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Modélisation graphique de la biodisponibilité des métaux pour le ver de terre

Graphical modeling of metal bioavailability to earthworm

Directrice de thèse : **Isabelle LAMY**
Co-encadrement de la thèse : **Mickaël Hedde**

Jury

M. Rodolphe GILBIN, Chercheur, IRSN, Cadarache	Rapporteur
M. Erik SMOLDERS, Professeur, Katholieke Universiteit, Leuven	Rapporteur
Mme Johanne NAHMANI, Chargée de recherche, CNRS, Montpellier	Examinatrice
M. Alexandre PÉRY, Chercheur, Directeur de l'école doctorale ABIES, Paris	Examinateur
Mme Hélène ROUSSEL, Docteure, ADEME, Angers	Invitée
Mme Isabelle LAMY, Directrice de recherche, INRA, Versailles	Directrice
M. Mickaël HEDDE, Chargé de recherche, INRA, Versailles	Co-directeur

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INRA UMR 251 PESSAC :
Physico-chimie et écotoxicologie des sols d'agrosystèmes contaminés
INRA Centre de Versailles-Grignon, RD10 - 78026 Versailles Cedex

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Abstract

Soil organisms like earthworms are exposed to trace metals in contaminated soils. Only a fraction of total metal content in the environment can interact with soil organisms and lead to toxicological effects. Risk assessment of contaminated soils needs to measure the extent of this interaction between metals and organisms (i.e. bioavailability). Bioavailability is however a complex process that cannot be measured directly. A three-step definition had been given: 1) environmental availability designates the metal pools in soil that can potentially be uptake by the organism, 2) environmental bioavailability reflects the process of uptake of the contaminant by the organism and 3) toxicological bioavailability indicates the effects of the contaminant on the organism. A number of chemical and biological methods exist to measure each of these steps but none of them was proven to be fully generic in realistic environmental contexts. *In situ*, soils are often lowly contaminated by multiple contaminants and the causal relationships between chemical and biological indicators in such conditions have to be established. In this thesis we propose a graphical model (structural equation model: SEM) to address the causal hypotheses implied by the three-step definition of bioavailability. A laboratory exposure experiment was designed to test a SEM of metal bioavailability to earthworm. We chose a target earthworm species, *A. caliginosa*, commonly found in temperate soils. An analysis of literature data was conducted to verify metal bioaccumulation in *A. caliginosa* was representative of other earthworm species. We further selected a wide panel of soils to explore a realistic gradient of metal exposure. Specific chemical and biological methods were selected to reflect each step of metal bioavailability in the model. Environmental availability was assessed by experimental (extractions) and theoretical (modeling) procedures. Metal quantifications in earthworms and in three subcellular compartments were conducted to constitute observed variables of environmental bioavailability. Finally, for toxicological bioavailability, biomarkers reflecting different processes by which metals exert their effects on earthworms were quantified. The strength of the relationships between single observed variables was investigated over the panel of soils. The results showed indicator- and metal-specific relationships and further highlighted the challenges of relating chemical and biological indicators when considering low levels of bioavailability. The conceptual SEM was then confronted to our empirical observations. The results identified sets of chemical and biological indicators verifying the causal assumptions of the three-step definition of bioavailability in field-contaminated soils. This study shows the relevance of the SEM approach, that goes beyond the imperfections of single observed variables to reflect complex theoretical entities, and represents a powerful and comprehensive framework to investigate bioavailability in realistic environmental context.

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Avant Propos

J'ai fait le choix de présenter ce manuscrit de thèse sur la base d'articles. Ces articles ont été conçus en lien direct avec les objectifs de ma thèse et constituent donc des chapitres à part entière du manuscrit. Ils représentent la manière dont j'ai articulé ce travail de thèse. L'ensemble du manuscrit est donc présenté en anglais et un résumé étendu est présenté à la fin du document. La chronologie de l'acquisition des donnéesa fait que le premier article publié sur mes travaux correspond au chapitre 4, tandis que les chapitres 3, 5 et 6 correspondent à des articles soumis ou en préparation. Afin de clarifier l'enchaînement du manuscrit, j'ai repris dans le chapitre 2 certains éléments du chapitre 4 qui méritaient d'être présenté en début de manuscrit.

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Introduction

Introduction

Soil contamination is a key environmental issue. In a recent report, the European Union estimated more than 2 million potentially contaminated sites over Europe (Van Liedekerke et al., 2014). The most frequent contaminants identified in this report are metallic trace elements or heavy metals. Trace elements are not only main contaminants in soil, but they also raise particular concern because contrarily to several organic pollutants, they are persistent in soils and past contaminations can still have an impact today.

Trace metals such as Cd, Pb and Zn are naturally present in soils at low concentrations due to the parent rock material. However, past and present human activities modified the concentrations of metals in soils. High pollution even resulted in both sanitary (widespread lead poisoning) and environmental issues (contamination of food chains). Mining activities, widespread use of fertilizers and sewage sludge, metallurgical industries, fuel combustion are among the main sources of heavy metals in soils (Alloway, 1995).

Remediation techniques have been successfully used to remove trace elements from soils (e.g. excavation, phytoremediation) but such procedures mainly apply in the case of local contamination. Diffuse metal contamination of soils cover large areas. For example, the Aznalcóllar mining incident in Spain contaminated 10,000 ha of farmland and wetland including a 900 ha area in the Doñana Natural Park in 1998 (Meharg et al., 1999). Diffuse metal contamination can also occur following atmospheric deposition of contaminated dusts (e.g. at the vicinity of smelting or mining industries) or sewage sludge spreading. In such cases, remediation procedures can hardly be applied and the strategy can be to adapt the current land use if the level of contamination is judged unacceptable.

This raises the question: what is an acceptable level of soil metal contamination? The total metal concentration in soil is poorly related to the risks encountered by humans and ecosystems. In soil, only a fraction of total metal can enter the organism and lead to an effect: this is a simple definition of the concept of bioavailability.

Soils are complex and heterogeneous systems at the interface of both geological and bio-

logical processes. The physical and chemical breakdown of the parent rock material combined with the activity of soil organisms (plant roots, micro-organisms, terrestrial invertebrates) that produce and recycle organic matters, gives rise to a highly complex and structured habitat. Soil consists of both mineral and organic particles of various size, shapes and chemical characteristics. Solid soil particles can bind metals. The binding affinity depends on the material, and is strongly influenced by pH and redox conditions (Alloway, 1995). Thus only a small proportion of total metals are found in soil solution, where they are considered to interact with soil organisms. Metal speciation designates the different forms (or species) of metals that can be found in soils. It can be completely different from one soil to the other and it is a crucial determinant of their bioavailability to soil organisms (Peijnenburg et al., 2007).

Earthworms are a key component of the soil habitat, often considered as ecosystem engineers. The key role of earthworms in soil formation and their beneficial effect on plant growth was already evidenced by Darwin (1881). In many terrestrial ecosystems, earthworms are the most abundant biomass. They contribute to a number of soil ecological functions. For instance, they participate to soil water regulation by modifying soil porosity (burrows and casts). They accelerate soil organic matter (SOM) degradation and humification and thus control the release of available nutrients for other soil organisms and the incorporation of OM into soil (Blouin et al., 2013).

Earthworms living in metal-contaminated soils can bioaccumulate metals up to higher concentrations than in the soil material. Indeed, not only do earthworms live in permanent contact with the soil, but they also feed on the soil. Earthworms can further be vulnerable to metal exposure. Abdul Rida and Bouché (1995) showed that the eradication of the earthworm genus *Scherotheca* in southern France resulted from its higher susceptibility to lead and copper. Decreased earthworms abundance at the vicinity of smelting works were often reported (Nahmani and Lavelle, 2002; Spurgeon and Hopkin, 1996b).

Because of their functional importance, earthworms are excellent bioindicators of soil quality. In metal-contaminated soils, assessing metal bioavailability to earthworms is therefore crucial within the framework of environmental risk assessment. Methods to estimate bioavailable concentrations of metals are needed and standardized methods are required to promote the integration of bioavailability into environmental risk assessment schemes (Harmsen, 2007). However, measuring bioavailability remains a major challenge because bioavailability is a complex and dynamic phenomenon that cannot be measured directly.

The definition of bioavailability was clarified during the recent years (Lanno et al., 2004; Peijnenburg et al., 1997; Harmsen, 2007). Bioavailability is viewed as a dynamic process

decomposed in three steps (1) a fraction of total soil metal is available or potentially available depending on physico-chemical processes (environmental availability), (2) a portion of available metals can be uptake by a given organism depending on physiological processes (environmental bioavailability) and (3) a fraction of the internalized contaminant accumulates and/or lead to an effect on the organism (toxicological bioavailability). Each step is assessed either by chemical methods (environmental availability) or biological methods (environmental and toxicological bioavailability). Recently this definition was proposed as a normalized framework to study bioavailability (ISO 17402, 2008).

