Assessing the risks of soil-associated metals to bats in England and Wales

Béatrice Véronique Hernout

Submitted for the degree of PhD

University of York

Environment Department

December 2013

Abstract

Bat populations around the world are in decline. This may be due to a range of pressures including exposure to environmental contaminants. However, little is known about the actual risks of contaminants, such as metals, to bat health. In light of this, the present study investigated the risks of soil-associated metals to bats.

A spatially-based modelling framework was developed in order to predict the risks of metals to 14 bat species in England and Wales. Lead was found to pose the greatest risk followed by copper, cadmium and zinc. The key factors driving the risk were the proportion of invertebrate orders in the bat diet, followed by the amount of food eaten and the predicted safe daily dose.

Monitoring data on metal concentrations in *Pipistrellus sp.* tissues were then developed to evaluate the modelling framework. Approximately 21% of the bats sampled contained residues of at least one metal high enough to cause toxicity. The monitoring data agreed with model predictions and showed Pb to pose the greatest risk, followed by Cu, Zn and Cd. Additionally, the model evaluation revealed that bats with high metal residues were generally found in areas predicted to be “at risk” by the model.

However, predictions were not perfect. One of the potential factors explaining this mismatch, namely bioaccessibility, was investigated further. Metal bioaccessibility from different insect orders to bats was assessed using an *In vitro* gastric model (IVGM). Bioaccessible fractions were significantly different across insect types. Inclusion of the bioaccessible fraction as a model input resulted in a change in the risk ranking for different species.

Overall the results showed that metals could well be contributing to declines in bat populations. The modelling framework developed could play an important role in determining the risks of metals to bats (and other wildlife species) more generally and in identifying areas where mitigation measures could be targeted to reverse these population declines.

List of contents

List of tables

List of figures

Acknowledgements

I am grateful to my supervisor, Prof. Alistair Boxall, for his advice and help during my Ph.D. I am also thankful to my co-supervisors Dr. Kathryn Arnold and Dr. Colin McClean for their sensible comments and advice. My project has benefited from a high quality training programme complemented by fruitful discussions. I would also like to thank Dr. James Aegerter for his advice and for lending me field work material. The help of Dr. John Lewis, Dr. Stéphane Pietravalle, Dr. Robert Weaver and Prof. Malcolm Cresser was also much appreciated, as was that of Malcolm Baxter and Mickael Walls, who assisted with chemical analysis.

I would like to acknowledge the European Union, which financially supported my project under the 7th Framework Programme (project acronym CREAM, contract number PITN-GA-2009-238148). I would also like to express my gratitude to all members of the CREAM project, and particularly Dr. Volker Grimm, for their enthusiastic and inspiring collaboration. Thanks to the whole EcoChemistry group at FERA, particularly Chris Sinclair, for helping to organise field and experimental work.

Thanks to the Veterinary Laboratory Agency for providing access to bat samples; the Museum of Natural History of Genève for providing a large number of publications on bat ecology; the National Soil Resources Institute for providing data on soil metal concentrations in England and Wales; and the Bat Conservation Trust for providing data on bat distributions in England and Wales.

Special thanks to Sara for her positive energy and having a spare bed for me; Annika for our endless discussions on putting the world to rights; and to all the other students from the team. It was worth moving to York to meet you all.

I would like to address special thoughts to my friend Nico, who always encouraged me to give my best, and certainly still does. Huge thanks to the “group” from “Plan de Cuques, Allauch, Château”. We have all been there for each other in particular moments, as old good friends would do.

Finally, deep thanks to my family, for supporting me, being so understanding, believing in me and for transmitting to me a passion for nature, wildlife and science. Thank you, Philou, for your huge patience, support and being, as you are, by my side.

Author's declaration

This thesis contains original work completed by myself as a PhD student at the Food and Environment Research Agency (York), and the University of York under the supervision of Professor Alistair Boxall (January 2010 – December 2013).

This research was funded by a Marie Curie fellowship which was financially supported by the European Union under the 7th Framework Programme (Project acronym CREAM, contract number PITN-GA-2009-238148).

Two scientific papers from the work have been published in international peer-reviewed journals. A further four publications are in development or have been submitted. The publications have been written by the candidate as leading author. However, it should be noted that the publications have gained in quality through suggestions, advice and editing from the co-authors. The current status of the publications is presented in Table 0.1.

While this thesis is based on the published and drafted papers, the manuscripts have been reworked, for inclusion in the thesis, so that they are presented in a consistent style and format, thus avoiding repetition of material.

Table 0.1: Status of the papers produced from the work conducted to develop this thesis with respect to the publication process.

Paper number	*Authors*	*Title*	*Status*	*Journal*
1	Hernout, B.V, Somerwill, K.E, Arnold, K.E., McClean, C.J, and Boxall, A.B.A.	A spatially-based modelling framework for assessing the risks of soil-associated metals to bats	Published in 2013	*Environmental Pollution*
2	Hernout, B.V., Pietravalle, S., McClean, C.J., Aegerter, J., Arnold, K.E., and Boxall, A.B.A.	Interspecies variation in exposure to trace metals for bats	In preparation	*Environmental Toxicology and Chemistry*
3	Hernout, B.V., McClean, C.J., Arnold, K.E. and Boxall, A.B.A.	Metal exposure may contribute to declines in bat populations	In preparation	*PLOS ONE*
4	Hernout, B.V., Bowman, S.R., Weaver, R., Jayasinghe, C. and Boxall, A.B.A.	Implications of differences in bioaccessibility for the assessment of risks of metals to bats	Submitted	*Environmental Science & Technology*
5	Hernout, B.V., Arnold, K.E., McClean, C.J., Grimm, V. and Boxall, A.B.A.	Predicting the threats of chemicals to wildlife: what are the challenges?	Published in 2011	*Integrated Environmental Assessment and Management*
6	Hernout, B.V., McClean, C.J., Arnold, K.E., and Boxall, A.B.A.	Is bat fur a valuable less invasive tool to monitor metal contamination?	In preparation	*The Science of the Total Environment*

Chapter 1

Introduction

Metals in the environment

Metals are naturally present in the environment. Due to their physio-chemical properties (e.g. resistance, resilience, strength, conducting properties, and thermal conduction potential), metals have been exploited and extracted (*via* mining, waste incinerators, combustion, oil and gas extraction processes, refineries) for use in industry, agriculture (e.g. plant fertilisers), construction, high technology products and for many other anthropogenic purposes (e.g. leaded gasoline, electrical appliances) (Hoffman *et al.,* 2001). Anthropogenic extraction and use can result in large quantities of metals being released into ecosystems and, as a result, metal emissions to the natural environment have been widespread across Europe since the industrial revolution (Hoffman *et al.,* 2001). As they are non-biodegradable, once released they persist in the environment. Several hundreds of thousands of metal contaminated sites are in urgent need of remediation in both Europe and the US (Reddy *et al.,* 2010; Panagos *et al.,* 2013). This number may be an underestimate however, as the monitoring of contaminated sites is widely heterogeneous across European countries (Panagos *et al.,* 2013). In England and Wales, around 300,000 hectares of land is estimated to be affected by metal contamination (Environment Agency, 2009).

Exposure pathways for wildlife

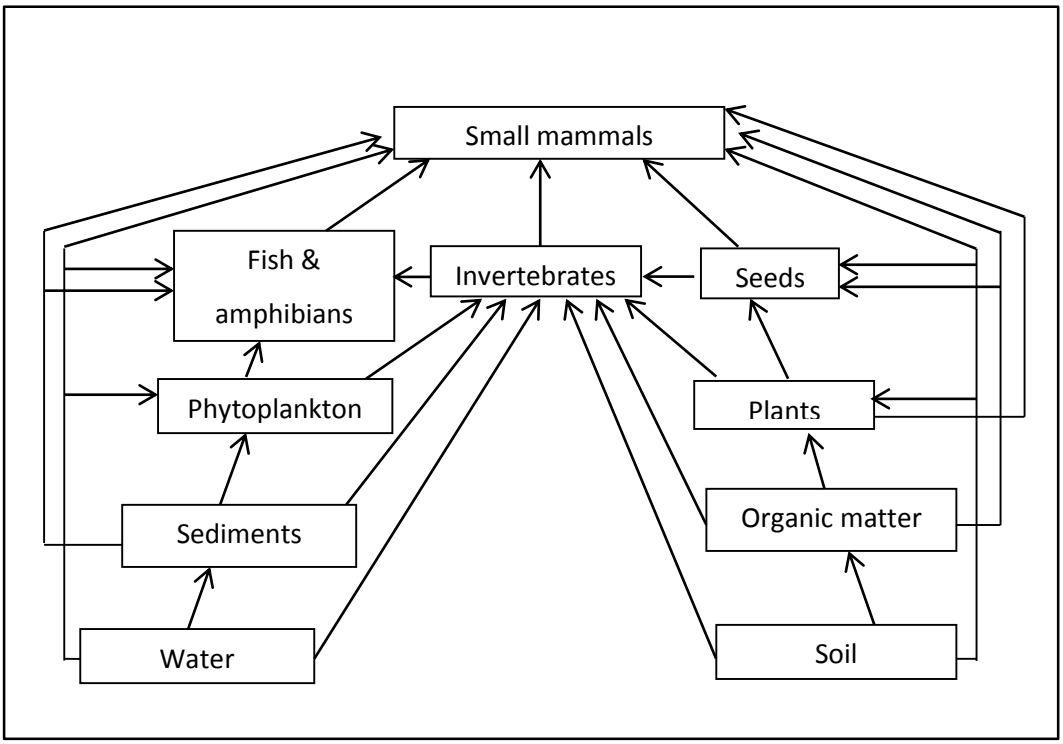
Wildlife can be exposed to metals *via* a range of possible pathways, including: inhalation, dermal exposure and ingestion (Sample and Suter, 1994). Determining the degree of exposure of terrestrial wildlife species to metals is particularly complex as the organisms are exposed to multiple media (e.g. soil, sediments) *via* a diverse range of activities (e.g. drinking, swimming, fishing, ingestion of food and soil, breathing) (Sample and Suter, 1994). In addition, most wildlife species are mobile and the exposure can occur within different habitats which are contaminated by several sources (Sample and Suter, 1994). In mammals,

a potential contamination pathway is the transfer of metals across the placental barrier from mothers to the embryo or from contaminated milk to the juveniles (Streit and Nagel, 1993). However, oral ingestion is usually considered as the main pathway of metal exposure for wild mammals in environmental risk assessment (ERA) studies which are performed to evaluate the risks of chemicals to the environment (Ma 1989; EFSA journal, 2009). In the remainder of this chapter, the uptake of metals into small mammals and potential effects of the accumulated metals are discussed.

Metal uptake into small mammals

Once introduced into ecosystems through anthropogenic activities, metals can be transported around the different environmental media and can be taken up by plants and animals (Heikens *et al.,* 2001). The different pathways of uptake of metals *via* ingestion into small mammals are presented in Figure 1.1.

Figure 1.1: Main exposure routes of exposure for mammals to metals in the environment *via* ingestion.



Uptake into invertebrates

Invertebrates are in close contact with water, sediments or soil layers depending on their life stage. In terrestrial ecosystems, metals can bind to the mineral fraction of soils, soil organic carbon or accumulate in microorganisms, plants and soil-dwelling animals (Vermeulen *et al.,*

2009). In aquatic ecosystems, metals can be transferred from sediments to benthic invertebrates and insect larvae feeding on phytoplankton and zooplankton (Linder and Joermann, 2001). The accumulation from soil surface layers and sediments into different invertebrate species has been widely documented (Heikens *et al.,* 2001; Goodyear and McNeill, 1999). Several studies have derived biota accumulation factors (BAF) (Hoffman *et al.,* 2001) which can be used to estimate concentrations in invertebrates from soil or sediment concentrations (Heikens *et al.,* 2001; Wang, 1987).

The uptake of a metal into invertebrates is influenced by the solubility, speciation and complexation of the metal. Uptake into organisms can also be influenced by environmental and biological factors. In aquatic systems, several environmental factors affect the uptake of metals including pH, temperature, alkalinity, hardness, inorganic ligands (Wang, 1987). Several abiotic factors such as pH, organic content, cation exchange capacity and clay content influence the uptake from soil into terrestrial invertebrates (Vijver *et al.,* 2003; Jelaska *et al.,* 2007; Beyer *et al.,* 1985). For example, previous studies have shown that metal concentrations in earthworms increase as the pH of study soils decreases (Nahmani *et al.,* 2007). Conversely, increases in organic matter content, the cation exchange capacity and soil cations, and clay size particles reduce the uptake of metals into invertebrates (Nahmani *et al.,* 2007). These observations are explained by the fact that the different parameters influence the sorption of metal ions to soil particles and the concentrations of free metal ions in solutions by complexation and therefore impact the potential metal availability to invertebrates (Nahmani *et al.,* 2007).

On a larger scale, environmental properties such as habitat type, landscape composition and diversity can also be important. For example, organic content and pH can vary across sites and habitat type, thus suggesting that the combination of abiotic factors and ecological conditions may influence the metal bioavailability and accumulation into organisms (Fritsch *et al.,* 2012; Vermeulen *et al.,* 2009; Kapusta *et al.,* 2003).

Biotic factors such as organism size, life stage, species type and dietary regime also play a role in metal uptake (Wang, 1987). Recently, the use of species traits to predict uptake of contaminants into organisms has been suggested to be included in ERA processes (Rubach *et al.,* 2012). Factors such as size related traits (e.g. biovolume, surface area, length and dry mass), trophic position, respiration mode and lipid content have all been shown to be correlated with the uptake of insecticide by aquatic invertebrates (Rubach *et al.,* 2012). These species traits may well also play an important role in the uptake of metals into terrestrial

invertebrates, although the importance of species traits in terms of uptake in terrestrial systems has not been extensively studied.

Uptake into other food items

Metals can be taken up by plants and seeds (Peralta-Videa *et al.,* 2009; Spitzer *et al.,* 1980), which can then be consumed by certain rodent species (Sheffield *et al.,* 2001). Metal uptake into fish and amphibians has also been demonstrated (Dallinger *et al.,* 1987; Moriarty *et al.,* 2013). As many small mammals species feed on fish (e.g. the fish-eating bat, *Myotis vivesi*) and amphibians (e.g. the Eurasian water shrew, *Neomys fodiens*; Otálora-Ardila *et al.,* 2013; Haberl, 2002), this provides a potential route of exposure from water bodies into wildlife. Direct ingestion of soil and organic matter (e.g. for shrews) and of contaminated water can also occur (Kaufman *et al.,* 2007).

Uptake from food types into small mammals

The chemical properties of the metal also influence the uptake from food types into small mammals. The gut chemistry and biochemistry of the mammalian species will influence the metal's bioavailability. Host factors such as age, gender and species are also important when it comes to the accumulation of metals in small mammals (Fritsch *et al.,* 2010). For example, Cd concentrations in small mammal tissues are thought to be affected by the age of the organism (Rudy, 2009). The mammalian feeding behaviour may also influence their metal exposure. In addition, mammals with different diets may also frequent different habitats (Dietz *et al.,* 2009).

Metal bioaccumulation in small mammals

Bioaccumulation has been defined as "the net accumulation of a substance by an organism as a result of uptake directly from all environmental sources and as a result of uptake from all routes of exposure" (Linder and Joermann, 2001). Evidence of metal accumulation has been shown in small mammals following oral exposure to high levels of metals (Ma and Talmage, 2001). When it comes to characterising bioaccumulation, the target organ bioaccumulation factor is widely used. This is defined as the ratio between the concentration in the kidneys and the dietary concentration (Ma and Talmage, 2001). Target organ bioaccumulation factors of 6-8 and 3-4 for Cd and Pb respectively, have been determined for the shrew (*Sorex araneus*) (after a food intake of earthworms, beetles and spiders) (Ma and Talmage, 2001). Other studies have also measured higher renal concentration of metals in

shrews and moles compared to in their food (earthworms) (Hendricks *et al.,* 1995; Ma, 1987). Metal accumulation in small mammals is likely to occur in heavily contaminated sites (e.g. in the vicinity of Pb smelters) in which metal residues may reach high levels (Ma and Talamage, 2001; Pankakoski *et al.,* 1994). For example, relationships between tissue concentrations, traffic densities and distance from highways have been found for the shrew (*Blarina brevicauda* and *Sorex araneus*) (Goldsmith and Scanlon, 1977; Chmiel and Harrison, 1981; Ma and Talmage, 2001).

Metal biomagnification

Biomagnification has been defined as a process whereby the concentration of a given element increases in organisms at higher trophic levels (Linder and Joermann, 2001). Evidence of metal biomagnification has been shown for mammals for many persistent contaminants. For example, in Arctic regions, marine mammals and polar bears (*Ursus maritimus*) have been shown to contain high concentrations of pesticides and persistent organic pollutants (DDT, PCBs etc.) due to atmospheric processes and the physio-chemical properties of the compounds (Ross and Troisi, 2001; Norstrom *et al.,* 1988). For metals, mercury, Cd and possibly Pb accumulate in terrestrial food chains into higher trophic levels (Mason and Wren, 2001). However, the literature on biomagnification of metals in the terrestrial ecosystem is relatively scarce compared to aquatic ecosystems.

Metal toxicity to small mammals

Essential and non-essential metals

Metals can be assigned to one of two categories: biologically essential and non-essential metals. Biologically essential metals (e.g. copper, nickel, iron, zinc) are necessary in organisms to ensure efficient functioning of the organism and correct functioning of cellular processes. For example, Cu plays an important role in biological electron and oxygen transport (Hoffman *et al.,* 2001). However, essential metals can also become toxic at high concentrations when their intake is higher than the organism's excretory and detoxification capacity. A deficiency in essential metals can also make organisms vulnerable to disease (Hoffman *et al.,* 2001).

Non-essential metals (e.g. Pb, Cd, Hg and selenium) can be tolerated at trace levels, but are generally considered toxic and can compete with essential metals for active sites (Hoffman

et al., 2001). Organisms can regulate metals and metal excretion depending on the chemical form in which they are available in the environment (Carravieri and Scheifler, 2013).

Effects on laboratory controlled small mammals

Metal accumulation can adversely affect organisms (Vermeulen *et al.,* 2009). Indeed, high metal concentrations in mammalian tissues are known to result in toxic effects in a variety of organs including the heart, bones, intestines, kidneys, reproductive and nervous systems (Clark and Shore, 2001). The majority of data on metal toxicity in mammals come from experimental studies using rodents (mice, voles and rats) bred under controlled conditions. Exposure to Cd results in histopathological lesions, embryotoxicity, weight loss and mortality (Sheffield *et al.,* 2001). Lead exposure can result in nephrosis, lesions of the kidneys, hypertension, low body weight, renal oedema, urinary dysfunction, lesions of reproductive organs and low haematocrit measurements (Ma and Talmage, 2001). Exposure to high concentrations of Zn can elicit a range of effects, including reductions in growth, death of foetuses and mortality (Ma and Talmage, 2001).

Effects on wild small mammals

A wide range of toxic symptoms have been observed in individuals from wild populations living on metal contaminated sites. The main effects induced by Cd exposure in the field are impacts on kidneys and liver (e.g. lesions, tubular cell degeneration in the kidneys, renal tubular dysfunction caused by inflammation and fibrosis), and cardiovascular and skeletal system damage (Ma and Talmage, 2001; Hunter and Johnson, 1982). The severity of the symptoms (lesions in the liver and kidney damage) has been found to correlate with the tissue Cd concentrations (Hunter and Johnson, 1982). Other symptoms such as lack of appetite, small body weight, mortality and histological renal changes have also been observed in individual shrews sampled near a Pb smelter (Ma and Talmage, 2001). Wild small mammals such as the wild house mouse (*Mus musculus*) and field voles (*Microtus agrestis*) have shown cytogenetic damage, effects on spermatogenesis and renal histopathological changes when exposed to metal contaminated sites (Ieradi *et al.,* 1996; Ma and Talmage, 2001).

Effects on a population scale

While data on metal-induced effects on individuals are relatively abundant, field studies focussing on the effects of metals on wild populations are largely lacking (Shore and Douben,

1994). As such, the incidence of symptoms at the population and community levels requires further investigation in order to understand the risks posed by metals to population viability. This research need is highlighted in ERA guidelines developed for pesticides and plant protection products for birds and mammals (EFSA Journal, 2009). For example, metal pollution can impact habitat suitability and reduce population size in rodent populations, as shown in Pennsylvania where the population size of wild rodents, living within a few km from a Zn smelter, was significantly lower than at more distant sites (Storm *et al.,* 1993).

Sensitivity of different species

The measurement of metal tissue residues in laboratory toxicity studies has been used to define critical toxic thresholds for small mammals (Ma, 1996; Chmielnicka *et al.,* 1989). However, caution should be taken when extrapolating data from laboratory studies to impacts on wild populations, as unrealistic doses and administration routes may have been used in the experiments and small mammals and different genders may not have the same tolerance to metals (see Chapter 6). For example, insectivorous species seem to be more tolerant than rodents to Cd and Pb-induced stress as they have a greater level of metallothionein production and, therefore, a greater detoxification capacity (Shore and Douben, 1994; Ma and Talmage, 2001). Moreover, insectivores such as the shrew and mole feed mostly on earthworms, spiders and isopods, which have a high metal bioaccumulation capacity and are also present in contaminated areas. In light of this, insectivorous species may accumulate more metals than rodents. For example, feeding shrews with a Cd daily dose similar to the mouse LD_{50} (Lethal concentration, 50%), showed no effects on survival rates, but did show effects on body weight (Ma and Talmage, 2001).

Metal contamination in bats

Among wildlife species, the scientific interest in chiropteran species has increased in recent years due to observed declines in populations of these species worldwide (Pennisi *et al.,* 2004). Several stressors (e.g. climate change, habitat fragmentation, roost loss, urbanisation and agricultural intensification, the increase in wind turbines, the pressure of disease and exposure to chemicals in the environment) have been suggested as potential causes of the observed declines in populations (Frick *et al.,* 2010; Jefferies, 1972; Jones *et al.,* 2009; Walker *et al.,* 2007; Wickramasinghe *et al.,* 2003). Indeed, the ecological importance of bats has been highlighted, since they are beneficial to ecosystems and agriculture. For example, the loss of

bats in North America has been estimated to cost around $4 billion/year due to agricultural losses (Boyles *et al.,* 2011).

Bats are expected to receive high chemical exposure as they are long-lived organisms and small flying mammals with a high metabolic rate (Hickey *et al.,* 2001). They consume more than 40% of their body weight each night during the feeding period. In Europe, bats are insectivorous and feed mostly on invertebrates which are susceptible to accumulating contaminants from plant material that they consume. Although a range of metal-induced toxic effects has been shown on mammalian species, only a few studies have focussed on the toxic effects of metals to bats, whereas the effect of organic compounds has received more interest (Hickey *et al.,* 2001). These few studies on bats have shown a wide range of symptoms observed in intoxicated bats. These symptoms, which occurred following exposure to Pb, Cd and Zn, included tremors, spasms, general slowness, lack of control in body movement, diarrhoea, excessive salivation, incoordination, inability to fly, muscle tremors, testicular necrosis, spermatogenesis, renal and hepatic inclusion bodies, histological lesions in different organs and tissues (kidneys, spleen, lungs, brain) and mortality (Clark and Shore, 2001; Dixit and Lohiya, 1974; Sutton and Wilson, 1983; Sutton and Hariono, 1987; Hariono *et al.,* 1993; Zook *et al.,* 1970; Hurley and Fenton, 1980). However, most of these studies have looked at fructivorous species.

The incidence of these symptoms in fructivorous species highlights the need for further investigations to identify the potential threat of contaminants to other species of bat at the population level. Many of the symptoms observed are related to reproduction. In addition, the transfer of metals from mothers to juveniles may alter the reproduction of bat populations (e.g. foetus and juvenile death) (Streit and Nagel, 1993; Lüftl *et al.,* 2003). Mortality in wild populations has been observed in Australia and in France following Pb exposure, probably due to high and localised contamination sources (e.g. use of rustproof paint). Although sublethal and lethal effects have been reported, metal tissue concentrations have not been measured to derive critical toxic levels for bats (Carravieri and Scheifler, 2013). In the South West of England, a monitoring study determining residue concentrations in bat tissues showed that around 5% of the pipistrelle (*Pipistrellus pipistrellus*) samples had renal residues at levels associated with acute Pb poisoning (Walker *et al.,* 2007).

Aims and objectives

The overall aim of this thesis was to explore the risks of soil-associated metals to insectivorous bats. The study focussed on Cd, Cu, Pb and Zn, which are the most investigated metals in the literature in terms of uptake. Indeed, uptake from soil or sediments into invertebrate species has been relatively well documented for these metals. The study investigated bat species living in England and Wales, and particularly the common pipistrelle (*Pipistrellus Pipistrellus*) bat, as this is the most common bat in Europe and the whole of the UK.

The investigations involved the development of: a spatially explicit modelling framework through which to assess the risks of soil-associated metals to bats in England and Wales, a monitoring dataset on metal concentrations in different bat tissues aimed at evaluating the model predictions, and the development of an *in vitro* gastric model (IVGM) to assess differences of bioaccessibility in insect types.

The specific objectives of the study were to:

- Develop a modelling framework to assess the risks of soil-associated metals to *Pipistrellus sp.* (*Pipistrellus pipistrellus* and *Pipistrellus pygmaeus*) bats in England and Wales.
- Extend the model to a larger number of species of bats living in the UK and assess the risks of metal contamination to these different species while developing an understanding of the drivers of risk in different species.
- Develop a monitoring dataset of actual metal concentrations in bat tissues to evaluate our model predictions and to assess the potential risk of metal contamination to bat health.
- Investigate the bioaccessibility of metals in different food items using an IVGM and assess the potential implications of these differences for ERA. The IVGM was developed for insectivorous bats to assess the metal bioaccessibility for different insect types.

This chapter (***Chapter 1***) provides a general introduction to the topic area and lays out the aims and objectives of the thesis.

Chapter 2 presents the development, parameterisation and application of the spatially explicit modelling framework to assess the risks of soil-associated metals (Cd, Cu, Pb and Zn) to *Pipistrellus sp.* bats in England and Wales. The metal uptake route considered in the model

is from soils into invertebrates and then into bats. Information on soil metal concentration, uptake into prey items, bat diet, foraging behaviour, bat distribution, and toxicity of metal to bats are all integrated into the modelling framework to assess exposure, following which the exposure predictions are compared with toxicological data to establish the level of risk.

In ***Chapter 3***, the modelling framework is extended to assess risks of metals to the health of 13 additional bat species present in the UK. Information on bat ecology (diet, foraging behaviour, bat distribution for the different bat species and the metal uptake into different insect prey items) are compiled. The relative exposure of the different bat species is discussed and the main ecological parameters driving risk are identified through a systematic evaluation of the model.

Chapter 4 presents the results of a monitoring campaign to assess the level of metals in bat (*Pipistrellus sp.*) tissues (kidneys and liver) collected across a gradient of soil metal pollution in England and Wales. Tissue concentrations are compared with toxicity data for small mammals to assess the potential concern for bat health. The developed dataset is also used to evaluate our modelling framework predictions and to verify whether the individuals identified as "at risk" by the model contained higher metal levels in their tissues than the bats not identified as at risk.

Chapter 5 presents studies which aimed to develop an IVGM to assess the metal bioaccessible fraction from insects to bats. The differences in bioaccessibility across different insect types (Coleoptera, Lepidoptera and Diptera) are then investigated using the *in vitro* system. The insects investigated comprised the major part of the diet of bats living in England and Wales. The implications of the observed differences in bioaccessibility, between insect orders, for ERA are explored.

Chapter 6 presents a discussion of the knowledge gained from the model development and the investigations based on actual bat and insect samples. The possible role of metal contamination as a factor involved in the observed bat population declines is also explored. The main limitations and issues encountered while developing and parameterising the food chain model to predict the threats of metals to wildlife species are discussed. Major remaining questions are identified and potential solutions to address these questions are detailed. This chapter ends with a final conclusion.

Chapter 2

A spatially-based modelling framework for assessing the risks of soil-associated metals to bats

Introduction

Populations of selected bat species in different regions of Europe are declining (Jones *et al.,* 2009). For example, in England, *Pipistrellus sp.* numbers declined by 62% between 1978 and 1987 (Stebbings, 1988). Similar declines have been reported for *Rhinolophus hipposideros*, *Rhinolophus ferrumequinum* and *Myotis myotis* in Central Europe (Dietz *et al.,* 2009). While these drastic declines can partly be explained by environmental and climatic changes (Frick *et al.,* 2010; Jones *et al.,* 2009), a number of anthropogenic factors are also believed to have played a role, including changes in water quality, roost loss, disturbance, urbanisation and industrialisation, agricultural intensification, the increase in wind turbines, disease pressures, pesticide use and metal contamination (Jefferies, 1972; Jones *et al.,* 2009; Walker *et al.,* 2007; Wickramasinghe *et al.,* 2003).

As discussed in Chapter 1, bats can be exposed to chemical contaminants through a variety of pathways, including consumption of contaminated water, inhalation and ingestion of contaminated prey (Allinson *et al.,* 2006; Clark and Shore, 2001). However, our knowledge of the potential impacts of chemical contaminants on bats is much less developed compared to other mammals such as shrews, mice and rats (Clark and Shore, 2001; Walker at al., 2007). Bats might be expected to receive relatively high and prolonged exposure to chemical contaminants as they are long-lived species for their body size, they can accumulate contaminants (Hickey *et al.,* 2001) and they also consume a large amount of prey each night which may have been exposed to contaminated soils and water bodies.

Metals are known to bioaccumulate in mammals and elicit a range of toxic effects, including anaemia and retarded gonad development, liver and kidney dysfunction and reduced growth and reproduction (Clark and Shore, 2001). Despite this, most studies detailing the effects of contaminants on bats have focussed on organic compounds (Hickey *et al.,* 2001). In the few studies which have investigated the toxicity of metals to bats, certain effects (tremors, spasms, general slowness, lack of control in body movement and mortality) have been reported following exposure to Pb (Clark and Shore, 2001; Hariono *et al.,* 1993; Sutton and Wilson, 1983), Cd (Clark and Shore, 2001) and Zn (Hurley and Fenton, 1980).

In light of the observed declines in some bat populations and the anticipated long-term exposure of bats to metal contamination, it would be valuable to develop a better understanding of the potential levels of exposure of bats to metals in the environment and of the potential effects on the health of bat populations.

One approach taken when it comes to understanding the potential impacts of metals on bat health is to use environmental models, such as food chain models, which integrate information on chemical occurrence, uptake into prey items, wildlife ecology and toxicity to predict the potential risks posed by contaminants to wildlife species. Food chain models mainly consist of calculating ingested doses of chemicals for wild birds and mammals (EFSA Journal, 2009). They can integrate complex ecological parameters and toxicity information which can be associated with long-term sublethal exposures. Initial measurements or estimations of chemical concentrations in media and food sources are used to predict chemical exposure (Linder and Joermann, 2001). The method consists of determining a risk characterisation ratio (RCR) based on the estimated intake concentration or body burden concentration and an effect level. The RCRs are then compared to a trigger value to determine whether the exposure presents an acceptable or an unacceptable risk for the target receptor. Further refinement of the risk assessment, possibly involving additional experimental studies, is required, with results indicating an unacceptable risk (EFSA, 2009). The main limitations met while predicting exposure risk to wildlife *via* food chain models are further detailed in Chapter 6.

In light of this, the present chapter describes a modelling framework for predicting exposure and the subsequent risks of soil-associated contaminants to wildlife. The application of the modelling framework is illustrated for populations of *Pipistrellus* species (*Pipistrellus pipistrellus* and *Pipistrellus pygmaeus*) exposed to soil-associated metals in England and Wales.

Methods

The modelling framework (Figure 2.1) is based on a simple risk assessment approach recommended by the European Food Safety Authority to assess the risks of pesticides to birds and mammals (EFSA Journal, 2009). The framework considers uptake of metals from soils into insects and then bats and was designed to predict the spatial exposure of bats to metals in England and Wales. The framework considers the potential effects of individual metals. The basic risk characterisation approach is described below. The parameterisation of the framework is then described, followed by the application of the framework to *Pipistrellus sp.* in England and Wales.

