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Relationships between metal compartmentalization and biomarkers in earthworms exposed to field-contaminated soils☆

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ABSTRACT

Partitioning tissue metal concentration into subcellular compartments reflecting toxicologically available pools may provide good descriptors of the toxicological effects of metals on organisms. Here we investigated the relationships between internal compartmentalization of Cd, Pb and Zn and biomarker responses in a model soil organism: the earthworm. The aim of this study was to identify metal fractions reflecting the toxic pressure in an endogeic, naturally occurring earthworm species (*Aporrectodea caliginosa*) exposed to realistic field-contaminated soils.

After a 21 days exposure experiment to 31 field-contaminated soils, Cd, Pb and Zn concentrations in earthworms and in three subcellular fractions (cytosol, debris and granules) were quantified. Different biomarkers were measured: the expression of a metallothionein gene (*mt*), the activity of catalase (CAT) and of glutathione-s-transferase (GST), and the protein, lipid and glycogen reserves. Biomarkers were further combined into an integrated biomarker index (IBR).

The subcellular fractionation provided better predictors of biomarkers than the total internal contents hence supporting its use when assessing toxicological bioavailability of metals to earthworms. The most soluble internal pools of metals were not always the best predictors of biomarker responses. *metallothionein* expression responded to increasing concentrations of Cd in the insoluble fraction (debris + granules). Protein and glycogen contents were also mainly related to Cd and Pb in the insoluble fraction. On the other hand, GST activity was better explained by Pb in the cytosolic fraction. CAT activity and lipid contents variations were not related to metal subcellular distribution. The IBR was best explained by both soluble and insoluble fractions of Pb and Cd.

This study further extends the scope of *mt* expression as a robust and specific biomarker in an ecologically representative earthworm species exposed to field-contaminated soils. The genetic lineage of the individuals, assessed by DNA barcoding with cytochrome c oxidase subunit I, did not influence *mt* expression.

1. Introduction

Terrestrial organisms exposed to metal-polluted soils can accumulate high amounts of trace metals in their tissues, especially soil-dwelling organisms like earthworms (Hendriks et al., 1995). Organisms can detoxify internal metals by partitioning the body burden into sequestered pools in which metals do not interact with the sites of toxic action (Lanno et al., 2004). Metal sequestration was shown to occur by two main pathways at the subcellular level: binding to metal-rich granules (MRG) and to heat stable proteins (e.g. metallothioneins) (Wallace and Lopez, 1996; Stürzenbaum et al., 2004). Both processes are supposed to decrease the toxicity of metals by reducing their potential to interact with the target sites of toxic action inside the organism. Subcellular fractionation procedure was proposed to evaluate internal metal compartmentalization in organisms (Wallace et al., 2003). The partitioning of tissue metal concentration into subcellular compartments may reflect 'toxicologically bioavailable' pools providing good descriptors of the effects of metals. This assumption however needs to be

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examined. Indeed, the relationships between internal compartmentalization of metals and biological endpoints such as biomarkers were rarely addressed (Jones et al., 2009; Colacevich et al., 2011). In a previous study, we have shown that the subcellular partitionings of Cd and Pb in earthworms were modified after an exposure to a gradient of metal availability in field-contaminated soils (Beaumelle et al., 2015) but the question whether a particular subcellular fraction of a given metal best reflects toxicological bioavailability (ISO 17402, 2008) remains to be addressed.

The partitioning of metals in up to five subcellular fractions can be obtained using serial centrifugations. The soluble or cytosolic fraction combines three compartments: (i) microsomes and organelles, (ii) heat denatured proteins (both of which are supposed to be indicative of toxic pressure) and (iii) heat stable proteins (assumed to contain metal binding proteins and assigned to a detoxified fraction). In earthworms, a large majority of internal Cd is retrieved from the whole soluble fraction where it is assumed to be mainly bound to metallothionein proteins, thus constituting a detoxified fraction (Conder et al., 2002). Contrarily to Cd, Pb retrived from the whole soluble fraction is not sequestered by metal binding proteins; the soluble fraction could thus be indicative of Pb toxicity to earthworm (Jones et al., 2009).According to the metal considered, the soluble fraction of internal metal can thus either be assigned to a detoxified (Cd) or a toxic (Pb) internal pool. Another isolated metal fraction, the insoluble fraction, can be further separated into two compartments of i) tissue, membranes and cellular debris (called debris hereafter) and ii) a compartment containing the MRG. The debris fraction is the more operational (Wallace et al., 2003; Jones et al., 2009) and was proposed to be associated to the metabolic needs of organisms. Indeed, most of the body burden of essential elements (Zn and Cu) in earthworms is retrieved from this fraction (Vijver et al., 2004, 2006; Huang et al., 2009). The MRG fraction is assigned to a detoxified pool of metals with a low capacity to store incoming metals after short-term exposure (Conder et al., 2002).

