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

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

Jane Andre[ab*], Stephen R. Stürzenbaum[c], Peter Kille[a], A. John Morgan[a] and Mark E. Hodson[b].

[a] Cardiff School of Biosciences, Cardiff University, Park Place, Cardiff, CF10 3US.

[b] Department of Soil Science, School of Human and Environmental Sciences, University of Reading, Whiteknights, Reading, RG6 6DW.

[c] King's College London, School of Biomedical & Health Sciences, Department of Biochemistry, Pharmaceutical Sciences Research Division, London, SE1 9NH

* Author for correspondence. Current address: Dr. Jane Andre, School of Health and Medicine, Division of Biomedical and Life Sciences, Lancaster University, Lancaster, LA1 4YQ

Abstract

Subcellular fractionation techniques were used to describe temporal changes (at intervals from T_0 to T_{70} 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 $CaPO_4$-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 (Knapen, *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 H_2O (Boisson, *et al.*, 1998) prior to them being oven dried at 30°C overnight, sieved to <2mm, then digested in boiling 16N HNO_3 (Morgan and Morgan, 1990) and analysed for major inorganic constituents by inductively coupled plasma - optical emission spectroscopy (ICP-OES; Perkin-Elmer Opitma 3000). Analysis of an in house certified reference material (a sewage sludge amended soil) indicated that 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.

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

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 intial 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 H_2O) 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 toxico-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 HNO_3 (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

earthworm. Blanks were included for each analyses and detection limits were calculated as 800 µg L^{-1}, 200 µg L^{-1} and 400 µg 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.

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

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.

Results

Soil analysis

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

Body mass changes

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

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.*

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.

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

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.

Acknowledgments. This work was performed during the tenure of a Natural Environment Research Council studentship (JA) registered at Reading University, and a research grant (F0 0407/AI) awarded by The Leverhulme Trust to AJM. We thank

445 Dr Trevor Piearce of Lancaster University for providing us with specimens of
446 *Lumbricus eiseni* for genotyping.
447

448 References

449 Andre, J., Charnock, J., Stürzenbaum, S.R., Kille, P., Morgan, A.J., Hodson, M.E.,
450 2009. Accumulated Metal Speciation in Earthworm Populations with
451 Multigenerational Exposure to Metalliferous Soils: Cell Fractionation and High-
452 Energy Synchrotron Analyses. Environmental Science and Technology 43, 6822-
453 6829.

454 Andre, J., King, R.A., Stürzenbaum, S.R., Kille, P., Hodson, M.E., Morgan, A.J.,
455 2010. Molecular genetic differentiation in earthworms inhabiting a heterogeneous Pb-
456 polluted landscape. Environmental Pollution 158, 883-890.

457 Arnold, R.E., Hodson, M.E., 2007. Effect of time and mode of depuration on tissue
458 copper concentrations of the earthworms *Eisenia andrei*, *Lumbricus rubellus* and
459 *Lumbricus terrestris*. Environmental Pollution 148, 21-30.

460 Arnold, R.E., Hodson, M.E., Charnock, J.M., Peijnenburg, W.J.G.M., 2008.
461 Comparison of the subcellular partitioning, distribution and internal speciation of Cu
462 between Cu-tolerant and naive populations of *Dendrodrilus rubidus* Savigny.
463 Environmental Science and Technology 42, 3900-3905.

464 Boisson, J., Mench, M., Sappin-Didier, V., Sold, P., Vangronsveld, J., 1998. Short-
465 term in situ immobilization of Cd and Ni by beringite and steel shots application to
466 long-term sludged plots. Agronomie 18, 347-359.

467 Buza, L., Young, A., Thrall, P. (2000). Genetic erosion, inbreeding and reduced
468 fitness in fragmented populations of the endangered tetraploid pea *Swainsona recta*.
469 Biological Conservation 93, 177-186.

470 Cain, D.J., Luoma, S.N., 1998. Metal exposures to native populations of the caddisfly
471 *Hydropsyche* (Trichoptera: Hydropsychidae) determined from cytosolic and whole
472 body metal concentrations. Hydrobiologia 386, 103-117.

473 Cain, D.J., Luoma, S.N., Wallace, W.G., 2004. Linking metal bioaccumulation of
474 aquatic insects to their distribution patterns in a mining- impacted river.
475 Environmental Toxicology and Chemistry 23, 1463-1473.

476 Campbell, P.G.C., Chapman, P.M., Hale, B.A., 2006. Risk assessment of metals in the
477 environment. Issues in Environmental Science and Technology 22, 101-131.