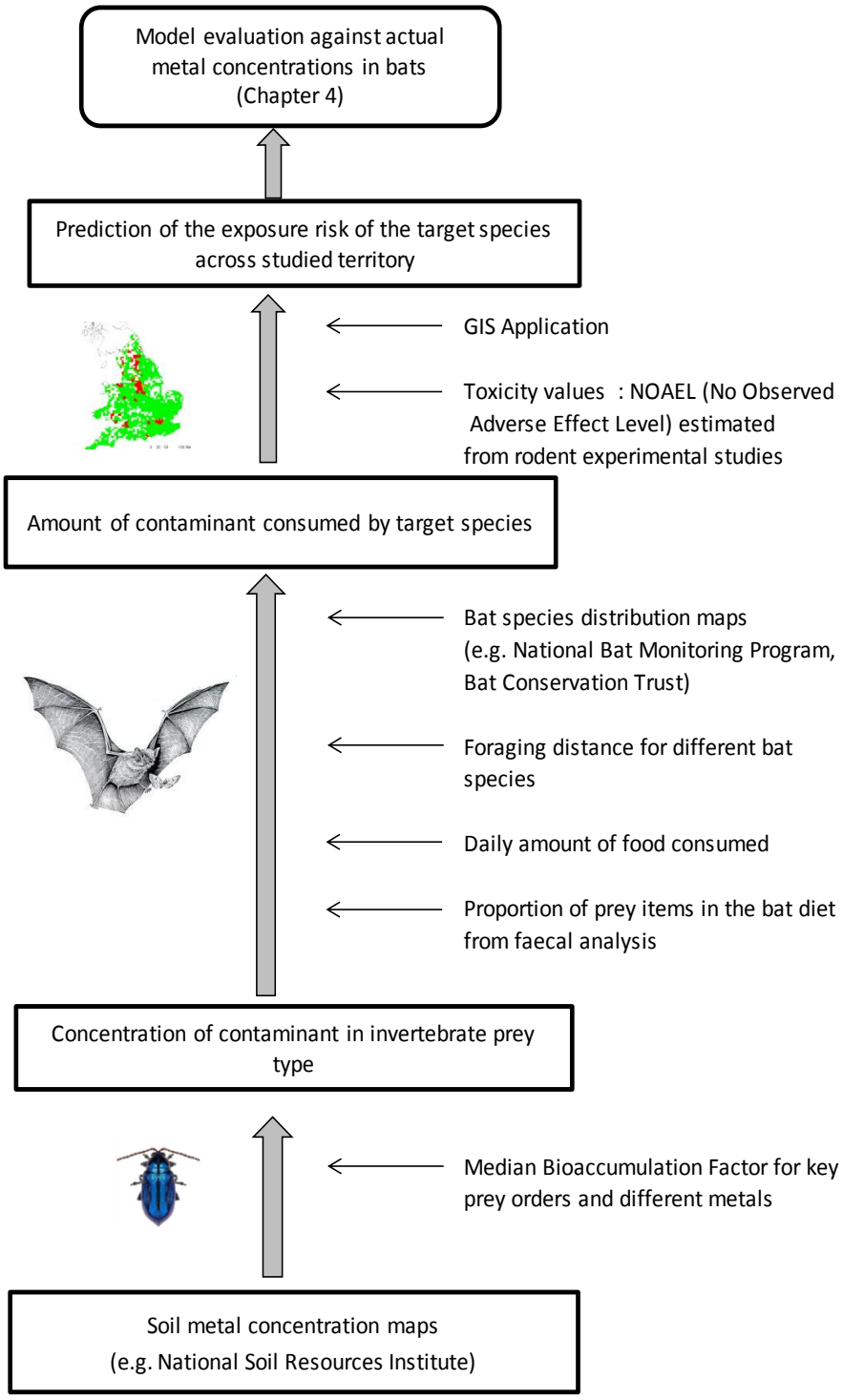
Basic risk characterisation approach

The model considers uptake of metals from soil into insects and subsequent feeding on the insects by bats. The overall daily oral exposure (E) of the bat to the metal is calculated using Equation 1. If a bat is feeding across a number of sites or areas with different metal concentrations, the concentration in each insect type can be calculated for each individual site and an overall E value can be estimated based on the distance that a bat covers to forage.

$$E = s\ X\ a \sum_{i}^{n} b_i\ x\ p_i \qquad \text{Equation 1}$$

where *E* is the overall oral dose in µg/g body weight/day, *s* is the concentration of the metal in the soil expressed in µg/g dry weight, *a* is the amount of food eaten in g dry weight/g body weight/day, *i* and *n* are different prey items, *b* is the biota accumulation factor for the metal from soil into an insect prey item, and *p* is the proportion of the diet accounted for by an individual prey item.

Figure 2.1: Main steps involved in the modelling framework. Key parameters and data sources are shown. The main model steps are represented in the boxes and the input data are listed on the right hand side of the figure.



In order to determine the potential risks of a metal to the exposed bats, daily oral dose is then compared with a 'safe' dose value to derive a risk characterisation ratio (RCR) (Equation 2).

$$RCR = \frac{E}{P} \qquad \text{Equation 2}$$

where *RCR* is the risk characterisation ratio, *E* is the daily dose of metal that a bat receives (µg/g body weight/day) and *P* is the predicted safe daily dose for the metal (µg/g body weight/day). The 'safe' dose is predicted from reported no observable effects doses by dividing the No observed adverse effect level (NOAEL) (µg/g body weight/day) by an uncertainty factor of five. This uncertainty factor was selected as it is commonly used in the regulatory assessment of the long-term risks of pesticides to birds and mammals species to account for uncertainties in the toxicological data (e.g. inter-laboratory difference, inter and intra species differences, differences in sensitivity of different life stages) (EFSA Journal, 2009). An *RCR* value of less than one would indicate that there is an acceptable risk to the exposed bats from metal exposure. *RCR* values greater than one indicate that bats in a region may be threatened by metal contaminants.

Model parameterisation

The exposure and risk models were parameterised for the Common (*Pipistrellus pipistrellus*) and Soprano pipistrelle (*Pipistrellus pygmaeus*) bats. These two species are very similar and have only recently been separated according to their echolocation call type and morphological features (Dietz *et al.,* 2009). The common pipistrelle is the most common bat in the UK (Dietz *et al.,* 2009). The two species are widespread in Europe and their ranges overlap each other (Dietz *et al.,* 2009). The literature relating to these two sibling species rarely makes clear reference to one in particular. As such, in the following sections we refer to the two species as *Pipistrellus sp*.

Diet of *Pipistrellus. sp* (p) (Equation 1)

A number of studies have explored the composition of bat diets; these studies typically use faecal analysis to determine the proportion of a particular prey item in the diet, usually at the order level. Several metrics have been used in these analyses e.g. percentage frequency, percentage occurrence, percentage items, percentage number and percentage volume using faecal pellets (Kervyn, 1996; Vaughan, 1997). For this study, we only selected studies where

dietary composition was expressed as a percentage volume. This method is believed to be more accurate than some of the other methods and is commonly used in the literature (Flanders and Jones, 2009). Based on the available data, in terms of percentage of volume (Pithartovà, 2007), the diet of the soprano pipistrelle is dominated by Diptera (flies) (67.33% of volume), followed by Hymenoptera (sawflies, wasps, bees, ants) (4%) and Hemiptera (true bugs) (3.67%). Using the literature data, it was only possible to characterise around 75% of the diet.

Biota accumulation factors (b) (Equation 1)

BAFs for uptake of Cd, Cu, Pb and Zn from the soil were obtained from the literature for Diptera, Hemiptera and Hymenoptera. BAF was expressed as the ratio between soil metal element concentration (dry weight) and the internal metal element concentration in invertebrates (dry weight). BAFs for each insect order were collected and a median value calculated for use in the model (Figure A1.1). The median value was chosen to minimise the impact of outlier values. As it was not possible to fully characterise the diet of the bats, a mean value was calculated for each metal from the results for the three orders and this mean value was used to estimate uptake of metals into the missing part of the diet.

Daily consumed amount (a) (Equation 1)

As experimental data were not available on the feeding rates of *Pipistrellus sp*. the amount of prey consumed per day was derived using an allometric equation based on a body mass gradient for small mammals (Nagy, 1987). The equation gives a food ingestion rate in g dry weight/g body weight/day. Using a mean mass of 4.9 g measured on common pipistrelle (Genoud and Christe, 2011), an ingestion rate of 0.177 g dry weight/g bw/day was obtained.

In order to evaluate the effectiveness of the allometry method, experimental data were obtained for two other bat genera, namely *Myotis* and *Eptesicus* which are slightly larger in size than *Pipistrellus sp*. The mean feeding rate, measured experimentally for these bats was 0.125 g dry weight/g bw/day (Table A1.1) (assuming 68.8% moisture contents in insects (EFSA Journal, 2009)) which corresponds favourably to the value of 0.160 g dry weight/g bw/day obtained from the allometric equation. The allometry method therefore appears to work reasonably well for estimating the feeding rate of bats and the value used in the model is conservative compared to the values measured experimentally.

Foraging distance

The foraging distance represents the mean distance between the roost and the feeding area (Davidson-Watts and Jones, 2006, cited by Dietz *et al.,* 2009). For the common pipistrelle, the hunting grounds are located close to the nursery roosts. In England, the average foraging distance is 1.5 km (Davidson-Watts and Jones, 2006, cited by Dietz *et al.,* 2009). A slight difference has been observed for the soprano pipistrelle, which normally hunts within an average of 1.7 km from the roost (Davidson-Watts and Jones, 2006, cited by Dietz *et al.,* 2009). In this study, the maximum average value found in the literature was used.

No observed adverse effect level (NOAEL)

The NOAEL is defined as the highest exposure where adverse effects are not seen in an experimental study. For standard laboratory mammal species such as rats and mice, data on toxicological effects and NOAELs are abundant in the literature. However, for other wildlife mammal species, fewer data are available and no toxicological data exist for British bat species or insectivorous bats. One approach to dealing with this lack of data is to extrapolate from the standard test organism data. For example, Sample *et al.*, (1996) used experimentally derived NOAELs for metals for rats and mink and estimated NOAELs for different wildlife mammal species by adjusting the dose according to differences in body size, as shown in Equation 3.

$$\mathrm{NOAEL_w} = \mathrm{NOAEL_t}\left(\frac{\mathrm{bw_t}}{\mathrm{bw_w}}\right)^{1/4} \qquad \text{Equation 3 (Sample } \textit{et al}\text{., 1996)}$$

where $NOAEL_w$ is the equivalent NOAEL for a wildlife species; $NOAEL_t$ is the NOAEL available for the test species; bw_t is the body weight of the test species and bw_w is the body weight of the wildlife species.

The toxicological endpoint used for the test species experiment was based on reproductive effects on rats for Cd, Pb and Zn and on mink for Cu (Sample *et al.,* 1996). Further details on the chronic experimental studies used by Sample *et al*. (1996) to derive NOAELs are mentioned in Table A1.2. Reproduction is known to address crucial impacts on population dynamics and to address ecological impacts over long term periods (Sample *et al.,* 1996). The approach proposed by Sample *et al.,* (1996) was used to estimate the chronic NOAELs for

Pipistrellus sp. based on a mean body weight of 4.9 g. NOAELs of 2.80, 44.18, 23.21 and 464.20 µg/gbw/day were obtained for Cd, Cu, Pb and Zn respectively.

Application of modelling framework to bat populations in England and Wales

The spatial analysis was conducted using a geographical information system (GIS) (ArcGis 9, Arcmap Version 9.3.1) in order to assess the variations in risk across England and Wales. Data on concentrations of metals in soils across England and Wales (*s*, Equation 1), derived following acid extraction, were obtained from the National Soils Resources Institute (NSRI). The NSRI soil data provide metal concentrations in soils at a 5 X 5 km square resolution. Soil data in the NSRI dataset were obtained by sampling in the centre of each grid cell for analyses to cover a 1:250000 map of England and Wales. For each cell, twenty-five cores of soil were taken at the nodes of a 4m grid within a 20 m x 20 m square centred on the OS 5-km grid-point. Two sets of data were used: the first set corresponded to samples obtained between 1979 and 1987 (5677 sites for the four metal elements studied), while the second included samples obtained between 1994 and 2003 (1681 sites for the four metal elements studied). The analytical method of extraction was the same for both datasets. Data from the more recent dataset were used in preference to data from the older dataset, and thus the older data were only used to fill gaps in the more recent dataset. Approximately 70% of the data from the first dataset was used in the model.

To run the spatial risk analysis, daily exposure values (*E*) were initially calculated for each grid square represented in the NSRI dataset and the equations and parameters described above. The results of the *E* calculations were then imported into GIS software along with the bat distribution data. The bat distribution data are expressed as a presence/absence in a 1:250 000 map where each 5 X 5 km cell grid mentions whether the bat has been detected on this area (presence) or not (absence). To address the effects had by foraging distance on exposure, we subdivided each 5 x 5 km cell into 100 smaller cells of 0.5 x 0.5 km and assumed that each sub-cell had the same metal soil concentration of the main cell in which it resided. We then assumed that an individual bat resided in each sub-cell and that it could feed within a circle around the centre of each sub-cell; the radius of which was defined by the bat foraging distance. Any sub-cells whose centres fell inside the radius of the circle were taken into account to calculate a mean amount of contaminant consumed by a bat feeding from sub-cells. By running this analysis for all 100 sub-cells in a full cell, it was possible to obtain an overall mean exposure value for a cell (Figure A1.2). *RCR* values were then estimated for each cell for each individual metal.

Bat distribution data (expressed as presence or absence) in England and Wales was then taken from the results of the National Monitoring Bat Program run by the Bat Conservation Trust (scale of resolution was 5 x 5 km squared). This data were overlaid on the predictions of *RCR* values. Risk maps and associated risk distribution diagrams were then produced for individual metals and metal mixtures.

Results and discussion

Mean concentrations in soils increased in the order Cd < Cu < Pb < Zn (Table 2.1). Metal concentrations varied widely (Table 2.1; Figure A1.3). Some areas of high concentration are associated with geological sources. However, much of the contamination results from the UK's industrial past, particularly from past mining activity, power generation and the metal industry (smelters, foundries and steel mills) (Blundell *et al.,* 2009). Since 1990, metal emissions have significantly decreased in the UK while concentrations of Zn and Cd in soils have declined (Review of transboundary air pollution (Rotap, 2009)). However, Cu and Pb concentrations may not decline for centuries and may still be increasing in some habitats (Rotap, 2009).

BAFs for Pb in each of the insect orders were substantially lower than for the other three metals (Figure A1.1). The low BAFs for Pb might be explained by the low water solubility of Pb and low uptake through the gut wall or by rapid excretion of the accumulated Pb (Janssen and Hogervorst, 1993). The high affinity of Pb for organic matter and other chemical constituents (such as iron and carbonate) of soils may also contribute to the low BAF values which were observed (Bidwell and Gorrie, 2006; Luoma, 1989).

BAFs, both within and across insect orders, varied by up to an order of magnitude while there was no one order which showed highest uptake for all metals. These differences might be explained by the species traits of the different insect groups. Dipteran larvae live in aquatic (fresh water), semi-aquatic or moist terrestrial environments and while some are herbivores, most feed on dead organic matter or parasitise other animals, especially vertebrates, molluscs, and other arthropods (Barnard, 2011). Hemiptera are phytophagous, predatory, generally feed on other insects or even small vertebrates, and are usually not associated with aquatic habitats (Barnard, 2011). Hymenoptera can be herbivorous, predatory or parasitic (Barnard, 2011). A number of recent studies have shown that species traits can significantly affect uptake of contaminants into invertebrates (Rubach *et al.,* 2012).

Table 2.1: Summary statistics obtained for different stages of the modelling framework (soil metal concentrations and metal concentration predicted in prey items) applied to assess the risks of soil-associated metals to *Pipistrellus sp*. in England and Wales.

			Min	Max	Mean	Median	SD
Soil metal concentrations (µg/g)		Cd	<0.05*	40.9	0.67	0.5	0.98
		Cu	<0.04*	1507.7	22.43	17.3	36.8
		Pb	<0.63*	17364.93	73.3	39	281.32
		Zn	<0.02*	3648	88.48	74	103.41
Metal concentration predicted in invertebrate order (µg/g)	Diptera	Cd	<0.04	30.06	0.49	0.37	0.72
		Cu	<0.03	1221.24	18.16	14.01	29.8
		Pb	<0.13	3646.64	15.39	8.19	59.07
		Zn	<0.02	2816.26	68.28	57.13	79.83
	Hemiptera	Cd	<0.03	20.74	0.34	0.25	0.5
		Cu	<0.05	1842.41	27.39	21.14	44.96
		Pb	<0.01	208.38	0.88	0.47	3.38
		Zn	<0.03	5107.2	123.83	103.6	144.76
	Hymenoptera	Cd	<0.04	36.07	0.59	0.44	0.87
		Cu	<0.01	438.74	6.52	5.03	10.71
		Pb	<0.02	520.95	2.2	1.17	8.44
		Zn	<0.02	2856.38	69.26	57.94	80.96
	Hypothetic order	Cd	<0.04	28.96	0.48	0.35	0.7
		Cu	<0.03	1167.46	17.36	13.4	28.49
		Pb	<0.05	1458.65	6.15	3.28	23.63
		Zn	<0.02	3593.28	87.12	72.89	101.85

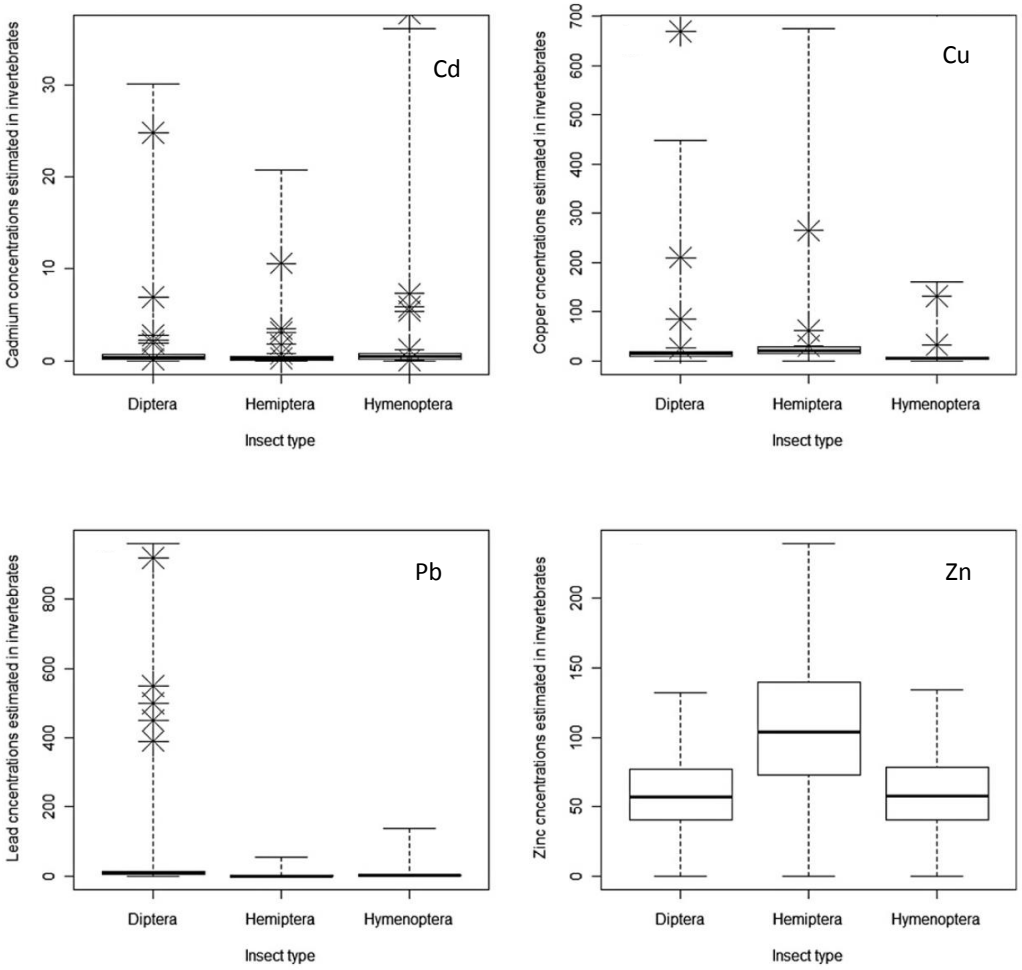
*Limit detection value in soil using EDTA extraction in µg/g (McGrath and Loveland, 1992).

The observed difference in uptake may also be explained by the variation in the properties of the soil and sediments used in the uptake studies. Soil and sediments properties such as pH, organic matter concentration and type, sulphur and carbonate contents, acid buffering capacity, cation exchange capacity and the presence of iron and manganese acid minerals are known to be very important in determining the bioconcentration of metals from soils into invertebrates and plants (John and Leventhal, 1995; Laskowski *et al.,* 1995; Morel, 1997). Only a small percentage of publications reviewing bioaccumulation analysed one or several of these properties in uptake studies on invertebrates. A number of studies have attempted to relate soil and sediment properties to uptake (Bendell Young, 1999; Bidwell and Gorrie, 2006; Vermeulen *et al.,* 2009). However, most of these studies have only focussed on the influence of one or two soil or sediment parameters and good relationships are not currently available for estimating uptake into the insect groups, which have been the focus of this

study. This demonstrates the need to work at least at the order level when performing modelling exercises of this type.

When these BAF values were applied to the soil data, estimated concentrations of the study metals in prey items increased in the order Cd < Pb < Cu < Zn (Table 2.1). Indeed, a comparison of predicted ranges of metal concentrations in the different insect orders with measured concentrations for Cd and Cu in invertebrates in the UK (Davison *et al.,* 1999; Dixit and Witcomb, 1983; Hunter *et al.,* 1987b; Figure 2.2) indicates that measured concentrations fall within the prediction ranges, thus giving some re-assurance that the estimated prey concentrations are realistic. No measured Zn and Pb concentrations in Hemiptera and Hymenoptera were found for the UK, meaning it was not possible to perform a comparison for these orders.

Figure 2.2: Distribution of concentrations of metals (µg/g dry weight) Cd, Cu, Pb, Zn in different insect orders, estimated by the model. Stars indicate measured concentrations in insects in the UK in µg/g (dry weight).



Summary data on bat daily doses and resulting RCRs are shown in Table 2.2. Risk maps and risk distribution diagrams for individual metal and metal mixture are shown in Figure 2.3. Pb was predicted to be the greatest threat to bat health, followed by Cu, Cd and Zn. Exposure to soil-associated Cd was predicted to pose a risk in 0.6% of the area where *Pipistellus sp.* occurs (Figure 2.3). Exposure to soil-associated Cu was predicted to pose a risk in 2.8% of the total area where *Pipistrellus sp*. occurs (Figure 2.3). Pb exposure risk was seen for 5.9% of the bat distribution (Figure 2.3). Only 0.5% of the bat distribution was predicted to be at risk from Zn exposure (Figure 2.3).

Table 2.2: Summary statistics obtained for different stages of the modelling framework (daily dose predictions and risk characterisation ratio) applied to assess the risks of soil-associated metals to *Pipistrellus sp*. in England and Wales.

	Daily dose prediction (µg/gbw/day)				**Risk Characterisation Ratio (RCR)**			
	Cd	Cu	Pb	Zn	Cd	Cu	Pb	Zn
Min	<0.01	<0.01	<0.02	0.00	<0.02	0.00	0.00	0.00
Max	5.25	212.27	504.17	548.03	9.38	24.02	108.61	5.90
Mean	0.09	3.21	2.14	13.38	0.16	0.37	0.45	0.15
Median	0.07	2.51	1.19	11.39	0.13	0.29	0.25	0.13
SD	0.11	4.87	6.88	14.01	0.21	0.58	1.60	0.16

While experimental data relating to the effects of metal exposure on bat health in England and Wales were not available, a limited amount of data were available on metal accumulation in bats in some of the regions predicted to be at risk. For example, Walker *et al.,* (2007) analysed renal metal concentrations in British bats in Devon and Cornwall. Pb had the highest median concentration (2.45 µg/g dw) and maximum (69.7 µg/g dw) dry weight renal concentration in Pipistrelle bats, followed by Cd (median concentration 1.42 µg/g dw and maximum concentration 29.1 µg/g dw) and Hg (median concentration 0.93 µg/g dw and 5.08 µg/g dw).

The modelling framework predicts that soil-associated metals in some areas of England and Wales are at levels which could be affecting bat health. It is therefore possible that soil-associated metals may be contributing to declines in bat populations in some regions of the UK. Due to limitations in our current knowledge and in order to maintain the simplicity of the approach, the modelling framework has made a number of assumptions which require testing in order to corroborate our risk predictions. In addition, it would be beneficial to validate parts of the model against wider datasets on the levels of metals in prey items and bats from sites across England and Wales. Some of the key limitations are discussed below.

The NSRI data were derived from samples obtained from the centre of each 5 x 5 km grid. Although we assumed that said value represented the level in the total grid, this may not be the case, particularly in areas with high heterogeneity in the soil characteristics.

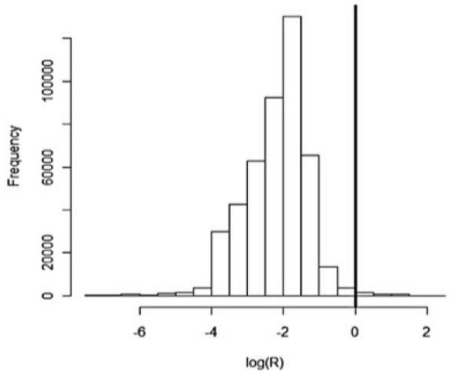
For pragmatic reasons we have used a median BAF, derived from studies on soils with very different characteristics, to estimate movement of metals from soils into insects. It is well known that the bioavailability of metals to soil-dwelling organisms is highly dependent on the underlying characteristics (e.g. pH, organic carbon content and type, cation exchange capacity) of the soil (John and Leventhal, 1995). While relationships have been developed

between metal uptake and soil properties for certain soil organisms, to the best of our knowledge, these relationships do not exist for the prey items considered in this study. By developing an understanding of the relationships between metal uptake and soil properties for key bat prey species, it would be possible to consider the effects of soil parameters on bat exposure. To some extent, the modelling framework could be adapted to other wildlife species, such as birds, since metals can be taken up by wild birds and affect their populations (Berglund *et al.,* 2011; Eeva *et al.,* 2009).

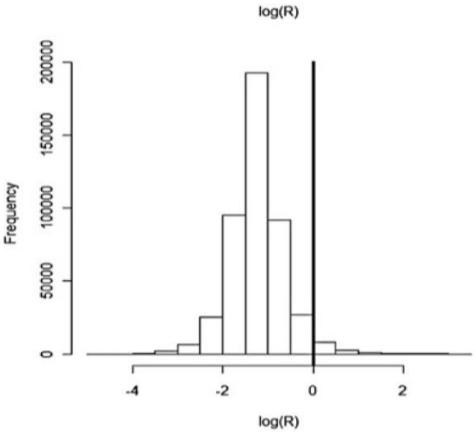
The amount of food consumed (*a*) has been derived from an allometric equation (Nagy, 1987). The equation has been validated by comparing experimental data (Table A1.1) and results given by the equation. The body mass used in the equation represents an average body for both species studied and has been measured in adult males and females not presenting signs of lactation or pregnancy. Bats have different food rate ingestion and weights according to their life stage (juvenile, male in spermatogenesis, pregnant female, lactating female) and their cycle (torpor or summer). As such, sensitivity to chemical exposure could vary depending on the life stage.

Figure 2.3: Risk maps and risk-frequency distributions for *Pipistrellus sp*. exposed to Cd, Cu, Pb, Zn. Grid cells coloured in black represent zones identified "at risk" (i.e. with an RCR > 1) by the modelling framework. Grid cells coloured in grey represent zones characterised as "not at risk" (i.e. RCR < 1). Grid cells coloured in white represent zones in which bats did not appear or where soil concentrations were not available. On the frequency distribution, the RCR value 1 is represented by a vertical bold black line.

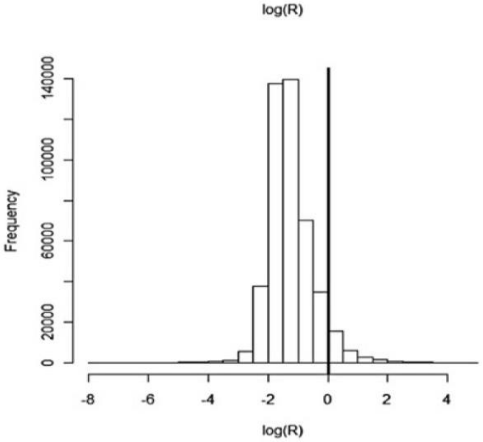
Cd



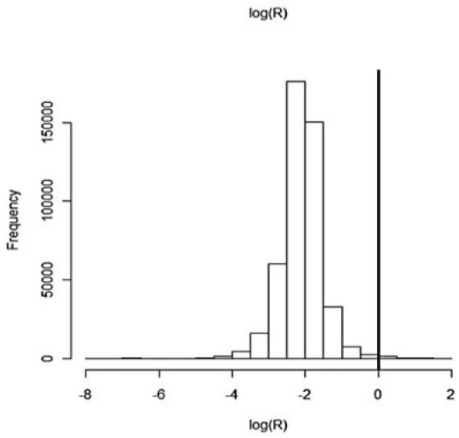
Cu



Pb



Zn



There is a limited amount of available data when it comes to the toxicity of metals to bats. Consequently, used NOAELs were derived by extrapolation, from standard mammalian test organisms (rat and mink in this study) to wildlife (Sample *et al*., 1996). This extrapolation was based on differences in body size alone, and thus caution should be used when interpreting the data as bats could be either more or less sensitive to metal exposure than standard mammalian organisms. Studies on other insectivorous mammals have indicated that these can be more tolerant to metals than rodents (Ma and Talmage, 2001). For example, shrews have been shown to be more tolerant to Cd exposure than mice (Ma, 1994, cited by Ma and Talmage, 2001). It is also known that many wildlife species in contaminated environments are able to tolerate high concentrations of certain metals (Ma and Talmage, 2001). Additional factors could also affect sensitivity to toxicants, including differences in the bioaccessibility of the metals from food items and differences in metabolic pathways and rates. A better understanding of the bioaccessibility of metals from insects compared to food items used in standard tests alongside the use of toxicokinetic and dynamic modelling approaches might help to improve the extrapolations from standard rodent test data.

As bats were exposed to a mixture of metals, it was also valuable to consider the risk arising from combined exposure. A review of publications on metal mixture interactions in mammals was performed as part of the current study. However, no clear relationship was found across the different studies. Some studies indicated that the assessed compounds are antagonistic; some showed additivity, whereas other studies showed synergism. Based on this, a decision was made not to estimate the risk arising for the metal mixtures. Indeed, additional investigations are needed in order to develop a better understanding of mixture interactions in wildlife species.

The study has focussed entirely on *Pipistrellus sp*. The sensitivity of other bat species could be very different due to e.g. differences in diet composition and food intake. For example, in the bat monitoring studies carried out by Devon and Cornwall (Walker *et al.,* 2007), highest median Pb concentrations were found in the tissues of the whiskered bat (*Myotis mystacinus*) with 4.05 µg/g dw and the brown long-eared bat (*Plecotus auritus*) with 3.38 µg/g dw. Higher median Cd concentrations were found in the Natterer's bat (*Myotis nattereri*) with 6.27 µg/g dw and the whiskered bat with 1.61 µg/g dw. As such, in Chapter 3 we extend the modelling framework to assess the risks of metals to a wider range of bat species which occur in the UK.

Chapter 3

Interspecies variation in exposure to trace metals for bats

Introduction

In the previous chapter, a spatial modelling framework was described and applied to estimate the risks from soil-associated metals to the health of populations of the common pipistrelle in England and Wales. Results indicated that Pb exposure in 6% of areas where *Pipistrellus sp.* reside would be high enough to affect bat health, while for Cd, 3% of areas would have concentrations high enough to affect bat health (Chapter 2). However, the study only looked at one bat species, with the relevance of the results to other species of bats unknown.

Other bat species could be at either greater or lower risk than *Pipistrellus sp.* due to differences in factors such as their spatial range, food intake and dietary composition. For example, bats which specialise in consuming prey with a high metal accumulation capacity, which have food intake and a spatial range restricted to polluted areas, might be expected to be more exposed than other species. Monitoring studies for metal residues in bats shows that renal metal concentrations differ across bat species, which may reflect differences in dietary exposure (Walker *et al.,* 2007). As observed for passerine birds, interspecific differences in metal exposure may be linked with their diet (Berglund *et al.,* 2011). For example, it was shown that the pied flycatcher (*Ficedula hypoleuca*) accumulated more metals than great tits (*Parus major*) as the diet composition of pied flycatchers is composed of a large proportion of insects from higher trophic levels than the great tits (Berglund *et al.,* 2011).

When using modelling frameworks of the type described in Chapter 2, it is important to understand the sensitivity of a framework to changes in model input parameters. This knowledge can be invaluable in informing the parameterisation process for a model.

Sensitivity analyses are strongly recommended for use in ERA as part of a good modelling practise (Schmolke *et al.,* 2010). The analyses examine how outputs vary as inputs change to understand how the risk predictions are dependent on the variability and the uncertainty of factors contributing to the risk (Grimm and Railsback, 2005; Risk assessment guidance for superfund, 2001). Simple methods can be used to analyse the sensitivity of a model by changing the input values in a systematic way to calculate the model output. The Monte Carlo approach is widely used for sensitivity analysis by running simulations with distributions of each input parameter to determine the effect on the risk estimate. More complex approaches, as used in this Chapter, involve complex mathematical and statistical techniques and can include the effect of the combination of several factors having different statistical distributions (Risk assessment guidance for superfund, 2001). Sensitivity analyses have been used previously in ecological modelling exercises. For example, sensitivity analyses have been recently applied to an agent based model, simulating skylark (*Alanda arvensis*) population response to landscape change (Parry *et al*., 2013). The authors identified which parameters were most important and should be focussed on in the model parameterisation process (Parry *et al.,* 2013).

In the work described in this Chapter, to improve our knowledge of the potential threat of metal contamination to bats, the modelling framework described in Chapter 2 was extended to predict risks of soil-associated metals to 14 UK bat species. The interspecies variability at risk was investigated for the different metals (Cd, Cu, Pb and Zn). Sensitivity analyses were also performed to identify which ecological factors from the model are the main drivers in determining species variability in exposure risk.

Methods

Risk of British bat species to metal exposure

The modelling framework method described in Chapter 2 was applied to estimate the risks of metals to 14 bat species present in the UK, namely: *Barbastella barbastellus, Eptesicus serotinus, Myotis bechsteinii, Myotis daubentonii, Myotis mystacinus, Myotis nattereri, Nyctalus leisleri, Nyctalus noctula, Pipistrellus sp. (Pipistrellus pipistrellus* and *Pipistrellus pygmaeus), Pipistrellus nathusii, Plecotus auritus, Plecotus austriacus, Rhinolophus ferrumequinum,* and *Rhinolophus hipposideros*. In order to run the framework it was necessary to collate information on the following: concentrations of metals in soils, soil-insect accumulation factors, bat diet, bat distribution and sensitivity to metal exposures.

Concentrations of Cd, Cu, Pb and Zn in soil in England and Wales were obtained from NSRI (National Soil Resources Institute) at a 5 x 5 km^2 resolution (Chapter 2). Ecological data on bats (bat diet composition, foraging distance and weight) were gathered from the literature (Tables 3.1 and 3.2) (Chapter 2). Daily food intakes and chronic NOAELs were based on the average bat weight for each species and were derived using the allometric relationships described by Nagy (1987) and Sample *et al*., (1996) (Table 3.2). Further details on the allometric derivation of the NOAEL are provided in Chapter 2 while details on the experimental studies used in this derivation are presented in Table A1.2. BAF data were obtained from the literature for each of the invertebrate orders listed in the bat diet for the four metals studied (Table 3.3). The bat distribution dataset (presence/absence data on a 5 x 5 km^2 resolution) was provided by the NBMP (National Bat Monitoring Programme) from the Bat Conservation Trust for each bat species. The spatial analysis was conducted using GIS (ArcGIS, ArcMap Version 9.3.1). The final output was RCR for each 5 x 5 km^2 cell (Chapter 2). The percentage of areas (species' distribution) at risk for each species and metal, as well as for the group of metals combined were derived from the number of cells where a species was found to be at risk (i.e. with an RCR ≥ 1) divided by the total number of cells in which the bat species was present (Chapter 2).

