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EXPOSURE ASSESSMENT OF METALS IN CAVE-DWELLING BATS

VOLUME 1

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Resumo

Populações de morcegos estão em declínio pelo mundo. As principais razões desse declínio foram atribuídas à influência antrópica, incluindo alterações climáticas, urbanização, atividades agrícolas e industriais. As atividades humanas causam o deslocamento das colónias de morcegos de cavernas e outras de outros abrigos naturais para minas abandonadas. Devido a isso, as minas tornaram-se áreas de interesse para a conservação de morcegos cavernícolas em todo o mundo. As bem documentadas propriedades persistentes dos metais em todos os compartimentos ambientais (bióticos e abióticos) podem comprometer as espécies que vivem nas minas, mesmo após o término das operações de extração. Assim, o objetivo deste estudo é entender se morcegos que habitam em minas abandonadas estão expostos a metais. Amostras não invasivas de pelo, membrana alar e guano de 140 indivíduos de quatro espécies de morcegos insetívoros e cavernícolas (*R. ferrumequinum*, *R. euryale*, *R. hipposideros* e *M. schreibersii*) foram coletadas em quatro minas abandonadas de extração de metais na região Norte e Centro de Portugal. Estas minas são utilizadas como locais de hibernação para as espécies de morcegos estudadas. Adicionalmente, amostras de solo, rocha e água foram também coletadas de cada mina para avaliar as possíveis vias de exposição. As amostras foram avaliadas em termos das concentrações de 13 metais essenciais e não essenciais (As, Ag, Cd, Co, Cr, Cu, Mn, Ni, Pb, Se, Zn, Sn e W) usando ICP-MS. Elevadas concentrações de metais foram encontradas nas quatro espécies de morcegos, tanto na membrana alar como no pelo, confirmando sua exposição aos metais. Concentrações mais altas foram encontradas na membrana alar do que no pelo. Foram encontradas diferenças entre *M. schreibersii* e as restantes espécies estudadas*,* com esta espécie a apresentar as maiores concentrações de metais. As diferenças entre as espécies podem ser explicadas pelas diferenças no comportamento de alimentar e no uso do habitat, ligadas às áreas urbanas e ao conteúdo de metais encontrados no solo. No entanto, os resultados não permitiram identificar as vias de exposições de forma conclusiva. Em comparação com outros estudos, as concentrações encontradas em morcegos cavernícolas foram maiores do que nas espécies de morcegos com outras preferências em termos de locais de hibernação e reprodução. Os resultados reforçam a importância da monitorização de metais em espécies cavernícolas que utilizam minas como abrigo, uma vez que exposição a metais constitui um risco potencial para estas espécies de morcegos.

Palavras-chave: Minas Abandonadas, Quirópteros, Insetívoros, Pelo, Membrana alar, Amostras não-invasivas.

Abstract

Bat populations seem to be decreasing all over the world. The main reasons for the decline have been attributed to anthropogenic influence, including climate change, urbanization, agricultural and industrial activities. Human activities cause the displacement of colonies from caves and other natural roosting areas into abandoned mines. Due to this, mines have become areas of interest for the conservation of cave-dwelling bats worldwide. The well documented persistent properties of metals in all environmental compartments (both biotic and abiotic), might compromise the biota living in mines even after operations have ceased. Thus, the aim of this study is to understand if cave-dwelling bats roosting in abandoned mines are being expose to metals. Non-invasive samples of fur, wing, and guano, of 140 individuals of four species of insectivorous cave-dwelling bats (*R. ferrumequinum, R. euryale, R. hipposideros,* and *M. schreibersii*) were collected on four abandoned metal mines in the north and center of Portugal, used as hibernation roosting sites for the species. Samples of soil, rock, and water were also collected from each mine to understand the origin of the exposure. The samples were evaluated in terms of the concentration of 13 essential and non-essential metals (As, Ag, Cd, Co, Cr, Cu, Mn, Ni, Pb, Se, Zn, Sn and W) using ICP-MS spectrophotometer. Metal concentrations were found in the four bat species in both wing and fur, confirming their exposure to the metals, with general higher concentrations found in wing membrane. Differences were found between *M. schreibersii* and the other bat species studied, with this species presenting the biggest metal concentrations. The differences between species might be explained by differences in foraging behavior and habitat use, linked to anthropogenic activities and metal content found in the environmental compartments. However, was not possible to identify clearly the routes of exposures present. In comparison with other studies, the concentrations found in cave-dwelling bats were higher than in bat species that have other behaviors and preference in terms of roosting sites. The results reinforce the importance of monitoring metals in species roosting in mines, since exposure to metals might constitute a potential risk for cave-dwelling bats.

Key words: Abandoned Mines, Chiroptera, Insectivorous, Fur, Wing Membrane, Non-invasive sampling.

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Chapter I General Introduction

i. Exposure Assessment: General Concepts

Along with the growth of technological, industrial, and agricultural advances in human societies, the emission of different substances into the environment has also risen (Järup, 2003). Some of these substances may cause severe damages to humans and the environment (Tchounwou, et al., 2012). Examples like the methylmercury discharges in the Japanese coasts that caused neurological problems to the fisher communities in Minamata or the well documented DDT that was responsible for an immense decline in bird populations in the United States drew attention to the connection between anthropogenic pollutants and the ecosystem's health. (Berglund & Järup, 2001). To address these type of problematic several tools have been developed to measure the ecological hazard human activities may have on the environment (Lioy, 1990).

The United States Environmental Protection Agency (EPA) defines risk assessment (RA) as "a process in which information is analyzed to determine if an environmental hazard might cause harm to exposed persons and ecosystems" (EPA, 2004). A risk merely constitutes the likelihood of an event happening or a substance causing a menace. RA performs four mayor steps during the process of evaluation, which start with the identification of the hazard, a dose-response assessment, a risk characterization, and the exposure assessment (Berglund & Järup, 2001), the last one being the main topic in this master thesis.

The exposure assessment performs a series of procedures to study the possible contact with pollutant (Merrill, 2008). For an organism to be considered exposed to a pollutant, contact with the boundaries between the environment and the inside of the body (skin, mouth, and nose) must occur (Lioy, 1990). Since exposure is considered a function of concentration and time several factors that might influence the toxicity levels once they enter the biota need to be taken into account. Among those factors we can define: exposure pathways, routes of exposure, duration and frequency of exposure and, concentration of exposure (Merrill, 2008). The exposure pathways refer to the environmental media and course a pollutant takes from the origin of emission to the biota, specifically water, soil, air, and/or food (Berglund & Järup, 2001). The route of exposure consists in the biological mechanism a pollutant uses to enter the body of an organism, which can be through inhalation, ingestion, dermal contact, through the placenta, the ears (in the case of noise), and through the eyes (in the case of UV rays) (Berglund & Järup, 2001; US EPA, 1992). The duration and frequency of the exposure is calculated to determine how often the exposure is occurring and for how long, being long term-exposures, short-term exposures, and cumulative exposures (total exposure over a determine time frame) (EPA, 2004; US EPA, 1992). The concentration of exposure refers to the amount of pollutant that is present in the exposure pathway at the moment the contact is occurring with the biota. However, not all the concentration found in the medium is going to enter an organism. The amount that actually enters the organism through the exposure routes is define as the dose (Berglund & Järup, 2001; EPA, 2004; EPA, 1992). In general, the sole presence of a contaminant in the environment (soil, water, or air) might not constitute a risk for the biota if exposure, in specific amounts is not occurring.

ii. Metals: Effects and Exposure

Overview and General Concepts

Throughout the years, and do to the industrial, domestic, and technological increase in their use, metals have become a global concern as environmental stressors due to their distinct chemical properties and characteristics. (Tchounwou, et al., 2012). Heavy metals are classified as metallic elements with a specific density higher than water (5 g/cm^3) (Järup, 2003). Although in biological related literature any metal that presents toxic properties is classified as a heavy metal, making them challenging to define (Allinson et al., 2006). As per some are considered essential nutrients due to their role in certain biochemical and physiological processes when present in specific concentrations in the growing medium, they take part in enzymatic activity and positively influence growth in livestock for example (Kochare, 2015; Tchounwou et al., 2012). This is the case with copper (Cu), zinc (Zn), iron (Fe), manganese (Mn), molybdenum (Mo), nickel (Ni), and cobalt (Co), which are labeled as micronutrients, and are considered metals when found in concentrations high enough to become toxic (Rengel, 1995).

Metals that are not considered essential for biological functions but are toxic to the biota in very low concentrations are referred to as trace metals. This include metals like arsenic (Ar), cadmium (Cd), chromium (Cr), lead (Pb), mercury (Hg), nickel (Ni) and, uranium (U) (Kibria, 2016). Metals like As, Cd, Pb, and Hg are of high concern in aquatic environments, and Pb, Hg, and Cd are the most reported metals to cause contamination (Govind, 2014).

Effects of Metals on the Environment

Metals are considered harmful pollutants of public and environmental concern (Järup, 2003). Metals, when released into the environment have the properties of causing alterations and hazardous effects that can impair the welfare of an ecosystem, and even cause death to individuals, categorized them as pollutants (Duruibe, et al., 2007). Metals have toxic, persistent and bio accumulative properties that cause a wide range of effects on the environment. They can be present and accumulate in all environmental compartments, meaning that the exposure to them by the biotic compartment is highly inevitable once they are released in jeopardizing quantities (Govind, 2014; Tchounwou et al., 2012).

In the case of toxicity in animals, with most studies performed on humans, small mammals and aquatic organisms, metals become toxic when they are not metabolized by the body and therefore get accumulated in the soft tissues (Kibria, 2016; Tchounwou et al., 2012). The effects they have on individuals vary greatly depending on many physical and biological factors, such as age, gender and, exposure. The exposure to them could be categorized as acute, chronic, or sub-chronic, each leading to different effects depending on the metal and the individual (Duruibe, et al., 2007). These effects in humans have been known to be toxic, neurotoxic, carcinogenic, mutagenic, or teratogenic (Duruibe, et al., 2007). Metals like Hg, Pb and Cd have been known to cause mental and neurological impairing, influencing the behavior in mammals and fishes. This jeopardizes their ability to mate, escape and avoid predators. Metals like Cr are also known to cause cancer (Govind, 2014). They are also able to alter several metabolic processes and influence the production of neurotransmitters. In addition, they are known to impair the function of the nervous, cardiovascular and endocrine systems, as well as impair the function of the colon, liver, kidneys and, skin (Kumar, 2014). Some metals can also damage and induce mutations in the DNA due to their genotoxic effect (Sánchez-Chardi & Nadal, 2007).

When metals are released in relevant amounts into the environment they can disrupt an entire ecosystem as their toxic properties can affect all organisms in a trophic chain due to bioaccumulation and biomagnification (Bergeron, et al., 2010; Govind, 2014; Sánchez-Chardi & Nadal, 2007). Bioaccumulation is the gradual growth in quantity of a substance in a living organism (Fernandes, et al., 2007; Govind, 2014; Weisbrod et al., 2009). When an individual absorbs a toxic chemical through the skin or intakes it, the amount becomes higher in the body than in the original source (environment and/or diet). As long as exposure continues (bioavailability of the metals in the environment) the concentration will continue to increase until it becomes life threatening for the individual (Fernandes et al., 2007; Govind, 2014; Weisbrod et al., 2009).

Along with bioaccumulation, the biomagnification properties of metals go beyond affecting selected organisms as they could act on all beings on a food web. Biomagnification is the process in which a substance increases in quantity and toxicity as it goes up the trophic chain. After the metals are absorbed by an organism at the bottom of the food chain they are transferred to a second, bigger organism that would theoretically eat several individuals of the original organism (in the case of marine chains) (Jakimska, et al., 2011). The metal quantity will increase in the second organism as well as the toxic properties making it dangerous for the animals higher up in the chain. However, it will vary between essential and non-essential metals, as some organisms will metabolized the essential metals and eventually excrete them (Atwell, et al., 1998; Gray, 2002; Laskowski, 1991).

