

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

Article

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1 Metal bioaccumulation and cellular fractionation in an epigeic
2 earthworm (*Lumbricus rubellus*): the interactive influences of
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4 mitochondrial genotype.

5
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Abstract

Subcellular fractionation techniques were used to describe temporal changes (at intervals from T₀ to T₇₀ days) in the Pb, Zn and P partitioning profiles of *Lumbricus rubellus* populations from one calcareous (M_{DH}) and one acidic (M_{CS}) geographically isolated Pb/Zn-mine sites and one reference site (C_{PF}). M_{DH} and M_{CS} individuals were laboratory maintained on their native field soils; C_{PF} worms were exposed to both M_{DH} and M_{CS} soils. Site-specific differences in metal partitioning were found: notably, the putatively metal-adapted populations, M_{DH} and M_{CS}, preferentially partitioned higher proportions of their accumulated tissue metal burdens into insoluble CaPO₄-rich organelles compared with naive counterparts, C_{PF}. Thus, it is plausible that efficient metal immobilization is a phenotypic trait characterising metal tolerant ecotypes. Mitochondrial cytochrome oxidase II (COII) genotyping revealed that the populations indigenous to mine and reference soils belong to distinct genetic lineages, differentiated by ~13%, with 7 haplotypes within the reference site lineage but fewer (3 and 4, respectively) in the lineage common to the two mine sites. Collectively, these observations raise the possibility that site-related genotype differences could influence the toxico-availability of metals and, thus, represent a potential confounding variable in field-based eco-toxicological assessments.

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

Introduction

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

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

toxicity and accumulated burden is difficult to evaluate due to the cellular compartmentalization of metals (Campbell, *et al.*, 2006; Vijver, *et al.*, 2006). Techniques such as analytical electron microscopy and synchrotron-based X-ray absorption spectroscopy have been used to some extent to characterize the ligand-binding speciation of metals and metalloids in invertebrate tissues (Cotter-Howells, *et al.*, 2005; Langdon, *et al.*, 2005; Arnold, *et al.*, 2008; Andre, *et al.*, 2009). However, a much more widely used method for segregating invertebrate metal burdens into operationally defined detoxified- and non-detoxified subcellular metal compartments is to differentially centrifuge tissue homogenates. To date, such studies have mainly concentrated on aquatic animals (Honeycutt, *et al.*, 1995; Wallace and Lopez, 1997; Conder, *et al.*, 2002; Wallace, *et al.*, 2003; Cain, *et al.*, 2004; Vijver, *et al.*, 2004), but there is a burgeoning body of publications on the assessment of metal partitioning in earthworms (Arnold *et al.*, 2008; Andre *et al.*, 2009; Huang, *et al.*, 2009; Vijver, *et al.*, 2006; Li, *et al.*, 2008; Jones, *et al.*, 2009).

Voets, *et al.*, (2009) reviewed some of the literature demonstrating that the cellular metal distribution patterns in indigenous invertebrate and vertebrate populations often differ from the distribution patterns observed in naive counterpart organisms exposed to metals in laboratory or field-based transplant experiments. Evidently both exposure history and genetic differentiation are biotic variables that can lead to modifications of the efficiency of metal detoxification by invertebrates (Wallace, *et al.*, 2003) as well as vertebrates (Knäpen, *et al.*, 2004). Morgan, *et al.* (2007) also noted that the genetic background of a population can confound biomarker assays, a further indication that the balance between the sensitive and detoxified metal pools can be altered by micro-evolutionary events. Given that comprehensive phylogenetic studies on earthworms

using mitochondrial and nuclear markers have recently revealed high intra-species genetic diversity (Velavan, *et al.*, 2007; Novo, *et al.*, 2008) and deeply divergent genetic lineages, possibly in some cases corresponding with cryptic species (King, *et al.*, 2008; Shepeleva, *et al.*, 2008; Pérez-Losada, *et al.*, 2009), it is a major omission that, to the best of our knowledge, no studies hitherto have explicitly attempted to describe the cellular partitioning of metals in field populations of earthworms with respect to exposure history and genotype. A recent report (Langdon, *et al.*, 2009) that populations of the species *Lumbricus rubellus* inhabiting abandoned arsenic mine sites have evolved resistance to the metalloid brings the omission into sharp focus.

