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Metal bioaccumulation and cellular fractionation in an epigeic earthworm (Lumbricus rubellus): the interactive influences of population exposure histories, site-specific geochemistry and mitochondrial genotype

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1 # Metal bioaccumulation and cellular fractionation in an epigeic
2 earthworm (*Lumbricus rubellus*): the interactive influences of
3 population exposure histories, site-specific geochemistry and
4 mitochondrial genotype.

5

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8

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24 Abstract

25 Subcellular fractionation techniques were used to describe temporal changes (at
26 intervals from T_0 to T_{70} days) in the Pb, Zn and P partitioning profiles of *Lumbricus*
27 *rubellus* populations from one calcareous (M_{DH}) and one acidic (M_{CS}) geographically
28 isolated Pb/Zn-mine sites and one reference site (C_{PF}). M_{DH} and M_{CS} individuals were
29 laboratory maintained on their native field soils; C_{PF} worms were exposed to both
30 M_{DH} and M_{CS} soils. Site-specific differences in metal partitioning were found:
31 notably, the putatively metal-adapted populations, M_{DH} and M_{CS}, preferentially
32 partitioned higher proportions of their accumulated tissue metal burdens into insoluble
33 $CaPO_4$-rich organelles compared with naive counterparts, C_{PF}. Thus, it is plausible
34 that efficient metal immobilization is a phenotypic trait characterising metal tolerant
35 ecotypes. Mitochondrial cytochrome oxidase II (COII) genotyping revealed that the
36 populations indigenous to mine and reference soils belong to distinct genetic lineages,
37 differentiated by ~13%, with 7 haplotypes within the reference site lineage but fewer
38 (3 and 4, respectively) in the lineage common to the two mine sites. Collectively,
39 these observations raise the possibility that site-related genotype differences could
40 influence the toxico-availability of metals and, thus, represent a potential confounding
41 variable in field-based eco-toxicological assessments.

42

43 Keywords: earthworms, Pb & Zn, subcellular fractionation, field & lab exposures,
44 genotyping

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49 Introduction

50 Direct toxic effects arise in metal-exposed organisms not as a consequence of the total
51 accumulated tissue metal burden *per se* but when the rate of uptake overtakes the
52 combined rates of excretion and detoxification, such that the internal metal-specific
53 concentration threshold of metabolically-available metal is exceeded (Rainbow, 2007;
54 Pan and Wang, 2008). The threshold concentration denoting the transition from no
55 adverse effect to an observable adverse effect for a given metal is referred to as the
56 critical body residue (CBR) (McCarthy and Mackay, 1993; Péry, *et al.*, 2005). Thus,
57 only a fraction of the body burden is toxicologically (re)active or available (Rainbow,
58 2002; Vijver, *et al.*, 2004). Organisms have evolved mechanisms to regulate the
59 bioreactivities of essential and non-essential metals (Campbell, *et al.*, 2006). In
60 general these initially entail binding and trafficking by chaperone molecules. Essential
61 cations may subsequently be delivered to physiologically labile intracellular storage
62 sites, classically exemplified by Ca^{2+}-storing endoplasmic reticulum regions, whilst
63 excess essential and non-essential cations can also either be excreted directly or
64 immobilized as insoluble products in specialized organelles often with long half-lives.
65 These structures possess diverse morphologies and matrix compositions (Hopkin,
66 1989) that are generically referred to as 'metal-rich granules' or 'concretions'
67 (Campbell, *et al.*, 2006).

68

69 Improved toxic effects prediction and ecological risk assessment would be likely
70 outcomes of a better knowledge of the fate and speciation of metal within sentinel
71 organisms (Vijver, *et al.*, 2006; Huang, *et al.*, 2009; Jones, *et al.*, 2009). Although
72 there is some evidence from studies on aquatic invertebrates that the toxico-available
73 metals are associated with the cytosolic (soluble) fraction (Perceval, *et al.*, 2006; Péry,
74 *et al.*, 2008), it is generally the case that the relationship between metal induced

