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How Do Richness and Composition of Diet Shape Trace Metal Exposure in a Free-Living Generalist Rodent, *Apodemus sylvaticus*

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1 How do richness and composition of diet shape trace
2 metal exposure in a free-living generalist rodent,
3 *Apodemus sylvaticus*.

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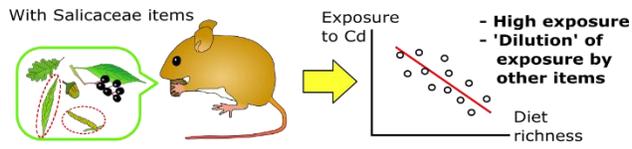
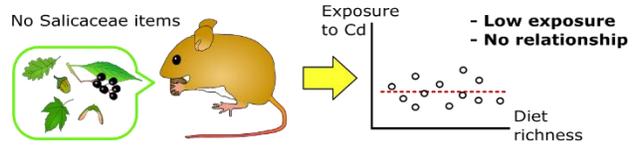
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13 **KEYWORDS**

14 Food identification; Metabarcoding; Trophic transfer; Wildlife; *Salicaceae*.

15 Abstract Art

TM contaminated site



16
17

18 **Abstract**

19 Exposure of terrestrial mammals to chemical contaminants like trace metals (TMs) is considered
20 to be mainly based on trophic transfer. Although relationships between TM transfer to animals
21 and identity of contaminated food have been studied, the variation of the TM transfer with
22 respect to diet diversity has been poorly documented. In this study, the oral exposure to TMs of
23 wood mice *Apodemus sylvaticus* was investigated with respect to both the number of different
24 items, i.e. diet richness, and the identity of items determined by metabarcoding from their
25 stomach content, i.e. diet composition. The results showed that consuming Salicaceae, a known
26 cadmium accumulator plant family, significantly increased exposure to cadmium and zinc.
27 However, an increase in diet richness minimized exposure to cadmium when mice consumed
28 Salicaceae items. This strongly suggests that TM accumulator items can lead to a high oral
29 exposure to TMs but that such high exposure due to TM accumulator items can be “*diluted*” by
30 diet richness due to other low accumulator items. Our results clearly indicate that both the
31 presence of certain items in the diet and diet richness are important determinants of exposure to
32 TMs in generalist animals, which matches the predictions of the “*diet dilution hypothesis*”.

33 **Introduction**

34 Trace metals (TMs) are naturally occurring substances in trace amounts, in a proportion below
35 0.1%, in natural media.¹ Although some of them are essential for life, TMs can pose risks to both
36 wildlife and human health at elevated concentrations resulting from anthropogenic activities.²
37 The assessment and the mitigation of environmental risk of TM contamination of ecosystems
38 require both a sound understanding and an accurate estimate of the exposure of free-living
39 organisms to TMs. The exposure of wild mammals to TMs occurs mainly through consumption
40 of contaminated food.³ The composition of diet has been focused on as an important aspect of
41 the variation of exposure of small mammals to TMs. For instance, earthworms or snails,
42 organisms that may accumulate TMs at high concentrations in their tissues, have been considered
43 to be important contributors for exposure to TMs in mammals consuming them.⁴⁻⁶ However,
44 mammals can consume simultaneously numerous different items. Although ingesting some metal
45 accumulator items leads to an increase in exposure, many other less TM accumulator items could
46 reduce total TM concentrations in the diet. A wide variety of items consumed may result in two
47 opposite effects: increased opportunity of consuming TM accumulators or reduced TM
48 concentrations of ingested food. However, few studies have assessed the accurate diet
49 composition and richness related to the TM contamination in mammals,⁷ and, to our knowledge,
50 no study have investigated the relationships between the oral exposure of mammals to TMs and
51 such diet richness.

52 The accurate determination of items actually consumed is a major challenge in wildlife diet
53 assessment because identification at high taxonomic resolution is almost impossible with
54 classical macro- or microscopy-based methods. However, current technological development
55 such as high-throughput next generation sequencing has led a rise of new DNA-based techniques

56 as a powerful approach for multispecies identification using degraded DNA extracted from wide
57 range of environmental samples, i.e. soil, water or feces (*eDNA metabarcoding*).^{8–12} The
58 efficiency of the eDNA metabarcoding has been demonstrated in diet analysis in various
59 mammals.^{13–17} This method is expected to identify in detail the diet composed of numerous items
60 in generalist small mammals like the wood mouse (*Apodemus sylvaticus*),^{18–21} a rodent widely
61 spread in Europe²² and frequently used for monitoring of environmental metal pollution,^{5,6,23–25}
62 and then to allow associating the oral exposure of the rodent to TMs and both the composition
63 and the richness of their diet.

64 The present study analyzed the relationship between exposure to TMs of wood mice captured
65 along a gradient of soil TM contaminations in a smelter-impacted area and their diet determined
66 by metabarcoding from their stomach content. We hypothesized that (1) the consumption of
67 (hyper-)accumulator items like earthworms induces a high exposure of small mammals to TMs
68 and that (2) such exposure is shaped by the diet richness due to a “dilution” effect where high
69 exposure related to accumulator items is reduced by the consumption of less accumulator items
70 in case of high diet richness.

71

72 **Materials and methods**

73 **Study Area.** The study area was located in Northern France (Noyelles Godault, Hauts-de-
74 France, France). Due to metal containing dust released from the former lead (Pb) and zinc (Zn)
75 smelter “Metaleurop Nord”, the soils surrounding the smelter are highly contaminated by TMs,
76 especially cadmium (Cd), Pb and Zn.^{26–29} Total TM concentrations in soils of woody habitats in
77 an area of 40km² around Metaleurop Nord range from 0.1 to 237 mg kg⁻¹ of dry soil for Cd, from
78 16 to 7331 mg kg⁻¹ for Pb, and from 44 to 7264 mg kg⁻¹ for Zn, which dramatically exceed the

79 concentrations in a reference site (0.9-2, 43-200 and 89-278 mg kg⁻¹ of dry soil for Cd, Pb, and
80 Zn, respectively) from the same region.³⁰ The present study was undertaken on seven sites of
81 500m x 500m, located along a soil contamination gradient and composed of three types of
82 dominant habitats (for details see Supporting Information Table S1). TM concentrations in soils
83 of woody habitats, expressed as mg kg⁻¹ of dry soil, were based on data published in Fritsch et
84 al.³⁰ and used for statistical analyses.

85

86 **Sample Collection. Rodent Trapping.** Wood mice were captured in spring (April) and in
87 autumn (September and October) 2012, in accordance with current French legislation about
88 ethics and use of animals in research. In each season and each site, 10 trap lines composed of 10
89 small break-back traps (3 m spaced each) were used with peanut as rodent's bait following
90 Fritsch et al.³¹ All trap lines were set in woody habitats and their position was geo-referenced.
91 The trap lines were checked in the morning for 3 consecutive days and re-set and/or re-baited, if
92 necessary. Captured animals were immediately frozen and stored at -20°C for further analyses in
93 the laboratory. For this study, 200 wood mice composed of 95 females, 103 males and two
94 individuals whose sex could not be identified were used. The sex-ratio was not significantly
95 different from 1:1 (chi-squared = 0.32, *p-value* = 0.57). One hundred and three mice were taken
96 in spring and 97 in autumn. The ratio between the two seasons was not significantly different
97 from 1:1 (chi-squared = 0.18, *p-value* = 0.67).