The present study had two main aims. First, to investigate the interactive influences of population exposure history and site-specific geochemistry on subcellular metal (Pb, Zn) and P partitioning by comparing two putative adapted *L. rubellus* populations sampled from geochemically contrasting disused Pb/Zn mines (one acidic and one calcareous, respectively) and maintained on their native soils with each other and with reference earthworms transferred experimentally to both polluted soils. Phosphorus partitioning was monitored because phosphate is recognised as the predominant counter-ion in earthworm Pb- and Zn-sequestering cellular compartments (Cotter-Howells, *et al.*, 2005). The second study aim was to use mitochondrial cytochrome oxidase II (COII) to genotype the three field populations. Andre, *et al.* (2010) observed site-specific differences in the tissue and subcellular partitioning profiles of *L. rubellus* populations indigenous to calcareous and acidic sites, respectively. Moreover, the authors reported that the two identified genetically distinct *L. rubellus* lineages were differentially distributed across a heterogeneous polluted landscape, with lineage ‘A’ predominating within a calcareous Pb/Zn-polluted ‘island’ and

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

Materials and Methods

Soil and earthworm collection and preparation

Soil and earthworms (mature, *L. rubellus*) were collected from one control site, Pontcanna Fields (C_{PF}) ST 165779 (GPS: 51:29.63122N 3:12.24983W) and two contaminated disused, metalliferous mine sites, Draethen Hollow (M_{DH}) ST 217877 (GPS: 51:34.96185N 3:7.88760W) and Cwmystwyth Stream (M_{CS}) SN 803748 (GPS: 52:21.48890N 3:45.54702W). At least ten soil samples (excluding the litter layer), taken from a 0-5cm depth, were randomly collected from the sampling areas, combined and mixed. The pH of all soils were measured in deionised H₂O (Boisson, *et al.*, 1998) prior to them being oven dried at 30°C overnight, sieved to <2mm, then digested in boiling 16N HNO₃ (Morgan and Morgan, 1990) and analysed for major inorganic constituents by inductively coupled plasma - optical emission spectroscopy (ICP-OES; Perkin-Elmer Optima 3000). Analysis of an in house certified reference material (a sewage sludge amended soil) indicated that the overall analytical error did not exceed 5.2%. In addition the calibration accuracy of the instrument was assessed through the analysis of an in-house matrix-matched standard and was within 10%. To provide an indication of the organic matter content, loss on ignition (LOI) was determined for each soil sample. 10g (dry weight) of each soil was weighed in a glass crucible and heated to 500°C overnight. The percentage weight reduction was then recorded.

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Sub-cellular fractionation

Thirty boxes containing 300g of contaminated M_{CS} soil and 30 boxes containing 300g of contaminated M_{DH} soil were established. The soils were wetted to 70% of their water-holding capacity and reference site (C_{PF}) earthworms were placed into 15 boxes (three individuals per box) of M_{CS} and 15 boxes of M_{DH} soil. Similarly, 3 native M_{DH} earthworms were placed into each of the remaining 15 boxes containing M_{DH} soil and 3 native M_{CS} earthworms into each of the remaining 15 boxes containing M_{CS} soil. At 1, 3, 14, 28 and 70 days of soil exposure, 3 boxes of each soil-earthworm combination (i.e. maximum 'n' per 'treatment' = nine) were selected at random and the earthworms depurated prior to freezing. They were depurated for an initial period of 48 hours on moistened filter paper (which was changed daily to prevent coprophagy), followed by 24 hours in a filter-paper free petri-dish (with de-ionised H₂O) to allow exudation of any filter paper consumed (Arnold and Hodson, 2007). The total exposure period extended to 10 weeks in order to allow sufficient time for the toxicologically available fraction to stabilize, as was shown to be the case in the lumbricid species *Eisenia fetida* with no previous history of metal exposure (Jones *et al.*, 2009). When required the earthworms were defrosted, weighed, homogenized in 0.01M Tris-HCl, pH 7.5, and fractionated as described in Arnold, *et al.*, (2008) into a soluble "C fraction" (cytosolic fraction including soluble proteins such as metallothionein and heat shock proteins) and separate insoluble "D" (metal-rich granules) and "E fractions" (tissue fragments, mitochondrial and gut contents) which for the purposes of this study were combined (see supplementary Figure 1). Individual fractions were digested in boiling 16N HNO₃ (Morgan and Morgan, 1990). Samples were made up to volume with ultra-pure water and analysed for major inorganic constituents by ICP-OES with resulting concentrations expressed as mg of metal per kg (wet weight) of

