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Fur: a non-invasive approach to monitor metal exposure in bats

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**Fur: a non-invasive approach to monitor metal exposure in bats**

**Abstract**

This paper presents a novel assessment of the use of fur as a non-invasive proxy to biomonitor metal contamination in insectivorous bats. Metal concentrations (cadmium, copper, lead and zinc) were measured using ICP-MS in tissues (kidneys, liver, stomach and stomach content, bones and fur) obtained from 193 Pipistrellus pipistrellus/pygmaeus bats. The bats were collected across a gradient of metal pollution in England and Wales. The utility of small samples of fur as an indicator of metal exposure from the environment was demonstrated with strong relationships obtained between the concentrations of non-essential metals in fur with concentrations in stomach content, kidneys, liver and bones. Stronger relationships were observed for non-essential metals than for essential metals. Fur analyses might therefore be a useful non-invasive proxy for understanding recent, as well as long term and chronic, metal exposure of live animals. The use of fur may provide valuable information on the level of endogenous metal exposure and contamination of bat populations and communities.

**1. Introduction**

Non-invasive sampling approaches such as fur, feather or claw are invaluable in field-based monitoring studies to understand levels of pollution in wildlife species, particularly for protected species, such as bats (Dauwe et al., 2000; D’Havé et al., 2006). These methods are less damaging and stress inducing than invasive approaches such as blood sampling or the taking of biopsies and allow the monitoring of both levels of pollution and the fitness of a given population over time without significantly affecting the fitness of individuals (D’Havé et al., 2006).

Hair sampling is highly suited as a non-invasive sampling technique as hair is easily accessible, stable, and its storage does not require restricted conditions (Appenzeller and Tsatsakis, 2012). Hair is particularly useful for monitoring metals as the sulphydryl group of
the keratin present in the hair matrix has the ability to bind metal cations (Beernaert et al., 2007). The organism can excrete metals in hair via their continuous contact with the bloodstream during growth (Beernaert et al., 2007). Hair analysis can provide reliable information on exposure to non-essential metals (e.g. Cd and Pb) as these are accumulated in storage organs and then be deposited in the hair as well as in the nails, teeth, urine, faeces and sweat (Kales and Christiani, 2005). Unlike bone and stomach content analysis which can provide measures of long and short-term exposure to metals, respectively, hair reflects exposure during the period in which the hair is growing (Kales and Christiani 2005). Hair analysis can indicate metal bioavailability from the environment (McLean et al., 2009; Nolet et al., 1994; Pereira et al., 2004; Marcheselli et al., 2010). Hair has been defined as a biomarker in human studies (Kales and Christiani, 2005) and there is evidence for its utility in mammalian wildlife studies (McLean et al., 2009; Pereira et al., 2006; Beernaert et al., 2007).

In wild mammals, positive correlations have been determined between concentrations of metals in hair and blood (Vermeulen et al., 2009) and concentrations in kidneys, liver and muscle (Beernaert et al., 2007; Pereira et al., 2006; D’Havé et al., 2006; Marcheselli et al., 2010).

Bats can be particularly exposed to contaminants due to their relatively long life and high food intake (Hickey et al., 2001). In a recent modelling exercise for Pipistrellus sp. in England and Wales, we predicted that around 6% of areas where bats reside to have Pb levels that pose a risk to bat health, 3% for Cu, followed by Cd (0.6%) and Zn (0.5%) (Hernout et al., 2013). The exposure risks of soil-associated metals have been predicted to vary across bat species (Hernout et al., 2015). In order to determine the role of metal pollution in bat population declines, monitoring studies combining determination of metal concentrations via less-invasive methods and demographic parameters would be useful. It is vital that the assay is sensitive enough such that the sample of hair required is small enough that its collection
does not cause lasting harm to the animal, for example, affecting thermoregulation. In bat monitoring programs using radio-tracking approaches, the attachment of a transmitter is usually between the scapulae and the fur in this region (approximately a patch 0.5 x 0.5 mm) is clipped to allow attachment of the transmitter (Womack et al., 2013; Kosonen, 2013). These fur clippings are currently discarded but might be used for further analyses. However, very little is known about the validity of using bat fur as a biomarker of metal exposure, particularly when comparing concentrations determined in fur versus internal organs. A recent study on the utility of bat fur to assess metal exposure using a relative small sample size (Flache et al., 2015). Only one study has correlated Pb concentrations in hair and different tissues of fruit bats (Pteropus species), but this was also from a restricted number of individuals and for a limited geographic area (Hariono et al., 1993).