98 *Stomach Content Extraction.* Stomach content (SC) was extracted with a spatula from each
99 rodent's body thawed at room temperature. After removing remaining bait, each SC was
100 homogenized and split into two aliquots. One aliquot (about 10 mg) was stored in 95 % ethanol
101 for the metabarcoding analysis. The other was used for measuring TM concentrations which

102 were used to estimate exposure of mice to metals *via* the food ingested within the last few hours.
103 The spatula was thoroughly cleaned with disposable tissues, then washed with ultra-pure water
104 (18.2 MΩ/cm² by Millipore Milli-Q Integral 3) and wiped off with other tissue between each
105 extraction.

106

107 **Metal Analysis.** The SCs for TM concentration analysis were dried at 50°C in an oven until
108 obtaining constant mass. The dried samples were digested in HNO₃ (67-69 %; Fisher Scientific
109 Bioblock, ultratrace quality Optima) with H₂O₂ (Fisher Scientific Bioblock) by using Digiprep
110 (SCP Sciences). The samples were then diluted by adding ultra-pure water (18.2 MΩ/cm²). TM
111 concentrations were measured with an inductively coupled plasma mass spectrometry (ICP-MS:
112 X Series II, ThermoFischer Scientific) and expressed as µg g⁻¹ of dry mass (DM). Analysis
113 accuracy was checked by using certified reference materials (INCT-OBTL-5: Oriental Basma
114 Tobacco Leaves). Mean and standard deviation of recovery rate for Cd, Pb and Zn were
115 respectively 107.2 ± 4.7, 83.6 ± 9.6 and 100.7 ± 9.1 %.

116

117 **Diet Assessment Using Metabarcoding Analysis.** *DNA Extraction, Amplification and*
118 *Sequencing.* The DNA extraction, amplification and the PCR purification were performed at
119 SPYGEN facilities (www.spygen.com). DNeasy Blood and Tissue Kit (Qiagen GmbH) was used
120 for extracting total DNA, following the manufacturer's instruction. DNA amplification was
121 performed with three sets of primers: primer targeting the P6 loop of the chloroplast *trnL* (UAA)
122 intron *g/h*³² was used for identifying general plant species, primer targeting a short fragment of
123 mitochondrial 16S gene (16S mtDNA) was for arthropod and mollusk DNA (*16SMAV-*
124 *F/16SMAV-R*)¹⁶, and primer targeting a short region of 16S mtDNA for earthworm (*ewD/ewE*)³³

125 (for details of those primers, see Supporting Information Table S2). PCR amplifications were
126 carried out on Applied Biosystems Veriti 96 Wells (Life Technologies). The amplification was
127 realized in a final volume of 25 μ L using 3 μ L of DNA extract. Two PCR replicates were
128 performed per each sample. For the amplification of arthropod and mollusc DNA, 2 μ M of a
129 blocking primer for mammal's DNA (*MamMAVBI*¹⁶) were added in the PCR mix. The
130 amplification mixture contained 1 U of AmpliTaq Gold DNA Polymerase (Applied Biosystems),
131 10 mM of Tris-HCl, 50 mM of KCl, 2.5 mM of MgCl₂, 0.2 mM of each dNTP, 0.2 μ M of group-
132 specific primers, 0.2 μ g/ μ L of bovine serum albumin (BSA, Roche Diagnostic) and ultra-pure
133 water (18.2 M Ω /cm²) to bring each sample to the final volume. The mixture was denatured at
134 95°C for 10 min, followed by 45 cycles of 30 s at 95°C, 30 s at 50°C for *trnL-g/h*, at 55°C for
135 *16SMAV-F/16SMAV-R* and at 58°C for *ewD/ewE* and 1 min at 72°C, followed by a final
136 elongation at 72°C for 7 min. Extraction and PCR negative controls were analyzed in parallel in
137 order to monitor potential contamination. After amplification, the samples were purified using a
138 MinElute PCR purification kit (Qiagen GmbH). Before sequencing, purified DNA was titrated
139 using capillary electrophoresis (QIAxcel; Qiagen GmbH). The purified PCR products were
140 pooled in equal volumes, to achieve an expected sequencing depth of 10,000 reads per sample.
141 Libraries were prepared using TruSeq Nano DNA genomic kit (Illumina) and a pair-end
142 sequencing (2x100 bp) was carried out with an Illumina HiSeq sequencer (Illumina) using
143 TruSeq SBS Kit v3 (Illumina) following the manufacturer's instructions. Library preparation and
144 sequencing were performed at Fasteris facilities (www.fasteris.com).

145 *Bioinformatics*. Reads were handled by the software Mothur pipeline.³⁴ Forward and reverse
146 reads were assembled in contig sequences. Sequences were filtered based on length (20-90bp for
147 *trnL-g/h*; 36-38bp for *16SMAV*; 69-81bp for *ewD/ewE*), homo-polymer (less than 10 nucleotides)

148 and no ambiguous nucleotides. After de-replication (count occurrence of each different
149 sequence), only unique sequences with a minimum count of 10 (sum of all samples) were used
150 for further analyses. The data was applied both to diet richness estimation and to identification of
151 food composition.

152 *Diet Richness Estimation.* Molecular Operational Taxonomic Units (MOTUs) were used for
153 measuring diet richness. Sequences were clustered for each primer with average neighbor
154 algorithm using Needlman-Wunsch distance. The cutoff values were chosen as effective
155 clustering on the basis of relationships between number of clusters and distance: 0.032, 0.042,
156 and 0.034 for sequences obtained from the primers *trnL-g/h*, *16SMAV* and *ewD/ewE*,
157 respectively. After using an occurrence of 100 as a threshold to remove background sequencing
158 data, the sequence data were converted into presence/absence of MOTUs because the number of
159 sequences from degraded DNA is likely not proportional to the biomass of each taxa really
160 eaten.³⁵

161 *Food Composition.* For the detail of food identification method, see Ozaki et al.³⁶ Briefly, each
162 sequence extracted from SCs (i.e., query sequence) was compared with reference sequences of
163 species recorded in the study area,³⁶ taken from the GenBank sequence database
164 (www.ncbi.nlm.nih.gov/genbank). When query sequence matched with more than one reference
165 sequence, query sequences were gathered into one group composed of the corresponding species,
166 and then gathered at the family level. The sequences were finally converted into
167 presence/absence of each plant family in SCs. Invertebrate resources in the field were identified
168 mainly at family level, which could not allow to identify invertebrate food. We used only the
169 presence/absence of arthropod and mollusk items (referred to as only “*arthropod*” hereinafter)
170 and earthworm items in SCs as food composition.

171
172 **Statistical Analysis.** Statistical analyses were carried out on the 200 mice. As TM
173 concentrations in SCs could be influenced both by soil TM contamination levels through food
174 and ingestion of soils,^{37,38} we aimed to reduce potential bias related to the level of environmental
175 contamination when quantifying oral exposure as follows. For each TM, a linear positive
176 relationship was observed between the concentrations in SCs and in soils of the woody habitat
177 where each mouse was captured (logarithmically transformed concentrations, *p-values* of
178 ANOVA < 0.05; for details see Supporting Information Figure S1). The residuals of these linear
179 models were referred to as “*exposure*” hereinafter and used in following statistical analyses.