Identification of key drivers of risk

A number of analyses were performed to identify the key factors driving the risk of metals to bats, as determined in the model. Distributions of selected model input parameters (Table 3.4) covering all species were used alongside the model to identify which of these were most important in determining the risk values calculated. The Emulator GEM-SA 1.1 (Gaussian Emulation Machine for Sensitivity Analysis, Kennedy 2005) was used to determine the effect of each individual input, or pairs of inputs on the output value.

The emulation process was as follows: given 100 points (selected to evenly cover the input parameter space), the original model was run to generate the corresponding 100 outputs. The emulator was then built using these inputs and output values. The emulator provides a simple function that approximates the original model. The sensitivity analysis was then applied with the emulator approximation (assuming a uniform distribution of all the variables within their range). The results are provided in a table comprising the parameters and their respective percentage contribution to the variability of the output. As the soil data comprised an upper tail with a few high concentrations and the parameter distributions were assumed to be uniform, the range of soil concentrations selected for sensitivity analyses was to cover only 95% and 99% of the soil data (Table 3.4). The different input parameters and their respective ranges are shown in Table 3.4.

The emulator cannot integrate spatial components and, as such, the spatial range in which the bat species reside was not included. The metal soil concentration ranges which occur within the bat distribution are different for each bat species. Thus, the analyses assumed a similar spatial distribution for all the bat species.

The differences in RCRs across metals and bat species were tested using the non-parametric Kruskal-Wallis test. In order to compare our risk predictions across species against metal tissue concentrations, we selected two publications studying several species of the same area (Austria and England; Lüftl *et al*., 2003, cited by Carravieri and Scheifler, 2013 and Walker *et al*., 2007, respectively).

Table 3.1: Bat diet composition expressed in percentage volume compiled from literature studies for different bat species living in the UK.

Bat species	Ortho-	Derma-	Hemi-	Coleo-	Dipt-	Tricho-	Lepido-	Hyme-	Arane-	Opili-	SUB total	Missing order	Total	References
A	-	-	-	-	6.30	0.10	90.66	-	0.60	-	97.66	2.34	100.00	[16]; [18]
B	-	-	4.40	27.70	57.60	-	4.80	1.30	-	-	95.80	4.20	100.00	[6]
C	7.68	17.57	-	32.83	12.50	-	24.47	-	-	-	95.05	4.95	100.00	[1]
D	-	-	14.63	1.67	72.34	-	-	-	-	-	88.64	11.36	100.00	[14]
E	-	-	1.64	-	46.01	19.35	-	3.87	29.13	-	100.00	0.00	100.00	[14]
F	20.70	0.21	18.01	34.58	19.46	0.41	1.66	0.83	4.14	-	100.00		100.00	[19]; [21]
G	-	-	2.00	14.00	46.30	-	32.85	3.00	-	-	98.15	1.85	100.00	[10]; [19]; [20]
H	-	-	3.82	27.28	17.30	12.00	21.32	0.04	0.60	-	82.36	17.64	100.00	[8];[9]; [13];[17]
I	-	-	5.83	5.00	73.67	2.33	-	-	4.05	-	90.88	9.12	100.00	[14]
J	-	-	3.67	-	67.33	-	-	4.00	-	-	75.00	25.00	100.00	[14]
K	-	7.40	-	8.00	29.20	8.60	27.20	-	4.20	13.40	98.00	2.00	100.00	[15]
L	-	-	0.20	6.17	5.20	1.50	83.90	-	-	-	96.97	3.03	100.00	[2]; [4]; [21]
M	-	-	0.16	28.85	17.11	2.43	41.43	10.02		-	100.00		100.00	[5]; [7]; [11] ; [12] ; [19]
N	-	-	1.00	1.40	42.60	1.30	44.05	0.55	-	-	90.90	9.10	100.00	[3]; [4]

Table 3.1: continued

The bat species are the following: A: *Barbastella barbastellus*, B: *Eptesicus serotinus*, C: *Myotis bechsteinii*, D: *Myotis daubentonii*, E: *Myotis mystacinus*, F: *Myotis nattereri*, G: *Nyctalus leisleri*, H: *Nyctalus noctula*, I: *Pipistrellus nathusii*, J: *Pipistrellus sp*. (*Pipistrellus Pipistrellus* and *Pipistrellus pygmaeus*), K: *Plecotus auritus*, L: *Plecotus austriacus*, M: *Rhinolophus ferrumequinum*, N: *Rhinolophus hipposideros*. The abbreviations for invertebrate orders are the following: Orthoptera (Ortho-), Dermaptera (Derma-), Hemiptera (Hemi-), Coleoptera (Coleo-), Diptera (Dipt-), Trichoptera (Tricho-), Lepidoptera (Lepido-), Hymenoptera (Hyme-), Araneida (Arane-) and Opiliones (Opili-).

References: [1]: Barataud *et al.,*(2010) ,[2]: Bauerova (1982), [3]: Bontadina *et al.,*(2008), [4]: Feldman *et al.,*(2000), [5]: Flanders and Jones (2009), [6]: Gajdosik and Gaisler (2004), [7]: Jones (1990), [8]: Jones (1995), [9]: Kanuch *et al.,*(2005)a, [10]: Kanuch *et al.,*(2005)b, [11]: Lugon (1996), [12]: Ma *et al.,*(2008), [13]: Mackenzie and Oxford (1995), [14]: Pithartova (2007), [15]: Rydell (1989), [16]: Rydell *et al.,*(1996), [17]: Rydell and Petersons (1998), [18]: Sierro and Arlettaz (1997), [19]: Vaughan (1997), [20]: Waters *et al.,*(1999) and [21]: Whitaker *et al.,*(1994).

Table 3.2: Input parameters values used for each bat species: foraging distance (km), body weight (g), daily amount (g dry weight/gbw/day) of food eaten and NOAEL (µg/gbw/day).

Bat species	Foraging distance (km) (Dietz, 2009)	Average weight (g) (Dietz, 2009)	Daily amount of food eaten (g dry weight/ gbw/day) (Nagy, 1987)	NOAEL (µg/gbw/day) (Sample *et al.,* 1996)			
				Cd	**Cu**	**Pb**	**Zn**
Barbastella barbastellus	4.50	8.50	0.161	2.44	38.57	20.27	405.32
Eptesicus serotinus	12.00	21.50	0.136	1.94	30.59	16.07	321.40
Myotis bechsteinii	2.50	8.50	0.161	2.44	38.57	20.27	405.32
Myotis daubentonii	15.00	8.00	0.162	2.48	39.16	20.58	411.51
Myotis mystacinus	2.80	5.50	0.173	2.72	43.01	22.60	451.92
Myotis nattereri	4.00	8.50	0.161	2.44	38.57	20.27	405.32
Nyctalus leisleri	4.20	15.50	0.144	2.10	33.19	17.44	348.79
Nyctalus noctula	26.00	25.50	0.132	1.86	29.31	15.40	307.97
Pipistrellus nathusii	6.50	8.00	0.162	2.48	39.16	20.58	411.51
Pipistrellus pipistrellus	1.70	4.90	0.177	2.80	44.27	23.26	465.16
Plecotus auritus	3.30	7.50	0.164	2.52	39.80	20.91	418.20
Plecotus austriacus	5.50	8.00	0.162	2.48	39.16	20.58	411.51
Rhinolophus ferrumequinum	2.10	21.00	0.137	1.95	30.77	16.16	323.29
Rhinolophus hipposideros	5.00	5.50	0.173	2.72	43.01	22.60	451.92

Table 3.3: Median BAFs values for each metal (Cd, Cu, Pb, Zn) and invertebrate order studied (Araneida, Coleoptera, Dermaptera, Diptera, Hemiptera, Hymenoptera, Lepidoptera, Opiliones, Orthoptera and Trichoptera) derived from literature studies.

	Araneida	Coleoptera	Dermaptera	Diptera	Hemiptera	Hymenoptera	Lepidoptera	Opiliones	Orthoptera	Trichoptera
Cd	3.125	0.525	0.134	0.735	0.507	0.882	0.890	3.591	0.238	0.318
Cu	0.047	0.037	0.039	0.210	0.012	0.030	0.038	0.083	0.005	0.295
Pb	0.894	0.999	0.003	0.810	1.222	0.291	0.449	0.557	1.303	0.783
Zn	0.553	1.011	0.055	0.772	1.400	0.783	0.136	0.780	1.166	3.286

Table 3.4: Input parameters and their ranges used in the sensitivity analysis to identify the key drivers of risks.

Parameters	**Range used in the sensitivity analysis**	
Concentration of the metal in the soil (µg/g dry weight). Range limited to 95 and 99% observations.	95% observations Cd: 0-1.6 Cu: 0-47.9 Pb: 0-194.9 Zn: 0-175.8	99% observations Cd : 0-3.2 Cu : 0-100.3 Pb : 0-514.6 Zn : 0-350.8
Safe daily dose for the metal (NOAEL/5) (µg/g body weight/day)	Cd: 0.37-0.56 Cu: 5.86-8.85 Pb: 3.08-4.65 Zn: 61.59-93.03	
Amount of food eaten (dry weight/g body weight/day)	Between bat species: 0.13-0.18	
Proportion of the diet accounted for an individual prey item (% volume)	Coleoptera Diptera Lepidoptera	0-1
	Araneidea Dermaptera Hemiptera Hymenoptera Opiliones Orthoptera Trichoptera	0-0.25

We calculated the mean soil concentrations associated with the spatial distribution and the feeding range for each species by using the method described in Chapter 2 (Figure A1.2). This spatial application was applied to map soil concentrations occurring within each bat distribution and does not take into account the diet or food intake. To explore the effects of species location (area in which the bat is living) and feeding range (area in which the bat is foraging), we compared the soil concentrations to which each species would be exposed using the non-parametric Kuskal-Wallis test.

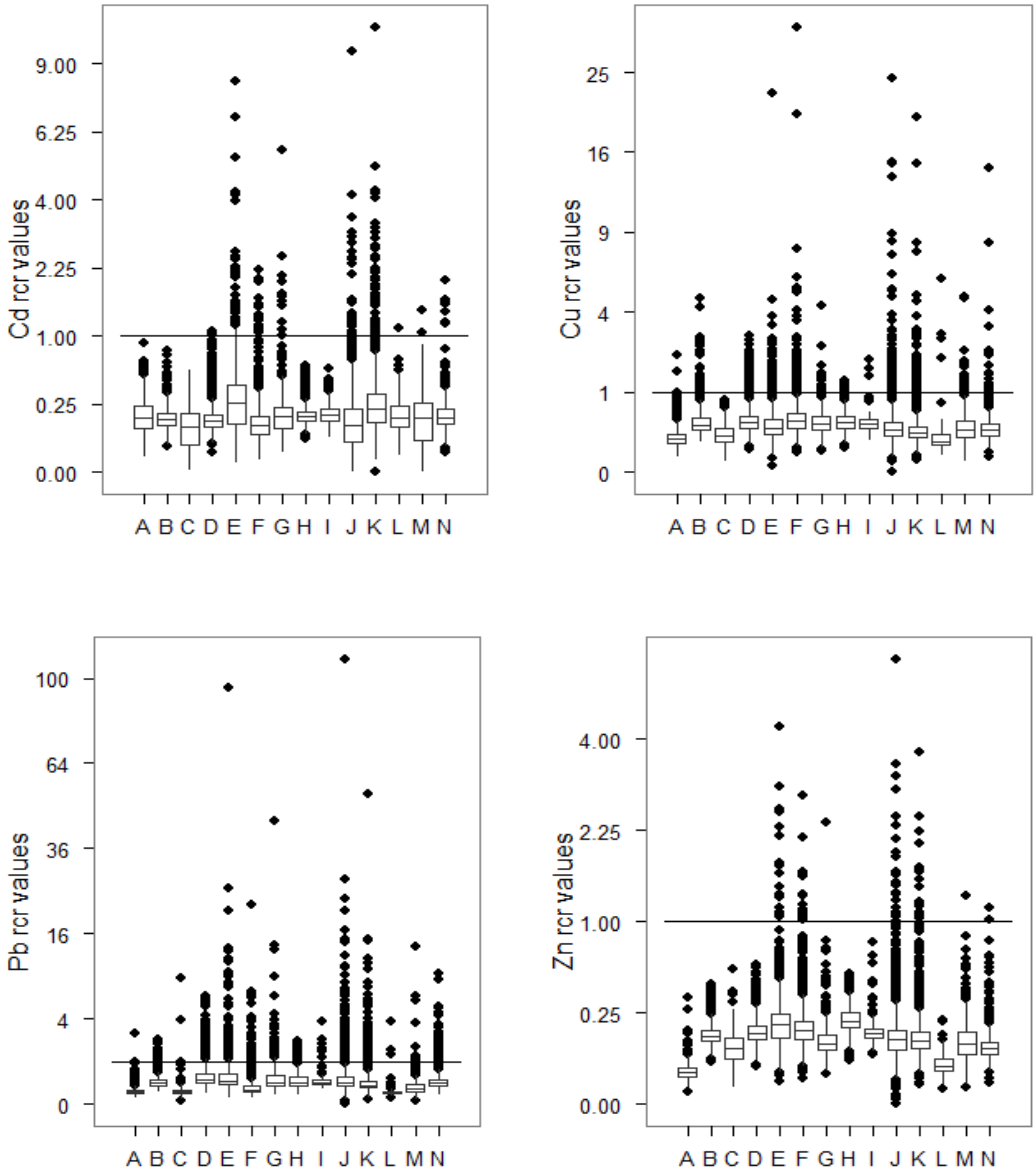
In addition, the soil metal concentrations within and outside the bat spatial distribution were compared to explore whether or not bat species "avoid" polluted areas. Metal concentrations data are not normally distributed and, therefore, the non-parametric Wilcoxon signed-rank test was used to explore this aspect.

Results

Risk of British bat species to metal exposure

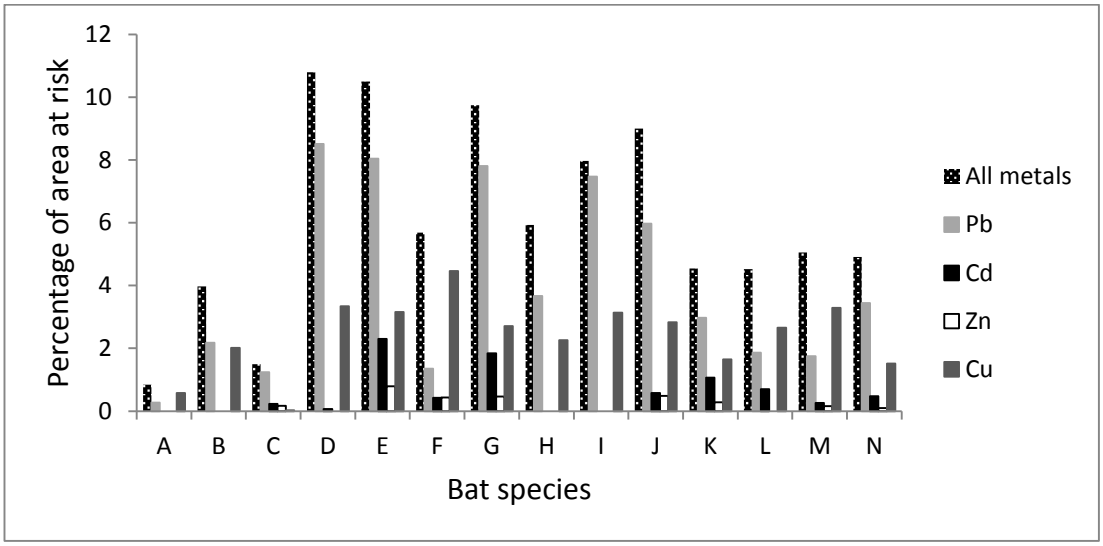
Median RCRs for all species were highest for Cu, followed by Pb, Cd and Zn (0.30, 0.23, 0.16 and 0.12 respectively) (Figure 3.1). The RCRs were significantly different across metals for each bat species (Kruskal-Wallis chi-squared 1893, 3331, 251, 8790, 1092, 5841, 958, 5648, 193, 5383, 4582, 307, 510, and 2922 for the bat species A, B, C, D, E, F, G, H, I, J, K, L, M, and N, respectively, df = 3, $p < 0.001$). *M. nattereri*, *N. noctula*, *M. daubentonii*, *P. nathusii*, *N. leisleri*, *E. serotinus*, and *M. mystacinus* appeared to be most exposed to Cu. *M. daubentonii*, *M. mystacinus, Pipistrellus sp, P. Nathusii, N. leisleri, and N. noctula;* for Cd, *M. mystacinus* and *P. auritus* appeared to be most exposed to Pb. *N. noctula*, *M. mystacinus*, *M. nattereri* and *M. daubentonii* were predicted to be most exposed to Zn (Figure 3.1). The RCRs were significantly different across bat species for each metal (Kruskal-Wallis chi-squared: 3175, 5180, 7830, and 8273, for Cd, Cu, Pb and Zn respectively, df = 13, $p < 0.001$).

Figure 3.1: Median risk characterisation ratio distributions for Cd, Cu, Pb and Zn for 14 bat species across their spatial distribution: A = *Barbastella barbastellus*, B = *Eptesicus serotinus*, C = *Myotis bechsteinii*, D = *Myotis daubentonii*, E = *Myotis mystacinus*, F = *Myotis nattereri*, G = *Nyctalus leisleri*, H = *Nyctalus noctula*, I = *Pipistrellus nathusii*, J = *Pipistrellus sp.*, K = *Plecotus auritus*, L = *Plecotus austriacus*, M = *Rhinolophus ferrumequinum*, N = *Rhinolophus hipposideros*. Black lines represent the threshold RCR of 1. The upper and lower whiskers extend from the hinge to the highest and the lowest values which are within 1.5 times the inter-quartile range. Above the upper whisker, RCR values are represented by points. The y axis has been square root transformed.



The overall risk (risk defined by the area having an RCR higher than one) (Chapter 2) by any one or more of the four metals studied can reach over 10% of the bat distribution (11.0 and 10.5%, *M. daubentonii* and *M. mystacinus* respectively) (Figure 3.2). Regarding the percentage of species' distribution at risk, contamination of Pb posed the greatest risk to all bats species with between 0.3 and 8.5% (*B. barbastellus* - *M. daubentonii*) of species' distribution determined to be at risk by the model. The next most important metal was Cu (0 – 4.5% (*M. bechsteinii -M. nattereri*), followed by Cd (0 – 2.3% ((*B. barbastellus, E. serotinus, N. noctula, P. nathusii*) - *M. mystacinus*)) and Zn (0 – 0.8% (*B. barbastellus, E. serotinus, M. daubentonii, N. noctula, P. nathusii, P. austriacus* - *M. mystacinus*) (Figure 3.2).

Figure 3.2: Percentage of species' distribution determined at risk from metals Cd, Cu, Pb and Zn and from all the metals combined for the 14 species studied. The bat species are the following: A: *Barbastella barbastellus,* B: *Eptesicus serotinus,* C: *Myotis bechsteinii,* D: *Myotis daubentonii,* E: *Myotis mystacinus,* F: *Myotis nattereri,* G: *Nyctalus leisleri,* H: *Nyctalus noctula,* I: *Pipistrellus nathusii,* J: *Pipistrellus sp.,* K: *Plecotus auritus,* L: *Plecotus austriacus,* M: *Rhinolophus ferrumequinum,* N: *Rhinolophus hipposideros.*

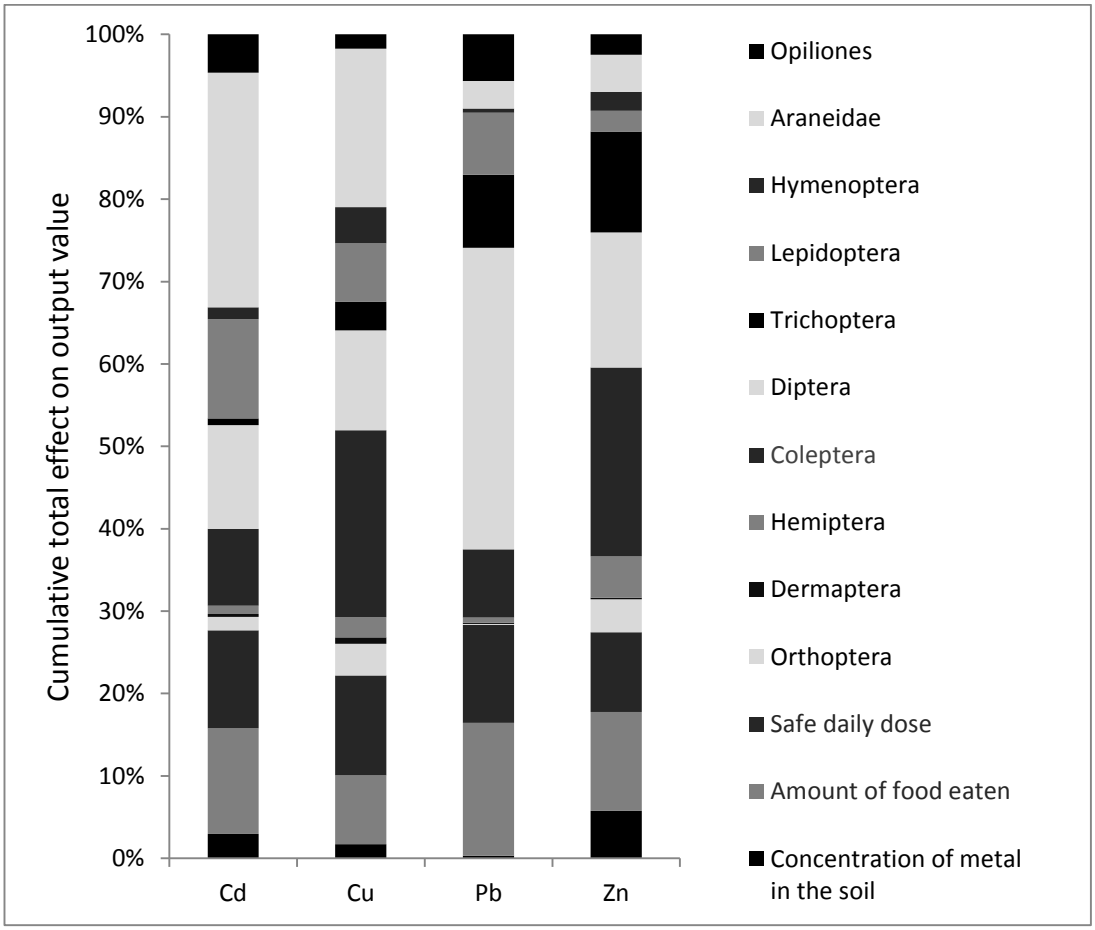


Identification of key drivers of risk

An initial evaluation of the effects of non-spatial input values on the model outcome was carried out. This evaluation included the range of soil concentrations covering 95% of the data. For all the metals, the proportion of Coleoptera and Diptera in a bat's diet were particularly important in determining a bat species' risk (Figure 3.3). The key drivers determining the risk of bats to Pb exposure were the proportion of dipteran species in the diet (contributing to 44% of the RCR), followed by the amount of food eaten (19%) and then the predicted safe daily dose (14%) (Figure 3.3). For Cu, the proportion of Coleoptera and Araneidae were found to be similar in terms of importance, contributing 28% and 24% to the

total effect respectively (Figure 3.3). The amount of food eaten and the proportion of Diptera were also important, with both of these parameters contributing 15% of the total effect (Figure 3.3). For Cd, the proportion of Araneidae was the most important factor (33%), followed by the proportion of Diptera (15%), the amount of food eaten (15%), the predicted safe daily dose (14%) and finally the proportion of Lepidoptera (14%) (Figure 3.3). For Zn, the most important factor was the proportion of Coleoptera (27%), followed by the proportion of Diptera (19%), Trichoptera (14%) and finally the amount of food eaten (14%) (Figure 3.3).

Figure 3.3: Cumulative effect for the different parameters given by the sensitivity analyses results (in percentage). The model parameters studied are: the proportion of invertebrates in the diet, for each invertebrate type (Opiliones, Araneidae, Hymenoptera, Lepidoptera, Trichoptera, Diptera, Coleoptera, Hemiptera, Dermaptera, Orthoptera), the safe daily dose (µg/g body weight/day), the amount of food eaten (g dry weight/g body weight/day) and the concentration of metal in the soil (µg/g dry weight) (95% of the soil data covered).



The second evaluation covered 99% of the soil data and, as expected, the soil had a greater contribution to the risk than when 95% of the soil data were covered. However, there were

slight differences between the two analyses and the soil concentration was not the most important parameter in both analyses (Figure A2.1).

To understand the importance of the location of a bat species and feeding range, the effects of the location in which the bat is living and foraging in terms of soil exposure concentrations in England and Wales were established. Although the concentrations of metals significantly differed across species (Figure 3.5) (Kruskal-Wallis chi-squared: 309, 972, 1891 and 371 for Cd, Cu, Pb and Zn, respectively, df = 13, $p < 0.0001$), the sensitivity analysis showed that metal concentration was not a determinant factor. As such, the effect of the different bat distributions and feeding ranges on the risk output would be minor.

Seven species (*B. barbastellus, P. auritus, P. Nathusii, Pipistrellus sp, P. austriacus E. serotinus and M. bechsteinii*) resided in areas (within the bat distribution) which had significantly lower soil concentrations than areas where these species were not found (outside the bat distribution) for at least one metal (Wilcoxon signed-rank test, $p < 0.05$) (Figure 3.5; Table A2.2).

Figure 3.4: Median metal soil concentrations (µg/g dw) associated with bat distribution and the foraging distance of each species. The bat species are the following: A: *Barbastella barbastellus,* B: *Eptesicus serotinus,* C: *Myotis bechsteinii,* D: *Myotis daubentonii,* E: *Myotis mystacinus,* F: *Myotis nattereri,* G: *Nyctalus leisleri,* H: *Nyctalus noctula,* I: *Pipistrellus nathusii,* J: *Pipistrellus sp.,* K: *Plecotus auritus,* L: *Plecotus austriacus,* M: *Rhinolophus ferrumequinum,* N: *Rhinolophus hipposideros.* The upper and lower whiskers extend from the hinge to the highest and the lowest values which are within 1.5 times the inter-quartile range. Above the upper whisker, soil concentration values are represented by points. The y axis has been square root transformed.

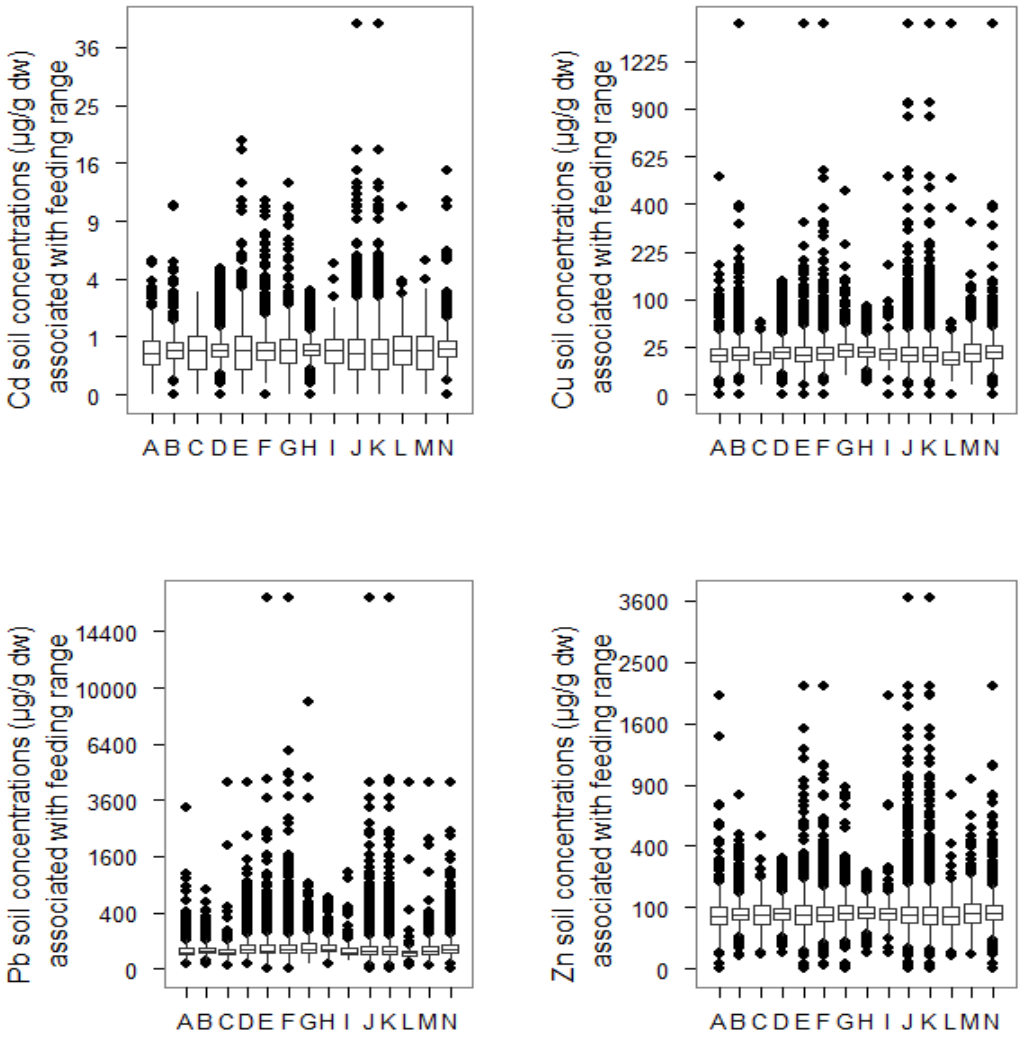
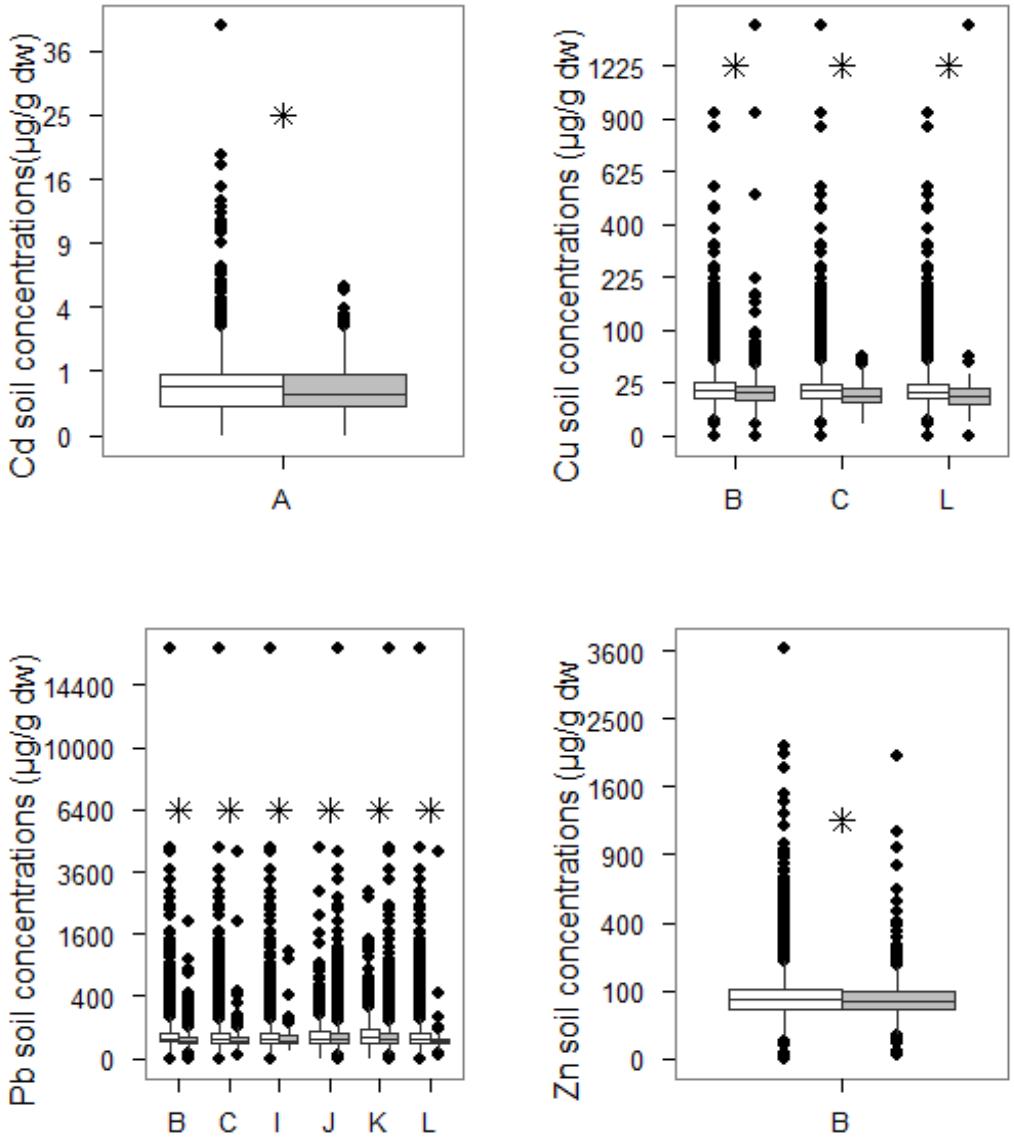


Figure 3.5: Median soil metal concentrations within (grey) and outside the bat ranges (white) for Cd, Cu, Pb and Zn for the different bat species studied (µg/g dw). The soil metal concentrations presented are significantly lower within (grey) than outside (white) the actual distribution. The bat species are the following: A: *Barbastella barbastellus*, B: *Eptesicus serotinus*, C: *Myotis bechsteinii*, I: *Pipistrellus nathusii*, J: *Pipistrellus sp*., K: *Plecotus auritus*, L: *Plecotus austriacus*. The upper and lower whiskers extend from the hinge to the highest and the lowest values which are within 1.5 times the inter-quartile range. Above the upper whisker, soil concentrations are represented by points. The y axis has been square root transformed.