Understanding how biomagnification works in terrestrial and marine chains is a dense process. As the most studied metal in marine food webs, mercury for example, is known to biomagnify in some subsets of a chain, however depending on the area of study organisms can have mercury but not through biomagnification. This is due to the different transfer mechanisms occurring at different levels of the food webs (Atwell et al., 1998). When it comes to terrestrial apex predators, due to the large interspecific variations in the assimilation of the metals in the animals, Laskowski (1991) concluded that there are several transfer pathways metals can use and that biomagnification is not a rule for all webs. Some studies suggest that bioaccumulation and biomagnification might be higher in terrestrial species, due to less diverse food chains, as oppose to pelagic species which have varied foot habits (Hsu, Selvaraj, & Agoramoorthy, 2006). Inclusive and extensive studies need to be performed to understand the behavior of metals in specific webs due to the nature of the metal and the complexity of the tropic chains.

Exposure of Metals: Sources and Routes

As metal contamination is naturally irreversible and toxic there is a worldwide environmental concern as exposure to them has risen dramatically over the years (Tchounwou et al., 2012). Exposure is possible due to the persistent properties of metals (Järup, 2003). Once released into the environment they can get in contact with the boundaries between organisms and their surroundings in a specific concentration for a defined interval of time, producing chronic, sub-chronic or acute exposures (Kumar, 2014). The contact can be through the airways, the skin, or the mouth (inhalation, ingestion, and dermal contact) as exposure routes. The sole presence of the metals in the environment will not be considered an exposure risk if contact with organisms is not occurring. (Berglund & Järup, 2001; Järup, 2003).

Metals can be released into the environment by both natural and anthropogenic pathways, with a clear understanding that anthropogenic activities constitute the main source. They can be released into or be present in air, soil, sediment, and water. If exposure occurs, they can also be present in biotic compartments (Dinis & Fiúza, 2011; Duruibe, et al., 2007; Järup, 2003).

Metals that are naturally present in soil include Cd, Zn, Pb, and Cu, which can be found in the earth´s crust, with higher than normal levels in sedimentary rocks. Metals like Zn, Pb, Fe, Ar, Co, and Na are present in rock's ores, but are only released during mining activities (Dinis & Fiúza, 2011; Duruibe, et al., 2007; Tchounwou et al., 2012). On air we can find metals like Cd and Hg which are present in volcanic activities and can me released during eruptions. Forest fires have also been classified as natural sources of cadmium in the air. While the main natural sources of mercury in the atmosphere are the degassing of the earth's crust and evaporation by certain bodies of water which contain mercury (Dinis & Fiúza, 2011; Govind, 2014). Metals that are released into the air can eventually deposit in water bodies and soil, with the ability of moving between abiotic compartments (Dinis & Fiúza, 2011).

The anthropogenic sources of metals vary greatly, as they originate from several activities which affect more than one environmental compartment at a time. When it comes to air, high quantities are released into the atmosphere mainly from metal industries where high temperatures are used during combustion, extraction, and processing (Nriagu & Pacyna, 1988). Such industries could be non-ferrous metal smelters (responsible for the highest release of Pb along with gasoline combustion), iron, zinc-cadmium, copper-nickel, steel production and pyrometallurgy industries in general, where several metals like Cr and Mn are released as bioproducts. Cd, Cu, Pb, Cr and Zn are widely use in the production of phosphate fertilizers and lubricants (Dinis & Fiúza, 2011; Hamers, et al., 2006; Järup, 2003; Nriagu & Pacyna, 1988; Tchounwou et al., 2012). While the highest releases of cadmium and mercury into the air have been recorded to originate from the production of pesticides. Other industries that highly contribute to the release into the air are the cement industry, mining, coal, wood and, oil combustion (the last three being industrial, commercial, domestic or to produce electricity) and waste incineration (Duruibe, et al., 2007; Järup, 2003a; Nriagu & Pacyna, 1988).

The main anthropogenic sources of metals in water bodies come from the direct or indirect discharges from industrial or domestic sources, through runoff and releases from storage and transport. Domestic wastewater effluents contribute highly to the release of As, Cr, Cu, Mn, and Ni; while coal-burning power plants, iron and steel plants, and sewage sludge contribute as well to the release of Pb, Cd, Se, and Hg. Mining highly impacts the water through acid drainage, erosion of soil and mine wastes, and contaminant leaching (Elaw, 2014; Nriagu & Pacyna, 1988).

In soil, industries may directly dispose biproducts of coal combustion, such as ash residues. Activities such as urban waste, sewage sludge, the production and use of pesticides and fertilizers, smelter slag and, agricultural and food wastes can highly contribute to the release of metals like Cu, Hg, Pb, and Zn into the soil. Activities such as mining can pollute wide areas of land, while releasing high amounts of metal biproducts as well (ELaw, 2014; Kochare, 2015; Nriagu & Pacyna, 1988).

It is important to note the high mobility of metals. As atmospheric fallouts and air/water exchanges can aid in the transferring of metals in between environmental compartments. This causes the exposure of metals through not only the environment (air, water, and soil) but through food, as the metals can accumulate in vegetation and in animals, increasing the risk of exposure.

iii. Bats

Overview on Bats

The animals from the order Chiroptera (bats) are the second most numerically diverse mammalians with more than 1200 species described worldwide, representing approximately 20% of all mammal species and are the only ones capable of flying. (Mattar & González, 2017). They are divided into two suborders, the Megachirpotera, which refers specifically to the Pteropodidae family or fruit bats; and the Microchiroptera which includes all the other bat families (Mickleburgh, et al., 1992). Around 75% of the Microchiroptera species are insectivorous, being able to capture up to 600 insects in an hour or even the equivalent to 40% up to 100% of their body mass in one night. The remaining species that are not insectivorous can feed on nectar, fruits, fish, blood, small mammals, flowers and amphibians (Hutson, et al., 2001; Nathusii, 2006).

They inhabit all places on Earth, except for the Antarctica, the Artic and some isolated islands, with a higher species diversity in the neotropical regions, playing important roles in all ecosystems they inhabit (Bernard, 2002). Insectivorous bats control populations of insects that are considered pests for agricultural activities, while other species aid in pollination and seed dispersal enhancing regeneration of habitats (Hutson et al., 2001).

Cave-dwelling bats import organic matter into caves, providing a special function that supports several invertebrate communities (Palmeirim & Rodrigues, 1992).

Nevertheless, even though bats provide numerous ecosystems services their populations have been declining worldwide due to anthropological stressors. The increase in human population has reduced the forest and woodland areas in both tropical and tempered areas, displacing several species of bats from their natural roosting sites (Mickleburgh, et al., 2002). Intensive agriculture also contributes to the loss of roosting areas and degradation of important areas for bats, as the need for wider areas of land for plantations has eliminated important connections between roosting and feeding areas like tree lines and hedgerows. Also, the use of pesticides, slash and burn techniques, and overall the activities that disturb their habitats and roosting sites have major implications in their populations (Mickleburgh, 2002).

Cave Dwelling Bats

Bats have a wide selection of habitats for roosting, with forest and woodlands as the key habitats for most species as the holes in trees, both alive and death, provide optimal conditions for temperature conservation and predator avoidance (Hutson et al., 2001). However, some species of bats are considered cave dwellers, as caves provide optimal sites for hibernation, courtship, raising of offspring, socializing, and protection from predators and weather (Tuttle & Moreno, 2005). A wide range of microclimates are present in caves, bats may select their preferred temperature while inside depending on the time of the year and on their biological need. Bats have high metabolic rates that come along with high water and heat loss. Along with their high losses of energy during flying, their exposed wing membranes and their small bodies causes them to lose as far as a third of their weight each night through transpiration. Caves with a high level of humidity can aid bats into avoiding dehydration (Kunz, 2006; Thomas, & Lumsden, 2003).

Due to the high disturbance of humans in natural caves, bats have been displaced and force to find new roosting places in abandoned mines. Some mines can provide similar conditions of temperature and humidity as natural caves, providing ideal environmental conditions for bats to breed in the summer, hibernate in winter and rest during migrations in spring and fall (Tuttle & Taylor, 1998).

iv. Aims

Currently there are 25 species of bats identified in mainland Portugal, of which all of them are insectivorous. However, all the species categorized as threatened are cave-dwellers at least one part of the year, either during hibernation or maternity. Due to human disturbances, several bat populations have been displaced from their original roosting sites into abandoned mines. The conservation Plan for Cave Dwelling Species published by Palmeirim & Rodrigues (1992), even suggest the closing of abandoned mines with methods that will allow bats to enter. However, metal mines even after abandoned can be focus points for metal contamination, that could persist and be present in the soil, vegetation, and water bodies in the nearby areas. Considering the population decline of bats, the fact that some species are using mines as permanent or temporary roosting sites and the well documented effects of metals on fauna, gives a reason to think that metals might be affecting bat population.

The aim of this thesis is to understand if bats using abandon mines as roosting areas are being expose to metal contamination by direct (food and water) or indirect (soil) pathways. To accomplish this aim, metal concentration into three biotic compartments, being them fur, wing, and guano, will be measured in four insectivorous bat species: *Rhinolophus euryale, Rhinolophus ferrumequinum, Rhinolophus hipposideros, and Miniopterus schreibersii* (Table 1). Three abiotic environmental compartments: rock, soil, and water inside the mine and in the nearby areas will also be sampled to understand the origin of the exposure in the mine.

Table 1 - Ecological and morphological characteristics of the target species.

Species	Family	Common Name	Conservation Status in Portugal[8] / IUCN Status[9]	Biomass	Body Length	Life Spam in the Wild	Habitat	Threats Factors
Rhinolophus euryale[1]	Rhinolophidae	Mediterranean Horseshoe Bat	Critically Endangered / Near Threatened	8 to 17.5 g	65 to 88 mm	13[5]	Occupies relatively large caves and mines at all times of the year, with groups living in buildings in the North of the country.	Low fertility characteristic of bats; focused on small number of sites; habitat degradation through destruction of shelters; pesticide use; high mortality due to road kills (low flight).
Rhinolophus ferrumequinum[2]	Rhinolophidae	Greater Horseshoe Bat	Vulnerable / Least Concern	17 to 34 g	57 to 71 mm	30.5[6]	Breeding colonies mainly housed in large buildings, also caves and mines for hibernation.	Habitat degradation through destruction of shelters and blockade of small mines by vegetation; pesticide use; high mortality due to road kills (low flight).
Rhinolophus hipposideros[3]	Rhinolophidae	Lesser Horseshoe Bat	Vulnerable / Least Concern	5 to 9 g	35 to 45 mm	29.4[5]	Can habitat in buildings (usually abandoned houses), caves and mines; hibernates in underground shelters	Habitat degradation through destruction of shelters and blockade of small mines by vegetation; pesticide use; high mortality due to road kills (low flight).
Miniopterus schreibersii[4]	Miniopteridae	Common Bentwing Bat	Vulnerable / Near Threatened	8 to 11 g	52 to 63 mm	22[7]	Exclusively cave-dwelling: it breeds and hibernates in caves and mines, rarely uses other shelters.	Low fertility characteristic of bats; focused on small number of sites; destruction and disruption of shelters and their disruption, particularly during breeding and hibernation period is greatest threats; landscape change; pesticide use.

References: [1]Juste & Alcaldé, 2016**;** [2]Piraccini, R. 2016; [3]Taylor, P. 2016**;** [4] Hutson, et al. 2008 ; [5]Gaisler et al. 2003 ; [6]Carey and Judge, 2000; [7]Wilkinson and South, 2002.; [8]Rainho, 2013; [9] IUCN, 2018

v. Thesis Framework

This thesis is divided in four chapters. This thesis is divided in four chapters, three main chapters plus Chapter IV that is the references cited in the thesis.