75 toxicity and accumulated burden is difficult to evaluate due to the cellular
76 compartmentalization of metals (Campbell, *et al.*, 2006; Vijver, *et al.*, 2006).
77 Techniques such as analytical electron microscopy and synchrotron-based X-ray
78 absorption spectroscopy have been used to some extent to characterize the ligand-
79 binding speciation of metals and metalloids in invertebrate tissues (Cotter-Howells, *et*
80 *al.*, 2005; Langdon, *et al.*, 2005; Arnold, *et al.*, 2008; Andre, *et al.*, 2009). However, a
81 much more widely used method for segregating invertebrate metal burdens into
82 operationally defined detoxified- and non-detoxified subcellular metal compartments
83 is to differentially centrifuge tissue homogenates. To date, such studies have mainly
84 concentrated on aquatic animals (Honeycutt, *et al.*, 1995; Wallace and Lopez, 1997;
85 Conder, *et al.*, 2002; Wallace, *et al.*, 2003; Cain, *et al.*, 2004; Vijver, *et al.*, 2004), but
86 there is a burgeoning body of publications on the assessment of metal partitioning in
87 earthworms (Arnold *et al*., 2008; Andre *et al*., 2009; Huang, *et al.*, 2009; Vijver, *et*
88 *al.*, 2006; Li, *et al.*, 2008; Jones, *et al.*, 2009).
89
90 Voets, *et al.*, (2009) reviewed some of the literature demonstrating that the cellular
91 metal distribution patterns in indigenous invertebrate and vertebrate populations often
92 differ from the distribution patterns observed in naive counterpart organisms exposed
93 to metals in laboratory or field-based transplant experiments. Evidently both exposure
94 history and genetic differentiation are biotic variables that can lead to modifications of
95 the efficiency of metal detoxification by invertebrates (Wallace, *et al.*, 2003) as well
96 as vertebrates (Knapen, *et al.*, 2004). Morgan, *et al.* (2007) also noted that the genetic
97 background of a population can confound biomarker assays, a further indication that
98 the balance between the sensitive and detoxified metal pools can be altered by micro-
99 evolutionary events. Given that comprehensive phylogenetic studies on earthworms

100 using mitochondrial and nuclear markers have recently revealed high intra-species
101 genetic diversity (Velavan*, et al.*, 2007; Novo*, et al.*, 2008) and deeply divergent
102 genetic lineages, possibly in some cases corresponding with cryptic species (King*, et*
103 *al.*, 2008; Shepeleva*, et al.*, 2008; Pérez-Losada*, et al.*, 2009), it is a major omission
104 that, to the best of our knowledge, no studies hitherto have explicitly attempted to
105 describe the cellular partitioning of metals in field populations of earthworms with
106 respect to exposure history and genotype. A recent report (Langdon*, et al.*, 2009) that
107 populations of the species *Lumbricus rubellus* inhabiting abandoned arsenic mine
108 sites have evolved resistance to the metalloid brings the omission into sharp focus.
109
110 The present study had two main aims. First, to investigate the interactive influences of
111 population exposure history and site-specific geochemistry on subcellular metal (Pb,
112 Zn) and P partitioning by comparing two putative adapted *L. rubellus* populations
113 sampled from geochemically contrasting disused Pb/Zn mines (one acidic and one
114 calcareous, respectively) and maintained on their native soils with each other and with
115 reference earthworms transferred experimentally to both polluted soils. Phosphorus
116 partitioning was monitored because phosphate is recognised as the predominant
117 counter-ion in earthworm Pb- and Zn-sequestering cellular compartments (Cotter-
118 Howells*, et al.*, 2005). The second study aim was to use mitochondrial cytochrome
119 oxidase II (COII) to genotype the three field populations. Andre*, et al.* (2010)
120 observed site-specific differences in the tissue and subcellular partitioning profiles of
121 *L. rubellus* populations indigenous to calcareous and acidic sites, respectively.
122 Moreover, the authors reported that the two identified genetically distinct *L. rubellus*
123 lineages were differentially distributed across a heterogeneous polluted landscape,
124 with lineage 'A' predominating within a calcareous Pb/Zn-polluted 'island' and

125 lineage 'B' predominating in an adjacent acidic polluted location. The present study
126 extended these previous observations through the novel combination of cell
127 fractionation and genotype analyses applied to geographically isolated populations.
128