478 Conder, J.M., Seals, L.D., Lanno, R.P., 2002. Method for determining toxicologically
479 relevant cadmium residues in the earthworm *Eisenia fetida*. Chemosphere 49, 1-7.

480 Corp, N., Morgan, AJ., 1991. Accumulation of heavy metals from polluted soils by
481 the earthworm *Lumbricus rubellus*: Can laboratory exposure of 'control' worms reduce
482 biomonitoring problems? Environmental Pollution 74, 39-52

483 Cotter-Howells, J., Charnock, J.M., Winters, C., Kille, P., Fry, J.C., Morgan, A.J.,
484 2005. Metal Compartmentation and Speciation in a Soil Sentinel: The Earthworm,
485 *Dendrodrilus rubidus*. Environmental Science and Technology 39, 7731-7740.

486 Haimi, J., Knott, K.E., Yliniemi, I., 2007. Does metal contamination affect clonal
487 diversity of the parthenogenetic earthworm *Dendrobaena octaedra*? European Journal
488 of Soil Biology 43, 268-272.

489 Harpending, H.C., 1994. Signature of Ancient Population-Growth in a Low-
490 Resolution Mitochondrial-DNA Mismatch Distribution. Human Biology 66, 591-600.

491 Heethoff, M., Etzold, K., Scheu, S., 2004. Mitochondrial COII sequences indicate that
492 the parthenogenetic earthworm *Octolasion tyrtaeum* (Savigny 1826) constitutes of
493 two lineages differing in body size and genotype. Pedobiologia 48, 9-13.

494 Honeycutt, M.E., Roberts, B.L., Roane, D.S., 1995. Cadmium deposition in the
495 earthworm *Eisenia fetida*. Ecotoxicology and Environmental Safety 30, 143-150.

496 Hopkin, S.P., 1989. Ecophysiology of metals in terrestrial invertebrates. Elsevier
497 Applied Science, Barking, UK.

498 Huang, R., Wen, B., Pei, Z., Shan, X.Q., Zhang, S., Williams, P.N., 2009.
499 Accumulation, Subcellular Distribution and Toxicity of Copper in Earthworm
500 (*Eisenia fetida*) in the Presence of Ciprofloxacin. Environmental Science and
501 Technology 43, 3688-3693.

502 Jones, R.P., Bednar, A.J., Inouye, L.S., 2009. Subcellular compartmentalization of
503 lead in the earthworm, *Eisenia fetida*: Relationship to survival and reproduction.
504 Ecotoxicology and Environmental Safety 72, 1045-1052.

505 King, R.A., Tibble, A., Symondson, W.O.C., 2008. Opening a can of worms:
506 unprecedented sympatric cryptic diversity within British lumbricid earthworms.
507 Molecular Ecology 17, 4684-4698.

508 Klerks, P.L., Bartholomew, P.R., 1991. Cadmium accumulation and detoxification in
509 a Cd-resistant population of the oligochaete *Limnodrilus hoffmeisteri*. Aquatic
510 Toxicology 19, 97-112

511 Knapen, D., Bervoets, L., Verheyen, E., Blust, R., 2004. Resistance to water pollution
512 in natural gudgeon (*Gobio gobio*) populations may be due to genetic adaptation.
513 Aquatic Toxicology 67, 155-165.

514 Kumar, S., Tamura, K., Nei, M., 2004. MEGA3: Integrated software for Molecular
515 Evolutionary Genetics Analysis and sequence alignment. Briefings in Bioinformatics
516 5, 150-163.

517 Langdon, C.J., Hodson, M.E., Arnold, R.E., Black, S., 2005. Survival, Pb-uptake and
518 behaviour of three species of earthworm in Pb treated soils determined using an
519 OECD-style toxicity test and a soil avoidance test. Environmental Pollution 138.

520 Langdon, C.J., Morgan, A.J., Charnock, J.M., Semple, K.T., Lowe, C.N., 2009. As-
521 resistance in laboratory-reared F1, F2 and F3 generation offspring of the earthworm
522 *Lumbricus rubellus* inhabiting an As-contaminated mine soil. Environmental
523 Pollution 157, 3114-3119.

524 Lentzsch, P., Golldack, J., 2006. Genetic diversity of *Aporrectodea caliginosa* from
525 agricultural sites in Northeast Brandenburg, Germany. Pedobiologia 50, 369-376.

526 Li, L.Z., Zhou, D.M., Wang, P., Luo, X.S., 2008. Subcellular distribution of Cd and
527 Pb in earthworm *Eisenia fetida* as affected by Ca^{2+} ions and Cd–Pb interaction.
528 Ecotoxicology and Environmental Safety 71, 632-637.