180 *Exposure to TMs and items consumed.* The exposure was compared between the two seasons,
181 and presence and absence of each identified item in SCs by the non-parametric Wilcoxon-Mann-
182 Whitney test. Among the identified items consumed by wood mice (for details see Ozaki et al.³⁶),
183 only items whose occurrence was higher than 10 in the diet of the 200 mice were used in this
184 comparison between presence/absence of items to ensure statistical power of tests: arthropods,
185 earthworms, Betulaceae (e.g. genera *Alnus*, *Betula*), Fagaceae (*Fagus*), Oleaceae (*Fraxinus*),
186 Poaceae (*Arrhenatherum*, *Holcus Lolium*, etc), Rosaceae (*Crataegus*), Sapindaceae (*Acer*),
187 Salicaceae (*Populus*, *Salix*), and the sequence group composed of Adoxaceae (*Sambucus*),
188 Asteraceae (*Hedera*) and Cornaceae (*Cornus*) items (referred to as “*group AAC*” hereinafter).
189 When exposure was significantly related to several variables tested, we used conditional
190 inference trees (CIT³⁹) for ranking their importance for the exposure to TMs. This is a recursive
191 binary partitioning analysis that studies significant univariate splits over all possible splitting
192 variables. The splitting variable the most significantly associated to response value will be
193 chosen. Those steps are recursively performed to the two split data, until no significant

194 difference are observed. Permutation tests by Strasser and Weber⁴⁰ are applied for the splitting
195 tests. In this study, CIT was performed across binary partitioning variables: season (spring or
196 autumn) and the presence/absence of the 10 types of items mentioned above. Sites were
197 considered to be random effects for exposure in the Wilcoxon-Mann-Whitney test and CIT
198 because of potential effects linked to each site. Those tests were executed for the residuals of the
199 random effect model, i.e. the model with only site as random effect. As all items were used as
200 partitioning variables without taking into account the result of each Wilcoxon-Mann-Whitney
201 test, *p-value* of CIT was not adjusted by Bonferroni correction.

202 If CIT suggested an influence of TM of hyper-accumulator items, we then checked whether
203 exposure was explained by the importance of the given items in the diet. In general, ascertaining
204 abundance information using metabarcoding of environmental DNA (eDNA) still lacks
205 consistent evidence due to multiple factors distorting the eDNA–biomass relationship, such as
206 difference in origin (species, organ, etc.) and stability of eDNA and primer bias in
207 amplification.⁴¹ This is particularly true when comparison of different taxa, identified using
208 different primers, is an objective. We calculated the proportion of DNA reads of hyper-
209 accumulator items suggested by CIT in the SC over the total number of DNA reads. In doing so,
210 we did not expect strong bias in comparison because (i) each hyper-accumulator was identified
211 using a single primer and (ii) we assumed that its DNA stability would remain comparable
212 among samples. We therefore assumed that changes in the biomass of a hyper-accumulator
213 ingested would be reflected by changes in the proportion of its DNA sequences over the total
214 number of DNA sequences.

215 Relationships between exposure and the proportion of sequences of the given hyper-
216 accumulator items within SCs containing the given items was analyzed using a linear mixed

217 model with the site as random effect. The variations explained by the fixed and random effects
218 were estimated by marginal R^2 (R^2_m) and conditional R^2 (R^2_c), respectively.⁴² Relationships
219 between the proportion of the given items and diet richness was also checked using a linear
220 mixed model.

221 *Diet of wood mice.* Co-occurrence of items, which could mislead into a fault interpretation in
222 the relationships between exposure and a given item consumed, was checked by CIT: Frequency
223 of occurrence of each plant item was assessed across season and the presence/absence of the
224 other plant items in SCs. In addition, relationship between occurrence of items and TM
225 concentration in soils was analyzed by generalized linear models with logit function for binary
226 data. Probability for occurrence of a given item was compared to a gradient of each TM
227 concentrations in soils. Significance of each model was checked by the likelihood-ratio test
228 compared to the null model.⁴³ Meanwhile, diet richness across season was checked by the
229 Wilcoxon-Mann-Whitney test. Each correlation between TM in soils and diet richness was
230 analyzed by the non-parametric Spearman correlation test.

231 *Exposure to TMs and diet richness.* Number of plant, arthropod, and earthworm MOTUs in SC
232 were defined as three independent diet richness, and the sum of the three richness as total diet
233 richness. A linear mixed model with the site as random effect was built and analyzed for the
234 exposure to each TM with respect to the total diet richness and to the three richness. The
235 relationships between the exposure, the diet richness, and the presence of specific items were
236 analyzed by model-based recursive partitioning (MOB⁴⁴). The principle of MOB is the same as
237 CIT, i.e. recursive binary partitioning of data by statistical approach, but each terminal node is
238 associated to a model rather than to a variable like CIT. After establishing a model and deciding
239 partitioning variables to involve, MOB assesses whether the model parameters are stable with

240 respect to partitioning variables. If there is a significant instability,^{45,46} the analysis selects the
241 partitioning variable associated with the highest instability and computes the split point (for
242 quantitative variables) or split class (for qualitative variables) which optimizes the parameter
243 stability. Those steps are repeated in the two split data, until instability is not significant. Finally,
244 significance of explanatory variables in each terminal node was assessed by a type III ANOVA
245 ($p\text{-value}_{node}$). In our case, MOB was executed on the basis of mixed linear models.⁴⁷ Partitioning
246 variables were the same as for CIT, and $p\text{-value}$ of partitioning test was not adjusted. The
247 variations explained by the fixed and random effects of the whole model were estimated by R^2_m
248 and R^2_c , respectively.⁴² Explanatory value of the fixed effects in a terminal node (R^2_{node}) was also
249 calculated.

250 All statistical analyses were computed using the statistical software R (ver. 3.4.2; R
251 Development Core Team). The mixed linear models were carried out based on “*lme*” function in
252 “*nlme*” package. CIT was performed by “*ctree*” function in “*partykit*” package, and MOB based
253 on linear mixed models was performed by “*lmertree*” function in “*glmertree*” package.

254

255 **Results**

256 **Diet of mice.** *Occurrence of items.* The frequency of occurrence of plant, arthropod and
257 earthworm items in stomach content of mice is 96.5, 72.5, and 27.0 %, respectively. No
258 significant difference between the two seasons was observed. Frequencies of occurrence of
259 Fagaceae, Oleaceae, Salicaceae, and Sapindaceae items were significantly higher in spring than
260 in autumn ($p\text{-value} < 0.001$ for each). Conversely, Poaceae and AAC group items were more
261 frequent in autumn ($p\text{-value} < 0.001$ for each). The details of the results are provided in
262 Supporting Information Table S3.