129 Materials and Methods

130 Soil and earthworm collection and preparation

131 Soil and earthworms (mature, *L. rubellus*) were collected from one control site,
132 Pontcanna Fields (C_{PF}) ST 165779 (GPS: 51:29.63122N 3:12.24983W) and two
133 contaminated disused, metalliferous mine sites, Draethen Hollow (M_{DH}) ST 217877
134 (GPS: 51:34.96185N 3:7.88760W) and Cwmystwyth Stream (M_{CS}) SN 803748 (GPS:
135 52:21.48890N 3:45.54702W). At least ten soil samples (excluding the litter layer),
136 taken from a 0-5cm depth, were randomly collected from the sampling areas,
137 combined and mixed. The pH of all soils were measured in deionised H_2O (Boisson,
138 *et al.*, 1998) prior to them being oven dried at 30°C overnight, sieved to <2mm, then
139 digested in boiling 16N HNO_3 (Morgan and Morgan, 1990) and analysed for major
140 inorganic constituents by inductively coupled plasma - optical emission spectroscopy
141 (ICP-OES; Perkin-Elmer Opitma 3000). Analysis of an in house certified reference
142 material (a sewage sludge amended soil) indicated that that the overall analytical error
143 did not exceed 5.2%. In addition the calibration accuracy of the instrument was assessed
144 through the analysis of an in-house matrix-matched standard and was within 10%. To
145 provide an indication of the organic matter content, loss on ignition (LOI) was
146 determined for each soil sample. 10g (dry weight) of each soil was weighed in a glass
147 crucible and heated to 500°C overnight. The percentage weight reduction was then
148 recorded.
149

Comment [MEH1]: Have to include this as otherwise the reviewer would expect a reference number to see which commercially available CRM was used. If you have the name or number of the CRM you used (normally something like SSXX I can add in the certified composition.

Comment [MEH2]: This reads correctly

150 Sub-cellular fractionation

151 Thirty boxes containing 300g of contaminated M_{CS} soil and 30 boxes containing 300g
152 of contaminated M_{DH} soil were established. The soils were wetted to 70% of their
153 water-holding capacity and reference site (C_{PF}) earthworms were placed into 15 boxes
154 (three individuals per box) of M_{CS} and 15 boxes of M_{DH} soil. Similarly, 3 native M_{DH}
155 earthworms were placed into each of the remaining 15 boxes containing M_{DH} soil and
156 3 native M_{CS} earthworms into each of the remaining 15 boxes containing M_{CS} soil. At
157 1, 3, 14, 28 and 70 days of soil exposure, 3 boxes of each soil-earthworm combination
158 (i.e. maximum 'n' per 'treatment' = nine) were selected at random and the
159 earthworms depurated prior to freezing. They were depurated for an intial period of 48
160 hours on moistened filter paper (which was changed daily to prevent coprophagy),
161 followed by 24 hours in a filter-paper free petri-dish (with de-ionised H_2O) to allow
162 exudation of any filter paper consumed (Arnold and Hodson, 2007). The total
163 exposure period extended to 10 weeks in order to allow sufficient time for the toxico-
164 available fraction to stabilize, as was shown to be the case in the lumbricid species
165 *Eisenia fetida* with no previous history of metal exposure (Jones *et al*., 2009). When
166 required the earthworms were defrosted, weighed, homogenized in 0.01M Tris-HCl,
167 pH 7.5, and fractionated as described in Arnold*, et al.*, (2008) into a soluble "C
168 fraction" (cytosolic fraction including soluble proteins such as metallothionein and
169 heat shock proteins) and separate insoluble "D" (metal-rich granules) and "E
170 fractions" (tissue fragments, mitochondrial and gut contents) which for the purposes
171 of this study were combined (see supplementary Figure 1). Individual fractions were
172 digested in boiling 16N HNO_3 (Morgan and Morgan, 1990). Samples were made up to
173 volume with ultra-pure water and analysed for major inorganic constituents by ICP-
174 OES with resulting concentrations expressed as mg of metal per kg (wet weight) of

175 earthworm. Blanks were included for each analyses and detection limits were calculated as

176 800 µg L^{-1}, 200 µg L^{-1} and 400 µg L^{-1} for P, Pb and Zn respectively. No certified reference

177 materials exist for use with this fractionation method but previous analysis of standard

178 additions were within range (10%), indicating good recovery in the matrix (Arnold et al.,

179 2008) and precision, calculated from repeat analyses of samples was < 5 %. Fresh,

180 depurated, weights of the worms sacrificed at each time interval were recorded prior

181 to fractionation; weight change over the exposure periods were taken as estimates of

182 physiological condition.