This research therefore investigated the suitability of bat fur as a non-invasive bioindicator of exposure to non-essential (i.e. Pb and Cd) and essential (i.e. Cu and Zn) metals. The evaluation was done using a dataset on metal contamination for a large number of bat individuals that were obtained from across a gradient of metal pollution across England and Wales.

2. Methods and Materials

2.1 Sample selection and processing

Adult males (n=193) of the Pipistrellus pipistrellus/pygmaeus species were obtained from across England and Wales (including five confirmed specimens of Pipistrellus pygmaeus). Metal can be transferred through lactation in females (Streit and Nagel, 1993) and metal (i.e. Cd) accumulation can correlate with the age of organisms (Rudy, 2009), therefore, we focused only on adult males. The individuals were selected from an archive of 3000 bats, collected in 2008, 2009 and 2010, and provided by the Animal Health and Veterinary
Laboratory Agency (AHVLA) (Surrey, England). Bats that were either found dead or died during rehabilitation, were submitted by bat conservation organizations and members of the public, working under license from Natural England where necessary. The bats were not killed for the purposes of this study. Bats were collected as part of the ongoing UK bat Lyssavirus surveillance system (McElhinney et al., 2013; Schatz et al., 2013). Bats were identified and after Lyssavirus screening, carcasses were kept in 40% formaldehyde solution by the AHVLA, a common preservation method for veterinary and museum samples, until metal concentration analysis was conducted.

To select the bats for analysis, data on metal concentrations in soils from the locations at which the 3,000 bats were found were acquired from the National Soil Resources Institute (NSRI) soil dataset (5 x 5 km resolution). The subsample of 193 bats was then selected to reflect the frequency distribution of soil metal concentrations across England and Wales (from the NSRI dataset). We first selected the samples to obtain similar frequency distribution from the soil concentrations of the locations in which bat were collected and the soil concentrations across the area of England and Wales, for each metal studied. Next, we purposefully included bats from soils with extreme concentrations of metals in soils to give complete spatial coverage across the area of England and Wales (Figure S1). We then tested for correlations between soil concentrations in the 5 x 5 km grid cell where a bat was collected and the specific tissue concentrations of that individual.

Prior to analysis, individuals were dissected to excise kidneys (n=191), liver (n=191), stomach (and stomach content) (n=168), fur (n=192) and bones (humerus, radius and femurs) (n=192). A small sample of 0.14 (± 0.19) g of fur (wet weight) was shaved using a ceramic scalpel, which corresponds to 8.6 x 10^-3 (± 5.5 x 10^-3) g of fur sample in constant dry weight. Fur samples were rinsed with Millipore water to avoid exogenous sources of contamination from our samples. The tissues were then dried until constant dry weight prior to extraction.
and analysis. Tissues in a bad conservation state or missing (previously extracted) have not been analyzed. An aliquot of formaldehyde (0.5 ml) (n=100) was also taken to quantify any metal that may have leached from the bat body into the preservative (See Supplemental information Text S1).

2.2 Quantification of metal concentrations

Metals (Cd, Pb, Cu and Zn) were selected for analysis since they are the most documented metals in several studies determining concentrations in bat tissues and risks of metals to bats (Carravieri and Scheifler, 2013; Clark and Shore, 2001; Hernout et al., 2013; Hernout et al., 2015). Prior to analyses by ICP-MS (Agilent 7500ce, Cheshire, UK), dried samples 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 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. Calibration standards were prepared in the same acid matrix.