Discussion

Some species seem to be more at risk to metal exposure than others (Figure 3.1, Figure 3.2). The different bat species investigated have different diets, foraging behaviour and spatial distribution. Diet composition appears to be the most important factor in determining the risk in our model (Figure 3.3). The proportions of Coleoptera and Diptera are particularly important in determining risk (Figure 3.3).

Of the species predicted to be most exposed to metals (Figure 3.1; Figure 3.2), significant declines in populations have been recorded for several of these species in the UK. For example, for the *P. Pipistrellus* species, significant declines have been recorded in the 1980s, while for *M. mystacinus* and *E. serotinus*, the declines have been more localised (e.g. in the southeast of England for *E. serotinus*) and associated with roost loss (Dietz *et al*., 2009). In addition, the *N. noctula* species, with higher median RCRs than most of the other species for Cu and Zn, has been added to the UK's biodiversity action plan since there is an increasing concern about a marked decline in its population (Dietz *et al*., 2009).

There has been no significant trend in population decline for these bat species as analysed by conservation bodies such as the NBMP (National Bat Monitoring Programme) (Dietz *et al*., 2009). This could be explained by a lack of good population monitoring data. For example, the IUCN Red list (2008) stated that the population trends remain unknown for *N. noctula* and *P. nathusius*. However, the IUCN Red List also highlights a wide range of threats (e.g. habitat loss, habitat fragmentation, disturbance, use of pesticides, use of insecticides, water quality changes etc.) on bat populations (IUCN Red List, 2008). The lack of good bat monitoring data does not allow us to link our risk predictions with recent population declines.

A comparison of model predictions with monitoring data on metal tissue concentrations measured in various European bat species (Lüftl *et al*., 2003, cited by Carravieri and Scheifler, 2013 and Walker *et al*., 2007) indicated that some of the bat species predicted to be at higher risk compared to other species, contain higher metal concentrations in their tissues than other species. For example, *M. mystacinus* and *Pipistrellus sp*. contained Pb renal concentrations around twice the median value for all the species studied (*E. serotinus*, *N. noctula*, *P. Nathusii*) (Lüftl *et al*., 2003, cited by Carravieri and Scheifler, 2013). For Cu, *N. noctula* contained the highest renal concentrations (34 µg/g dw) amongst other species (*E. serotinus*, *M. mystacinus*, *Pipistrellus sp*., and *R. hipposideros*) and was predicted to be the second species most at risk from Cu (based on median RCR) (after *M. nattereri* for which the concentration has not been determined) (Lüftl *et al.*, 2003, cited by Carravieri and Scheifler,

2013). From the bats predicted to be the most exposed to Zn, *M. mystacinus*, contained Zn concentrations (76.5 μg/g dw) higher than the mean (74 μg/g dw) obtained from all the species studied (*E. serotinus*, *N. noctula*, *Pipistrellus sp*. and *R. hipposideros*) (Lüftl *et al*., 2003, cited by Carravieri and Scheifler, 2013). For Cd, *M. mystacinus*, *P. auritus* and *N. noctula* appeared to be the most exposed and contained higher or similar Cd concentrations in their kidneys (1.2, 0.8, 0.8 for *M. mystacinus*, *P. auritus* and *N. noctula*, respectively) than the mean (0.8 μg/g dw) for all the species studied (*Pipistrellus sp*., *P. auritus*, *E. serotinus*, *M. mystacinus*, *N. noctula*, *R. hipposideros*) (Lüftl *et al*., 2003 cited by Carravieri and Scheifler, 2013 and Walker *et al*., 2007).

Indeed, although the metal concentrations in kidneys have not been determined for all bat species (Carravieri and Scheifler, 2013; Walker *et al*., 2007) investigated in our study, it was possible to distinguish similar patterns. It is particularly important to highlight *M. mystacinus*, as it was predicted to be the most exposed to all metals in our study and has been shown to globally contain higher metal concentrations in kidneys compared to other species investigated in the Carravieri and Scheifler (2013) study. Further monitoring studies are encouraged to determine the actual metal concentrations in bats across Europe so as to verify whether certain species are more exposed than others and, therefore, to potentially validate our predictions against a monitoring dataset comprising a wider range of bat species.

Coleoptera, Diptera and Lepidoptera are the most important prey items composing bat diets and vary greatly in proportion across the different bat diets (Table 3.1). The sensitivity analysis highlighted that these prey items are important in determining risk (Table 3.3). The BAF values for different orders can explain some of our sensitivity analysis results. For example, the model is not as sensitive to the proportion of Lepidoptera in the diet compared to the proportions of Diptera and Coleoptera. Lepidoptera generally have a lower BAF compared to Diptera and Coleoptera (Figure 3.3; Table 3.3). The only exception was for Cd, where a higher percentage total effect was observed for Lepidoptera (14%, against 11% for Coleoptera) (Figure 3.3). This can be explained by a higher BAF for Cd for Lepidoptera than Coleoptera (Table 3.3). In addition, Trichoptera and Araneidae had relatively higher BAFs for some metals (3.3 for Zn and 3.1 for Cd, for Trichoptera and Araneidae respectively) compared to other orders. Although the proportion of Trichoptera and Araneidae in the diet was lower than other orders, the total effect on the model output was within the same range of Coleoptera and Diptera (Figure 3.3).

Bat distribution and foraging distance were not included in the sensitivity analyses. These parameters could have a major impact on the metal soil concentrations to which a species is exposed. However, since the metal soil concentrations did not appear to be the most important parameter in our study (Figure 3.3), we believe that the analyses performed can provide satisfactory information pertaining to the model's sensitivity.

The differences in metal soil concentrations within and outside the bat distributions (Figure 3.5, Table A2.2) may indicate that the species (*B. barbastellus*, *P. auritus*, *P. Nathusii*, *Pipistrellus sp*, *P. austriacus*, *E. serotinus* and M. *bechsteinii*) are avoiding polluted areas. However, a large number of species resided in areas with higher mean soil concentrations than areas where the bats did not occur, thus meaning that the distribution of these species cannot be linked to an avoidance of polluted areas (Figure 3.5, Table A2.2).

Other factors may explain interspecific differences. The model used an allometric relationship to derive sensitivity data for each species, which only accounts for size differences. However, specific differences in metal sensitivity may occur due to differences in detoxification processes. For example, it has recently been shown that different passerine bird species regulate their oxidative stress differently after metal exposure (Rainio *et al.,* 2013). The activity of the antioxidant enzymes varies across these bird species (Rainio *et al.,* 2013). It may be that interspecific differences occur in metal detoxification and regulation across bat species. Another study has shown interspecific differences in metal accumulation levels across several small mammal species (Fritsch *et al.*, 2010). The accumulation in small mammals also varied within a group consuming a similar diet, thus suggesting that the traditional differentiation based on the trophic group may mislead the interpretation of the results (Fritsch *et al.*, 2010). The authors indicated that interspecific differences in metal accumulation may be related to the physiological (e.g. metabolic rate, digestive characteristics influencing metal bioaccessibility, excretion rate, etc.) and behavioural characteristics (diet composition, habitat preferences, etc.) of the organism (Fritsch *et al.*, 2010). These interspecific differences may also explain the variation of risk predicted across species in our study.

Our results show that the proportion of food items consumed per bat species is relatively important, and as such, further research could utilise technologies such as OMICSs or stable isotopes to accurately investigate wildlife diet composition (Matranga and Corsi, 2012). Indeed, small and soft body items may be neglected using traditional methods in diet analysis (Vaughan, 1997; Robinson and Stebbings, 1993). In addition, metal bioaccumulation in

invertebrates needs to be better understood since it can influence exposure model predictions. Bioaccumulation studies are often only based on a limited number of species cultured experimentally and then generalised to a higher taxonomical level. Metal uptake can also be influenced by species traits (Rubach *et al.*, 2012). In addition, more information on the metal bioaccessibility from invertebrates may help to refine exposure predictions (Kaufman *et al.*, 2007).

Developing the model further could involve a refinement in metal exposure estimations by including more detailed data on food availability, foraging and habitat use, temporally and spatially across different habitats. Indeed, these factors have emerged as important when it comes to addressing wildlife exposure assessments (Schipper *et al*., 2012; Vermeulen *et al.*, 2009). Additional sources of contamination such as sediments could also be included as well as the consideration of the different life stages of the invertebrates. For example, the larval stages of Diptera are aquatic and, as such, may represent a contamination pathway between the aquatic and the terrestrial ecosystem (Reinhold *et al*., 1999).

This study identified bat species at risk from metal contamination in England and Wales and interspecific variation in risk to metal exposure. Particular conservation and management actions could be orientated on bat species and areas identified as at risk by the model. The sensitivity analyses highlighted the importance of diet composition, amount of food ingested and toxicity data for the target species. While sensitivity analyses are emphasised and required by many institutions (e.g. Health Canada Contaminated Sites Division, 2005; European food safety authority, EFSA Journal, 2009) in ERA, the literature remains scarce. In light of this, further studies employing sensitivity analysis in food chain modelling are encouraged. These studies can also help to prioritise research needs on key factors driving contamination risk.

The work presented in this chapter and Chapter 2 was entirely modelling-based. In order to lend weight to the conclusions of the model predictions for the different bat species, it would be valuable to evaluate the model using real data on metal risks to bats in England and Wales. Therefore, the next chapter describes the findings of a field monitoring study for bats in England and Wales and uses this data to evaluate the modelling framework.

Chapter 4

Monitoring of residues of metals in bat tissues and model evaluation

Introduction

The results reported in Chapters 2 and 3 indicate that bat species in England and Wales are exposed to metals within unacceptable ranges, particularly areas of their distribution. These predictions are particularly relevant since bat populations declines have been observed in different regions of the world (Dietz *et al.,* 2009; Stebbings, 1988). However the model predictions are computed values and numerous assumptions have been made, including:

1. Median BSAFs used in the model are representative of uptake from the natural environment in England and Wales into invertebrates;
2. Dietary data reflect the actual diets for bats in England and Wales;
3. Metal bioavailability from food items to bats is assumed to be 100%;
4. Sensitivity of bats to metal exposure can be estimated from toxicity studies on other species; and
5. The RCRs are reflective of metal exposure risks for bats.

These and other assumptions are discussed in more detail in Chapter 6. However, considering all of these assumptions, it would be beneficial to evaluate the model predictions against real monitoring data in order to give users the results and verify the level of confidence in the modelling framework's predictions.

In order to evaluate the model, experimental monitoring data are ideally needed for England and Wales. An ideal dataset for model evaluation would include information on actual residues in bat tissues, pathological data and information on the health of bat populations in different areas. While data on metal-induced effects on individuals are relatively abundant, field studies detailing the effects of metals on wild populations are largely lacking (Shore and

Douben, 1994). A number of studies have been performed to assess residues of metals in different bat species in different regions (Carravieri and Scheifler, 2013). The ranges of metal concentrations (Cd, Cu, Pb and Zn) measured in European insectivorous bats are summarised in Table 4.1.

The available data on residues in bats are restricted to either analyses of only a small number of individuals for small or large areas or to the analysis of a large number of individuals for a small area. Much of the European data are available only for countries in mainland Europe. Therefore, the available data are not optimum for use in the evaluation of our modelling framework presented in Chapter 2.

As such, in the present study we determined levels of metals in different tissues of insectivorous bats from across England and Wales. We focussed on *Pipistrellus sp.*, which has a wide distribution across Europe and which was the focus of our modelling. In this study we first presented a large national-scale dataset of trace metal concentrations in different organs of bats.

Information from the tissue analyses were then compared to critical toxic concentration levels for small mammals so as to establish the toxicological pressure of metals on the bat populations. Finally, we used the results to evaluate the performance of the modelling framework to determine its utility as a tool for risk assessment and management.

Table 4.1: Median metal concentrations in liver and kidneys of European bats taken from Carravieri and Scheifler (2013). The concentrations are expressed in µg/g on a dry weight. To transform the results, which were initially expressed in wet weight, we assumed that concentrations expressed in dry weight are four times higher than wet weight values.

	Species	Median concentrations (min-max) in µg/g dw.		Sample	Country
		Liver	Kidneys		
Cadmium	*Pipistrellus pipistrellus*	1.53[a]	-	14	Germany
	Myotis mystacinus	-	1.61	17	England
	Myotis nattereri	-	6.27	13	
	Pipistrellus sp.	-	1.42	172	
	Plecotus auritus	-	0.83	59	
	Eptesicus serotinus	-	0.432 (<LD* – 7.896)	10	Austria
	Myotis emarginatus	-	0.140 (0.004 – 11.656)	11	
	Myotis mystacinus	-	1.216 (<LD* – 24.208)	26	
	Nyctalus noctula	-	0.768 (0.308 – 5.160)	11	
	Pipistrellus kuhlii	-	0.808 (0.028 - 16.000)	23	
	Pipistrellus pipistrellus	-	0.588 (0.056 – 11.268)	43	
	Rhinolophus hipposideros	-	0.412 (0.012 – 1.552)	5	
	Vespertilio murinus	-	1.452 (0.860 – 5.228)	6	
	Myotis dasycneme	-	0.17	6	Netherlands
Copper	*Eptesicus serotinus*	-	22.88 (13.60 – 44.40)	10	Austria
	Myotis emarginatus	-	20.60 (11.00 – 474.04)	11	
	Myotis mystacinus	-	32.44 (9.16 – 152.2)	26	
	Nyctalus noctula	-	34.28 (18.36 – 58.16)	11	
	Pipistrellus kuhlii	-	33.76 (16.64 – 81.52)	23	
	Pipistrellus pipistrellus	-	33.72 (13.24 – 144.72)	43	
	Rhinolophus hipposideros	-	29.36 (17.52 – 71.24)	5	
	Vespertilio murinus	-	22.12 (10.48 – 32.40)	6	

<LD: value inferior to detection limit (Lüft *et al*., 2003, cited by Carravieri and Scheifler, 2013).

Table 4.1: Continued.

	Species	Median concentrations (min-max) in µg/g dw.			Sample	Country
		Liver	Kidneys			
Lead	*Pipistrellus pipistrellus*	2.95	-		14	Germany
	Myotis mystacinus	-	4.05		17	England
	Myotis nattereri	-	1.16		13	
	Pipistrellus sp.	-	2.45		172	
	Plecotus auritus	-	3.38		59	
	Eptesicus serotinus	-	1.492	(0.696- 3.848)	10	Austria
	Myotis emarginatus	-	4.836	(1.828-14.000)	11	
	Myotis mystacinus	-	4.668	(0.524- 55.240)	26	
	Nyctalus noctula	-	1.624	(1.248 – 3.516)	11	
	Pipistrellus kuhlii	-	3.456	(1.664 – 10.200)	23	
	Pipistrellus pipistrellus	-	3.828	(1.540 – 63.416)	43	
	Rhinolophus hipposideros	-	3.388	(1.496 – 9.272)	5	
	Vespertilio murinus	-	2.704	(1.340 – 5.660)	6	
	Myotis daubentonii	-	1.28		6	Czech Republic
	Myotis myotis	1.52	1.20		33/32	
	Pipistrellus nathusii	-	1.60		6	
	Pipistrellus pipistrellus	1.32	0.52		23/23	
	Pipistrellus pygmaeus	0.84	2.16		05/07	

<LD: value inferior to detection limit (Lüft *et al*., 2003, cited by Carravieri and Scheifler, 2013).

Table 4.1: Continued.

	Species	Median concentrations (min-max) in µg/g dw.			Sample	Country
		Liver	Kidneys			
Zinc	*Eptesicus serotinus*	-	211.6	(89.6 – 490.0)	10	Austria
	Myotis emarginatus	-	472.4	(138.0 – 2391.2)	11	
	Myotis mystacinus	-	306	(112.8 – 3283.6)	26	
	Nyctalus noctula	-	100.8	(54.4 – 135.2)	11	
	Pipistrellus kuhlii	-	163.2	(56.0 – 1702.0)	23	
	Pipistrellus pipistrellus	-	212.4	(68.0 – 1760.0)	43	
	Rhinolophus hipposideros	-	654	(101.2 – 1558.4)	5	
	Vespertilio murinus	-	158	(107.6 – 357.6)	6	
	Myotis daubentonii	-	1.44		6	Czech Republic
	Myotis myotis	1.36	1.64		33/32	
	Pipistrellus nathusii	-	3.40		6	
	Pipistrellus pipistrellus	1.12	1.88		23/23	
	Pipistrellus pygmaeus	1.2	2.52		05/07	

<LD: value inferior to detection limit (Lüft *et al*., 2003, cited by Carravieri and Scheifler, 2013).

Materials and methods

Sample collection and processing

Pipistrelle adult male bats (n=193), mainly of the *Pipistrellus pipistrellus* species and a few specimens of the sibling *Pipistrellus pygmaeus*, (n=5) species were obtained from different areas in England and Wales. The common pipistrelle (*Pipistrellus pipistrellus*) bat is widely distributed across Europe, including the whole of the UK. Only males were selected since females can transfer metals through lactation (Streit and Nagel, 1993) and, as such, metal concentrations determined in females may not reflect the entire amount of accumulated contaminant. Adult individuals were selected in order to maximise the chances of detecting concentrations above the limit of detection, since metal accumulation is correlated with the age of organisms (Rudy, 2009). Bats, found dead or fatally injured, were collected in 2008, 2009 and 2010, and were kept in 40% formaldehyde solution prior to analysis. The analyses were conducted in 2012.

Bats were selected from an archive of 3000 bats provided by the Veterinary Laboratory Agency (Surrey, England). Bat locations were recorded for each individual. Firstly, the metal concentrations in soils from the bat locations were extracted from the National Soil Resources Institute (NSRI) soil dataset, which has data on soil concentrations across England and Wales at a 5 x 5 km^2 resolution, and a frequency distribution of concentration data was developed. A subsample of 193 bats was then selected to give a frequency distribution similar to the overall frequency distribution of the soil concentrations for the four metals studied. We purposefully included bats from soils with extreme concentrations of metals and tried to select bats with a view to giving complete spatial coverage across the area of England and Wales.

Prior to analysis, individuals were weighed and were then dissected to excise kidneys, liver, stomach (and stomach content), fur and bones (humerus, radius and femurs). Fur samples were obtained from the area between the scapulae. In many radio tracking studies, this area is clipped to attach transmitters (Womack *et al.,* 2013). However, in the present study, this particular area was sampled in order to determine whether fur can be used as a less-invasive to monitor levels of metal exposure in bats. The results of this assessment will be reported elsewhere. The tissues were oven dried until having reached constant dry weight and taken for analysis. Tissues in a poor conservation state or missing (previously extracted) and empty stomachs were not analysed, which explains why the number of samples (N) may not equate

to the total number of bats analysed. An aliquot of formaldehyde (0.5 ml) was also taken to quantify any metal which may have leached from the bat body into the preservative (Appendix 3).

Sample analyses: Quantification of metal concentrations.

Prior to analyses by ICP-MS (Agilent 7500CE, Cheshire, UK), dried samples (organs, fur, bones) were digested on a hot block at 100°C for 1 hour in 1 ml of nitric acid, followed by another hour at 100°C following the addition of 0.2 ml of hydrogen peroxide. Digests were made up to a fixed volume of 10 ml with Millipore water to obtain a final digest containing 10% acid.

A constant number of the internal standards (chemically similar to the analyte) was added into each tube. Quantification was performed by internal standardisation where the analyte signals and the internal standard signals were compared. The calibration curve was also used for corrections by normalising the response of the analyte to the response of the internal standard. This method determines accurate concentrations and corrects for drift (changes in sensitivity over time) and matrix effects (sample-related changes in sensitivity).

Quality assurance and quality control

Each analytical batch contained 1 spike, 4 blanks and 2 certified reference materials (bovine liver BCR 185R and spinach NCS ZC73013). Results for the spike sample showed a good recovery, while blank concentrations were below detection limits. The reference material results were within the acceptable range for Pb and Zn and had an average variation of 11% (absolute values) of the certified concentrations for all metals (Appendix 3).

Data analyses

Concentrations of metals in bat tissues were expressed as dry weight concentrations. Due to the variation in sample size, the LOD was calculated for each tissue type and metal. Non-detected concentrations were assigned a value of half the limit of detection. To be able to compare our results with previous studies (Lüftl *et al.,* 2003; Pikula *et al.,* 2010), we assumed that concentrations expressed in dry weight were four times (Clark and Shore, 2001) higher than wet weight values. Metal concentrations in bat tissue (for Cd, Cu, Pb and Zn) were not normally distributed (Shapiro test: $p < 0.001$).

In order to establish whether or not a bat was at risk from metal contamination, we compared measured concentrations in the liver and kidney with previously derived critical

toxic threshold concentrations for Pb and Cd and with upper level concentrations for Cu and Zn. The critical toxic levels for Pb and Cd were associated with structural and functional kidney damage (Chmielnicka *et al.*, 1989; Ma, 1996). The association of tissue residues and the effects on a long term population level in small mammals is unknown. By definition, there is no critical toxic threshold for essential metals such as Cu and Zn, although the upper level of metals in small mammals has been proposed for use in risk assessment. The Cu upper range was provided from a review of numerous studies on shrews, voles and mice, whereas the Zn data came from a more limited dataset (Ma and Talmage, 2001; Schleich *et al.,* 2010).

Model evaluation

Model predictions were taken from the work presented in Chapter 2. The model evaluation comprised two steps. We first compared tissue concentrations in two groups separated by their risk location status as defined by the model ("at risk" and "not at risk"). Unacceptable risk was defined in the model when a grid cell had a RCR exceeding the threshold value of one (Chapter 2). The hypothesis asserted that bat individuals found in areas predicted to be at risk would have higher internal concentrations of metals in their tissues than bats obtained from areas predicted to be not at risk. The receiver-operating characteristic (ROC) was used to compare monitoring data with model predictions (Platts *et al.,* 2008). The receiver-operating characteristic (ROC) curve provides a good visualisation between high sensitivity and high specificity which can vary when discriminating between two groups of data separated by a threshold value. Data analyses were performed with the software R version 2.12.1. Secondly, the percentage of bats predicted to be at risk (percentage of area predicted at risk on the overall bat distribution) in our previous spatial modelling work was also compared to the proportion of bats identified as at risk based on measured residue data and threshold values.

Results

Trace metal elements concentrations

Bats (193 adult male *Pipistrellus sp.*) were obtained from across a pollution gradient in England and Wales between 2008 and 2010 (Figure A3.1). Bats were dissected to obtain kidneys, liver, stomach, fur and bones, and these were analysed for metals through inductively coupled plasma mass spectrometry (ICP-MS). The highest concentrations in all tissues were seen for Zn followed by Pb, Cu and then Cd. (Table 4.2; Figure 4.1). Additional

results for bones, fur and stomach are included in Figure A3.2, but are not discussed further as no critical toxic threshold has been defined for these tissues.

Table 4.2: Median and maximum metal concentrations (µg/g dry weight) measured in kidneys and liver of *Pipistrellus sp*. in this study and previous studies. Toxic and upper range values from other studies are used to compare our tissue concentrations.

Metal	**Bat tissue**	**Statistics**	**Metal concentrations measured in *Pipistrellus sp*. tissues (µg/g dry weight)**					**Toxic threshold or upper range values**
			This study	Walker *et al.*, 2007	Lüftl *et al.*,2003	Pikula *et al.*, 2010	Streit and Nagel, 1993	
	Country		England and Wales	England	Austria	Czech Republic	Germany	
	Sample size		193	172	43	23	14	
Cd	Kidneys	Median	0.03	1.42	0.59	-	-	105.00[a]
		Max	1.51	29.1	11.27	-	-	
	Liver	Median	0.03	-	-	-	-	
		Max	12.98	-	-	-	-	
Cu	Kidneys	Median	12.89	-	33.72	-	-	30.00[b]
		Max	134.05	-	144.72	-	-	
	Liver	Median	10.69	-	-	-	-	30.00[b]
		Max	70.99	-	-	-	-	
Pb	Kidneys	Median	0.76	2.45	3.83	0.52	-	25.00[c]
		Max	367.22	69.7	63.42	-	-	
	Liver	Median	0.35	-	-	1.32	2.95	10.00[c]
		Max	5039.93	-	-	-	-	
Zn	Kidneys	Median	18.05	-	212.40	1.88	-	274.00[d]
		Max	354.17	-	1760.0	-	-	
	Liver	Median	18.79	-	-	1.12	-	465.00[d]
		Max	5205.31	-	-	-	-	

References: [a] Chmielnicka *et al.*, 1989; [b] Ma and Talmage, 2001; [c] Ma, 1996; [d] Schleich *et al*., 2010.

Trace metal element toxicity

The comparison of concentrations of metals in the liver and kidneys to toxicological threshold values (Table 4.1), indicates that 21% of the bats analysed had concentrations of one or more metals which exceeded toxic thresholds (Figure A3.3). Bats containing tissue residues associated with toxic effects were located in both rural and urban areas (Figure A3.3). Pb was the most toxicologically important metal with 7 (kidney) - 11 (liver)% of the bats containing concentrations above the threshold. The range for Cu was 4 - 9% and for Zn 0.5 – 5.2%. Concentrations of Cd in all bats were well below toxic thresholds.

Model evaluation

The comparison of risk categories obtained using the model with measured metal concentrations in tissues showed that concentrations of Pb (in kidneys and liver), Cu (in liver) and Zn (in kidneys) were significantly higher in the 'at risk' group than in the 'not at risk' group (Table 4.3; Figure 4.2). Comparison of the proportions of bats predicted to be at risk using the model with predictions and proportions at risk based on metal tissue concentrations indicated that, with the exception of Cd, the model slightly underestimates the risk of the metals (Table 4.3). For Pb, the model predicted that 5.9% of the bat distribution would be at risk whereas the tissue data suggested that between 7 and 11% of bats analysed had tissue levels associated with adverse effects on small mammals. For Cu, the model prediction was 2.8% and the proportion based on tissue analysis was 4-9%. For Zn and Cd, the model indicated that 0.5-0.6% of animals are at risk whereas the analytical data indicated that no bats had toxic residues of Cd, while for Zn, 0.5-5% of bats had toxic residues.

Figure 4.1: Median metal concentrations in kidneys and liver (n=191 for both organs) for Pb, Cu, Zn and Cd (µg/g dry weight). The y axis has been square root transformed. Black lines represent critical toxic threshold or maximum upper range level found in literature. The upper and the lower whiskers extend from the hinge to the highest and the lowest values which are within 1.5 times the inter-quartile range.

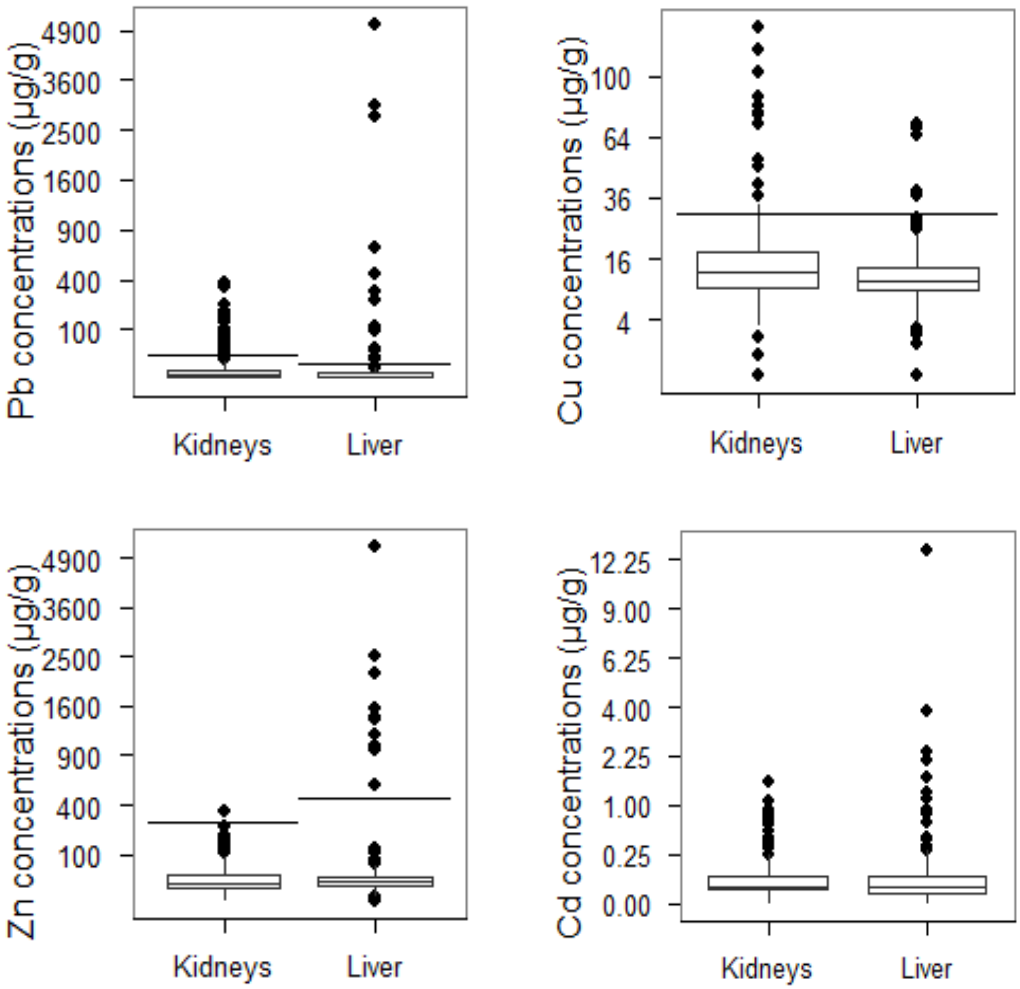
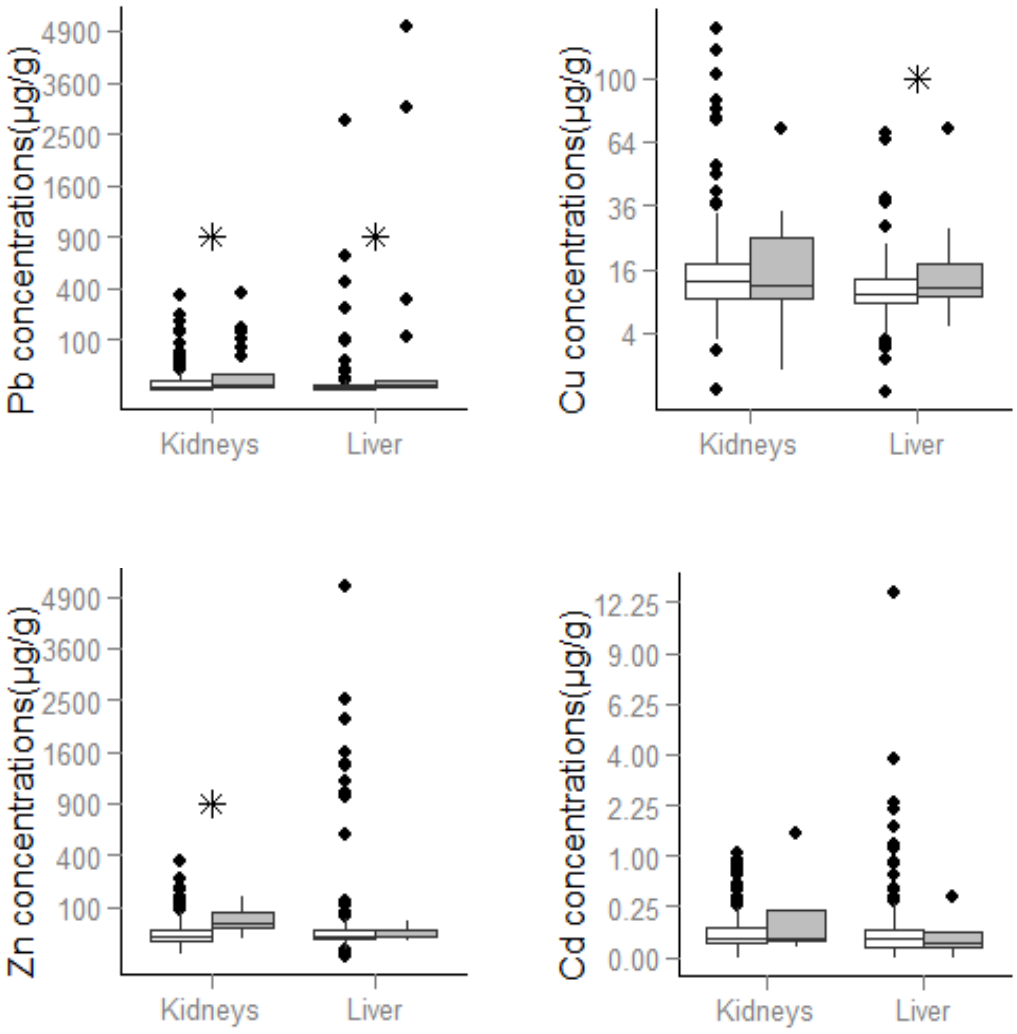


Table 4.3: ROC analyses (ROC AUC) results for tissue metal concentrations (kidneys and liver) between two groups of bats found in an area predicted to be at risk (G2) and in areas predicted not to be at risk (G1). The ROC AUC is the area under the receiver-operating characteristic curve. * Indicates a significant difference (P < 0.05). Total number of observations (N) and the median tissue concentrations (µg/g dw) are mentioned for both groups.

