat in *P. maniculatus* and *P. leucopus* were reduced in comparison with the community structure of small mammals from a reference site (Coolon et al., 2010).

Environmental contaminants may affect population size. K. L. Phelps and K. McBee (2010) studied the community structure of small mammals at a mining area and an ore processing area within TCSFS and compared that structure with the community structure at two uncontaminated sites to investigate ecotoxicological effects on community composition and population size at TCSFS. The population diversity of small mammals was less at TCSFS (Phelps & McBee, 2010).

Bone tissue

Bone is a supportive connective tissue consisting of osteoclasts, osteoblasts, and cellular matrix. The hard tissue of bone consists of collagen fibers and essential minerals including calcium, magnesium, and phosphate ions (Bilezikian, Raisz, & Rodan, 2002). Minerals deposited on flexible collagen make bone a hard tissue. The mineralized osseous tissue gives bone a three-dimensional internal structure, and this hard tissue gives bone rigidity. Bone also has soft tissues including marrow, endosteum, periosteum, nerves, blood vessels and cartilage. Bone tissue is divided into two major types: the

compact bone and the hard spongy bone tissues. The rigid tissue of bone constitutes part of the endoskeleton of vertebrates (Bilezikian et al., 2002).

Physiologically, bone's main functions are protection, support, movements, and transduction. Bone protects most organs of the body such as the brain, heart, lungs, and gut tissues. Bone marrow performs a significant function in the circulatory system: it produces red blood cells (RBC) and white blood cells (WBC) as well as platelets, and stores minerals (Bilezikian et al., 2002). In addition, bone moves skeletal muscles, tendons, ligaments and joints. Moreover, bone is an active tissue that is involved in metabolic processes for bone mineralization, osteoblast formation, and resorption (Youness, Mohammed, & Morsy, 2012).

The bone resorption process is enhanced by reactive oxygen species (ROS) which are released by osteoclasts through oxidative-reductive processes (Youness et al., 2012).

The excessive accumulation of ROS inhibits and suppresses bone formation and enhances bone resorption (Youness et al., 2012). ROS accumulation and antioxidant defense affect bone strength and metabolism (Youness et al., 2012).

Youness et al. (2012) investigated the effects of cadmium on thirty female Sprague Dawley rats (*Rattus norvegicus*). The rats, 3 months old, ingested 50 mg Cd/L as $CdCl_2$ in drinking water for three months. Serum samples were used to analyze concentrations of calcium, phosphorus, parathyroid hormone (PTH), 1,25 dihydroxy Vitamin D_3, osteocalcin, as well as alkaline phosphate activity. The results in this study showed significant elevation in serum phosphate, Ca, and PTH concentrations, and this

elevation was associated with significant reduction in osteocalcin, vitamin D_3 concentrations, and bone alkaline phosphatase activity (Youness et al., 2012).

Markers of oxidative stress and bone mineral density were investigated in postmenopausal women 60-78 years old. Serum samples were taken to analyze 8-hydroxy-2`-deoxyguanosine (8-OHdg) levels (Baek et al., 2010). The results showed that bone disease and fracture were enhanced by oxidative stress that resulted from the imbalance between the oxidative and antioxidative processes and bone mineral density (BMD) (Baek et al., 2010). Bone possesses metabolic processes, a mineralized bone matrix, and stores growth factors such as insulin-like growth factors. Also, bone transforms growth factor, morphogenetic proteins, and acts to buffer blood pH by balancing both absorbing and releasing alkaline salts (Steele & Bramblett, 1988).

Moreover, bone is involved in detoxification in multiple ways. Bone tissue stores heavy metals and other foreign elements, and the body has the ability to remove these elements from the blood stream and reduce the risk from these elements to other tissues by excreting them via excretion processes. Finally, bone regulates phosphate metabolism by releasing fibroblast growth factor –23 (FGF-23), which reduces phosphate reabsorption in kidneys (Bilezikian et al., 2002).

Microarchitecture effects of bone tissue

Cadmium effects on bone formation and resorption

Cadmium toxicity is one of the environmental problems that affects human health. The risks of cadmium toxicity include bone dysfunction and osteoporosis (Youness et al., 2012). Cadmium toxicity can cause a decrease in bone formation and an increase in bone resorption which are associated with imbalance in osteoblasts and osteoclasts (Åkesson et

al., 2006). Cadmium is an oxidant agent that produces ROS such as hydrogen peroxide, hydroxyl radicals, and superoxide ions that are involved in organ damage and oxidative stress. The organs damaged the most by cadmium include bones, liver, kidney, brain and testes (Chang et al., 1996; Ognjanović et al., 2010). Cadmium exposure disrupts enzymatic and non-enzymatic activity which inhibits the antioxidative enzymes and decreases non-enzymatic antioxidant activity (Ognjanović et al., 2010). The other effects of cadmium exposure include the depletion in bone mineral density (BMD) and acceleration of bone fracture (Järup & Åkesson, 2009).The mechanism of cadmium toxicity on bone can reduce renal function, Vitamin D activation, Ca absorption, and bone mineralization (Järup, 2002). Renal dysfunction results from cadmium exposure which decreases structure as well as function of renal proximal tubules such as mitochondrial and 1,25 $(OH)_2$ D_3 biosynthesis (Youness et al., 2012). Youness et al. recorded significant elevation of serum calcium, phosphorous, and parathyroid hormone levels of female laboratory rats (Sprague Dawley), which were administrated $CdCl_2$. Significant depletion in vitamin D, osteocalcin, and alkaline phosphate levels were recorded.

Moreover, cadmium toxicity affects bone metabolism by disrupting hydroxyapatite formation (Uriu et al., 2000). Disrupted bone metabolism impacts calcium metabolism and bone calcification (bone formation) (Brzóska & Moniuszko-Jakoniuk, 2005). Cadmium combines with the hydroxyapatite crystals and bone proteins which results in bone weakness and fragility (Blumenthal, Cosma, Skyler, LeGeros, & Walters, 1995; Oda et al., 2001). Cadmium exposure also disrupts Vitamin D metabolism and causes vitamin D deficiency and bone damage (Chang et al., 1996).

The elevation of PTH and reduction of 1, 25$(OH)_2$ D can be observed in renal failure patients (Nogawa et al., 1987). The exposure to cadmium induces acidosis with significant changes in bone mineralization and metabolism processes (Clarke, Wynne, Wilson, & Fitzpatrick, 1995). Furthermore, their results indicated that the decrease in bone formation and resorption damage can occur because of renal dysfunction and disruption in vitamin D and PTH metabolism (Chang et al., 1996).

The histological investigation of Youness et al. (2012) showed reduction in wide bone thickness, width of central Haversian canals, erosive cavities, and low numbers of osteocytes. This study also recorded thinning and separation in trabecular bone. Youness et al. (2012) concluded that the administration of 2.0 mg/kg $CdCl_2$ for 13 weeks caused depletion of bone mass and dilation of Haversian canals in laboratory rats. These findings are supported by the results of C. Wang et al. (2011) which recorded the elevation of bone resorption and reduction of bone formation resulting in osteoporosis, and noted reduction of trabecular bone thickness and increase in cortical width and porosity.

Lead effects on bone formation and resorption

Lead exposure is an environmental issue due to its effects on human health. Lead exposure affects bone formation and resorption in all age groups. Bone is one of the major tissues that accumulates and stores lead for long periods of time (Pounds, Long, & Rosen, 1991). Skeletal system formation and resorption regulation depend on signaling cascades. Lead prevents second messenger's activity such as Ca^{2+} by blocking Ca^{2+} and Ca^{2+}-ATPase binding which results in increase of intracellular Ca^{2+} concentration (Florea et al., 2013). The subcellular mechanism for effects of lead ion can be observed in its ability to compete and substitute for essential ions such as Zn^{2+} and Ca^{2+}. This

mechanism occurs in the metal-binding domains of transcription factors and kinases (Bouton & Pevsner, 2000). Lead ions bind and activate cellular calcium signaling peptides such as calmodulin (Goldstein & Ar, 1983). Lead disrupts intracellular Ca^{2+} transport and release in adrenal chromaffin cells; lead toxicity results in decline in Ca^{2+} signaling processes in bone and cartilage tissues (Campbell, Rosier, Novotny, & Puzas, 2004; Puzas, Campbell, O'Keefe, & Rosier, 2004; Sun, Tian, Tomsig, & Suszkiw, 1999). The results of lead on growth plate are skeletal dysfunction and reduced bone strength. Lead suppresses collagen I and II gene expression and mRNA synthesis. Also, lead disrupts second messenger signaling, decreases proliferation, and increases proteoglycan synthesis in chondrocytes of growth plate (Hicks et al., 1996).

Histological changes can be seen in bone affected by lead toxicity. The histomorphometric analyses of rat and mice skeletal models showed unsystematic bone architecture and thickness of growth plate after exposure to lead (González-Riola et al., 1997). In early adulthood, lead exposure disrupts growth plate function and delays bone growth rate (Campbell et al., 2004). The effects of lead exposure included decrease in bone density and increase in bone resorption, which inhibits long bone growth in mice and rats exposed to Pb (González-Riola et al., 1997).

Besides the effects of lead exposure on Ca^{2+} second messenger signaling, lead disrupts parathyroid hormone (PTH)-like protein and transforming growth factor (TGF)-β, as well as the function of AP-1, NF-kβ, and JNK signaling pathways (Puzas et al., 2004). These signals participate in reduction in the chondrocytes matrix in addition to extracellular signal-regulated protein kinase (ErK-1) and mitogen-activated protein kinase (MAPK) signaling pathways that have a role in chondrogenesis (Mengshol,

Vincenti, Coon, Barchowsky, & Brinckerhoff, 2000). Puzas et al. (2004) reported that lead exposure increasesed chondrogenesis processes and inhibited endrochondral ossification processes, which inhibit bone formation and accelerate bone fracture by increasing early stages of differentiation and blocking late stages of bone transition.

Lead causes toxic and carcinogenic effects on bone that disrupt osteoblast metabolism (Angle, Thomas, & Swanson, 1993). For example, osteonectin secretion was inhibited by lead ions in the osteogenic sarcoma 17/2.8 cells of rat models. In addition, lead had effects on gene expression for alkaline phosphatase and type I collagen (Klein & Wiren, 1993). Lead decreased insulin-like growth factors, vitamin D, and calcium levels of transformed bone cells; this indicated the alteration of calcium homeostasis (Puzas et al., 2004). Moreover, lead exposure affected osteoclast structure and function. These effects resulted from the accumulation of inclusion bodies in both the nucleus and cytoplasm of osteoclast cells. The presence of lead in bone matrix increased bone resorption, and a few in vitro studies recorded that the stimulation of osteoclast activity was increased by lead ion (Miyahara et al., 1995).

Women's estrogen status is a significant factor which makes them more sensitive to lead exposure. Elevations of blood lead levels were recorded in Hispanic women at early menopause age compared with women at late postmenopause. High blood lead levels in menopausal women caused bone loss (Symanski & Hertz-Picciotto, 1995). In addition to the lead release from the skeletal system during menopause and bone resorption in women, other systems such as nervous and circulatory systems release lead and contribute to high blood lead levels, which impact bone formation (Puzas et al., 2004). Beier et al. (2013) found that long term exposure to Pb caused significant changes

in skeletal parameters of rats which included reduction in gene expression, reduction of bone mass, and osteoporosis. This study reported other effects which included reduction in gene expression such as osteoblastic genes *Coll*, alkaline phosphatase (ALP), and osteocalcin (OC) genes. In addition, the same study reported increased induction of cell differentiation to adipocytes (adipogenesis) and inhibition of bone nodule formation by lead exposure.

Zinc effects on bone formation and resorption

Zinc is important for several biochemical pathways in the bone formation process. Zinc has significant effects on bone formation and mineralization (Yamaguchi & Weitzmann, 2011). Average zinc concentration in bone is 100-200µg/g which represents 30% of the total body zinc content (Hambidge, Casey, & Krebs, 1986). Zinc performs essential functions in the human skeleton system as a cofactor for multiple enzymes such as alkaline phosphatase, which is important for bone mineralization and collagen formation (Ryz, Weiler, & Taylor, 2009).

Previous studies found that zinc had significant roles in bone formation during the fetal and postnatal periods (Yamaguchi, 2010). Zinc plays a significant role in human endochondral bone formation and mineralization (Leek et al., 1984). Zinc influences bone formation, resorption, and skeleton growth through osteoblast synthesis and bone formation, as well as suppression of osteoclast synthesis and bone resorption (Yamaguchi & Weitzmann, 2011).

Zinc is involved in bone formation through signaling pathways and enhancing gene expression. Zinc increases the osteogenetic function that stimulates proliferation and osteoprotegerin (OPG) activity in osteoblast culture (Liang et al., 2012). OPG is one of

the tumor necrosis factor (TNF) receptors that are expressed in the skeleton system. OPG increases bone mineral density and decreases bone damage (Gori et al., 2000). The study by Liang et al. (2012) tested multiple zinc concentrations in mice cell culture at (0, 10, 30, 50, 70, 110, 130, and 150 µM), after 24 and 48 hours. The study recorded that cell proliferation and OPG expression were optimal at 50 µM of zinc concentration treatment (Liang et al., 2012). Zinc and protein kinase C activator increased OPG expression. However, OPG expression was decreased when osteoblasts were treated with Protein Kinase C inhibitor (PKC) (Liang et al., 2012).

Bone and metal detoxification

Bone acts as an internal source of metals and accumulates heavy metals such as cadmium and lead (Chang et al., 1996). During certain physiological changes such as pregnancy, lactation, and menopause, bone accumulates toxic metals in the extracellular bone matrix. Bone's ability to accumulate lead decreases blood lead concentration (Chang et al., 1996). Because bone removes toxic metals and foreign substances from circulation, it reduces damage to organs such as liver, kidney and pancreas.