Chapter I corresponds to a general introduction, describing the main concepts and topics on the document. It starts introducing the concept of exposure assessment and its importance as part of the environmental assessment tool. Later on, it is followed by an overview on metals, its definition, their main anthropological and natural sources and its behavior and effects on the environment. It finished with a general over view on bats, with a specific section on cave-dwelling bats, reasons for their population decline and reason of their displayed from natural roosting areas to abandoned mines.

Chapter II targets the main goal of the thesis, which is to understand if cave-dwelling bats living in abandoned mines are being exposure to possible metal contamination left from the operation activities. It includes the procedure used for the experiment, describes the study area, the sampling, and the final findings of the research.

Chapter III discusses the results of the research with the intention to answer the questions made on the aim of the document. It provides a conclusion on the matter combining the results found with existing literature.

Chapter II Exposure Assessment of Metals in Cave-Dwelling Bats

i. Introduction

Bats, as the second most diverse mammals, are distributed all over the world, with isolated Oceanic islands, the Antarctica, and the Arctic as exceptions. Their wide distribution, in addition to their ability to fly, allow them to fill an extensive range of ecosystem niches. Making them key agents in environmental and ecosystems services such as pollination, seed dispersion, and pest (insect) control (Hutson et al., 2001; Jones et al., 2009; Nowak R.M, 1994). However, bat populations seem to be decreasing worldwide (Mickleburgh et al. 2002; Jones et al. 2009; Pikula et al. 2010; Hernout et al. 2013; Hernout et al. 2015; Zukal et al. 2015; O'Shea et al. 2016; Hernout et al. 2016;). Due to its importance in several ecosystems, the need to understand the reasons behind their population decline becomes essential (Jones et al., 2009; Zukal et al., 2015; Nowak, 1994). The main reasons for the decline of the bat populations are mostly human related. Climate change, urbanization, agricultural and industrial activities, diseases such as the white fungus (*Pseudogymnoascus destructans*) commonly known as the white nose syndrome, have all been accounted for (Jones et al., 2009; Mickleburgh et al., 2002; Zukal et al., 2015). Human activities have also cause the displacement of colonies from caves and other natural roosting areas into abandoned mines. Currently mines are key areas for hibernating during winter, rearing young during summer, and they may also be used as temporary havens during spring and fall for several species of cave-dwelling bats. (Tuttle & Taylor, 1998). This makes mines key areas for bat conservation. In the specific case of Portugal, special measures are implemented to protect the mines when operations are ceased. Specifically to permit the entrance of bats, as all cave dwellers are categorized as "Vulnerable", with the *R. euryale* categorized as "Critically Endangered" (Palmeirim & Rodrigues, 1992).

The effects of mining activities in the environment have been well documented. Metal contamination in soil and water may persist in the area, and even spread with runoffs, erosion, and wind to other areas, even after the closure of the mine. Metals have the ability to persist in all environmental compartments, including the biotic compartment if bioavailable, with the ability to bioaccumulate and biomagnify on living beings (Zocche et al., 2010; Jung & Thornton, 1996; Li et al., 2014; López, 2015; Navarro et al., 2008).

Accumulation through ingestion is greater on animals placed on higher levels in the food chains (Fernandes et al., 2007). This, plus the high metabolic capacities also associated to top predators and the slow reproductive rates of bats, place insectivorous bats, specifically, in a rather vulnerable situation regarding its response to pollutants, including metals (Jones et al, 2009). In other words, they will show effects faster than animals in lower levels and due to its slow reproduction rate, the populations will take longer to recover. Taking into account that bats are occupying abandoned mines as new roosting areas and the persistent properties of metals, raises the question that cave-dwelling bats might be expose to them. If they are, metals might be included as one of the reasons for the populations declines.

However, few studies have been performed to address the effects of metals on bats, and even fewer studies have specifically target cave-dwelling bats roosting in abandoned mines. Even when metal pollution has been known to influence on bat's diversity, relative abundance, population structure and flight activity (Sameeh et al., 2016; Zukal et al., 2015). DNA damage in blood cells has also been attributed to metal contamination (Zocche et al., 2010). A study performed in England and Wales concluded that at least 21% of the individuals sampled contained metal concentrations high enough to manifest in toxic effects (Hernout et al., 2016). Zukal et al. (2015) made an overview of 52 papers published regarding bats as bioindicators of metal pollution, in which only one was focus on metals in bats roosting in mines. Zocche et al. (2010), performed the first ecotoxicological study on bats roosting on mines in Brazil, concluding that the levels of metals were indeed higher in the bats living in the mines as oppose to the ones living in the control area.

The major aim of this study is to determine is cave-dwelling bats living in abandoned mines in Portugal are being expose to metals. This will be determining by measuring the concentration of 13 metals in fur, wing membrane, and guano of four species of insectivorous bats. Due to the conservation status of bats only non-lethal samples only were used, as they have been validated to be representative of metal concentration (Mina, 2017). Sampling of environmental abiotic compartments (soil, water, and, rock) will also be performed to better understand the routes and pathways used by the metals.

The working hypothesis seeks to answer the question: Are cave dwelling bats living in abandoned mines being expose to metals? Additionally, we will provide new information needed to determine if the exposure constitutes a potential risk for bat populations in Portugal.

ii. Materials and Methods

Study Area

The collection of the samples was performed in four abandoned mines in the north of Portugal (Figure 1). The mines Regoufe, Adória, Carviçais and Coelhoso were sampled during the month of March 2018, period corresponding to the end of the winter season and thus the end of hibernation.

A total of 140 individuals were sampled for fur, wing membrane and guano, from four different insectivorous species: 57 individuals of *Rhinolophus ferrumequinum* (greater horseshoe bat), 42 individuals of *Miniopterus schreibersii* (common bentwing bat), 22 individuals of *Rhinolophus euryale* (Mediterranean horseshoe bat) and 19 individuals of *Rhinolophus hipposideros* (lesser horseshoe bat*)*.

The mines were selected from the report *Análise Dos Dados Do Programa De Monitorização De Abrigos Subterrâneos De Importância Nacional De Morcegos (1988-2012)*[1] (AIN) (ICNF, 2014), prepared by the Portuguese *Instituto da Conservação da Natureza e das Florestas*[2] (ICNF). The AIN takes into account specific criteria from the *Critérios De Avaliação Dos Abrigos De Morcegos De Importância Nacional*[3](ICNF, 2013) to consider a roosting area as of natural importance such as the conservation status of the species found, based on the *Livro Vermelho dos Vertebrados de Portugal*[4] (Cabral et al., 2005) and the ecological characteristics of each species regarding its ability to adopt different roosting areas (ICNF, 2013). In the case of Regoufe, it is considered of national

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[1] English Translation: Data Analysis of the Monitoring Program of Bat's Underground Roosting Areas of National Importance

[2] English Translation: Institute for the Conservation of Nature and Forestry

[3] English Translation: Evaluation Criteria of Bat's Roosting Areas of National Importance

[4] English: Translation: Red Book of Vertebrates of Portugal

importance due to the presence of *R. ferrumequinum* during its hibernation period. Coelhoso is consider of natural importance during the hibernation season of the *R. hipposideros* and the *R. ferrumequinum.* Adória is consider of natural importance during the hibernation season of the *R. euryale* and the *R. ferrumequinum*. While Carviçais shelters the *M. schreibersii*, as well during its hibernation season (Table 2). The mines may shelter other species aside from the ones mentioned during other periods of ecological importance according to the AIN. However, those criteria are the ones of interest for this document as they are the species found during the biological sampling, no other species were found (ICNF, 2014). Table 2 depicts the locations of the mines, the metals extracted during operation, the species and the number of individuals sampled and the season they are present. The specific location of the mines can be visualized in Figure 1.

Table 2 - Location, species and number of individuals sampled in the four surveyed mines

Mine	Location/ District	Metals extracted during operation	Species (# of Individuals sampled)	Season species are present
Regoufe	Arouca/ Aveiro	Tungsten, Tin	*R. ferrumequinum* (21) *M. schreibersii* (2) *R. hipposideros* (1)	Hibernation
Adória	Ribeira de Pena/ Vila Real	Tungsten, Tin	*R. euryale* (21) *R. ferrumequinum* (1)	Hibernation
Carviçais	Torre de Moncorvo/ Bragança	Tungsten, Tin	*M. schreibersii* (40) *R. ferrumequinum* (18) *R. hipposideros* (1) *R. euryale* (1)	Hibernation
Coelhoso	Bragança/ Bragança	Tin	*R. hipposideros* (17) *R. ferrumequinum* (17)	Hibernation

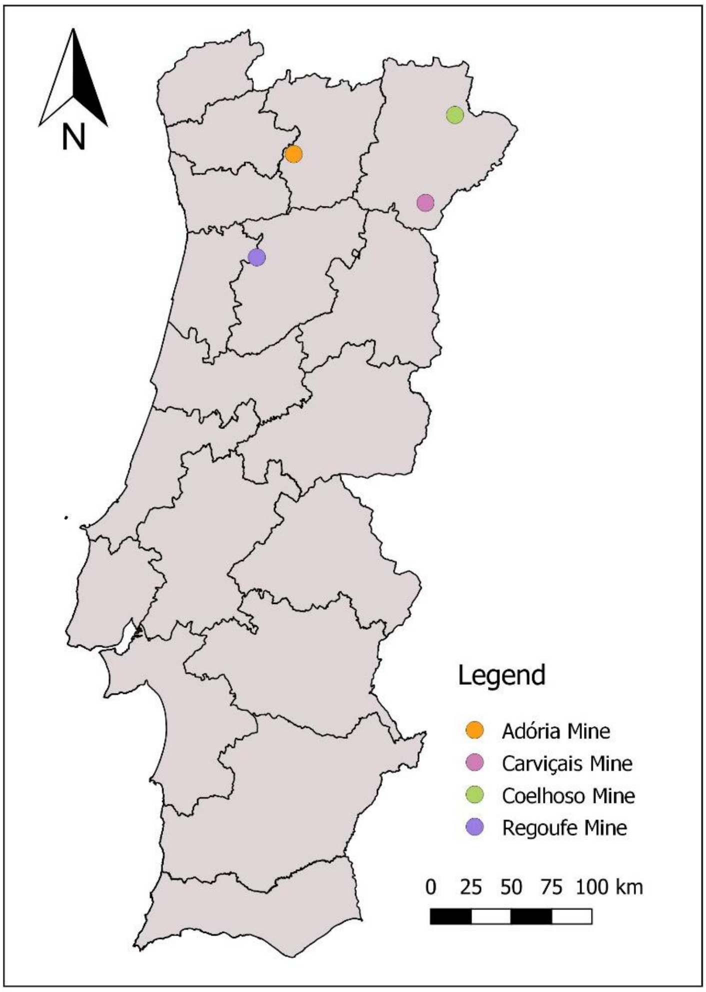

Figure 1 - Map of the study area, depicting the sampling mines

The sampling was divided in biological (fur, wing and guano) and environmental (soil, rock, and water) compartments and was performed with the aid of the Centre for Research and Technology of Agro-Environment and Biological Sciences (CITAB), Laboratory of Applied Ecology from the University of Trás-os-Montes e Alto Douro.

Biological Sampling and Preparation

The collection of the individuals was performed using a harp-net trap placed in the entrance of each mine before sunset. Due to foraging behavior, sunset and sunrise represent peak activity periods at the entrance of roosting area, including the mines. A canvas catch bag was attached at the bottom of the harp-net's frame to collect the bats as they leave the site. In entrances were the harp-net could not be placed due to field conditions the capture was made by hand. The bats were then placed inside individual cloth bags and hang by a string. After sunset they were taken out one by one for identification and sampling.