Bat tissue	Statistics	Cd	Cu	Pb	Zn
Kidneys	ROC AUC	0.52	0.51	0.67*	0.81*
	Median tissue concentrations G1, Median tissue concentrations G2 (µg/g dw)	0.03, 0.04	12.93, 12.74	0.65, 1.69	17.74, 51.45
Liver	ROC AUC	0.57	0.62*	0.68*	0.52
	Median tissue concentrations G1, Median tissue concentrations G2 (µg/g dw)	0.03, 0.02	10.23, 12.45	0.31, 1.05	18.73, 20.41
N Group 1, N Group 2		187, 4	162, 29	166, 25	184, 7

Figure 4.2: Differences in tissue metal (Pb, Cu, Zn and Cd) concentrations between bats found in areas predicted to be "at risk" (grey) and areas predicted not be to risk (white) (in µg/g dry weight). The bat tissues analysed are kidneys and liver (n=191 for both organs).* Indicates a significant difference (P < 0.05) (ROC analyses). The y axis has been transformed with a square root transformation. The upper and the lower whiskers extend from the hinge to the highest and the lowest values which are within 1.5 times the inter-quartile range.



Discussion

Trace metal elements concentrations

This study focussed on tissue metal concentrations of bats from England and Wales. Median soil concentrations for the two countries were 0.67, 22.4, 73.30 and 88.48 mg/kg for Cd, Cu, Pb and respectively. The values were within a factor of 2-5 of mean concentrations in other European countries and in N. America (Lado *et al.,* 2008; Shacklette and Boerngen, 1984; Smith *et al.,* 2005). We would therefore expect levels of exposure of bats to metals in other regions of the world to be similar.

Maximum renal concentrations for Pb in our study were around 5-fold higher than in the few previous studies detailing metal residues in *Pipistrellus sp*. bats (Table 4.2). For Cu our results were similar to values reported in the literature, whereas for Cd and Zn, we observed lower maximum concentrations than in other studies (Table 4.2). The commonly observed order of concentrations of the different trace metals in small mammals is Zn > Cu > Pb > Cd (Schleich *et al.,* 2010), as also seen in our study when metals are ranked based on median measured concentrations (Table 4.2). Generally speaking, our median concentrations were lower than those from previous studies in which renal concentrations of metals were measured on *Pipistrellus sp*. (Table 4.2). The differences between our results and previous data are probably explained by the fact that our study is a country wide survey which includes sites with very high and very low concentrations of metals, whereas other studies have focussed on much smaller areas with a history of metal contamination. For example, a study by Walker *et al*. (2007) included bats just from SW England while bats from our sample that had been obtained from this area generally had lower median concentrations than for all the bats analysed (with renal median concentrations: 0.04; 11.30; 0.59 and 15.32 µg/g for Cd, Cu, Pb and Zn respectively, n=5). In addition, our Pb maximum concentrations are associated with study sites in areas where soil is highly polluted with Pb (i.e. the Pennines, which comprises Pb bearing deposits which were extensively mined in the past).

Trace metal element toxicity

The percentages of bats in which concentrations of metals exceeded toxic thresholds were appreciable and suggest that a significant proportion of the bat population in England and Wales may be affected by metal exposure. Laboratory-based studies indicate that the higher concentrations we observed in bat tissues could cause damage to the kidneys of small mammals (Ma and Talmage, 2001). While there is limited available data regarding the effects

of metals on wild bats, studies in Australia and France have suggested that Pb exposure can cause mortality in individual bats and affect population levels (Carravieri and Scheifler, 2013). The percentage observed for Pb was higher than in the study of Walker *et al.* (2007) (Between 7 and 11% in our study against 5% in Walker *et al.* (2007)). As previously discussed, variation in contamination levels where the bats were found may explain these differences, since our study considered a representative range of soil contamination in England and Wales (including highly contaminated areas), whereas Walker *et al.* (2007) focussed on a smaller area.

Model evaluation

Results for Cu (liver), Pb (kidneys, liver) and Zn (kidneys) suggested that the model can identify areas where the health of populations of *Pipistrellus sp.* is at risk from metal exposure. While the model appeared to partly distinguish between bats with toxic residues and those without, giving similar estimations of the proportion of the population at risk, the modelling results were not perfect (e.g. not all the bats predicted by the model to be "at risk" had tissue concentrations exceeding toxic threshold). Differences between the model predictions and our residue level results can be explained by many limitations in the modelling approach, including the level of spatial and temporal resolution of the available metal soil concentration data, the effects of soil parameters on uptake into food items, the spatial and quantitative uniform availability of prey items and the bioavailability of metals from the food items into the bats. Some of these limitations are discussed in more detail in Chapter 2 and Chapter 6.

In addition, it is also important to highlight that the results of our model outputs and the tissue concentrations are not directly comparable as the model determines a risk based on the level of oral exposure while the assessment based on monitoring data considers internal exposure. The endpoints used to determine the risk were also different. The modelling framework used a derived no-observed effect for small mammals using the reproduction as endpoint (e.g. reduction of foetal implantations, foetal survivorship, offspring weights) (Chapter 2) whereas the toxic thresholds used in this chapter were associated with kidney damage (Chmielnicka *et al*., 1989; Ma, 1996). Despite this limitation, the levels of agreement we have found should encourage similar monitoring studies across a wider range of species and from others regions. Indeed, due to differences in diet composition, the potential contamination could vary widely between bat species.

In order to obtain better estimates of risk, the modelling framework would need to be further developed to account for factors such as the effect of soil parameters on metal uptake, differences in bioaccessibility of metals for different insect orders, and to include additional ecological information such as data on the selection of foraging areas, the prey availability and the landscape composition; all of which could have a significant impact on the transfer of trace metals to vertebrates (Scheifler *et al.,* 2012; Fritsch *et al.,* 2012). Despite these limitations, the modelling framework did surprising well and provided a good estimation of the range of bats predicted to be at risk, thus showing that exposure risk can be modelled by integrating soil concentrations and diet data. As such, the existing framework is, in the absence of monitoring data, a helpful tool when it comes to predicting risk of metals to wildlife, and may assist future decision making processes. Further modelling studies could address the potential impact of metal contamination at a population level including different life stages. The population's fitness and reproduction ability may be affected by metal contamination at different life stages. For example, metals can be actively transferred by lactation in bats and are known to accumulate across the age range (Streit and Nagel, 1993; Rudy, 2009). The modelling frameworks may well represent a valuable tool for ERA and could be extended to a wider range of wildlife species and pollutants. In a context of diverse environmental stressors affecting wildlife populations, the modelling framework and our analytical studies show the importance of metal contamination on bat populations.

Importance of metals in the context of declining bat populations

A substantial proportion of the bats contain residues of metals high enough to cause toxicity. The metal residues' toxic thresholds were based on a kidney damage endpoint while our risk model predictions were based on a reproductive endpoint; which is known to address crucial impacts on population dynamics and ecological impacts over long term periods (Sample *et al*., 1996). Bats are exposed to a large range of environmental stressors (e.g. climate change, white nose syndrome in North America) (Sherwin *et al.,* 2013; Blehert 2012), thus meaning that a better understanding of stressor interactions could be beneficial to bat conservation. Alongside other factors, metal exposure could, therefore, be playing an important role in the continuing decline of bat populations observed in countries with a legacy of mining and heavy industries. This study has focussed on the analysis of adult males of only one species. It was deemed likely that metal concentrations (and hence toxicity) would differ in females and juveniles and in other species. Our previously developed model is able to partly distinguish between bats with toxic levels of metals and those with levels of less concern. The model therefore provides a useful tool for extending this work to other species and other regions.

The model may also help in identifying areas within a region posing a risk, so that remediation measures can be targeted, which could in turn help mitigate the declines in populations. Metal pollution is not exclusive to the UK and concerns other parts of Europe and the US (Lado *et al.,* 2008; Shacklette and Boerngen, 1984). As such, metal contamination may affect several communities of bats worldwide.

In the following chapter we explore one of the factors which could potentially explain the mismatch between model predictions and experimental observations, namely bioaccessibility.

Chapter 5

Implications of differences in bioaccessibility for the assessment of risks of metals to bats

Introduction

Food chain models, such as that presented in Chapter 2, are often used to determine the potential risks of chemicals to wildlife species in ERA (European Food Safety Authority, EFSA Journal, 2009). In these models, oral ingestion is usually recognised as the major route of contaminant uptake into wildlife such as birds and mammals. Ingested chemicals are known to dissolve to different degrees in the gastrointestinal environment of different organisms, meaning that their availability for absorption into the blood stream, through the intestinal wall, will also vary (Ruby *et al.,* 1999). In light of this, many food chain models use, as model input, data on the assimilation efficiency of a chemical in the species of interest (EFSA Journal, 2009).

One approach when it comes to the production of data on assimilation efficiency is to perform *in vivo* studies on the bioavailability of the contaminant of interest in the species of interest. These studies typically involve the measurement of contaminants in the blood and body tissue of living animals dosed with known amounts of a chemical. These studies are expensive, time consuming and raise ethical issues (Kaufman *et al.,* 2007). An alternative is to use *in vitro* methodologies. *In vitro* models simulating human digestion have been developed and validated with *in vivo* data, especially for Pb and arsenic (Ruby, 2004). *In vitro* Physiological Based Extraction Tests (PBET) are now recommended in safety testing of toys to evaluate the bioaccessibility of metals to children (Ruby, 2004). The concept has also been used in a few environmental studies to assess the bioaccessibility of metal to wildlife species (Kaufman *et al.,* 2007; Ollson *et al.,* 2009,).

For ERA it is usually assumed that bioaccessibility is similar for different food items within similar food matrices (e.g. invertebrates, seeds) (EFSA Journal, 2009). However, contaminants are known to have different bioaccessibilities in different soil types (Ruby *et al.,* 1999), from different plant types (grass and forbs) (Ollson *et al.,* 2009), vegetables (Hu *et al.,* 2013), fish and meat (Laird *et al.,* 2009). These studies have tended to focus on a single metal. Moreover, no data are available on differences in bioaccessibility for different insect types. This may be important for ERA since a large number of wildlife species, such as bats and birds, feed on invertebrates and their diet composition can vary significantly.

In this chapter, we present the development of a two compartment (stomach and small intestine) *in vitro* model of the bat digestive system and then use this to explore how the bioaccessibility of metals (Cd, Cu, Pb and Zn) varies across different insect orders which are important in bat diets. The results are then used in the exposure modelling framework presented in Chapter 2 to explore the implications of the findings for ERA.

Materials and methods

Soil and insect collection

To avoid metal contamination, all equipment used to collect the insects in the field and to sort the insects was acid-cleaned prior to use. Soil and invertebrate samples were collected from 7 sites in England, selected using the National Soil Resources Institute (Cranfield University, UK) soil metal distribution maps, to cover a gradient of soil metal pollution**.** At each site**,** soil samples were collected from the top surface to 10 cm depth with an auger from a central point and from four additional sampling locations, each 100m further North, East, West and South of the central point. These samples were then consolidated for analysis. Whenever a targeted sampling point was inaccessible, an alternative sampling strategy was applied as described by McGrath and Loveland (1992). When the location was inaccessible, deviation from the targeted point was permitted of 100 m north, then east, then south and finally west. If the sampling point was still inaccessible, further deviation was permitted alongside 200m and then 400 m, following the same rotation (McGrath and Loveland, 1992). The sampling point was abandoned if the described strategy failed (McGrath and Loveland, 1992). Prior to analysis, the soil samples were oven dried at 105° C, ground and sieved through a 2mm mesh to homogenise the samples.

The insect sampling and collection regime was designed to obtain the main orders found in the diet of 14 bat species in the UK (see Chapter 3), namely Coleoptera (beetles), Diptera

(flies) and Lepidoptera (moths) (Table 3.1). Insects were collected over the whole bat feeding season from April until September 2012 (composed of 4 trapping sessions of 10 days evenly distributed from April to September; a supplementary inter-session was effectuated for Diptera and Coleoptera to compensate the lack of insects collected during the first session). Different trapping approaches were used for the different insect groups. Each site was set up with the following: 10 Pitfall traps (for Coleoptera) along a transect which was positioned randomly within the soil sampling area (each pitfall trap was separated by 10m); one Malaise trap, which was positioned at the central point (for Diptera); and two Heath traps (for Lepidoptera), which were placed randomly in the soil sampling area and were active overnight (2 nights per sampling sessions). Sample containers in the Malaise traps and pitfall traps contained water and one drop of detergent to break the surface water resistance. Moths were frozen as soon as possible at -20°C after collection. Collected insects were sieved and rinsed with ultrapure Milli-Q water (Merck Millipore). The insects were identified and classified into the different taxonomic groups at the order level. Each invertebrate group was also separated into two groups: one for total metal analysis and one for the *in vitro* bioaccessibility testing. Samples were frozen at -20°C until further analysis.

Prior to analysis, samples were dried at 80°C until having reached a constant dry weight in order to avoid any bias induced by different moisture content between the total and the IVGM concentrations.

Bioaccessibility fraction

The IVGM is a two-compartment phase model, reproducing both gastric (P1) and intestinal (P2) phases (Appendix 4, details on P1 and P2 solutions). In both phases, digestive juices were artificial, with their composition based on mammalian physiology. The bioaccessible fraction was determined as the metal mobilised from the samples into the digestive juices. The IVGM simulates two compartments: stomach and small intestine. These compartments were mimicked as the main digestion process occurs in the stomach and the small intestine. The stomach compartment is more acidic and, as such, an IVGM only composed of the stomach compartment might overestimate the metal bioaccessible fraction. Including a small intestine compartment more accurately represents the metal fraction available for absorption through the epithelium wall. In addition, metals such as Pb are known to mainly be absorbed in the small intestine (Oomen *et al.,* 2003b).

In order to develop a realistic IVGM for the bat, a review of the literature was performed to identify the appropriate test parameters such as the food-to-fluid ratio, the enzymes used,

the body temperature, the pH and the food transit time. As bats have a high metabolism, a food-to-fluid ratio of 40:1 was selected (Moriarty *et al.,* 2012). The selected digestive solutions included pepsin, which is the main component of insectivorous bat gastric juice (Scillitani *et al.,* 2005), and chitinase, which is also an active and important enzyme in the bat digestive tract (Whitaker *et al.,* 2004). The body temperature of active bats was maintained between 32 and 36°C (Herreid and Schmidt-Nielsen, 1966) while the pH levels of insectivorous mammal species were 1.5 and 7 in P1 and P2, respectively (Kaufman *et al.,* 2007). These values were used to set temperature and pH parameters for our IVGM. Finally, a total 5 hours of digestion (1h and 4h in P1 and P2, respectively) was selected as the incubation period, as this is the food transit time measured in the bat *Myotis myotis* (fed with American cockroaches marked with paint) before it excretes 71% of the total mass as faeces (Stalinski, 1994). *Myotis myotis* is an insectivorous bat as are all bat species distributed in the UK.

The gastric solution P1 (Appendix 4, details on P1 and P2 solutions) was prepared with pepsin and warmed at 34°C in an ultrasonic bath. The digestion was started by adding 20ml of gastric solution P1 at a pH 1.5 (Kaufman *et al.,* 2007; Ruby *et al.,* 1996) to tubes containing 0.5g (dry weight) of insects. The mixture was shaken at 275 rpm in a controlled temperature (34°C) shaker for 1 h (Moriarty *et al.,* 2012). The intestinal phase P2 was started by adjusting the pH of the samples to 7 (Moriarty *et al.,* 2012) with Na_2CO_3 solution. Bile extracts, pancreatin (Ruby *et al.,* 1996; Moriarty *et al.,* 2012) and chitinase were added to the sample solutions. The mixture was incubated and shaken at 275 rpm in a controlled temperature shaker for a further 4 h. Following this, the samples were centrifuged 10 min at 3661 g to separate the chyme and the digested matrix and were filtered using a 0.45 µm syringe filter (Moriarty *et al.,* 2012). The supernatant was analysed through the use of inductively-coupled plasma mass spectrometry (ICP MS). All material used to contain and manipulate samples or solutions were previously acid-washed with 1N HNO_3 and thoroughly rinsed with ultrapure water to avoid metal contamination (Appendix 4, details on reference materials).

Metal analyses

The soil (n=7), invertebrates (Diptera n=32, Coleoptera n=33, Lepidoptera n=23) and bioaccessible fraction (n=21) samples were analysed in triplicate. The differences in sample size were due to the variability of insect trapping success. Soil (0.1 g) was digested in 5 ml aqua-regia for 6 hours at 105°C. Once cool, the sample was diluted to 10 ml with purified water and the solution was mixed using a Vortex mixer. The solution was left to stand

overnight. An aliquot of 50µl of this solution was taken by pipette and 5 ml of internal standard was added and mixed with a vortex mixer (Appendix 4, details on reagents).

For the *in vitro* test, the supernatant (2 ml) was taken for each sample. The supernatant and the invertebrate samples analysed for total metal concentrations (not run through IVGM) were digested on a hot block at 100°C for 1 hour with 1 ml of HNO_3 (15.7 M), and another hour at 100°C with 0.2 ml of hydrogen peroxide. Digests were made up to a fixed volume of 10ml with Millipore water to obtain a final digest containing 10% (v/v) acid.

Quantification was performed by internal standardisation (chemically similar to the analyte) where the analyte signals and the internal standard signals were compared. The calibration curve was also used for corrections by normalising the response of the analyte to the response of the internal standard. This method can determine accurate concentrations and correct for drift (changes in sensitivity over time) and matrix effects (sample-related changes in sensitivity). Metal concentrations were determined with ICP-MS (Agilent 7500CE, Cheshire, UK).

Determination of BAFS, IVGM concentrations and bioaccessible fraction

The BAFs for each of the insect orders were calculated as the ratio of the metal concentrations in invertebrates (in µg/g dw) to the metal concentration in soils (in µg/g dw). Median BAFs were obtained for different insect orders. The IVGM concentration (in µg/g dw) was defined as the concentration present in the insect digested through the IVGM method and was calculated as the amount present in the chyme (or supernatant) (µg/l) multiplied by the final digest volume (l) and divided by the initial sample weight of insects (g dw). The bioaccessible fraction was calculated for each of the prey items as the amount present in the insect digest and extracted with the IVGM method. This was divided by the total metal concentrations, and multiplied by 100.

Risk comparison

The modelling framework (Chapter 2) was used to establish the implications of the observed differences in bioaccessibility for ERA. In order to achieve this, we included the bioaccessible fraction as a parameter in the model simulations. The revised modelling was done for all 4 metals and for all of the 14 British bat species studied in Chapter 3. As we only had data on bioaccessibility in Lepidoptera, Coleoptera and Diptera, in the event that another insect order

was a component of the diet of a bat species, the mean fraction for the three tested orders was assumed.

RCRs, obtained using the bioaccessibility correction were compared to the results obtained in Chapter 3 which assumed that 100% of the metal ingested was bioavailable. To compare the two corrected and non-corrected mode outputs and assess the importance of the bioaccessible fraction, the percentage of RCR variation between the models was calculated. This can indicate the relative impact of the bioaccessible fraction on the output value across species. In addition, the RCR outputs were ranked in descending order (highest RCR: 1) for both models. The variation in rank was calculated as the rank from the "initial" model minus the rank from the "corrected" model. As a consequence, elevated rank variations indicated that the inclusion of the bioaccessibility correction resulted in a large change in risk for a particular bat species. A higher positive rank difference would indicate that the risk for the bat species has increased compared to the other species while a high negative rank difference would indicate that the risk has decreased for this particular species compared to other species.

Both models used the NOAEL as a toxic threshold value to determine whether or not the risk was acceptable for bat health. This NOAEL was derived for bats from experimental studies using oral dose ingestion which are, therefore, not 100% bioavailable (Sample *et al.,* 1996). No data were found on a toxic threshold integrating the bioaccessibility factor. Consequently, the RCRs were expected be lower for the model integrating the bioaccessible fraction ("corrected model") than for the model assuming a 100% bioaccessibility of the fraction of metal ingested ("initial model").

Statistics

Total and IVGM concentrations were compared using the non parametric Wilcoxon-Mann Whitney test. IVGM concentrations across food types were compared with the non parametric Wilcoxon-Mann Whitney test. Correlations between total and IVGM concentrations were determined with the Spearman test. Statistical analyses were performed with the software R version 2.12.1.

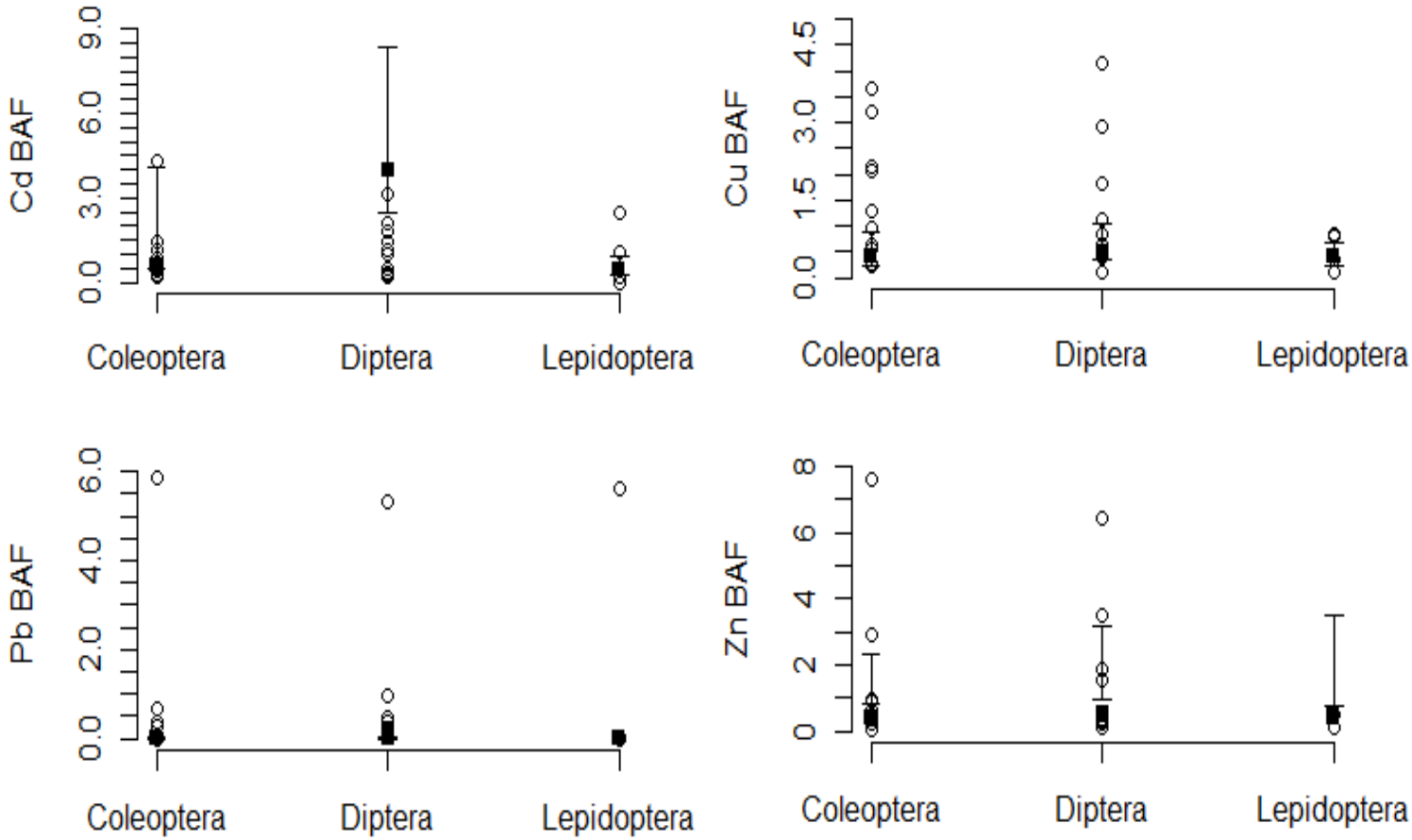
Results and discussion

Bioaccumulation factors

Metal bioaccumulation factors (BAFs) were the highest for Cd and Diptera, followed by Zn-Lepidoptera, Zn-Diptera and Zn-Coleoptera. BAFs were the lowest for Pb, and in all the insect orders studied. The variation in BAFs was observed both within and across insect orders (Figure 5.1).

These differences might be explained by the species traits of the different insect groups. Dipteran larvae are generally bottom-dwelling organisms in sediments or can live in moist terrestrial environments, and whereas some are herbivores, most feed on dead organic matter or parasitise other animals (Barnard, 2011). Coleoptera often feed on plants, fungi and detritus, and can also predate on other invertebrates. Most coleopteran species have life cycles occurring in the terrestrial ecosystem (Barnard, 2011). Moth caterpillars mostly feed on plant material, eating fruit or seeds, and a very few are carnivorous (Barnard, 2011). Species traits (e.g. size related traits such as bio-volume, surface area, length and dry mass, trophic relation, respiration mode, lipid content etc.) are known to be correlated with the uptake of insecticides by aquatic invertebrates (Rubach et al., 2012). As such, species straits may also influence the uptake of metals in terrestrial invertebrates and explain differences in uptake across species. Our BAF results were generally in agreement with the findings of previous studies (Figure 5.1). The exception was Cd in Diptera, where we obtained higher BAFs than in previous work. Most prior studies on metal uptake in Diptera have focussed on dipteran larvae as bottom-dwelling organisms in sediments and lepidopteran caterpillars feeding on leaves, whereas the current study included adult Diptera and moths, with a focus on versus soil concentrations. However, most of our measured BAFs did not differ considerably from the published studies, and the Cd concentrations measured in emergent midges were slightly lower in the study by Reinhold et *al*. (1999).

Figure 5.1: Median BAFs across study sites (▪) and BAFs from literature (○) for the different insect orders (Coleoptera, Diptera and Lepidoptera) (Literature studies are referred to in Appendix 4). Error bars represent the maximum BAF values measured in this study across the different sites.



Metal bioaccessible fraction

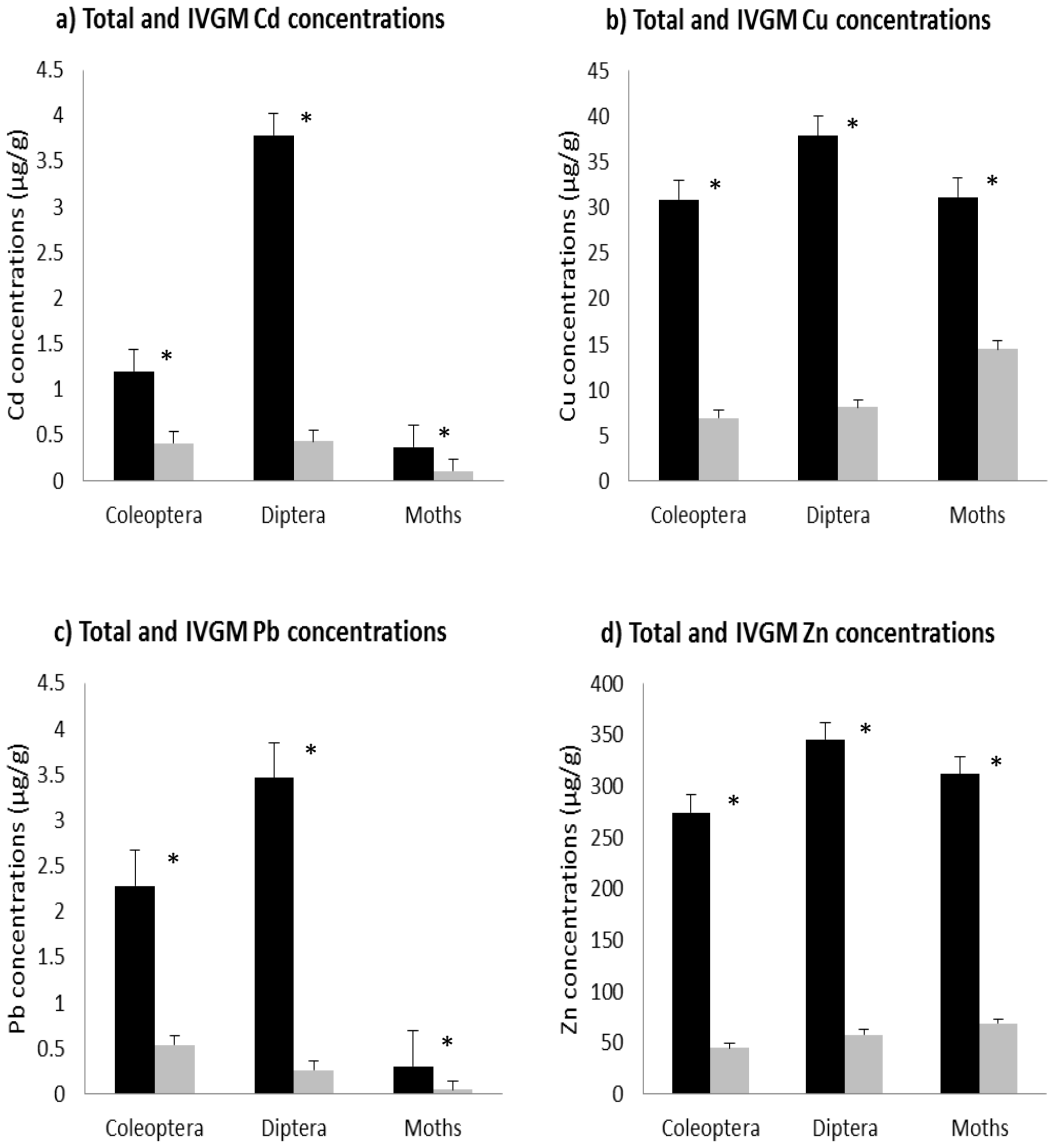
Metal bioaccessible fractions were significantly lower than the total measured amounts of metals in the insects (Figure 5.2); they ranged from 9% (Pb-Diptera) to 72% (Cd-Coleoptera) (Table 5.1) and were significantly different across insect types (except for Cu: Coleoptera-Diptera; Pb: Coleoptera-Diptera and Zn: Diptera-Lepidoptera) (Table A4.1). Bioaccessibility within an order was correlated to the measured total concentration in the insects (Spearman test, Rho = 0.94, 0.90 and 0.83 for Coleoptera, Diptera and Lepidoptera, respectively, $p < 0.0001$). Kaufman *et al.,* (2007) also observed highly significant correlations between the total concentrations in a food item and the bioaccessible concentration.

Table 5.1: Average bioaccessible fraction (in %) and standard deviation (in brackets) for the different insect orders and metals studied.

Bioaccessible fraction (%)	Cd	Cu	Pb	Zn
Coleoptera (n=7)	72 (263.2)	23 (7.0)	39 (93.6)	17 (3.8)
Diptera (n=7)	10 (0.1)	21 (0.1)	9 (0.1)	17 (0.1)
Lepidoptera (n=7)	25 (0.2)	50 (0.3)	17 (0.1)	25 (0.1)

The bioaccessible fraction was higher from Coleoptera, followed by Lepidoptera and Diptera (averaging 38%, 29% and 14%, respectively) (Table 5.1; Table A4.1). This may indicate a higher metal exposure for species feeding exclusively on coleopteran prey, compared to those feeding primarily on moths or Diptera. Differences in species traits, chemistry of the organisms, their volume and the ratio of soft to dry body parts across insect orders may influence the bioaccessibility and, therefore, explain the differences in bioaccessibility across insects. However, little is known about the drivers of bioaccessibility across different food matrices and further research should investigate the influence of species traits of invertebrates in metal uptake in terrestrial ecosystems. Interestingly, Diptera accumulated more non-essential metals than the other orders (total metal concentrations) (Figure 5.2) but did not show a higher bioaccessible fraction, thus suggesting that a large proportion of metal accumulated would not have been taken up in its present form by the next trophic level (Ruby *et al.*, 1996).

Figure 5.2: Differences in mean total (black) and IVGM concentrations (grey) in µg/g dry weight measured for a) Cd, b) Cu, c) Pb and d) Zn. * Indicates a significant difference (Wilcoxon-Mann Whitney test, P < 0.05) between the two extraction methods. The error bar represents standard errors.



A similar study investigating Pb bioaccessibility from earthworm samples obtained a higher bioaccessible fraction, averaging 77% (52-100%), (Kaufman *et al.,* 2007) than measured in our study. Lumbricidae are often referred to as high metal accumulators (Heikens *et al.,* 2001) and total metal concentrations measured in earthworms tend to be higher than in some insects orders (e.g. average of Pb concentrations in earthworms tissue: 730 µg/g (Kaufman *et al.,* 2007). In addition, the insects studied here may be regulating metal concentrations across their holometabolous life cycles by successive moulting and the shredding of the exoskeleton (Krantzberg and Stokes, 1988; Vermeulen *et al.,* 2009; Lodenius *et al.,* 2009; Nieminen *et al.,* 2001). We should also note that Kaufman *et al.* (2007) did not include the intestinal phase and as Pb mobilisation occurs in the acidic conditions of the stomach, the bioaccessibility fraction would have been higher than using our IVGM.

The differences in bioaccessible fraction within insect orders can be explained by the metal uptake in the insects studied. The uptake can be influenced by the diet, the physiochemical properties of the food content, kinetics, and life cycles (Heikens *et al.,* 2001; Vermeulen *et al.,* 2009). Indeed, previous studies have proposed the integration of detailed information regarding species traits, such as feeding behaviour, and the life stage of invertebrates in risk assessment (Heikens *et al.,* 2001; Rubach *et al.,* 2012).