183

Comment [MEH3]: If you want to convert these into an equivalent mg per kg wet weight so that they are the same units as the results you report you need to assume a mass of earthworm digested and use this, together with the volume of digestate to calculate them

184 Mitochondrial Cytochrome Oxidase II (COII) genotyping

185 *L. rubellus* earthworms from the M_{DH} (n=22), M_{CS} (n=32) and C_{PF} (n=29) sites, and

186 single *L. castaneus* and *L. eiseni* (from uncontaminated sites in Lancashire, England)

187 were collected by digging and hand-sorting. The animals were transported back to the

188 laboratory in their native soil and depurated (Arnold and Hodson, 2007). A short

189 length (approximately 3cm) of posterior segments was clipped from all *L. rubellus*

190 individuals, and genomic DNA was extracted using DNAzol reagent (Invitrogen Ltd.,

191 Paisley, UK). Forward (5'-TAGCTCACTTAGATGCCA) and reverse (5'-

192 GTATGCGGATTTCTAATTGT) *L. rubellus*-specific primers were used to amplify

193 the cytochrome oxidase II (COII) gene, prior to an Exo-SAP-IT PCR clean-up and

194 sequencing using ABI PRISM® BigDye v3.1 Terminator Sequencing technology

195 (Applied Biosystems, USA) as described by Andre, *et al.*, (2010). Raw sequence

196 traces were confirmed using Finch TV before being imported into Mega v3.1 (Kumar,

197 *et al.*, 2004) for alignment and tree construction. The distance-based neighbour

198 joining (NJ) algorithm (Saitou and Nei, 1987), using p-distance, was used to estimate

199 tree topology and calculate branch lengths.

200

201 Results

202 Soil analysis

203 Tables 1 and 2 show the concentration of Pb, Zn and P in soil and earthworms
204 sampled from the metalliferous M_{CS} and M_{DH} and reference C_{PF} sites, as well as the
205 percentage body weight change over the full extent of the exposure period. The Pb
206 and Zn soil concentrations were highest at the calcareous M_{DH} site; acidic M_{CS} soil
207 was only mildly contaminated, but contained significantly higher Pb and Zn
208 concentrations than the reference C_{PF} soil. Phosphorus concentration was significantly
209 higher in C_{PF} reference soil than in the two metalliferous soils. C_{PF} earthworms
210 maintained higher whole body P concentrations after 70 days of exposure to both
211 metalliferous soils when compared with their M_{DH} and M_{CS} counterparts. Mean total
212 earthworm tissue Pb and Zn levels to some extent reflected the corresponding soil Pb
213 and Zn concentrations, although it is noteworthy that the worms indigenous to the
214 acidic M_{CS} site had a Pb bioaccumulation factor of greater than 1 (based upon dry-
215 weight values, data not shown).

216

217 Body mass changes

218 Mortality was evident across all treatment groups and the mean fresh weights of
219 earthworms, including C_{PF} worms on their 'own' unpolluted reference soil, decreased
220 considerably over the exposure period. These observations indicate that a degree of
221 stress mediated by dietary restriction and/or metal toxicity was experienced by all
222 earthworms in our experimental regime.

223

224 Sub-cellular fractionation

225 *Lead*: Following a ten-week exposure period, the *ex-situ* partitioning profiles were
226 similar for both indigenous and naive introduced earthworms exposed to the same soil
227 (Figure 1). Significant increases in Pb concentration were seen in the soluble (C) and
228 insoluble (D+E) fractions of all worms exposed to M_{DH} soil, and C_{PF} individuals
229 exposed to M_{CS} soil. M_{CS} individuals only demonstrated a slight increase in insoluble
230 Pb. Destroying the physical integrity of the field soils by indiscriminate sampling,
231 drying, sieving, homogenisation, and re-hydrating appears to have released more
232 metal for uptake into earthworm tissues above the corresponding equilibrated field
233 levels. Pb was found to preferentially partition into the non-soluble or detoxified
234 (D+E) fraction in all earthworm/soil combinations (Figure 1B). Plotting the time
235 course partitioning data with the soluble fraction Pb values expressed as a percentage
236 of the whole body Pb concentration values (Figure 2) revealed differences in the
237 efficiencies of incorporating Pb into the detoxified fraction between indigenous
238 worms and naive worms introduced into the metalliferous soils. Specifically, and
239 consistently over the entire exposure period, the proportion of Pb within the sensitive
240 soluble fraction of M_{CS} earthworms was proportionately less than that in C_{PF}
241 earthworms maintained on M_{CS} soil (Figure 2A). A similar efficiency difference was
242 found between M_{DH} and C_{PF} earthworms, but only after 10-weeks of exposure (Figure
243 2B); at earlier intervals no difference was apparent in Pb partitioning between these
244 two populations. Naïve earthworms accumulated Pb linearly in all three fractions over
245 the duration of the exposure period. In contrast, after 28 days M_{DH} earthworms
246 preferentially partitioned the majority of accumulated metal into the insoluble
247 (detoxified) fraction. As the concentrations of Cu, Ni, and Sr did not change
248 appreciably over the 10 week exposure period (data not shown), this implies that the