Bone resists metal toxicity and degradation more than renal cells. Loss of bone mineral density in humans increases with Cd, Pb, and Hg toxicity (Järup, 2002). There is an association between the induction of MT, renal failure, and depletion in bone density. Bone density decreases as a result of calcium, phosphate, and protein leakage from renal cells that are affected by metal toxicants (Alfvén, Järup, & Elinder, 2002). High MT concentrations, renal damage, and bone malformation have been recorded in adult bottlenose dolphins (*Tursiops aduncus*) in South Australia that were exposed to Cd, Cu, Zn, Hg, Pb and Se (Lavery et al., 2009).

Nutrient deficiency effects on bone formation

Nutrients are important for bone growth and development. Adequate nutrients supply bone with energy, amino acids, essential minerals, vitamins, proteins and ions that are required for bone formation (Prentice et al., 2006). Decreasing nutrients during childhood and adolescence can increase osteoporosis risk. Organic nutrients such as carbohydrates, proteins, lipids, and vitamins are important for bone formation. Inorganic nutrients include dietary minerals, water, and oxygen, which are considered to be essential elements due to their necessity in building human bone and the whole body. The essential minerals for bone formation include calcium, magnesium, zinc, copper, and iron (Okyay et al., 2013). These minerals represent the main contents of bone structure and they are important for collagen, protein, and matrix syntheses (Gulekli, Davies, & Jacobs, 1994).

Nutrient deficiencies can cause health problems such as osteoporosis, which is a common risk problem in postmenopausal women (Johnell & Kanis, 2005; Okyay et al., 2013). Moreover, macroelements such calcium have significant roles in sustaining bone metabolism and mineralization (Nieves, 2005). Bone stores calcium as a crucial element which conjugates with phosphorous for bone formation and inhibits bone resorption (Prentice et al., 2006). There is a significant relationship between calcium intake and bone density in children and adults (Chevalley et al., 1994). Low dietary calcium can be a reason for bone weakness, and calcium supplementation is used to treat older people with bone weakness (Chevalley et al., 1994).

Calcium deficiency can cause hypocalcemia, and inhibit matrix formation and mineralization. Low calcium levels increase both bone resorption and parathyroid gland

size (Stauffer, Baylink, Wergedal, & Rich, 1973). Also, calcium deficiency has been shown to decrease bone density and increase parathyroid hormone secretion in laboratory female Sprague Dawley rats (Goldie & King, 1984).

Vitamin D deficiency can increase bone resorption, and decrease intestinal calcium absorption (Baylink, Stauffer, Wergedal, & Rich, 1970). Beside calcium, zinc is a crucial nutrient for human bone growth and development (Yamaguchi, 2010). Zinc increases osteoblast formation and osteoclast inhibition. There are other essential elements including iron, copper, and magnesium that maintain mineral metabolism (Yamaguchi, 2010). Zinc concentration is high in the osteoid layer which makes zinc content in the skeleton the major source of zinc for the whole body (Herzberg, Foldes, Steinberg, & Menczel, 1990).

Recently, studies have documented that zinc plays a role in maintaining bone homeostasis, and it is used as a therapy for osteoporosis problems (Yamaguchi, 2010).

Zinc is known as an anabolic factor for bone metabolism, and it stimulates fracture healing. Calcium concentration, alkaline phosphate activity, DNA contents, and fracture healing were increased significantly in the femoral-diaphysis of laboratory rats that were orally administrated 100mg/kg zinc acexamate daily for 28 days (Igarashi & Yamaguchi, 1999).

Zinc deficiency decreases bone formation and mineral density. Zinc levels decrease in serum and bone in adolescence due to use for physiological functions (Walker & Black, 2004). Zinc depletion in osseous tissue may cause osteoporosis and bone fractures in adolescents (Ilich J, 2000). A study by Leek et al. (1984) recorded that

zinc deficiency was observed in monkeys during the early postnatal period. This study recognized significant depletion in bone formation, mineralization, and maturation in monkeys during the same period of time. Zinc deficiency was found to cause bone weakness and abnormal skeleton in fetal and postnatal development (Hsieh & Navia, 1980). Adequate dietary zinc is essential in adolescence because it reduces osteoporosis risks (Ryz et al., 2009).

Twenty-four nine-week old Sprague Dawley rats were used to test the correlation of zinc deficiency in bone and adolescent age. Rats were kept on <1 mg/kg Zn diet, 5 mg/kg diet, and the control group was kept on 30 mg/kg diet for nine weeks. The study investigated serum minerals and femur morphometry, serum osteocalcin, C-terminal peptide, femur zinc, and skeleton densitometry of young adults (Ryz et al., 2009). Mineral density in spinal bone was 8% in the 1 mg/kg Zn diet group. Mineral density was 14% lower in spine but not other skeleton parts such as tibia and femur. Serum osteocalcin was 33% lower with 5 mg/kg diet; femur zinc was 57%, and 56-88% lower with 5 mg/kg diet in comparison with the control group (Okyay et al., 2013; Ryz et al., 2009). Furthermore, zinc deficiency causes problems in immune function, bone growth, and many cellular enzymatic functions are coupled with zinc, including RNA and DNA synthesis (Chang et al., 1996). Cook-Mills and Fraker (1993) found that the main effects of zinc deficiency were thymic atrophy and lymphopenia. Zinc did not decrease the effect of cadmium on blood parameters, liver, kidney, testes, or spleen function in rats treated with $ZnCl_2$ and $CdCl_2$ (Rhman, Bakhiet, & Adam, 2011).

Vitamins C, D, and K are involved in crystal and collagen formation in bone surface, cartilage, bone metabolism, and growth plate of long bones (Prentice et al.,

2006). Vitamin D deficiency is common in children with little sunshine exposure. Maternal nutrients, such as Vitamin D, C, P, Mg, and K, are essential for maintaining bone mineral density during pregnancy (Prentice et al., 2006). In contrast, the study in pregnant Gambian and Indian women showed no correlation between Ca and Vitamin D intake and child bone mineral density (BMD), weight, and length (Raman, Rajalakshmi, Krishnamachari, & Sastry, 1978). According to the European Society for Clinical and Economic Aspects of Osteoporosis and Osteoarthritis (ESCEO), vitamin D supplementation is recommended in the range of 800-1000 IU/day for optimal health (Rizzoli et al., 2013). A deficiency in Vitamin D decreases calcium absorption and causes low circulatory calcium which results in high PTH levels. The elevation in PTH levels increases bone loss and fracture risks (Nieves, 2005).

In addition to Vitamin D, Vitamin C, K, and A are essential cofactors for bone development which enhance collagen, hydroxyproline, and hydroxylysine synthesis. Vitamin C deficiency decreases bone mineral density, while a high amount of Vitamin C decreases bone loss and fractures (Nieves, 2005). Vitamin K is another cofactor for bone development, and it is a fat soluble vitamin. It has roles in bone metabolism, blood coagulation factors, and it reduces calcium excretion, bone resorption, and fracture risks (Booth, 1997; Nieves, 2005).

Excess intake of Vitamin A from retinol increases hip fractures (Nieves, 2005). Phosphorous is important for bone formation and mineralization. However, high levels of phosphorous with low calcium intake cause bone loss and hyperthyroidism (Whiting, Boyle, Thompson, Mirwald, & Faulkner, 2002). Low phosphorus intake decreases osteoblasts' function and increases bone resorption (Heaney & Nordin, 2002).

Potassium ion influences calcium homeostasis and excretion. Low potassium increases calcium excretion. Adequate potassium intake increases BMD and decreases bone resorption (Demigne, Sabboh, Remesy, & Meneton, 2004).

Magnesium ions are involved in enzymatic reactions and protein synthesis. Magnesium deficiency is associated with low calcium, and Vitamin D resistance with health problems such as muscle cramp and irregular heartbeat. Fluoride is another element that is essential for bone and dental development. However, high fluoride intake increases the risk of hip fractures and causes fluorosis (Nieves, 2005).

Toxic effects of heavy Metals on wildlife animals

Metals from natural sources and from human activities can cause serious damage on wildlife (McBee & Bickham, 1988; Ritchard F Shore 2001; Talmage & Walton, 1991). Tissue samples of river fish including northern hog sucker (*Hypentelium nigricans*), river carpsucker (*Carpiodes carpio*), largescale sucker (*Catostomus macrocheilus*), and mountain sucker (*C. platyrhynchus*) were collected from four sites downstream of smelters in Trail, BC, Canada (reference sites) and E. Helena, MT; Herculaneum, MO; and Glover, MO to investigate the effects of lead on blood hemoglobin, and delta-aminolevulinic dehydrogenase (ALAD) activity (Schmitt, Caldwell, Olsen, Serdar, & Coffey, 2002). Animals in this study showed low ALAD activity, and high Pb concentration downstream in all sites was higher than at reference sites. In addition, Pb and Zn concentrations in liver, blood, and whole carcass, hemoglobin levels, and ALAD activity showed differences among species from different sites (Schmitt et al., 2002).

Macroinvertebrate samples were collected from lead-zinc mining areas in southeastern Missouri to investigate effects of heavy metal mining on aquatic invertebrates. In comparison with reference sites, macroinvertebrate biotic conditions and responses in mining sites correlated with downstream metal levels (cadmium, nickel, lead, and zinc) (Poulton et al., 2010).

Immune response in the white-footed mouse (*P. leucopus*) from a site contaminated with heavy metals including cadmium, copper, lead, mercury, and zinc was compared to mice collected from three reference sites (Biser, Vogel, Berger, Hjelle, & Loew, 2004). The results showed that spleen weight differed among animals; however, age had a greater effect on the differences in this species than exposure to metals at the site of capture (Biser et al., 2004).

Another study showed that relative body weight, plasma parameters (glutamic oxaloacetic transaminase, GOT; glutamate pyruvate transaminase, GPT; creatinine), and genotoxic parameters (frequency of micronuclei), along with bioaccumulation of heavy metals (Pb, Hg, Cd, Fe, Zn, Cu, Mn, Mo, and Cr), were all significantly different in wood mouse (*Apodemus sylvaticus*) specimens from a polluted landfill site in comparison with reference site specimens, although intensity of response varied among metals (Sanchez-Chardi, Penharroja-Matutano, Oliveira Ribeiro, & Nadal, 2007). Sánchez-Chardi and Nadal (2007) found no difference in body masses of greater white-toothed shrew (*C. russula)* collected from the Garraf Landfill compared to reference site animals, but did see a significant increase in frequency of micronuclei in the blood of individuals from the landfill. Sánchez-Chardi and Nadal (2007) used histopathology, blood parameters (white blood cells, red blood cells, hemoglobin, and hematocrate), hepatic enzyme activity, and

genotoxicity (frequency of micronuclei) to assess physiological effects on the greater white-toothed shrew (*Crocidura russula*) collected from a deactivated pyrite mine in northeastern Spain. Histological alterations in hepatic tissue, such as apoptosis and necrosis, were evident in *A. sylvaticus* and *C. russula* that were collected from the Garraf Landfill, a site polluted with heavy metals in northeastern Spain. Tubular necrosis and inflammation were more apparent in *A. sylvaticus* than in *C. russula.* There were no histological changes observed in lungs, pancreas, spleen, gonads, esophagus, intestines, or adrenal glands in either species compared to reference site animals (Sánchez-Chardi, Peñarroja-Matutano, Borrás, & Nadal, 2009).

***Peromyscus leucopus* as bioindicator species**

The most common species of small mammal at TCSFS is the white-footed mouse (*Peromyscus leucopus*) (Phelps & McBee, 2010), a species that also serves as a good model in research as a biomonitor (Husby, Hausbeck, & McBee, 1999; Husby & McBee, 1999; Levengood & Heske, 2008; Phelps & McBee, 2010). Even though *P. leucopus* has been the subject of a number of different studies of effects of environmental contaminants, response of metallothioneins has not been investigated in this species to our knowledge.

The genus *Peromyscus* has a wide distribution in North America (King & Mammalogists., 1968; Langer & Giessen, 2007; Wozencraft, Wilson, & Reeder, 2005). Members of the genus *Peromyscus* are terrestrial, nocturnal, small mammals inhabiting both temperate and tropical regions of North America. *P. leucopus*, the white-footed mouse, is found throughout most of the eastern United States. White-footed mice are omnivorous, depending on grains, seeds, fruit, and insects for their nutrition (King &

Mammalogists., 1968). White-footed mice are brownish to grayish with a dark mid dorsal stripe, white ventral area, ears covered with short dark hair, white feet in dorsal view, and a relatively short tail (Lackey et al., 1985); their mass is 15- 25 g, averaging 23 g; length is 150-205 mm (Aguilar, 2002). White-footed mice typically live for six months to one year in the wild and can have 2 to 9 pups per litter with a gestation period of 22-28 days (King & Mammalogists., 1968; Lackey, Huckaby, & Ormiston, 1985). This species has been well studied in terms of its ecology, physiology, and biogeography (Montgomery, 1989). Its near ubiquitous distribution in North America, relatively short generation time, high reproductive potential, and small home range give it excellent potential as a biomonitor (McBee & Bickham, 1988; Ritchard F Shore 2001; Talmage & Walton, 1993).

