

Synthesis of earthworm trace metal uptake and bioaccumulation data: role of soil concentration, earthworm ecophysiology, and experimental design

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1 **Synthesis of earthworm trace metal uptake and bioaccumulation data: role of soil**
2 **concentration, earthworm ecophysiology, and experimental design**

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25 **Abstract**

26 Trace metals can be essential for organo-metallic structures and oxidation-reduction in
27 metabolic processes or may cause acute or chronic toxicity at elevated concentrations. The
28 uptake of trace metals by earthworms can cause transfer from immobilized pools in the soil to
29 predators within terrestrial food chains. We report a synthesis and evaluation of uptake and
30 bioaccumulation empirical data across different metals, earthworm genera, ecophysiological
31 groups, soil properties, and experimental conditions (metal source, uptake duration, soil
32 extraction method). Peer-reviewed datasets were extracted from manuscripts published before
33 June 2019. The 56 studies contained 3513 soil-earthworm trace metal concentration paired data
34 sets across 11 trace metals (As, Cd, Cr, Cu, Hg, Mn, Ni, Pb, Sb, U, Zn). Across all field and
35 laboratory experiments studied, the median concentrations of Hg, Pb, and Cd in earthworm
36 tissues that were above concentrations known to be hazardous for consumption by small
37 mammals and avian predators but not for Cu, Zn, Cr, Ni, and As. Power regressions show only
38 Hg and Cd earthworm tissue concentrations were well-correlated with soil concentrations with
39 $R^2 > 0.25$. However, generalized linear mixed-effect models reveal that earthworm
40 concentrations were significantly correlated with soil concentrations for log-transformed Hg, Cd,
41 Cu, Zn, As, Sb ($p < 0.05$). Factors that significantly contributed to these relationships included
42 earthworm genera, ecophysiological group, soil pH, and organic matter content. Moreover,
43 spiking soils with metal salts, shortening the duration of exposure, and measuring exchangeable
44 soil concentrations resulted in significantly higher trace metal uptake or greater bioaccumulation
45 factors. Our results highlight earthworms are able to consistently bioaccumulate toxic metals (Hg
46 and Cd only) across field and laboratory conditions. However, future experiments should
47 incorporate greater suites of trace metals, broader genera of earthworms, and more diverse

- 48 laboratory and field settings generate data to devise universal quantitative relationships between
- 49 soil and earthworm tissue concentrations.

50 **Keywords**

51 Bioconcentration; soil pollution; toxic metals; heavy metals; environmental toxicology

52

53 **Capsule**

54 The meta-analysis revealed that while concentrations affected earthworm Cd and Hg
55 concentrations, earthworm properties, soil properties, and experimental design, source of metal,
56 and exposure duration significantly affected trace metal uptake.

57

58

59 **1. Introduction**

60 Trace metals are generally considered to include metal and metalloid elements that occur
61 at abundances < 0.1% of the Earth's crust (Adriano, 2001). Some trace metals, such as chromium
62 (Cr), copper (Cu), zinc (Zn), manganese (Mn), and nickel (Ni) are essential for organisms due to
63 their roles in folding of organo-metallic structures such as enzymes and proteins and regulating
64 oxidation-reduction in metabolic processes (Hooda, 2010). Other trace metals, such as arsenic
65 (As), cadmium (Cd), mercury (Hg), lead (Pb), antimony (Sb), and uranium (U) are non-essential
66 and can cause acute or chronic toxicity when accumulated even in small quantities by plants and
67 animals (Adriano, 2001; Kabata-Pendias, 2010). Trace metals are naturally present in the
68 terrestrial environments at low concentrations but may occur at higher concentrations due to
69 lithology (Peng et al., 2004; Maleri et al., 2008; Tijani et al., 2006) but more frequently from
70 local and regional pollution from smelters (Morgan and Morgan, 1990; Filzek et al 2004; Dai et
71 al., 2004; Nannoni et al., 2011), roads (Pagotto et al 2001), agricultural soil amendments

72 (Centofanti et al 2016), mining activities (Wang et al., 2009; Duarte et al., 2014). Urban areas
73 with non-point source pollution can also be substantially contaminated with trace metals.

74 Earthworms are one of the most important soil fauna due to their size and biomass
75 compared to other soil fauna, and their ability to physically, biologically and chemically alter
76 soils (Scheu 1987; Bohlen et al., 2004; Sizmur and Hodson, 2009; Sizmur et al 2011). The
77 uptake of trace metals by earthworms is of importance not only because of the potential toxicity
78 to the earthworms themselves, but also for trophic transfer of metals from soils to predators and
79 detritivores in terrestrial food webs (e.g. Talmage and Walton, 1993; Nahmani et al 2007;
80 Richardson et al 2016). It is generally agreed earthworms can take up metals through ingestion of
81 soils and dermally by exposure to dissolved metals in soil pore water (Vijver et al., 2003).
82 Spurgeon and Hopkins (1999) showed that while earthworms were capable of regulating their
83 internal tissue concentrations of essential metals, such as Cu and Zn, at an equilibrium level, the
84 tissue concentrations of non-essential metals, such as Pb and Cd, do not reach equilibrium since
85 earthworms lack specific excretion mechanisms for these elements. Regulation of tissue
86 concentrations is also dependent on earthworm-specific physiological processes, such as the
87 excretion of metals by the calciferous glands or retention of metals within chloragogenous
88 tissues, where Zn and Pb are associated with 'Type A' phosphate-rich insoluble granules and Cu,
89 Cd and Hg are associated with 'Type B' sulfur-rich metallothionein-like proteins (Fischer and
90 Molnar 1993; Spurgeon and Hopkin 1999; Fraser et al., 2011; Karaca et al., 2010).

91 From the large number of studies on the bioaccumulation of trace metals by earthworms,
92 their tissue concentrations are considered a reliable indicator of trace metal bioavailability in
93 soils (Ma 1987; Suthar et al., 2008; Pérès et al., 2011). Linear and logarithmic equations have
94 been developed and analyzed in aggregate to estimate uptake of metal by earthworms by

95 Neuhauser et al., (1995), Sample et al., (1999), and Nahmani et al., (2007). As stated by
96 Nahmani et al., (2007) “Much work has been carried out on accumulation of metals by
97 earthworms in soils... Yet it is still not possible to predict with a high degree of confidence the
98 body burden of an earthworm...” The limitations to our capability to interpret and extrapolate
99 results from studies is due to the wide range of experimental conditions, with discrepancies
100 between laboratory conditions and the ‘real world’ environment. The literature contains many
101 studies that have focused on Lumbricidae earthworms under laboratory conditions. Moreover,
102 there are contradictions in soil to earthworm trace metal relationships reported. For example, soil
103 concentrations of Cu, Zn, or Pb were significantly correlated with earthworm tissue
104 concentrations ($R^2 > 0.50$) in some studies (e.g. Neuhauser, 1995; Sample et al., 1999; Ma 2004;
105 Alvarenga et al., 2013) while others reported no significant correlation and low explanatory
106 power ($R^2 < 0.25$) (e.g. Nahmani et al., 2007; Wang et al., 2012; Richardson et al., 2015;
107 González-Alcaraz et al., 2018). Thus, there is a need to undertake a synthesis of literature data
108 sets to further identify additional experimental design, soil, and earthworm properties hindering
109 universal relationship equations with strong predictive power.