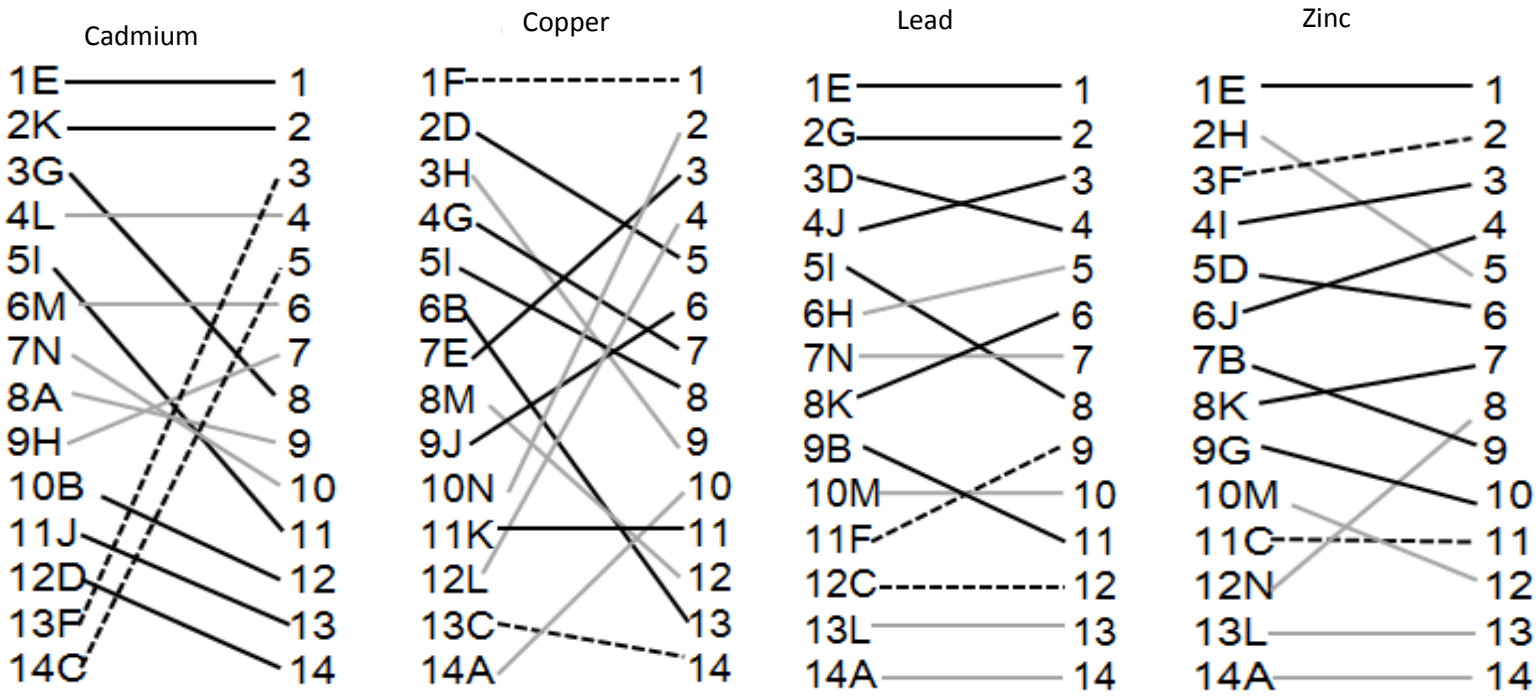
A few studies have also shown differences in bioaccessibility across food types: vegetation (with a difference of 12%, as bioaccessible fraction between grass and forbs) (Ollson *et al.,* 2009), vegetables (with differences of 57%, 45%, 26% and 25% across vegetable types for Cd, Cu, Pb and Zn, respectively for the gastric bioaccessibility) (Hu *et al.,* 2013) and fish and meat (mercury bioaccessibility ranging from 0.5 to 93.9) (Laird *et al.,* 2009). Factors explaining these differences, aside from the metal uptake, include the metal's chemical properties (e.g. distribution among cellular component and binding properties) and the nature of tissue (protein composition, moisture and fat content) (Metian *et al.,* 2009). The subcellular distribution in the tissue has been shown to influence the metal bioaccessible fraction in marine fish species (He *et al.,* 2010). As investigated for soil matrices, any factors influencing metal solubility, dissolution and transport may have an impact on the bioaccessible fraction (Ruby *et al.,* 1999; Oomen *et al.,* 2002). Further work could be undertaken on the active transport mechanisms of metals across the epithelium gut cells with biological experimental studies using the *in vitro* differentiated intestinal cells (Caco 2-cells) (Oomen *et al.,* 2003a).

Implications for risk assessment

The RCR variation between the two models indicated that the introduction of bioaccessible fraction can influence the risk across bat species. Large differences in percentage of RCR variation were observed, yielding a difference of 27, 23, 9 and 8% across bat species for Cu, Cd, Pb and Zn respectively (Table A4.2). Variations between species were also observed when looking at the change in risk ranking position. The average variation in risk rank was 4 positions in the rank for Cu, 3 for Cd, and 1 for Pb and Zn (average of absolute values) (Figure 5.3, Table A4.2). The highest changes occurred for Cd and Cu, which are the metals with the highest bioaccessibility fractions for all insects (averages of 36% and 31% for Cd and Cu respectively against 21% and 19% for Pb and Zn respectively). The highest variations were observed in *Myotis nattereri* and *Myotis bechsteinii* for Cd, with a change of 10 places, followed by *Rhinolophus hipposideros* and *Plecotus austriacus* for Cu with a change of 8 places (Figure 5.3, Table A4.2). Other bat species showed their relative risk of contamination increasing: e.g. *Barbastella barbastella* and *Myotis mystacinus* for Cu (Figure 5.3, Table A4.2).

The species for which risk ranking increased for Cd and Cu feed mostly on Coleoptera and Lepidoptera, respectively (Figure 5.3, Table 3.1). These observations are explained by the fact that the two orders had the highest bioaccessibility fractions for Cd and Cu: 72% (Cd-Coleoptera) and 50% (Cu-Lepidoptera). These results suggest that the initial model would have underestimated their risk in comparison to other species. In contrast, the inclusion of the bioaccessibility drives down the risk for some species: e.g. *Nyctalus leisleri and Pipistrellus nathusii* for Cd and *Eptesicus serotinus* for Cu (Table A4.2). All these species feed mostly on Diptera (Figure 5.3, Table 3.1), which is the order with lower metal bioaccessibility.

Figure 5.3: Change in risk rank position (indicated by the numbers) for different bat species (indicated by letters) before (left hand size) and after (right hand side) the inclusion of the bioaccessibility factor in the model for the different metals studied. Different lines correspond to the main prey item in the bat diet: Diptera (dark line), Lepidoptera (grey line), and Coleoptera (dashed line). The bat species are the following: A: *Barbastella barbastellus*, B: *Eptesicus serotinus*, C: *Myotis bechsteinii*, D: *Myotis daubentonii*, E: *Myotis mystacinus*, F: *Myotis nattereri*, G: *Nyctalus leisleri*, H: *Nyctalus noctula*, I: *Pipistrellus nathusii*, J: *Pipistrellus sp*., K: *Plecotus auritus*, L: *Plecotus austriacus*, M: *Rhinolophus ferrumequinum*, N: *Rhinolophus hipposideros*.



Recommendations for ERA

The relative ranking of bats at risk changes when bioaccessibility (of metal cations) is included as a component of the modelling framework. Due to the variation of prey items in the diet composition across species and the significant variation of metal bioaccessibility in insects, the risk predictions have drastically changed for some bat species. Using the traditional ERA approach by assuming 100% metal bioavailability in food matrices may overestimate the actual risk. In our previous study, the model output results compared to metal residues in bat tissues showed that the bats with high metal residues were significantly found in areas predicted to be “at risk” by the model . However, some bats predicted to be “at risk” by the model did not contain high enough tissue concentrations to affect their health. These results suggest that the trigger value used in the model to characterise an area as “at risk”, or not, was low. In light of this, the model may have slightly overestimated the proportion of bats “at risk” (Chapter 2). In ERA, a large diversity of invertebrates and food matrices comprises the diet of wildlife species. Other than a potential overestimation, a traditional ERA approach may be misleading in prioritising the protection and the management of wildlife species. As such, we strongly recommend consideration of bioaccessibility as a model component in ERA.

Chapter 6

General Discussion

Drastic declines in wildlife species are being observed across the globe (Groom *et al.,* 2006). These declines are explained by factors such as changes in land use and climate, but also exposure to chemicals in ecosystems (Groom *et al.,* 2006). For example, in areas of South Asia, the near extinction of populations of vultures was attributed to the use of diclofenac as a veterinary treatment (Oaks *et al.,* 2004).

While the risks of a few classes of chemicals (e.g. pesticides) to wildlife are relatively well studied and understood, for many other substances we have limited knowledge on their potential pressures on wildlife. As such, there is an urgent need to develop approaches through which to identify the potential exposure of wildlife to these chemicals and their impacts on the health of wildlife populations. One approach is to use well designed ecological monitoring studies, supplemented by controlled ecotoxicological investigations. However, these approaches can be extremely costly and time-consuming. Therefore, it would be beneficial to prioritise substances and scenarios for investigation beforehand, such as food-chain modelling (Smart *et al.,* 2006; EFSA Journal, 2009).

Over the last decades, bat populations have declined worldwide (Dietz *et al.,* 2009; Stebbings, 1988). Indeed, potential stressors on bat populations have been identified as climate change, water quality change, roost loss, urbanisation and agricultural intensification, increase in wind turbines, zoonosis and exposure to chemicals in the environment (Frick *et al.,* 2010; Jefferies, 1972; Jones *et al.,* 2009; Walker *et al.,* 2007; Wickramasinghe *et al.,* 2003). Studies on bats and environmental contaminants have tended to focus on the effects of organic compounds. Bat exposure to organic chemicals has been associated with declines in a number of bat species in selected regions. However, knowledge on the potential impact of metals on bats remains scarce, despite the fact that metals are known to elicit adverse effects on small mammals (Hickey *et al.,* 2001). A few experimental studies have shown that exposure of bats to metals can elicit a range of symptoms including tremors, spasms, general

slowness, lack of control in body movement and possibly mortality following exposure to Pb, Cd and Zn (Clark and Shore, 2001; Hariono *et al.,* 1993; Hurley and Fenton, 1980; Sutton and Wilson, 1983).

Metal pollution became widespread during the industrial revolution. In England and Wales, a large number of land sites are contaminated by metals and metalloids (Environment Agency, 2009). A number of studies have explored the uptake of soil associated metals into invertebrates and plants. Once mobilised into organisms, metals can move up through the food chain into taxa such as insectivorous mammals and birds (Ma and Talmage, 2001; Fristch *et al.,* 2012). It is therefore likely that bats will be exposed to metals *via* ingestion of contaminated invertebrate items.

In light of this, the studies described in the present thesis were conducted to provide a better understanding of metal contamination in bat populations. This chapter begins with a brief summary of the findings of the different components of the thesis and then moves on to discuss the implications of the findings for ERA and chemical risk management. Finally, major gaps in the knowledge which have been identified during the work programme are discussed and recommendations are provided regarding priorities for future work on the topic. While the work focussed on bats and metals, the modelling framework in the future could be parameterised to assess risks to other wildlife species for other classes of chemical in other regions.

Key findings of the experimental chapters

A series of studies was performed to better understand the transfer of trace metal elements from soil associated metals to bats. It is widely recognised that food ingestion is the major exposure route of contaminant uptake into wildlife. Investigations using modelling and experimental studies have therefore focussed on understanding the transfer of metals through a simplified food chain, namely soil to invertebrates to bats and the potential impacts of metal contamination on bat populations.

There is only a limited amount of available data regarding the exposure of bats to metals and their potential effects on the health of bat populations. In ERA, ecological models are often used to evaluate the potential effects of contaminant on wildlife. As such, in Chapter 2, a spatially explicit modelling framework was developed and parameterised in order to assess the risks of soil-associated metals to bats. The framework integrated information on metal concentrations in soils, uptake into prey items, bat ecology and toxicity. The framework was

applied to *Pipistrellus sp.* bat species on a national scale in England and Wales. Lead was found to pose the greatest risk, followed by Cu, Cd and Zn. The modelling framework was then extended to a larger number of bat species present in the UK in Chapter 3. While variation in risks were observed across the different bat species, Pb was still found to be the metal posing the greatest risk to all of the 14 bat species studied, followed by Cu, Cd and Zn.

The most important ecological factors driving risks across bat species were identified in Chapter 3 using sensitivity analysis. For all metals, the proportion of invertebrate orders (in particular Coleoptera and Diptera) comprising the bat diet was particularly important in determining bat exposure risks, followed by the amount of food eaten and the predicted safe daily dose. Chapter 2 and Chapter 3 brought together information on how to predict risks from soil associated metals to several bat species residing in England and Wales. Chapter 3 identified which bat species are more exposed to metals than others species, and more specifically in which areas are the bat species most exposed. Important ecological parameters driving the risks were also identified in this chapter. Other than potentially being used in ERA and targeted remediation actions, the framework can also be adapted to a wider range of receptor wildlife species and contaminants. An important further step was to evaluate the model predictions based on monitoring data.

In Chapter 4, a large monitoring dataset on metal tissue concentrations was compiled by analysing metal concentrations in the tissues of 193 *Pipistrellus sp.* bats. Measured concentrations were compared to critical toxic concentrations in order to assess the risk. Approximately 21% of the bats sampled contained residues of at least one metal high enough to elicit toxic effects. Lead was found to pose the greatest risk (7-11% of the bats with toxic residues concentrations), followed by Cu (4-9%), Zn and Cd. A comparison of risk levels obtained using the modelling framework with our measured metal concentrations in tissues showed that the bats with high Pb, Cu and Zn residues were generally found in areas predicted to be “at risk” by the model. This gives some confidence in the model predictions and suggests that such modelling frameworks could be extended to other geographical situations, pollutants and target species, providing a potentially valuable tool for ERA. The overall study suggests that metal contamination could affect bat populations in England and Wales. However, the model predictions were not perfect, thus meaning that further work was carried out on one of the many factors which could explain differences between the model predictions and the monitoring data, namely bioaccessibility.

In food chain modelling and ERA, bioaccessibility is usually assumed to be similar within the different food matrices. Differences in metal bioaccessibility across several insect orders were investigated in Chapter 5. An IVGM simulating the gastric and intestinal conditions of insectivorous bats was used to investigate the proportion of metals mobilised in the digestive juices from different invertebrate types. The bioaccessible fraction was higher for Coleoptera, followed by Lepidoptera and Diptera (with averages of 38%, 29% and 14% respectively). The bioaccessible fractions were significantly different across insect types (except for a few cases). When comparing the model predictions before and after having included the bioaccessible fraction as a model component, the risk predictions changed and the ranking of bat species, based on median RCRs, was altered. The change in risk rank position for different bat species before and after the inclusion of the bioaccessibility in the model varied in both directions: increasing or decreasing. These changes varied according to the diet of the bats and the bioaccessibility of their prey items. Bats feeding mostly on insects with a high metal bioaccessibility had their risk rank increased whereas bats feeding mostly on insects with a low metal bioaccessibility had their risk rank decreased.

In the next sections, we discuss some of the key questions and uncertainties arising from the work described in this thesis for the assessment and management of the risks of contaminants to wildlife species.

Major questions and potential solutions

Is the exposure pathway identified the most important one? – We have focussed on the exposure of bats to soil-associated metals *via* food. Other sources of exposure are possible, including water and sediment, wastewater treatment works and landfill sites. Contaminants may also be taken up dermally, by inhalation and by drinking water. By mapping out potential exposure pathways for various bat species to different chemicals in different regions and linking this to ecological and chemical fate data and transport, it should be possible to identify the key routes of exposure.

What is the spatial and temporal level of resolution of chemical contamination needed? – We have used data on metal concentrations at a 25 km^2 resolution from the 1970s-1980s. A finer resolution may be required to establish risks. Concentrations may also change in time. For example, Pb concentrations have declined since the removal of Pb as an anti-knocking agent in gasoline. It would be useful to understand drivers of the variability in concentrations of chemicals at different scales over time. To do this, monitoring surveys could be carried out on a limited number of sites, with the results then being extrapolated to the broader

landscape. Further modelling studies could include a finer scale of metal concentrations in the soil.

Which data should be used for estimating uptake from the environment into food items? – Many publications are available on the uptake of metals from soils into different invertebrate species. Uptake is affected by species traits, life stage and the environmental characteristics (e.g. soil properties). However, the available datasets are not consistent or comprehensive. None of the metals which we have studied have a full set of data on bioaccumulation in key prey species within a taxonomic order in the range of soil types likely to be encountered in the UK. By understanding relationships between species traits, life stages, soil properties, ecology and uptake, it should be possible to estimate the concentrations of a chemical in a prey item at any location. This understanding could be developed through studies on the diversity of key invertebrate species for multiple chemicals in a range of soil types present in different landscapes. Further research could investigate the effects of soil properties on uptake into invertebrates. These effects could also be considered in the framework. Invertebrates have different life stages and the uptake of metal may be different according to their life stage. In addition, emergent insects may be an important pathway for metals from aquatic to terrestrial ecosystems. The inclusion of sediments, alongside soil, as a source of metal exposure for different life stages of insects would create a more ecologically relevant framework. The availability of the food items could vary spatially and temporally. These variations could be modelled by integrating the availability of food items across different habitat types.

What do wildlife eat? – Data on the amount of food eaten by bats is available from studies on captive individuals and from energetic models. Dietary composition data has been derived from analysis of faecal material and is expressed in different ways (e.g. number, volume or mass composition). The relationship of this data to the real environment is questionable. Traditional techniques of diet composition introduce several biases such as the underestimation of soft bodied items. Improved diet composition data might be obtained using "omics" technologies or stable isotope studies. The foraging behaviour and the density of the prey items could also be integrated according to the different habitat types

How bioaccessible is a contaminant from the prey? – No bioaccessibility data were available for metals in bats, thus meaning that we initially chose the worst case scenario approach and assumed 100 % availability. *In vivo* studies could provide data but are expensive and difficult to justify ethically. Data may be available from studies on rodents, which can be extrapolated

to other wildlife. In light of this, we performed preliminary investigations to develop and apply *in vitro* systems to understand the bioaccessibility of metals from different food items. The inclusion of bioaccessibility as a model component may provide a more accurate estimation of the daily dose ingested. This also changes the order of risk ranking for different species. We recommend further work on this methodology in order to understand the relationships between the *in vitro* results and *in vivo* bioaccessibility and the application of the method to other species, food matrices and contaminant classes.

What is the degree of uptake of the bioaccessible fraction? - Our investigations focussed on the bioaccessible fraction, which is the metal fraction available for absorption into the blood stream, through the intestinal wall. However, this may not reflect actual uptake into an organism. The metal fraction which is actually taken up into the systemic circulation could be investigated using a Caco-2 cell line, which consists of culturing epithelium gut cells (Oomen *et al*., 2003a). The transport of a bioaccessible metal fraction through epithelium gut cells would provide a better estimation of the metal bioavailability (Oomen *et al*., 2003a). Other studies have also explored the metal speciation during the uptake of metals across the intestinal wall. Further investigations on metal bioavailability and speciation are therefore encouraged.

How do you assess the toxicity of a contaminant to your test organism? – No data was available on the toxicity of metals to bats, and thus we assumed that the bats had the same sensitivity as a rodent where toxicological data were available (Chapter 2). While data could be generated from *in vivo* studies, a more ethical approach in the case of heavily protected species, such as bats, would be to improve the extrapolations from rat and mouse data using toxicokinetic and toxicodynamic modelling methods.

How do we evaluate the modelling framework? – It is critical that models are evaluated against empirical data. We used experimental monitoring data to attempt to evaluate the modelling framework. However, a range of complementary approaches could be used to evaluate the exposure component of the modelling framework, including: non-invasive sampling (e.g. of feathers, fur, nails, blood samples etc.) of free-living animals. The measuring biomarkers and pathology in wild animals could enable an evaluation of the toxicological predictions from the modelling framework. The data from our monitoring study have already been used to evaluate whether fur could be used as a non-invasive tool for characterising the metal exposure of bats. The results are very promising and are currently being written up as a scientific publication.

How to interpret our risk results? – Our risk predictions are based on risk characterisation calculations (Chapter 2). The main uncertainties related to the input parameters determining the amount of contaminant consumed are detailed in Chapter 2. The safe daily dose was predicted using an allometric equation to derive NOAEL from test species to wildlife species. The endpoint was based on reproductive effects observed beyond the NOAEL doses (e.g. reduction of foetal implantations, foetal survivorship, offspring weights, foetal growth rates and augmentation of foetal resorptions and mortality of the offspring) (Table A1.2). We also used an uncertainty factor to account for uncertainties in the toxicological data (e.g. inter-laboratory difference, inter and intra species differences, differences in sensitivity of different life stages). The addition of the uncertainty factor has therefore reduced our NOAEL value. As such, the term of risk in this study is generalised to RCR values higher than 1 (threshold value). Whilst our risk calculation is in accordance with the guideline of the regulatory assessment of the long-term risks of pesticides to birds and mammals species (EFSA Journal, 2009) and provides valuable information on risk contamination to bats, it may also reflect uncertainties. In addition, caution should be taken when reading the risk predictions. Our risk predictions are indicated in terms of percentage of area at risk (number of grid cells with an RCR >1 divided by the total number of grid cells within the bat distribution) which should not be confused with the percentage of the individual bats at risk within their population.

The risk results in Chapter 4 were determined by comparing tissue metal concentrations against critical thresholds. For Cd and Pb, the thresholds values were based on data using an endpoint associated with structural and functional kidney damage of small mammals (Chmielnicka et al., 1989; Ma, 1996). Although there may be interspecies differences in sensitivity to chemicals, we assumed that bats exceeding these threshold values would present the same symptoms. As there is no critical toxic threshold for essential metals (Cu and Zn), we used the upper level of metals found in literature data for small mammals to compare our tissue concentrations (Ma and Talmage, 2001; Schleich et al., 2010). After taking this into consideration, we assumed that bats containing higher tissue concentrations than the upper levels might present severe symptoms of metal contamination.

What are the ecological effects of exposure to metals? – This cannot be answered with the approach used in this study, which focussed on metal exposure, but can be explored using an individual-based population model developed according to the guidelines for Good Modelling Practice which are currently developed in the CREAM project (http://cream-itn.eu; Grimm *et al.,*2009; Schmolke *et al.,* 2010). In order to understand the implications of metal

contamination on a population scale of wildlife species, more monitoring data are needed to understand the dynamics of the populations for the different species. By integrating these data in population models which include population growth, mortality, age structure and density dependence, we could extrapolate knowledge from an individual level to a population level by estimating the impact of contaminant on the survival of populations (Wang and Grimm, 2010). The implications of population models would be important in ERA, wildlife conservation and management programme.

Population modelling is part of higher-tier assessment studies which can help to further investigate the risk involved in chemical contamination. Other higher-tier assessments such as refining the food chain model, investigating other exposure routes (e.g. drinking water, inhalation) and the avoidance of wildlife to chemicals, and collecting data from field studies can verify whether the effects are within acceptable ranges (EFSA Journal, 2009).

How do metals interact with other stressors? – The context here is one of global change, involving the actions of several stressors on wildlife populations such as habitat fragmentation, habitat loss, climate change, urbanisation, zoonosis and the emergence of environmental contaminants. In light of this, a better understanding of the interactions of multiple stressors on wildlife species is needed. For example, links between high metal contamination and the high prevalence of parasitism have been found for the lesser horseshoe bat (*Rhinolophus hipposideros*) and the protozoa Eimeria, for voles and the cestode (*Paranoplocephala dentata*) and red fox (*Vulpes vulpes*) infested with cestodes (*Mesocestoides* spp.) and nematodes (*Toxascaris leonina*) (Afonso *et al*., 2012; Jankovska *et al*., 2009, Jankovska *et al*., 2010). Further research is needed regarding the relationships of metal exposure and the prevalence of pathogens.

Are our findings applicable to other regions? – This study highlighted that metal contamination may be playing a role in bat population declines. Further monitoring studies in other countries and for other bat species could verify whether metal contamination has been involved in these declines worldwide.

Conclusions

The use of modelling frameworks, of the type developed and explored in this thesis, is valuable for identifying chemicals and scenarios which might pose a risk to wildlife health. The comparison of experimental monitoring data with model predictions indicated that the framework which was used is able to partly distinguish between bats which are at risk and those which are not at risk. Model predictions and experimental data also indicated that exposure levels for bats to metals in the environment are high enough to cause toxicological effects. Metals could, therefore, be considered as one of the potential stressors contributing to the continuing declines in bat populations which are being observed in different regions of the world.

However, the model predictions are not yet perfect due to the fact that many assumptions were made in the development and application of the modelling framework and due to a lack of knowledge in some areas. While our work on bioaccessibility, presented in Chapter 5, begins to address one of the knowledge gaps, there are many more questions which need answering, as discussed above. By performing targeted research to address some of the questions listed above, it should be possible in the future to further develop the modelling framework and to better assess the threats to wildlife. This work will need to be highly co-ordinated and involve environmental chemists, toxicologists, soil scientists, ecologists and modellers.

Appendix 1

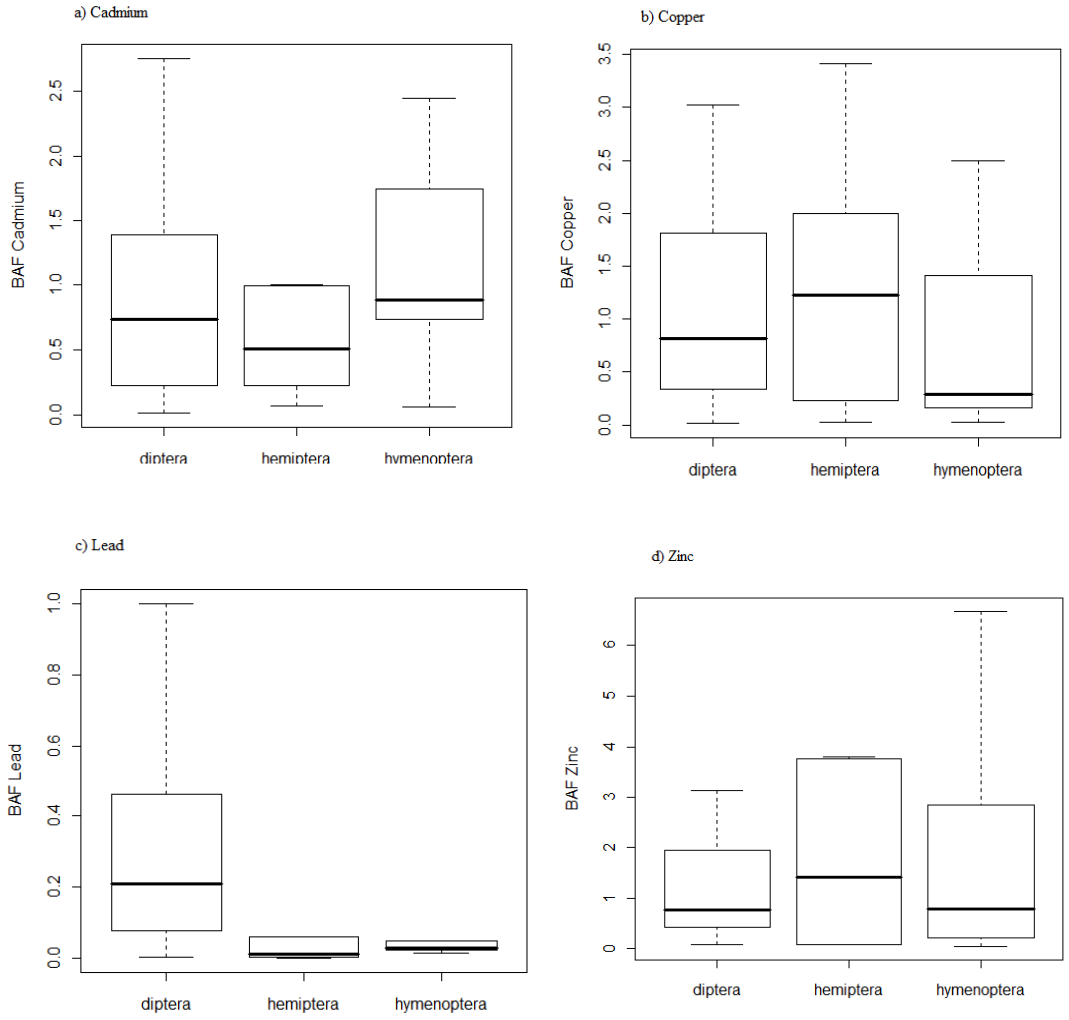
Table A1.1: Daily consumed amount (g/gbw/day wet weight) of insects by bats found in the literature.

Species	State	DCA (g/gbw/day) (wet weight)	Note	Reference
Myotis lucifugus	Pregnant female	0.317	Field study	Anthony and Kunz, 1977
	Lactating female	0.484		
	Juvenile	0.286		
Myotis velifer	Adult female (mean)	0.152	Field study	Kunz, 1974
	Adult male (mean)	0.117		
Myotis lucifugus	Male and female	0.150	Captive bats	Coutts *et al.,* 1973
Eptesicus fuscus	Male and female	0.240		
Myotis daubentonii	Female (pregnancy period)	0.860	High success model	Encarnaçao and Dietz, 2006
	Male	0.439		
	Female (Post lactating period)	0.516		
	Male (Spermatogenesis period)	0.964		
	Female (Pregnancy period)	0.462	Low success model	
	Male	0.231		
	Female (Post lactating period)	0.284		
	Male (Spermatogenesis period)	0.530		
		0.402	Mean value based from literature	This study

Table A1.2: Details on experimental studies used to derive the NOAEL in Sample *et al.* (1996) study.

	Cd	**Cu**	**Pb**	**Zn**
Test species	Rat	Mink	Rat	Rat
Study duration	6 weeks including critical lifestage: chronic	357 days: chronic	Over a year including critical lifestage: chronic	Days 1-16 of gestation: chronic
Endpoint	Reproduction	Reproduction	Reproduction	Reproduction
Exposure route	Oral gavage	Oral in diet	Oral in diet	Oral in diet
Rationale	Beyond the NOAEL dose of 1mg/kg/day, the reduction of foetal implantations, foetal survivorship and the augmentation of foetal resorptions were observed.	Beyond the NOAEL dose of 11.7 mg/kg/day, supplemental Cu increased the percentage mortality of mink kits.	Beyond the NOAEL dose of 8 mg/kg/day, the reduction of offspring weights and kidney damage were observed in the young.	Beyond the NOAEL dose of 160 mg/kg/day, the augmentation of foetal resorptions and reduction of foetal growth rates were observed.
Reference	Sutou *et al*., 1980b; cited by Sample *et al*., 1996.	Aulerich *et al*., 1982; cited by Sample *et al*., 1996.	Azar *et al*., 1973; cited by Sample *et al*., 1996.	Schlicker and Cox 1968; cited by Sample *et al*., 1996.

Figure A1.1: BAF values for each insect prey order. Boxplots of BAF data between different metals a) Cd, b) Cu, c) Pb and d) Zn and different order of insects Diptera, Hemiptera and Hymenoptera.



References:

Anderson *et al.,* 1978; Bendell-Young, 1999; Beyer *et al.,* 1985; Bidwell and Gorrie, 2006; Croisetière *et al.,* 2006; Davison *et al.,* 1999; Del Toro *et al.,* 2010; Desrosiers *et al.,* 2008; Dixit and Witcomb, 1983; Gillis *et al.,* 2006; Hare *et al.,* 2001; Hare and Campbell, 1992; Harrahy and Clements, 1997; Hunter *et al.,* 1987a; Hunter *et al.,*; 1987b; Péry *et al.,* 2007; Rabitsch, 1995; Reinhold *et al.,* 1999; Robinson *et al.,* 2007; Roth, 1993.

Figure A1.2: Foraging distance application illustrated. Figure illustrating the foraging distance application used in the modelling framework. Squares represent metal soil concentrations with initial resolution (5X5 km) (a). The resolution is changed in a finer resolution (0.5 km X 0.5 km) (b). A mean value is calculated for subcells comprising a circle (1.7km diameter) (c). The mean value will be attributed to the square containing the circle centre (c). Final result after foraging distance application: a new value is calculated according to surrounding area values (d).

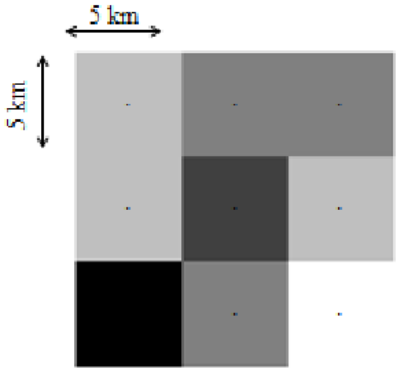


Figure 2a) Squares are representing metal soil concentrations with initial resolution. Points are representing square's centres.

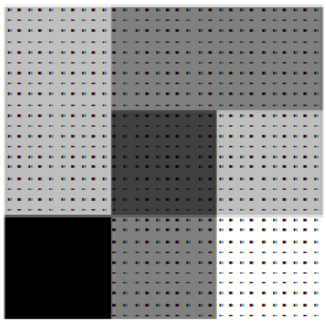


Figure 2b) Points are representing centres of squares with a finer resolution (0.5km X 0.5 km).

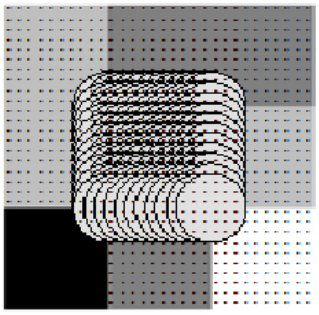


Figure 2c) Circles (1.7km diameter) are showing squares taken into account to calculate the mean. The mean value will be calculated between all the squares for those whose centre is located in the circle. The central area is given as an example.

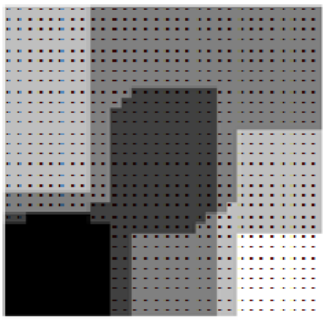


Figure 2d) Final result after foraging distance application: a new value is calculated according to surrounding area values.

Figure A1.3: Soil metal concentration maps. Soil metal concentration maps for the four metals studied in µg/g dry weight: a) Cadmium, b) Copper, c) Lead and d) Zinc. Cadmium soil concentrations are ranged from 0.05 to 1.5 (grey cells) and 1.5 to 41 (black cells). Copper soil concentrations are ranged from 0.04 to 70 (grey cells), and 70 to 1507.7 (black cells). Lead soil concentrations are ranged from 0.63 to 250 (grey cells), and 250 to 17365 (black cells). Zinc soil concentrations are ranged from 0.02 to 200 (grey cells), and 200 to 3648 (black cells). The white cells represent areas for which soil concentrations data were not available.