To collect wing membrane samples, 4mm biopsy punch were used, twice on both wings, for a total of 4 wing punches. Prior to taking the wing samples, each biopsy punch was sterilized with acetone and an open flame; punching up to 4 individuals before losing sharp and replaced. The tissue sample was taken close to the body specifically between the leg and the fifth finger (dactylopatagium major). Each sample was placed in a sterile Eppendorf's and label accordingly. A total of 138 wing samples were collected.

For the hair collection sterilized stainless steel scissors were used. An approximate amount equivalent to $1cm^2$ was cut from the area between the scapulae. The hair was stored in sterile Eppendorf's and labeled accordingly. A total of 139 fur samples were collected.

The guano was collected during the biological sampling. When the bats are collected and temporary placed in cloth bag they defecate. This guano was collected using tweezers and placed in sterile Eppendorf's. A total of 83 fresh guano samples were collected. All biological samples were stored at -20ºC until metal extraction in the laboratory.

After collection the hair and wing samples were cleaned in a three-step process to ensure the removal of all external contamination. First, they were rinsed once with detergent Triton X-100 diluted at 1%. Second, the were rinsed twice with acetone and three times with ultra-pure water. The excess of liquid was removed with the aid of tissue paper. The samples were then dried for 72 hours at a temperature of 45°C, and weighted (Mina, 2017).

Environmental Sampling

The environmental sampling consists in the collection of soil, rock, and water. The soil sampling was performed in the entrance of the mine and in the back areas in non-flooded locations, for a total of eight soil samples, two per mine. The samples were collected with an approximate depth of 10 cm (avoiding the first 2 cm of soil). Rock was removed from the walls with the aid of a pick, one sample per mine. All samples were placed in a sterilized labeled container. The water samples were taken from the nearby water bodies and from the inside of the mines. All environmental samples were kept at -20ºC for preservation. The water samples were all filtered by centrifugation to remove impurities. The rock, and soil samples were dried for 72 hours at a temperature of 45°C and weighted before the metal extraction.

Metal Extraction and Quantification

For the extraction procedure, the samples were mixed with 65% nitric acid at and placed for 12 hours at a temperature of 150°C in pressure pumps PDS-6 systems (Loft fields Analytical Solutions, Neu Eichenberg, Germany). After the 12 hours all the organic material is disintegrated in the acid, which is diluted to a 10% with ultra-pure water, and only the metals remain. The quantities of acid and water were different depending on the type of sample and weight to preserve a 10% acidity in the final solution. For wing and fur a ratio of 0.5mL acid/2.75mL ultra-pure water was used for a final volume of 3.25mL. Since fresh guano contains silicates difficult to disintegrate, a ratio of 1mL nitric acid/5.5mL ultra-pure water was used for a final volume of 6.5mL. For the environmental samples collected inside of the mine (rock, soil, and guano) a ratio of 2mL nitric acid/ 11 mL ultra-pure water was used for a final volume of 13mL. For the water samples, the ultra-pure water was replaced by the filtered water collected from the mines (an amount of 2.75mL per sample) and 0.5mL of nitric acid was added (Mina, 2017).

To quantify the amount of metals an ICP-MS spectrophotometer (Model iCAP Q, Thermo Fisher Scientific, Bremen, Germany) was used. The target metals to be read were Arsenic

(As), Silver (Ag), Cadmium (Cd), Cobalt (Co), Chromium (Cr), Copper (Cu), Manganese (Mn), Nickel (Ni), Lead (Pb), Selenium (Se), Zinc (Zn), Tin (Sn) and Tungsten (W) for all the samples. Even though only Sn and W were mined, other metals are always released during mining activities. Ultra-pure water blanks were used to ensure the procedure was done contamination free. A standard solution was prepared using multielement certified reference material (92091, Periodic table mix 1 for ICP, Sigma-Aldrich) for As, Cd, Cr, Co, Cu, Pb, Mn, Ni, Se, Ag, and Zn. For Sn and W unimetal material was used. For the calibration of the ICP-MS a 5-point calibration curve (50ppb, 20ppb, 10ppb, 2ppb and a blank) was done for each element for the biological samples. For soil, a 7-point calibration curve (2000ppb, 1000ppb, 100ppb, 20ppb, 10ppb, 2ppb, and a blank) was used. The detection limits obtained for each metal were 0.02104µg/g for Cr, 0.0146µg/g for Mn, 0.00374µg/g for Co, 0.05344µg/g for Ni, 0.02416µg/g for Cu, 0.14506µg/g for Zn, 0.02126µg/g for As, 0.03812µg/g for Se, 0.00282µg/g for Ag, 0.00254µg/g for Cd, 0.01134µg/g for Sn, 0.00994µg/g for W, and 0.03494µg/g for Pb. All metal concentrations detected were read in ppb by the ICP-MS but converted to µg of metal/g of the samples dry weight for the statistical analysis.

Data Analysis

To analyze the data collected an initial data exploration was performed using R 3.3.2 (R Development core team 2017). The data exploration consisted in graphing each metal concentrations using the variables of species, sample type (i.e. fur, wing membrane and guano) and location with dot plots, histograms, boxplots and scatterplots. Previous to data exploration and analysis, all the metals concentration values were log transformed to visualize and model the data since it ranges over several orders of magnitude. Additionally, the log transformation will also allow for a normal distribution, and to reduce heterogeneity in the residuals.

Pearson's Correlations were performed between each metal concentrations in the samples collected (fur vs wing, fur vs guano, wing vs guano) to confirm their independence.

Using a Linear mixed model (LMM), the sample types (fur and wing; fixed factors), the species (*R. ferrumequinum, R. hipposideros, R. euryale*, and *M. schreibersii*; fixed factors) and the location of the mines (Regoufe, Adória, Carviçais, and Coelhoso; fixed factors) were analyzed to understand their effect on the metal concentrations (response variable). The animal's ID was included as random factors in all the analyses. Potential interactions between the explanatory variables were also analyzed. Specifically, the interactions between species and location; and species and sample type.

The variable guano was included in a separate LMM to test if it could explain the concentrations found in the sample types (wing and fur), as a proxy for food. For this LMM, guano, species (*R. ferrumequinum, R. hipposideros, R. euryale*, and *M. schreibersii*) and metal (Cr, Mn, Co, Ni, Cu, Zn, As, Se, Ag, Cd, Sn, W, and Pb) were used as fixed factors to test their effect on the sample types (response variables). The individuals were included as random effects. Potential interactions between the explanatory variables were also analyzed. Specifically, the interactions between species and metal; metal and guano; species and guano; and species, metal, and guano.

After fitting each LMM, model validation was performed by checking heteroscedasticity and normality on the residuals.

The outputs were then expressed using the estimated mean and standard error (*i.e.* Mean±SE), unless otherwise stated; as well as the value of t and the p-values of the pairwise contrasts when differences between the response variables were identified. The statistical tests were considered significant when $p < 0.05$. For the LMM the software IBM.SPSS® Statistics 23 was used.

iii. Results

Overall, the metal presenting the highest concentrations was Zn, for the three biological sampling types (fur, wing, and guano). Fur followed the total median concentration pattern of Zn>Mn>Cu>Cr>Ni>As>Pb>Se>Sn>Co>W>Ag>Cd. The sequence of wing for the median concentrations was Zn>Cu>Cr>Mn>Ni>Pb>As>Sn>Se>Co>W>Cd>Ag. For guano the sequence was Zn>Mn>Cu>As>Pb>Ni>Cr>Se>W>Co>Sn>Cd>Ag. When focusing on species the concentration patterns changed. In the *M. schreibersii* the concentrations on fur follow a similar order as the total median concentrations except for As>Se>Ni>Pb>Cr>Sn. In the case of wing, almost the same sequence is followed as in the general results, with the exception of Se>Sn. The *R. ferrumequinum* follow a similar sequence as the total concentrations of fur except for Cu>Mn and Pb>Se>As. For wing a similar pattern was followed as compared to the total median concentrations, with the exception of Ni>Mn. The *R. euryale* follow the sequence Zn>Cu>Ni>Mn>Cr> Pb>Se>As>Sn>Co>Ag>W>Cd for fur. For wing it follows a similar patterned as the total median results, with the exception of Ni>Mn and Sn>As. The *R. hipposideros* follows the same patterned as the total median concentrations of fur with the exception of Cu>Mn and Pb>As. As for wing the sequence follow was Zn>Cr>Cu>Ni>Mn>Pb>Sn> Se>As>Co>W>Cd>Ag (Tables 3 and 4). In the case of guano, the sequences are the same in the four species (Figure 2).

No correlation was found between the biological samples collected (fur, wing, and guano) (Figure 3).

Table 3 - Metal concentration (µg/g dry weight) in fur of the four bat species sampled.

FUR								
	Miniopterus schreibersii		***Rhinolophus euryale***		***Rhinolophus ferrumequinum***		***Rhinolophus hipposideros***	
	Median	**Range**	**Median**	**Range**	**Median**	**Range**	**Median**	**Range**
Cr	3.55	(0.05 – 30.67)	7.78	(2.15 – 47.87)	3.55	(1.12 – 22.03)	9.24	(2.08 – 45.57)
Mn	74.75	(0.05 – 123.19)	8.58	(3.48 – 78.18)	4.13	(0.97 – 27.34)	13.90	(1.52 – 54.34)
Co	0.92	(0.002 – 3.53)	0.27	(0.04 – 2.68)	0.098	(0.03 – 0.44)	0.25	(0.05 – 1.30)
Ni	5.55	(0.02 – 30.84)	8.81	(2.71 – 27.30)	2.67	(1.21 - 33.08)	5.59	(1.81 – 38.34)
Cu	34.22	(0.15 – 131.42)	42.65	(22.81 – 131.49)	14.84	(8.20 – 59.10)	31.18	(8.43 – 105.45)
Zn	252.5	(140.80 – 414.8)	121.93	(82.43 – 442.38)	103.81	(79.42 – 219.82)	223.56	(88.68 – 410.29)
As	12.86	(0.02 – 71.88)	1.23	(0.48 – 55.85)	1.00	(0.35 – 10.55)	3.92	(0.59 – 20.39)
Se	7.28	(1.83 – 13.77)	1.36	(0.55 – 5.66)	1.39	(0.53 – 8.55)	3.17	(0.34 – 5.29)
Ag	0.12	(0.001 – 0.67)	0.13	(0.03 – 0.24)	0.022	(0.007 – 0.09)	0.03	(0.009 – 0.13)
Cd	0.06	(0.002 – 0.26)	0.01	(0.008-0.45)	0.014	(0.005 – 0.066)	0.04	(0.005 – 0.21)
Sn	1.00	(0.26 – 3.28)	0.83	(0.42 – 2.22)	0.41	(0.16 – 1.76)	1.24	(0.20 – 2.89)
W	0.1867	(0.074 – 0.74)	0.11	(0.07 – 0.92)	0.092	(0.03 – 0.60)	0.18	(0.05 – 1.54)
Pb	4.02	(1.26 – 21-62)	2.68	(1.17 – 22.71)	1.78	(0.43 – 25.19)	5.29	(0.58 – 36.54)

Table 4 - Metal concentration (µg/g dry weight) in wing of the four species sampled