263 The frequency of occurrence of each item was also dependent on the presence of other items.
264 In spring, the frequency of occurrence of Salicaceae items in SCs was significantly and
265 negatively related to the presence of Sapindaceae items (Supporting Information Figure S2a; *p*-
266 *value* < 0.001). The frequency of occurrence of Salicaceae items was also significantly and
267 negatively related to the presence of the group AAC items when Sapindaceae items were absent
268 in spring (*p*-*value* < 0.024). This trend was similar for the frequency of occurrence of
269 Sapindaceae items: in spring, the frequency of occurrence of Sapindaceae items was significantly
270 lower when Salicaceae items were present in SCs, followed by the presence of the group AAC
271 items only when Salicaceae items were absent (Supporting Information Figure S2b; *p*-*value* <
272 0.001 and = 0.019, respectively). The frequency of occurrence of Fagaceae items in autumn was
273 significantly higher when Betulaceae items were present in SCs (Supporting Information Figure
274 S2c; *p*-*value* < 0.001). Frequency of occurrence of Oleaceae items was significantly higher when
275 Sapindaceae items were present in SCs (Supporting Information Figure S2d; *p*-*value* < 0.01).
276 When Sapindaceae items were absent, the frequency of occurrence of Oleaceae items was higher
277 when Fagaceae items were present in SCs (*p*-*value* = 0.01).

278 Along the gradient of soil TM concentration, the occurrence of both arthropod and earthworm
279 items did not significantly differ (data not shown). The occurrence of both Salicaceae and
280 Fagaceae items significantly reduced along the gradient of soil TM concentrations only in spring
281 (Supporting Information Figure S3a and S3b; *p*-*value* < 0.05 for each of the three TMs in soils),
282 whereas the occurrence of Sapindaceae items significantly increased along the gradient
283 (Supporting Information Figure S3c and S3d; *p*-*value* < 0.05) in both spring and autumn. The
284 occurrence of the group AAC items significantly increased along the gradient only in autumn
285 (Supporting Information Figure S3e; *p*-*value* < 0.05).

286 *Diet richness.* Plant, arthropod and earthworm diet richness ranged from 0 to 6 MOTUs
287 (median: 2), from 0 to 9 (median: 2), and from 0 to 3 (median: 0), respectively. Total diet
288 richness, as well as both plant and arthropod diet richness, were significantly higher in autumn
289 than in spring (data not shown, $W = 3463$, $p\text{-value} < 0.001$; $W = 3646$, $p\text{-value} < 0.001$; $W =$
290 4190.5 , $p\text{-value} = 0.04$, respectively). No significant correlation between the three diet richness
291 values was observed, either in spring or in autumn (Supporting Information Figure S4a and S4b).
292 Only plant diet richness in spring significantly decreased along the gradient of TMs in soils
293 (Spearman's ρ from -0.26 to -0.19, $p\text{-value} < 0.05$ for the three TMs; Figure S4a).

294 **Exposure to TMs and items consumed.** Cd, Pb and Zn concentrations in SCs ranged from
295 0.03 to 47.1 (median: 0.93), from 0.03 to 194.2 (median: 3.70) and from 5.3 to 337.5 (median:
296 71.1) $\mu\text{g g}^{-1}$ DM, respectively. The TM concentration in SCs and the exposure were significantly
297 higher in spring than in autumn for the three TMs ($p\text{-value} < 0.05$; for details see Supporting
298 Information Figure S5). The exposure to Cd was significantly higher when Salicaceae items were
299 present in SCs and significantly lower when AAC plants were present in SCs ($W = 2559$ and
300 5986 ; $p\text{-value} < 0.05$, respectively; Supporting Information Figure S6a). The exposure to Zn was
301 significantly higher when Salicaceae items or Oleaceae items were present in SCs ($W = 3073$
302 and 524 ; $p\text{-value} < 0.05$, respectively; Figure S6b). The exposure to Pb was not influenced by
303 any item consumed.

304 CIT analysis revealed that season was the most important variable influencing the exposure of
305 rodents to Cd ($p\text{-value} < 0.001$), followed by the presence of Salicaceae items in SC in spring
306 (Figure 1a; $p\text{-value} = 0.01$). The exposure to Cd was the highest when Salicaceae items were
307 present in SC in spring, followed by when Salicaceae item were absent in spring, and the lowest
308 in autumn. For Zn, season was the most important variable ($p\text{-value} < 0.01$), and the presence of

309 Salicaceae items in SC determined the exposure to Zn only in autumn (Figure 1b; p -value <
310 0.05). The exposure to Zn was the highest in spring, followed by when Salicaceae items were
311 present in SCs in autumn, and the lowest when Salicaceae item were absent in SCs in autumn.
312 No significant partitioning variable was observed for exposure to Pb.

313 Only exposure to Cd was positively explained by the proportion of sequence number of
314 Salicaceae items (Figure 2a; p -value < 0.05). R^2_m and R^2_c were 0.079 and 0.251, respectively.
315 The proportion of sequence number of Salicaceae items was negatively correlated with diet
316 richness of plant (Figure 2b; p -value < 0.01; $R^2_m = 0.154$; $R^2_c = 0.280$).

317 **Exposure to TMs and diet richness.** Exposure to Cd was significantly and negatively
318 correlated, although weakly, only with the diet richness of earthworms (p -value = 0.04) but not
319 with other richness values ($R^2_m = 0.021$ and $R^2_c = 0.103$; data not shown). The relationship
320 between exposure to Cd and the total diet richness was the most significantly conditioned by the
321 presence/absence of Salicaceae items in SCs (the first oval in the upper part of Figure 3a; p -value
322 < 0.001). When Salicaceae items were present in SCs, the exposure was negatively correlated
323 with the diet richness (the scatter plot in the lower part of Figure 3a linked to “Salicaceae” by the
324 line with “yes”; p -value_{node} < 0.01, $R^2_{node} = 0.184$). Season also significantly conditioned the
325 relationship only for SCs in which Salicaceae items were absent (p -value < 0.01). In spring, the
326 presence of earthworm items significantly conditioned the relationship for SCs in which
327 Salicaceae items were absent (p -value = 0.03). In autumn, the presence of Poaceae items
328 significantly conditioned the relationship for SCs in which Salicaceae items were absent (p -value
329 = 0.03). When both Salicaceae and Poaceae items were absent in SCs in autumn, the exposure
330 was positively correlated with the diet richness (p -value_{node} = 0.04, $R^2_{node} = 0.110$). The other
331 cases showed no significant relationship between the exposure and the total richness. The

332 relationship between exposure to Cd and the three diet richness values was the most significantly
333 conditioned by presence/absence of Salicaceae items in SCs (Figure 3b; $p\text{-value} < 0.001$). The
334 exposure was negatively correlated with diet richness of plant when Salicaceae items were
335 present in SCs ($p\text{-value}_{node} < 0.001$, $R^2_{node} = 0.220$). Season significantly conditioned the
336 relationship when Salicaceae items were absent in SCs ($p\text{-value} = 0.03$) but the exposure showed
337 no significant relationship with any diet richness. R^2_m and R^2_c of the models for the total richness
338 were 0.196 and 0.275 and for the three richness were 0.191 and 0.276, respectively.

339 No significant relationship was observed between the exposure to Zn and both the total and the
340 three richness. However, the relationship between the exposure to Zn and the total diet richness
341 was significantly conditioned by season (Figure 3c; $p\text{-value} < 0.01$). The exposure was positively
342 correlated with the total diet richness in autumn ($p\text{-value}_{node} = 0.04$, $R^2_{node} = 0.106$). The
343 relationship between the exposure to Zn and the three diet richness was significantly conditioned
344 by season ($p\text{-value} < 0.01$), followed by the presence of Betulaceae items in SC in autumn
345 (Figure 3d; $p\text{-value} = 0.02$). The exposure to Zn was significantly and negatively correlated with
346 the diet richness of earthworm when Betulaceae items were present in SCs in spring ($p\text{-value}_{node}$
347 $= 0.01$, $R^2_{node} = 0.687$; it is noteworthy that only two SCs among 12 ones contained 2 different
348 earthworm items). R^2_m and R^2_c of the models for the total richness were 0.060 and 0.187 and for
349 the three richness were 0.098 and 0.196, respectively.