Appendix 2

Figure A2.1: Cumulative effect for the different parameters given by the sensitivity analyses results. The model parameters studied are: the proportion of invertebrates in the diet for each invertebrate type (Opiliones, Araneidae, Hymenoptera, Lepidoptera, Trichoptera, Diptera, Coleoptera, Hemiptera, Dermaptera, Orthoptera), the safe daily dose (µg/g body weight/day), the amount of food eaten (g dry weight/g body weight/day) and the concentration of metal in the soil (µg/g dry weight) (99% of the soil data covered).

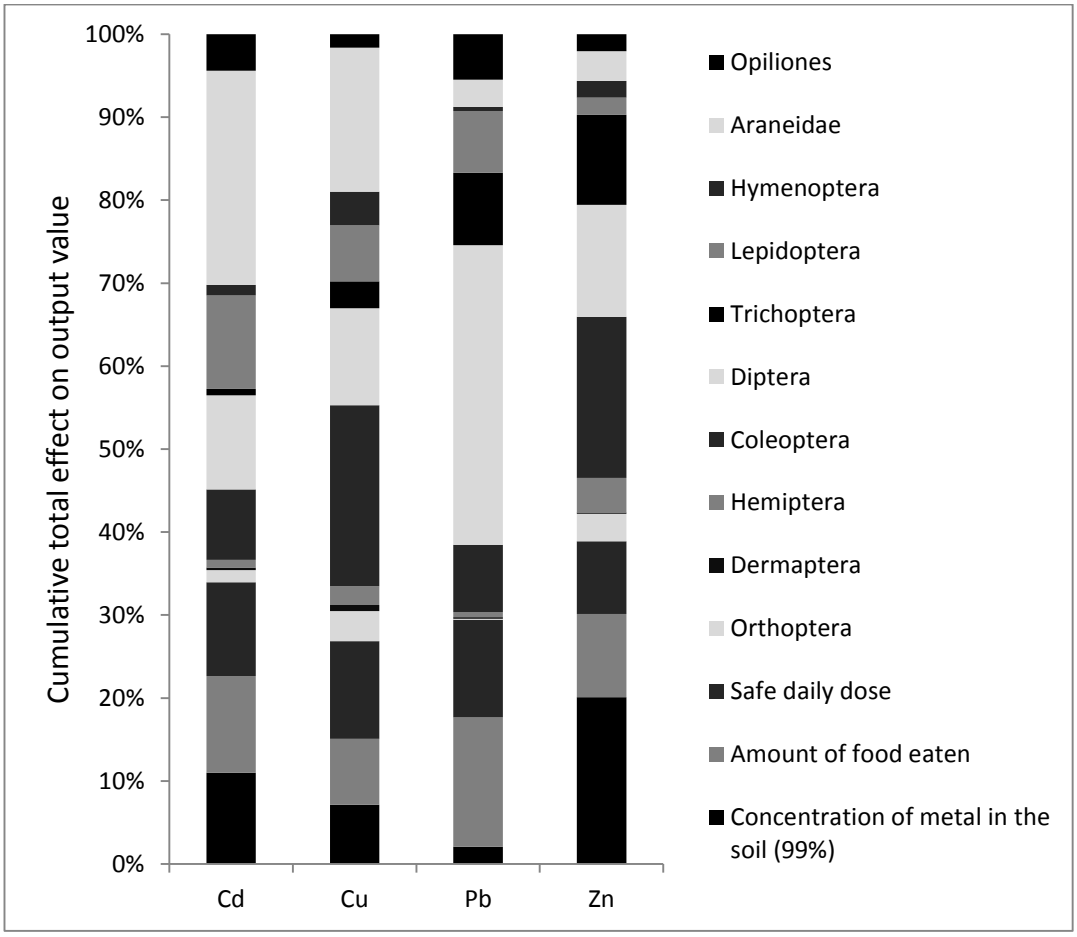


Table A2.2: Statistics comparison between the soil concentrations (µg/g dw) within and outside the bat distribution. The Wilcoxon test (W) and the median values of soil concentrations (µg/g dw) within the bat distribution (M1) and outside the bat distribution (M2) are presented.

Bat species	Statistics	Cd	Cu	Pb	Zn
A *Barbastella barbastellus*	W	1616814*	1546672*	1345317*	1603720*
	M1, M2	0.40, 0.60	16.15, 17.50	32.52, 40.00	71.17, 74.70
B *Eptesicus serotinus*	W	3030011	2614959*	2325515*	2858988*
	M1, M2	0.50, 0.50	16.00, 17.75	34.00, 41.00	73.00, 75.00
C *Myotis bechsteinii*	W	710498.5	535279.5*	576185.5*	710499.5
	M1, M2	0.60, 0.50	14.40, 17.50	34.00, 39.00	79.00, 74.00
D *Myotis daubentonii*	W	3973855	4086353*	4163732*	4086948*
	M1, M2	0.50, 0.50	17.70, 16.85	40.00, 38.00	75.68, 73.00
E *Myotis mystacinus*	W	3800058*	3780119*	4095058*	3885667*
	M1, M2	0.60, 0.50	17.90, 17.00	43.00, 37.00	79.00 , 72.00
F *Myotis nattereri*	W	4048335	3912995	4031918	4035446
	M1, M2	0.60, 0.50	17.40, 17.30	39.00, 39.00	75.00, 74.00
G *Nyctalus leisleri*	W	1291279*	1507456*	1366473*	1344767*
	M1, M2	0.60, 0.50	20.70, 17.00	44.00, 39.00	79.00, 74.00
H *Nyctalus noctula*	W	3935032	4329690*	4175796*	4207633*
	M1, M2	0.50, 0.60	18.20, 16.69	40.00, 38.00	76.00, 72.68
I *Pipistrellus nathusii*	W	273877.5	280630	210365*	275459
	M1, M2	0.60, 0.50	18.20, 17.30	31.00, 39.00	81.00, 74.00
J *Pipistrellus pipistrellus*	W	2998828	3127886*	2802473*	3317946*
	M1, M2	0.50, 0.50	17.60, 16.50	38.30, 41.00	76.00 , 69.00
K *Plecotus auritus*	W	2099179	2193413*	1826860*	2295664*
	M1, M2	0.50, 0.50	17.50, 16.15	38.00, 46.00	75.70, 68.00
L *Plecotus austriacus*	W	306041	210799.5*	200495.5*	283107.5
	M1, M2	0.60, 0.50	13.10, 17.40	29.00, 39.00	71.00, 74.00
M *Rhinolophus ferrumequinum*	W	1657160*	1692736*	1621986	1712497*
	M1, M2	0.60, 0.50	19.40, 17.10	41.01, 39.00	80.70, 73.67
N *Rhinolophus hipposideros*	W	3040685*	3150993*	3103538*	3208757*
	M1, M2	0.60, 0.50	18.69, 17.00	42.00, 38.00	81.00, 72.30

Appendix 3

Formaldehyde aliquot analyses

Median trace metal concentrations determined in the preservative solution (40% formaldehyde) were: 0.02; 21.44; 0.68 and 10.73 µg/g dw for Cd, Cu, Pb and Zn respectively (n= 100 aliquots of formaldehyde from 100 different bat individuals). The aliquot was previously oven dried to obtain formaldehyde concentrations on a dry weight basis to compare them with our tissue concentrations. These results were lower than the mean of the medium tissue concentrations for Pb, Zn and Cd (25, 8 and 5 times respectively) and were higher for Cu (2 times higher). This suggests that the metal extraction for Pb, Zn and Cd is negligible, whereas a possible Cu extraction from the formaldehyde solution may have occurred. It may therefore be the case that certain concentrations were underestimated, although a correction based on a quantitative value cannot be defined.

Quality assurance and quality control

Average spike recoveries were 101, 98, 99 and 99% for Cd, Cu, Pb and Zn respectively. The median blank results were below detection limits (Mean of minimum LOD being: 0.009, 0.043, 0.015 and 0.603 for Cd, Cu, Pb and Zn, respectively). The reference material results were within the acceptable range for Pb for NCS ZC73013 and Zn for BCR 185R. The average percentage of variation from the certified concentrations were -7, -10, -15 and -0.2 for Cd, Cu, Pb and Zn, respectively for BCR 185R; and 22, -6, 2 and 24 for Cd, Cu, Pb and Zn respectively for NCS ZC73013.

Figure A3.1*:* Map showing the locations where the 193 bats analysed were collected.

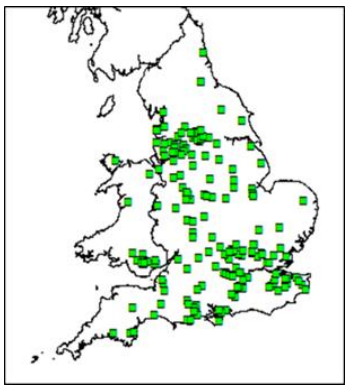


Figure A3.2: Median metal concentrations in bones (n=192), fur (n=192) and stomach (n=168) (in µg/g dry weight) for Pb, Cu, Zn and Cd. The y axis has been transformed with a square root transformation. The upper and the lower whiskers extend from the hinge to the highest and the lowest values which are within 1.5 times the inter-quartile range.

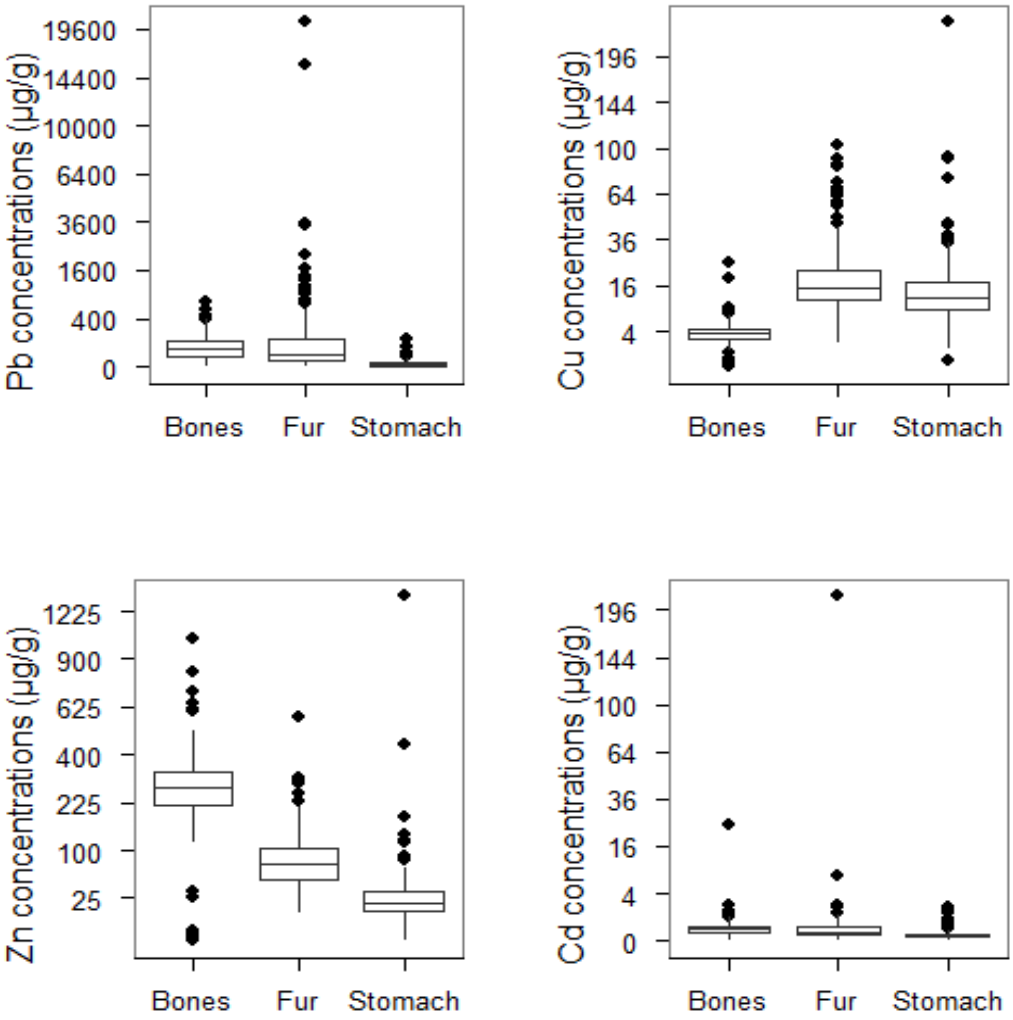
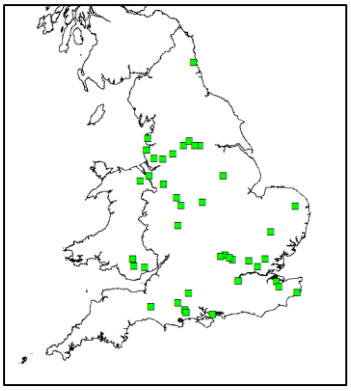


Figure A3.3: Map showing the location of the bats presenting toxic residues (n=41).



Appendix 4

Quality assurance / Quality control

Soil samples

Spike recovery: Average spike recoveries measured were 95%, 92 %, 89% and 89% for Cd, Cu, Pb and Zn respectively.

Batches of soil samples were analysed with three standard reference materials: BCR 143 over fertilised soil, BCR 141R calcareous loam soil and IAEA-SL-1 lake sediment. The certified values were (in µg/g) 31.1, 236.5, 1333, 1272 for Cd, Cu, Pb and Zn respectively for BCR 143; 14±0.4; 46.4±1.8; 57.2±1.2; 283±5 for Cd, Cu, Pb and Zn respectively for BCR 141R and 0.26±0.05; 30±6; 37.7±7.4; 223±10 for Cd, Cu, Pb and Zn respectively for IAEA-SL-1. Results were within acceptable range for IAEA-SL-1 and Cd, Cu and Pb. The average percentages of variation from the certified concentrations were -10%, -17%, -9%, -16% for Cd, Cu, Pb and Zn respectively for BCR 143 and -9%, -7%, -10% and -8% for Cd, Cu, Pb and Zn respectively for BCR 141R and 8%, -1%, -1% and 7% for Cd, Cu, Pb and Zn respectively for IAEA-SL-1.

For each batch, four blanks were run. Median blank values for Cd, Cu, Pb and Zn were below the minimum detection limits for each batch (< 158, 412.6, 96.3, 1588.1 ng/g for Cd, Cu, Pb and Zn respectively).

Field triplicates were analysed. Average standard deviations were 0.2, 8.5, 17.2, and 21.5 for Cd, Cu, Pb and Zn respectively.

Invertebrate samples

Spike recovery: Average spike recoveries measured were 101%, 101%, 95% and 94% for Cd, Cu, Pb and Zn respectively.

Batches of invertebrate samples were analysed with two standard reference materials: bovine liver BCR 185R and spinach NCS ZC73013. The acceptable ranges of the certified values were (in µg/g) 0.544±0.017; 277±5; 0.172±0.009; 138.6±2.1 for Cd, Cu, Pb and Zn respectively for BCR 185R and 150±25; 8.9 ± 0.4; 11.1± 0.9; 35.3±1.5 for Cd, Cu, Pb and Zn respectively for NCS ZC73013. Results were within acceptable range for Cu and Pb for NCS ZC73013. Average percentages of variation from the certified concentrations were -8%, -4%,

16% and 8% for Cd, Cu, Pb and Zn respectively for BCR 185R and 22%, 1%, 4% and 43% for Cd, Cu, Pb and Zn respectively for NCS ZC73013.

Due to the variation of sample size, the LOD was calculated for each sample. For each batch, four blanks were run. Median blank values for Cd, Cu, Pb and Zn were below average minimum detection limits for each batch (< 2.6, 42.4, 5.0, 219.0 ng/g for Cd, Cu, Pb and Zn respectively).

Field triplicates were analysed. The Cd mean standard deviations were 1.3, 0.6 and 0.4 for Diptera, Coleoptera and moths respectively. The Cu mean standard deviations were 4.9, 7.8 and 10.4 for Diptera, Coleoptera and moths respectively. The Pb mean standard deviations were 1.3, 2.2 and 0.3 for Diptera, Coleoptera and moths respectively. The Zn mean standard deviations were 38.1, 52.1 and 131.0 for Diptera, Coleoptera and moths respectively.

Bioaccessibility extraction

For each batch, analytical triplicates, 1 spike, 4 blanks and 2 Standard Reference materials were included.

Average spike recoveries measured were 105, 108, 94 and 114% for Cd, Cu, Pb and Zn respectively.

Batches of invertebrate samples were analysed with two certified reference materials: Bovine liver BCR 185R and Spinach NCS ZC73013. Results were within acceptable range for Zn for NCS ZC73013 and Cd and Zn for BCR 185R. Average percentage of variation from the certified concentrations were -2.6, -11.4, -14.8 and 0.4% for Cd, Cu, Pb and Zn respectively for BCR 185R and 26.9, -16.1, -8.3 and -2.4 % for Cd, Cu, Pb and Zn respectively for NCS ZC73013. Median blanks were below detection limits (Mean LOD being: 0.11, 8.06, 1.22 and 53.45 for Cd, Cu, Pb and Zn respectively).

Cd mean standard deviations of triplicates were 0.6, 0.6 and 0.7 for Coleoptera, Diptera and moths respectively. Cu mean standard deviations of triplicates were 1.3, 1.3 and 1.1 for Coleoptera, Diptera and moths respectively. Pb mean standard deviations of triplicates were 0.7, 0.7 and 0.7 for Coleoptera, Diptera and moths respectively. Zn mean standard deviations of triplicates were 6.2, 6.2 and 4.2 for Coleoptera, Diptera and moths respectively.

For the batches of bioaccessibility extraction, positive and negative controls were performed as a blank test to evaluate the method.

6 Positive controls Amount of Certified Reference material BCR 185R (500 mg) digested with the same method as the invertebrate samples (with enzymes). Mean concentrations were 1.7, 178.1, 1.5 and 119.5 µg/g for Cd, Cu, Pb and Zn respectively.

5 Negative controls Amount of Certified Reference material BCR 185R (500 mg) digested with the same method to that of invertebrate samples but without enzymes. Mean concentrations were 0.2, 46.21, 0.07 and 51.69 µg/g for Cd, Cu, Pb and Zn respectively. As mean concentrations determined from positive controls were 9, 4, 22 and 2 (for Cd, Cu, Pb and Zn respectively) fold higher than mean concentrations determined from negative controls, the enzyme activity during the bioaccessibility procedure is shown.

4 Digestion of blanks following the IVGM procedure Blanks digested with the same method to that of invertebrate samples (with enzymes). Mean concentrations were 0.004, 0.47, 0.06 and 11.16 for Cd, Cu, Pb and Zn respectively. These concentrations were approximately 10, 9, 10 and 2 times lower (for Cd, Cu, Pb and Zn respectively) than the "normal" blank test performed.

P1 and P2 solutions recipes

The gastric phase (P1) comprised: 950 ml/l deionised water, 1.25 g pepsin (from porcine stomach mucus: Sigma-Aldrich activity of 800-2500 units/mg), 0.50 g citrate (Fisher Chemical Co), 0.50 g malate (Aldrich Chemical Co), 420 µl lactic acid (Synthetic syrup), 500 µl acetic acid (Fisher Chemical co). Na_2CO_3 was added for each tube in order to adjust the pH to 7 for the second phase extraction. A chitinase solution was made up in 1ml of deionised water for the second phase extraction with chitinase from *Streptomyces griseus* (5 UN, Sigma Aldrich) and chitinase from *Trichoderma viride* (25 g, Sigma Aldrich). Concentrated HCl was used to adjust the pH to 1.5. 35 mg of bile extract (B8631 porcine, Sigma-Aldrich), 10 mg pancreatin (P1500 porcine, Sigma Aldrich) and 10 µl of chitinase solution were added per tube for the second phase extraction (P2).

Reagents used for metal digestion

Aqua regia (4 HCl: 1 HNO_3) (HCl Aristar sp gr 1:18) and HNO_3 (69% Aristar for trace analyses VWR Prolabo) were used for the digestion of soil samples and HNO_3 and H_20_2 (30% Analar Normapur BDH Embarasafe) were used for the digestion of invertebrate samples and the *in vitro* test.

References corresponding to Figure 5.1

Anderson *et al.,* 1978; Bendell-Young 1999; Beyer *et al.,* 1985; Bidwell and Gorrie, 2006; Croisetière *et al.,* 2006; Davison *et al.,* 1999; Desrosiers *et al.,* 2008; Dixit and Witcomb, 1983; Gillis *et al.,* 2006; Gongalsky, 2006; Hare and Campbell, 1992; Hare *et al.,* 2001; Harrahy and Clements, 1997; Hunter 1987; Hunter *et al.,* 1987b; Janssen and Hogervorst, 1993; Jelaska *et al.,* 2007; Milton *et al.,* 2002; Péry *et al.,* 2007; Prince *et al.,* 2001; Purchart and Kula, 2007; Rabitsch 1995; Reinhold *et al.,* 1999; Roth 1993; Schipper *et al.,* 2008; Stone *et al.,* 2002; van Straalen and van Wensem, 1986; van Straalen *et al.,* 2001; Vandecasteele *et al.,* 2003; Vermeulen *et al.,* 2009; Vijver *et al.,* 2003; Zhang *et al.,* 2009; Zhuang *et al.,* 2009.

Table A4.1: Wilcoxon test results (W) for IVGM concentrations between insect types (Significance level defined <0.05).

Bioaccessibility	**Coleoptera (n=7)**				**Diptera (n=7)**			
	Cd	Cu	Pb	Zn	Cd	Cu	Pb	Zn
Lepidoptera (n=7)	W=275*	W=18*	W=369*	W=97*	W=349*	W=36*	W=380*	W=156
Diptera	W=138*	W=152	W=237	W=115*				

Table A4.2: Percentage of variation in mean risk characterisation ratios between the initial model and the corrected model. These percentages of variation were determined for the 14 bat species studied. The variation in rank order of risk characterisation ratios were determined. The risk characterisation ratios were previously ranked in a decreasing order. The bat species are the following: A: *Barbastella barbastellus*, B: *Eptesicus serotinus*, C: *Myotis bechsteinii*, D: *Myotis daubentonii*, E: *Myotis mystacinus*, F: *Myotis nattereri*, G: *Nyctalus leisleri*, H: *Nyctalus noctula*, I: *Pipistrellus nathusii*, J: *Pipistrellus sp*., K: *Plecotus auritus,* L: *Plecotus austriacus*, M: *Rhinolophus ferrumequinum*, N: *Rhinolophus hipposideros*.

Bat sp.	Cd		Cu		Pb		Zn	
	Percentage of variation in RCR	Variation in rank	Percentage of variation in RCR	Variation in rank	Percentage of variation in RCR	Variation in rank	Percentage of variation in RCR	Variation in rank
A	-76	-1	-55	4	-85	0	-79	0
B	-79	-2	-82	-7	-91	-2	-87	-2
C	-63	9	-72	-1	-82	0	-83	0
D	-84	-2	-76	-3	-90	-1	-83	-1
E	-68	0	-71	4	-85	0	-80	0
F	-61	10	-74	0	-84	2	-83	1
G	-80	-5	-77	-3	-90	0	-85	-1
H	-74	2	-79	-6	-86	1	-86	-3
I	-80	-6	-77	-3	-89	-3	-83	1
J	-80	-2	-74	3	-88	1	-80	2
K	-70	0	-70	0	-85	2	-82	1
L	-74	0	-58	8	-83	0	-80	0
M	-74	0	-76	-4	-87	0	-86	-2
N	-78	-3	-66	8	-87	0	-80	3

Table A4.3: Ranges and standard deviation of total and IVGM concentrations (μg/g dry weight) for the different metal and the insects studied.

		Coleoptera		**Diptera**		**Lepidoptera**	
		IVGM	Total	IVGM	Total	IVGM	Total
Cd	Range	0.0-1.8	0.4-3.6	0.1-1.2	2.1-7.4	0.0-0.4	0.2-0.6
	SD	1.1	1.1	0.4	1.8	0.1	0.1
Cu	Range	4.5-10.2	21.1-46.2	6.1-12.4	31.6-49.1	10.7-24.2	17.2-40.9
	SD	2.2	9.4	2.4	5.7	7.3	7.6
Pb	Range	0.0-1.9	0.5-8.4	0.1-0.6	1.4-6.0	0.0-0.1	0.2-0.5
	SD	1.1	2.6	0.1	1.6	0.0	0.1
Zn	Range	27.3-51.6	223.3-342.3	40.4-81.2	293.3-391.8	44.1-114.7	116.6-367.3
	SD	10.4	35.8	15.6	30.5	35.9	89.2

Glossary

BAF: Biota accumulation factor

EFSA: European Food Safety Authority

ERA: Environmental risk assessment

GEM-SA: Gaussian emulator machine for sensitivity analysis

GIS: Geographical information system

ICP-MS: Inductively coupled plasma mass spectrometry

IVGM: *In vitro* gastric model

LD50: Median lethal concentration

LOD: Limit of detection

NOAEL: No observed adverse effect level

NSRI: National Soil Resources Institute

P1: Gastric phase of the IVGM

P2: Intestinal phase of the IVGM

PBET: Physiologically based extraction test

PCB: Polychlorinated biphenyl

RCR: Risk characterisation ratio

ROC: Receiver operating characteristic

List of references

Afonso, E., Tournant, P., Scheifler, R., Roué, S., Raoul, F., and Giraudoux, P., 2012. Spatial variability of biological and chemical contaminants in nurseries of lesser horseshoe bat: preliminary results in Franche-Comté (Eastern France). *Proceedings of 60th Annual International Conference of the Wildlife Disease Association*, Québec, Canada.

Allinson, G., Mispagel, C., Kajiwara, N., Anan, Y., Hashimoto, J., Laurenson, L., Allinson, M., and Tanabe, S., 2006. Organochlorine and trace metal residues in adult southern bent-wing bat (*Miniopterus schreibersii bassanii*) in Southern Australia. *Chemosphere*, 64: 1464-1471.

Anderson, R.V., Vinikour, W.S., and Brower, J.E., 1978. The distribution of Cd, Cu, Pb and Zn in the biota of two fresh water sites with different trace metal inputs. *Holarctic Ecology*, 1(4): 377-384.

Anthony, E.L.P., and Kunz, T.H., 1977. Feeding strategies of the little brown bat, *Myotis lucifugus*, in Southern New Hampshire. *Ecology*, 58: 775-786.

Barataud, M., Grandemange, F., Duranel, A., and Lugon, A., 2010. Etude d'une colonie de mise-bas de *Myotis bechsteinii* Kuhl, 1817. Sélection des gîtes et des habitats de chasse, régime alimentaire et implications dans la gestion de l'habitat forestier. *Le Rhinolophe*, 18: 83-112.

Barnard, P.C., 2011. The Royal Entomological Society Book of British Insects. Wiley Blackwell eds.

Bauerova, Z., 1982. Contribution to the trophic ecology of the grey long-eared bat, *Plecotus austriacus*. *Folia Zoologica*, 31(2): 113-122.

Bendell-Young, L.I., 1999. Application of a kinetic model of bioaccumulation across a pH and salinity gradient for the prediction of cadmium uptake by the sediment dwelling chironomidae. *Environmental Science & Technology*, 33(9): 1501-1508; DOI 10.1021/es980680u.

Berglund, A.M.M., Koivula, M.J., and Eeva, T., 2011. Species and age-related variation in metal exposure and accumulation of two passerine bird species. *Environmental Pollution*, 159: 2368-2374.

Beyer, W.N., Pattee, O.H., Sileo, L., Hoffman, D.J., and Mulhern, B.M., 1985. Metal contamination in wildlife living near two zinc smelters. *Environmental Pollution Series A-Ecological and Biological*, 38: 63-86.

Bidwell, J.R., and Gorrie, J.R., 2006. The influence of salinity on metal uptake and effects in the midge *Chironomus maddeni*. *Environmental Pollution*, 139(2): 206-213; DOI 10.1016/j.envpol.2005.05.017.

Blehert, D.S., 2012. Fungal disease and the developing story of bat white-nose syndrome. *PLoS Pathogens*, 8(7); DOI: 10.1371/journal.ppat.1002779.

Blundell, A., Hannam, J.A., Dearing, J.A., and Boyle, J.F., 2009. Detecting atmospheric pollution in surface soils using magnetic measurements: A reappraisal using an England and Wales database. *Environmental Pollution*, 157: 2878-2890.

Bontadina, F., Schmied, S. F., Beck, A., and Arlettaz, R., 2008. Changes in prey abundance unlikely to explain the demography of a critically endangered Central European bat. *Journal of Applied Ecology*, 45(2): 641-648.

Boyles, J.G., Cryan, P.M., McCracken, G.F., and Kunz, T.H., 2011. Economic importance of bats in agriculture. *Science*, 332(6025): 41-42.

Carravieiri, A., and Scheifler, R., 2013. Effets des substances chimiques sur les chiroptères: synthèse bibliographique. *Le Rhinolophe*, 19: 1-46.

Chmiel, K.M., and Harrison, R.M., 1981. Lead content of small mammals at a roadside site in relation to the pathways of exposure. *Science of the Total Environment*, 17: 145-154.

Chmielnicka, J., Halatek, T., and Jedlinska, U., 1989. Correlation of cadmium-induced nephropathy and the metabolism of endogenous copper and zinc in rats. *Ecotoxicology and Environmental Safety*, 18: 268-276.

Clark, D.R., and Shore, R.F., 2001. "*Chiroptera*", in: Shore, R.F., Rattner, B.A. Ecotoxicology of wild mammals (Eds), John Wiley & Sons, New York, pp 159-215.

Coutts, R.A., Fenton, M.B., and Glen, E., 1973. Food intake by captive *Myotis lucifugus* and *Eptesicus fuscus* (Chiroptera: Vespertilionidae). *Journal of Mammalogy*, 54: 985-990.

Croisetière, L., Hare, L., and Tessier, A., 2006. A field experiment to determine the relative importance of prey and water as sources of As, Cd, Co, Cu, Pb, and Zn for the aquatic invertebrate *Sialis velata*. *Environmental Science and Technology*, 40: 873-879.

Dallinger, R., Prosi, F., Segner, H., and Bach, H., 1987. Contaminated food and uptake of heavy metals by fish: a review and a proposal for further research. *Oecologia*, 73: 91-98.

Davison, G., Lambie, C.L., James, W.M., Skene, M.E., and Skene, K.R., 1999. Metal content in insects associated with ultramafic and non-ultramafic sites in the Scottish Highlands. *Ecological Entomology*, 24(4): 396-401; DOI 10.1046/j.1365-2311.1999.00213.x.

Del Toro, I., Floyd, K., Gardea-Torresdey, J., and Borrok, D., 2010. Heavy metal distribution and bioaccumulation in Chihuahuan desert rough harvester ant (*Pogonomyrmex rugosus*) populations. *Environmental Pollution*, 158: 1281-1287.

Desrosiers, M., Gagnon, C., Masson, S., Martel, L., and Babut, M.P., 2008. Relationships among total recoverable and reactive metals and metalloid in St. Lawrence River sediment: Bioaccumulation by chironomids and implications for ecological risk assessment. *Science of the Total Environment*, 389: 101-114.

Dietz, C., Von Helversen, O., and Nill, D., 2009. Bats of Britain, Europe and Northwest Africa. A&C Black Publishers, London.

Dixit, S.S., and Witcomb, D., 1983. Heavy-Metal Burden in water, substrate, and macroinvertebrate body tissue of a polluted river Irwell (England). *Environmental Pollution Series B Chemical and Physical*, 6: 161-172.

Dixit, V.P., and Lohiya, N.K., 1974. Histological changes in testis of the non-scrotal mammal, *Rhinopoma kinneari* (Wrougton) following the administration of cadmium chloride. *Indian journal of experimental biology*, 12: 200-202.

Eeva, T., Hakkarainen, H., and Balskii, E., 2009. Local survival of pied flycatcher males and females in a pollution gradient of a Cu smelter. *Environmental Pollution*, 157: 1857-1861.

EFSA Journal, 2009. Risk assessment for birds and mammals, Guidance of EFSA. 7, 358 [Available online: www.efsa.europa.eu.]

Encarnação, J.A., and Dietz, M., 2006. Estimation of food intake and ingested energy in Daubenton's bats (*Myotis daubentonii*) during pregnancy and spermatogenesis. *European Journal of Wildlife Research*, 52: 221-227.

Environment Agency, 2009. Dealing with contaminated land in England and Wales. A review of progress from 2000-2007 with Part 2A of the Environmental Protection Act. 39.

Feldman, R., Whitaker, J.O., and Yom-Tov, Y., 2000. Dietary composition and habitat use in a desert insectivorous bat community in Israel. *Acta Chiropterologica*, 2: 15-22.

Flanders, J., and Jones, G., 2009. Roost use, ranging behaviour, and diet of greater horseshoe bats (*Rhinolophus ferrumequinum*) using a transitional roost. *Journal of Mammalogy*, 90(4): 888-896.

Frick, W.F., Reynolds, D.S., and Kunz, T.H., 2010. Influence of climate and reproductive timing on demography of little brown myotis *Myotis lucifugus*. *Journal of Animal Ecology*, 79: 128-136.

Fritsch, C., Coeurdassier, M., Faivre, B., Baurand, P., Giraudoux, P., Van den Brink, N.M., and Scheifler, R., 2012. Influence of landscape composition and diversity on contaminant flux in terrestrial food webs: A case study of trace metal transfer to European blackbirds *Turdus merula*. *Science of the Total Environment*, 432: 275–287.

Fritsch, C., Cosson, R.P., Coeurdassier, M., Raoul, F., Giraudoux, P., Crini, N., de Vaufleury, A., and Scheifler, R., 2010. Responses of wild small mammals to a pollution gradient: Host factors influence metal and metallothionein levels. *Environmental Pollution*, 158: 827-840.

Gajdošík, M., and Gaisler, J., 2004. Diet of two Eptesicus bat species in Moravia (Czech Republic). *Folia Zoologica*, 53(1): 7-16.