WING								
	Miniopterus schreibersii		***Rhinolophus euryale***		***Rhinolophus ferrumequinum***		***Rhinolophus hipposideros***	
	Median	**Range**	**Median**	**Range**	**Median**	**Range**	**Median**	**Range**
Cr	54.93	(14.63 – 931.54)	105.34	(40.81 – 1675.77)	88.36	(30.20 – 2474.56)	231.36	(34.07 – 1980.70)
Mn	48.32	(22.43 – 775.49)	50.72	(23.83 – 1243.65)	39.75	(15.76 – 1508.86)	155.28	(20.51 – 1289.73)
Co	1.58	(0.60 – 9.63)	1.38	(0.64 – 25.20)	1.37	(0.54 – 33.09)	2.72	(1.17 – 21.12)
Ni	33.50	(12.60 – 629.52)	67.09	(21.03 – 1898.40)	45.24	(17.48 – 1313.99)	165.41	(28.86 – 1474.53)
Cu	95.98	(25.56 – 318.33)	137.42	(70.11 – 453.18)	108.80	(31.82 – 823.60)	206	(74.18 – 645.21)
Zn	162.65	(67.13 – 1530.08)	196.15	(78.68 – 3855.95)	132.13	(39.51 – 2235.94)	520	(178.8 – 2215.6)
As	5.27	(2.32 – 58.77)	3.71	(1.47-22)	5.17	(2.11 – 51.53)	3.28	(1.94 – 7.57)
Se	1.77	(0.65 – 22.89)	2.25	(0.91 – 9.59)	2.55	(0.25 – 14.50)	4.85	(1.19 – 7.90)
Ag	0.08	(0.05 – 0.32)	0.1	(0.08 – 0.36)	0.09	(0.04 – 0.89)	0.15	(0.51 – 0.24)
Cd	0.13	(0.05 – 2.37)	0.14	(0.071 – 0.82)	0.16	(0.05 – 1.80)	0.17	(0.10 – 0.59)
Sn	1.58	(0.77 – 12.71)	3.93	(1.09 – 7.70)	2.58	(0.32 – 24.88)	8.12	(1.12 – 13.79)
W	0.73	(0.27 – 4.47)	1.35	(0.32 – 5.05)	1.23	(0.32 – 4.44)	1.08	(0.57 – 3.39)
Pb	13.75	(5.40 – 67.29)	20.02	(10.41 – 234.35)	15.53	(5.54 – 142.91)	34.09	(12.89 – 136.01)

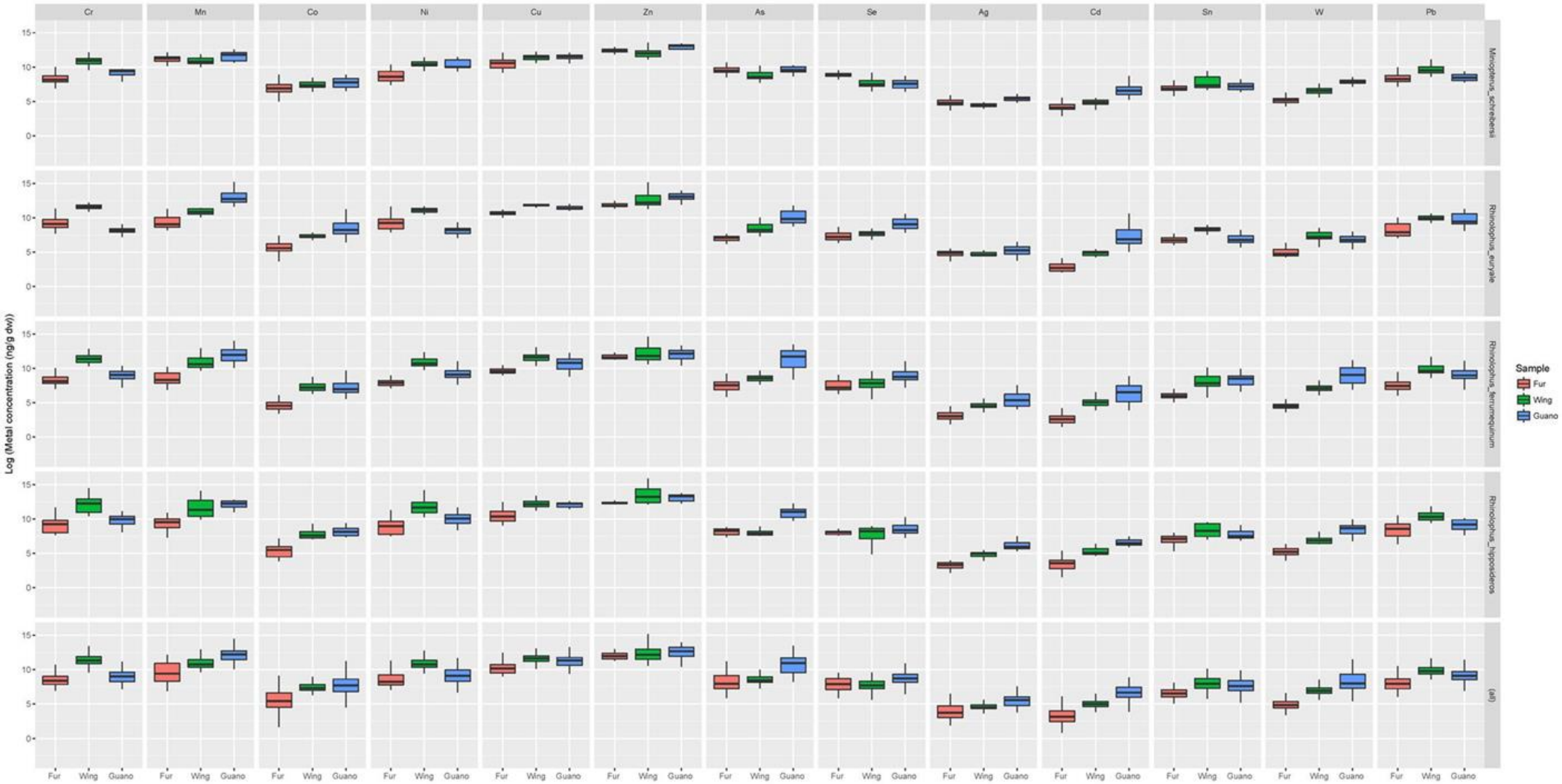

Figure 2 - Boxplots of the log-transformed metal concentrations (ng/g dw) in fur, wing, and guano, for the four bat species sampled.

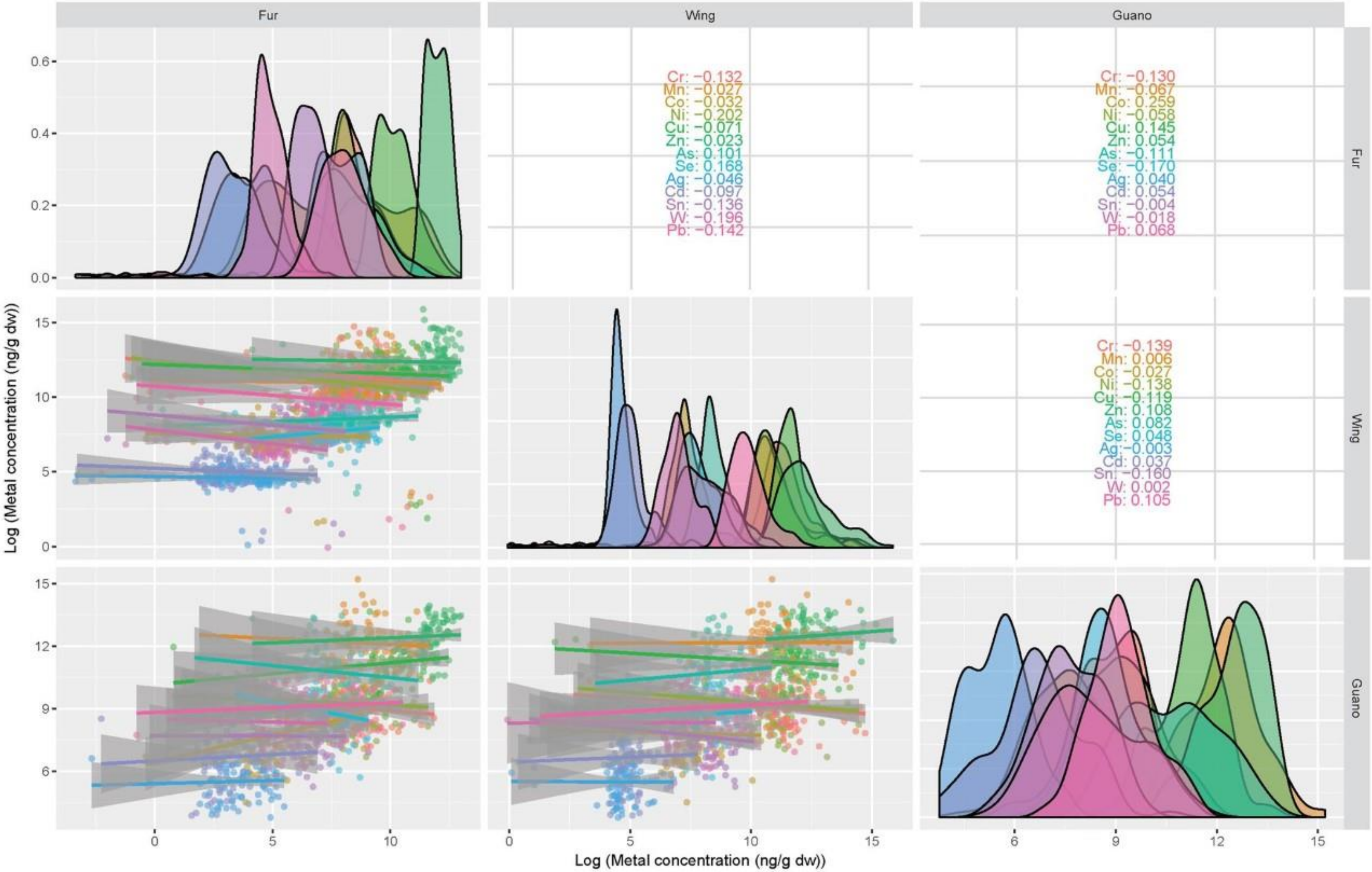

Figure 3 - Density plots, Pearson correlations, and scatterplots (with regression line and confidence intervals in grey) by metal and sample type, focus in comparing Wing vs Fur, Wing vs Guano, and Fur vs Guano

The results showed that there was no significant different between the metal concentration regarding the sampled mines. Differences, however, were found regarding the sampled species. These differences were specifically in both essential (Co, Zn, Mn, and Se), and non-essential metals (As, Ag, and, Cd). Significant differences between sample type (wing and fur) were found in all metals except for Co and Se. Regarding the interaction between location and species, a significant difference was only found in the metals Zn and Se. The interaction between specie and sample type was significantly different for all metals in all species (Table 5).

Mn differences regarding species, sample type and its interaction lay in the low values found in the fur of the *R. ferrumequinum* (1.61±0.28 log µg/g dw; $t_{(260)}$= -5.910; p =0.001) and the *R. hipposideros* (2.64±0.50 log µg/g dw; $t_{(260)}$= -2.763; p =0.031) specifically in comparison with the high values in the *M. schreibersii* (4.36±0.37) and the overall high concentrations found in wing. With the *R. ferrumequinum* presenting the lowest values of Mn in fur. Regarding the concentrations of Mn found in wing, there is no significant difference between species (p-values>0.05).

In the case of As, the differences regarding species, sample type and its interaction lay in the high values of As in the *M. schreibersii.* Concerning fur, the *M. schreibersii* (2.862±0.3 log µg/g dw) presents higher values than the *R. euryale* (0.932±0.422 log µg/g dw; $t_{(258)}$=-3.729; p=0.001), the *R. ferrumequinum* (0.592±0.228 log µg/g dw; $t_{(258)}$=-6.029; p<0.001), and the *R. hipposideros* (1.372±0.454 log µg/g dw; $t_{(258)}$=-2.738; p=0.026). In wing, the *M. schreibersii* (2.311±0.299 log µg/g dw) presents a significantly higher difference than the *R. hipposideros* (0.726±0.496 log µg/g dw; $t_{(258)}$:-2.736; p =0.04). However, the concentration of As in fur for the M. schreibersii was higher than in wing.