350 No significant relationship was observed between the exposure to Pb and both the total and the
351 three richness. There was no significant conditioning factor for exposure to Pb.

352

353 **Discussion**

354 Our study shed light on the role of diet richness and composition of food on the exposure to
355 TMs in a generalist species. Our results revealed that high diet richness reduces exposure to
356 TMs, particularly in case of consumption of at least one hyper-accumulator taxa, which however
357 depends on both the metal considered and type of items consumed.

358 Consuming plant items belonging to Salicaceae family significantly raised the trophic
359 exposure of wood mice to both Cd and Zn. Although precaution should be taken in interpreting
360 relationships between number of DNA sequences and biomass consumed (e.g. Deiner et al.⁴¹), a
361 high proportion of Salicaceae family plants in food, which may be considered as a proxy of
362 greater biomass of Salicaceae family plants in the SC, also increased exposure to Cd. Our
363 findings indicated that consuming Salicaceae plants items increased trophic exposure of mice to
364 Cd. Salicaceae family actually includes taxa accumulating Cd to high concentrations in their
365 tissues like leaves (e.g. genera *Salix* and *Populus*),⁴⁸ and their importance on the exposure of
366 mammals to Cd has been suggested. For instance, Nolet et al.⁴⁹ showed positive correlations
367 between Cd concentrations in tissues (hairs and kidneys) of beavers *Castor fiber* and in their
368 main food item, bark of genera *Salix* and *Populus*. These genera also accumulate Zn. Brekken
369 and Steinnes⁵⁰ argued that genera *Salix* and *Populus* could be the most important Cd and Zn
370 contributors for moose *Alces alces* in a metal-contaminated area. Migeon et al.⁵¹ reported higher
371 concentrations of Cd and Zn in both leaves and stems of genera *Salix* and *Populus* than in the
372 other woody species around Metaleurop Nord. The authors also demonstrated their high
373 bioconcentration factors (i.e. ratio of metal concentrations in leaves and in soils) which reached 2
374 and 1.2 for Cd and Zn, respectively. Our study confirmed that Salicaceae items were obviously
375 important contributors of Cd and, to a lesser extent of Zn, to wood mice Furthermore, our results
376 demonstrated that high Cd concentration due to Salicaceae items was “diluted” by other plant

377 items, potentially less Cd accumulators than Salicaceae items. Such phenomenon was postulated
378 by Boyd⁵² under the name of “*diet dilution hypothesis*”: trophic exposure of vertebrate
379 herbivores to TMs by high metal accumulator plants would be diluted by consuming low
380 accumulator resources. Indeed, high diet richness of plant might lead to dilute the proportion of
381 Salicaceae items in SCs in our analyses. While this possibility has been suggested in other field
382 studies (e.g. Martens and Boyd⁵³), we clearly demonstrated, to our knowledge for the first time,
383 the validity of this hypothesis under field conditions.

384 However, we failed to identify potential contributor of exposure to Pb. Migeon et al.⁵¹
385 demonstrated an interspecific variation of Pb concentrations in plant tissues over our study area,
386 although less contrasted than for Cd or Zn. One possibility could be an effect of soil ingested by
387 mice. Quantity of soil ingested by small rodents represents only a few percent of dry mass in the
388 diet, which was quantified to be maximum of 2% for the white-footed mouse.³⁷ Lead
389 bioconcentration factors of woody plants in our study area were very low, ranging from 0.005 to
390 0.11,⁵¹ suggesting a low mobility of Pb towards plants. The same pattern was observed over this
391 area for invertebrates (earthworms, mollusks, arthropods) which exhibited low biota-to-soil
392 concentration ratios for Pb (mostly lower than 0.1, and lower than for Cd and Zn).^{54,55} It is thus
393 possible that a large part of Pb in SCs could be derived from soils and that it may obscure any
394 effect of dietary richness or composition on Pb concentration in SCs. Effects of geophagy on
395 exposure to TM and/or accumulation of TM in animals remain as a further issue that
396 undoubtedly deserves attention.

397 On the other hand, consuming invertebrate items, especially earthworms that are well-known
398 as high TM accumulator species,⁵⁶ did not increase the exposure of wood mice to TMs in our
399 study. Accumulation patterns in earthworms exposed to soils can be strongly modified by

400 environmental factors like pH or organic matter content in soils,⁵⁷⁻⁵⁹ which may explain the
401 discrepancy between our results and the literature emphasizing the importance of earthworms on
402 the exposure of mammals to TMs.^{5,6,60} Further, trophic availability of metals accumulated in the
403 various diet items can be different according to their sequestration forms in item tissues, which
404 may also or rather lead to such discrepancies with literature.⁶¹ Moreover, some invertebrates
405 show high metal accumulation capacity, e.g. snails, isopods, or spiders,⁶²⁻⁶⁶ whereas others, e.g.
406 Coleoptera,⁶⁵ accumulate less metals in their tissues. For instance, Fritsch et al.⁵⁴ showed that
407 trophic transfer of TMs to blackbirds *Turdus merula* was less important in habitats linked with a
408 diet based on earthworms than in habitats linked with a diet based on other invertebrates in the
409 surroundings of Metaleurop Nord. They also demonstrated close to or even higher transfer
410 factors (i.e. metal concentration in items divided by metal concentration in soils) of some other
411 invertebrate taxa than earthworms for Cd and Pb. In the present study, invertebrate items could
412 not be specified further than large categories like “arthropods and mollusks” or “earthworms”,
413 and our low taxonomic resolution of invertebrate items could make unclear the role of animal
414 matters on the TM exposure of mammals.

415 Few studies have focused on the relationship between exposure to TMs and diet richness. To
416 our knowledge, only Orłowski et al.⁷ showed significant correlations between the number of
417 items in the diet of nestling rooks *Corvus frugilegus* and accumulation of TMs in their tissues. In
418 that study, Pb concentrations in the liver were negatively correlated with the number of vegetal
419 items, while Cd concentrations were positively correlated with the number of animal items.
420 However, oral exposure to TMs is not always correlated with TM accumulation in tissues. In the
421 study of Godwin et al.⁶⁷ for example, TM concentrations in stomach contents and in tissues of
422 tree swallow *Tachycineta bicolor* nestlings of the age of 14 days showed no correlation for Ni,

423 Sr, and Zn, negative correlations for Mo and Cu, and correlations depending on year and/or
424 tissue for other elements including Cd and Pb. TM accumulation in animal tissues is determined
425 by several other factors than oral exposure, like species, age, sex and exposure by other routes.⁶⁸
426 Assimilation of elements via oral route is modulated in intestines by composition of micro- and
427 macro-nutrients, such as iron or calcium.^{3,69} Metal speciation is also another factor affecting
428 intestinal assimilation of TMs. Chunhabundit et al.⁷⁰ reported higher intestinal assimilation of Cd
429 in human intestinal cell from animal-based food than from vegetal-based food. Taking into
430 account those nutrient factors modulating bioavailability of TMs, diet diversity and composition
431 possibly play different roles over the exposure to TMs and the TM accumulation.