529 Lock, K., Janssen, C.R., 2001. Zinc and cadmium body burdens in terrestrial
530 oligochaetes: Use and sigificance in environmental risk assessment. Environmental
531 Toxicology and Chemistry 20, 2067-2072.

532 Mathews, L.M., 2006. Cryptic biodiversity and phylogeographical patterns in a
533 snapping shrimp species complex. Molecular Ecology 15, 4049-4063.

534 McCarthy, L.S., Mackay, D., 1993. Enhancing ecotoxicological moedeling and
535 assessment. Environmental Science and Technology 27, 1719-1728.

536 Morgan, A.J., Kille, P., Sturzenbaum, S.R., 2007. Microevolution and ecotoxicology
537 of metals in invertebrates. Environmental Science and Technology 41, 1085-1096.

538 Morgan, A.J., Morris, B., 1982. The accumulation and intracellular compartmentation
539 of cadmium, lead, zinc and calcium in two earthworm species (*Dendrobaena rubida*
540 and *Lumbricus rubellus*) living in highly contaminated soil. Histochemistry and Cell
541 Biology 75, 269-285.

542 Morgan, J.E., Morgan, A.J., 1989a. The effect of lead incorporation on the elemental
543 composition of earthworm (Annelida, Oligochaeta) chloragosome granules.
544 Histochemistry 92, 237-241.

545 Morgan, J.E., Morgan, A.J., 1989b. Zinc sequestration by earthworm (Annelida:
546 Oligochaeta) chloragocytes. Histochemistry and Cell Biology 90, 405-411.

547 Morgan, J.E., Morgan, A.J., 1990. The distribution of cadmium, copper, lead, zinc
548 and calcium in the tissues of the earthworm *Lumbricus rubellus* sampled from one
549 uncontaminated and four polluted soils. Oecologia 84, 559-566.

550 Morgan, J.E., Morgan, A.J., 1998. The distribution and intracellular
551 compartmentalisation of metals in the endogeic earthworm *Aporrectodea caliginosa*
552 sampled from an unpolluted and a metal contaminated site. Environmental Pollution
553 99, 167-175.

554 Morris, B., Morgan, A.J., 1986. Calcium-lead interactions in earthworms:
555 Observations on *Lumbricus terrestris* sampled from a calcareous abandoned lead
556 mine site. Bulletin of Environmental Contamination and Toxicology 37, 226-233.

557 Novo, M., Velavan, T.P., AlmodOvar, A., Schulenburg, H., Diaz-CosIn, D.J.,
558 Michiels, N.K., 2008. Microsatellite markers for the drought-resistant earthworm
559 *Hormogaster elisae*. Molecular Ecology Resources 8, 901-903.

560 Pan, K., Wang, W.X., 2008. The subcellular fate of cadmium and zinc in the scallop
561 *Chlamys nobilis* during waterborne and dietary metal exposure. Aquatic Toxicology
562 90, 253-260.

563 Peles, J.D., Towler, W.I., Guttman, S.I., 2003. Population genetic structure of
564 earthworms (*Lumbricus rubellus*) in soils contaminated by heavy metals.
565 Ecotoxicology 12, 379-386.

566 Perceval, O., Couillard, Y., Pinel-Alloul, B., Campbell, P.G.C., 2006. Linking
567 changes in subcellular cadmium distribution to growth and mortality rates in
568 transplanted freshwater bivalves (*Pyganodon grandis*). Aquatic Toxicology 79, 87-98.

569 Pérez-Losada, M., Ricoy, M., Marshall, J.C., Domínguez, J., 2009. Phylogenetic
570 assessment of the earthworm *Aporrectodea caliginosa* species complex (Oligochaeta:
571 Lumbricidae) based on mitochondrial and nuclear DNA sequences. Molecular
572 Phylogenetics and Evolution 52, 293-302.

573 Péry, A.R.R., Béthune, A., Gahou, J., Mons, R., Garric, J., 2005. Body residues: a key
574 variable to analyze toxicity tests with *Chironomus riparius* exposed to copper-spiked
575 sediments. Ecotoxicology and Environmental Safety 61, 160-167.

576 Péry, A.R.R., Geffard, A., Conrad, A., Mons, R., Garric, J., 2008. Assessing the risk
577 of metal mixtures in contaminated sediments on *Chironomus riparius* based on
578 cytosolic accumulation. Ecotoxicology and Environmental Safety 71, 869-873.

579 Pfenninger, M., Schwenk, K., 2007. Cryptic animal species are homogeneously
580 distributed among taxa and biogeographical regions. BMC Evolutionary Biology 7,
581 121.