A constant amount of the internal standards (rhodium and indium) was added to all tubes. Quantification was performed by internal standardization where the analyte signals and the internal standard signals were compared. Then the calibration curve was used to convert the analyte signal into concentration values. This method determines accurate concentrations and corrects for drift (changes in sensitivity over time) and matrix effects (sample-related changes in sensitivity). Standard quality insurance procedures were employed including analysis of method blanks, spiked samples and certified reference materials (bovine liver BCR 185R and spinach NCS ZC73013). (See Supplemental information Text S2).

2.3 Data analyses

Metal concentrations in bat tissue and soil (for Cd, Cu, Pb and Zn) were expressed as dry weight concentrations. These were not normally distributed (Shapiro-Wilk test: p<0.001)
Due to the variation in sample size between analyses, the detection limit (DL) was calculated for each tissue type and metal. For statistical analyses, metal concentrations below detection limit were replaced by an estimated value using the log-probit regression method (Helsel 1990, Sinha et al. 2006), implemented by the US EPA in the software ProUCL 5.0.00 (Singh and Maichle, 2013). Around 31%, 0.1%, 6% and 7% of the data were below DL for Cd, Cu, Pb and Zn, respectively. Tissue and soil concentrations of metal were ln-transformed prior to analysis. To determine the strengths of the associations between metal concentrations contained in fur versus the other tissues, and fur versus soil concentrations, we used Pearson correlation tests. As multiple correlation tests were applied, the p-values were adjusted using the Holm-Bonferroni method. The number of pairs (n) across the associations was not equal since tissues in a poor conservation state or missing (previously extracted) have not been analyzed. The results are presented in the Table 1, the strongest relationships ($r > 0.40$) were selected for representation (Figure 1), and the other relationships ($r < 0.40$) are presented in Figure S2. Further details on the statistical results can be found in Table S1 (Supplemental information). Data analyses were performed with the software R version 2.12.1.

### 3. Results and discussion

We observed significant positive relationships between concentrations of metals in fur and concentrations in the other internal tissues studied (stomach, kidneys, liver and bones) (Table 1, Figure 1, and Figure S2). The exceptions were observed for Zn and liver, for which the relationship was negative and non-significant, and Zn and kidneys, and Pb and liver for which the relationships were non-significant (Table 1, Figure S2). Similar results have been observed for several other mammal species. For example, positive relationships between fur and kidneys, liver and bones Pb concentrations were measured in fruit bats (Hariono et al., 1993). Nolet et al. (1994) showed positive relationships between metal concentrations in...
beaver hair and the bark of Salicaceae, which is their main food item. Positive correlations between concentrations in hair have been found with concentrations in kidneys and liver for Pb and Cd on the wood mice (Apodemus sylvaticus) (Tête et al., 2014; Beernaert et al., 2007) and for Pb and Cu in the European hedgehog (Erinaceus europaeus) (D’Havé et al., 2006). We found stronger positive correlations for non-essential than essential metals (Table 1, Figure 1, Figure S2), which can be due to their regulation by homeostatic mechanisms (D’Havé et al., 2006) and can suggest an effective regulation process of essential metals for bats, as described for mammals (McLean et al., 2009).