Establishing clear relationships between chemical and biological indicators of bioavailability has proved difficult when applied to field-contaminated soils because a number of confounding factors intervene (Smolders et al., 2009). Confounding factors are biotic and abiotic factors that can interfere with the relationships between dose and effects. Usually such factors are minimized in laboratory exposure experiments in order to clarify the dose-effect relationship. A number of studies used artificial soils and artificial contamination with high metal concentrations to study metal uptake by or metal effect on organisms. The pertinence of chemical and biological indicators of bioavailability to earthworm is rarely evaluated over wide ranges of environmentally relevant exposures.

In field-contaminated soils, the cause-effect relationships between the presence of available metals in soil, the uptake (or absorption) processes and the effects of metals remain to be established. Traditional statistical tools cannot explicitly address a causal relationship. In ecology, scientific frameworks to investigate causal assumptions and complex systems have emerged in the literature. Graphical models in particular, have received much attention because they offer a means to test complex sets of causal hypothesis and to confront theories to empirical observations (Grace et al., 2012). Graphical models were seldom used to study metal bioavailability to soil organisms. And yet, they could provide a pertinent framework to address the structural and causal implications of the definition of bioavailability.

In this thesis, a structural equation model (SEM) of metal bioavailability to earthworm was designed and tested. The aim was to confront the three-step theory of bioavailability to empirical observations made over a range of field-contaminated soils, and subsequently to support the selection of an appropriate set of chemical and biological methods to assess metal bioavailability to earthworm. The first chapter reviews the potentialities of graphical models such as SEM in the context of bioavailability assessment. A structural model of bioavailability is proposed, and several methods to assess each step of metal bioavailability to earthworm are presented. The second chapter details the experimental design. The specification of the conceptual model is

presented, and we further argument the choice of the target species and of the series of soils considered to conduct a laboratory exposure experiment. The third, fourth and fifth chapters focused on the relationships between individual measurements of each step of bioavailability. In these three chapters, the objective was to identify particular measurements more robust than others to evaluate metal bioavailability in field-contaminated soils. The relationships between environmental availability and environmental bioavailability viewed by several chemical and biological methods is treated in Chapter 3. The links between environmental availability and toxicological bioavailability are investigated in Chapter 4. Chapter 5 focused on the relationships between environmental bioavailability and toxicological bioavailability. The sixth chapter addresses the structural relationships between environmental availability, environmental bioavailability and toxicological bioavailability in a comprehensive SEM of metal bioavailability to earthworm.

Chapter 1

Chapter 1

Synthesis

If the definition of bioavailability has reached a consensus among scientists (Lanno et al., 2004; Peijnenburg and Jager, 2003; Harmsen, 2007) there is a vast number of methods to assess it. In order to promote the integration of bioavailability into risk assessment of metals in the environment, it is important to identify generic measurements of metal bioavailability.

In this synthesis a new framework of analysis is proposed to clarify the causal relationships between chemical and biological measurements of bioavailability. We argue that graphical modeling can participate to this clarification and we review suitable methods to assess metal bioavailability to earthworms relatively to the three-step definition given in ISO 17402, 2008.

1.1 Graphical modeling of metal bioavailability

Graphical models are more and more used in natural sciences and especially in ecology. They allow to address cause-effect relationships, quantify direct and indirect effects, and to represent theoretical unmeasured variables (latent or construct variables) using several observed (or manifest) variables. Graphical models are thus useful to confront a well-established theory to empirical observations. Structural equation models (SEM) are one example of graphical models. We will first review several interesting features of this model relatively to the study of metal bioavailability and propose a structural model of metal bioavailability.

1.1.1 What is structural equation modeling ?

In SEM it is possible to address explicitly causal relationships. A definition of a causal relationship of X on Y is that any change in X will result in a change in Y, but that Y can change

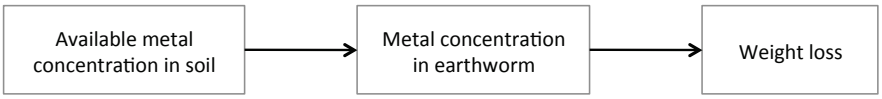


Figure 1.1: Path diagram: Example of hypothetical causal relationship

without a resulting change in X (Pearl et al., 2009). Causal relationships can thus be viewed as directed or asymmetrical relationships. In graphical models, directed relationships are represented in directed graphs by unidirectional arrows called paths. Figure 1.1 shows a simplified graphical model of metal bioavailability. If a metal is available in the soil, earthworm can absorb it, which will affect its growth. Directed graphs such as the one depicted Figure 1.1 translate hypothetical causal relationships into a mathematical (graphical) language. The structural relationships between variables are reflected by their patterns of covariation. The directed graph can thus be translated into a model-implied covariance structure that is compared to the observed covariance structure in order to decide whether the hypothetical causal relationships are consistent with empirical data or not (Shipley, 2002). In the example (Fig.1.1), there is no path between availability and weight loss; the model assumes that weight loss is only affected by metal availability if the earthworm absorbs metals. It means that if we could block experimentally metal absorption by earthworms, there would be no relationship between the available metal concentration and weight loss: weight loss and available metal concentration are independent conditioning on metal absorption. If the model is correct, the conditional independence between weight loss and available metal concentration will be mirrored by a statistical independence in observational data.

Another key feature of SEM is the possibility to model unobserved variables (latent or construct variables) that are indirectly reflected by several observed variables (indicators or manifest variables). They present many advantages in ecology and social sciences, where a number of variables are complex constructs that cannot be adequately represented using a single measurement (Pugesek and Tomer, 2003). Construct variables are used to represent the true value of a parameter, or an underlying cause (Grace et al., 2010). For example, the body size of an earthworm can be measured by several different indicators: fresh weight, total body length, or even a visual estimate of the weight (Figure 1.2). The covariance among the different manifest variables formulates the latent variable, body size, which can be subsequently regressed on other variables in structural models. The use of several imperfect/indirect measurements to construct a latent variable reinforces the validity of the latent to reflect the theoretical variable of interest compared to single indicator construct. Variance in each of the observed variable that is unrelated to the latent variable will constitute the

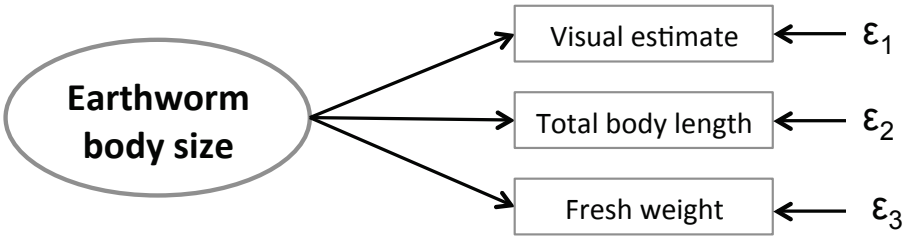


Figure 1.2: A measurement model of earthworm body size viewed as a latent variable (ellipse) reflected by several measurements (rectangles)

residual error variance, and will not be used in a regression of the latent variable to another. This is a main advantage over statistical analysis such as linear regression, because the latter assumes that single manifest variables are measured without error. Contrarily to multiple linear regression in which correlated explaining variables can result in biased coefficients, in SEM the correlation between the manifest variables of a construct reinforces its validity and precision (Pugesek and Tomer, 2003; Grace et al., 2010).

Using latent variables in SEM, the analysis no longer focuses on relating single variables that imperfectly reflect theoretical entities, but rather manipulates theoretical entities reflected by several measurements in a structural manner. Measurement models describe the relationships between latent and manifest variables. An example is shown Figure 1.2. By convention, ellipses represent latent variables and rectangles represent observed variables. The direction of the paths indicates the causal effect of the latent on the manifest variables. Path coefficients (arrows) quantify the magnitude of this effect. The ϵ terms are the measurement errors. They represent all the other unmodeled causes of variation in the observed variables, and reflect the imperfection of the observed variable to reflect the construct.