110 Despite a vast body of literature, there remain a number of unanswered questions about
111 how soil properties (i.e. metal concentration, pH, organic matter) influence the bioaccumulation
112 of metals by earthworms (Dai et al., 2004; Ma 2004; Karaca et al., 2010) and a universal
113 quantitative relationship between soil trace metal concentrations and earthworm tissue
114 concentrations is lacking. As a prime example, measuring bioaccumulation may be problematic
115 because the straightforward calculation of bioaccumulation factor (BAF; synonymous with
116 bioconcentration factor), involving the ratio of dry weight tissue concentrations by soil
117 concentrations, can be affected by the method used to measure soil concentrations. Furthermore,

118 experimental design artefacts or generalizations may result from the conditions under which
119 trace metal bioaccumulation studies have been conducted. Bioaccumulation has been well-
120 studied for a limited set of trace metals (Pb, Cu, Zn, Cd) but similar relationships may not hold
121 true for many other trace metals. Experiments have been undertaken using a wide range of soil
122 types, ranging from very organic rich soils to support *E. fetida* (e.g. Suthar and Singh 2009
123 utilized a soil composed of up to 80% cow dung) or artificial soils, such as the OECD standard
124 soil, with <10% organic matter (Nahmani et al., 2007). Soils are frequently spiked in the
125 laboratory with metal salts (Nahmani et al., 2007), which may not adequately represent trace
126 metal concentrations associated with organic matter and secondary oxides of field-contaminated
127 soils. Lastly, the duration of exposure adopted in laboratory bioassays can be far shorter than at
128 the time expected for attenuation to occur (Sheppard et al., 1997; Nahmani et al., 2007).

129 The purpose of this study is to synthesise data on trace metal uptake and bioaccumulation
130 by earthworms, similar to previous meta-analyses but include additional experimental design
131 variables to evaluate broader patterns. This meta-analysis set out to revisit the soil-earthworm
132 uptake paradigm in our first question and explore three additional questions centered on soil-
133 earthworm properties and experimental design aspects. (1) To what extent are trace metals taken
134 up and bioaccumulated by earthworms across a broad range of earthworm genera ? (2) Does soil
135 pH, soil organic matter, genera and earthworm ecophysiological groups influence earthworm
136 uptake and bioaccumulation of all trace metals, or only specific metals? (3) Do experimental
137 design variables (e.g. source of metals, exposure duration) artificially influence the
138 bioaccumulation of trace metals in earthworms? (4) Which soil extraction methods are most
139 appropriate for quantifying bioaccumulation of trace metals by earthworms? The answers to
140 these four questions are needed to coalesce conflicting findings of earthworm metal

141 bioaccumulation to move towards the generation of universally applicable relationships between
142 soil and earthworm trace metal concentrations.

143

144 **2. Methods**

145 2.1 Search Protocol

146 Our meta-analysis utilized the rich-body of ISI-Web of Science listed literature
147 concerning trace metals in soils and their uptake by earthworms, ranging from laboratory
148 conditions to field experiments. The literature search of peer-reviewed publications published
149 before June 2019 reporting results on bioaccumulation of trace metals by earthworms was
150 performed using the ISI-Web of Science research database (e.g. Van Groenigen et al., 2014,
151 2019). We used the following search term:

152 ((TS=(earthworm\$ AND soil AND (trace metal\$ OR heavy metal\$ OR micronutrient\$
153 OR potentially toxic element\$ OR metal\$) AND (*bioaccum* OR biocon*) NOT
154 vermicompost*))).

155 The search yielded 267 studies that contained the desired search terms in their titles,
156 abstracts, keywords, and KeyWords Plus, which are words and phrases frequently used in the
157 references of an article. Studies not written in English were not included in these results.

158 2.2 Study selection

159 Studies were screened by carefully reading all 267 abstracts to determine suitability of
160 the query search results. Studies that included an experimental treatment that may influence
161 bioaccumulation rate such as soil sterilization, fungi or bacterial amendments, addition of
162 pesticides were not included. Studies that focused on non-mineral soil media were not included
163 such as sewage sludges, organic horizons, and subaqueous soils. Soils that utilized metal

164 treatments, such as sludges, metal salts, or contaminated soils from other areas were included in
165 our study. A total of 119 full-texts were acquired for further inspection. Studies that were unable
166 to be used in our study had one or more of the following issues: failure to report data in
167 accessible format (e.g. data across treatments or sampling sites were not reported, or only
168 reporting aggregate data), missing data set (e.g. soil concentrations not reported), failing to
169 mention depuration of earthworms, not reporting concentrations as dry weight. Authors of recent
170 studies (after 2005) focusing on several trace metals were contacted for data sets but all requests
171 were unsuccessful. We excluded 63 studies of the 119 full texts screened and only 56 studies
172 (Supplemental Table 1) met our criteria for use in our meta-analysis. All data are available in
173 supplementary material. Field studies included in our meta-analysis included different
174 ecosystems (forests, grasslands, agroecosystems), several climatic biomes (temperate,
175 continental, tropical and subtropical), and multiple types of experimental designs (indoor and
176 outdoor pot experiments, field plots of contaminated, uncontaminated, urban and preserved
177 ecosystems).

178

179 2.3 Data collection and extraction

180 Important study metadata were collected (Year Published, First Author Last Name, Metal
181 source as described in the study), earthworm information (Earthworm family, genera, species),
182 experimental design (uptake duration, extraction method and instrumental used for trace metal
183 analysis, treatments or site name, and number of replicates) and chemical data (%SOM, pH, As,
184 Cd, Cr, Cu, Hg, Mn, Ni, Pb, Sb, U, Zn soil and earthworm tissue concentrations). Data were
185 extracted from the 56 studies by transcription when presented in tables while data represented
186 graphically was extracted manually using PlotDigitizer Version 2.6.6, released April 27th, 2014

187 (<http://plotdigitizer.sourceforge.net>). The 56 studies contained 951 soil-earthworm trace metal
188 concentration paired data sets, with 3513 data points across 11 trace metals (As, Cd, Cr, Cu, Hg,
189 Mn, Ni, Pb, Sb, U, Zn).

190

191 2.4 Statistical Analyses

192 Descriptive statistics were calculated using MATLAB (Mathworks, Natick, MA, USA)
193 For the figures and in text data, average values are given \pm 1 standard error of the mean. BAF
194 were calculated as the ratio of earthworm to soil trace metal concentrations using values obtained
195 from each study without log-transformation. Descriptive statistics for the pooled soil, earthworm,
196 and BAFs are given in Table 1. Power regressions, also commonly referred to as log-linear
197 regressions, were used to quantify the relationship between soil concentrations and earthworm
198 tissue concentrations in MATLAB. Linear regressions were not used because of their sensitivity
199 to higher concentration values over lower concentration values. Soil concentrations, earthworm
200 concentrations, and bioaccumulation factors were log-transformed and analyzed for normality
201 using the Lilliefors test (Lilliefors, 1967). Earthworm trace metal concentrations and
202 bioaccumulation values were compared across earthworm genera, metal sources, and
203 experimental design conditions, where applicable using generalized linear mixed-effect models
204 (GLMMs) in MATLAB.