In Ag, the differences regarding species, sample type and its interaction are found specifically in the fur concentrations, as there was no difference found in the concentrations in wing regarding species. In fur the highest concentrations are found, with no significant difference, in the *M. schreibersii* (-1.877±0.243 log µg/g dw), followed by the *R. euryale* (-2.264±0.343 log µg/g dw). The *R. euryale* presents significantly higher difference than the *R. ferrumequinum* (-3.853±0.186 log µg/g dw; $t_{(260)}$=-4.074; p<0.001) and the *R. hipposideros* (-3.605±0.372 log µg/g dw; $t_{(260)}$=-2.65; p=0.026). While the *M.*

schreibersii presents significantly higher concentrations than the *R. ferrumequinum* (3.853±0.186 log µg/g dw; $t_{(260)}$=-6.455; p<0.001).

In Cd, the differences regarding species, sample type and its interaction are found specifically in fur, as no difference was found in the concentrations in wing between species. The *M. schreibersii* (-2.265±0.273 log µg/g dw) presented significantly higher concentrations of Cd in fur than the *R. ferrumequinum* (-4.248±0.209 log µg/g dw; $t_{(261)}$=-5.77; p<0.001), *R. hipposideros* (-3.742±0.418 log µg/g dw; $t_{(261)}$=-2.96, p=0.014), and *R. euryale* (-3.752±0.386 log µg/g dw; $t_{(261)}$= -3.15, =0.009).

For Sn there is a significant different between wing (1.074±0.178 log µg/g dw; $t_{(261)}$ = 9.715; p <0.001) and fur (-0.388±0.178 log µg/g dw; $t_{(261)}$=-9.715; p <0.001) in general. There is no significant difference between the species and the concentrations in each sample type. The *R. ferrumequinum* (-1.024±0.245 log µg/g dw; $t_{(261)}$=-2.653; p=0.051) has lower concentrations of metal in fur regarding the other species, *M. schreibersii* (0.043±0.391 log µg/g dw; $t_{(261)}$= 2.653; p=0.051) for example, yet the differences are not significantly different with any specie.

Regarding W, the differences between sample type and the interaction of specie with sample type are explained by the high concentrations of wing (0.183±0.146 log µg/g dw; $t_{(259)}$= 16.126; p <0.001) in relation to fur (-1.817±0.146 log µg/g dw; $t_{(259)}$= -16.126; p<0.001). The concentrations of fur show no significant difference between species. The *R. euryale* (0.907±0.369 log µg/g dw; $t_{(259)}$=3.069; p =0.014) showed difference in wing concentration in relation to the *M. schreibersii* (-0.482±0.262 log µg/g dw; $t_{(259)}$= -3.069; p= 0.014) The *R. ferrumequinum* (0.474±0.199 log µg/g dw; $t_{(259)}$= 2.906; p =0.020) also showed significantly higher concentrations in wing than the *M. schreibersii* (-0.482±0.262 log µg/g dw; $t_{(259)}$= -2.906; p =0.020).

For Pb, a significant difference was found regarding wing (2.831±0.229 log µg/g dw; $t_{(260)}$=9.972; p <0.001) and fur (1.073±0.218 log µg/g dw; $t_{(260)}$ =-9.972; p <0.001) in general. However, even though a significant difference was found in the interaction between type and species overall, this was not link to a specific difference in a species.

Cr showed significant difference between wing (4.682±0.215 log µg/g dw; $t_{(260)}$= 15.502; p <0.001) and fur (1.850±0.215 log µg/g dw; $t_{(260)}$=-15.502; p<0.001). Even though *R.*

ferrumequinum (1.34±0.295 log µg/g dw) has the lowest concentration of Cr in fur the difference between species is not significant. The differences between concentrations in wing among species was also found to be not significant as well.

In Co there is a significant difference between wing (0.639±0.191 log µg/g dw; $t_{(260)}$=13.48; p <0.001) and fur (-1.345±0.182 log µg/g dw; $t_{(260)}$ =-13.48; p <0.001) Regarding its interaction with specie, wing has no significantly different concentrations. The highest concentrations of Co in fur are found in the *M. schreibersii* (0.139±0.307 log µg/g dw) with significantly higher concentrations than the *R. ferrumequinum* (-2.18±0.234 log µg/g dw; $t_{(260)}$= -6.006; p <0.001) and the *R. hipposideros* (-1.499±0.471 log µg/g dw; $t_{(260)}$= -2.913, p =0.019). Also, significant differences were found between *R. euryale* (-0.926±0.433 log µg/g dw; $t_{(260)}$= 2.545; p =0.046) and *R. ferrumequinum* (-2.180±0.234 log µg/g dw; $t_{(260)}$= -2.545; p =0.046) which has the lowest concentrations.

Regarding Ni, significant differences were found between sample type, due to the lower concentrations of fur (1.764±0.213 log µg/g dw; $t_{(260)}$= -13.69; p <0.001) in contrast with wing (4.233±0.212 log µg/g dw; $t_{(260)}$= 13.693; p <0.001) in general.

In the case of Cu, there are significant differences between the general concentrations of fur (3.317±0.211 log µg/g dw; $t_{(260)}$ =-8.083; p <0.001) and wing (4.762±0.211 log µg/g dw; $t_{(260)}$ =8.083; p <0.001), with higher concentrations on wing. No difference regarding species were found.

Regarding Zn, differences were found between species, type, the interaction between them and the interaction between specie and location. Concerning location and specie, the significant differences can be seen between the *R. ferrumequinum* in Carviçais (5.37±0.157 log µg/g dw) which contain higher concentrations, than the ones living in Coelhoso (4.539±0.159 log µg/g dw; $t_{(257)}$= -3.717; p =0.001) and Regoufe (4.764±0.145 log µg/g dw; $t_{(257)}$= -2.834; p =0.025). Significant differences can also be seen in the high concentrations of the *M. schreibersii* living in Regoufe (6.364±0.464 log µg/g dw; $t_{(257)}$=2.43; p =0.016), as oppose to the ones living in Carviçais (5.208±0.105 log µg/g dw; $t_{(257)}$=-2.43; p =0.016). The values of wing (5.746±0.145 log µg/g dw; $t_{(257)}$= 5.003; p <0.001) were significantly higher than the concentrations of fur (5.120±0.145 log µg/g dw; $t_{(257)}$= -5.003; p <0.001). Differences in wing concentrations were found between the *R. hipposideros* (6.587±0.354 log µg/g dw; $t_{(257)}$= 3.08; p =0.014) and the *R. ferrumequinum* (5.338±0.197 log µg/g dw; $t_{(257)}$= -3.08; p =0.014). Regarding fur, differences in concentration can be seen in the high concentrations on the *M. schreibersii* (5.850±0.259 log µg/g dw) compared to the *R. euryale* (4.596±0.365 log µg/g dw; $t_{(257)}$=-2.80; p =0.027) and the *R. ferrumequinum* (4.752±0.198 log µg/g dw; $t_{(257)}$= -3.37; p=0.005).

In Se, several patterns were found based on specie and its interaction with location and sample type. Regarding location and specie, significant differences can be found between the high concentrations of *R. hipposideros* roosting in Carviçais (1.694±0.527 log µg/g dw; $t_{(258)}$= 3.342; p =0.003) in comparison to Regoufe (-1.204±0.688 log µg/g dw; $t_{(258)}$= -3.342; p =0.003). Difference were also found between the *R. hipposideros* roosting in Coelhoso (0.966±0.128 log µg/g dw; $t_{(258)}$= 3.099; p =0.004), to the ones roosting in Regoufe (-1.204±0.688 log µg/g dw; $t_{(258)}$= -3.009; p =0.004). Same can be seen with the *R. ferrumequinum* living in Carviçais (1.351±0.126 log µg/g dw; $t_{(258)}$=4.879; p <0.001) in comparison to Regoufe (0.516±0.116 log µg/g dw; $t_{(258)}$= -4.879; p<0.001). Opposite trends occur with the *M. schreibersii*, which presents significantly higher concentrations in Regoufe (2.839±0.417 log µg/g dw; $t_{(258)}$= 3.712; p <0.001) as oppose to Carviçais (1.258±0.084 log µg/g dw; $t_{(258)}$= -3.712; p <0.001). In the interaction between specie and sample type differences can be seen in both fur and wing. *M. schreibersii* (2.636±0.227 log µg/g dw) presents higher concentrations of Se in fur as oppose to *R. euryale* (0.710±0.286 log µg/g dw; $t_{(258)}$= -5.27, p <0.001), *R. ferrumequinum* (0.471±0.154 log µg/g dw; $t_{(258)}$= -7.89, p <0.001), and R hipposideros (0.625±0.298 log µg/g dw; $t_{(258)}$= -5.37; p <0.001). Regarding wing, the *M. schreibersii* (1.461±0.220 log µg/g dw; $t_{(258)}$= 2.871; p =0.27) presents higher significant values than the *R. hipposideros* (0.346±0.320 log µg/g dw; $t_{(258)}$= -2.87; p =0.027) (Table 5).

Table 5 - Summary of the linear mixed effects model analysis for the different metals measured, depicting F value, degrees of freedom and the p-value. The number marked in green correspond to p-values < 0.05. The metals marked in grey correspond to the metal classified as essential and the metal in white correspond to non-essential metal.

Metal	**Location**				**Species**				**Sample type**				**Location*Species**				**Species*Sample type**			
	F	df1	df2	p	F	df1	df2	p	F	df1	df2	p	F	df1	df2	P	F	df1	df2	p
Cr	1.226	3	260	0.286	1.024	3	260	0.383	218.41	1	260	0.000	1.674	4	260	0.156	3.333	3	260	0.020
Mn	0.952	3	260	0.416	4.779	3	260	0.003	66.182	1	260	0.000	1.595	4	260	0.176	15.974	3	260	0.000
Co	0.944	3	260	0.420	4.952	3	260	0.000	0.691	1	260	0.599	0.691	4	260	0.599	17.880	3	260	0.000
Ni	1.627	3	260	0.183	1.548	3	260	0.203	162.754	1	260	0.000	0.826	4	260	0.510	4.920	3	260	0.002
Cu	0.448	3	260	0.719	0.533	3	260	0.660	52.589	1	260	0.000	0.270	4	260	0.897	5.038	3	260	0.002
Zn	2.225	3	257	0.086	6.679	3	257	0.000	20.831	1	257	0.000	3.732	4	257	0.006	4.635	3	257	0.004
Se	2.251	3	258	0.083	9.646	3	258	0.000	2.397	1	258	0.123	10.30	4	258	0.000	31.703	3	258	0.000
As	0.745	3	258	0.526	4.952	3	258	0.002	5.249	1	258	0.023	1.770	4	258	0.135	17.491	3	258	0.000
Ag	0.517	3	260	0.671	6.032	3	260	0.001	33.583	1	260	0.000	0.826	4	260	0.509	26.436	3	260	0.000
Cd	0.430	3	261	0.732	5.017	3	261	0.002	162.943	1	261	0.000	1.724	4	261	0.145	16.993	3	261	0.000
Sn	0.155	3	261	0.926	0.851	3	261	0.467	78.644	1	261	0.000	0.654	4	261	0.625	7.467	3	261	0.000
W	0.335	3	259	0.800	1.126	3	259	0.339	237.741	1	259	0.000	2.207	4	259	0.069	9.832	3	259	0.000
Pb	0.780	3	260	0.506	0.630	3	260	0.596	88.057	1	260	0.000	0.998	4	260	0.409	5.005	3	260	0.002

Regarding the use of guano to explain the concentrations of metals in the sample types, the results were overall not significant for wing ($F_{(1-948)}$=1.192; p =0.275) as well as for fur ($F_{(1-928)}$= 0.004; p =0.948). In wing, no significant difference were found in species ($F_{(3-958)}$= 0.339; p =0.797); the interaction between species and metals ($F_{(36-928)}$= 0.518; p=0.992); the interactions between metal and guano ($F_{(12-928)}$= 0.806; p =0.645); the interactions between species and guano ($F_{(3-928)}$= 0.441; p =0.724); and the interactions between species, guano and metal ($F_{(36-928)}$= 0.473; p =0.997). In the case of fur no significant difference were found in species ($F_{(3-958)}$= 0.451; p =0.717); the interaction between species and metals ($F_{(36-928)}$= 1.360; p =0.078); the interactions between metal and guano ($F_{(12-928)}$= 1.070; p =0.382); the interactions between species and guano ($F_{(3-928)}$= 0.157; p =0.925); and the interactions between species, guano and metal ($F_{(36-928)}$=1.217; p =0.180).