Heavy metals' effects on species of wild animals have been studied in Old World wildlife such as *Apodemus sylvaticus*. It is possible that *P. leucopus* and a similar but unrelated species such as *A. sylvaticus* will show similar responses to environmental contaminants. Wood mouse (*A. sylvaticus*) is distributed throughout Europe and Great Britain except in northern Scandinavia and Finland; throughout central and southwestern Asia; east of the Altai and Himalayan mountains; and in northwestern Africa (Nowak, 1991). *A. sylvaticus* lives in grassy woodlands and forests. It is 60-150 mm in length, 14-20 g in mass, and has a hairy tail. Its fur is soft, grayish brown, or brown with yellow, red, or sandy color in the dorsal area, and the ventral area is white or light gray with yellow spots. This species has large ears and eyes and white feet. Females produce 4 to 7 pups per litter, and the gestation period is 21-26 days. Similar to *P. leucopus*, the diet of *A. sylvaticus* includes roots, grains, seeds, berries, nuts, grasses, fruits, and insects

(Nowak, 1991). Members of the genus *Apodemus* have been used extensively in field studies of effects of environmental contamination in Europe (Fritsch et al., 2010; Ritchard F Shore 2001; Sánchez-Chardi & Nadal, 2007; Sánchez-Chardi et al., 2009). Organisms that have evolved independently in different parts of the world but occupy similar niches are considered ecological equivalents (Montgomery, 1989).

Peromyscus leucopus and *Apodemus sylvaticus* are considered ecological equivalents because of their similar size, morphology, physiology, life-history strategies, habitat choices, and diet (Grant, 1970; Montgomery, 1989). Both *Peromyscus* and *Apodemus* have high densities in fragmented forests more than in large forests and respond in similar ways to food availability and decreased predator pressure (Diaz, Santos, & Telleria, 1999).

Metallothioneins

Protein synthesis is one of the main functions in the biological cells. The liver produces multiple kinds of plasma proteins such as albumin and metallothionein (MT) (Šveikauskaitė, Šulinskienė, Sadauskienė, & Ivanov, 2014). The levels of circulating plasma and tissue proteins are used as indicators to the main cellular and physiological functions such as metallothionein synthesis (Dawson & Bortolotti, 1997; Marcotte et al., 1999; Sonne et al., 2009).

Metallothioneins are a group of essential proteins in biological cells. Metallothionein protein is a scavenger protein that binds with metals such as lead, cadmium and zinc. Metallothioneins are low molecular weight, cysteine-rich proteins that assist in metal detoxification. MT functions primarily in the liver and kidney, and these organs have a higher capacity for binding many metals and organic chemicals than other

organs. Blood circulation in these organs enhances this function by active transport and protein binding (Casarett et al., 2008). MTs have a high affinity to metal ions such as Zn, Cd, Cu, Pb, and Ag. MT synthesis is induced by high concentrations of metal ions (Chang et al., 1996).

Heavy metal ions like Cd, Cu, and Zn increase MT synthesis by inducing transcription of the metallothionein genes (Chang et al., 1996); therefore, MT is one of the biomarkers often used to indicate exposure to heavy metals. MT is composed of sulfur and three protein subunits that strongly bind with Cd, Pb, Zn and other heavy metals. After heavy metals have been absorbed across the gut, lungs, or skin (Sorensen, Nielsen, & Andersen, 1993), they are sequestered by the liver and bound by MT, then transported through the circulatory system in plasma to the kidneys where the metals are cleared by renal tubules.

The MT capacity can decrease the risk of high level of heavy metals. MT-Pb and MT-Cd complexes are formed in the liver and transferred to the kidney where they are filtered and reabsorbed in the proximal tubules of the nephrons (Chang et al., 1996; Sonne et al., 2009). Metallothionein is involved in detoxification function by binding with the metal ions and different enzymes of endothelial cells (Chang et al., 1996). The MT-Cd complex is broken in tubular cells by lysosomes, and the unbound Cd ions induce MT synthesis in renal tubules to bind with Cd ions (Chang et al., 1996). Cd accumulates in kidney tubules, where it inhibits Zn-dependent enzymes (e.g., leucine-aminopeptidase) that carry out renal proteins and release Cd ions in urine. Protein molecules are catabolized and excreted in the urine, or they are reabsorbed in the renal tubules, resulting in high proteinemia (Massaro, 1997). MT-Zn binding increases Zn distribution in the

liver and decreases Zn distribution in other tissues (Taubeneck, Daston, Rogers, & Keen, 1994).

Although the majority of work on metallothionein in wildlife has been done on aquatic species including fish, mollusks, and invertebrates (Amiard, Amiard-Triquet, Barka, Pellerin, & Rainbow, 2006; Kito, Tazawa, Ose, Sato, & Ishikawa, 1982), metallothionein induction has been documented in wild mammals exposed to metal contaminants. Metal concentrations for Cu, Cd, and Zn and metallothionein-binding capacity in liver, kidney, and brain tissues were significantly different among three species of arctic seals: ringed seal (*Phoca hispida*), harp seal (*Phoca groenlandic),* and hooded seal (*Cystophora cristata*). MT metal-binding capacity was highest in the kidneys compared to liver and brain for all species, indicating variable sensitivity among organs in these species (Sonne et al., 2009).

Seven small mammal species—wood mouse (*A. sylvacticus*), bank vole (*Myodes glareolus*), common shrew (*Sorex araneus*), pygmy shrew (*Sorex minutus*), common pine vole *(Microtus subterraneus*), greater white–toothed shrew (*C. russula*), and field vole (*Microtus agrestis*)—were collected from an area heavily contaminated by Cd, Pb, Zn, and Cu in Northern France (Metaleurop-Nord smelter) to analyze the correlation between heavy metal concentrations in liver and kidney and metallothionein concentrations. Cd concentration increased in liver and kidney with age in all species. The relationship between concentrations of Cd and Pb in soils and in tissues of animals was not significant, but concentrations of metals in liver and kidney correlated with increasing metallothionein levels among species. The relationship between levels of metals in soils and metallothionein induction in liver and kidney was different among species. *A.*

sylvaticus showed a significant relationship between metallothionein concentration and soil contamination; however, metallothionein concentration increased with increasing soil metal levels in liver and decreased in kidney. In *M. glareolus*, *S. araneus*, and *S. minutus*, metallothionein concentrations in both tissues were positively correlated with concentration of metals in soils (Fritsch et al., 2010). In wild species, total protein concentrations were analyzed in both female and male American kestrels (*Falco sparverius*) (Dawson & Bortolotti, 1997). The study showed higher significant plasma protein concentrations (g/dl) in females than in males ($F_{[1,512]}=67.41$, $P < 0.0001$) (Dawson & Bortolotti, 1997). The authors in this study suggested that the variation in total protein concentrations between wild females and males is associated with the variations in their physical conditions such as the period of prelaying egg in females and the incubation period in males.

In conclusion, heavy metals have an effect on environmental conditions in addition to human lives. The heavy metals mining at TCSFS was considered by the USEPA due to the health problems for humans and the risk effects on the wildlife populations. High concentrations of cadmium, lead and zinc in soil were recorded in TCSFS by the USEPA (ODEQ, 2003 ; United States Environmental Protection Agency USEPA, 2005). Heavy metals target nervous, respiratory, circulatory, and excretory systems and affect their functions. Bone is also influenced by heavy metals such as cadmium and lead. Exposure to lead and cadmium affects bone formation, resorption, and bone mineral density (Youness et al., 2012). Additionally, renal malfunction was recorded after exposure to heavy metals (Chang et al., 1996).

The white-footed mouse (*Peromyscus leucopus*) is one of the common species at TCSFS (Phelps & McBee, 2010). This species also serves as a good model in this kind of research and in this particular study as a physiological and ecotoxicological biomonitor (Husby et al., 1999; Husby & McBee, 1999; Levengood & Heske, 2008; Phelps & McBee, 2010). Metallothioneins have a high affinity for metal ions such as Cd, Pb, and Zn (Sonne et al., 2009). The study presented here analyzed the concentrations of the most common heavy metals in TCSFS in soil and kidney tissues. Bone microarchitectural evaluation and alterations of MT-1 in the common species *P. leucopus* at this area also were analyzed. Metallothionein is one of the appropriate biomarkers to investigate heavy metals exposure.

CHAPTER III

METHODOLOGY

The study included three different sites. Tar Creek Super Fund Site (TCSFS) at Beaver Creek and two reference sites, Sequoyah National Wildlife Refuge (SNWR) and Oologah Wildlife Management Area (OWMA). Soil samples were collected from these sites for metal analysis (lead, cadmium and zinc) in November and December, 2012. The TCSFS has a large population of white-footed mice (*Peromyscus leucopus*) which were used in this study to analyze the influence of heavy metals on the physiological alteration in kidney tissues. Both SNWR and OWMA are good sites to compare with TCSFS because no mining has been recorded in these sites. The use of two reference sites rather than just one provides a more accurate comparison to the highly contaminated TCSFS. In addition, these two sites are geographically distant. Other reasons to choose these sites were to compare relationships among soil metal levels, tissue metal levels, and metallothionein induction in *P. leucopus* species, which is present in all three sites.

Soil sampling

Soil samples were collected from each site following the procedure that is described by USEPA (United States Environmental Protection Agency USEPA, 2005). Random design was used to collect soil samples in each site separately. The distances by meter between samples were selected by assessing random numbers and drawing from a selection of random numbers. The position of sites was detected with the help of Eterx Vista CX Garmin and Google Earth program using a personal i-Phone to detect the directions on Google Maps. Each sampling location was recorded (Appendix). A coin flip was used to choose direction, either east or west side, from the first soil sample site. Moreover, a meter scale was used to measure the distances between soil samples. Eight duplicate soil samples were collected from each site. Each sample was labeled as T1-1, T1-2, T2-1, T2-2, etc. where T identifies TCSFS, the first numeral (1-8) indicates the number of the sample, and the second numeral (1-2) points to the original or the duplicate. Samples were collected from each position with a 10% HCl acid–washed metal scoop to a depth of 18-20 cm.

Soil samples were collected in labeled plastic bags and sealed for transfer to the lab. Samples were homogenized and weighed separately. Soil samples were dried in separate 10% HCl acid-washed and labeled polypropylene plates at room temperature for two weeks. Also, dried soil samples were sieved twice using first 1mm sieve size, No. 18 (USA. Standard Test Sieve) followed by a second 250 µm opening sieve (Fisher Scientific Company, Pittsburgh, Pennsylvania). Samples were saved in labeled acid-washed glass bottles.

Laboratory metal analysis

Soil digestion

Soil samples were prepared in the trace mineral laboratory of the Nutrition Sciences Department using microwave digestion. All soil samples from Tar Creek Super Fund Site (TCSFS) and the two reference sites, Sequoyah National Wildlife Refuge (SNWR) and Oologah Wildlife Management Area (OWMA) were weighed separately using the same digital balance and labeled polypropylene plates. Soil samples were digested using microwave digestion (Milestone, Inc, Shelton, Connecticut) according to the EPA procedure. Following the protocol for microwave digestion, all soil sample sizes were 0.5 g. For all soil samples, 0.5 g of soil was put in the labeled microwave vessel. Double distilled trace element grade nitric acid (99.999%) was purchased from Fisher Scientific Company (Pittsburgh, Pennsylvania). Microwave vessels were transferred to the hood and 10 ml of HNO_3 was added to the soil sample in each vessel. The soil and acid solution was swirled slowly to mix soil with acid. A teflon cover was placed on the teflon vessel, pushed down and an adaptor was placed on the flat part of the Teflon cover. Teflon indicator ring was placed on the cover and pushed shut. Vessels were then introduced into the polypropylene microwave rotor. Each indicator was closed tightly with a torque wrench. A thermo couple was placed into the reference vessel No.1 that contained a blank laboratory (control) sample, the microwave door was closed, and the machine was switched on.

The program was set on the USEPA Method Number 5031A. Samples were heated for 50 minutes at 100° C. Next, the segments were pulled gently from the microwave after 5 minutes because they were hot and opened slowly using a torque

wrench to release the pressure from the vessels. Samples were transferred to the hood to open the vessels cover and yellow acid fumes evaporated from the vessels. Acid solution from vessels 10 ml was poured into a plastic 15 ml tube purchased from VWR. Vessels were washed with 5 ml double distilled water, and water was added to the first tube.

All samples were digested following the same process. The digested soil samples were centrifuged at 1200 g for 10 minutes. The samples were decanted into a new tube (2nd tube) gently and slowly to avoid solution contamination. The first tube that contained the soil was disposed separately. The second tube for each digested sample was labeled as stock that was used for metal analysis.

0.2 ml of the digested soil solution in 10 ml total volume samples were diluted by adding double distilled water (DDW) to 0.20 ml sample solution in the new tube (3rd tube). This tube of each sample was used for the Inductively Coupled Plasma-Mass Spectroscopy (ICP-MS) instrument (Perkin Elmer) to analyze metal (Pb, Zn, and Cd) concentrations in each sample separately.

Instrument calibration

ICP-MS dilution standard

Analyses of Pb, Cd, and Zn concentrations in soil from both contaminated and uncontaminated sites were conducted in the OSU Nutrition Science laboratory by ICP-MS according to USEPA method 6010 (United States Environmental Protection Agency USEPA, 1996). The ICP-MS instrument was calibrated to ensure stability and consistency of all results. Before running samples by ICP-MS, five standard dilutions for ICP-MS were diluted. Internal standard (Terbium) solution (Perkin Elmer, Shelton, Connecticut) and double distilled water (DDW) were used for calibration and dilutions.

All sample volumes were run in ICP-MS as 0.1 µl sample diluted to 10 ml (DDW) and add 20 µl (IS). Sample tubes were vortexed for 20 seconds before analysis.