1.1.2 How structural equation modeling can apprehend bioavailability ?

Bioavailability is a concept for which there is no generic definition, and no single measurement (Harmsen, 2007). Depending of their background (chemist or biologist), scientists used different definition of bioavailability. Harmsen, 2007 distinguished the flux-based approach, in which bioavailability is viewed as the flux of contaminants to biota (Peijnenburg and Jager, 2003) from the content-based approach, in which bioavailability designates the fraction of total contaminants in soil with which the organism actually interact (Lanno et al., 2004). As the true rate of the uptake process cannot be measured directly, Harmsen, 2007 highlighted

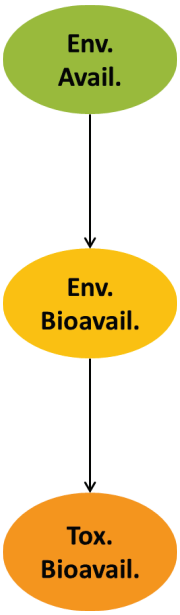


Figure 1.3: Structural model of bioavailability describing the causal relationships between environmental availability, environmental bioavailability and toxicological bioavailability. Each step is viewed as a latent variable (ellipses)

the importance of the content determination in bioavailability assessments.

In the end, the unified framework provided by Harmsen (2007) and ISO 17402 (2008) makes it possible to relate empirical characterizations to the complex theoretical entity that is bioavailability. Environmental availability (or availability) is assessed by chemical methods that are indirect measurements of bioavailability (Lanno et al., 2004). Indeed, only the organism can determine whether a chemical is bioavailable. Environmental bioavailability and toxicological bioavailability are measured by biological methods. They include determination of the amount of contaminants in the organism (direct measures of bioavailability according to Lanno et al., 2004) and determination of the effects of contaminants on the organism (viewed as indirect biological measures of bioavailability by Lanno et al. (2004)). The effects cover biological responses at different levels of biological organization, from genes to populations.

The conceptual definition of bioavailability implies causal relationships between environmental availability, environmental bioavailability and toxicological bioavailability. If more and more studies addressed the three steps simultaneously (e.g. Smith et al., 2010; Spurgeon et al., 2006) the structural aspect of bioavailability is not apprehended by traditional statistical methods. Furthermore, each step of bioavailability is a concept that cannot be measured directly but for which there exist several different measurements. Individually, these meth-

ods are often questioned as to their validity and robustness to reflect bioavailability. For example, bioaccumulation was criticized because it is a complex integrative measurement, resulting from both uptake and excretion processes (Luoma and Rainbow, 2005).

The three-step definition of bioavailability could thus be adequately represented using SEM. Each step of bioavailability is viewed as a latent variable, and there is a flow of causation from environmental availability to environmental bioavailability and to toxicological bioavailability. This structural model is depicted on figure 1.3.

If SEM cannot prove causation (Grace et al., 2012), the causal structure *per se* is not under investigation. Indeed, for decades, toxicologists and ecotoxicologists provided mechanistic and dynamic models that repeatedly proved dose-response relationships in controlled conditions. However, two crucial questions remain to be addressed (i) if chemical and biological methods still verify this causal structure in field ecosystems, i.e. if they are robust enough to assess metal bioavailability in the field, and (ii) on which chemical and biological methods in particular should dose-effect relationships be based in environmental risk assessment.

The structural model depicted Figure 1.3 can be applied to multiple contaminants and multiple species. The application of this model to the bioavailability of metals to earthworm depends on the observed variables chosen to reflect the three latent variables. A review of the literature was conducted to identify pertinent chemical and biological assessments that could be included in the model.

1.2 How to assess soil metal availability?

1.2.1 Soil metal availability

In soil, metals interact with the different soil constituents (clay, organic matters, metal (hydr)oxides, carbonates). Various metal species with different reactivity are found in soils. In the soil solution, metals exist as free ion, organic and inorganic complexes, as well as in colloids and suspended particulate material (Hooda, 2010). Metals in the soil solution are considered to be the 'actual' available fraction for a number of soil organisms (Harmsen, 2007). However, a large majority of the total metal content is associated to the solid phase. Several chemical mechanisms are involved in metal binding to the solid constituents of soil: cation exchange, specific adsorption, precipitation and complexation to organic matters (Alloway, 1995; Hooda, 2010). Desorption processes are governed by the soil properties (pH, organic matters content and quality, clay minerals) and determine bioavailability. Desorption

processes increases the probability of uptake by organisms and are considered as the first step of bioavailability: environmental availability (Peijnenburg and Jager, 2003).

Chemical methods seeking to quantify available metal pools are therefore crucial within the assessment of metal bioavailability, as they integrate the effect of soil physico-chemical characteristics. A number of extraction procedures were developed to fulfill this goal, with an initial emphasis on the assessment of metal availability to plants (Alloway, 1995). Weak extractions using water or salt solutions ($CaCl_2$, $Ca(NO_3)_2$, etc.) quantify the soil solution and exchangeable metal pools (Hooda, 2010). Dilute acid solutions (0.43 M HNO_3, CH_3COOH) extract the soil solution and exchangeable pools, and partially dissolves carbonates, (hydr)oxides and metals bound to organic matter. Chelating agents (ethylenediamine tetraacetic acid: EDTA, diethylenetriamine pentaacetic acid DTPA) dissolve the elements complexed with organic matters and fixed on iron and manganese hydroxides (Rauret, 1998).

Complementarily to experimental methods, theoretical models that calculate metal speciation or predict dissolved or free ion metal contents are used to assess metal bioavailability (Ge et al., 2005). Semi-mechanistic models were developed by Sauvé et al. (2000) to predict Cd, Cu, Ni, Pb and Zn dissolved concentrations. Such models rely on soil total metal content, pH and organic matter content as explaining variables. They provided acceptable predictions of metal concentrations in soil solution (Groenenberg, 2011). Geochemical speciation models are more complex mechanistic models that can be used to calculate metal speciation in both soil solution and on soil surfaces. Based on thermodynamic constants and a detailed characterization of soil constituents and solution, they notably quantify free ion concentrations, as well as organically bound metal contents (Weng et al., 2001; Dijkstra et al., 2004; Groenenberg, 2011).

Earthworms live in permanent contact with the soil solution and breathe through their skin. It is therefore often assumed that they are primarily exposed to metals through dermal exposure. Vijver et al. (2003) experimentally sealed earthworm mouth and concluded that oral uptake was negligible compared to dermal uptake. Saxe et al., 2001 draw similar conclusions based on modeling results, but they may have neglected the fraction of metal available for oral uptake by considering it was the water soluble fraction at gut pH (pH 7). Earthworms are permanently exposed to the soil via ingestion. As they digest SOM, it is possible that the digestion processes release a fraction of organically-bound metals. Morgan et al., 2004 provided evidence that the main metal uptake route was through the alimentary canal using immunohistochemical methods to localize metals in the tissues of earthworms living in heavily contaminated soils.

Table 1.1: Results of several studies relating soil metal concentrations to earthworm metal concentrations. y: relationship between internal (int) and extractable metal contents (extr), o: no relationship, cor: correlation, tot: soil total metal concentration