582 Pinceel, J., Jordaens, K., Van Houtte, N., De Winter, A.J., Backeljau, T., 2004.
583 Molecular and morphological data reveal cryptic taxonomic diversity in the terrestrial
584 slug complex *Arion subfuscus/fuscus* (Mollusca, Pulmonata, Arionidae) in continental
585 north-west Europe. Biological Journal of the Linnean Society 83, 23-38.

586 Rainbow, P.S., 2002. Trace metal concentrations in aquatic invertebrates: why and so
587 what? Environmental Pollution 120, 497-507.

588 Rainbow, P.S., 2007. Trace metal bioaccumulation: Models, metabolic availability
589 and toxicity. Environment International 33, 576-582.

590 Saitou, N., Nei, M., 1987. The Neighbor-Joining Method - a New Method for
591 Reconstructing Phylogenetic Trees. Molecular Biology and Evolution 4, 406-425.

592 Shepeleva, O.A., Kodolova, O.P., Zhukovskaya, E.A., Striganova, B.R., 2008.
593 Genetic diversity of populations of the earthworm *Lumbricus rubellus*
594 (Hoffm.)(Oligochaeta, Lumbricidae). Biology Bulletin 35, 170-177.

595 Spurgeon, D.J., Hopkin, S.P., Jones, D.T., 1994. Effects of cadmium, copper, lead and
596 zinc on growth, reproduction and survival of the earthworm *Eisenia fetida* (Savigny):

597 Assessing the environmental impact of point-source metal contamination in terrestrial
598 ecosystems. Environmental Pollution 84, 123-130.

599 Sturmbauer, C., Opadiya, G.B., Niederstatter, H., Riedmann, A., Dallinger, R., 1999.
600 Mitochondrial DNA reveals cryptic oligochaete species differing in cadmium
601 resistance. Molecular Biology and Evolution 16, 967-974.

602 Sturzenbaum, S.R., Kille, P., Morgan, A.J., 1998. The identification, cloning and
603 characterisation of earthworm metallothionein. FEBS Letters 431, 437-442.

604 Sturzenbaum, S.R., Winters, C., Galay, M., Morgan, A.J., Kille, P., 2001. Metal ion
605 trafficking in earthworms. Identification of a cadmiun-specific metallothionein. The
606 Journal of Biological Chemistry 276, 34013-34018.

607 Susuki, K.T., Sunaga, H., Aoki, Y., Hatakeyama, S., Sugaya, Y., Sumi, Y., Suzuki,
608 T., 1988. Binding of cadmium and copper in the matfly *Baetis Thermicus* that inhabit
609 a river polluted with heavy metals. Comparative Biochemistry and Physiology C
610 Comparative Pharmacology 91C, 487-492.

611 Velavan, T.P., Schulenburg, H., Michiels, N., 2007. Development and
612 characterization of novel microsatellite markers for the common earthworm
613 (*Lumbricus terrestris L.*). Host–Parasite Interactions 7, 24.

614 Vijver, M.G., Van Gestel, C.A.M., Lanno, R.P., Van Straalen, N.M., Peijnenburg,
615 W.J.G.M., 2004. Internal metal sequestration and its ecotoxicological relevance: a
616 review. Environmental Science and Technology. 38, 4705-4712.

617 Vijver, M.G., van Gestel, C.A.M., van Straalen, N.M., Lanno, R.P., Peijnenburg,
618 W.J.G.M., 2006. Biological significance of metals partitioned to subcellular fractions

within earthworms (*Aporrectodea Caliginosa*). Environmental Toxicology and Chemistry 25, 807-814.

Voets, J., Redeker, E.S., Blust, R., Bervoets, L., 2009. Differences in metal sequestration between zebra mussels from clean and polluted field locations. Aquatic Toxicology 93, 53-60.

Wallace, W.G., Lee, B.G., Luoma, S.N., 2003. Subcellular compartmentalistion of Cd and Zn in two bivalves. 1. Significance of metal-sensitive fractions (MSF) and biologically detoxified metal (BDM). Marine Ecology Progress Series 249, 183-197.

Wallace, W.G., Lopez, G.R., 1997. Bioavailability of biologically sequestered cadmium and the implications of metal detoxification. Marine Ecology Progress Series 1997, 149-157.

Figure Legends

Figure 1

Time course of Pb 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. Pb concentrations (per unit wet weight of earthworm) are presented as mean ±S.E. (maximum 'n' =9). C_{PF} refers to the reference site at Pontcanna Fields, M_{CS} the metalliferous acidic site Cwmystwyth Stream and M_{DH} the metalliferous calcareous site, Draethen Hollow.

Figure 2

Time course of subcellular Pb distribution in *Lumbricus rubellus* sampled from three 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.