249 temporal changes in the concentrations of Pb and Zn in the subcellular fractions were
250 not directly linked to the loss of whole-worm weight over this period.
251
252 *Zinc*: The temporal partitioning profiles of Zn resemble those of Pb, with indigenous
253 worms with multi-generational histories of metal exposure (M_{DH} and M_{CS}) and naive
254 worms with no previous field history of exposure (C_{PF}) each sequestering Zn
255 primarily in the insoluble (D+E) fraction, and restricting the cytosolic soluble Zn
256 fraction within relatively narrow limits (Figure 3). Zn uptake in both the soluble and
257 insoluble fractions by naive C_{PF} earthworms exposed to M_{DH} soil occurred in a linear
258 fashion during the entire exposure period, whereas after 28 days of exposure M_{DH}
259 earthworms appeared to preferentially partition Zn into the insoluble (detoxified)
260 fraction. The similarities between Pb and Zn partitioning extended to the comparative
261 efficiency of restricting the metals to the detoxified compartment in indigenous versus
262 introduced populations (Figure 4): the proportion of Zn present in the soluble fraction
263 was appreciably lower in earthworms from the heavily polluted M_{DH} site at all time
264 points compared with that in C_{PF} worms introduced to the M_{DH} soil; the proportion of
265 soluble fraction Zn in M_{CS} worms was appreciably lower than in C_{PF} worms
266 maintained on M_{CS} soil at three time points (3, 14, and 70 days).
267
268 *Phosphorus*: In both indigenous mine-site populations maintained on their 'own'
269 soils, and in naive worms introduced to the metalliferous soils, a fairly steady
270 redistribution of P from the soluble cytosolic phase to the insoluble compartment
271 occurred during the ten-week exposure period (cf. Figures 5A and 5B). A
272 considerably higher insoluble P concentration was measured in indigenous and

273 introduced earthworms exposed to M_{DH} soil compared with the two treatment groups
274 exposed to the significantly less polluted M_{CS} soil (Figure 5).
275
276 Cytochrome oxidase II (COII) genotyping
277 The phylogenetic structure of the study populations was assessed using the
278 mitochondrial cytochrome oxidase II (mtDNA COII) gene sequence data of
279 individuals sampled from the three sites. Good quality COII nucleotide sequences
280 (304bp) were aligned from 85 *L. rubellus* earthworms and from individuals of *L.*
281 *castaneus* and *L. eiseni* to rule out the possibility of misidentification. Only functional
282 COII sequences, with no stop or nonsense codons in the reading frame, were used.
283 Intra- and inter-site evolutionary relationships were phylogenetically analysed (Figure
284 6), with the tree constructed using the distance-based neighbour joining (NJ)
285 algorithm based upon p-distance. Only one representative of each site and haplotype
286 are shown and the resulting tree topology was well supported by bootstrap analyses.
287 The sampled *L. rubellus* individuals could be resolved into two distinct genetic
288 lineages (lineage A and B, respectively), with a mean inter-lineage mtDNA sequence
289 divergence of 13%.
290
291 C_{PF} earthworms grouped exclusively within lineage A, and comprised 7 distinct
292 haplotypes, with a between-haplotype diversity of 1 to 4% (Figure 6). In comparison,
293 the M_{DH} and M_{CS} populations derived from mine-associated soils belonged
294 predominantly to the lineage B genotype, and comprised 3 (M_{DH}) and 4 haplotypes
295 (M_{CS}) exhibiting between-haplotype diversity of 1 to 2%, respectively. Only one
296 individual from each mine site had lineage A genotype signatures. The genetic
297 distance between *L. rubellus* and two other *Lumbricus* species (*L. castaneus* and *L.*