Metal pool/ Extractant	Earthworm species	relationship [int] - [extr] (y/ø)	observed relationships	Statistical analysis	number of soils	Field / Bioassay	Exposure duration (days)	Extraction method	Reference
pore water	*E. fetida*	Cd y	-	R^2	9	Bioassay spiked soils	21	extraction through a hollow fiber membrane	Li et al. 2009
	E. andrei	As, Cr, Cu, Zn ø Cd, Ni, Pb y	-	R^2 (0.27-0.41)	19	Bioassay	21	centrifugation of field wet soil	Janssen et al. 1997
	E. fetida	Ba, Cu, Mg, Mn, Pb, Tl y	R2 [tot] > R2 [extr]	R^2 (0.40-0.80)	8	Bioassay	42	centrifugation	Nahmani et al. 2007
	L. rubellus, A. caliginosa	Cd y	-	R^2 (0.46-0.53)	15	Field	-	centrifugation	Hobbelen et al. 2006
	L. rubellus	Cu, Zn, Pb y	R2 [tot] > R2 [extr]	R^2 (0.11-0.22)	> 30	Field	-	calculation (Sauvé et al. 2000)	Veltman et al. 2007
	5 species	Cd, Cu, Zn, Pb ø	with [tot]	correlation	2	Field	-	centrifugation	van Vliet et al. 2005
	A. caliginosa	Cu y Cu ø	and with [tot] not with [tot]	R^2 (0.66-0.92) R^2	22 5	Bioassay field soils Bioassay spiked soils	28	suction over an acetate filter	Vijver et al. 2007
water-soluble	5 species	Fe, Ni, Mn, Cu, Cd, Pb, Ca and Mg ø (based on observation)	maximal internal concentrations while minimal water-extractable metals	-	4	Field	-	water 1/5 (soil/solution)	van Straalen et al. 2001
	A. rosea *A. caliginosa*	As, Cu ø Cd, Pb, Sb, Zn y	-	R^2 (0.34-0.52)	15	Field	-	step 1 of a sequential extraction	Nannoni et al. 2011
	A.caliginosa *L.rubellus*	Cd, Zn ø Pb y (inverse relationship	-	PCA	3	Field	-	step 1 of a sequential extraction	Becquer et al. 2005
NH_4NO_3	*A. caliginosa*	Cd, Pb ø Cu y	cor [tot] > [extr]	correlation (0.96)	8	Bioassay	28	1 M NH_4NO_3	Gaw et al. 2012
KNO_3	*E. andrei*	Bi, Cd, Cr, Cu, Ni, Pb, Zn ø	-	-	9	Bioassay	7 and 28	0.01 M KNO_3	Berthelot et al. 2008
KCl	*A.caliginosa* *L.rubellus*	Cd y Pb y (inverse relationship Zn ø	-	PCA	3	Field	-	step 2 of a sequential extraction, 100 mM KCl	Becquer et al. 2005
$CaCl_2$	*E. andrei*	As, Cd, Cu, Pb y	-	R^2 (0.25-0.68)	19	Bioassay	21	0.01 M $CaCl_2$	Janssen et al. 1997
	L. rubellus, A. caliginosa	Cd y (L.rubellus) ø (A.caliginosa) Cu, Zn ø	- -	R^2 (0.4)	15	Field	-	0.01 M $CaCl_2$	Hobbelen et al. 2006
	5 species	Cd, Cu, Zn, Pb ø	significant with [tot]	-	2	Field	-	0.01 M $CaCl_2$	van Vliet et al. 2005
	E. fetida	Cd, Cu, Zn y Pb ø	-	R^2 (0.45-0.86)	11	Bioassay	28	0.5 M $CaCl_2$	Lee et al. 2009
	E. fetida	Pb ø based on	Pb is bioaccumulated but not available according to	-	2	Bioassay	56	0.01 M $CaCl_2$	Bernard et al. 2010
	A. caliginosa	Cd, Pb ø Cu y	cor [tot] > [extr]	correlation (0.95)	8	Bioassay	28	0.01 M $CaCl_2$	Gaw et al. 2012
	L. rubellus	Cu y Zn ø	-	R^2 (0.92)	4	Bioassay	30	not communicated	Simonsen & Scott-Fordsmand 2004
	A. corticis *D. japonica*	Cu, Zn, Cd, Pb ø	significant with [tot]	R^2	30	Field	-	0.01 M $CaCl_2$	Kamitani et al. 2007

1.2.2 Chemical assessments of soil metal availability

Finding which chemical method better reflect available metal pools for earthworm has been the subject of many studies. One of the main approaches was to correlate internal metal concentrations with available metal contents. In this review, we collected the results obtained in the literature with such an approach (Table 1.1). We indicated for each article whether a correlation could be find, the strength of the correlation, the earthworm species under study, whether the correlation was observed *in situ* or in the laboratory, on how many sites/plots and a brief description of the chemical method conducted.

Metals readily available in the soil solution (dissolved metals, pore water, water-soluble) or weakly bound to soil particles (exchangeable) were often found correlated to metal concentrations in earthworms (Table 1.1). Water and weak salt extractions were therefore recognized as pertinent indicators of environmental availability relatively to earthworm. However, Table 1.1 also shows that the correlations between earthworm metal concentrations are often stronger with soil total metal concentrations than with easily extractable ones. Moreover, within a same study, the relationships are often specific of a given metal, or only significant at a given site. Finally, several publications have not successfully related easily extractable metal concentrations with internal metal contents in earthworms. These contrasted results point to the fact that the chemical assessments of weakly bound metal pools are not fully generic to explain internal metal concentrations in earthworms. No clear pattern can be distinguished either according to the species or metal considered. However, total metal concentrations are very often better descriptors of internal concentrations.

Several studies reported relationships between internal concentrations and DTPA- or EDTA-extractable amounts of metals (Kamitani and Kaneko, 2007; Dai et al., 2004; Lee et al., 2009). Using a sequential extraction procedure, Becquer et al. (2005) showed that internal Cd, Pb and Zn were related to organically-bound metals and bound to Mn and Fe hydroxides. Moreover, recent studies have developed a procedure of extraction that simulate the enzymatic gut content of earthworm (SEG: simulated earthworm gut test). They showed good relationships between SEG-extractable metals and internal concentrations in earthworms (Ma et al., 2009; Gaw et al., 2012; Smith et al., 2010). The extent of the uptake of metals through the intestinal pathway could be the reason why internal concentrations are so often better correlated to total concentrations in soil than to easily extractable amounts of metals (Table 1.1).

Harmsen, 2007 recommended to separate an 'actual' available fraction corresponding to the dissolved fraction, a 'potentially' available fraction corresponding to the maximum

amount that can be released and a non-available fraction. In the case of earthworms, the 'actual' available fraction could be assessed by pore water or weak salt extractions while for the potentially available fraction there is no consensual method.

1.2.3 Relationships between soil metal availability and earthworm response to metal exposure

One the one hand, soil total metal content do not reflect toxicological effects but on the other hand, earthworm tissue metal content is better predicted by soil total metal concentration. Several authors suggested that total internal concentrations were not pertinent to predict toxicity (Van Straalen et al., 2005; Luoma and Rainbow, 2005). Earthworms can bioaccumulate large amounts of metals in their tissue but only a portion of the total body burden causes toxicological effects. Like other organisms, earthworms can sequester metals under inert forms that does not interact with sites of toxic action (Vijver et al., 2004). Other biological endpoints than total internal content may be more appropriate to identify pertinent indicators for environmental availability.

The free ion concentration is often considered as the best predictor of metal toxicity to organisms (Qiu et al., 2014; Oste et al., 2001). This assumption historically emerged from scientists working on aquatic environments and gave rise to the free ion approach model (FIAM) and biotic ligand model (BLM) in which the concentration of a contaminant at a given target site in the organism (biotic ligand) governs metal toxicity (Paquin et al., 2002). The binding to the biotic ligand is modeled based on free ion concentration and takes into account cation competition for the target site (Di Toro et al., 2001). Terrestrial BLM (t-BLM) were developped for soil microorganisms, plants and invertebrates (Thakali et al., 2006). Based on the rationale that earthworms are primarily exposed to the soil solution, terrestrial BLM were applied to earthworms (Steenbergen et al., 2005; Thakali et al., 2006), and free ion approaches were used to predict earthworm survival rate (Qiu et al., 2014). Such an approach assumes that free ion metals in the soil solution are directly available to earthworms, but neglects other potentially available metal pools that can be available after soil has passed through the earthworm gut. In addition, free ion approaches and t-BLM were mostly applied after unrealistic earthworm exposure (sand, artificially contaminated soils with narrow ranges of soil properties (Steenbergen et al., 2005; Qiu et al., 2014)). Their pertinence in the context of field-contaminated soils remain to be addressed.

Context-dependent relationships between toxicological endpoints and chemical extractions are found in the literature, consistently with the approach with internal contents. In

highly contaminated soils, earthworms growth and reproduction were not significantly related to metal availability assessed by $CaCl_2$ extractions (Arnold et al., 2003; Smith et al., 2010). However, other authors demonstrated the opposite (Daoust et al., 2006; Owojori et al., 2010). Using DTPA, Owojori et al. (2010) observed that the extractable metal contents were related to earthworm biomass, survival and reproduction, Daoust et al. (2006) however found otherwise.

The relationships between available metal concentrations and biological endpoints in earthworms remain unclear. A number of chemical extractions (e.g. $CaCl_2$, NH_4NO_3, EDTA) are however routinely used in the literature. The identification of a proper method that best explains variations in earthworm biological endpoints is further challenged by the correlations between metal concentrations obtained after different extractions or modeling procedures. It is noteworthy that the ability of chemical methods to explain variations in other endpoints than the total body burden was rarely addressed; even though body burdens are not considered to properly reflect metal bioavailablity.

1.3 Biomarkers as indicators of metal bioavailability to earthworm?

1.3.1 Biomarkers: definition and position within the concept of metal bioavailability

Biomarkers are biological responses related to the exposure to or the toxic effect of an environmental chemical (Scott-Fordsmand and Weeks, 2000). They can cover a number of biological levels of organization, even if the term commonly implies responses at the subindividual level (Lagadic et al., 1994; Spurgeon et al., 2005). Biomarkers were proposed as interesting tools within the framework of environmental risk assessment because they represent early warning signals. Indeed, as they precede the effects at the life cycle level, biomarkers may offer the potential to be able treat a contaminated site before any adverse effect on the populations occur (Svendsen et al., 2004). Within ISO 17402, 2008, different measurements of toxicological bioavailability were proposed (mortality, reproduction, etc.).