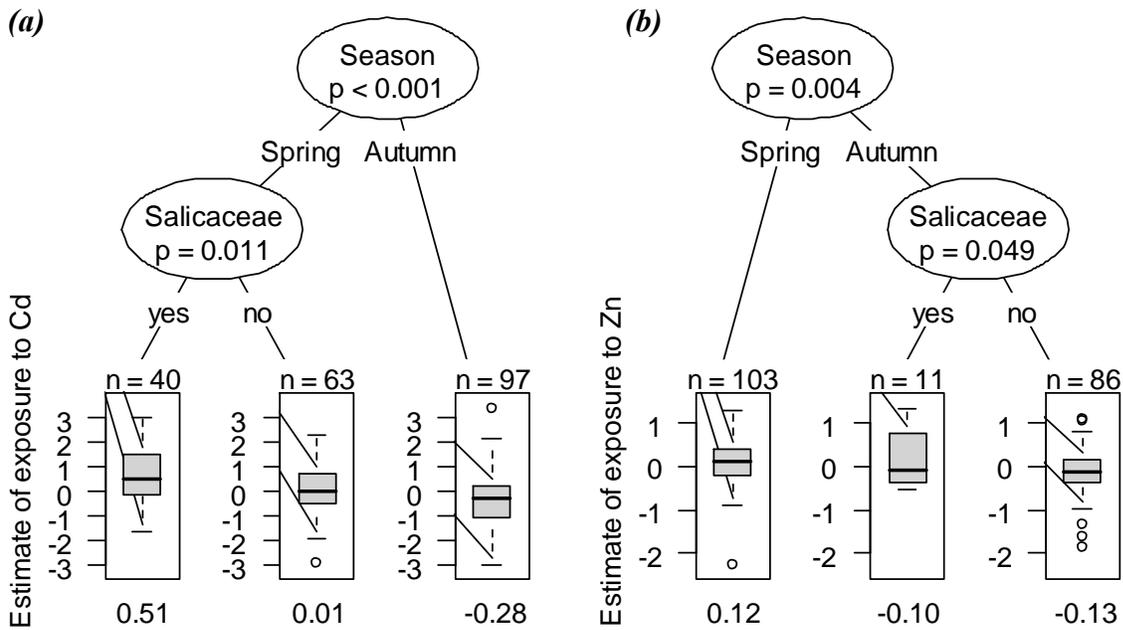
432 Even though an important functional role of diet richness on the exposure to TMs is shown
433 here, further investigations should be carried out for elucidating the variation of the oral
434 exposure in space and time. First, metal concentrations in both invertebrates and plants are
435 largely variable according to their organ, size, season, age and/or developmental stage,^{71,72} which
436 are undetectable by DNA based method. Although the metabarcoding is a powerful food
437 identification technique compared to the classical microscopic method or the nitrogen and carbon
438 isotopic approach, accessing information other than taxonomy of items, such as quantity
439 ingested, organs consumed and their TM concentrations, should be included in further studies.
440 Second, mechanisms underlying the relationship between the diet dilution and the diversity of
441 available food in the field remain unclear. The causes and consequences of food composition and
442 food selection depend on the foraging behavior of a given animal, which is also determined by
443 several factors such as balance between nutrients and toxins in food or avoidance of risks by
444 parasites and/or predators.⁷³ In our study, occurrences in SCs of Salicaceae and Sapindaceae, two
445 plant families preferentially consumed by wood mice,³⁶ were likely to switch along the gradient

446 of soil contamination: Occurrence in SCs of Salicaceae items decreased, whereas occurrence of
447 Sapindaceae items increased. This result could be due to a change in the preference of wood
448 mice for Salicaceae items along the gradient of soil TM contamination.³⁶ The composition of
449 available food in the field and the soil TM contamination may also be considered as to be
450 important factors determining the foraging behavior. Although our finding supported the diet
451 dilution hypothesis, the generality of this phenomenon can depend on several biological and
452 environmental conditions, which should be studied further.

453 To conclude, the level of exposure of small mammals to TMs is influenced not only by the
454 presence of certain items in the diet but also by the diet richness. The functional role of the diet
455 richness on exposure to TMs observed in the present study concurs with the diet dilution
456 hypothesis. Challenging such hypotheses about the functional role of biodiversity on the trophic
457 transfer of pollutants and investigating trait-based mechanisms are possible if ingested food is
458 identified in details. Although the metabarcoding approach allows identifying the taxonomy of
459 food under field conditions, associating life history traits and chemical composition of each item
460 and clarifying mechanisms of foraging behavior of animals will improve chemical risk
461 assessment in wildlife. Further perspectives also deal with the quantification of trophic links to
462 address the functional significance of trophic interactions for the transfer of matter, energy, and
463 essential elements versus the transfer of toxicants, and the evolutionary trade-offs between
464 acquiring nutrients but being exposed to toxic substances.

465

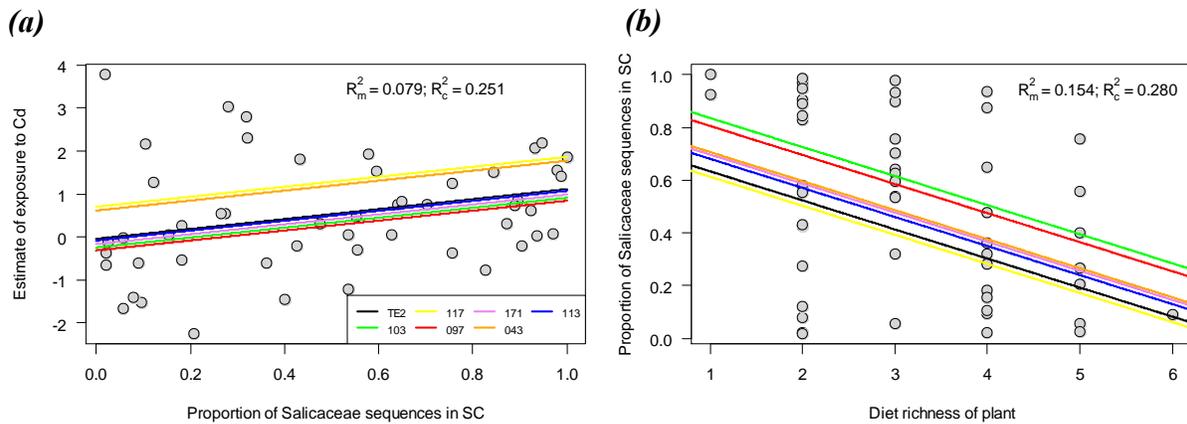
466 **Figure 1.** Conditional inference trees (CIT) for (a) the exposure to Cd and (b) the exposure to
 467 Zn, i.e. residuals of linear model between logarithmically-transformed concentrations of TMs in
 468 stomach content and in soils ($\mu\text{g g}^{-1}$ DM). Partitioning variables significantly selected for splits
 469 (univariate p -value < 0.05) are listed in inner nodes (represented as ovals) with p -value and their
 470 splitting criteria are listed on the lines connecting variables. Presence or absence of a given item
 471 in stomach content of wood mice is represented by “yes” or “no”, respectively. Boxplot in
 472 terminal node describes the residuals of the given TM for each final classification group with
 473 sample size, and median of the classification group is mentioned below.