298 *eiseni*) was calculated as 18.1%, thus indicating that the *L. rubellus* field populations
299 were correctly assigned.
300
301 Discussion
302 Abandoned metal mine soils in the UK and elsewhere harbour locally adapted
303 earthworm populations with innate abilities to tolerate phenomenally high internal
304 body loads of certain metals. For example, earthworms evidently thrive in field soils
305 contaminated to degrees exceeding by an order of magnitude the exposure level that
306 severely compromises reproduction in spiked laboratory soils (Spurgeon*, et al.*, 1994).
307 That these are residents and not immigrants from less-contaminated surrounding soil
308 is one way of interpreting the ‘patchy’ pattern of genotype distributions observed in *L.*
309 *rubellus* across geochemically heterogeneous metalliferous landscapes (Andre*, et al.*,
310 2010). A number of published studies provide mechanistic insights concerning the
311 modes of metal detoxification within discrete subcellular compartments in these
312 chronically exposed natural populations (Morgan and Morris, 1982; Morris and
313 Morgan, 1986; Morgan and Morgan, 1989a; 1998; Sturzenbaum*, et al.*, 2001).
314 However, evidence of phenotypic differences at the behavioural, physiological and
315 molecular levels between populations that have undergone multiple generations of
316 exposure and their counterparts with no comparable metal exposure history in their
317 native habitat remain sparse. Therefore, by comparing the subcellular partitioning
318 profiles amongst earthworm populations native to contaminated and clean sites,
319 further inferences into the metal management strategies of putatively adapted
320 ecotypes may be gained.
321

322 A Cd-resistant ecotype of the freshwater oligochaete *Lumbriculus hoffmeisteri* has
323 been shown to possess enhanced Cd accumulation efficiency (Klerks and
324 Bartholomew, 1991) and a concomitant reduction in the amount of trophically-
325 available Cd (Wallace and Lopez, 1997). Such integrated duality is also expressed for
326 Pb in at least one of the earthworm populations, M_{DH}, examined in the present study.
327 Specifically, Pb partitioning profiles for M_{DH} individuals showed a much lower
328 absolute level of soluble Pb (approximately 57% after 70 days exposure), when
329 compared to naive C_{PF} earthworms exposed to the same M_{DH} polluted soil. This
330 population appears to have evolved a capability to limit Pb toxico-availability
331 possibly through modifications of components of Ca^{2+} transporting pathway, such as
332 the sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA) (Andre*, et al.*,
333 2010). The notion is supported by plotting the soluble Pb fraction of M_{DH} worms as a
334 percentage of the total body load; although the total body burden increases over the 10
335 week exposure period on their native soil, there is a significantly lower proportion of
336 the Pb burden distributed in the soluble fraction compared with that found in C_{PF}
337 reference site worms maintained in the laboratory for the same period on M_{DH}
338 metalliferous soil. A similar differential was also recorded in the proportional Pb
339 content of the soluble fractions of M_{CS} and C_{PF} worms at all time intervals of
340 laboratory exposure to M_{CS} soil. This phenomenon of increasing metal concentrations
341 in earthworms from metal-contaminated soils maintained on their 'own' soils in the
342 laboratory has been reported by others (e.g. Corp and Morgan, 1991). It is not easily
343 explained in the cases of metal that are not especially redox active other than as a
344 consequence of the destruction of the physical structure of soil, with a release of
345 previously stabilized metal into the 'bioavailable' pool, i.e. a partial reversal of the
346 'ageing' process. The indication that Pb bioreactivity is reduced in earthworms

347 indigenous to Pb-contaminated soils was reinforced by the finding that the weight loss
348 experienced by reference C_{PF} earthworms maintained on the two studied metalliferous
349 soils exceeded that experienced by earthworms native to the field soils. These data
350 highlight the crucial role that intracellular components and machinery play in
351 facilitating the efficient delivery of metals to intracellular compartments where they
352 are sequestered in insoluble states.

353

354 Several publications have shown that lysosome-like chloragosomes within the
355 chlorogogenous tissue (possessing some functional similarities to vertebrate
356 hepatocytes) are the main metal-sequestering organelles. Chloragosomes represent
357 phosphate-rich storage compartments for group A, O-seeking, metals (Morgan and
358 Morris, 1982; Morgan and Morgan, 1989a; b; 1998). Andre*, et al.*, (2009) investigated
359 the ligand speciation of Pb within whole earthworms using synchrotron- based XAS
360 analysis, and obtained XANES spectra that unambiguously revealed that *L. rubellus*
361 with a protracted population history of Pb exposure preferentially sequester the metal
362 as insoluble pyromorphite [$Pb_5(PO_4)_3Cl$] and $Pb_3(PO_4)_2$. Given this fact, the
363 observation in the present study that intracellular P speciation shifts appreciably in all
364 exposures over the entire 10 week period from a relatively soluble to less soluble state
365 presumably to associate with intruding Pb is functionally logical.