Tissue digestion

Frozen kidney biopsies of *Peromyscus leucopus* were provided from the collection of vertebrates in the OSU Zoology Department. Kidney samples were saved in a liquid nitrogen tank before subsampling. The kidneys were subsampled into two parts: one part was cut in a plastic petri dish placed on wet ice using stainless-steel scalpel to one tenth of whole tissue and saved in plastic micro tubes for metallothionein-1 analysis and then returned back to the freezer at -80^0 C without thawing. The second part of the kidney was saved in plastic microtubes and refrigerated at -4^{0}C prior to digestion for metal analysis.

Kidney samples were weighed using a digital balance. Small microwave tubes were used for kidney sample digestion (Milestone, Inc, Shelton, Connecticut). Each sample was weighed and put in small microwave tubes; 1 ml of concentrated (99.999) HNO_3 was put in the tube and then 0.15 ml of H_2O_2 was added to the same tube. The program was set on USEPA Method Number 5031A. The microwave operation required 50 minutes to finish digestion at the temperature of 100° C. Next, the vessels were pulled gently from the microwave after 5 minutes. The segments were opened slowly using a torque wrench to release the pressure from the vessels. Samples were transferred to the hood to open the vessels cover, and yellow acid evaporated from the vessels.

Metal Analysis

ICP-MS

An inductively coupled plasma-mass spectroscopy (ICP-MS) instrument (Perkin Elmer) was calibrated to ensure stability and consistency of all results. Before samples were run, five standard dilutions for ICP-MS were prepared. All sample volumes were analyzed by ICP-MS as 80 µl sample with 10 µl Internal standard (Terbium) solution (Perkin Elmer, Shelton, Connecticut) and complete volume by DDW up to 5 ml. Sample tubes were vortexed for 20 seconds before analysis by ICP-MS.

Bone microarchitecture

Bone skeleton samples of *P. leucopus* were provided from the vertebrate collection from the OSU Zoology Department. Eight useable skeletons of *P. leucopus* from each site were selected for bone analysis.

The lumbar 2, 3, 4, 5 vertebra section of each skeleton was excised and the L_4 was scanned using a high resolution computed tomography system or micro-CT scanner (µCT 40, Scano Medical AG, Zurich, Switzerland). This section of L_4 vertebra was scanned in approximately 3 hours in a 16 mm plastic tube sample holder. The lumbar 4 vertebra was detected in each skeleton sample and saved as a 3-D image. The trabecular bone in the 3-D images of L_4 was contoured in a 300-400 µCT slide image. L_4 slices were contoured every 10 slices when the detection of spogiosa begins and ends from the growth plate (90-550). The threshold for evaluation was set as 350 (gray scale, zero-1000) for all slides. The trabecular bone was contoured to measure trabecular thickness (mm), trabecular number (mm^{-1}), and trabecular volume as a bone volume fraction (bone

volume/tissue volume %) for individual lumbar vertebra. The 3-D images of results were evaluated, and the data set was exported to evaluate and analyze the results.

Tissue homogenization

Peromyscus leucopus kidney samples were homogenized using Invitrogen MT protocols. One tenth of each sample was crushed in liquid nitrogen and transferred into a plastic pre-chilled 5 ml culture tube. Ten µl of protease inhibitor (Sigma Aldrich, St. Louis, Missouri, Cat. No. P-88340) was mixed with 1 ml phosphate buffered saline (PBS) and cooled using wet ice. Tissues were sonicated at low speed for ~20 seconds, and transferred into micro centrifuge tubes and centrifuged at 14,000xg at 4°C for 15 minutes. The supernatants were removed and four 80 µl aliquots were pipetted to vials for storage. Samples were stored at -80°C for metallothionein-1 analysis using ELISA procedures.

Total protein analysis

Total protein concentrations of kidney specimens were determined by Modified Lowry method using a commercial protein assay kit (Thermo Scientific, Waltham, Massachusetts) (Lowry, Rosebrough, Farr, & Randall, 1951). Homogentated kidney specimens were diluted 20X using Phosphate Buffer Saline (PBS). The standard curve was plotted for each bovine serum albumin (BSA) value. The concentrations of total protein (µg/ml) in homogenated kidney specimens were detected using a standard curve made with bovine serum albumin. The plot included the absorbance (nm) of the standards against the standards concentration (µg/ml). The concentrations (µg/ml) of diluted specimens were detected at OD 750 (nm) and were analyzed using Prism version 6.

Enzyme-linked immunosorbent assay (ELISA)

An ELISA technique was used to determine metallothionien-1 (MT-1) concentrations in kidney samples from *Peromyscus leucopus*. A commercial mouse *Mus musculus* MT-1 kit (Uscn, Life Science Inc., Houston, Texas) was used following manufacture protocols general procedure. Samples were tested to determine appropriate dilutions for analyses. The MT-1 concentrations were detected in a microplate reader at an OD of 450 (nm). Metallothionein-1 concentrations were expressed as microgram per mg of protein

Statistical analyses

Data were first compiled using Excel (Microsoft) and were analyzed using PROC GLM, MEANSAND SMEANS with PROC CORR, SAS 9.3. Means and standard error of mean were calibrated in SAS. This study determined the correlation between heavy metal concentrations and the biomarkers of toxicity in kidney and bone tissue to detect the effects of heavy metals and detoxification function response. It also examined specimens collected from a contaminated site (TCSFS) and compared them with reference sites (SNWR & OMWA).

A P value of < 0.05 was considered significant. Correlation between bone microarchitecture results and kidney mineral concentrations were analyzed using Pearson Correlation Coefficients. P values were ≤ 0.05, 005, 0.005.

CHAPTER IV

HEAVY METAL CONTAMINATION IN SOIL AND THE CORRELATION BETWEEN BONE MICROARCHITECTURE AND KIDNEY HEAVY METAL CONCENTRATIONS OF *PEROMYSCUS LEUCOPUS* FROM TAR CREEK SUPERFUND SITE

Introduction

Tar Creek Superfund site (TCSFS), Ottawa, OK is located in northeastern Oklahoma near the Kansas-Oklahoma border. TCSFS covers a 40-square mile area, and it is one of the Tri-State Mining District (Oklahoma, Kansas, and Missouri) sites. These sites include territories of ten tribal nations and several communities such as Quapaw Nation, Picher, Cardin, North Miami, and Commerce (Agency for Toxic Substances and Disease Registry, 2013). Lead and zinc ores were mined at TCSFS from the early 1900s to the late 1970s. During World II, the Tri-State Mining District produced Zn ore which constituted 75% of the United States total Zn production (Weidman, Williams, & Anderson, 1932).

The Oklahoma Department of Environmental Quality (ODEQ) recorded that TCSFS is the most challenging site in Oklahoma (Hughes, 2014). In 1983, the USEPA listed this site as a superfund site, and it received federal funding for research and health screening and for remediation (United States Environmental Protection Agency USEPA, 2005). Flotation chat waste was disposed in tailing ponds or in piles; these piles sometimes contained high levels of lead and other heavy metals (Agency for Toxic Substances and Disease Registry, 2013). Mines were found under water tables, and the retention ponds received water that was pumped from the mines (United States Environmental Protection Agency USEPA, 2005).

The U.S Army forces of Engineers and the U.S Geological Survey estimated that TCSFS contains 75 million tons of chat. Agency for Toxic Substances and Disease Registry (ATSDR) recorded the major pathways of exposure to lead contamination at TCSFS as contaminated air, contaminated water, contaminated food resources, and contaminated soil. Such human health problems as respiratory illness, liver dysfunction, and reproductive and renal failure can occur after exposure to these pathways (Agency for Toxic Substances and Disease Registry, 2013).

Heavy metals such as cadmium, lead and zinc at TCSFS were studied because of their effects on human health and their accumulation in small mammals' bodies (Sanchez-Chardi et al., 2007). Numerous human health issues have been documented after exposure to cadmium because of its ability to substitute other metals and nutrients such as zinc (Beyersmann & Hartwig, 2008). Cadmium, lead and zinc in soil sediments were measured at Beaver Creek and Douthat Settling pond within the TCSFS. Soil

sediments were 440-540 mg/kg for lead and 20-56 mg/kg cadmium and 3000-9300 mg/Kg for zinc at TCSFS (Moeller, 2004).

Cadmium disrupts protein synthesis, metabolism function, and other metalloenzymes (Chang et al., 1996). Cadmium accumulates in small mammals' bodies in tissues such as liver and kidney in *Apodemus sylvaticus, Clethrionomys glarelolus, Crocidura russula,* and *Microtus agrestis* (Johnson, Roberts, Hutton, & Inskip, 1978; Sanchez-Chardi et al., 2007).

Lead is a non-essential metal, and it is one of the abundant toxic metals at TCSFS. Lead exposure can result in acute and chronic toxic effects. Lead accumulates in wild mammals' in tissues such as kidney, liver, and bone. In smelter and mining sites, lead and zinc were recorded in wood mice (*A. sylvaticus*); bank voles (*C. glareolus*), and field voles (*M. agrestis*). The results showed high lead concentrations in bones; 42-68% of total lead found in body tissues was contained in bone (Johnson et al., 1978). Johnson et al. (1978) concluded that bones accumulate more lead than liver and kidneys.

Bone as a connective tissue has different sizes, shapes, and structures that serve important functions. Mineralized bone is the osseous tissue that gives bone rigidity. Bone includes blood vessels, nerves, cartilage, bone marrow, and endosteum. As a supportive tissue, bone works to protect different body organs such as brain, heart, and other organs. Bone tissue is one of the tissue markers that indicate xenobiotic and metal exposure. Bone tissue accumulates heavy metals such as cadmium and lead (Chang et al., 1996).

Studying bone microarchitecture helps to evaluate the toxic effects on bone after exposure to heavy metals. Bone dysfunction and osteoporosis are reported as toxic effects

of exposure to cadmium (Youness et al., 2012). Significant decrease in bone density and the presence of osteopenia has been recorded in women who are exposed to cadmium from environmental sources (Chang et al., 1996; Engström et al., 2012). Enzymatic and non-enzymatic activities in bone are disrupted by cadmium exposure (Ognjanović et al., 2010). Bone damage and kidney problems can be caused by short and long term exposure to cadmium (Alfvén et al., 2002). Urinary and blood cadmium levels can be used as markers of cadmium exposure and cadmium body burden (Alfvén et al., 2002).

Lead exposure decreases bone mineral density (BMD) which can cause osteoporosis (Campbell et al., 2004). High exposure to lead is associated with lower BMD and bone mass in children. Campbell and collagenous clarified how lead exposure targeted bone growth plates in children and inhibited parathyroid hormone related peptide (PTH rp) (Campbell et al., 2004). Another study recorded lower bone density in rats after exposure to lead for a long period of time (Puzas et al., 2004). Lead exposure inhibits osteoblast function (Chang et al., 1996; Puzas et al., 2004).

Zinc is another trace metal which was considered in this study. Zinc has important function in bone formation, turnover, and metabolism (Bekheirnia et al., 2004; OSHA, 2012). Most previous studies investigated zinc deficiency and bone growth. This study aimed to measure zinc concentrations in soil as an environmental source and bone microarchitecture of *Peromyscus leucopus* as a small mammal biomonitor species. Zinc as a cofactor plays essential roles in other tissue and enzyme functions that are important for bone mineralization and development such as alkaline phosphate and collagenase (Bekheirnia et al., 2004)

The objectives of the current study were 1- To determine the heavy metals concentrations (Cd, Pb, and Zn) in soil samples and kidney specimens of *Peromyscus leucopus* collected from TCSFS, BC and reference sites. 2- To determine the correlation between bone microarchitecture of L_4 and heavy metals concentrations of kidney specimens of *p. leucopus* collected from TCSFS, BC and reference sites.

Material and methods

Study site

Soil samples were collected from the beaver Creek of the Tar Creek Superfund site (TCSFS, BC) and two reference sites, Sequoyah National Wildlife Refuge (SNWR) and Oologah Wildlife Management Area (OWMA) following the procedure that is described by USEPA (United States Environmental Protection Agency USEPA, 2005). A random design was used to collect soil samples in each site separately. Position of collection sites was detected with the help of Eterx Vista CX Garmin and a Google Earth program using a personal i-phone to identify the directions on Google Maps. Samples were collected to a depth of 18-20 cm from each position with a metal scoop which was rinsed with 10% HCl acid (Castaldi, Santona, & Melis, 2005), then sealed in plastic bags. In the laboratory, soil samples were dried in polypropylene plates at room temperature for two weeks. Dried soil samples were sieved twice using first a 1 mm and then a 250 µm sieve (Fisher Scientific Company, Pittsburgh, Pennsylvania). Samples were stored in acid-washed glass bottles.

Laboratory metal analysis

Soil digestion

Soil samples were digested using a microwave digestion protocol (Milestone, Inc, Shelton, Connecticut) specified by USEPA Method Number 5031 A (Kingston, Walter, Chalk, Lorentzen, & Link, 1997; United State Environmental Protection Agency USEPA, 1986). All soil samples were weighed separately in polypropylene plates. Microwave vessel No.1 with a laboratory control sample was used as a standard for probe detection and drawing a standard curve. Subsequent soil samples were digested in vessels No. 2-8. Soil samples of 0.5 g were used and 10 ml of concentrated (99.999%) HNO_3 were added to the soil sample in each vessel. Samples were heated for 50 minutes to 100° C Vessels were decanted into plastic tube and rinsed with double distilled water. Tubes were centrifuged at 1200 g for 10 minutes, and supernants decanted gently and diluted for mineral analysis by ICP-MS.

Instrument calibration

ICP-MS dilution standard

Soil analysis

Pb, Cd, and Zn concentrations in soil and kidney specimens from both contaminated and uncontaminated sites were determined by ICP-MS according to USEPA method 6010 (United States Environmental Protection Agency USEPA, 1996). Terbium was used as internal standard.

