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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24 <u>Abstract</u>

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 <u>Keywords</u>: earthworms, Pb & Zn, subcellular fractionation, field & lab exposures,

44 genotyping

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Introduction

49 <u>Introduction</u>

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

3

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   <u>Materials and Methods</u>

130   <u>Soil and earthworm collection and preparation</u>

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

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

184 <u>Mitochondrial Cytochrome Oxidase II (COII) genotyping</u>

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

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

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

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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

10

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

11

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   <u>Cytochrome oxidase II (COII) genotyping</u>

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 <u>Discussion</u>

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

15

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

16

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

445 Dr Trevor Piearce of Lancaster University for providing us with specimens of

446 *Lumbricus eiseni* for genotyping.

447

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630

631 <u>Figure Legends</u>

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.