Two categories of biomarkers are distinguished: biomarkers of exposure and of biomarkers of effect. Biomarkers of effect are directly related to the risk of adverse health effects, while biomarkers of exposure precede any adverse health effects and are directly related to the exposure to chemicals (Forbes et al., 2006). According to this definition, biomarkers of

exposure can be considered as direct measures of metal bioavailability (Lanno et al., 2004; Eason and O'Halloran, 2002). They quantify the bioactive fraction of the pollutants, i.e. toxicological bioavailability (Scott-Fordsmand and Weeks, 2000).

A number of biomarkers have been shown to respond to metal contamination in earthworms: expression of the gene coding metallothionein (MT) (Bernard et al., 2010; Spurgeon et al., 2004), stability of lysosomal membranes (NRRT : neutral red retention time, Svendsen and Weeks, 1997), genotoxicity assessed by the Comet assay (Reinecke and Reinecke, 2004), enzymes activites (e.g. implied in the response to oxidative stress (Łaszczyca et al., 2004)), energy reserves (Holmstrup et al., 2011), surface cast production (Leveque et al., 2013). Several reviews already exist on earthworm biomarkers (Scott-Fordsmand and Weeks, 2000; Lionetto et al., 2012).

The purpose of the present synthesis is not to give a catalogue of biomarkers but to highlight the gaps of knowledge that remain to be addressed in order to confirm their pertinence as indicators of metal bioavailability. We identified four main gaps of knowledge related to: (i) the relationships between metal exposure and biomarker responses (ii) the assumption that metal exposure has a greater impact on biomarkers response than the effect of confounding factors, notably soil characteristics, (iii) the nature of biomarkers responses in field-contaminated soils that combine low levels of contamination and the presence of multiple metals.

1.3.2 Link between biomarkers responses and metal exposure

Most studies on biomarkers in earthworms exposed to metal contamination focused on the dose-response relationships considering total metal concentrations and not body loads or metal availability measurements (e.g. Reinecke and Reinecke, 2004).

A number of studies demonstrated that the expression of MT gene (coding for a protein involved in detoxification mechanisms of Cd, Cu, and Zn) increased with metal contamination (e.g. Brulle et al., 2007). Only two studies, however, demonstrated significant correlation between internal metal concentrations and the expression of MT. Spurgeon et al. (2004) showed a relationship with Cu body loads. Galay-Burgos et al. (2005) showed MT expression was correlated to internal Cu and Cd concentrations. Both these studies were spiking experiment with elevated concentrations of Cd (up to 180 ppm in Galay-Burgos et al. (2005) and to 800 ppm in Spurgeon et al. (2004)) and Cu (up to 180 ppm (Galay-Burgos et al., 2005) and 640 ppm (Spurgeon et al., 2004)). These two studies considered the earthworm species *Lumbricus rubellus* (Hoffmeister, 1843). The stability of lysosomal membranes (NRRT) was

shown to decrease with metal contamination in several earthworm species (Spurgeon et al., 2000; Svendsen et al., 2004). Several studies tested if NRRT was correlated to internal metal contents. Van Gestel et al. (2009) found significant relationship with internal Cu concentration in earthworms while Berthelot et al. (2009) found the opposite in multi-contaminated soils. The Comet Assay that measures genotoxicity was correlated to internal As contents in *Lumbricus terrestris* (Linnée) (Button et al., 2010). Fourie et al. (2007) have however shown no relationship with Cd body loads in several species (but Cd is not highly genotoxic). Holmstrup et al. (2011) correlated glycogen contents with internal metal concentrations and demonstrated significant relationships with Al and Ni but not with Cu, Cd or Pb. Concerning the activity of enzymes involved in the response to oxidative stress, Lukkari et al. (2004), for example, demonstrated that GST (glutathione-s-transferase) activity was not correlated to internal metal concentration in earthworm collected from field contaminated soils, while EROD (Ethoxyresorufin-O-deethylase) activity was positively correlated to Cu, Zn, Al and Fe contents.

Together, these various results are in agreement with the previous conclusions and with the rationale that internal metal contents do not reflect the effects of metal (Luoma and Rainbow, 2005). Overall, it seems that only the studies that considered soils spiked with elevated concentrations of metals were able to demonstrate significant correlations. More importantly, considering a few number of soils or treatments makes it difficult to detect significant effects of internal concentrations on biomarkers.

1.3.3 Effect of confounding factors: focus on soil characteristics

To be considered as a valid measurement of metal bioavailability, a biomarker needs to be weakly affected by confounding factors (such as temperature, season, weight etc.). According to Svendsen et al. (2004) the influence of confounding factors on biomarkers response needs to be minimal or well-characterized. Field studies demonstrated that metals were not always the most important factors affecting biomarkers in earthworms. Pérès et al. (2011) showed that the expression of the gene coding for MT responded to other factors than metals. Łaszczyca et al. (2004) showed that the activities of several enzymes involved in the response to oxidative stress were sensitive to the season. Energy reserves levels were also demonstrated to be sensitive to temperature and fertilization (Overgaard et al., 2009; Bednarska et al., 2013). The effect of confounding factors can greatly adverse the conclusions withdrawn from studying the responses of biomarkers. It is therefore very important to determine which are the most influent and how much they change biomarkers response.

Among confounding factors, soil properties are of crucial interest with regards to research on metal bioavailability because they influence both metal availability and earthworm biology and ecology. Soil pH, OM, metal (hydr)oxides and clay are the most important determinants for metal speciation, and hence metal availability (Sauvé et al., 2000). Soil characteristics can thus exert indirect effects on biomarker responses via their influence on metal speciation. Physico-chemical characteristics are also key factors controlling metal uptake by soil organisms (Thakali et al., 2006; Van Gestel, 2008). Peijnenburg et al. (1999) and Giska et al. (2014) showed that the uptake rates of metals in earthworm were determined by the pH. Nahmani et al. (2009) however found no clear pattern between soil properties and uptake rate constants.

Soil parameters can also affect biomarkers response more directly via changing earthworms physiology and behavior. However, this topic is rarely addressed. It is known that soil properties affect earthworms abundance *in situ*. The most important parameters are pH, SOM contents and soil texture (Curry, 2004). Earthworms are rarely present beneath a pH of 3.5. SOM are the food base for the earthworm and are thus vitally important. Soil texture play an important role. Equilibrated soil textures are more favorable than sandy soils and higher energy expenditure can be expected in compact medium (Lavelle, 1988).

Several studies demonstrated that these soil properties affected metal toxicity for earthworms. For example, Owojori et al. (2010) showed that increased clay content was associated to decreased mortality of *Eisenia fetida* (Savigny 1826) in Cu contaminated soils. Daoust et al. (2006) demonstrated the influence of pH, SOM and clay content over Cu toxicity (mortality) on *E. fetida*. Irizar et al. (2014) found Cd toxicity was modulated by SOM in *E. fetida*.

1.3.4 Biomarker responses in field-contaminated soils

In the case of diffuse pollution, soils are often moderately or lowly contaminated. Moreover, the process of ageing lead to decrease bioavailability of metals over time in field-contaminated soils (Lock et al., 2006). In addition, field contaminated soils are most of the time contaminated by mixtures of metals. The question arises whether biomarkers can indicate metal bioavailability in such a context.

First, their response to low doses is often hormetic. Calabrese, 2008 defined hormesis as "a biphasic dose-response phenomenon characterized by a low-dose stimulation and a high-dose inhibition". In the case of earthworms and metal contamination, several studies reported such hormetic-like response of biomarkers. Earthworms growth and reproduction

was shown to be stimulated by low levels of metals in both soil and worms (Svendsen and Weeks, 1997; Spurgeon et al., 2005; Spurgeon et al., 2004; Ma, 2005). Galay-Burgos et al., 2005 reported biphasic hormetic-like response of MT expression in earthworms exposed to Cd and Cu. In addition, the activity of enzymes involved in the response to oxidative stress (CAT, GST, SOD (superoxide dismutase)) was demonstrated to exhibit such hormetic dose-response (Łaszczyca et al., 2004; Zhang et al., 2009). Overall, there is little information on the hormetic effects of metals on earthworm biomarkers (Zhang et al., 2009). The fact that a biomarker increases at low doses means that the contaminant is both environmentally and toxicologically available. Within a bioavailability framework, the low-dose stimulation can thus be indicative while this is not the case for the assessment of harmful effects or risk.

Biomarkers may further respond to multiple metals in field-polluted soils. This characteristic of the field context is again leading to the expectation of a complex response of biomarkers. The toxic effects of mixture of metals are more and more addressed in ecotoxicology studies (Jonker et al., 2005). The effect of different metals interacting in an additive, synergistic or antagonistic manner is generally investigated using artificial exposure experiments (e.g Khalil et al., 1996; Lock and Janssen, 2002). Such studies are valuable to address the mechanisms underlying the complex effects of mixtures. However the prevalence of those interactions within more complex systems such as field-contaminated soils also need to be investigated.