205 For the GLMMs, earthworm metal concentrations and soil concentrations were log-
206 transformed, continuous variables (soil trace metal concentrations, %SOM, and pH) were treated
207 as fixed effects and categorical (ecophysiological group, earthworm genera, duration of metal
208 uptake and source of trace metals) as random effects. The GLMM model consisted of Normal
209 Distribution and Maximum pseudo likelihood fit method. This GLMM configuration was

210 selected based upon the paired data distribution, residual plots, and Akaike information criterion
211 (AIC) values. Results for the GLMM analyses are given in Table 2. Interactions among the
212 experimental design variables (ecophysiological group, earthworm genera, duration of metal
213 uptake and source of trace metals) were explored for log-transformed earthworm tissue
214 concentrations for metals with the most robust data sets (Cd n = 579, Cu n = 608, Pb n =593, and
215 Zn n = 601). To test for data set biases in earthworm tissue concentrations among earthworm
216 genera, uptake duration groups, and ecophysiological groups, an N-Way ANOVA with post-hoc
217 t-tests were performed using MATLAB.

218

219 **3. Results and Discussion**

220 *3.1.1 Earthworm trace metal concentrations*

221 Our meta-analysis of 56 studies shows that earthworms are able to bioaccumulate
222 potentially hazardous concentrations of many toxic metals. Median earthworm tissue
223 concentrations of Hg, Pb, and Cd were above concentrations found to be hazardous for
224 consumption of rodents and fowl by the United States National Research Council (Table 1)
225 (NRC, 2006). Moreover, mean and median earthworm tissue concentrations show
226 bioaccumulation of Zn, Ni, and As near levels that may be hazardous to small mammals and
227 avian (Table 1). The extent to which earthworms bioaccumulate trace metals is influenced both
228 by the regulation of internal tissue concentrations by earthworms (Spurgeon and Hopkin 1999;
229 Karaca et al., 2010; Natal-da-Luz et al., 2011) and by the chemical bioavailability of the trace
230 metals in the soil they inhabit (Bradham et al 2006; Natal-da-Luz et al., 2011).

231 Most of the 56 studies included in our meta-analysis focused on contaminated soils. From
232 Table 1 however, we observe that median soil concentrations for Hg, Cd, Cu, Zn, Mn, Cr, Ni,

233 As, and U are not greater than background concentrations (Table 1). Most median soil
234 concentrations fell within the range of typical soil concentrations for trace metals as reported by
235 Adriano (2001) and Kabata-Pendias and Mukherjee (2007). However, arithmetic mean and third
236 quartile (Q3) of soil concentrations were substantially elevated above background soil
237 concentrations for Hg, Pb, Cd, Cu, Zn, and Sb (Table 1). Many of the trace metal concentrations
238 in samples were near background due to their role as a control soil in experiments that also
239 included contaminated soils, or where soil was collected from non-point source contaminated
240 sites. The elevated trace metal concentrations are from sites that have historical legacies of
241 smelting (e.g. Nannoni et al., 2001; Zhang et al., 2009), agricultural soils following application
242 of biosolids and sewage (e.g. Liu et al., 2005; Centofanti et al., 2016), or former mining
243 operations (e.g. Morgan and Morgan, 1990; Sizmur et al., 2011; Wang et al., 2018). Elevated
244 concentrations in soils were also observed in soils artificially amended in the laboratory using
245 metal salts (e.g. Dang et al., 2015). Mean Hg, Pb, Sb, and U soil concentrations were skewed far
246 above the interquartile range (Table 1), indicating some experimental designs utilized
247 concentrations that far exceed values commonly found in the environment.

248 To explore the role of soil concentrations on earthworm trace metal uptake, we used
249 power also referred to as log-linear regressions, as opposed to linear regressions, to avoid bias
250 towards higher concentrations with larger numbers. Power regressions showed that the soil
251 concentrations strongly predict uptake of Hg and Cd in earthworm tissues ($p < 0.01$, $R^2 > 0.35$,
252 Supplemental Figure 1). Soil concentrations of the other metals, Cu, Cr, Pb, Zn, Ni, Mn, and As,
253 as well as Sb and U not shown in Supplemental Figure 1, did not predict earthworm uptake,
254 explaining less than 20% of the variation in tissue concentrations ($R^2 < 0.20$; $p > 0.05$;
255 Supplemental Figure 1). These results agree with previous studies that found Hg and Cd soil

256 concentrations drive uptake across several earthworm species (e.g. Richardson et al., 2015; Da
257 Silva et al., 2016; González-Alcaraz et al., 2018; Wang et al., 2019). Moreover, the results agree
258 with previous studies that found soil concentrations did not drive Cu, Zn, or Pb earthworm tissue
259 concentrations (e.g. Nahmani et al., 2007; Wang et al., 2012; Richardson et al., 2015; González-
260 Alcaraz et al., 2018).

261 We further investigated the influence of soil concentrations on earthworm tissues
262 concentrations using generalized linear mixed effect models (GLMM). The model was structured
263 as [Earthworm] = 1 + Ecophysiological Group + Genera + [Soil] + Metal Source + Uptake
264 duration + pH + organic matter, where all variables were categorical except for soil
265 concentrations, pH, and organic matter. GLMM results show that when source of metal, duration
266 of exposure to metals, and soil parameters are taken into account, soil concentrations were
267 significantly correlated with earthworm tissue concentrations for Hg, Cd, Cu, Zn, As, Sb, and U
268 (Table 2, $p < 0.05$). From these results, we hypothesised that the contrasting findings of these
269 studies were due to differences in the earthworm species adopted and other differences in
270 experimental design across the 56 studies. These issues are further explored in the following
271 sections.

272

273 *3.1.2 Earthworm trace metal concentrations across ecophysiological groups and genera*

274 Earthworm tissue concentrations were significantly different between ecophysiological
275 groups for Hg, Pb, Cd, Zn, Sb, and U using GLMM ($p < 0.05$) but not for Cu, Mn, and Cr.
276 Epigeic earthworms had significantly higher Hg, As, and Sb tissue concentrations than the other
277 ecophysiological groups (Figure 1). Epi-endogeic earthworms had similar concentrations as
278 endogeic earthworms for Pb, Cd, Cu, Zn, Mn, Cr, Ni, As, and U (Figure 1), but had significantly

279 lower Hg concentrations than endogeic earthworms. These results suggest that no
280 ecophysiological group consistently achieves higher or lower trace metals concentrations.
281 Furthermore, the uptake of several metals were not influenced by ecophysiological group at all,
282 hinting that choice of food (i.e. mineral soil vs litter) or dermal contact does not affect their
283 uptake. Lastly, differences in trace metal tissue concentrations between ecophysiological groups
284 may be influenced by additional variables not considered such as variations between species
285 within a group.