Genoud, M., and Christe, P., 2011. Thermal energetics and torpor in the common pipistrelle bat, *Pipistrellus pipistrellus* (Vespertilionidae: Mammalia). *Comparative Biochemistry and Physiology, Part A*, 160: 252-259.

Gillis, P. L., Reynoldson, T.B., and Dixon, D.G., 2006. Metallothionein-like protein and tissue metal concentrations in invertebrates (Oligochaetes and Chironomids) collected from reference and metal contaminated field sediments. *Journal of Great Lakes Research*, 32: 565-577.

Goldsmith, C.D., and Scanlon, P.F., 1977. Lead levels in small mammals and selected invertebrates associated with highways of different traffic densities. *Bulletin of Environmental Contamination and Toxicology*, 17: 311-316.

Gongalsky, K.B., 2006. Bioaccumulation of metals by soil-dwelling insects in a uranium production area. *European Journal of Soil Biology*, 42: S180-S185.

Goodyear, K.L., and McNeill, S., 1999. Bioaccumulation of heavy metals by aquatic macro-invertebrates of different feeding guilds: a review. *Science of the Total Environment*, 229: 1-19.

Grimm, V., Ashauer, R., Forbes, V., Hommen, U., Preuss, T.G., Schmidt, A., van den Brink , P. J. , Wogram, J. and Thorbek. P., 2009. CREAM: A European project on mechanistic effect models for ecological risk assessment of chemicals. Environmental Science and Pollution Research, 16: 614-617.

Grimm, V., and Railsback, S.F., 2005. "*Analysing Individual-based models*" in Individual based modelling and ecology. Princeton series in theoretical and computional biology, 312-346.

Groom, M. J., Meffe, G. K., and Carroll, C. R., 2006. Principles of conservation biology (3rd Edition). Sunderland, Mass: Sinauer Associates.

Haberl, W., 2002. Food storage, prey remains and notes on occasional vertebrates in the diet of the Eurasian water shrew, *Neomys fodiens*. *Folia Zoologica*, 51(2): 93-102.

Hare, L., and Campbell, P.G.C., 1992. Temporal variations of trace metals in aquatic insects. *Freshwater Biology*, 27(1): 13-27.

Hare, L., Tessier, A., and Warren, L., 2001. Cadmium accumulation by invertebrates living at the sediment-water interface. *Environmental Toxicology and Chemistry*, 20: 880-889.

Hariono, B., Ng, J., and Sutton, R.H., 1993. Lead concentrations in tissues of fruit bats (*Pteropus sp.*) in urban and non-urban locations. *Wildlife Research*, 20: 315-320.

Harrahy, E.A., and Clements, W.H., 1997. Toxicity and bioaccumulation of a mixture of heavy metals in *Chironomus tentans* (Diptera: Chironomidae) in synthetic sediment. *Environmental Toxicology and Chemistry*, 16: 317-327.

He, M., Ke, C., and Wang, W., 2010. Effects of cooking and subcellular distribution on the bioaccessibility of trace elements in two marine fish species. *Journal of Agricultural and Food Chemistry*, 58(6), 3517-3523; DOI 10.1021/jf100227n.

Health Canada Contaminated Sites Division, 2005. Procedures for the use of risk assessment under part XV.1 of the environmental protection act. Ontario ministry of the environment.

Heikens, A., Peijnenburg, W.J.G.M., and Hendricks, A.J., 2001. Bioaccumulation of heavy metals in terrestrial invertebrates. *Environmental Pollution*, 113(3): 385-393; DOI 10.1016/S0269-7491(00)00179-2.

Hendricks, A.J., Ma, W.C., Brouns, J.J., de Ruiter-Dijkmnan, E.M., and Gast, R., 1995. Modelling and monitoring of organochlorine and heavy metal accumulation in soils, earthworms, and shrews in Rhine-delta floodplains. *Archives of Environmental Contamination and Toxicology*, 29: 115-127.

Herreid II, C.F., and Schmidt-Nielsen, K., 1966. Oxygen consumption, temperature, and water loss in bats from different environments. *American Journal of Physiology*, 211(5): 1108-1112.

Hickey, M.B.C., Fenton, M.B., MacDonald, K.C., and Soulliere, C., 2001. Trace elements in the fur of bats (Chiroptera: Vespertilionidae) from Ontario and Quebec, Canada. *Bulletin of Environmental Contamination and Toxicology*, 66: 699-706.

Hoffman, D.J., Rattner, B.A., Scheunert, I., and Korte, R., 2001. "*Environmental contaminants*" Ecotoxicology of wild mammals, R.F. Shore, B.A. Rattner, eds John Wiley & Sons, New York, 1-48.

Hu, J., Wu, F., Wu, S., Cao, Z., Lin, X., and Wong, M.H., 2013. Bioaccessibility, dietary exposure and human risk assessment of heavy metal from market vegetables in Hong Kong revealed with an *in vitro* gastrointestinal model. *Chemosphere*, 91(4): 455-461; DOI 10.1016/j.chemosphere.2012.11.066.

Hunter, B.A., and Johnson, M.S., 1982. Food chain relationships of copper and cadmium in contaminated grassland ecosystems. *Oikos*, 38: 108-177.

Hunter, B.A., Johnson, M.S., and Thompson, D.J., 1987a. Ecotoxicology of copper and cadmium in a contaminated grassland ecosystem .1. Soil and vegetation contamination. *Journal of Applied Ecology*, 24: 573-586.

Hunter, B.A., Johnson, M.S., and Thompson, D.J., 1987b. Ecotoxicology of copper and cadmium in a contaminated grassland ecosystem. 2. Invertebrates. *Journal of Applied Ecology*, 24: 587-599.

Hurley, S., and Fenton, M.B., 1980. Ineffectiveness of fenthion, zinc phosphide, DDT and two ultrasonic rodent repellers for control of populations of little brown bats (*Myotis lucifugus*). *Bulletin of Environmental Contamination and Toxicology*, 25: 503-507.

Ieradi, L.A., Cristaldi, M., Mascanzoni, D., Cardarelli, E., Grossi, R., and Campanella, L., 1996. Genetic damage in urban mice exposed to traffic pollution. *Environmental Pollution*, 92: 323-328.

IUCN Red List, 2008. The IUCN Red list of threatened species. [Available online: http://www.iucnredlist.org/.]

Jankovska, I., Miholova, D., Bejcek, V., Vadlejch, J., Sulc, M., Szakova, J., and Langrova, I., 2009. Influence of parasitism on trace element contents in tissues of red fox (*Vulpes vulpes*) and its parasites *Mesocestoides spp*. (Cestoda) and *Toxascaris leonina* (Nematoda). *Archives of Environmental Contamination and Toxicology*, 58: 469-477.

Jankovska, I., Miholova, D., Langrova, I., Bejcek, V., Vadlejch, J., Kolihova, D., and Sulc, M., 2009. Influence of parasitism on the use of small terrestrial rodents in environmental pollution monitoring. *Environmental pollution*, 157: 2584-2586.

Janssen, M.P.M., and Hogervorst, R., 1993. Metal accumulation in soil arthropods in relation to micro-nutrients. *Environmental Pollution*, 79(2): 181-189.

Jefferies, D.J., 1972. Organochlorine insecticide residues in British bats and their significance. *Journal of Zoology*, 166: 245-263.

Jelaska, L.S., Blanusa, M., Durbesić, F., and Jelaska, S.D., 2007. Heavy metal concentrations in ground beetles, leaf litter, and soil of a forest ecosystem. *Ecotoxicology and Environmental Safety*, 66 (1): 74-81.

John, D.A., and Leventhal, J.S., 1995. "*Bioavailability of metals*" in duBray, E.A., ed., Preliminary compilation of descriptive geoenvironmental mineral deposit models: U.S. Geological Survey Open-File Report 95-831, p. 10-18.

Jones, G., 1990. Prey selection by the greater horseshoe bat (*Rhinolophus ferrumequinum*): optimal foraging by echolocation? *Journal of Animal Ecology*, 59: 587-602.

Jones, G., 1995. Flight performance, echolocation and foraging behaviour in noctule bats *Nyctalus noctula*. *Journal of Zoology*, 237(2): 303-312.

Jones, G., Jacobs, D.S., Kunz, T.H., Willig, M.R., and Racey, P.A., 2009. Carpe noctem: the importance of bats as bioindicators. *Endangered Species Research*, 8: 93-115.

Kaňuch, P., Janeckova, K., and Krištín, A., 2005a. Winter diet of the noctule bat *Nyctalus noctula*. *Folia Zoologica*, 54(1-2): 53-60.

Kaňuch, P., Krištín, A., and Krištofík, J., 2005b. Phenology, diet, and ectoparasites of Leisler's bat (*Nyctalus leisleri*) in the Western Carpathians (Slovakia). *Acta Chiropterologica*, 7(2): 249-258.

Kapusta, P., Sobczyk, L., Rozen, A., and Weiner, J., 2003. Species diversity and spatial distribution of enchytraied communities in forest soils: effects of habitat characteristics and heavy metal contamination. *Applied Soil Ecology*, 23: 187-198.

Kaufman, C.A., Bennett, J. R., Koch, I., and Reimer, K.J. , 2007. Lead bioaccessibility in food web intermediates and the influence on ecological risk characterization. *Environmental Science & Technology*, 41(16): 5902-5907; DOI 10.1021/es062443u.

Kennedy, M.C., 2005. GEM-SA, Version 1.1. Software: Gaussian Emulation Machine for Sensitivity Analysis. [Available online: http://www.tonyohagan.co.uk/academic/GEM/index.html.]

Kervyn, T., 1996. Le régime alimentaire du grand murin *Myotis myotis* (Chiroptera : Vespertilionidae) dans le sud de la Belgique. *Cahiers d'Ethologie*, 16: 23-46.

Krantzberg, G., and Stokes, P.M., 1988. The importance of surface adsorption and pH in metal accumulation by chironomids. *Environmental Toxicology and Chemistry*, 7(8): 653-670; DOI 10.1002/etc.5620070807.

Kunz, T.H., 1974. Feeding ecology of a temperate insectivorous bat (*Myotis velifer*). *Ecology*, 55: 693-711.

Lado, L.R., Hengl, T., and Reuter, H.I., 2008. Heavy metals in European soils: A geostatistical analysis of the FORGES Geochemical database. Geoderma, 148: 189-199.

Laird, B.D., Shade, C., Gantner, N., Chan, H.M., and Siciliano, S.D., 2009. Bioaccessibility of mercury from traditional northern country foods measured using an *in vitro* gastrointestinal model is independent of mercury concentration. *Science of the Total Environmental*, 407(23): 6003-6008; DOI 10.1016/j.scitotenv.2009.08.014.

Laskowski, R., Niklinska, M., and Maryanski, M., 1995. The dynamics of chemical elements in forest litter. Ecology, 76: 1393-1406.

Linder, G., and Joermann, G., 2001. "*Assessing hazard and risk of chemical exposures to wild mammals: food-chain analysis and its role in ecological risk assessment*" in

Ecotoxicology of wild mammals, R.F. Shore, B.A. Rattner, eds John Wiley & Sons, New York, 635-670.

Lodenius, M., Josefsson, J., Heliövaara, K., Tulisalo, E., and Nummelin, M., 2009. Cadmium in insects after ash fertilization. *Insect Science*, 16(1): 93-98; DOI 10.1111/j.1744-7917.2009.00259.x.

Lüftl, S., Freitag, B., Deutz, A., and Tataruch, F., 2003. Concentrations of heavy metals in European bats (Microchiroptera). *Fresenius Environmental Bulletin*, 12: 353-358.

Lugon, A., 1996. Ecologie du grand rhinolophe *Rhinolophus ferrumequinum* (Chiroptera, Rhinolophidae) en Valais (Suisse): Habitat, régime alimentaire et stratégie de chasse. MSC Dissertation, Université de Neuchâtel, Switzerland.

Luoma, S.N., 1989. Can we determine the biological availability of sediment-bound trace elements? *Hydrobiologia*, 176-177: 379-396.

Ma, J., Liang, B., Zhang, S., and Metzner, W., 2008. Dietary composition and echolocation call design of three sympatric insectivorous bat species from China. *Ecological Research*, 23(1): 113-119.

Ma, W.C., 1987. Heavy metal accumulation in the mole, *Talpa europaea*, and earthworms as an indicator of metal bioavailability in terrestrial environments. *Bulletin of Environmental Contamination and Toxicology*, 39: 933-938.

Ma, W.C., 1989. Effects of soil pollution with metallic lead pellets on lead bioaccumulation and organ/body weight alterations in small mammals. *Archives of Environmental Contamination and Toxicology,* 18: 617-622.

Ma, W.C., 1996. "*Lead in mammals*" in Environmental Contaminants in Wildlife Interpreting Tissue Concentrations, eds Beyer W, Heinz G, Redmon-Norwood A (CRC Press Inc, London).

Ma, W.C., and Talmage, S., 2001. "*Insectivora*" in Ecotoxicology of wild mammals, eds Shore RF, Rattner BA (John Wiley & Sons, New York), pp 123-158.

Mackenzie, G.A., and Oxford, G.S., 1995. Prey of the noctule bat (*Nyctalus noctula*) in East Yorkshire. *Journal of Zoology*, 236: 322-327.

Mason, C.F., and Wren, C.D., 2001. "*Carnivora*" in Ecotoxicology of wild mammals, R.F. Shore, B.A. Rattner, eds John Wiley & Sons, New York, 315-370.

Matranga, V., and Corsi, I., 2012. Toxic effects of engineered nanoparticles in the marine environment: Model organisms and molecular approaches. *Marine Environmental Research*, 76: 32-40.

Mc Grath, S.P., and Loveland, P.J., 1992. The soil geochemical atlas and England and Wales. Blackie academic & professional (Glasgow).

Metian, M., Charbonnier, L., Oberhaënsli, F., Bustamente, P., Jeffree, R., Amiard, J.C., and Warnau, M., 2009. Assessment of metal, metalloid, and radionuclide bioaccessibility from mussels to human consumers, using centrifugation and simulated digestion methods coupled with radiotracer techniques. *Ecotoxicology and Environmental Safety*, 72(5): 1499-1502; DOI 10.1016/j.ecoenv.2008.10.009.

Milton, A., Johnson, M.S., and Cook, J.A., 2002. Lead within ecosystems on metalliferous mine tailings in Wales and Iraland. *Science of the Total Environment*, 299(1-3): 177-190; DOI: 10.1016/S0048-9697(02)00253-X.

Morel, J.-L., 1997. "*Bioavailability of trace elements to terrestrial plants*." In Tarradellas, J., Bitton, G., Rossel, D. (eds) Soil ecotoxicology. Lewis Publishers, Boca Raton, pp 141-176.

Moriarty, M.M., Koch, I., and Reimer, K.J., 2012. Arsenic speciation, distribution and bioaccessibility in shrews and their food. *Archives of Environmental Contamination and Toxicology*, 62(3): 529-538; DOI 10.1007/s00244-011-9715-6.

Moriarty, M.M., Koch, I., and Reimer, K.J., 2013. Arsenic species and uptake in amphibians (*Rana clamitans* and *Bufo americanus*). *Environmental Science: Processes and Impacts*, 15: 1520-1528; DOI 10.1039/C3EM00223C.

Nagy, K.A., 1987. Field Metabolic Rate and Food Requirement Scaling in Mammals and Birds. *Ecological Monographs*, 57: 111-128.

Nahmani, F., Hodson, M.E., and Black, S., 2007. A review of studies performed to assess metal uptake by earthworms. *Environmental pollution*, 145: 402-424.

Nieminen, M., Nuorteva, P., and Tulisalo, E., 2001. The effect of metal on the mortality of *Parnassius apollo* larvae (Lepidoptera: Papilionidae). *Journal of Insect Conservation*, 5(1): 1-7; DOI 10.1023/A:1011371119290.

Norstrom, R.J., Simon, M., Muir, D.C.G., and Schweinsburg, R., 1988. Organochlorine contaminants in Arctic marine food chains: identification, geographical distribution and temporal trends in polar bears. *Environmental Science & Technology*, 22: 1063-1071.

Oaks, J.L., Gilbert, M., Virani, M.Z., Watson, R.T., Meteyer, C.U., Rideout, B.A., Shivaprasad, H.L., Ahmed, S., Iqbal, M.J., Arshad, C.M., Mahmood, S., Ali, A., and Khan, A.A., 2004. Diclofenac residues as the cause of vulture population decline in Pakistan. *Nature*, 427: 630–633.

Ollson, C.A., Kock, I., Smith, P., Knopper, L.D., Hough, C., and Reimer, K.J., 2009. Addressing arsenic bioaccessibility in ecological risk assessment: a novel approach to avoid overestimating risk. *Environmental Toxicology and Chemistry*, 28(3): 668-675; DOI 10.1897/08-204.1.

Oomen, A.G., Hack, A., Minekus, M., Zeijdner, E., Cornelis, C., Schoeters, G., Verstraete, W., Van de Wiele, T., Wragg, J., Rompelberg, C. J.M., Sips, A.J.A.M., and Van Wijnen, J.H., 2002. Comparison of five *in vitro* digestion models to study the bioaccessibility of soil contaminants. *Environmental Science & Technology*, 36 (15), 3326-3334; DOI 10.1021/es010204v.

Oomen, A.G., Rompelberg, C.J.M., Bruil, M.A., Dobbe, C. J.G., Pereboom, D. P.K.H., and Sips, A.J.A.M., 2003b. Development of an *In Vitro* digestion model estimating the bioaccessibility of soil contaminants. *Archives of Environmental Contamination and Toxicology*, 44(3): 281-287; DOI 10.1007/s00244-002-1278-0.

Oomen, A.G., Tolls, J., Sips, A.J.A.M., and Groten, J.P., 2003a. In vitro intestinal lead uptake and transport in relation to speciation. *Archives of Environmental Contamination and Toxicology*, 44: 116-124.

Otálora-Ardila, A., Herrera, G.L., Flores-Martínez, J.J.M., and Voigt, C.C., 2013. Marine and terrestrial food sources in the diet of the fish-eating myotis (*Myotis vivesi*). *Journal of Mammalogy*, 94(5): 1102-1110.

Panagos, P., Van Liedekerke, M., Yigini, Y., and Montanarella, L., 2013. Contaminated Sites in Europe: Review of the Current Situation Based on Data Collected through a European Network. *Journal of Environmental and Public Health*, 2013: 1-11; DOI 10.1155/2013/158764.

Pankakoski, E., Koivisto, I., Hyvärinen, H., Terhivuo, J., and Tähkä, K.M., 1994. Experimental accumulation of lead from soil through earthworms to common shrews. *Chemosphere*, 29: 1639-1649.

Parry, H.R., Topping, C.J., Kennedy, M.C., Boatman, N.D., and Murray, A.W.A., 2013. A Bayesian sensitivity analysis applied to an Agent-based model of bird population response to landscape change. *Environmental Modelling & Software*, 45: 104-115.

Pennisi, L.A., Holland, S.M., and Stein, T.V., 2004. Achieving bat conservation through tourism. *Journal of Ecotourism*, 3(3): 193-207.

Peralta-Videa, J.R., Lopez, M.L., Narayan, M., Saupe, G., Gardea-Torresdey, J., 2009. The biochemistry of environmental heavy metal uptake by plants: implications for the food chain. *The International Journal of Biochemistry and Cell Biology*, 41(8-9): 1665-1677.

Péry, A.R.R., Ducrot, V., Geffard, A., and Garric, J., 2007. Do differences between metal body residues reflect the differences between effects for *Chironomus riparius* exposed to different sediments? *Chemosphere*, 66: 397-403.

Pikula, J., Zukal, J., Adam, V., Bandouchova, H., Beklova, M., Hajkova, P., Horakova, J., Kizek, R., and Valentikova, L., 2010. Heavy metals and metallothionein in vespertilionid bats foraging over aquatic habitats in the Czech Republic. *Environmental Toxicology and Chemistry*, 29: 501-506.

Pithartovà, T., 2007. Feeding ecology of four bat species (*Myotis daubentonii*, *Myotis mystacinus*, *Pipistrellus nathusii*, and *Pipistrellus pygmaeus*): diet structure and seasonal dynamics in syntopic populations. *Vespertilio*, 11: 125-173. [In Czech with English abstract].

Platts, P.J., McClean, C.J., Lovett, J.C., and Marchant, R., 2008. Predicting tree distributions in East African biodiversity hotspot: model selection, data bias and envelope uncertainty. *Ecological Modelling*, 218: 121-134.

Prince SPM, W.P., Senthilhumar, W.P., and Subburam, V., 2001. Mulberry-silkworm food chain- a templet to assess heavy metal mobility in terrestrial ecosystems. *Environmental Monitoring and Assessment*, 69: 231-238.

Purchart, L., and Kula, E., 2007. Content of heavy metals in bodies of field ground beetles (Coleoptera, carabidae) with respect to selected ecological factors. *Polish Journal of Ecology*, 55(2): 305-314.

Rabitsch, W.B., 1995. Metal accumulation in arthropods near a lead/zinc smelter in Arnoldstein, Austria .1. *Environmental Pollution*, 90: 221-237.

Rainio, M.J., Kanerva, M., Salminen, J.-P., Nikinmaa, M., and Eeva, T., 2013. Oxidative status in nestlings of three small passerine species exposed to metal pollution. *Science of the total Environment,* 454-455: 466-473.

Reddy, K.R., Danda, S., Yukselen-Aksoy, Y., and Al-Hamdan, A.Z., 2010. Sequestration of heavy metals in soils from two polluted industrial sites: implications for remediation. *Land Contamination and Reclamation*, 18(1): 13-23.

Reinhold, J.O., Hendriks, A.J., Slager, L.K., and Ohm, M., 1999. Transfer of microcontaminants from sediment to chironomids, and the risk for the Pond bat *Myotis dasycneme* (Chiroptera) preying on them. *Aquatic Ecology*, 33(4): 363-376; DOI 10.1023/A:1009958028204.

Review of Transboundary air pollution (Rotap), 2009. "Heavy metals" in Review of transboundary air pollution. Acidification, eutrophication, ground level ozone and heavy metals in the UK. [Available online: http://www.rotap.ceh.ac.uk]

Risk assessment guidance for superfund, 2001. Process for conducting probabilistic risk assessment, Appendix A. Volume 3 Part A, pp 37. [Available online: http://www.epa.gov/oswer/riskassessment/rags3adt/pdf/appendixa.pdf]

Robinson, Jr G.R, Sibrell, P.L., Boughton, C.J., and Yang, L.H., 2007. Influence of soil chemistry on metal and bioessential element concentrations in nymphal and adult periodical cicadas (*Magicicada spp*.). *Science of the Total Environment*, 374: 367-378.

Robinson, M.F., and Stebbings, R.E., 1993. Food of the serotine bat, *Eptesicus serotinus* – is faecal analysis a valid qualitative and quantitative technique? *Journal of Zoology*, 231(2): 239-248.

Ross, P.S., and Troisi, G.M., 2001. "*Pinnipedia*" in Ecotoxicology of wild mammals, R.F. Shore, B.A. Rattner, eds John Wiley & Sons, New York, 371-426.

Roth, M., 1993. Investigations on lead in the soil invertebrates of a forest ecosystem. *Pedobiologia*, 37: 270-279.

Rubach, M.N., Ashauer, R., Buchwalter, D.B., De Lange, H.J., Hamer, M., Preuss, T.G.,Töpke, K., and Maund, S.J., 2012. A framework for traits-based assessment in ecotoxicology. *Integrated Environmental Assessment and Management*, 7(2), 172-186; DOI 10.1002/ieam.105.

Ruby, M.V., 2004. Bioavailability of Soil-Borne Chemicals: abiotic assessment tools. *Human and Ecological Risk Assessment*, 10(4): 647-656; DOI 10.1080/10807030490484291.

Ruby, M.V., Davis, A., Schoof, R., Eberle, S., and Sellstone, C.M., 1996. Estimation of lead and arsenic bioavailability using a physiologically based extraction test. *Environmental Science & Technology*, 30(2): 422-430; DOI 10.1021/es950057z.

Ruby, M.V., Schoof, R., Brattin, W., Goldade, M., Post, G., Harnois, M., Mosby, D.E., Casteel, S.W., Berti, W., Carpenter, M., Edwards, D., Cragin, D., and Chappell, W., 1999. Advances in evaluating the oral bioavailability of inorganics in soil for use in human health risk assessment. *Environmental Science & Technology*, 33(21): 3697-3705; DOI 10.1021/es990479z.

Rudy, M., 2009. Correlation of lead, cadmium and mercury levels in tissue and liver samples with age in cattle. *Food Additives & Contaminants. Part A, Chemistry, analysis, control, exposure & risk assessment*, 26(6): 847-853.

Rydell, J., 1989. Food habits of northern (*Eptesicus nilssoni*) and brown long-eared (*Plecotus auritus*) bats in sweden. *Holarctic ecology*, 12: 16-20.

Rydell, J., and Petersons, G., 1998. The diet of the noctule bat *Nyctalus noctula* in Latvia. *Zeitschrift für Säugetierkunde*, 63: 79-83.

Rydell, J., Natuschke, G., Theiler, A., and Zingg, P.E., 1996. Food habits of the barbastelle bat *Barbastella barbastellus*. *Ecography*, 19(1): 62-66.

Sample, B.E., and Suter, G.W., 1994. Estimating exposure of terrestrial wildlife to contaminants. Environmental Sciences Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee.

Sample, B.E., Opresko, D.M., and Suter, G.W., 1996. Toxicological benchmarks for Wildlife: 1996 Revision. Oak ridge National Laboratories: Health Sciences Research Division pp 217.

Scheifler, R., Fritsch, C., Bervoets, L., and van den Brink, N., 2012. INSPECT: Integration of spatially explicit risks of contaminants in Spatial Planning and Land Management. Final project report p: 80.

Schipper, A.M., Wijnhoven, S., Baveco, H., and van den Brink, N.W., 2012. Contaminant exposure in relation to spatio-temporal variation in diet composition: A case study of the little owl (*Athene noctua*). *Environmental Pollution*, 163: 109-116; DOI 10.1016/j.envpol.2011.12.020.

Schipper, A.M., Wijnhoven, S., Leuven, R.S.E.W., Ragas, A.M.J., and Hendriks, A.J., 2008. Spatial distribution and internal metal concentrations of terrestrial arthropods in a moderately contaminated lowland floodplain along the Rhine River. *Environmental Pollution*, 151: 17-26.

Schleich, C.E., Beltrame, M.O., and Antenucci, C.D., 2010. Heavy metals accumulation in the subterranean rodent *Ctenomys talarum* (Rodentia: Ctenomyidae) from areas with different risk of contamination. *Folia zoologica*, 59(2): 108-114.

Schmolke, A., Thorbek, P., DeAngelis, D.L., and Grimm, V., 2010. Ecological modelling supporting environmental decision making: a strategy for the future. *Trends in Ecology and Evolution*, 25: 479-486

Scillitani,G., Zizza, S., Liquori, G.E., and Ferri, D., 2005. Histochemical and immunohistochemical evidence for a gradient in gastric juice production in the greater horseshoe bat, *Rhinolophus ferrumequinum* (Schreber, 1774). *Acta Chiropterologica*, 7(2): 301-308; DOI 10.3161/1733-5329(2005)7[301:HAIEFA]2.0.CO;2.

Shacklette, H.T., and Boerngen, J.G., 1984. Element concentrations in soils and other surficial materials of the conterminous United States. USGS Professional paper 1270.

Sheffield, S.R., Sawicka-Kapusta, K., Cohen, J.B. and Rattner, B.A., 2001. "*Rodentia and Lagomorpha*" in Ecotoxicology of wild mammals, R.F. Shore, B.A. Rattner, eds John Wiley & Sons, New York, 215-314.

Sherwin, H.A., Montgomery, W.I., and Lundy, M.G., 2013. The impact and implications of climate change for bats. *Mammal Review*, 43(3): 171-183.

Shore, R.F., and Douben, P.E.T., 1994. "*The ecotoxicological significance of cadmium intake and residues in terrestrial small mammals."* in Ecotoxicology and Environmental Safety, 29, 101-113.

Sierro, A., and Arlettaz, R., 1997. Barbastelle bats (*Barbastella spp*.) specialize in the predation of moths: implications for foraging tactics and conservation. *Acta Oecologica*, 18(2): 91-106.

Smart, R., Northing, P., and Boxall, A.B.A., 2006. A review of models and methods for final tier (Tier 3) ecological risk assessment (SC030003). Central Science Laboratory Report, Sand Hutton, UY.

Smith, D.B., Cannon, W.F., Woodruff, L.G., Garrett, R.G., Klassen, R., Kilburn, J.E., Horton, J.D., King, H.D., Goldhaber, M.B., and Morrison, J.M., 2005. Major- and trace- element concentrations in soils from two continental-scale transects of the United States and Canada. Open file report USGS.

Spitzer, E., Lott, J.N.A., and Vollmer, C.M., 1980. Studies of metal uptake, especially iron, into preotein bodies of *Capsella* and *Lycopersicon* seeds. *Revue canadienne de botanique*, 58(11): 1244-1249.

Stalinski, J., 1994. Digestion, defecation and food passage rate in the insectivorous bat *Myotis myotis. Acta Theriologica*, 39(1): 1-11.

Stebbings, R. E. (1988). "Conservation of European bats." Christopher Helm, London.

Stone, D., Jepson, P., and Laskowski, R., 2002. Trends in detoxification enzymes and heavy metal accumulation in ground beetles (Coleoptera: carabidae) inhabiting a gradient of pollution*. Comparative Biochemistry and Physiology*, 132: 105-112.

Storm, G.L., Yahner, R.H., and Bellis, E.D., 1993. Vertebrate abundance and wildlife habitat suitability near the Palmerton zinc smelters, Pennsylvania. *Archives of Environmental Contamination and Toxicology*, 25: 428-437.

Streit, B., and Nagel, A., 1993. Heavy metal transfer by lactation in a bat colony. *Fresenius Environmental Bulletin*, 2: 168-173.

Sutton, R.H., and Hariono, B., 1987. Lead poisoning in flying foxes (Chiroptera: Pteropodidae). *Australian Mammalogy*, 10: 125-126.

Sutton, R.H., and Wilson, P.D., 1983. Lead poisoning in grey-headed Fruit Bats (*Pteropus poliocephalus*). *Journal of Wildlife Diseases*, 19: 294-296.

van Straalen, N.M., and van Wensem, J., 1986. Heavy metal content of forest litter arthropods as related to body-size and trophic level. *Environmental Pollution Series A, Ecological and Biological*, 42(3): 209-221.

van Straalen, N.M., Butovsky, R.O., Pokarzhevskii, A.D., Zaitsev, A.S., and Cornelis Verhof, S., 2001. Metal concentrations in soil and invertebrates in the vicity of a metallurgical factory near Tula (Russia). Pedobiologia, 45: 451-466.

Vandecasteele, B., Lauriks, R., De Vos, B., and Tack, F.M.G., 2003. Cd and Zn concentration in hybrid poplar foliage and leaf beetles grown on polluted sediment-derived soils. *Environmental Monitoring and Assessment*, 89: 263-283.

Vaughan, N., 1997. The diets of British bats (Chiroptera). Mammal Review, 27(2): 77-94.

Vermeulen, F., van den Brink, N.W., D'Havé, H., Mubiana, V.K., Blust, R., Bervoets, L., and De Coen, W., 2009. Habitat type-based bioaccumulation and risk assessment of metal and As contamination in earthworms, beetles and woodlice. *Environmental Pollution*, 157(11): 3098-3105; DOI 10.1016/j.envpol.2009.05.017.

Vijver, M., Jager, T., Posthuma, L., and Peijnenburg, W., 2003. Metal uptake from soils and soil-sediment mixtures by larvae of *Tenebrio molitor* (L.) (Coleoptera). *Ecotoxicology and Environmental Safety*, 54(3): 277-289.

Walker, L.A., Simpson, V.R., Rockett, L., Wienburg, C.L., and Shore, R.F., 2007. Heavy metal contamination in bats in Britain. *Environmental Pollution*, 148: 483-490.

Wang, M., and Grimm, V., 2010. Population models in pesticide risk assessment: Lessons for assessing population-level effects, recovery, and alternative exposure scenarios from modeling a small mammal. *Environmental Toxicology and Chemistry*, 29(6): 1292-1300.

Wang, W., 1987. Factors affecting metal toxicity to (and accumulation by) aquatic organisms — Overview. *Environment International*, 13(6): 437-457.

Waters, D., Jones, G., and Furlong, M., 1999. Foraging ecology of Leisler's bat (*Nyctalus leisleri*) at two sites in southern Britain. *Journal of Zoology*, 249(2): 173-180.

Whitaker Jr, J.O., Dannelly, H.K., and Prentice, D.A., 2004. Chitinase in insectivorous bats. *Journal of Mammalogy*, 85(1): 15-18.

Whitaker, J.O., Shalmon, B., and Kunz, T.H., 1994. Food and feeding habits of insectivorous bats from Israel. *Zeitschrift für Säugetierkunde*, 59: 74-81.

Wickramasinghe, L.P., Harris, S., Jones, G., and Vaughan, N., 2003. Bat activity and species richness on organic and conventional farms: impact of agricultural intensification. *Journal of Applied Ecology*, 40: 984-993.

Womack, K.M., Amelon, S.K., and Thompson III, F.R., 2013. Resource selection by Indiana bats during the maternity season. *Journal of Wildlife Management*, 77(4): 707-715.

Zhang, Z-S., Lu, X-G., Wang, Q-C., and Zhend, D-M., 2009. Mercury, Cadmium and Lead biogeochemistry in the soil-plant-insect system in Huludao City. *Bulletin of Environmental Contamination and Toxicology*, 83: 255-259.

Zhuang, P., Zou, H., and Shu, W., 2009. Biotransfer of heavy metals along a soil-plant_insect-chicken food chain: field study. Journal of Environmental Sciences, 21: 849-853.

Zook, B.C., Sauer, R.M., and Garner, F.M., 1970. Lead poisoning in Australian fruit bats (*Pteropus poliocephalus*). *Journal of the American Veterinary Medicine Association*, 157: 691-694.