Several reasons may thus prevent the use of biomarkers to assess toxicological bioavailability in field-contaminated soils. An approach commonly used to study the response of biomarkers is to consider one single reference soil, several treatments with high doses of total metals, and often artificially contaminated soils. If this approach is needed to understand the underlying mechanisms, it is equally important to study the response of biomarkers in more complex gradients of soils. In field-contaminated soils, low doses, multiple contamination and soil parameters may interact at a broader scale of observation and lead to very different results than when considering simplified systems.

1.4 Environmental bioavailability, a key step in the causal model

Environmental bioavailability represents the mediator between soil metal availability and toxicological bioavailability. Within a causal framework, it is thus a key component. Bioavailability is a dynamic process (Peijnenburg and Jager, 2003). Toxicokinetics approaches quan-

tify uptake and elimination rates, bioaccumulation and time to reach steady-state internal concentration. A number of authors used the uptake rate constants to assess metal bioavailability to earthworm (Peijnenburg et al., 1999; Van Straalen et al., 2005). Toxicokineics approaches were mostly conducted in the laboratory, following exposure to artificial soils, artificial contamination and using the model earthworm species (*E. fetida*) (Giska et al., 2014). But more and more studies have considered field-polluted soils (Nahmani et al., 2009; Peijnenburg et al., 1999) and more representative earthworm species (Giska et al., 2014). Nahmani et al. (2009) and Peijnenburg et al. (1999) found variable uptake rate constants in *E.andrei* and *E.fetida*. Nahmani et al. (2009) concluded that it was difficult to extrapolate uptake rates from one soil to the other, and the uptake kinetics parameters were soil-specific.

Bioaccumulation of metals in organism is an integrative assessment of chemical exposure in contaminated environments. Luoma and Rainbow, 2005 however highlighted its variability and its complexity. Indeed, bioaccumulation is the resultant of both uptake and excretion processes. Van Straalen et al. (2005) showed bioaccumulation was less pertinent than uptake rate to assess metal bioavailability to earthworm. Two main drawbacks are usually pointed out: (1) bioaccumulation does not address the dynamic nature of bioavailability and (2) it is not a good predictor for the effects of metals on organisms (Luoma and Rainbow, 2005). Nevertheless, bioaccumulation is routinely reported in papers addressing metal bioavailability and effects on earthworms. Moreover, measuring metal tissue concentration is crucial for risk assessment of metal biomagnification (Vandecasteele et al., 2004).

Within the definition of bioavailability, it is unclear if metal tissue concentration is a resultant of environmental bioavailability or of toxicological bioavailability. In ISO 17402, 2008, "accumulation" is positioned within the toxicological bioavailability step, but it is unclear if accumulation designates the internal metal content or the fact that the metal is accumulated at higher concentrations than in an organism unexposed to metal pollutants. Lanno et al., 2004 indicated that metal concentration in the organism was intermediary between environmental and toxicological bioavailability. Indeed, increasing metal concentrations in earthworm is the result of uptake processes. Thus, within the causal definition of bioavailability, internal metal concentration is caused by uptake processes but is not necessarily the cause of the organism toxic response.

One of the reasons why internal metal content is not a good predictor of the effects of metals on organism is that organism protect themselves from the side-effects of metals by sequestration in certain tissue of their body (Vijver et al., 2004). Similarly to metals in soil, only a fraction of total internal metals can lead to an effect. Earthworms compartmentalize metals, for example, in the chloragogenous tissue and in the posterior alimentary

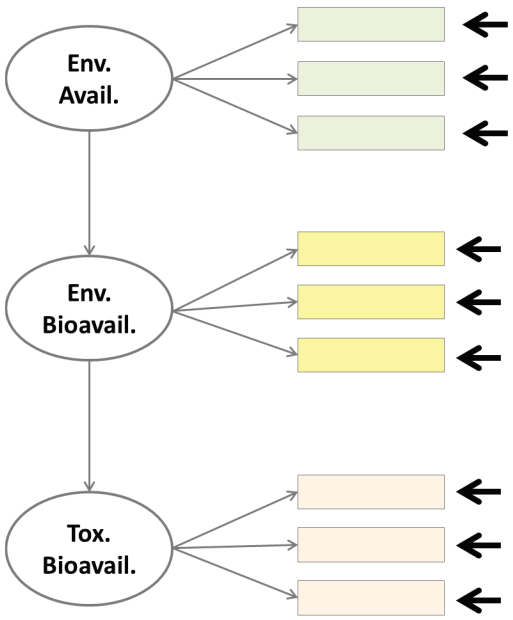
canal (Morgan et al., 2004; Andre et al., 2009). At the subcellular level, two major pathways of detoxification are metal binding to proteins (e.g. MT Stürzenbaum et al., 2004) and precipitation into insoluble metal concretions (metal-rich granules) (Vijver et al., 2004). Subcellular fractionation procedures partition metal body burden into operationally defined fractions notably cytosol, metal-rich granules and a fraction containing tissue, intact cells and cell membranes (called debris hereafter) following differential centrifugations. They were developed on aquatic organisms (Wallace et al., 1998) and applied to earthworms (Vijver et al., 2007; Jones et al., 2009). If metal concentrations in certain subcellular compartments are considered to reflect toxicologically bioavailable metal pools, it was rarely proved in the case of earthworms. Moreover, the subcellular distribution is metal-specific and several fractions combine both toxic and detoxified forms of metals (Vijver et al., 2004).

Several studies considered the time-variation of metal concentrations in subcellular compartments in earthworms (Jones et al., 2009; Li et al., 2009; Arnold et al., 2008). The relationship between subcellular partitioning and soil availability was only reported for Cu in earthworm (Vijver et al., 2007). Moreover, the subcellular partitioning was rarely related to biological endpoints. The added value of metal subcellular partitioning to assess metal bioavailability could be addressed, notably by determining its variations in ranges of field-contaminated soils (Jones et al., 2009). Metal concentrations in subcellular fractions are the result of uptake processes such as the total internal contents. However, within a causal definition of bioavailability, metal concentrations in certain fractions can be considered to have causal effects on toxicological bioavailability. Similarly to soil available concentrations, metal contents in certain subcellular fractions could be defined as toxicologically available, while toxicological bioavailability is the resultant of the interaction of such internal pools with biological processes.

1.5 Concluding remarks

There are a number of methods to assess metal bioavailability to earthworm but not one in particular was proven relevant in field-contaminated soils. Few studies have considered bioavailability as a three-step process, and a tool to articulate the three steps is lacking. Graphical models such as SEM could be used to clarify the structural relationships between environmental availability, environmental bioavailability and toxicological bioavailability.

Chapter 2



Chapter 2

Experimental design

In this thesis, the objective to test a SEM of bioavailability determined the experimental design. In this chapter we explain on which basis our experimental design was conducted. We decided to focus on three trace elements (Cd, Pb and Zn) because (i) they are frequently found in field-contaminated soils, (ii) they have distinct chemical properties in soil (Cd and Zn are more mobile in soil than Pb) and (iii) they have distinct physiological and toxicological properties for earthworms (Cd and Pb being toxic elements with no known biological function while Zn is an oligo-element). Our objective was to focus on realistic environmental exposures. Soil organisms living in field-contaminated soils have developed adaptation strategies and tolerance that render difficult to evaluate metal bioavailability to indigenous organisms living in contaminated environments (Posthuma and Van Straalen, 1993). This thesis being a primary approach to test the potentialities of SEM to reflect metal bioavailability, we decided to conduct a laboratory exposure of naive earthworms to field-contaminated soils.

The experimental design was firstly guided by the requirements of the SE model. In the first section of this chapter, SEM assumptions and requirements are reviewed and we explain how they determined our experimental choices. Since bioavailability is species dependent the SE model was applied to a target earthworm species. The objective being to assess realistic exposure conditions, we considered an earthworm species commonly found in the field. In the second section of this chapter, the choice of the target species, *(Aporrectodea caliginosa* (Savigny 1826), is discussed. Earthworms were exposed to a wide panel of field-contaminated soils in order to test the SE model. A gradient of environmental availability was created by selecting a series of field-contaminated soils exhibiting jointly ranges of total metal concentration in soils and of several soil properties that govern metal speciation. In the third section of the chapter, the selection of the series of field-contaminated soils is discussed.

The last section gives explanation for the choice of important experimental conditions for laboratory exposure of earthworms to soil.