286 Our GLMM analysis indicates that earthworm tissue concentrations varied among
287 earthworm genera for most metals (Hg, Pb, Cd, Cu, Zn, Mn, Sb, U) (Table 2). When focusing on
288 specific earthworm genera, our analysis only compared genera where $N > 10$ for at least five of
289 the metals analyzed in this study. Thus, comparisons for *Diplocardia*, *Drawidia*, *Pontoscolex*,
290 *Octolaision* and *Sparganophilus* were not included in this study due to small sample sizes. There
291 does not appear to be any genera most adept at bioaccumulating all metals, as differences among
292 genera were metal specific. For example; *Eisenia* had significantly higher As and Hg
293 concentrations than all other genera; *Aporrectodea*, *Dendrodrilus*, *Eisenoides*, and *Lumbricus*
294 had the highest Pb concentrations, and; *Dendrodrilus* had the highest Cd concentrations ($p <$
295 0.05 ; Supplemental Table 3). Moreover, several of the trace metals (e.g. Cu, Zn, and U) for
296 which tissue concentrations were significantly affected by genera in the GLMM (Table 2) had
297 similar tissue concentrations across most genera ($p > 0.10$; Supplemental Table 3). There are
298 important within-genus differences to take into consideration. First, earthworms within the same
299 genus can have very different feeding and burrowing habits (e.g. anecic *Lumbricus terrestris* and
300 epi-endogeic *Lumbricus rubellus*). Second, earthworms within the same genera may inhabit
301 different soils affecting their exposure to trace metal concentrations. Lastly, physiological

302 differences such as their length and surface area of folds within their intestines and excretion
303 capabilities influence metal concentrations in their tissues (Morgan and Morgan, 1990; Morgan
304 and Morgan, 1992; Spurgeon and Hopkins 1999).

305

306 *3.2.1 Earthworm bioaccumulation factors*

307 Our meta-analysis of 56 studies shows that earthworms consistently bioaccumulated Hg,
308 Cd, and Zn on the basis of Q1 and Median BAFs > 1.0 (Table 1) and power regressions
309 (Supplemental Figure 1). Earthworms were able to bioaccumulate Pb, Cu, Cr, Ni, Sb, As, and U
310 only under certain circumstances, on the basis of Q3 BAFs > 1.0 (Table 1) and power regressions
311 (Supplemental Figure 1). The limited bioaccumulation of Pb, Cu, Cr, Ni, Sb, and U, were likely
312 driven by two specific conditions: highly elevated soil concentrations with reduced uptake tissue
313 concentrations due to saturation and very low soil concentrations with low earthworm uptake
314 causing BAFs to not exceed 1.0. When examining soil concentrations and BAFs in XY space in
315 Supplemental Figure 2, it is clear that As, Cd, Cr, Cu, Hg, Mn, Ni, and Zn have significantly
316 higher BAF when soil concentrations are low ($p < 0.05$, R^2 ranged between 0.14 and 0.78). Lead
317 BAF was not significantly affected by soil concentration ($p > 0.10$, $R^2 = 0.00$). One mechanism
318 for decreasing BAFs with increasing soil concentration is mistaken scavenging as limiting
319 essential elements (such as Hg for Se in Richardson et al., 2015) and increased regulation and
320 excretion at elevated concentrations to maintain homeostasis (such as Mn for Ca in Morgan et al
321 2007).

322 We investigated the importance of earthworm type (ecophysiological group, genera) and
323 soil properties (soil extraction methods, soil pH, SOM), accounting for differences in
324 experimental design (source of metals, uptake duration) using generalized linear mixed-effect

325 models (GLMMs). The GLMM for BAF was structured as [Earthworm] = 1 + Ecophysiological
326 Group + Genera + Soil Extraction Method + Metal Source + Uptake duration + pH + organic
327 matter, where all variables were categorical except for pH and organic matter. Since soil
328 concentrations are used to calculate BAF, they cannot be added to the model. The BAF GLMM
329 results are given in Table 3 and described and interpreted in the following sections.

330

331 3.2.2 Earthworm bioaccumulation factor across genera and ecophysiological groups

332 GLMMs revealed that earthworm genera was a significant factor influencing BAF for all
333 trace metals. Our BAFs in Supplemental Table 3 show some genera bioaccumulated metals at
334 higher rates than others but no specific genus consistently bioaccumulated the highest
335 concentration of all trace metals. For example, Lumbricidae genera (*Allobophora*, *Aporrectodea*,
336 *Dendrobaena*, and *Dendrodrilus*) all bioaccumulated Pb, Cd, Cu, Zn, and Ni at greater rates than
337 Megascoelidae genera *Metaphire* and *Pheretima* group (Supplemental Table 3, $p < 0.05$). Similar
338 to the GLMMs for earthworm tissue concentrations, our analysis only compared genera where N
339 > 10 for at least five of the metals analyzed in this study, thus, *Diplocardia*, *Drawidia*,
340 *Pontoscolex*, *Octolasion* and *Sparganophilus* were not included. Moreover, comparisons
341 between genera are limited as earthworms within the same genus can have different feeding and
342 burrowing habits and may also be influenced by their preferred soil physiochemical properties.
343 In addition, there are physiological differences between earthworms to consider. For example,
344 the substantially reduced calciferous glands of *Megascolecidae* compared to earthworms of
345 Lumbricidae (both *Aporrectodea* and *Lumbricus*) may influence the assimilation and
346 bioaccumulation of trace metals.

347 Earthworm ecophysiological groups had different BAFs for most metals (Table 3).
348 Endogeic earthworms had significantly higher BAFs for Pb, Cd, Cr, Sb than all other groups
349 from GLMMs ($p < 0.05$; Figure 1). Further, epigeic earthworms had significantly higher BAFs
350 for Hg, Cu, Ni, and As than all other ecophysiological groups from GLMMs ($p < 0.05$; Figure 1).
351 Lastly, anecic earthworms had the lowest BAFs for Hg, Pb, Cd, Ni, and Sb from GLMMs ($p <$
352 0.05). BAFs can be high for epigeic earthworms due to high metal concentrations in the organic
353 rich soils they inhabit at high densities and endogeic earthworms can live in low organic matter
354 soil found in urban areas and point source polluted sites such as smelters (e.g. Morgan and
355 Morgan, 1990). Anecic earthworms consume fresh plant litter that typically have lower trace
356 metal concentrations than the partially decomposed organic matter consumed by epigeic and
357 endogeic earthworms (Bohlen et al., 2004; Karaca et al., 2010; Richardson et al., 2015).
358 Moreover, anecic earthworms can perform ‘external’ rumen digestive actions, in which they re-
359 ingest previously digested soils to consume fungal grazers and colonizing microbial communities
360 (Lavelle et al 1994). Epi-endogeic earthworms did not have BAFs resembling endogeic or
361 epigeic earthworms, highlighting their adaptive feeding behavior (Figure 1). Additional studies
362 are required to investigate comparability across metal concentrations, the earthworm diets, and
363 field versus laboratory conditions, all of which can influence trace metal bioaccumulation and
364 retention in their tissues.

365

366 *3.3.1 Experimental design – Source of metals*

367 The bioavailability of metals is strongly dependent on its phase in soil, as metals present
368 in native silicates or forged-alloyed metals by humans are generally unavailable for immediate
369 uptake by earthworms while exchangeable or dissolved forms are readily available for uptake.