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

earthworm. Blanks were included for each analyses and detection limits were calculated as 800 $\mu\text{g L}^{-1}$, 200 $\mu\text{g L}^{-1}$ and 400 $\mu\text{g L}^{-1}$ for P, Pb and Zn respectively. No certified reference materials exist for use with this fractionation method but previous analysis of standard additions were within range (10%), indicating good recovery in the matrix (Arnold et al., 2008) and precision, calculated from repeat analyses of samples was < 5 %. Fresh, depurated, weights of the worms sacrificed at each time interval were recorded prior to fractionation; weight change over the exposure periods were taken as estimates of physiological condition.

Mitochondrial Cytochrome Oxidase II (COII) genotyping

L. rubellus earthworms from the M_{DH} (n=22), M_{CS} (n=32) and C_{PF} (n=29) sites, and single *L. castaneus* and *L. eiseni* (from uncontaminated sites in Lancashire, England) were collected by digging and hand-sorting. The animals were transported back to the laboratory in their native soil and depurated (Arnold and Hodson, 2007). A short length (approximately 3cm) of posterior segments was clipped from all *L. rubellus* individuals, and genomic DNA was extracted using DNAzol reagent (Invitrogen Ltd., Paisley, UK). Forward (5'-TAGCTCACTTAGATGCCA) and reverse (5'-GTATGCGGATTTCTAATTGT) *L. rubellus*-specific primers were used to amplify the cytochrome oxidase II (COII) gene, prior to an Exo-SAP-IT PCR clean-up and sequencing using ABI PRISM[®] BigDye v3.1 Terminator Sequencing technology (Applied Biosystems, USA) as described by Andre, et al., (2010). Raw sequence traces were confirmed using Finch TV before being imported into Mega v3.1 (Kumar, et al., 2004) for alignment and tree construction. The distance-based neighbour joining (NJ) algorithm (Saitou and Nei, 1987), using p-distance, was used to estimate tree topology and calculate branch lengths.

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 naïve 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 naïve 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

temporal changes in the concentrations of Pb and Zn in the subcellular fractions were not directly linked to the loss of whole-worm weight over this period.

Zinc: The temporal partitioning profiles of Zn resemble those of Pb, with indigenous worms with multi-generational histories of metal exposure (M_{DH} and M_{CS}) and naive worms with no previous field history of exposure (C_{PF}) each sequestering Zn primarily in the insoluble (D+E) fraction, and restricting the cytosolic soluble Zn fraction within relatively narrow limits (Figure 3). Zn uptake in both the soluble and insoluble fractions by naive C_{PF} earthworms exposed to M_{DH} soil occurred in a linear fashion during the entire exposure period, whereas after 28 days of exposure M_{DH} earthworms appeared to preferentially partition Zn into the insoluble (detoxified) fraction. The similarities between Pb and Zn partitioning extended to the comparative efficiency of restricting the metals to the detoxified compartment in indigenous versus introduced populations (Figure 4): the proportion of Zn present in the soluble fraction was appreciably lower in earthworms from the heavily polluted M_{DH} site at all time points compared with that in C_{PF} worms introduced to the M_{DH} soil; the proportion of soluble fraction Zn in M_{CS} worms was appreciably lower than in C_{PF} worms maintained on M_{CS} soil at three time points (3, 14, and 70 days).

Phosphorus: In both indigenous mine-site populations maintained on their ‘own’ soils, and in naive worms introduced to the metalliferous soils, a fairly steady redistribution of P from the soluble cytosolic phase to the insoluble compartment occurred during the ten-week exposure period (cf. Figures 5A and 5B). A considerably higher insoluble P concentration was measured in indigenous and

introduced earthworms exposed to M_{DH} soil compared with the two treatment groups exposed to the significantly less polluted M_{CS} soil (Figure 5).