474

475

476 **Figure 2.** (a) Exposure of the wood mice to Cd along the gradient of proportion of Salicaceae
477 items' sequence number in stomach content, considered as a proxy of biomass consumed and (b)
478 the proportion of Salicaceae items' sequences along the gradient of diet richness of plant. Plotted
479 points indicate stomach contents. Values predicted from our models are indicated by lines.
480 Different color lines represent different sites (random effect). Variation explained by fixed and
481 random effects are respectively explained by marginal and conditional R^2 (R^2_m and R^2_c) in the
482 figures.

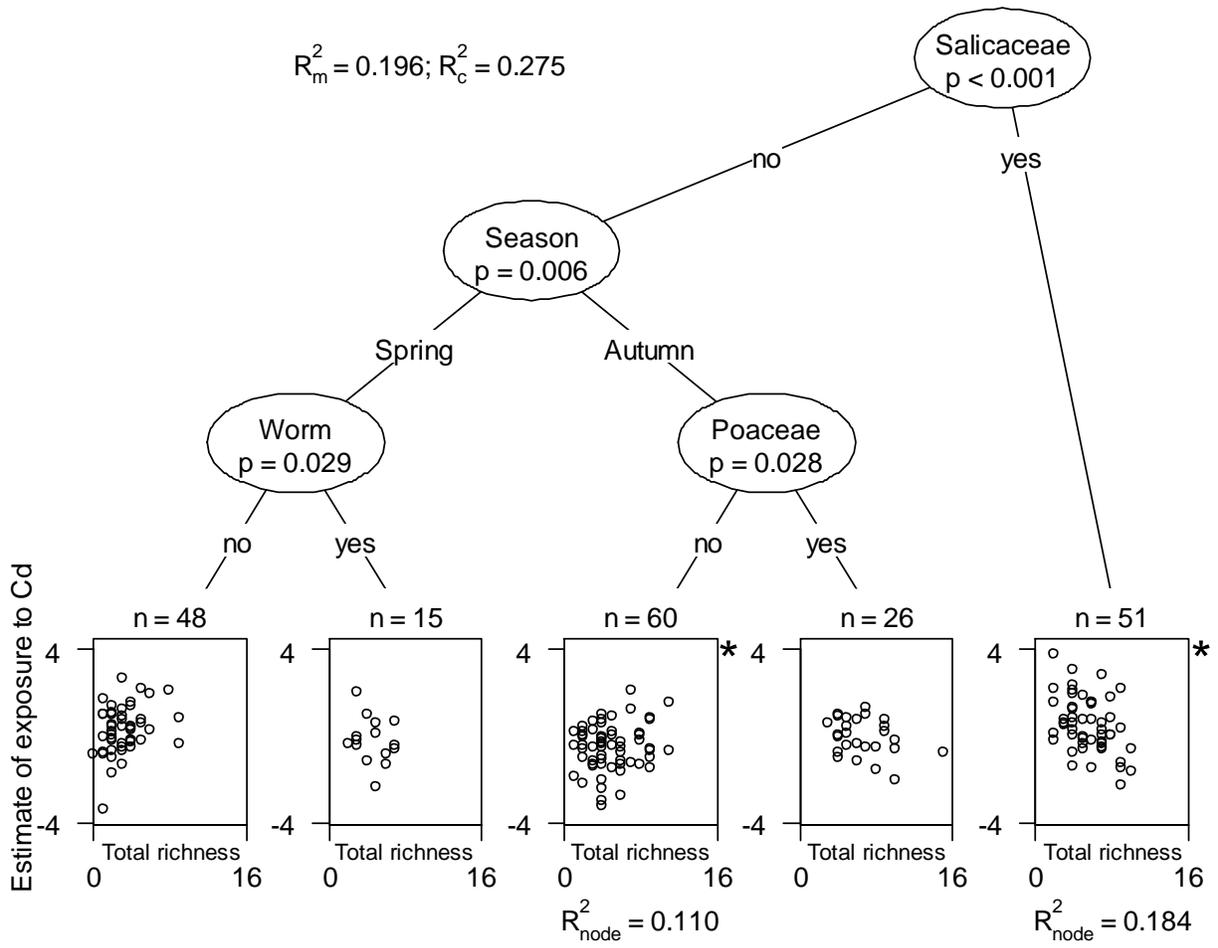


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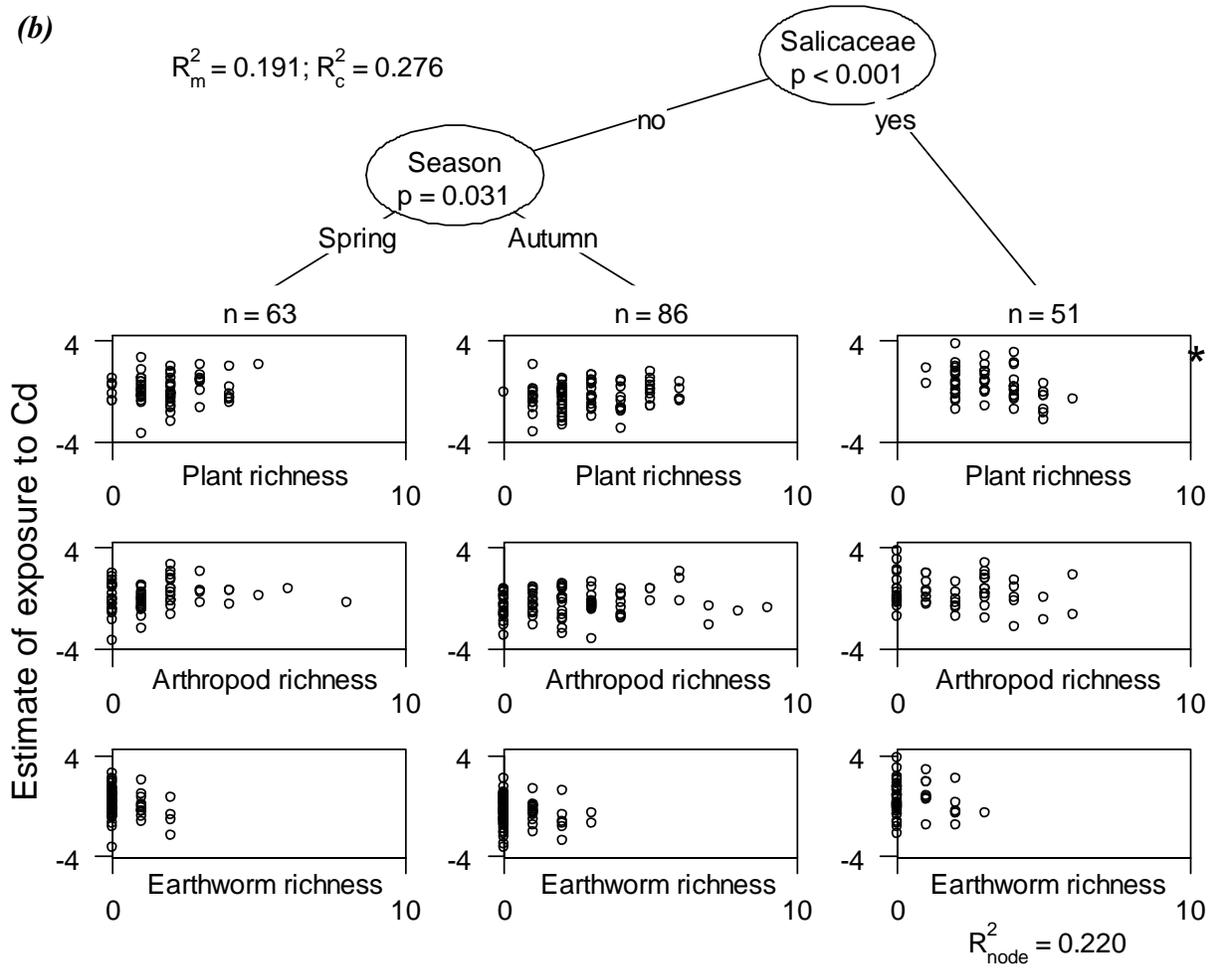
485 **Figure 3.** Model-based recursive partitioning (MOB) on mixed linear models for the exposure to
486 TMs, i.e. residuals of linear model between logarithmically-transformed concentrations of TMs
487 in stomach content and in soils ($\mu\text{g g}^{-1}$), with respect to either three diet richness of plant,
488 arthropod and earthworm MOTUs or the sum of them (i.e. total diet richness): exposure to Cd
489 with respect to the total diet richness (a) and to the three diet richness (b), exposure to Zn with
490 respect to the total diet richness (c) and to the three diet richness (d). Partitioning variables
491 significantly selected for splits (univariate *p-value* < 0.05) are listed in inner nodes (represented as
492 ovals) with *p-value* and their splitting criteria are listed on the lines connecting variables.
493 Presence or absence of a given item in stomach content of wood mice is represented by “yes” or
494 “no”, respectively. Scatter plots in terminal nodes describe relationships between the response
495 variable (y axis) and each explanatory variable (x axes, from bottom line to top, earthworm,
496 arthropod and plant diet richness). An asterisk (*) denotes significant correlation by type III
497 ANOVA. R^2 of the linear model for partitioned data (R^2_{node}) is mentioned below only if
498 significant correlation was observed. The variation explained by the fixed and the random effects
499 (R^2_{m} and R^2_{c} , respectively) are mentioned at the top of the figure.