370 Frequently studies focus on one type of metal source and have not compared how the source of a
371 metal affects the interpretation of uptake and bioaccumulation results. Using GLMMs, we found
372 that the source of metals significantly impacted earthworm tissue concentrations for Hg, Pb, Cd,
373 Cu, Zn, Cr, Ni, and As (Table 3). We further examined this effect in Figure 2 to determine if
374 there were any trends among types of metal sources. Our results show that earthworms
375 inhabiting soils affected by mining activities, smelting, laboratory spiking, and non-point source
376 pollution (e.g. urban soils) had higher tissue concentrations of Hg, Cd, Cu, Cr, Ni, and As than
377 earthworms exposed to background soil concentrations found in pristine environments (Figure
378 2). Further, we observed that earthworms in agricultural soils exhibited tissue concentrations
379 similar to, or below, the tissue concentrations of earthworms exposed to background soil
380 concentrations for Pb, Zn, and Mn (Figure 2). We therefore conclude that the source of metal
381 increased uptake of trace metals by earthworms rather than not simply elevated concentrations
382 pollution consistently results in.

383 GLMMs showed that the source of metal can significantly influence BAFs, which may be
384 the result of experimental design. Experiments using laboratory spiking methods, where a metal
385 salt is added to a soil, produced BAFs that were significantly higher than background BAFs for
386 Hg, Cu, Ni, and As (Figure 2). However, this effect was not consistent since laboratory spiking
387 generated a very low Pb BAFs and did not affect Cd, Zn, Mn, and Cr BAFs, compared to
388 background BAFs (Figure 2). Mining and smelting activities did not produce significantly higher
389 BAFs for Pb, Cd, Zn, Mn, and Ni when compared to background BAFs but did generate
390 inconsistent positive and negative effects on BAFs for As, Cr, Cu, Hg, and U (Figure 2). We
391 hypothesise that soil properties and concentration of laboratory spiking method can generate
392 artefacts for testing bioaccumulation due to differences in complexation, sorption, and

393 precipitation (Kumpiene et al 2008). The high solubility of trace metals applied by laboratory
394 spiking can result in higher dissolved concentrations in the soils to which the earthworms are
395 exposed (Nahmani et al., 2007), which may be unrealistic when compared to natural systems that
396 have had longer for the soil to ‘age’ and the dissolved concentration is allowed to come into
397 equilibrium with the adsorbed or precipitated phase.

398 Soil properties were important variables influencing BAFs for some metals. Da Silva et al
399 (2016) spiked low pH soils (pH 4) with high concentrations of Hg, creating a large bioavailable
400 Hg pool and high BAFs while Wijayawardena et al., (2017) spiked high pH soils (pH 5 – 8.5)
401 with Pb, creating a large insoluble, unavailable Pb reservoir with low earthworm BAFs when
402 assessed for total soil Pb. Soil concentrations in highly contaminated systems may be elevated to
403 the point that where BAFs are low even though tissue accumulation is high. One example is soil
404 near mining and smelting operations. In these systems, high concentrations in the soil drive high
405 accumulation in earthworm tissues but the BAF remains low because it is defined as the ratio of
406 tissue to soil concentrations. Non-point source pollution did not have significantly different
407 BAFs than background BAFs for most metals: As, Cd, Cu, Cr, Hg, Pb, Mn, and Ni, and Zn,
408 (Figure 2). These results suggest that using a source of metal contamination that best mimics
409 natural systems can recreate natural bioaccumulation pathways of metal uptake while still
410 generating elevated earthworm tissue metal concentrations. Laboratory spiking of soils with trace
411 metals or using point source polluted sites from mining or smelting has the potential to generate
412 experimental artefacts when findings are applied to non-point source polluted sites (e.g. degraded
413 areas or urban areas) and limit broad applicability of results.

414

415 *3.3.2 Experimental design – Exposure duration to metals*

416 The duration that earthworms are exposed to a soil can influence the bioaccumulation of
417 metals, as earthworm require time to attenuate to soil metal concentrations through soil ingestion
418 (see Spurgeon and Hopkin 1999) and passive diffusion across their skin (Vijver et al 2003). Our
419 GLMM analysis shows earthworm tissue concentrations for Hg, Pb, Cd, Cu, Zn, and As were
420 significantly influenced by the duration of exposure to the soils (Table 2). Although one would
421 expect the longest duration to cause the greatest uptake of metals, this was not always the case.
422 Longer exposure durations to Cd and Zn produced the highest earthworm tissue concentrations
423 (Figure 3), but short and medium duration experiments generated the highest concentrations of
424 Hg, As, and Sb (Figure 3).

425 Our GLMM analysis showed BAFs for Hg, Pb, Cd, Cu, Zn, and As were significantly
426 influenced by the duration of exposure to the soils (Table 3). Short duration experiments (< 2
427 weeks) generated the lowest BAFs for Hg, Pb, Cd, Cu, Zn and Sb compared to entire life
428 durations (Figure 3). Medium duration experiments (3 to 6 weeks) generated low BAFs for Pb,
429 Cd, and Sb and high BAFs for Hg, Cr, Ni, and As compared to entire life durations (Figure 3).
430 Similarly, long duration experiments (6 to 20 weeks) were more closely aligned with entire life
431 studies for some metals (Cd, Cu, Zn, Mn) but also generated metals with significantly higher (Cr,
432 Ni) or lower (Pb, As) BAFs compared to entire life studies (Figure 3).

433 These results highlight that duration of experiments can also limit interpretations from
434 laboratory-based experiments to field experiments. As with comparisons of earthworm tissues
435 among experiment durations, several factors regarding duration of exposure could be responsible
436 for the effect. First, short experiments can use concentrations that negatively impact their health
437 and alters physiology and behavior, or are lethal but their short duration allows for survival.
438 Second, earthworms may be unable to attenuate to a dynamic equilibrium of tissue trace metal

439 concentrations (particularly for essential elements) within the experimental duration (Spurgeon
440 and Hopkins 1999). Lastly, there may be covariance with the metal source as short duration
441 experiments with high soil metal concentrations typically use soils spiked with metal salts which
442 are highly bioavailable (Nahmani et al, 2007). Thus, experiment duration may be an important
443 variable or covary with other variables and additional field-based studies are needed uptake and
444 bioaccumulation under natural conditions.

445 *3.3.3 Experimental design – Extraction method impact on BAFs*

446 There are dozens of standardized extraction and digestion methods to assess trace metals
447 in soils with varying purposes, ranging from assessing mobility, exchangeability, inorganic
448 sorption, organic complexation, precipitation within secondary oxides, silicate forms, and total
449 concentrations (Rao et al., 2008). The choice of extraction procedure may meet specific research
450 aims for evaluating soil, but may affect comparability when calculating BAFs. Our GLMM
451 found that soil extraction method significantly biased BAFs for most metals: Pb, Cd, Cu, Zn, Cr,
452 Ni, and As (Table 3).