Cytochrome oxidase II (COII) genotyping

The phylogenetic structure of the study populations was assessed using the mitochondrial cytochrome oxidase II (mtDNA COII) gene sequence data of individuals sampled from the three sites. Good quality COII nucleotide sequences (304bp) were aligned from 85 *L. rubellus* earthworms and from individuals of *L. castaneus* and *L. eiseni* to rule out the possibility of misidentification. Only functional COII sequences, with no stop or nonsense codons in the reading frame, were used. Intra- and inter-site evolutionary relationships were phylogenetically analysed (Figure 6), with the tree constructed using the distance-based neighbour joining (NJ) algorithm based upon p-distance. Only one representative of each site and haplotype are shown and the resulting tree topology was well supported by bootstrap analyses. The sampled *L. rubellus* individuals could be resolved into two distinct genetic lineages (lineage A and B, respectively), with a mean inter-lineage mtDNA sequence divergence of 13%.

C_{PF} earthworms grouped exclusively within lineage A, and comprised 7 distinct haplotypes, with a between-haplotype diversity of 1 to 4% (Figure 6). In comparison, the M_{DH} and M_{CS} populations derived from mine-associated soils belonged predominantly to the lineage B genotype, and comprised 3 (M_{DH}) and 4 haplotypes (M_{CS}) exhibiting between-haplotype diversity of 1 to 2%, respectively. Only one individual from each mine site had lineage A genotype signatures. The genetic distance between *L. rubellus* and two other *Lumbricus* species (*L. castaneus* and *L.*

eiseni) was calculated as 18.1%, thus indicating that the *L. rubellus* field populations were correctly assigned.

Discussion

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

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

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

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

Due to its biological essentiality it is predictable that invertebrates are able to regulate intracellular Zn levels to a considerable degree. Chromatographic observations demonstrate that this may be achieved through Zn binding to a variety of low- and 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

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

harbour impoverished earthworm communities, in many instances no more than two taxonomically accepted representatives of the epigeic ecophysiological group.

In conclusion, the ability of an adapted population to tolerate the prevailing stress-evoking conditions in severely polluted habitats most probably involve heritable and integrated combinations of physiological, morphological and behavioural modifications. Thus, it is plausible to hypothesise that a metal tolerant earthworm population has evolved efficient mechanisms of detoxification that feature an enhanced immobilisation capacity coupled to a relative reduction in the metal sensitive fraction as an important component of their holistic adaptive arsenal. However, inferences about population-specific adaptation based on subcellular metal partitioning profiles should be drawn with a measure of caution because of the possibility that they could be attributable to lineage-specific traits that are independent of chronic metal exposure. This is illustrated by the findings of (Heethoff, *et al.*, 2004) that the parthenogenic earthworm *Octolasion tyrtaeum* is differentiated into two lineages differing significantly in body size. Such findings, together with those arising from the present study, raise the spectre that field-based eco-toxicological assessments that utilise earthworms, particularly those emanating from discriminating ‘omics’ measurements, might benefit from the elimination of a potential confounding biotic variable through prior genotyping of all individuals to establish that they possess some genetic background equivalence.

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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 $\pm$ 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

the soluble metal concentration expressed as a percentage of the total body concentration. The Pb levels shown represent fractions extracted from M_{CS} and C_{PF} earthworms in M_{CS} soil (A) and M_{DH} and C_{PF} earthworms in M_{DH} soil (B). [See Fig. 1 for dataset error bars and Fig. 1 legend for site identifiers.]

Figure 3

Time course of Zn partitioning into soluble (A) and non-soluble (B) cellular fractions in *Lumbricus rubellus* sampled from three populations and maintained on two mine-associated metal contaminated soils. Zn concentrations (per unit wet weight of earthworm) are presented as mean $\pm$ S.E. (maximum 'n' =9). [See Fig. 1 legend for site identifiers.]

Figure 4

Time course of subcellular Zn distribution in *Lumbricus rubellus* sampled from three populations and maintained on two field mine-associated metal contaminated soils, with the soluble metal concentration expressed as a percentage of the total body concentration. The Zn levels shown represent fractions extracted from M_{CS} and C_{PF} earthworms in M_{CS} soil (A) and M_{DH} and C_{PF} earthworms in M_{DH} soil (B). [See Fig. 1 for dataset error bars and Fig. 1 legend for site identifiers.]

Figure 5

Time course of P partitioning into soluble (A) and non-soluble (B) cellular fractions in *Lumbricus rubellus* sampled from three populations and maintained on two field-derived metal contaminated soils. P concentrations (per unit wet weight of

667 earthworm) are presented as mean $\pm$ 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.