Bone microarchitecture

µCT analysis

Eight skeletons of *P. leucopus* from each site were provided from the vertebrate collection from the Zoology Department at OSU. The lumbar 2, 3, 4, 5 vertebra section of each skeleton was excised and the L_4 was scanned using a high resolution computed tomography system or micro-CT scanner (µCT 40, Scano Medical AG, Zurich, Switzerland). The lumbar 4 vertebra was detected in each skeleton sample and saved as a 3-D image. The trabecular bone in the 3-D images of L_4 was contoured in a 300-400 µCT slide image. L_4 slices were contoured every 10 slices when the detection of spogiosa begins and ends from the growth plate (90-550). The threshold for evaluation was set as 350 (gray scale, zero-1000) for all slides. The trabecular bone was contoured to measure trabecular thickness (mm), trabecular number (mm^{-1}), and trabecular volume as a percent of bone volume fraction (bone volume/tissue volume) for individual lumbar vertebra. The 3-D images of results were evaluated, and the data set was exported to evaluate and analyze the results.

Statistical analyses

This study examined soil samples collected from a contaminated site, TCSFS, BC and compared with reference sites (SNWR & OMWA) and Metal concentrations in kidney were correlated with bone parameters. Pearson's Correlation Coefficients were determined for all samples taken together and by individual sites using PROC GLM, MEANSAND SMEANS with PROC CORR, SAS, V 9, 3. Values of $P<0.05$ were taken as significant.

Results

Soil samples from contaminated site, at Tar Creek Superfund Site (TCSFS, BC), and two reference sites were compared. Mean concentrations of Zn, Cd, Pb mg/kg in soil samples are presented in Table 4.1. The results showed that zinc concentrations (mean±SE) in TCSFS (14083±1826) were higher ($P<0.0001$) than in the two reference sites (20±2, 53±5) respectively.

Cadmium concentrations in soil samples in TCSFS and the two reference sites were sharply different. In TCSFS, as a contaminated site, higher significantly cadmium concentrations (48±04 mg/kg) were recorded than those at the two reference sites, (0.06 ±0.01, and 0.15 ±0.03 mg/kg).

As expected, Pb concentrations in TCSFS soil samples (1132±278 mg/kg) were higher ($P<0.0001$) than in the two reference sites (2.3±0.33, 6.4±1.1 mg/kg). In summary, the analysis of variance showed that there were significant ($P\leq0.0001$) differences between the TCSFS site and the two reference sites' dependent variables (Cd, Pb, and Zn)

Bone microarchitecture relation to heavy metals for combined sites

Trabecular bone microarchitecture parameters for the lumbar vertebrae (L_4) and kidney metal concentrations (Cd, Pb, and Zn) in *Peromyscus leucopus* were analyzed to detect correlations (n=24) (Table 4.2). Microcomputed tomography evaluation results of bone total volume ratio (BVTV) showed correlations with kidney Zn ($r=0.53$, ≤ 0.05). Also, kidney lead concentration and bone connectivity density were correlated ($r=0.46$, $P \leq 0.05$) showing that lead affect bone parameters.

Bone microarchitecture relation to heavy metals by individual sites

Correlation between bone parameters and kidney mineral concentrations by individual site also were examined and Pearson's correlation coeffecients are presented in (Tables 4.3-4.5). In TCSFS (Table 4.3), cadmium concentrations were negatively correlated with trabecular bone number ($r=-0.67$, $P \leq 0.05$), and Pb concentration was positively correlated with trabecular bone separation ($r=0.72$, $P \leq 0.05$).

The results showed no correlation between bone parameters and mineral concentrations. However, the results showed correlation between minerals. Cd and Zn (0.63, $P \leq 0.05$) and Zn and Pb (0.98, $P \leq 0.0005$) were correlated at OWMA (Table 4.4). The data from SNWR for L_4 vertebra parameters and mineral concentration showed no significant correlations. The results analysis by site showed correlation between Pb and Zn (0.67, $p \leq 0.05$) at SNWR (Table 4.5).

Discussion

Environmental toxicology field studies showed several impacts and physiological alterations due to their contact with the contaminants. The present study used specimens collected from the TCSFS, BC contaminated area and two reference sites (OWMA & SNWR). As expected, the concentrations of heavy metals (Cd, Pb, and Zn) in soil at TCSFS, BC were higher than at the reference sites. This study also analyzed mineral concentrations (Cd, Pb, and Zn) of kidney and the correlations between metal concentrations and biomarkers such as bone parameters in the biomonitor species *Peromyscus leucopus.*

Several studies have determined that TCSFS is a highly contaminated site with Cd, Pb, and Zn. Mineral analysis of soil sample results showed the same findings of heavy metal contamination at TCSFS compared to reference sites (OWMA & SNWR) as other researchers. Moeller (2004) recorded the elevation of cadmium, lead and zinc in soil sediments at Beaver Creek and Douthat Settling pond at TCSFS. Lead concentrations were 440-540 mg/kg and cadmium concentrations were 20-56 mg/kg while zinc concentrations were 3000-9300 mg/kg. The Oklahoma Department of Environmental Quality (ODEQ) and USEPA recorded that TCSFS is the most challenging site in Oklahoma because of the extensive heavy metals contamination at that site (Hughes, 2014; United States Environmental Protection Agency USEPA, 2005).

Large amounts of chat at TCSFS and extensive amounts of Cd, Pb, and Zn from mining and acid water were reported from the 1900's through the 1960's (Oklahoma Department of Environmental Quality, 2003). Heavy metals Cd, Pb, and Zn in tailings and yard soil at Tar Creek National Priorities list Superfund site in Oklahoma were analyzed in order to reduce metal and restore vegetation in this area (Brown, Compton, & Basta, 2007). Brown et al. (2007) recorded that Pb concentration in the tailing materials was higher (4003±2654 mg/kg) than yard soil (623±21 mg/kg). The total Cd concentration in yard soil was not significantly high (25.5±5.75 mg/kg) compared to tailing (28.7± 12.6 mg/kg). Also, they found no significant differences between Zn concentration in tailings (6830±3720 mg/kg) and yard soil (5308±1070 mg/kg). Schaider, Senn, Brabander, McCarthy, and Shine (2007) documented Cd, Pb, and Zn availability and bioaccessability in mine waste at TCSFS to determine heavy metals exposure,

transport, and bioavailability. The results showed high concentration of Cd (42±10 mg/kg), Pb (650±360 mg/kg), and Zn (9100±2500 mg/kg).

Soil as a large natural source can contain any contaminant in the environment. Soil samples are used to analyze metal concentrations in TCSFS and other reference sites. The use of soil in the current study is to determine the presence of environmental contaminants in biological source and to examine the alteration in physiological parameters of *P. leucopus* as a biomonitoring species.

The present study observed high concentrations of heavy metals in soil samples that were collected from TCSFS. The lumbar vertebra L_4 of *P. leucopus* evaluated by µ-CT revealed some correlations between bone parameters and mineral concentrations in kidney specimens. The lack of significant differences could have resulted from a limited sample size (n=8 from each site). Correlations between bone microarchitecture variables appeared to be higher in TCSFS, BC samples than reference sites.

This study used adult mice, which are more exposed to the contaminants due to their age. We expect to observe variations in bone parameters, which may result from their habitat, environmental contaminants and variable ages. Previous studies have documented major effects on bone density and osteoporosis effects that resulted from cadmium and lead exposure in human (Puzas et al., 2004; Youness et al., 2012).

Lavery et al. (2009) investigated heavy metal effects on bone density, other bone parameters, renal damage, and metallothionein (MT) concentrations of South Australian bottlenose dolphins (*Tursiops aduncus*). The results showed Cd, Zn, and Cu in *Tursiops*

aduncus liver as well as renal damage. Bone parameters of two individuals of *Tursiops aduncus* showed dysfunctions, renal damage, and high levels of MT (Lavery et al., 2009).

Bone is one of the most commonly targeted tissues by lead (Pounds et al., 1991). Lead toxicity effects on bone cellular levels cause alterations at the cellular level. These effects include changes in circulating hormone 1, 25-dihydroxyvitamin D_3 that regulates bone functions (Pounds et al., 1991; Puzas et al., 2004). Significant heavy metal concentrations (Cd, Cu, Ni, Pb, Zn, and Fe) were observed in femoral bone of bank voles (*Myodes glarelus*) and voles (*Microtus arvallis*) from a polluted area in Slovakia (Martiniaková, Omelka, Jancova, Stawarz, & Formicki, 2011). The results showed significant correlation between Cd and Ni (r=0.52), a strong relation between Pb and bone weight (r=0.53), and significant relation between Fe and osteons' vascular canal size (r=0.55). Martiniaková et al. (2011) concluded that heavy metal accumulation increased in femora bone of *Myodes glarelus* and *Microtus arvallis* at the Kolíňany polluted site in Slovakia. Also, Martiniaková, Omelka, Jancova, Stawarz, and Formicki (2010) recorded significant heavy metals concentrations in *Apodemus flavicollis* and *Apodemus sylvaticus* at another polluted site in Slovakia. Although slight heavy metal accumulations were recorded in femora, the study observed no changes in femora's bone weight, and the length of both species.

Exposure to $CdCl_2$ may cause renal failure through renal proximal tubules such as mitochondrial and 1, 25 $(OH)_2$ D3 biosynthesis (Youness et al., 2012). Bone resorption and negative health effects can increase in women after middle age due to exposure to low levels of cadmium in the diet (Åkesson et al., 2006). According to the a study of women in southeast China in an area heavily polluted by cadmium, cadmium affected

bone formation and turnover were affected through indirect effects on vitamin D_3 metabolism (H. Wang et al., 2003). Bone mineral density and other bone parameter changes can be influenced by age. Legrand et al. (2000) recorded several vertebra fractures in a male patient of 52 years of age with lumbar osteopenia. These findings are evaluated through X-ray absorptiometry and bone microarchitecture changes of L_2 and L_4 trabecular bone. Bone resorption is associated with the inhibition of osteoblast function, and the studies reported this inhibition associated with the lead effects on cellular functions and regulation such as 1, 25–dihydroxyvitamin D_3 (Pounds et al., 1991). Heavy metal toxicity reduces the function of micro and macro nutrients such as Zn, phosphate, and calcium which are the main components for bone strength and density.

Table 4.1: Zinc, cadmium and lead concentrations (mean±SE) of soil samples collected from Tar Creek Superfund site (Beaver Creek, BC), Oologah Wildlife Management Area (OWMA), and Sequoyah National Wildlife Refuge (SNWR).

Mineral /Site	n	SNWR	OWMA	BC
Zn (mg/kg)	16	20.0±1.9[b]	52.6±5.0[b]	14083.9±1825.8[a]
Cd(mg/kg)	16	0.06±0.01[b]	0.15 ±0.03[b]	48.04±3.98[a]
Pb(mg/kg)	16	2.3 ±0.3[b]	6.4±1.1[b]	1132 ±278[a]

Means in a row not sharing the same superscript are significantly different from each other (P<0.0001).

Table 4. 2: Pearson's Correlation coefficents for trabecular bone microarchitecture parameters of L_4 and kidney metal concentrations from *Peromyscus leucopus* collected at Tar Creek Superfund site (Beaver Creek, BC), Oologah Wildlife Management Area (OWMA), and Sequoyah National Wildlife Refuge (SNWR).

Variable	1	2	3	4	5	6	7	8
1)BVTV	_	_	_	_	_	_	_	_
2)ConnD	0.49 *	_	_	_	_	_	_	_
3)TbN	0.67***	0.88***	_	_	_	_	_	_
4)TbTh	0.37	-0.41	-0.29	_	_	_	_	_
5)TbSp	-0.66***	-0.77***	-0.96***	0.25	_	_	_	_
6)Kid-Cd	-0.30	-0.02	-0.30	-0.16	0.39	_	_	_
7)Kid-Pb	0.12	0.46*	0.35	-0.14	-0.29	0.29	_	_
8)Kid-Zn	0.53**	- 0.05	0.04	-0.30	-0.14	-0.05	0.17	_

P≤0.05* P≤0.005** P≤0.0005***

Abbreviations: BVTV=Bone Volume/Total Volume (bone volume fraction); ConnD=Connectivity density; TbTh= Trabecular thickness; TbSp= Trabecular separation; and TbN= Trabecular number.

Table 4. 3: Pearson's correlation coefficents for trabecular bone microarchitecture parameters of L_4 and kidney metal concentrations from *Peromyscus leucopus* collected at Tar Creek Superfund Site (TCSFS) Beaver Creek. (n=8).

Variables	1	2	3	4	5	6	7	8
1)BVTV	_	_	_	_	_	_	_	_
2)ConnD	0.81*	_	_	_	_	_	_	_
3)TbN	0.87**	0.95***	_	_	_	_	_	_
4)TbTh	0.19	-0.31	-0.28	_	_	_	_	_
5)TbSp	-0.82*	-0.89**	-0.98***	0.31	_	_	_	_
6)Kid-Cd	-0.49	-0.52	-0.67*	0.21	0.72*	_	_	_
7)Kid-Pb	0.58	0.21	0.41	0.46	-0.51	-0.43	_	_
8)Kid-Zn	-0.24	-0.21	0.01	-0.47	-0.14	-0.13	0.22	_

P≤0.05* P≤0.005** P≤0.0005***

Abbreviations: BVTV=Bone Volume/Total Volume (bone volume fraction); ConnD=Connectivity density; TbTh= Trabecular thickness; TbSp= Trabecular separation; and TbN= Trabecular number.