453 In Supplemental Figure 3, we compared BAFs calculated from five categories of
454 extraction methods: water soluble being the least exhaustive, exchangeable focusing on cation
455 exchangeable metals using a salt (e.g. CaCl_2 or MgCl_2), extractable using an organic ligand (e.g.
456 EDTA or DTPA) or weak acid (dilute nitric acid or acetic acid), pseudototal digestion (e.g.
457 concentrated HNO_3 , HCl , H_2SO_4 or some combination), and total digestions (HF , HClO_4 ,
458 H_3PO_4). Our analysis shows that pseudototal and total digestions consistently produced BAFs
459 that were similar for all metals (Supplemental Figure 3). Using exchangeable or extractable soil
460 concentrations consistently generated higher BAFs than pseudototal or total digestion methods
461 (Supplemental Figure 3). However, BAFs measured using water soluble phases produced BAFs

462 similar to pseudototal or total digestion for some metals (e.g. Cd, Cu, Ni) but also generated
463 significantly higher BAFs for other metals (Zn and As).

464 The impact of the soil extraction method on BAFs has two important ramifications for
465 considering if metals are bioaccumulated and to what extent. First, using BAFs relies on the
466 assumption that >1.0 means metals are actively bioaccumulated by earthworm physiologically
467 but this analysis shows extraction method can affect these results. For example, Cu and Ni BAFs
468 measured with pseudototal and total digestions are <1.0 , suggesting they are not actively
469 bioaccumulated. However, if exchangeable and extractable concentrations are used to calculate
470 BAFs for Cu and Ni, then BAFs are >1.0 and they are considered actively bioaccumulated. We
471 recommend using BAFs for pseudototal and total digestions, as other extraction procedures may
472 overestimate BAFs through underestimating soil metal concentrations. Second, if other soil
473 extraction methods are desired, the assumption of 1.0 being an inflection point of
474 bioaccumulation may need to be reconsidered and a new point dependent on the soil extraction
475 method would be warranted. However, we argue that authors should avoid this later framework
476 for consistency in the literature.

477 3.3.4 Interactions among experimental design and data set biases

478 Our N-Way ANOVA analysis found significant interactions among earthworm genera,
479 uptake duration, and ecophysiological groups for Cd, Cu, Pb, and Zn (Supplemental Table 2). As
480 a prime example, litter-feeding and dwelling earthworms of the genus *Eisenia fetida* were
481 consistently used in shorter duration laboratory experiments than mineral soil dwelling,
482 earthworms genera conducted for their entire lifetimes under field conditions. This is simply due
483 to the fact that *Eisenia fetida* are a preferred model soil dwelling laboratory organism due to their

484 short life cycle, maturation in ~50 days, ease of care on organic wastes, and ability to reproduce
485 and live in high densities (OECD 1984).

486 In spite of our efforts to include a diverse array of studies on bioaccumulation, it is
487 important to note key limitations and biases in our data set. First, trace metal data were primarily
488 Cd, Cu, Pb and Zn data ($n > 500$), while metals such as Cr, Hg, Mn, Ni, Sb, U were reported less
489 often ($n < 200$). Second, data from agricultural areas and non-point source polluted sites are
490 underreported ($n < 0$ to 51) compared to areas near mining and smelting activities ($n = 70$ to
491 150). Third, Lumbricidae were overrepresented (mean across trace metals $n = 41\%$) compared to
492 Megascolecidae (mean $n = 10\%$) and Glossoscolecidae (mean $n = 6\%$). Fourth, anecic
493 earthworms (mean across trace metals $n = 10\%$) were understudied compared to epigeic (mean n
494 $= 44\%$), endogeic (mean $n = 23\%$), epi-endogeic (mean $n = 23\%$) earthworms. Lastly, our study
495 did not utilize the breadth of studies examining toxicokinetics, commonly due to additional
496 treatments affecting uptake and excretion rates. Thus, our study primarily utilized organisms that
497 spent their entire life cycle in the soil (mean across trace metals $n = 73\%$) as opposed to shorter
498 exposure durations.

499

500 **4. Conclusions**

501 The uptake and bioaccumulation of trace metals is important for ecotoxicological
502 research to ensure earthworm predators are not at risk of toxicity and an underappreciated aspect
503 of soil biogeochemistry. Our study demonstrated that specific metals, such as Hg, Cd, and Zn are
504 taken up and bioaccumulated across earthworm genera. Other metals, such as Pb, Cu, Ni, As, can
505 also be taken up and bioaccumulated under certain conditions. Traditionally, we consider the
506 primary driver of trace metals in earthworm tissues to be their respective soil concentrations.

507 However, many other factors play a role in uptake, particularly for metals where earthworm and
508 soil concentrations were poorly correlated: Pb, Cu, Zn, Mn, Cr, Ni, As, Sb, and U. These
509 additional factors can be environmental conditions, which include, but are not limited to, genus
510 of earthworm, ecophysiological group, soil pH, and organic matter content. Moreover,
511 anthropogenic activities can also control the uptake and bioaccumulation of trace metals through
512 different trace metal sources (e.g. non-point source pollution, smelting, mining). Unfortunately,
513 the manner by which we study uptake and bioaccumulation of trace metals can generate artefacts
514 that limit generalizability of results from many studies. Experimental design limitations include
515 the spiking soils with substantially elevated concentrations of metals in the laboratory, reduction
516 of the duration of exposure before full effects may be realized, and underestimating total metal
517 concentrations with weak extraction procedures.

518

519

520 **5. Research Needs**

521 *5.1 Reporting full trace metal sets*

522 Many studies only report values for a limited set of trace metals and determining co-
523 variance and element competition has remained largely unexplored. When possible, reporting
524 full sets of trace metals analyzed with appropriate QA/QC should be a standard. Measurement of
525 a consistent suite of metals aids researchers interested in other trace metals and also provides
526 insights into whether metals are co-varying or co-bioaccumulated by earthworms. We
527 recommend that researchers utilizing Atomic Absorption Spectroscopy, Inductively Coupled
528 Plasma Optical or Atomic Emission Spectrometry, and Inductively Coupled Plasma Mass
529 Spectrometry measure As, Cd, Cu, Pb, Ni, and Zn as primary suite of common inorganic soil

530 contaminants. A secondary suite consisting of Co, Cr, Sb, Sn, U, and W are proposed as a suite
531 of emerging and site-specific pollutants that may be elevated in earthworms, but the literature
532 severely lacks data on these metals. The measurement of Hg should only be done with either a
533 direct mercury analyzer or an established ICP-MS protocol. Lastly, data should be published in
534 accessible formats. Tables with metal concentrations for each treatment, each site, or lowest
535 applicable treatment unit so data can be further interpreted. Cumulative figures and in-text
536 reporting are not recommended for promoting accessible data. With widely available
537 supplemental data submissions with publishers and data repositories, researchers must consider
538 making their data available for future studies to build upon.