2.1 Specification and assumptions of the structural equation model

Step 1 Model specification

The first step in SE modelling is to construct a path diagram (or directed graph) reflecting our understanding of the causal processes involved. Because each step of bioavailability can be viewed as a latent variable reflected by different measurements, a structural model (relationships between latent variables) was specified based on the widely accepted theory of bioavailability (Lanno et al., 2004; Harmsen, 2007) and was described earlier (Chap.1 p. 8). This structural model can be applied to a number of organisms and of contaminants. The measurement model (relationships between latent and manifest variables) was specified by selecting several chemical and biological indicators reflecting bioavailability of metals to earthworm based on a review of the literature (Chap.1 p. 12). In SEM, several assumptions are made and several requirements need to be fulfilled. Those constraints and assumptions guided the specification of the measurement model and the experimental design.

Measurement model of environmental availability

As previously highlighted, earlier studies showed that earthworms were exposed to soil metals by both dermal uptake (Vijver et al., 2003) and ingestion (Morgan et al., 2004). Environmental availability could thus be modeled as a combination of measurements that assess 'actual' and 'potentially' available metals.

In SEM, there is a difference between latent variables (that have causal effect on their manifest variables) and composite variables (unmeasured variables that represent heterogeneous collections of causes) (Grace and Bollen, 2006). If we were to test the assumption that environmental availability is the result of, for example, metals bound to soil organic matters, readily extractable by a weak extractant and free ion concentration, a composite variable that summarize the effects of the three metal concentrations would be appropriate. The are several ways to recognize a composite from a latent variable. First the manifest variables of a latent are consistently correlated, because if the latent variable change, all

manifest variables will change as well (the manifest variables are caused by the latent). The manifest variables of a composite variable do not need to be correlated because they cause the unmeasured variable. Second, the definition of a latent variable does not change if we remove or add a manifest variable (Grace and Bollen, 2008). For example, in a measurement model representing environmental availability as a latent reflected by free ion concentration, readily extractable content and metal bound to soil organic matter concentration, removing metal concentration bound to soil organic matters change the definition of the latent variable. Worst, it is not certain that the underlying cause of covariation between those last three observed variables is really environmental availability and not the total soil metal concentration.

The use of composite variable raises several statistical issues (Grace and Bollen, 2006). Composite variables are basically multiple regressions with observed variables as the explaining variables. In cases where observed variables are correlated, the path coefficients will be biased, as in multiple regression (Shipley, 2002). Yet metal concentrations in soil are often correlated, sometimes strongly correlated (e.g. EDTA-extractable and total concentrations). Furthermore, one of the main advantages of latent variables is the possibility to explicitly take into account the influence of measurement errors on observed variables (Chap.1, p.8), and this is not possible with composite variables (Grace and Bollen, 2006).

For our SE model, two distinct latent variables can be considered to reflect environmental availability: one reflecting desorption processes, and reflected by loosely bound metal concentrations (in soil solution or easily extractable by weak extractants) and another reflecting sorption processes, and reflected by more strongly bound metal concentrations (bound to organic matters digested by earthworms).

Both experimental and modeling methods that have been successfully related to earthworms bioaccumulation and effects in the literature were selected as manifest variables. Two chemical extractions were selected: $CaCl_2$ as a weak extractant reflecting easily extractable metals and EDTA as a chelating agents which can compete with strong binding sites in soil and assigned to metals bound to organic matters. Two theoretical calculations of metal specific pools were further considered: a semi-mechanistic model predicting total dissolved metal contents in the soil solution (Sauvé et al., 2000) and a geochemical mechanistic model predicting soil metal speciation (thus both loosely and strongly bound metal concentrations).

Measurement model of environmental bioavailability

Although the uptake rate kinetics is the most direct measurement of environmental bioavailability, we have seen that it is not clear if they bring additional information compared to internal concentrations to account for Cd, Pb and Zn uptake in earthworms exposed in the laboratory for short term duration. Furthermore we will see further in this section that SEM testing needs large sample size, difficult to combine with multiple measurements of internal concentrations over time (toxicokinetics experiments).

As we have seen earlier, the tissue metal concentration was criticized as it is an imperfect measurement of metal bioavailability, and because its relationships with toxicological bioavailability is not straightforward. The SEM framework, however, can tackle both these drawbacks. First, the measurement error term integrates external influences (other than environmental bioavailability) on bioaccumulation, making it possible to address explicitly the imperfection of internal metal concentration to reflect environmental bioavailability. Second, in the SE model of bioavailability, there is not causal path between internal metal concentration and toxicological bioavailability, toxicological bioavailability is caused by environmental bioavailability, of which internal concentration is only an observed manifestation.

For a measurement model to be identified it is necessary to have several manifest variables. Otherwise, the variance of the measurement error is assumed to be 0 meaning that the observed variable perfectly reflects the latent variable. As stated earlier, this is not acceptable for internal metal concentration and environmental bioavailability. Therefore we decided to consider additional metal contents in earthworms as manifest variables of environmental bioavailability. Subcellular fractionation was developed as a mean to better relate internal metal contents with the effects of metals (Vijver et al., 2004). Within the ISO conceptual framework, it means that they should increase with soil metal availability, and with metal uptake. In the SE model, we therefore considered both total internal concentrations and concentrations in three subcellular compartments: cytosol, debris (tissue, membranes and intact cells) and granules as manifest variables for environmental bioavailability.

It is noteworthy that metal concentration in certain compartments (e.g. Pb in the cytosol) are considered to be indicative of the toxic pressure (Jones et al., 2009). As such, one could assume that Pb concentration in the cytosol is rather a manifest variable for toxicological bioavailability. Again, within the SEM framework, a manifest variable is the resultant of its latent variable. We defined toxicological bioavailability as the internal portion of metals that affect biomarkers at several levels of biological organization. Under this definition, Pb concentration in the cytosol would rather be the cause of toxicological bioavailability. In the

SE model, we used metal concentrations in the subcellular fractions differently: considering that they reflected metal uptake. The question of the relationship with biological effects was treated in chapter 5 (p.99).

Measurement model of toxicological bioavailability

Different biomarkers were chosen as manifest variables for toxicological bioavailability. As highlighted in the introduction, biomarkers are supposed to reflect bioavailability, but few studies demonstrated their pertinence.

In this thesis, the choice of biomarkers was based on the will to represent different levels of biological organization (molecular, cellular and individual). It is widely accepted that the measurement of a single biomarker is insufficient to reflect the stress associated with metal exposure. Stress is a multidimensional concept (Van Straalen, 2003). Multi-biomarker approaches using a battery of biomarkers from different levels of biological organization, revealed more appropriate to reflect the stress response of earthworms after metal exposure (Asensio et al., 2013; Berthelot et al., 2009).

The expression of the gene coding for the metallothionein protein, notably involved in Cd detoxification, was chosen as a manifest variable in the measurement model of toxicological bioavailability. Several studies demonstrated its pertinence to reflect metal exposure in earthworms (Bernard et al., 2010; Spurgeon et al., 2004).

The activity of enzymes involved in the response to oxidative stress are often used as biomarkers of metal exposure. Enhanced glutathione-s-transferase (GST) and catalase (CAT) activities have been reported in earthworms exposed to metals (Saint-Denis et al., 2001; Lukkari et al., 2004; Maity et al., 2008; Berthelot et al., 2008). However, the response was often transient (i.e. rapid return to the basal level, (Lukkari et al., 2004; Maity et al., 2008; Berthelot et al., 2008)). And a number of confounding factors were shown to affect the activity of these enzymes (Saint-Denis et al., 1998; Arnaud et al., 2000). In the SE model, we considered GST and CAT activities as manifest variables of toxicological bioavailability.

Organisms are assumed to modify their energy reserves to cope with metal exposure (Spurgeon and Hopkin, 1996a). Differences in energy allocation could lead to decreased growth and reproduction (Moolman et al., 2007; De Coen and Janssen, 2003). Compared to MT expression and CAT and GST activities, energy reserves bring insights into earthworms physiology (energy status). Energy reserves (lipid, glycogen and protein contents) were selected as manifest variables in the model.

Weight loss after exposure was chosen as well. Several studies showed this biomarker was related to soil metal contamination, availability and internal metal concentrations (Smith et al., 2010; Ming et al., 2012). However, in other studies, there was no significant weight loss in earthworms exposed to contaminated soils (Vijver et al., 2006).

The conceptual SEM of bioavailability considering environmental availability as the 'actual' available metal pools is shown Figure 2.1.

Step 2 Translation of the conceptual model into structural equations

A second step in SEM is the translation of the causal model into structural equations. It is important to precise that this translation is imperfect. Pearl et al., 2009 stated that the main reason for the confusion between causation and correlation was that the sign '=' in a mathematical equation cannot reflect a causal assumption. Mathematical equations reflect symmetrical relationships: For example, $y = 0.5x + 3$ can also be written $x = 2y - 3$. Say y is the concentration of metal in earthworm and x is the concentration of metal in soil, and we wish to represent a causal relationship from x to y. The first equation reflect our causal understanding, but the second equation can be misinterpreted to mean that the metal concentration in earthworm influences the metal concentration in soil.