Table 4.4: Pearson's correlation coefficents for trabecular bone microarchitecture parameters of L_4 and kidney metal concentrations from *Peromyscus leucopus* collected at collected from Oologah Wildlife Management Area (OWMA), (n=8).

Variable	1	2	3	4	5	6	7	8
1)BVTV	_	_	_	_	_	_	_	_
2)ConnD	-0.02	_	_	_	_	_	_	_
3)TbN	0.18	0.75*	_	_	_	_	_	_
4)TbTh	0.50	-0.78*	-0.67*	_	_	_	_	_
5)TbSp	-0.07	-0.62	-0.97***	0.70*	_	_	_	_
6)Kid-Cd	0.25	-0.009	0.49	-0.21	-0.63	_	_	_
7)Kid-Pb	-0.19	0.02	0.20	-0.16	-0.26	0.22	_	_
8)Kid-Zn	-0.19	0.03	0.29	-0.25	-0.39	0.63*	0.98***	_

P≤0.05* P≤0.005** P≤0.0005***

Abbreviations: BVTV=Bone Volume/Total Volume (bone volume fraction); ConnD=Connectivity density; TbTh= Trabecular thickness; TbSp= Trabecular separation; and TbN= Trabecular number.

Table 4. 5: Pearson's correlation coefficents for trabecular bone microarchitecture parameters of L_4 and kidney metal concentrations from *Peromyscus leucopus* collected at Sequoyah National Wildlife Refuge (SNWR) , (n=8).

Variables	1	2	3	4	5	6	7	8
1)BVTV	_	_	_	_	_	_	_	_
2)ConnD	0.48	_	_	_	_	_	_	_
3)TbN	0.62	0.91***	_	_	_	_	_	_
4)TbTh	0.67*	-0.14	0.06	_	_	_	_	_
5)TbSp	-0.58	-0.92***	-0.98***	-0.04	_	_	_	_
6)Kid-Cd	0.10	-0.14	-0.06	0.27	0.05	_	_	_
7)Kid-Pb	-0.19	0.30	0.41	-0.34	-0.41	-0.0002	_	_
8)Kid-Zn	-0.17	0.02	0.12	-0.46	-0.21	0.07	0.67*	_

P≤0.05* P≤0.005** P≤0.0005***

Abbreviations: BVTV=Bone Volume/Total Volume (bone volume fraction); ConnD=Connectivity density; TbTh= Trabecular thickness; TbSp= Trabecular separation; and TbN= Trabecular number.

CHAPTER V

KIDNEY CONCENTRATIONS OF LEAD, ZINC AND CADMIUM WERE NOT CORRELATED WITH METALLOTHIONEIN-1 IN *PEROMYSCUS LEUCOPUS* FROM TAR CREEK SUPERFUND SITE

Introduction

Heavy metals are toxic ions that affect human organ functions such as liver and kidney functions (Shore & Rattner, 2001). With chronic exposure, lead and cadmium can accumulate in kidneys and cause renal dysfunction (Chang et al., 1996). When lead and cadmium accumulate in organs in vivo, they induce detoxification functions. Cadmium damages kidneys and reduces the reabsorption function in the proximal tubules (Damek-Poprawa & Sawicka-Kapusta, 2003).

Also, cadmium disrupts calcium metabolism which causes osteomalacia (Kido et al., 1993). Unbound cadmium is more toxic than bound cadmium (Chang et al., 1996). Cadmium has high binding capacity with metallothionein protein (Sonne et al., 2009).

Lead ions accumulate primarily in kidneys and affect function of other metals such as zinc and iron (Damek-Poprawa & Sawicka-Kapusta, 2003). Previous studies defined heavy metals toxicity based on binding affinity to essential protein groups such

as amino and sulfhydryl groups (Durnam & Palmiter, 1981; Ishida, Stupp, Turcios-Ruiz, Williams, & Espinoza, 2012)

Protein synthesis is one of the main functions used as a biomarker for heavy metals exposure, and metallothionein (MT) is one of the most common biological detoxification biomarkers. Metallothionein is measured in biomonitor mammals as a metal-binding protein (Fritsch et al., 2010).

MT is a cysteine-rich, low molecular weight protein that is important in physiological functions (Ishida et al., 2012). MT protein was discovered in 1957 by Margoshes and Valee in horse renal cortex (Margoshes & Vallee, 1957). MT has seven metal binding sites with the α-domain region binding to four metals and the β-domain binding to three metals. The α-domain contains 11-12 cysteines and the β-domain consists of 9 cysteine amino acids. MTs mainly localize in cell cytoplasm and organelles such as the nucleus and lysosomes of kidney cells (Sigel, Sigel, & Sigel, 2009).

MT is classified based on different characteristics such as molecular weight, encoding gene, chromosomes, amino acid sequences, metal binding, and other functions among different animal species (Thirumoorthy et al., 2011).

Historically, the first classification of MT was done by Fowler, Hildebrand, Kojima, and Webb (1987) based on the MT primary structure. MT is classified into three classes. Class I is based on the MT proteinaceous characteristic in the cysteine location, which is highly conserved in mammals such as the horse. Studies showed that there are some differences in mollusca and crustaceans which belong to this class such as crabs and lobsters. Class II involves proteinaceous MT in animal groups that lack the similarity

with the mammalian group. Class III classification involves non-proteinaceous MTs such as plant-metal-binding peptides (Phylochelatins).

Another classification of MT isoforms classified MT into major and minor groups. The major group includes MT-1 and MT-2 which have identical structure and similar metal binding capacity for ions such as cadmium and zinc, while the minor group includes MT-3 and MT-4 which involve specific functions regarding cell type (Thirumoorthy et al., 2011). For example MT-3 is classified as unique in terms of its function in nerve cells as a Growth Inhibitory Factor. According to this classification, we can track the gene structures differentiation within MT isoforms.

The final classification form discussed here is based on MT gene structure. Vertebrate MT is classified into 4 isoforms, which include MT1, MT2, MT3, and MT4 in addition to 13 MT-like proteins (Sigel et al., 2009). Human MT proteins are categorized based on gene structure such as MT1 isoform, which has many genes such as MT-1A, MT1B, MT1E, MT1F, MT1G, MT1H, and MT1X; MT2a and MT2b gene, MT3 gene and MT4 gene (Sigel et al., 2009; Sonne et al., 2009). Based on MT functions, both MT1 and MT2 genes are expressed in liver and kidney tissues and are abundant in these tissues related to the main function for heavy metal detoxification (Sigel et al., 2009).

In addition to the liver and kidney, MT1 and MT2 genes are expressed in other soft tissues like testis, pancreas, and blood lymphocytes that involve detoxification functions. MT3, which exists in human and other vertebrates' brain and nervous tissues, functions as an Inhibitory Growth Factor (IGF). MT4 is the most abundant protein in

keratinized epithelia tissues to maintain Cu homeostasis in these tissues (Sonne et al., 2009).

MT proteins have many crucial functions in human and other living organisms including heavy metals detoxification and protection against oxidative stress.

MT has a metal binding capacity in that MT has the ability to bind many metals including cadmium, zinc, mercury, copper, arsenic, silver, lead and other metals. This binding mechanism occurs through a chronological, not a cooperative mechanism. The ability of MT to exchange other metals like Cd, Pb, and Cu with zinc explains how MT reduces metal toxicity (Davis & Cousins, 2000). Metals are absorbed through the respiratory and digestive system and are transported to other soft tissues via blood. MT has the ability to capture and bind with metals from the plasma (e.g. Cd ions), and due to the fact that the MT has low molecular weight, it is easily to be filtered by the kidney glomerular membrane; then Cd is released via urine and MT is reabsorbed by proximal renal tubules (Sigel et al., 2009).

Different studies show that MTs are able to repair Cd^{2+} poisoned proteins and keep homeostasis by metal ion exchange, and this function can protect protein from metal toxicity. Metal concentrations such as Cu, Cd, and Zn and MT-binding capacity in liver, kidney, and brain tissues were significantly different among three species of arctic seal (Sigel et al., 2009). MT metal-binding capacity was the highest in the kidneys compared to the liver and brain for all three species indicating variable sensitivity in these organs among these species (Sonne et al., 2009). MTs in marine animals respond differently to metals due to the role of metal biokinetics- MT binding, dissolving-uptake rate, diet, and

efflux rate (W.-X. Wang & Rainbow, 2010). The second main mechanism deals with homeostasis functions of MT including detoxification and metal storage mechanisms at the cellular level such as zinc metabolism and metal accumulation by MT protein. Cells produce apometalloproteins such as zinc finger proteins and enzymes which have metal binding capacity (Davis & Cousins, 2000).

Moreover, MT protein has an antioxidant function related to its structure. MT has a protective function against oxidant and electrophile exposure that otherwise react with sulfahydryl groups in the MT molecule (Ruttkay-Nedecky et al., 2013). Cysteine residues in MTs can detect and capture oxidant radicals such as superoxide and hydroxyl radicals (Davis & Cousins, 2000). The cysteine will be oxidized to cystine, and metals that are bound to cysteine will be released to the cell cytoplasm. MT-Zn binding gives MT redox cycle properties, by which the cysteine can induce MT oxidoreductive properties (Ruttkay-Nedecky et al., 2013). Thornalley and Vašák (1985) observed that MT-1 in rabbit liver cells that contain Cd and Zn worked as a scavenger to reduce free hydroxyl (OH) and superoxide (O^{-2}) radicals that are produced by the xanthine oxidase reaction. Moreover, MT structure has a cysteinyl thiolate group which gives MT protein the ability to scavenge hydroxyl radicals (Ruttkay-Nedecky et al., 2013).

Another functional mechanism of MT discussed here has to do with its inducement by stresses like microbial infection and physical stress. In addition, MT works as an anti-tumor factor. Studies show that any problem of MT expression and function may lead to malignant transformation of cells and increase the expression of MTs in breast, colon, kidney, liver, skin (melanoma) and other cancers (Chang et al., 1996). Davis and Cousins (2000) found low levels of MT expression in hepatocellular

carcinoma and liver adenocarcinoma. Conclusions derived from the findings of these studies highlight the fact that high MT expression explains MT resistance to chemotherapy drugs as anti-tumor factors. MT levels appear low in malignant tissue because of the influence of MT on zinc binding domain of the p53 tumor suppressor and because DNA methylation may suppress MT expression in tumors independently of MT factor (MTF) or other mediators of MT expression.

MT gene structure includes three exons constituting, which includes, Cys-rich N-terminal domain, the central spacer and the Cys-rich C-terminal domain. Number and position of Cys-residues inside the two terminal domains represent useful molecular regions to classify the MT genes into different types (Davis & Cousins, 2000). Therefore, the main differences in MT genetic organization between humans and other species include the presence of additional cysteine amino acids, number of exons and introns, DNA duplication, molecular weight and Cys residues. The promoter of specific sequences regulates MT expression, metal response elements (MRE), glucocorticoid response elements (GRE), GC-rich boxes, and basal level elements (BLE). These differences show MT diversity in taxonomic range which represents high-heterogeneity and less homology sequence; however, the homology is found within some taxonomic groups such as the differences among vertebrate species (Davis & Cousins, 2000). MT binds three Zn (II) ions in its ß- domain and four Zn ions in the ơ-domain. MT gene is regulated by metals through (MRE) sequence of DNA in the MT promoter. The Metallothionein Transcriptional Factor-1 (MTF-1) is a multiple finger protein which is stimulated by Zn ion presence (Davis & Cousins, 2000; Sonne et al., 2009).

Moreover, a number of studies show that Zn and Cd MT binding make MT more resistant to proteolysis (Davis & Cousins, 2000). MT synthesis is similar among species, but there are some differences in gene expression in tissues. For example, both MT1 and MT2 express in liver and kidney tissues and they are abundant in these tissues related to the main function for heavy metal detoxification (Sigel et al., 2009). MT proteins vary considerably among different species according to the cysteine residues, domains, and central structure (Tanguy, Mora & Moraga, 2001). The genomic differences between humans and other species result from the differences in gene organization (Sigel et al., 2009). Some studies on the MT genes structure and functions compared results with previous studies and reported that cystein rich and metal binding make the conservation between all species with some differences (Sigel et al., 2009).

Additionally, the variability among MTs function is based on the mechanism of the specific molecular structure and function of each one (Sigel et al., 2009; Sonne et al., 2009). MT expression in response to metal exposure is regulated by the zinc finger transcription factor (MTF-1) that binds with MT MRE (Sonne et al., 2009). Metal exposure inhibits MRE and that disrupts MT transcription. However, MTF-1 can be activated by Zn ion and interact with other proteins that regulate MT transcription (Davis & Cousins, 2000). At the late G1 phase and early S Phase, MT expression is on the highest level and mRNAs for MT-1 and MT-2 genes remain stable by metal induction. However, MT gene transcription is inhibited by DNA methylation (Sonne et al., 2009).

MT works as a reservoir for apometalloproteins that function as transcription factors. Acquired zinc exchanges faster to support zinc metabolism, storage and donation. This mechanism is supported by enzymatic activity like apo-carbonic anhydrase and apo-

carboxypeptidase. Moreover, MT enhances metal exchange function by oxidized glutathione (GSSG) and 1:1 Zn-glutathione (GSH) complex. This mechanism enhances Zn transfer from MT to other apometalloproteins and this pathway implies MT induction and gene expression by metal oxidants and electrophiles and other cellular zinc sensitive processes, proliferations, and apoptosis events (Sonne et al., 2009).