The use of several biomarkers to explore the effects of metals on organisms is generally recommended in order to reflect the multidimensionality of the stress response (van Straalen, 2003; Berthelot et al., 2009). For the purpose of risk assessment, indices that summarize and integrate the response of multiple biomarkers, such as latent variables in structural equation models (Beaumelle et al., 2016), or integrated index for biomarker response (IBR (Beliaeff and Burgeot, 2002)), are useful tools. Many biomarkers respond to biochemical or molecular subcellular parameters, thus individual biomarkers may better respond to metal concentration in subcellular fraction than to total earthworm metal concentration. On the other hand, different biomarkers may not respond to the same subcellular fraction of a given metal, as they can reflect different mechanisms of effects of metals. Moreover, in field-contaminated soils where multiple metals often co-occur, biomarkers can be affected by different metals (Beaumelle et al., 2014). Because earthworms use different accumulation strategies according to the metal, the relationships between biomarker responses and internal accumulation in earthworms exposed to multi-contaminated soils could be complex. As a result, it is not certain that a particular subcellular fraction can be indicative of earthworm stress response to metal exposure.

Among the three ecomorphological groups of earthworms (epigeic, endogeic and anecic), endogeic worms live in constant contact with, and feed mostly on soil. In contaminated soils, they are thus potentially more exposed to metals than epigeic and anecic species, notably the ecotoxicological test species *Eisenia andrei* and *Eisenia fetida* (Van Vliet et al., 2005; Suthar et al., 2008). Within endogeic earthworms, the species *Aporrectodea caliginosa* (Savigny) is widely distributed being one of the most abundant species in temperate regions (Pérez-Losada et al., 2009). *A. caliginosa* is an attractive sentinel species which has been used in several environmental studies (Klobučar et al., 2011). However, *A. caliginosa* is known to be a species complex and confusion between species is often observed. Pérez-Losada et al. (2009) investigated phylogenetic relationships and species boundaries within the *A. caliginosa* species complex in Europe, showing that at least five distinct species belong to this species complex. Biomarker responses may vary from one species to another and it is essential to consider the use of biomarkers in an evolutionary context (Pauwels et al., 2013).

In this context, the aim of this study was to identify subcellular metal compartments indicative of toxic pressure in an ecologically relevant earthworm (*A. caliginosa*) exposed to environmentally relevant metal exposure levels in soil. The present study was part of a larger project whose aim was to challenge the measurements of metal bioavailability to earthworms in field-contaminated soils. After investigating the relationships between soil metal pools and biomarkers (Beaumelle et al., 2014), and between soil metal pools and internal metal pools (Beaumelle et al., 2015), a structural equation model (SEM) was used to investigate the general causal relationships between soil metal availability, metal accumulation in earthworms and biomarkers through the use of latent variables reflected by several observed variables (Beaumelle et al., 2016). The present study went more in depth into the relationships between internal metal pools and biomarkers taking into account the effect of individual metals on biomarkers.

Before investigating the relationships between biomarkers in earthworm and Cd, Pb and Zn concentrations in three subcellular fractions, we ensure that the individuals belonged to the same species through DNA barcoding using the sequence of the cytochrome c oxidase subunit I (COI) mitochondrial gene (Pérez-Losada et al., 2012). The three subcellular fractions considered for metal analysis were the global soluble, debris and granules fractions. Biomarkers representing distinct processes by which metals can exert their effects on earthworms were considered: (i) the expression of the gene coding the metallothionein 2 protein, previously demonstrated to increase with Cd exposure (Spurgeon et al., 2004; Bernard et al., 2010; Brulle et al., 2011), (ii) the activity of two enzymes involved in the response to oxidative stress (catalase: CAT and glutathione-S-transferase: GST) that are expected to increase in earthworms exposed to metals (Saint-Denis et al., 2001; Lukkari et al., 2004), and (iii) the energy status assessed by lipid, glycogen and protein contents, expected to decrease because of the energy cost associated to metal exposure (Holmstrup et al., 2011). Biomarkers were further combined into an integrated biomarker index (IBR) to seek for a global assessment of earthworm response to internal metal concentrations in the three selected subcellular compartments.

2. Material and methods

2.1. Exposure experiment

Thirty one field contaminated and uncontaminated soils from the North of France were selected in order to have joint variations in both soil metal total concentrations and soil physicochemical characteristics that influence metal speciation (mainly pH, CEC, soil texture and OM content). Soil samples were collected in four contaminated sites (Mortagne (Mo), Metaleurop (Me), Pierrelaye (Pi) and Triel (Tr)) and four uncontaminated sites (Closeaux (Cl), Yvetot (Yv), Feucherolles (Fe) and Bannost (Ba)) whose detailed description can be found in Beaumelle et al. (2014). The total Cd, Pb and Zn concentrations in the studied soils ranged from 0.2 to 8.3 mg Cd/kg, from 19.6 to 491.0 mg Pb/kg and from 40.0 to 1004.2 mg Zn/kg. The selected soils covered wide ranges of soil