366

367 Due to its biological essentiality it is predictable that invertebrates are able to regulate
368 intracellular Zn levels to a considerable degree. Chromatographic observations
369 demonstrate that this may be achieved through Zn binding to a variety of low- and
370 high-molecular weight molecules (Susuki*, et al.*, 1988; Cain and Luoma, 1998; Lock

371 and Janssen, 2001). Homeostatic systems operate to not only sequester and detoxify
372 excess Zn but, when needed, to release Zn in order to meet the cells physiological
373 requirements. This system is undoubtedly at work in earthworms from M_{DH} as,
374 despite considerable increases in total body load, they demonstrate the ability to
375 maintain their intracellular soluble Zn content within relatively narrow limits. Again,
376 the phosphate-rich chloragosomes are implicated in Zn storage and detoxification
377 alongside a less well characterised sulphur-rich organelle, the cadmosome
378 (Sturzenbaum*, et al.*, 1998). The involvement of chloragosomes and cadmosomes in
379 excess Zn sequestration has been corroborated by XAS analyses, with XANES
380 spectra indicating that Zn binds to both O- and S-donating ligands (Andre*, et al.*,
381 2009).
382
383 Cryptic or sibling species are typically found in taxa that thrive in complex,
384 heterogeneous, environments and have been discovered by genotyping fauna
385 inhabiting diverse marine, freshwater, and terrestrial habitats (Sturmbauer*, et al.*,
386 1999; Pinceel*, et al.*, 2004; Mathews, 2006; Pfenninger and Schwenk, 2007). The *L.*
387 *rubellus* population indigenous to the unpolluted field site, C_{PF}, belongs exclusively to
388 lineage A and can be resolved into 7 haplotypes, whilst the two geographically
389 isolated mine-site populations both belong to the genotypically distinct lineage B
390 comprised of 3 and 4, respectively, distinct haplotypes. The number of *L. rubellus*
391 populations examined was too restricted to draw firm conclusions regarding
392 microevolutionary genealogies, but the higher intra-lineage diversity of the C_{PF}
393 sample is indicative of a relatively stationary population that has undergone multiple
394 introductions and bottleneck episodes during its evolutionary history (Harpending,
395 1994). It is tempting to interpret the comparatively narrow genetic diversity within the

396 lineage B inhabitants of the mine sites as a hallmark of stress-driven genetic erosion
397 processes (natural selection, genetic drift, inbreeding) having acted upon these
398 populations. Genetic erosion can certainly accompany small fragmented populations
399 (Buza *et al*., 2000) such as those found inhabiting the 'islands of toxicity' that typify
400 abandoned metal mine sites. However, the genetic erosion notion as an explanation of
401 the genetic structure of mine-associated earthworm populations should be tempered
402 with the knowledge that calculations from genetic parameters lead to the conclusion
403 that lineage A (with an inter-stadial expansion time of ~250 000 years BP) is
404 appreciably 'older' than lineage B (expansion time of ~17000 years BP) (Andre*, et*
405 *al.*, 2010) and may have had the opportunity to evolve more genetic richness. Peles*, et*
406 *al.* (2003) suggested that certain alleles and genotypes in *L. rubellus* may be more
407 sensitive to the effects of heavy metals because the frequency of both differed
408 significantly at polymorphic loci between populations inhabiting sewage
409 contaminated and reference soils. Conversely, Haimi*, et al.* (2007) reported that metal
410 contamination did not significantly impact upon clonal diversity in the earthworm
411 *Dendrobaena octaedra*. Analogous inter- or intra-lineage conclusions cannot firmly
412 be drawn from the present study on *L. rubellus*. Whether or not the two deeply
413 divergent *L. rubellus* lineages warrant the status of (cryptic) species must await
414 further genetic and breeding evidence. Nevertheless, it is noteworthy that Lentzsch
415 and Golldack (2006) observed that species richness not ambient soil conditions was
416 the overriding factor affecting intraspecific diversity and genotype abundance in the
417 earthworm *Aporrectodea caliginosa*, thus ostensibly supporting the hypothesis that
418 ecological niches are colonised at a species level prior to local population-level
419 adaption. The shallow soils often associated with abandoned metal mines usually