Beside metal exposure, MT transcription is induced by environmental oxidative stressors which modulate cellular signal transduction cascades (Sigel et al., 2009). Zinc activates and induces more MT expression to control oxidative stress. Anti-oxidant response elements (ARE) enhance MT expression in response to reactive oxygen species and they work as synergistic elements with (MRE). The elements are activated by signal transducers and activators of transcription proteins (STAT) by cytokine signaling (Davis & Cousins, 2000).

Moreover, two GRE sequences 17 kb upstream in the 5′flanking region of mouse MT promoter regulate and induce MT expression in response to glucocorticoid hormones and cytokines through protein kinases in hepatocytes. MT promoter includes 1-2 elements that start or enhance transcription rates like Sp1, AP-1, and AP-2. Cyclic-AMP regulation may be mediated through AP-2. Interleukins (IL)-1, cAMP and glucagon molecules increase MT mRNA levels and zinc metabolism in hepatocytes (Davis & Cousins, 2000).

Material and methods

Kidney sampling

Frozen kidneys samples of *Peromyscus leucopus* species were provided from the vertebrate collection in the Zoology Department at OSU. Kidney samples were saved in a

liquid nitrogen tank before subsampling. The kidneys were subsampled into two parts: one part was cut in a petri dish on wet ice by stainless-steel scalpel to approximately one tenth of the whole tissue and saved in labeled micro tubes for metallothionein-1 analysis and then returned back to the freezer at -80°C without thawing. The second part of the kidney was stored in microtubes and kept in the refrigerator at -4°C for metal analysis.

Tissues digestion

Microwave acid digestion

Kidney samples were weighed using a digital balance. Small microwave tubes were used for kidney sample digestion (Milestone, Inc, Shelton, Connecticut). Each sample was weighed; 1 ml of concentrated (99.99) HNO_3 was put in the tube and then 0.15 ml of H_2O_2 was added to the same tube. The program was set for the USEPA Method Number 5031 A. The microwave operation took 50 minutes to finish digestion at a temperature of 100° C. One ml of acid solution was transferred into labeled microtubes for metal analysis.

Metal analysis

ICP-MS

Pb, Cd, and Zn concentrations in kidney specimens from both contaminated and uncontaminated sites were determined by ICP-MS according to USEPA method 6010 (United States Environmental Protection Agency USEPA, 1996). Terbium was used as internal standard.

Metallothionein-1 analysis

Tissue homogenization

Peromyscus leucopus kidney samples were homogenized using Invitrogen MT protocols (Uscn, Life Science Inc., Houston, Texas, 2015). Each sample was crushed in liquid nitrogen and transferred into a pre-chilled 5 ml culture tube. Ten µl of protease inhibitor (Sigma Aldrich, St. Louis, Missouri, Cat. No 78441B) was mixed with 1 ml phosphate buffered saline (PBS) and cooled using wet ice. Tissues were homogenized at low speed for ~20 seconds, and transferred into micro centrifuge tubes and centrifuged at 14,000 xg at 4°C for 15 min. The supernatants were removed and four 80 µl aliquots were stored at -80°C for total protein analysis and for metallothionein-1 analysis using ELISA procedures.

Total protein analysis

Lowry method

Total protein concentrations in kidney specimens of *Peromyscus leucopus* were determined using a commercial modified Lowry protein assay kit (Thermo Scientific, No., 23240, Rockford, Illinois) (Lowry et al., 1951). Samples were diluted using Phosphate Buffered Saline (PBS). The concentrations (µg/ml) of diluted specimens were detected at OD 750 nm and were analyzed on Prism program, 6 (GraphPad Software) against a bovine serum albumin standard curve.

Enzyme-linked immunosorbent assay (ELISA)

ELISA was used to determine metallothionien-1 (MT-1) concentrations in kidney samples of *Peromyscus leucopus*. A commercial mouse (*Mus musculus)* MT-1 kit was used following the manufacturers, protocol (Uscn Life Science Inc, Huston, Texas).

Samples were diluted in PBS as appropriate for the standard curve dilutions and MT-1 concentrations were measured with a microplate reader at 450 nm. MT-1 concentrations were expressed as microgram per mg of protein. The plot was based on the absorbance (nm) of the standards against the standard concentrations (pg/ml).

Results

Metal concentrations in kidney

Kidney specimens of *Peromyscus leucopus* were provided from the OSU Zoology Vertebrate Collection. The samples were collected from a contaminated site, Beaver Creek (BC) at Tar Creek Superfund Site (TCSFS) (n=18), and two reference sites Oologah Wildlife Management Area (OWMA) (n=15), and Sequoyah National Wildlife Refuge (SNWR) (n=16). Metal concentrations (Zn, Cd, and Pb) in kidney samples (mg/gm) are presented in (mean±SE) Table (5.1). The zinc concentrations in kidney samples (mg/gm) were compared between contaminated TCSFS (BC) and reference sites (OWMA & SNWR), and the results showed no significant differences.

Cadmium concentrations (mg/gm) in kidney samples in TCSFS and reference sites were significantly different. In TCSFS as a contaminated site, higher cadmium concentrations (4.62±0.71 mg/gm) were recorded than at the two reference sites ($P \leq 0.0005$), and the results showed no differences between the two reference sites, SNWR (0.53 ±0.06) and OWMA (0.53 ±0.10) mg/gm. As expected, Pb concentrations in kidney samples of TCSFS samples (0.57±0.10 mg/gm) were higher than in the two reference sites, SNWR and OWMA (0.05±0.01 and 0.04±0.01 mg/gm) respectively.

The body weight of *P. leucopus* was variable in this study. However, there were no statistically differences in body weight between contaminated and uncontaminated sites (Table 5.4).

The statistical analysis (one way analysis of variance followed by mean separation test) showed that there are significant differences between TCSFS site and the reference sites' dependent variables (Cd and Pb) mean) ($P \leq 0.0005$). Means were significantly different between contaminated site and uncontaminated sites (Table 5.1).

Metallothionein-1 concentrations in kidney

Kidney specimens of *Peromyscus leucopus* were used to determine total protein concentration. The results did not show a statistically significant correlation between heavy metal concentrations and metallothionein-1 in kidney specimens of *P. leucopus* from TCSFS or from reference sites. There were no significant differences in metallothionein-1 concentrations (µg/mg) at TCSFS and reference sites SNWR and OWMA as shown in Table 5.2. Variability in dose and length of exposure to the heavy metal in those three sites may be a reason that the metallothionein-1 concentrations did not show significant differences in this species of wild small mammals. MT-1 concentration (µg/mg protein) in *P. leucopus* specimens from TCSFS and SNWR and OWMA showed high variability and this variability was analyzed in mineral concentration (Cd, Pb, and Zn) of these three sites. The statistical analysis showed no significant differences between MT-1 µg/mg protein at the contaminated site (TCSFS) (0.15±0.07) and reference sites (SNWR & OMWA) (0.08±0.02& 0.22±0.06) respectively. The results also showed no statistically significant correlation between kidney mineral concentrations and metallothionein concentrations (Table5.3).

Discussion

Heavy metals (Cd, Pb, and Zn) accumulate in vital organs such as the liver and kidneys. *Peromyscus leucopus* is one of the biomonitor species that is used by environmental, physiological, and ecotoxicological studies to predict and assess the effects and alterations on human health. This study analyzed the heavy metals concentrations from frozen kidney specimens of *P. leucopus* that were collected from contaminated site and compared with reference sites.

Heavy metal concentration findings in this study were in agreement with previous studies. In Australia, an area highly contaminated with Cd, Cu, Hg, Zn, Pb, and Se caused severe problems of metal toxicity. The evidence showed renal damage in Australian bottlenose dolphins (*Tursiops aduncus*) that were collected from the contaminated site in south Australia (Lavery et al., 2009). Fritsch et al. (2010) studied the effects of heavy metals and accumulation in small wild mammals such as the wood mouse (*Apodemus sylvacticus)*, bank vole (*Myodes glareolus*), common shrew (Sorex araneus), pygmy shrew (*Sorex minutus*), common pine vole (*Microtus subterraneus*), greater white–toothed shrew (*Crocidura russula*), and field vole (*Microtus agrestis*) in North France surrounding the Metaleurop-Nord smelter, a contaminated area with Cd, Pb, and Zn. They reported a significant relationship between the presence of heavy metals in soil and the levels of heavy metals in small mammals' liver and kidney in different species. Heavy metal accumulations were recorded in shrews more than the rodent species.

Oxidative stress status, clinical markers, and blood lead, and zinc levels were examined in Zn-Pb mine workers and in a healthy control group (Malekirad et al., 2010).

Pb blood levels recorded in the worker group were within normal levels (0.9-3µg/dl). However, the Zn-Pb mine workers' results showed elevation in blood Zn and Pb, activity of superoxide dismutase, myeloperoxidase, glutathione and reductase as well as lipid peroxidation comparing with the control group. Severe clinical problems were reported in the worker group such as memory impairment, insomnia, headache, fatigue, deafness, agitation, tremors, stress, and reduced concentration. However, the results showed lower DNA damage in Zn-Pb workers than in the control group. Malekirad et al. (2010) concluded that the exposure to the combination of Pb and Zn resulted in decreasing Pb toxicity in Zn-Pb workers. Some parameters of *Peromyscus leucopus* like tooth abnormalities, genetic structure, and ano-genital distance have already been examined for these animals (Hays, 2010), and another study investigated the population dynamics and demographics (Phelps & McBee, 2010).

Small mammals are close to the environmental contaminants which may be related to their vegetation and habitat. Read and Martin (1993) found high heavy metal accumulation in shrews, low Cd accumulation in pygmy shrews (*Sorex minutus)*, and high Cd accumulation in the common shrew *(Sorex araneus)*. Their study analyzed the environmental conditions with physiological evidence and biomarker differences that were correlated to contaminant effects and bioaccessibility such as consumption, accumulation, and excretion.

Metal levels such as Cu, Cd, and Zn were increased in liver of striped red mullet (*Mullus surmuletus*) and golden grey mullet (*Liza aurata*) that collected from Kastela Bay, Middle Damatia (Filipović & Raspor, 2003). The elevation of liver metal levels was significantly correlated with age and weight parameters in these species.

Physiological alterations and responses were recorded in the elevation of MT levels in small mammals. Although MT analysis has been conducted on numerous vertebrate and invertebrate aquatic species, there has been little work done on metallothioneins in small mammals from contaminated sites in North America.

Exceptions are the Old World species, the greater white-toothed shrew (*Crocidura russula)*, the bank vole (*Myodes glareolus*), and the wood mouse (*Apodemus sylvaticus*) (Fritsch et al., 2010). *A. sylvaticus* shows the greatest ecological similarity to *P. leucopus* and provides an important point of comparison with this study. Fritsch et al. (2010) found that metallothionein levels increased with increasing soil metal levels in the liver and decreased in the kidneys in *M. glareolus*, *S. araneus*, and *S. minutus*. Cadmium absorption and MT induction were increased in kidneys, liver and duodenum of laboratory mice during lactation (Solaiman, Jonah, Miyazaki, Ho, & Bhattacharyya, 2001). Comparing with the control group, protein synthesis in the liver of mice treated in vitro increased after $NiCl_2$ injection for 14 days, however, a Pb $(CHCOO)_2$ injection group did not show any change in protein synthesis (Šveikauskaitė et al., 2014). This shows that the increase in protein concentrations in $NiCl_2$ may relate to the elevation of metallothionein and other protein concentrations; the Pb $(CHCOO)_2$ injection group may be adapted to the Pb $(CHCOO)_2$ toxicity (Šveikauskaitė et al., 2014).

Metallothionein protein has been analyzed in aquatic wild environment. Metallothionein and metal concentrations were analyzed in the liver, brain, and kidneys of wild fish species, striped red mullet (*Mullus surmuletus*) and golden grey mullet (*Liza aurata*) (Filipović & Raspor, 2003). The results in this study showed statistical differences in metallothionein concentrations in liver and kidney tissues, and the

metallothionein concentration results were higher in *M. surmuletus* kidney samples (31.1±8.0µg mg^{-1} protein). Filipović and Raspor (2003) concluded that there was a significant positive correlation between metal concentrations in *M. surmuletus* liver and metallothionein concentrations such as Zn value, and the liver results showed 69% correlation between Cu value and metallothionein induction. Their study recorded no statistically significant correlation between metal concentrations and metallothionein in brain and kidney samples of *M. surmuletus* and *Liza aurata* species. Liver has the main detoxification function more than other organs to produce metallothionein and reduce metal toxic effects (Filipović & Raspor, 2003; Olsson, Gerpe, & Kling, 1999).

Alterations in populations' genetic structure, mutation, or reduction in fecundity may occur due to contaminant exposure (Bickham, Sandhu, Herbert, & Chikhi). Hays (2010) did not record an increase in the frequency of structure chromosomal aberrations, but the study recorded alterations in genetic structure in the *P. leucopus* population from TCSFS.

The present study did not record statistical differences in kidney metallothionein-1 concentrations but did record some variations. In addition to the wild conditions and physical stresses, these differences may relate to animal, age, weight, and sex. It may be better to analyze metallothionein concentrations in both liver and kidney tissues because liver is the first organ to detoxify heavy metals.

Metallothionein-1 and heavy metal concentrations did not show a significant correlation, and we assumed that the elevation in Zn concentration exposure decreased Cd and Pb toxicity and MT-1 induction at TCSFS.

Table 5.1: Kidney Zinc, cadmium and lead concentrations (mean±SE) from *Peromyscus leucopus* collected at Tar Creek Superfund site (Beaver Creek, BC), Oologah Wildlife Management Area (OWMA), and Sequoyah National Wildlife Refuge (SNWR).