(a)



500

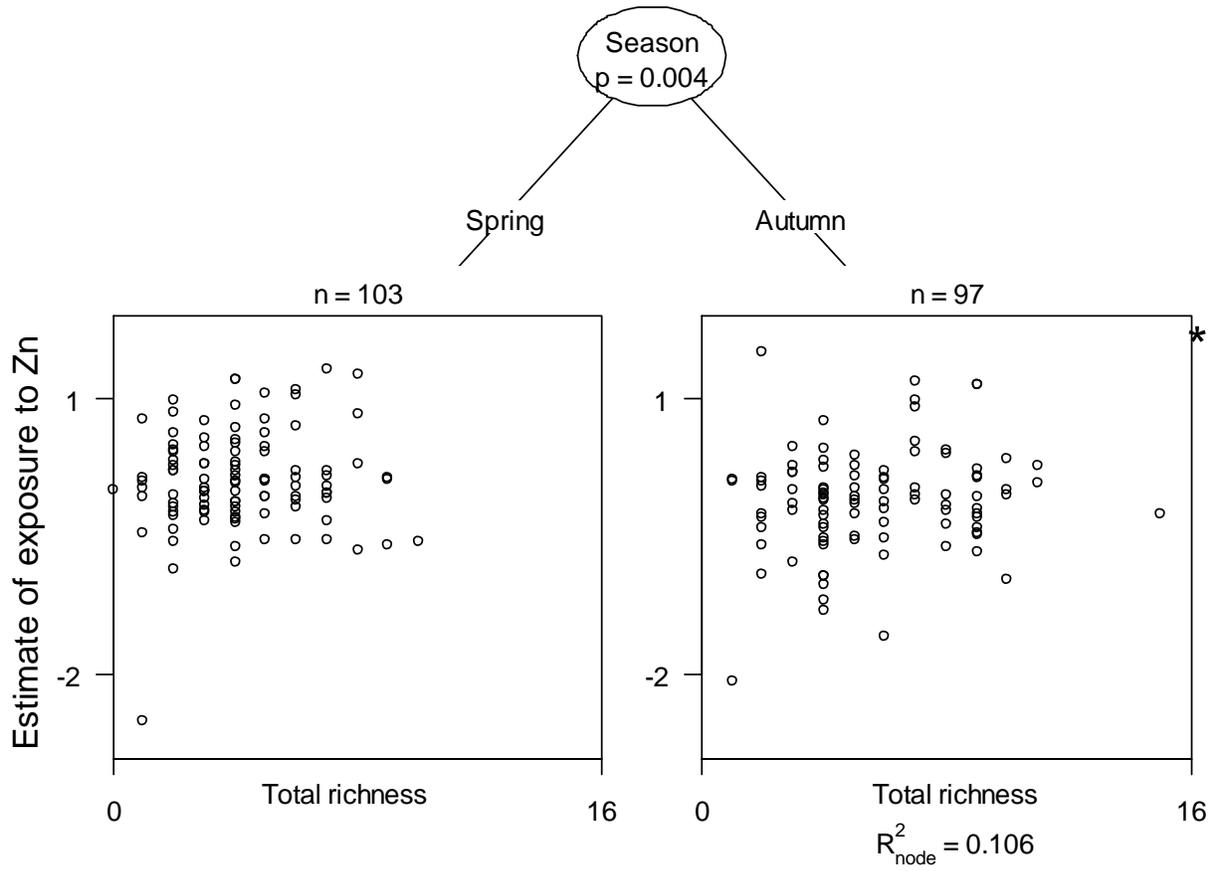
(b)



501

(c)

$$R_m^2 = 0.060; R_c^2 = 0.187$$



502

504 **Supporting Information**

505 Figure S1: Relationships between concentrations of TMs in SCs and in soils.

506 Figure S2: Conditional inference tree for frequency of occurrence of Salicaceae, Sapindaceae,
507 Fagaceae and Oleaceae items in SCs of wood mice.

508 Figure S3: Predicted probabilities for occurrence of items consumed in SCs along a gradient of
509 Cd concentration in soils by generalized linear models.

510 Figure S4: Correlation matrix for TM concentrations in soils and dietary richness of plant,
511 arthropod and earthworm MOTUs.

512 Figure S5: Concentrations of the three TMs in SCs of wood mice and exposure to the TMs in
513 spring and autumn.

514 Figure S6: Significant differences in exposure to TMs between the presence/absence of plants
515 items in SCs.

516 Table S1: Information about each study site about TM contamination and landscape dominant
517 habitats.

518 Table S2: List of primers used for identifying food.

519 Table S3: List of frequency of occurrence of items in SCs.

520

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524 **Notes**

525 # both authors contributed equally to supervising this work

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536

537 AUTHOR CONTRIBUTIONS

538 F.R. and R.S. conceived the study; S.O., R.S., C.F. and F.R. designed the study; R.S., C.F., F.M.,
539 T.C. and F.R. performed field samplings and laboratory treatments; B.V. performed
540 bioinformatics treatments; S.O. performed the statistical analyses; S.O., F.R., R.S. and C.F.
541 interpreted the results. S.O. wrote the manuscript under the supervision of all authors.

542

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