Bones, kidneys and liver are characterized as biomarkers of long-term and chronic exposure of contaminants for small mammals (Cooke 2011; Ma 2011). Our results indicate strong correlations between concentrations in fur and stomach and bones for Cd, and between concentrations in fur and stomach and bones for Pb (Table 1). Stronger positive associations were found between fur and bones than between fur and kidneys or fur and liver concentrations, for Cd and Pb (Table 1, Figure 1, Figure S2). These results are similar to the findings of Hariono et al. (1993) who worked with fruit bats. Kidneys and liver have a great potential to accumulate metals and are therefore, widely used as bioindicators to determined pollution level (Cooke 2011; Ma 2011). In contrast to bones, kidneys and liver can regulate their concentrations via level of metallothionein production and have a detoxification capacity (Shore and Douben, 1994; Ma and Talmage, 2001). In addition, renal concentrations of Pb have shown to reach a steady state in mammals in the later subadult stage of their development (Ma 2011), as shown in life-time exposed shrews (Sorex araneus) (Ma and van der Voet, 1993). In contrast to kidneys and liver, non-essential metals that bind to bone matrix are not readily accessible into the bloodstream (Ma, 2011). For example, the half time of Pb is around 10-30 years in bones and it is measured in months for soft tissues (Ma, 2011; Kales and Christiani, 2005). In addition, excessive Zn may be stored in bones and/or interacts with
other elements as suggested in Sanchez-Chardi and Lopez-Fuster (2009). Thus, concentrations in bones indicate a long-term and chronic metal exposure, particularly for non-essential metals (Cd and Pb). Our positive relationships between concentrations in bones, kidneys and liver and fur suggest that bat fur samples can be used as a biomarker of chronic metal exposure for bats, particularly for non-essential metals.

Our positive relationships between ingested food (stomach and stomach content) and hair contamination (Table 1, Figure 1) indicate that concentrations in fur can be used as a biomarker of recent sources of metal exposure, measured on a large number of individuals. The strong positive relationship between metal concentrations, particularly for Pb, followed by Cd, Cu and Zn, in fur and stomach contents can confirm the assumption that food is an exposure route of metal contamination for bats. For humans, consumption of animal-derived foodstuffs such as beef, among several other factors (i.e. drinking water, smoking habits, alcohol conditions and health condition) has also been shown to be the main exposure pathway for metal exposure based on the analysis of human hair (Pereira et al., 2004).

Non-significant positive correlations were observed between Cd concentrations in fur and in the soil of the area where the bat was collected (Table 1, Figure S2). A study on marsupial insectivores and rodents has shown good positive correlations between non-essential metal (Cd and Pb) concentrations in hair and soil sampling in the trapping areas, but not for essential metals (McLean et al., 2009). The strengths of the positive relationships were higher than in our study. This difference could be due to the high mobility and the large home range (which can reach 50 hectares) of Pipistrellus pipistrellus (Joint Nature Conservation Committee 2007) compared to the mammalian species studied in McLean et al. (2009). Concentrations in soils may not accurately reflect the environmental exposure and bioavailability of metals for bats, as many environmental and biological factors are playing a role in the exposure of contaminants through the food chain, such as: uptake from media (i.e.
soils) into prey items; the environmental factors affecting this uptake (e.g. pH, organic matter content); the oral exposure of the target species; the bioavailability and bioaccessibility of food items into predators; the foraging behavior, age and reproductive stage of the target species, etc. (Hernout et al., 2011). We would advocate that fur is a biomarker of the bioavailable fraction of metal, and a poor indicator of the total metal pollution in media, such as soil. Further spatial analysis could integrate habitat preferences as a potential factor influencing metal bioaccumulation in tissues of bats, as seen in other small terrestrial mammal species (Fritsch et al., 2010).

