Soil Biology & Biochemistry 42 (2010) 1566–1573

Contents lists available at ScienceDirect

Soil Biology & Biochemistry

journal homepage: www.elsevier.com/locate/soilbio

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

[a] Cardiff School of Biosciences, Cardiff University, Park Place, Cardiff, CF10 3US, UK
[b] Department of Soil Science, School of Human and Environmental Sciences, University of Reading, Whiteknights, Reading, RG6 6DW, UK
[c] King's College London, School of Biomedical & Health Sciences, Department of Biochemistry, Pharmaceutical Sciences Research Division, London, SE1 9NH, UK

ARTICLE INFO

Article history:
Received 18 March 2010
Received in revised form
20 May 2010
Accepted 28 May 2010
Available online 8 June 2010

Keywords:
Earthworms
Pb & Zn
Subcellular fractionation
Field & lab exposures
Genotyping

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.

1. 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

* Corresponding author. Current address: School of Health and Medicine, Division of Biomedical and Life Sciences, Bailrigg, Lancaster University, Lancaster, LA1 4YQ, UK.
E-mail address: j.andre@lancs.ac.uk (J. Andre).

0038-0717/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.soilbio.2010.05.029

and synchrotron-based X-ray absorption spectroscopy have been used to some extent to characterise 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.

2. Materials and methods

2.1. 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: 51:29.63122 N 3:12.24983 W) and two contaminated disused, metalliferous mine sites, Draethen Hollow (M_{DH}) ST 217877 (GPS: 51:34.96185 N 3:7.88760 W) and Cwmystwyth Stream (M_{CS}) SN 803748 (GPS: 52:21.48890 N 3:45.54702 W). At least ten soil samples (excluding the litter layer), taken from a 0–5 cm 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 <2 mm, then digested in boiling 16 N 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. 10 g (dry-weight) of each soil was weighed in a glass crucible and heated to 500 °C overnight. The percentage weight reduction was then recorded.

2.2. Sub-cellular fractionation

Thirty boxes containing 300 g of contaminated M_{CS} soil and 30 boxes containing 300 g 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 h on moistened filter paper (which was changed daily to prevent coprophagy), followed by 24 h in a filter-paper free petri-dish (with deionised 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 0.01 M 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 Fig. 1). Individual fractions were digested in boiling 16 N 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 $\mu g\ L^{-1}$, 200 $\mu g\ L^{-1}$ and 400 $\mu g\ L^{-1}$ for P, Pb and Zn respectively. No certified reference materials exist for use with this fractionation method but previous analysis of standard additions