420 harbour impoverished earthworm communities, in many instances no more than two
421 taxonomically accepted representatives of the epigeic ecophysiological group.
422
423 In conclusion, the ability of an adapted population to tolerate the prevailing stress-
424 evoking conditions in severely polluted habitats most probably involve heritable and
425 integrated combinations of physiological, morphological and behavioural
426 modifications. Thus, it is plausible to hypothesise that a metal tolerant earthworm
427 population has evolved efficient mechanisms of detoxification that feature an
428 enhanced immobilisation capacity coupled to a relative reduction in the metal
429 sensitive fraction as an important component of their holistic adaptive arsenal.
430 However, inferences about population-specific adaptation based on subcellular metal
431 partitioning profiles should be drawn with a measure of caution because of the
432 possibility that they could be attributable to lineage-specific traits that are independent
433 of chronic metal exposure. This is illustrated by the findings of (Heethoff*, et al.*,
434 2004) that the parthenogenic earthworm *Octolasion tyrtaeum* is differentiated into two
435 lineages differing significantly in body size. Such findings, together with those arising
436 from the present study, raise the spectre that field-based eco-toxicological assessments
437 that utilise earthworms, particularly those emanating from discriminating 'omics'
438 measurements, might benefit from the elimination of a potential confounding biotic
439 variable through prior genotyping of all individuals to establish that they possess
440 some genetic background equivalence.
441
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447

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630

631 Figure Legends

632 Figure 1

633 Time course of Pb partitioning into soluble (A) and non-soluble (B) cellular fractions
634 in *Lumbricus rubellus* sampled from three populations and maintained on two mine-
635 associated metal contaminated soils. Pb concentrations (per unit wet weight of
636 earthworm) are presented as mean ±S.E. (maximum 'n' =9). C_{PF} refers to the
637 reference site at Pontcanna Fields, M_{CS} the metalliferous acidic site Cwmystwyth
638 Stream and M_{DH} the metalliferous calcareous site, Draethen Hollow.

639

640 Figure 2

641 Time course of subcellular Pb distribution in *Lumbricus rubellus* sampled from three
642 populations and maintained on two mine-associated metal contaminated soils, with

643 the soluble metal concentration expressed as a percentage of the total body
644 concentration. The Pb levels shown represent fractions extracted from M_{CS} and C_{PF}
645 earthworms in M_{CS} soil (A) and M_{DH} and C_{PF} earthworms in M_{DH} soil (B). [See Fig.
646 1 for dataset error bars and Fig. 1 legend for site identifiers.]
647
648 Figure 3
649 Time course of Zn partitioning into soluble (A) and non-soluble (B) cellular fractions
650 in *Lumbricus rubellus* sampled from three populations and maintained on two mine-
651 associated metal contaminated soils. Zn concentrations (per unit wet weight of
652 earthworm) are presented as mean ±S.E. (maximum 'n' =9). [See Fig. 1 legend for
653 site identifiers.]
654
655 Figure 4
656 Time course of subcellular Zn distribution in *Lumbricus rubellus* sampled from three
657 populations and maintained on two field mine-associated metal contaminated soils,
658 with the soluble metal concentration expressed as a percentage of the total body
659 concentration. The Zn levels shown represent fractions extracted from M_{CS} and C_{PF}
660 earthworms in M_{CS} soil (A) and M_{DH} and C_{PF} earthworms in M_{DH} soil (B). [See Fig. 1
661 for dataset error bars and Fig. 1 legend for site identifiers.]
662
663 Figure 5
664 Time course of P partitioning into soluble (A) and non-soluble (B) cellular fractions in
665 *Lumbricus rubellus* sampled from three populations and maintained on two field-
666 derived metal contaminated soils. P concentrations (per unit wet weight of

667 earthworm) are presented as mean ±S.E. (maximum ‘n’ = 9). [See Fig. 1 legend for
668 site identifiers.]
669
670 Figure 6
671 Phylogenetic tree based on p-distance of the cytochrome oxidase II mitochondrial
672 gene of 87 *Lumbricus rubellus* individuals from the contaminated sites M_{DH}
673 (triangles) and M_{CS} (circles) and the reference site C_{PF} (squares). *L. castaneus* and *L.*
674 *eiseni* individuals (open squares) are included. Two *L. rubellus* lineages are apparent,
675 termed lineage ‘A’ (light grey) and ‘B’ (dark grey), respectively, with a mean inter-
676 lineage mtDNA sequence divergence of approximately 13%. Tree topology was well
677 supported by bootstrap analyses. Only one representative of each haplotype at the
678 particular sites and are shown; numbers in parentheses indicate the numbers of
679 individuals of a given haplotype.