Despite the fact that good positive correlations were obtained between concentrations of metals in fur and several tissues, the correlations were not perfect. The variability might be explained by a range of variables such as age, diet and moulting which can impact the levels of metals contained in fur or hair. For example, Cd accumulation has been associated with age in small mammals (Goyer, 1996; cited by Ma and Talmage, 2001) We did not know the exact age of the individuals, which can reach up to 16 years for Pipistrellus pipistrellus (observed maximum age) (Dietz et al., 2009). In our study, only adult males were analyzed, probably reducing the variability in measured concentrations. Radio tracking, as well as OMICS (e.g. genomics, proteomics or metabolomics) and stable isotope studies, combined with hair analyses could provide better information on the foraging habitats and diet intake of bats. As hair bioaccumulates metal during the growing phase, moulting effects could be an important variable. Depending on the moulting stage, higher (before the moult) or lower (after the moult) concentrations could be measured in small mammals (Beernaert et al., 2007; Fraser et al., 2013). For adult bats, the moult occurs once a year, generally in the late summer (summer-fall) (Dietz, 2009). However, moulting cycles can be variable among species, sex and age classes (Fraser, 2013). As it was not possible to identify the month of death of the individual, but only the year, we could not assess the impact of moultng stage on hair metal content.
Further studies could investigate the relationships between contaminants concentrations in fur and other tissues, considering detailed individual factors, such as: aging, moulting phase, gender, habitats and diet, as well as inter-species factors. These information could refine our knowledge on the use of fur as a biomarker and provide the optimal conditions to biomonitor bats for exogenous contamination.

Formaldehyde is a widely used fixative solution for Museum’s collection, as we have used in our study. The potential leaching of metals (Cu and Cd) has been illustrated in invertebrate, fish and mammals tissues (Gellein et al., 2008; Hendrickx et al., 2003; Quan et al., 2002;). Formalin may oxidize to formic acid and lower the pH of the solution, resulting in an extraction of metals from the biological samples into the preservative medium (Simmons, 2014). Whereas the potential extraction of metals into the preservative solution may occur, others studies have presented contradictory results regarding the potential effects of the solution. These studies have not presented differences between the concentrations of metals in samples preserved in formaldehyde versus in fresh or frozen samples (of bovine liver and human tissues) (Bischoff et al., 2008; Bush et al., 2012; Theron et al., 1974). Others studies have presented higher concentrations of Cd, Cu, Pb and Zn in samples preserved in formaldehyde compared to frozen specimens (in arthropod and fish tissues) (Gibbs et al., 1974; Hendrickx et al., 2003). The potential interpretation of these results was the dissolution of tissues stored in formaldehyde resulting as a decrease of the dry weight of the samples, and thus, an increase of metal concentrations was determined in the invertebrate tissues (Hendrickx et al., 2003). Further studies are needed on the effects of the preservative solution on chemical analysis, and caution may be taken while interpreting the results. Therefore, when possible, fresh or frozen samples are preferred for metal analysis.

Another methodological bias could be involved by the potential external contamination in our fur samples. Although, the external contamination of metal could have been washed while
stored in the preservative solution, which could have the same effect than a washing using ethanol or acetone solution as used in Little et al., 2015 and Nam et al., 2012. Further studies should investigate the efficiency of the different washing solutions for metal analysis using fur and hair samples. A possible external contamination may have occurred, and therefore, overestimation of our concentrations of metals in fur samples cannot be excluded.

In conclusion, our data suggest that fur is suitable for monitoring long-term, chronic as well as recent exposure, particularly for non-essentials metals (Pb and Cd). Fur was an optimal biomarker for Pb, based on our strong positive relationships of concentrations determined with fur and stomach and bone samples. The use of fur is also strongly recommended to monitor potential accumulation in kidneys (for Pb, Cd and Cu), bones (for Cd and Zn), and stomach for (Cd, Cu and Zn). Despite inter-individual variations, potential underestimation (leaching in the preservative solution), and/or overestimation (external contamination for the fur samples) of our concentrations of metals due to methodological bias, we believe that the results presented here demonstrated that fur analysis could be a useful indicator for understanding the exposure of bat populations to metals. This approach could provide a valuable tool in further developing our understanding of the importance of metals as a driver for some of the observed declines in bat populations seen around the globe.
Acknowledgements

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Ethical approval: The authors declare that they have no conflict of interest. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted. This article does not contain any studies with human participants performed by any of the authors.
References


ProUCL 5.0.00 User Guide document (EPA/600/R-07/038)
**Table and figure legend**

**Table 1**: Relationships between bat fur and internal organs (kidneys, liver, stomach and stomach content, bones) and soil concentrations. Pearson’s coefficients (r) were used to evaluate the strength of these associations. As multiple correlation tests were applied, the p values were adjusted using the Holm-Bonferroni method. Asterisks (*) indicates significant correlation (p<0.05); (ns) indicates a non-significant correlation; and (n) indicates the sample size.