Arsenic reported the highest concentrations in soil, while Mn reported the highest concentrations in rock. In the case of Coelhoso, the concentrations of metals in soil follow the sequence As > Mn > Cu > Zn > W > Se > Co > Cr > Sn > Ag > Pb > Ni > Cd. Regoufe follows the sequence As > Mn > Zn > W > Sn > Se > Cu > Pb > Ni > Cr > Cd > Co > Ag for soil concentrations. Adoria's metal concentrations on soil follow the sequence Mn > Pb > Zn > As > Cu > Se > Ni > Cr > W > Co > Sn > Ag > Cd. Carviçais follows the sequence Cu > As > Mn > Ni > Zn > Co > Cr > Se > W > Pb > Cd > Sn > Ag.

Regarding the metal concentrations in rock the sequence followed for Coelhoso is Mn > As > Cu > Zn > Co > Se > Ni > Cr > Pb > Sn > W > Cd > Ag. For Regoufe the sequence is Mn > As > Zn > Sn > W > Se > Pb > Cu > Cr > Ni > Cd > Co > Ag. For Adoria the concentrations follow the sequence Mn > Zn > Se > Pb > As > Cu > Co > Sn > Cr > W > Ni > Cd > Ag. Carviçais follows the sequence As > Se > Cr > Mn > Cu > Zn > Pb > Sn > Ni > Co > W > Ag > Cd.

In water all the concentrations found of all metals in the four mines were lower than 0.1 ug/l (Table 6).

Table 3 - Metal concentrations in the environmental compartments sampled (i.e. soil, rock, and water) in the four surveyed mines.

Metal Concentrations in Soil

Mine		**Cr**	**Mn**	**Co**	**Ni**	**Cu**	**Zn**	**As**	**Se**	**Ag**	**Cd**	**Sn**	**W**	**Pb**
Coelhoso	Mean	10.06	232.32	10.27	5.22	93.60	27.69	6984.43	16.84	7.17	0.44	8.61	27.66	6.01
	Standard Error	1.03	76.51	5.70	0.57	9.60	3.04	3754.05	0.18	0.01	0.08	0.86	2.84	0.25
Regoufe	Mean	1.08	434.50	0.62	1.21	4.61	136.69	435.60	7.23	0.04	0.64	8.19	11.58	4.21
	Standard Error	0.18	41.41	0.44	0.01	0.18	59.56	403.93	2.97	0.02	0.41	1.04	2.51	1.06
Adoria	Mean	5.48	184.59	2.52	5.95	36.39	88.86	46.99	33.68	0.19	0.13	1.19	3.20	114.07
	Standard Error	3.58	4.85	0.33	3.92	0.72	12.98	0.03	5.22	0.04	0.05	0.19	0.84	84.56
Carviçais	Mean	22.71	241.94	52.19	92.70	4240.96	60.26	1463.43	20.14	0.38	0.91	0.48	3.22	3.17
	Standard Error	6.37	161.29	48.51	85.70	4113.04	39.31	561.63	13.49	0.12	0.60	0.16	2.11	0.29

Metal Concentrations in Rock

Mine		**Cr**	**Mn**	**Co**	**Ni**	**Cu**	**Zn**	**As**	**Se**	**Ag**	**Cd**	**Sn**	**W**	**Pb**
Coelhoso	Mean	19.61	595.54	31.72	22.03	77.56	67.43	109.58	22.50	0.17	0.20	3.62	2.18	4.247
	Standard Error	1.69	1.72	0.80	1.26	1.77	0.055	14.91	1.35	0.002	0.007	0.22	0.18	0.47
Regoufe	Mean	1.77	440.43	0.09	1.72	3.76	118.43	187.18	6.70	0.017	0.88	13.35	6.68	4.00
	Standard Error	0.47	46.44	0.014	0.96	1.30	10.27	33.29	1.85	0.006	0.12	1.13	0.65	0.17
Adoria	Mean	1.24	122.71	1.66	1.03	11.64	79.75	17.49	32.86	0.22	0.34	1.44	1.19	28.14
	Standard Error	0.05	11.32	0.07	0.07	0.52	4.277	1.96	5.45	0.002	0.04	0.11	0.25	1.29
Carviçais	Mean	12.79	11.74	0.75	2.31	10.91	9.67	49.32	33.51	0.30	0.09	2.89	0.39	2.97
	Standard Error	0.04	0.21	0.002	0.02	0.24	0.03	0.29	0.39	0.02	0.001	0.06	0.05	0.05

Metal Concentrations in Water

Mine		**Cr**	**Mn**	**Co**	**Ni**	**Cu**	**Zn**	**As**	**Se**	**Ag**	**Cd**	**Sn**	**W**	**Pb**
Coelhoso	Mean	0.002	0.04	0.004	0.02	0.09	0.09	0.01	0.0002	<0.001	0.0002	<0.001	0.003	0.002
	Standard Error	0.0002	0.015	0.002	0.0006	0.04	0.008	0.003	<0.001	<0.001	<0.001	<0.001	<0.001	0.0001
Regoufe	Mean	0.0005	0.004	0.001	0.003	0.008	0.04	0.04	<0.001	<0.001	0.0003	<0.001	0.003	0.001
	Standard Error	<0.001	0.0005	<0.001	<0.001	0.0019	0.01	0.01	<0.001	<0.001	<0.001	<0.001	<0.001	0.0003
Adoria	Mean	0.002	0.007	0.0003	0.009	0.02	0.05	0.01	0.0003	<0.001	<0.001	0.0001	0.008	0.003
	Standard Error	0.002	0.002	<0.001	0.004	0.0049	0.03	0.004	<0.001	<0.001	<0.001	<0.001	0.005	0.001
Carviçais	Mean	0.001	0.13	0.01	0.02	0.11	0.06	0.008	0.0006	<0.001	0.0003	<0.001	0.003	0.002
	Standard Error	0.0003	0.06	0.01	0.014	0.10	0.03	0.002	0.0005	<0.001	0.0002	<0.001	<0.001	0.0002

iv. Discussion

Are cave-dwelling bats roosting in abandoned mines exposed to metals?

Metal concentrations were found in the four bat species in both wing and fur, confirming their exposure to the metals. Quantification of metal concentrations on bat's wings have only been performed on one other study (Mina, 2017). In comparison with the concentrations found in Mina (2017), the results in our study showed higher median values in Cr, Mn, Co, Ni, Cu, Zn, As, Cd, and Pb. Our study is the first to use the wing membrane of bats to assess Ag, Sn, and W. It is also the first study to use wing membrane to quantify metal exposure in living bats, and the first in Portugal to use non-lethal samples to evaluate metal exposure on live bats. When comparing the fur results of our study to Mina (2017), our study depicted higher concentrations in Cr, Mn, Ni, Cu, As, and Pb. In Mina (2017), the samples were collected from carcasses of *Hypsugo savii, Nyctalus leisleri, Pipistrellus pipistrellus,* and *Pipistrellus pygmaeus,* product of collision in wind farms in the north of Portugal. The difference in metal quantities might be explain with the difference in sampling locations and difference in species studied. The bats studied in Mina (2017) are not known to be cave-dwellers. The *Hypsugo savii* is known to roost in rock crevices, rarely choosing caves, and when so, it stays in crevices in the entrance only (Alcalde, et al., 2009; Benda, et al., 2003). The *Nyctalus leisleri* roosts mainly in tree holes (Marques, et al., 2006). *Pipistrellus pygmaeus* mainly uses buildings as roosting sites and occasionally hollow trees and rock crevices (Dietz, et al., 2009; Benda, et al., 2003). The *Pipistrellus pipistrellus* mainly uses crevices on trees and buildings, and among the four is the only one who might be found dwelling in a cave during winter (Dietz, et al., 2009; Benda, et al., 2003; Rainho, et al. 2013). In the specific case of Portugal, no mines or caves of natural importance have been selected due to the presence of this species (ICNF, 2014). In comparison, the cave-dwelling species in our study, which roost in mines of Portuguese national importance, seem to have higher metal concentrations in both fur and wing than the species studied in Mina (2017). Only one other study was found targeting the metal concentrations on bats roosting specifically in abandoned mines (Zocche et al., 2010). Higher concentrations of Cr, Ni, Cu, and Pb were found in the liver of the bats sampled in the abandoned mine as opposed to the ones in the control area. DNA damage was also identified in the bats roosting in the mined area. Exposure of metals in other mammal species has already been identified in abandoned mines. Roberts et al. (1978) analyzed the concentrations of lead in small mammals living in abandoned non-ferrous mining areas. These animals presented higher concentrations of lead in almost all tissues (except for muscle) than the ones living in a control area and other lead-contaminated sites. Roberts & Johnson (1978), also reported higher concentrations of metals in the total body of the vole *Peromyscus maniculatus* in a mined area as oppose to a control area. Even though, not a lot of studies have been performed in abandoned mines, the ones that have been performed all conclude the importance of monitoring the biota that now occupies those areas, as exposures have been confirmed.

Significant differences between species were detected in the concentrations of metals in the four bat species, with a significant interaction between species and sample type. The high inter-specific differences have been established in several studies, explaining how the variation can be due to differences in foraging behavior and selected preys (insects living in mining areas may accumulate metals, becoming a route of exposure); habits use and degree of synanthropy (biota living in urban areas are known to accumulate metals); and/or physiological differences among species (difference in excretion and metabolic rates) (Flache, Becker, et al., 2015; Flache, Czarnecki, Düring, Kierdorf, & Encarnação, 2015; Hernout et al., 2016; Hickey, Fenton, Macdonald, & Soulliere, 2001; O'Shea, 2001; Walker et al., 2007). The toxicokinetic and toxicodynamic of non-essential metals could also play an important role when being metabolized or excreted by the bats (Flache et al., 2015). In the case of wing membrane significance between species was only found in Se, Zn, As and W. For Zn, the highest concentration was found in the *R. hipposideros* and for Se the highest concentration was found in the *M. schreibersii*. For As and W the highest concentrations were found in the *M. schreibersii* and the *R. euryale* respectively. Regarding fur, the *M. schreibersii*, was the specie with the highest accumulation of all non-essential metals: As, Ag, Cd, Sn. For the essential metals the *M. schreibersii* presented the highest concentrations in Mn, Co, Zn and Se. In comparison, the *M. schreibersii* is the most exposed species of the four sampled. In our study the biggest group of *M. schreibersii* was found in Carviçais, which also corresponds to the mine with the highest concentrations of Cr, Mn, Co, Ni, Cu and Cd in soil of the four sampled locations. In the case of rock, Carviçais reported the highest concentrations in Se and Ag out of the four. This might indicate a possible transfer of the metals to this bat species. Although a group of *R. ferrumequinum* was also found in Carviçais they did not report concentrations as high as the *M. schreibersii.* The *M. schreibersii* is the only species, out of the four studied, that doesn´t belong the family Rhinolophidae. This might indicate differences in metabolic rates between the two families. The high accumulation of metals in the *M. schreibersii*, as compared to the other species sampled, can also be due to its dietary composition, its degree of synanthropy, and its habitat selection. The *M. schreibersii* are known to feed in urban areas (Hutson, et al., 2008; ICN, 2006). This high degree of synanthropy makes them rather vulnerable to all sorts of contaminants (Zukal et al., 2015). In the study performed by Flache et al. (2015) the bat species roosting in urban areas, as oppose to the ones roosting in cultured areas and water bodies, presented the higher levels of metal concentrations in fur. This was also seen in the study by Hariono et al. (1993) in which the highest concentrations of Pb were found in fruit bats living in urban areas. The *M. schreibersii* prey mainly on moths and black butterflies (Lepidoptera), flies and mosquitoes (Diptera), and beetles (Coleoptera) (Hutson, et al., 2008; ICN, 2006). Making it the species with the biggest diet diversity out of the species sampled. Both proportion and variety of prey have been recorded to be of great relevance in bioaccumulation of metals in bats, hence the diversity of prey may increase the chances of exposure (Hernout, et al., 2015). The order Coleoptera has the highest bioaccessibility, followed by Lepidoptera and Diptera (Hernout, et al., 2015), all orders that the *M. schreibersii* feeds on. Among the four species, the *M. schreibersii* is the only migratory species. According to Palmeirim et al. 1999 the highest recorded movement in Portugal has been 240km. Its high mobility makes it difficult to identify punctual exposure but makes the species more vulnerable to different contamination sources. Specifically, because the species is exclusively cave-dwelling and due to the anthropogenic interventions in natural caves, their habitat is reduced to abandoned mines, which could represent a focal point of metal contamination (ICN, 2006). As oppose to the *M. schreibersii*, the *R.*