539

540 *5.2 Exploring earthworms beyond E. fetida, L. terrestris, and L. rubellus*

541 The abundance of studies on *L. terrestris* and *E. fetida* are not a surprise as they have
542 been considered model organisms for laboratory study. In our study, *E. fetida* (N = 141/951), *L.*
543 *terrestris* (N = 67/951), and *L. rubellus* (N = 104/951) were the three most commonly studied
544 species of earthworms, constituting 33% of the earthworms studied. While this is advantageous
545 for reproducibility when studying molecular scale processes, physiological responses, and
546 genetic processes, it severely limits application to field studies where hundreds of species are
547 understudied. Moreover, the focus on *E. fetida* is problematic as it is a small, organic-rich soil
548 dependent earthworm, most commonly studied under laboratory conditions, and data focused on
549 this earthworm skew results towards their preferred type of soil environment. Our GLMM results
550 show that pH and organic matter can significantly impact earthworm tissue concentrations and
551 bioaccumulation of trace metals. Thus, additional studies on uptake and bioaccumulation of
552 endogeic and epi-endogeic earthworms are needed and should consider being conducted at the

553 earthworm community-level. Moreover, further studies on Asiatic and American earthworms of
554 the families *Megascolecidae*, *Acanthodrilidae*, *Moniligastridae*, and *Glossoscolecidae* are
555 required to further our understanding.

556

557 *5.3 Earthworm field studies at larger scales*

558 Most field studies have focused on limited point-source polluted sites. However, this
559 causes a lack of field scale studies investigating soils at the ecosystem level and their influence
560 on earthworm uptake and bioaccumulation of trace metals. More regional to continental scale
561 studies are needed to accurately capture the influence of soil properties (e.g. pH, SOM, texture,
562 structure) and environmental parameters (e.g. soil moisture, temperature) on metal uptake and
563 bioaccumulation. In addition, changes to metal cycling in the environment can influence many
564 other properties important at the global scale (e.g. organo-metalloid disruption releasing DOC,
565 leaching of nutrients decreasing plant growth).

566

567 *5.4 Earthworms in agricultural settings*

568 Another effect of focusing on laboratory soils with amended trace metal concentrations,
569 is a lack of data on background metal concentrations across earthworm genera. Field and
570 laboratory studies are needed to determine background, natural, or uncontaminated concentration
571 data for earthworms. Due to the limited background data, it is difficult to assess if earthworms
572 are exhibiting contaminated or polluted trace metal concentrations or if these are differences due
573 to their physiology.

574

575

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580
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Bioaccumulation factor		Q1	0.20	4.22	0.11	0.30	1.50	0.11	0.24	0.11	0.06	0.56	0.96	
Median		1.11	9.49	0.31	0.67	3.44	0.25	0.50	0.40	0.14	1.25	2.09		
Mean		140.4	25.4	67.3	12.1	13.4	0.64	2.40	6.36	21.24	1.48	9.83		
Metal	N	Ecophysiological Group	5.17 Earthworms	2.13	1.41 Soil concentration	1.45	1.17 Metal Source	0.57 Uptake duration	1.25 Soil pH	1.55 Soil organic matter	4.94	1.96 Model AIC	6.02	
As	329 Observations	n.s.	<0.01	(+)	<0.01	608	<0.01	(+)	<0.01	n.s.	(+)	<0.01	338	601
Cd	580	<0.01	<0.01	(+)	<0.01		<0.01	(+)	<0.01	n.s.	(-)	<0.01	379	

†Background soil

concentrations are from reported values from Adriano (2001), Smith et al., (2014) and Kabata-pendias and Mukherjee (2007).
 ‡Concentrations from Mineral Tolerance of Animals: 2005 by the United States National Research Council, NRC(2006).

Table 2 Model output p-values from generalize linear mixed effect models for earthworm trace metal tissue concentrations across 56 aggregated studies for random and fixed variables. (+) indicates a positive effect and (-) indicates a negative effect of a variable. Akaike information criterion (AIC) values for selecting each model are also given.

Cr	96	n.s.	<0.01	n.s.	<0.01	n.s.	(+)<0.01	n.s.	65
Cu	608	n.s.	<0.01	(+)<0.01	<0.01	(+)<0.01	n.s.	(-)<0.01	295
Hg	200	<0.01	<0.01	(+)<0.01	<0.01	(+)<0.01	n.s.	(-)<0.01	175
Mn	143	n.s.	<0.01	n.s.	n.s.	n.s.	(+)<0.01	n.s.	102
Ni	165	n.s.	<0.01	n.s.	<0.01	n.s.	(+)<0.01	n.s.	117
Pb	593	<0.01	<0.01	n.s.	<0.01	(+)<0.01	n.s.	(+)<0.01	988
Sb	90	<0.01	<0.01	(+)<0.01	n.s.	n.s.	n.s.	n.s.	48
U	112	<0.01	<0.01	(+)<0.01	n.s.	n.s.	(-)<0.01	n.s.	49
Zn	601	<0.01	<0.01	(+)<0.01	<0.01	(+)<0.01	n.s.	n.s.	317

Table 3 Model output p-values from generalize linear mixed effect models for earthworm BAF values across the 56 aggregated studies for random and fixed variables. (+) indicates a positive effect and (-) indicates a negative effect of a variable. Akaike information criterion (AIC) values for selecting each model are also given.

Metal	N	Ecophysiological Group	Earthworm Genera	Soil Extraction Method	Metal Source	Uptake duration	Soil pH	Soil organic matter	Model AIC
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As	329	n.s.	<0.01	<0.01	<0.01	(+)<0.01	n.s.	n.s.	487
Cd	580	<0.01	<0.01	<0.01	<0.01	(+)<0.01	(-)<0.01	(-)<0.01	488
Cr	96	n.s.	<0.01	<0.01	n.s.	n.s.	n.s.	n.s.	95
Cu	608	<0.01	<0.01	<0.01	<0.01	(+)<0.01	n.s.	(-)<0.01	680
Hg	200	<0.01	<0.01	n.s.	<0.01	(+)<0.01	n.s.	n.s.	230
Mn	143	n.s.	<0.01	n.s.	n.s.	n.s.	(+)<0.01	n.s.	129
Ni	165	n.s.	<0.01	<0.01	n.s.	n.s.	n.s.	n.s.	137
Pb	593	<0.01	<0.01	<0.01	<0.01	(+)<0.01	n.s.	n.s.	1077
Sb	90	<0.01	<0.01	n.s.	n.s.	n.s.	n.s.	n.s.	76
U	112	<0.01	<0.01	n.s.	n.s.	n.s.	(-)<0.01	n.s.	79
Zn	601	<0.01	<0.01	<0.01	<0.01	(+)<0.01	n.s.	(-)<0.01	699