**Figure 1**: Relationships between log-transformed metal concentrations in bat fur and kidneys (Pb), stomach and stomach content (Pb, Cd and Cu) and bones (Pb and Cd) (µg/g dw). The trend line indicates the linear regression.

**Table 1**

<table>
<thead>
<tr>
<th>Metal</th>
<th>Kidneys (n=191)</th>
<th>Liver (n=190)</th>
<th>Stomach and stomach content (n=167)</th>
<th>Bones (n=191)</th>
<th>Soil (n=192)</th>
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<td>Cd</td>
<td>0.37*</td>
<td>0.23*</td>
<td>0.54*</td>
<td>0.53*</td>
<td>0.10 (ns)</td>
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<tr>
<td>Cu</td>
<td>0.39*</td>
<td>0.24*</td>
<td>0.41*</td>
<td>0.25*</td>
<td>0.09 (ns)</td>
</tr>
<tr>
<td>Pb</td>
<td>0.42*</td>
<td>0.10 (ns)</td>
<td>0.75*</td>
<td>0.72*</td>
<td>0.04 (ns)</td>
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<tr>
<td>Zn</td>
<td>0.19 (ns)</td>
<td>-0.07 (ns)</td>
<td>0.39*</td>
<td>0.36*</td>
<td>0.04 (ns)</td>
</tr>
</tbody>
</table>
Supplemental information

Text S1. Formaldehyde aliquot analyzes
Median trace metal concentrations determined in the preservative solution (40% formaldehyde) were: 0.43; 630.43; 21.60 and 351.07 µL/L for Cd, Cu, Pb and Zn, respectively (n= 100 aliquots of formaldehyde from 100 different bat individuals). It may therefore be the case that certain concentrations were underestimated, although a correction based on a quantitative value cannot be defined.

Text S2. Quality assurance and quality control (QA-QC)
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, 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.

The average spike recovery 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.
Table S1: Statistical results of the Shapiro (W), Pearson correlation (r) and holm Bonferroni tests, with their p values, applied on our dataset of metal concentrations in the different bat tissues and soil concentrations (NSRI dataset).

<table>
<thead>
<tr>
<th>Metal</th>
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<th>Stomach</th>
<th>Bones</th>
<th>Soil</th>
<th>Fur</th>
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<td></td>
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<td>Bonferroni Adjusted p value</td>
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<td>Shapiro test W</td>
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<td>P value</td>
<td>&lt; 2.2e-16</td>
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<td>&lt; 2.2e-16</td>
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<td>Pearson r p value</td>
<td>0.19</td>
<td>-0.07</td>
<td>0.39</td>
<td>0.36</td>
<td>0.04</td>
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<td>Bonferroni Adjusted p value</td>
<td>0.02119200</td>
<td>0.69200000</td>
<td>0.00000112</td>
<td>0.00000112</td>
<td>0.69200000</td>
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Figure S1: Histograms presenting the distributions of the soil concentrations of metals determined in the whole range of England and Wales (light grey) and the locations where the bat samples have been collected (dark grey). The soil concentrations of metals values were extracted from the NSRI dataset.

Figure S2: Relationships between log-transformed metal concentrations in bat fur and kidneys (Cd, Cu and Zn), liver (Cd, Cu, Pb and Zn), stomach and stomach content (Zn), bones (Cu and Zn), and soil (Cd, Cu, Pb and Zn) (µg/g dw). The trend line indicates the linear regression.