ferrumequinum presented the lowest concentrations of Mn, As, Ag, Cd, Co, Cd, Zn and Se in fur out of the four species, even though a large group was found in the same mine. In comparison, the *R. ferrumequinum* has the highest biomass of the four species, and a bigger average body size than the *M. schreibersii*. This might explain the low concentrations due to the possible differences in the metabolic rates of both species. Their habitat selection and foraging behavior, are to be taken into account as well. They feed mostly on beetles (Coleoptera) and moths (Lepidoptera) in open areas such as pastures, shrublands, and woodlands. Since they forage away from urban areas and its mobility is rather low (engaging in short distances between hibernation and maternity roosting sites) their possibilities of ingesting contaminated prey are reduced (Piraccini, R., 2016; Esp et al., 1999). It is important to note that all the species studied only use the mines for hibernation during the winter season. Bats are animals with a rather high mobility, being able to travel several kilometers per night in search for suitable foraging areas (Zukal, et al., 2015). In the case of the *R. ferrumequinum* it was the only species found in the four mines. This makes it complicated to locate a specific contamination source.

The *R. hipposideros* presented the highest concentrations of Zn in fur. Even though, Zn is an essential metal necessary for regular body functions, its accumulation through the food chain is possible (Flache et al., 2015). The order Diptera presents the highest bioaccumulation factor for Zn out of the insects' bats normally feed on (Hernout et al., 2015). The *R. hipposideros* preys almost exclusively of midges (Diptera), craneflies (Diptera) and some moths (Lepidoptera), making the order Diptera their main food source. Also, adult midges are known to contain high concentrations of Zn when the environment they emerge from has zinc as well (Goodyear, et al., 1999). The foraging behavior of this species usually takes place exclusively within woodland areas, while open areas, such as pastures and riverside zones, are avoided. They are also sedentary, with differences of only 20 km from hibernation to maternity sites. (Zahn et al. 2008, Lino et al. 2014; Esp et al., 2001). Hence the *R. hipposideros* presenting the highest concentrations of Zn can be explain due to dietary composition, considering they don't forage in urban areas and their mobility is quite low when compared to the other species. However, the *R. hipposideros* was mainly found in Coelhoso, which reported the lowest concentrations of zinc in soil out of the four mines, and the second lowest concentration in rock. The *R. euryale* presented the highest concentration of W in wing. This result was not expected, as well. This species mostly feeds on moths (Lepidoptera) and beetles (Coleoptera). They are also categorized as a sedentary species, using caves almost all year round, with recorded distances traveled from hibernation to maternity sites of only 41 km in Portugal (Esp et al., 1999). Their habitat preference for caves might explain its high concentrations of W, as a continuous exposure in the mines used might occur. Still in our study, the *R. euryale* was found in Adoria, which consequently presented the lowest levels of W in soil out of the four mines and the second lowest concentration in rock.

Nevertheless, a direct link between the location that species were collected from and the metal concentrations found in fur and wing was not established. The environmental compartments (soil, rock, and water) sampled alone, do not explain the differences in concentrations found in wing and fur on the different bat species, as all the individuals were collected from mining areas. Even though relevant concentrations of metals were found in soil and rock in the four mines, they might not be bioavailable, hence not constituting a direct exposure pathway for the bats through dermal contact. The fact that metals are naturally present in soil, with emphasis in the essential metals (like Zn), makes it complicated to conclude any risk or exposure based solely on soil measures (Hernout et al., 2016). Roberts & Johnson, (1978) studied the transfer of Pb, Zn, and Cd thought a terrestrial food chain in an abandoned mine. The study found high concentrations of metals in soil and in invertebrates, however, the total body amount of metals found in small mammals was not consistently higher than the levels in their estimated diets, presenting even lower amount of Pb in the mined area than in the control area. Thus, heavy metal pollution is not necessarily linked directly to the concentrations found in soil, but rather to the transference potential of the metals and differential mobility within a food chain. In our study the interaction between the species found and location confirmed differences in only two essential metals. The *R. ferrumequinum* in Carviçais had higher concentrations of Zn and Se, than in the other mines. The *M. schreibersii* presented higher concentrations of Zn in Regoufe and the *R. hipposideros* presented higher concentrations of Se in Carviçais. The high concentrations of Zn in Regoufe can also be seen in the sampled soil and rock, as this mine presented the highest concentrations in both compartments of the four locations. The highest concentration of Se in rock was also found in Carviçais, while in soil it was the second highest concentration. However, these results might be biased due to the uneven distribution of the species per mine. In Regoufe, only two individuals of *M. schreibersii* were collected, while only one individual of *R. hipposideros* was collected in Carviçais. Regarding water, extremely low concentrations were found for all metals in all mines, making ingestion of water as a direct route of exposure in the mines unlikely.

As a direct exposure, specifically through the trophic chain, the bioavailability of metals to insects is influenced by the properties of the soil, which can, in any case allow or impair accumulation due to stabilization of the metals. (Hernout et al., 2013; John Leventhal, 1995; Navarro et al, 2008). Studies have recorded how pH, quantity and quality of organic matter and carbonate content, to name a few, are of high relevance in determining the bioavailability of metals from soils and sediments to insects (Hernout et al., 2013). Insectivorous bats are expose mainly through bioaccumulation through the tropic chain. In this study, since insects were not collected, guano was used to test if it could significantly explain the metal concentrations as a proxy for food intake. Martin (1992) concluded that metals in guano could represent the amount of metals found in the insects, as some parts of them are not fully digested by the bats and hence excreted. The amounts of metals found in guano could reflect how the different insect orders bats prey on is linked to the amount of metals accumulated, as some orders are more expose to metals than others. In the case of non-essential metals, higher concentrations were expected in guano than in tissue sampled (Zukal et al., 2015). Relevant concentrations of metals were found in the guano collected in our study, yet, the results showed the relationship between the concentrations of guano and the sampled wing and fur was not significant. Nevertheless, more studies would need to be perform on soil and insects to confirm bioavailability through the tropic chain in the mines alone and identify a possible bioaccumulation and/or biomagnification.

Is Metal Exposure a Potential Risk for Bat Populations?

Significant higher concentrations were found in wing in the non-essential metals, except for As, which showed higher concentrations in fur. This might be because arsenic contents in mammals are mostly present in organs that are involved in excretion and sequestration (Erry et al., 2005). Making fur a more relevant reservoir of arsenic in the body of bats than wing membrane. For the essential metals, wing presented higher concentrations than fur in Cr, Co, Cu, Zn and Ni. The high concentrations of metals in wing compared to fur might be explain due to the permanent exposure of wing to the environment. Absorption through skin is one of the known routes of exposure metals use to enter the body (Heikens, et al., 2001; Tchounwou et al., 2012; Vinodhini & Narayanan, 2008; Zukal, et al. 2015). Although, no studies have been performed directly on the wing membrane of bats, absorption through skin in small mammals and humans has already been recorded (Wester et al. 1992; Hostynek 2003; Larese et al. 2007). Also, when using animal fur for contamination assessments, molting periods should be taken into consideration, as this could explain the low amounts in fur as compared to wing membrane. Bats shed their hair once a year during late summer, approximately between July and August (Dietz et al., 2009). Period in which the metal concentrations found might differ, as concentrations prior to molting will be higher than the ones found after or during the molting period (Flache, Becker, et al., 2015). The fur collected in this study was sampled during the month of March, prior to the molting period. Since hair is being renewed annually it might represent accumulation in shorter periods of time or a more acute exposure. As oppose to wing membrane, that regenerates only when wounded or due to natural wear, providing useful information about a possible chronic metal exposure over a more extended period (Faure, Re & Clare, 2009; Weaver et al., 2009). A comparison between fur and wing during different seasons might be needed to further understand the time span of the exposure.

The use of fur as a non-invasive tool to assess heavy metal concentration in bats has been validated by several studies. Correlations have linked the amount of metals found in fur to internal organs. Hernout, et al. (2015) observed significant correlations between the concentrations found in fur of Cd and Cu to the ones found in stomach, kidneys, liver, and bones on *Pipistrellus pipistrellus/pygmaeus* bats. Significant correlations in Pb were also found between fur and kidneys, stomach, and bones. Concentrations of Zn in fur, were correlated with stomach, and bones. Hariono (1993), found correlations between the concentrations of Pb in fur with bones and teeth on fruit bats, concluding it could be representative of a long-term exposure. The study also found a correlation between fur and kidney and liver. Mina (2017) also found correlations between wing membrane to other organs, specifically As in bone, brain and liver; Cd in bone; Mn in liver, brain, and heart. Fur was found to correlate with liver in As; bone, brain, heart and liver in Co; liver in Cu; bone and liver in Pb; and Co correlated in fur with all internal organs. What constitutes a concentration of metals in fur (and wing) that indicates toxicity in bats is still uncertain, as a threshold has yet to be establish (Hariono et al. 1993). However, the correlation of them with other organs allows comparisons to be made to a certain extend when assessing effects. In the study performed by Zocche et al., (2010), DNA damage was found in the bats roosting in the abandoned mine. The concentration of metals found in the liver of the species are similar (and even lower) to the concentrations found in fur and wing in our study. Thus, the species in our study might be at risk of suffering DNA damage. Literature has already established the exposure and risk abandoned mines constitute to the biota. The fact we reported concentrations for all the metals analyzed in both fur and wing, and that we were able to compare them with another study supports the idea that cave-dwelling bats might be at risk due to their exposure to metals.

Chapter III Conclusion

Conclusion

Metal concentrations were found in all the bat species sampled in both wing and fur, confirming their exposure to the metals. Higher concentrations were found in wing probably due to its continuous exposure to the environment and due to the molting periods bat's fur undergoes. Differences in the metal concentrations were found between the species collected. Yet, the routes of exposure are still unclear. The *M. schreibersii* presented the highest metal concentrations out of the four species. With the high concentrations of As, Ag, Cd, Sn, Mn, Co, Zn and Se in fur; and Se and As in wing. This might be due to its foraging behavior and habitat use linked to urban areas; differences in metabolic rates; and/or the fact that the biggest colony was found in Carviçais, which was the mine the reported the highest concentration of Cr, Mn, Co, Ni, Cu and Cd in soil. The *R. ferrumequinum* presented the lowest concentrations of Mn, As, Ag, Cd, Co, Cd, Zn and Se in fur, even though a large group was also found roosting in Carviçais. Metal concentrations in soils and rock may not always reflect the environmental exposure and bioavailability of the metals for bats. Many environmental and biological factors play a role in the exposure of contaminants through the food chain. In comparison with other studies, the concentrations found in cave-dwelling bats were higher than in bat species that select alternative roosting sites. The results reinforce the importance of monitoring metals in species roosting in abandoned mines for the conversation of bats. In addition, metals need to be considered as a possible risk for cave-dwelling bats.

Chapter IV References

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