Mineral /Site	n	SNWR	OWMA	BC
Zn (mg/g)	18	28.4±4.6 [a]	18.5±3.8 [a]	23.1±3.3 [a]
Cd(mg/g)	15	0.53 ±0.06[b]	0.53 ±0.08 [b]	4.62±0.71 [a]
Pb(mg/g)	16	0.05±0.01 [b]	0.04 ±0.01 [b]	0.57±0.10 [a]

Means in a row not sharing a superscript are significantly different from each other ($P<0.0001$).

Table 5.2: Metallothionein concentration's (mean±SE) of kidney from *Peromyscus leucopus* collected from Tar Creek Superfund site (Beaver Creek, BC), Oologah Wildlife Management Area (OWMA), and Sequoyah National Wildlife Refuge (SNWR).

MT-1(µg/mg protein)	n	Mean±SE	Minimum	Maximum
Beaver Creek (BC)	14	0.15±0.07[a]	0.02	0.1
Oologah Wildlife Management Area (OWMA)	15	0.08±0.02[a]	0.00	0.3
Sequoyah National Wildlife Refuge (SNWR)	14	0.22±0.06[a]	0.04	0.7

Abbreviations: Mt-1 /(µg/mg protein)= metallothionein-1 per mg protein (µg/mg).

Means in a row sharing the same superscript are not significantly different from each other.

Table 5.3: Pearson's correlation Coefficients between kidney metal concentrations (Cd, Pb, and Zn) and Metallothionein-1 (µg/mg) in *Peromyscus leucopus* collected at Tar Creek Superfund site (Beaver Creek, BC), Oologah Wildlife Management Area (OWMA), and Sequoyah National Wildlife Refuge (SNWR).
TCSFS (BC), and reference sites (n=43-44).

Variable	1	2	3	4
1) Kid-Zn	_	_	_	_
2) Kid-Cd	-0.00	_	_	_
3) Kid-Pb	-0.06	0.59***	_	_
4) Mt-1 (µg/mg)	-0.09	-0.11	-0.04	_

P≤0.05* P≤0.005** P≤0.0005***

Abbreviation: Mt-1= metallothionein-1 per mg protein (µg/mg). Kid= kidney.

Table 5.4: Body weight (g) (mean±SE) of *Peromyscus leucopus* collected from Tar Creek Superfund site (Beaver Creek, BC), Oologah Wildlife Management Area (OWMA), and Sequoyah National Wildlife Refuge (SNWR).

Weight/g	n	Mean±SE	Minimum	Maximum
Beaver Creek (BC)	17	25.6±0.8[a]	19.0	31.0
Oologah Wildlife Management Area (OWMA)	16	23.6±1.3[a]	16.0	32.0
Sequoyah National Wildlife Refuge (SNWR)	16	23.4±1.2[a]	15.0	34.0

Means in a row sharing the same superscript are not significantly different from each other.

CHAPTER VI

SUMMARY AND CONCLUSIONS

TCSFS is a highly contaminated site with heavy metals (Cd, Pb, and Zn), and the species that have been used in this study is one of the most common species at the site. This study found higher metal concentration in soil samples from TCSFS compared with the two reference sites. However, the bone microarchitecture analyses of *Peromyscus leucopus* L_4 vertebra of contaminated and uncontaminated sites did not show high correlation between bone parameters and metal concentration in the kidney. The elevation of Zn concentration and the combination of heavy metals exposure may decrease Cd and Pb toxicity. This study raises the possibility that this species *P. leucopus* adapted to the heavy metal exposure.

The body weight of *P. leucopus* as a wild mammal was variable in this study. However, there were no statistically significant differences in body weight between contaminated site and uncontaminated sites. In addition, the time and dose of exposure are difficult to determine in this species as a field animal. This study has wide implications because responses to chronic exposure to heavy metals in *Peromyscus leucopus* may help predict impacts on the physiological processes of other exposed

species and human health. Heavy metal accumulations at TCSFS impact both human and wildlife species. The increase of Cd, Pb, and Zn concentrations in TCSFS soil samples were the main reason for the elevation of these metals in kidneys of small wildlife mammals based on their habitat and food sources.

This study showed a correlation between metals exposure, accumulation, physiological alterations, and responses in small mammals. The analysis of heavy metal and metallothionein concentrations in the kidney of small mammal's tissues may compromises the picture on environmental quality and physiological alteration. Bone microarchitecture evaluation and kidney mineral concentrations clarified the relationship between environmental chronic exposure and effects on small mammal species.

The metallothionein concentration assessment could be a great indicator to the metal contamination in the environment (Atli & Canli, 2007). In the current study, the contaminated site (TCSFS) showed significant elevation in metal concentrations in soil samples, kidney specimens, and L_4 trabecular bone separations of *P. leucopus*. These findings confirmed the relationship between the physiological functions and environmental conditions. The measurement of a specific biomarker is a very important approach to determine the specific endpoint of concern and to reach conclusions that help human health and environmental sustainability.

The present study established the contaminants' effects on small mammals in the wildlife. The results of this study were unbiased and there were no constraints or limitations on the small mammals as with lab conditions. However, due to the field

conditions, this study had several uncontrolled variables such as field contaminant uptake, dose and length of exposure to the heavy metals, and animal age.

For future studies, analyzing the bone microarchitecture and the heavy metal exposure of wildlife small mammals, it could be better to conduct a laboratory study to analyze control of uncontaminated small mammals and treat different groups by different heavy metal combinations such as Zn, Cd and Pb to determine the effect of combination exposures. Future research on heavy metal effects on the physiological alterations and detoxification functions might be better conducted under combined lab and field conditions using different tissues such as blood plasma, brain and liver. It may be beneficial for future research in environmental physiology and ecotoxicology to add more information about the antioxidant and homeostasis cellular functions. Little research has been conducted concerning other metallothionein inductions and functions such as MT3 and MT4; therefore, analyzing these MTs will be an important future project using *P. leucopus* and human samples. To the best of my knowledge, this is the first study to provide information regarding MT-1 concentration and bone microarchitecture evaluation in *P. leucopus.*

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APPENDICES

Table 1: Shows soil samples collecting directions on google map
TCSFS=Tar Creek Super Fund Site =Beaver Creek (BC)
OWMA= Oologah Wildlife Management Area
SNWR=Sequoyah National Wildlife Refuge

Sample	Site	Date	Map
T1-1	TCSFS(BC)	11/21/2012	36° 55′ 52″N,
T1-2	TCSFS(BC)	11/21/2012	94° 45′44′W
TT2-1	TCSFS(BC)	11/21/2012	36° 56′ 22″N,
T2-2	TCSFS(BC)	11/21/2012	94° 45′37′W
T3-1	TCSFS(BC)	11/21/2012	36° 55′ 37″N,
T3-2	TCSFS(BC)	11/21/2012	94° 45′41′W
T4-1	TCSFS(BC)	11/21/2012	36° 55′ 37″N,
T4-2	TCSFS(BC)	11/21/2012	94° 45′41′W
T5-1	TCSFS(BC)	11/21/2012	36° 56′ 19″N,
T5-2	TCSFS(BC)	11/21/2012	94° 45′34′W
T6-1	TCSFS(BC)	11/21/2012	36° 56′ 18″N,
T6-2	TCSFS(BC)	11/21/2012	94° 45′34′W
T7-1	TCSFS(BC)	11/21/2012	36° 56′ 18″N,
T7-2	TCSFS(BC)	11/21/2012	94° 45′34′W
T8-1	TCSFS(BC)	11/21/2012	No service
T8-2	TCSFS(BC)	11/21/2012	No service
O1-1	OWMA	11/21/2012	36° 41′ 25″N,
O1-2	OWMA	11/21/2012	95° 37′50′W
O2-1	OWMA	11/21/2012	36° 41′ 25″N,
O2-2	OWMA	11/21/2012	95° 37′56′W
O3-3	OWMA	11/21/2012	36° 41′ 27″N,
O3-2	OWMA	11/21/2012	95° 37′44′W

Acknowledgements reflect the views of the author and are not endorsed by committee members or Oklahoma State University.

O4-1	OWMA	11/21/2012	36° 41′ 18″N,
O4-2	OWMA	11/21/2012	95° 36′29′W
O5-1	OWMA	11/21/2012	36° 41′ 25″N,
O5-2	OWMA	11/21/2012	95° 37′50′W
O6-1	OWMA	11/21/2012	36° 41′ 18″N,
O6-2	OWMA	11/21/2012	95° 36′29′W
O7-1	OWMA	11/21/2012	36° 41′ 2″N,
O7-2	OWMA	11/21/2012	95° 37′44′W
O8-1	OWMA	11/21/2012	36° 41′ 18″N,
O8-2	OWMA	11/21/2012	95° 36′29′W
S1-1	SNWR	12/8/2012	35° 26. 089` N,
S1-2	SNWR	12/8/2012	049° 58. 429` W
S2-1	SNWR	12/8/2012	-
S2-2	SNWR	12/8/2012	-
S3-1	SNWR	12/8/2012	35° 26. 884` N,
S3-2	SNWR	12/8/2012	49° 58. 450` W
S4-1	SNWR	12/8/2012	35° 26. 909` N,
S4-2	SNWR	12/8/2012	049° 58. 470` W
S5-1	SNWR	12/8/2012	35° 26. 885` N,
S5-2	SNWR	12/8/2012	49° 58. 460` W
S6-1	SNWR	12/8/2012	35° 26. 889` N,
S6-2	SNWR	12/8/2012	49° 58. 472` W
S7-1	SNWR	12/8/2012	35° 26. 893` N,
S7-2	SNWR	12/8/2012	49° 58. 475` W
S8-1	SNWR	12/8/2012	35° 26. 890` N,
S8-2	SNWR	12/8/2012	49° 58. 480` W

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FIGURES

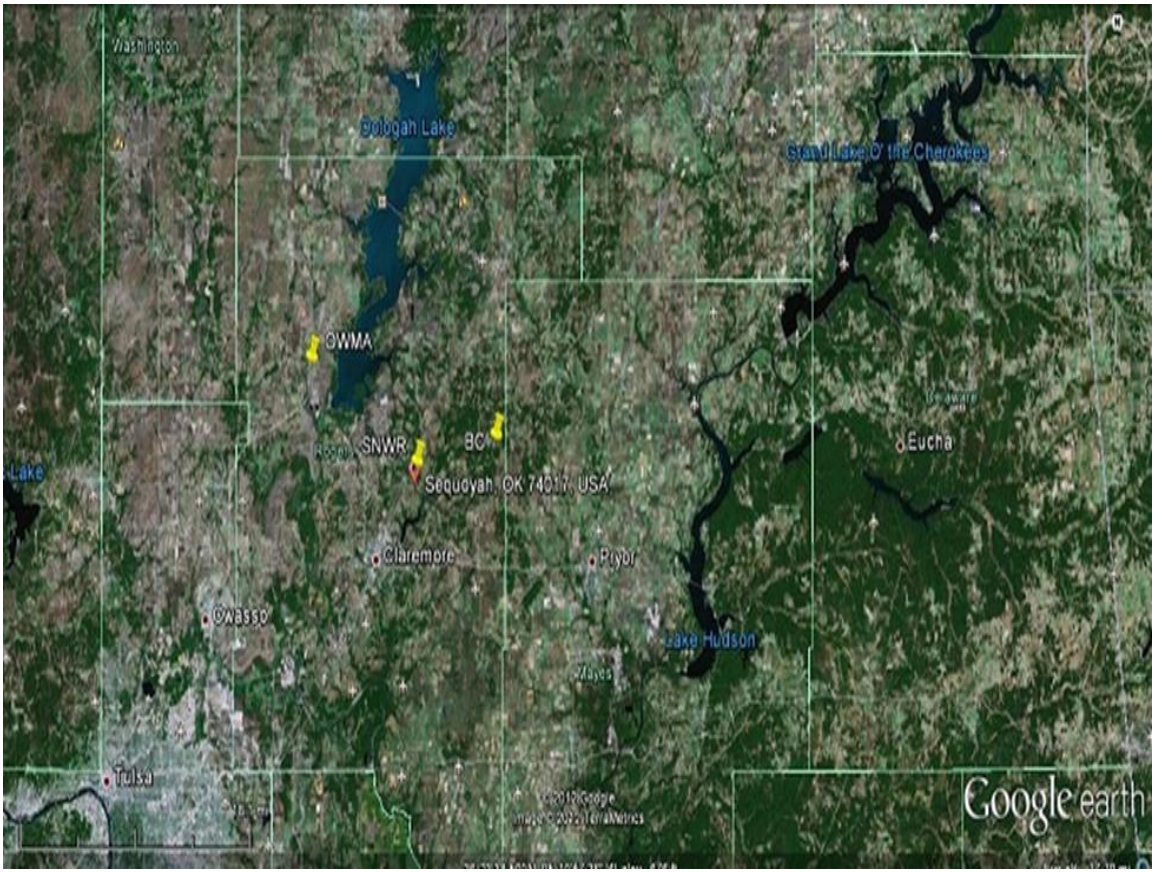

Figure 1: Study sites were labeled on Google Earth, Oolaga wild life management area (OWLA), Sequoyah national wildlife refuge (SNWR) and contaminated site at TCSFS, Beaver creek (BC).

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Figure 2: Beaver Creek in Ottawa County, Oklahoma Trapping site of *Peromyscus leoucopus* and soil sampling site (Polluted site). Eight random soil samples were collected from 100 m^2 (K. L. Phelps, 2006).

Figure 3: Oologah Wildlife Management Area in Nowata County, Oklahoma. Eight random soil samples were collected from 100 m^2 (K. L. Phelps, 2006).

Figure 4: Sequoyah national wildlife refuge (SNWR), Oklahoma. Eight random soil samples were collected from 100 m^2

VITA

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