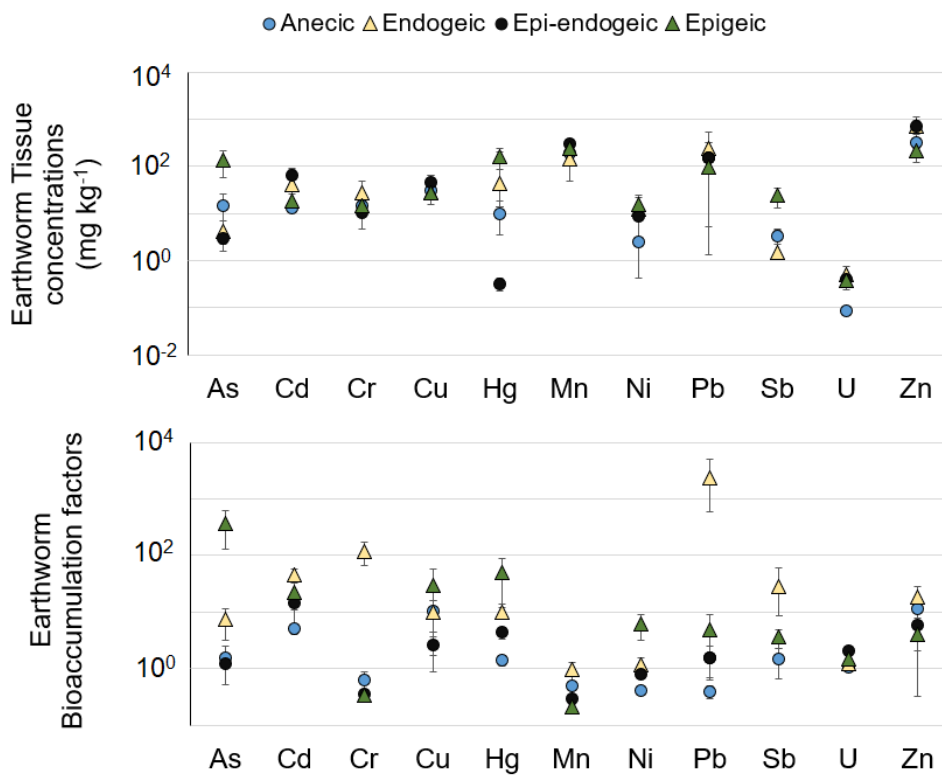


Figure 1 Earthworm trace metal concentrations and bioaccumulation factors (tissue concentrations divided by soil concentrations) across the 57 studies. Error bars are ± 1 standard deviation. N for each plot is given in the supplemental materials.

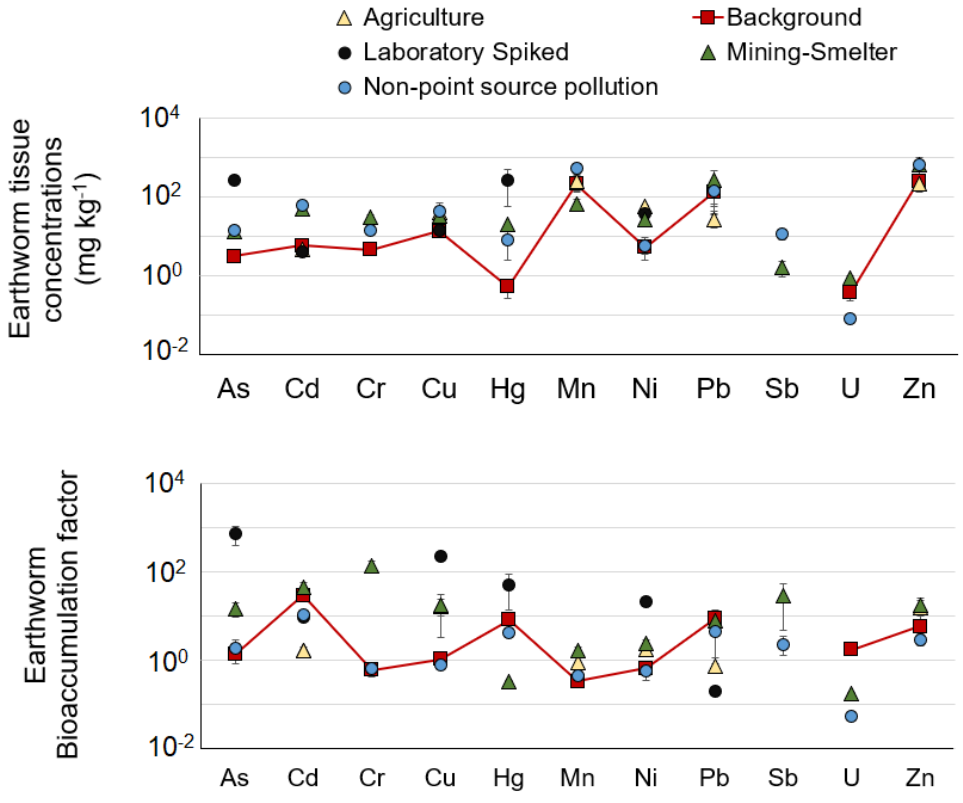
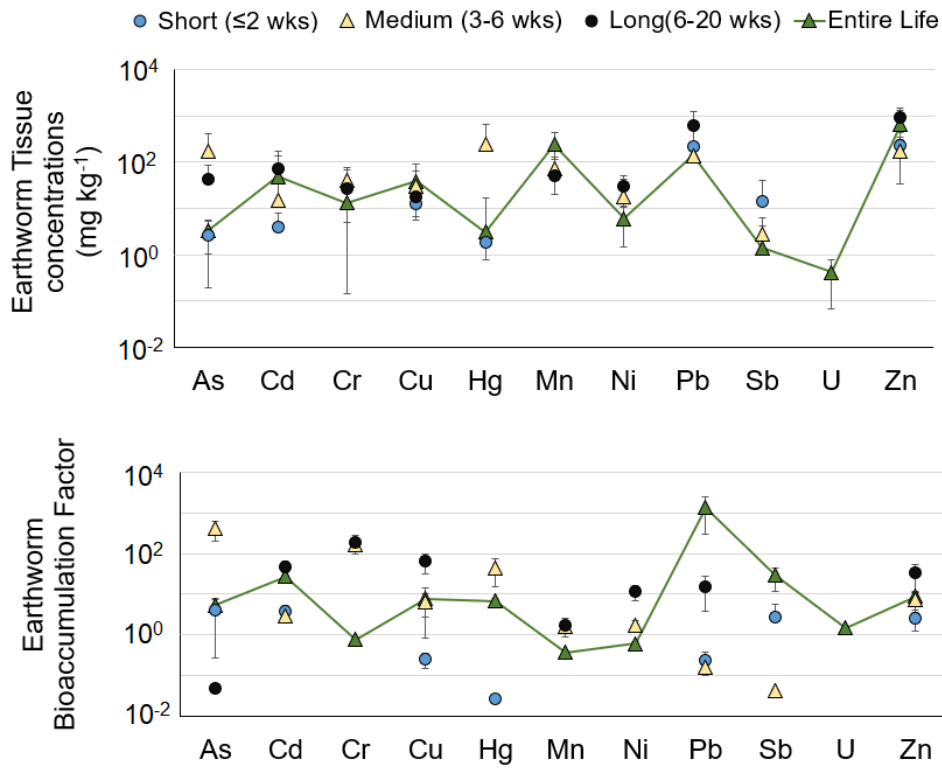


Figure 2 Plots of average earthworm tissue concentrations and bioaccumulation factors examined across types of metal sources for 56 studies. Error bars are ± 1 standard deviation. N for each plot is given in the supplemental materials.

1



2

3

4 Figure 3 Earthworm trace metal concentrations and bioaccumulation factors (tissue
5 concentrations divided by soil concentrations) examined by duration of metal exposure across
6 the 56 studies. Error bars are ± 1 standard deviation. N for each plot is given in the supplemental
7 materials.

8