The translation of causal assumption into a set of testable assumptions against our observations is therefore imperfect because it needs '=' signs instead of '→' (i.e. asymmetrical effect). This can lead to misinterpretation of SEM results if the causal system is not well understood. And this is the reason why SEM cannot prove causation but rather facilitates the investigation of causal relations (Grace et al., 2012). In our SE model, the conceptual definition of bioavailability is clearly established and there is no doubt on the direction of causality.

The structural equations can have different forms. In general, the relationships are assumed to be linear. In the equations derived from the directed graph, the parameters to be estimated are the path coefficients and error variances. We give two examples: Internal metal concentration C_{int} depends on environmental bioavailability EB, the coefficient a quantifies the magnitude of this effect. Internal metal content is also influenced by other factors accounted for in ϵ. ϵ is a random variable following a Gaussian distribution and for which we need to estimate the variance (σ_{int})

$$C_{int} = a.EB + \epsilon_{int} \qquad \epsilon_{int}\ N(0, \sigma_{int})$$

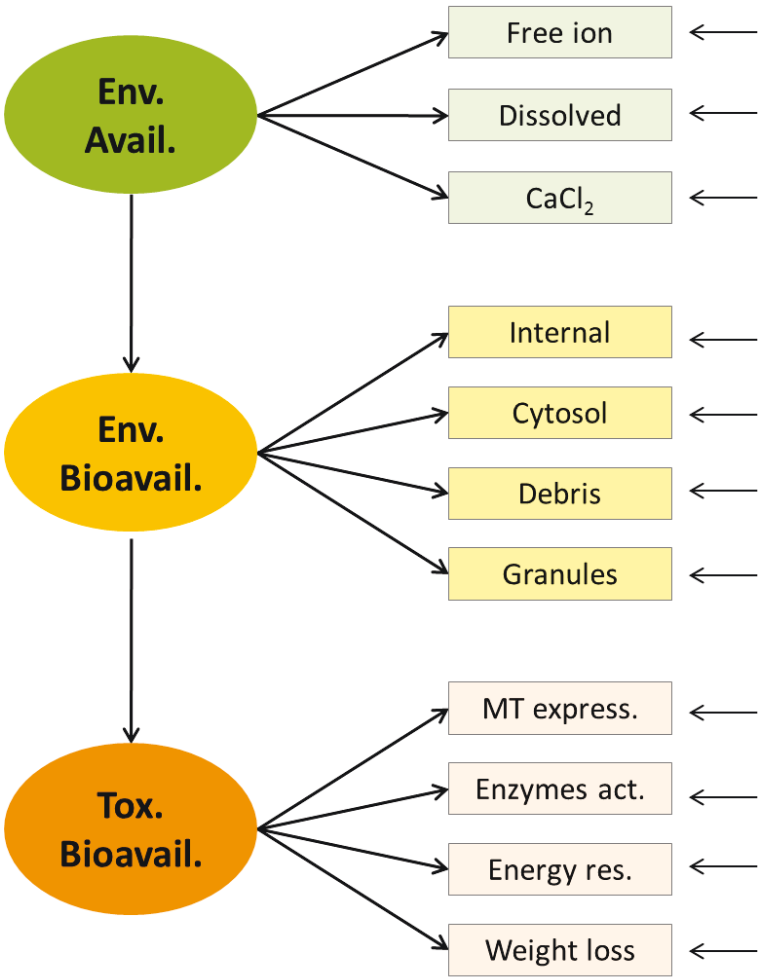


Figure 2.1: A structural equation model (SEM) of metal bioavailability for earthworms. Latent variables are shown in ellipses, observed variables are shown in rectangles. Unidirectional arrows indicate cause-effect relationships. Environmental availability, apprehended as the actual availability, is reflected by metal concentration obtained after $CaCl_2$ extraction, semi-mechanistic model of dissolved concentration and geochemical model of free ion content. Environmental bioavailability can be reflected by metal concentration in total earthworm (internal) and in three subcellular fractions (cytosol, debris and granules). Toxicological bioavailability is indirectly measured by biomarkers from different levels of biological organization; expression of the gene coding metallothionein protein (MT expression), energy reserves, activities of two enzymes involved in the response to oxidative stress (GST and CAT) and weight loss.

Another example for a latent variable is TB that is regressed on EB with a path coefficient b quantifying the magnitude of the effect, and as for internal metal content, the latent has other sources of variation accounted for in the ζ term, and for which the model will estimate the variance σ_{TB}

$$TB = b.EB + \zeta_{TB} \qquad \zeta_{TB}\ N(0, \sigma_{TB})$$

Step 3 Model-implied variance-covariance matrix derived from the structural equations

The proposed conceptual model represented by the path diagram (step 1) and equations (step 2) makes assumptions about the relationships between the modeled variables, that have specific implications for their variance and covariances (Hershberger et al., 2003).

In the conceptual model of bioavailability, it is assumed that TB is independent from EA (environmental availability) if EB is blocked (i.e. there can be an effect of metals only if earthworm absorbed metals), and hence there is no covariance between TB and EA when EB is constant. A central device in SEM is called directed-separation (d-separation). d-separation is a graphical condition that translate causal assumptions in directed graphs into statistical information (conditional independencies) (Shipley, 2002). In our model, TB is d-separated from EA given EB, MT expression is d-separated from total internal metal content given both TB and EB, etc. All d-separation statements are reflected by statistical independencies (null covariances) in the model. If the model is correct, then the observed covariance structure will also reflect such statistical independencies (Shipley, 2002). Thus the third step in SEM approach is to derive the model-implied variance covariance matrix given the equations of step (2) and the d-separation statements.

Step 4 Estimation of the parameters

To be estimated with unique values, parameters must be identified. For that, it is necessary to have as many known pieces of information as parameters to estimate. The most commonly used procedure to verify if the model is identified is the t-rule;

$$t \leq n(n+1)/2$$

where n is the number of observed variable in the model and t is the number of free parameters.

In our SEM of bioavailability Figure 2.1, there are 2 path coefficients in the structural model, 14 path coefficients in the measurement model and 17 error variances to be estimated. The t-rule was verified: ($t = 33$ and $n = 14$, $t < 105$). Although the t-rule is necessary, under-identification can still arises in models that verify this rule. Models with too many paths, multicollinearity and small sample size can prevent identification. Therefore the complexity of the model needs to be adapted to the data (Grace et al., 2012).

Parameters are quantified by minimizing the difference between observed and model-implied covariance matrices. The estimation method depends on the assumptions about the distribution of the observed variables. Usually, the maximum likelihood (ML) estimation method is conducted. However this method requires multivariate normal distribution of the data. As this is rarely verified, new methods, particularly the robust ML estimator (Satorra and Bentler, 2001), are more and more used in ecology to handle non-normal data (e.g. Borer et al., 2012). This procedure is also suitable in case of moderate sample size (Rosseel, 2012).

Step 5 Assessing the fit of the model

At this step, one typically addresses whether the model-implied and observed covariances matrices are close to each other. The most commonly used approach is to use the Chi square goodness-of-fit statistic, which tests the null hypothesis that there is no difference between observed and model-implied covariance.

Low sample size would increase the chance to reject a model even though it is true (Shipley, 2002). There are no general rules to determine the appropriate sample size. However rules of thumbs are reported in the literature, for example Bentler stated that it was necessary to have at least 5 times more observations than parameters to estimate. Applying this rule to the SE model of metal bioavailability to earthworms, it means 33 x 5 = 165 observations are necessary.

Based on the *a priori* conceptual model and on the constraints inherent to SEM, we designed a laboratory experiment to a wide panel of soils. We decided as a compromise to adapt the sample size required by the model to a more reliable and feasible design. Indeed, the majority of the manifest variables involved in the model required replicated measures to insure the quality of the data. The experiment therefore included 155 individual observations providing 31 mean values to test the SE model. As we were interested in the robustness of the

causal relationships, we assumed that if causal relationships were verified in our experiment with a limited statistical power, it would reinforce their robustness.

The drawback of the moderate sample size is that further exploration of more complex models integrating, for example, soil parameters in the SEM of bioavailability cannot be conducted.

2.2 Choice of the target earthworm species

Earthworms are particularly interesting to study the bioavailability of metals (Nahmani et al., 2007a). Indeed, they are exposed to trace elements because they live in constant contact with the soil matrix, they accumulate metals (e.g. Spurgeon and Hopkin, 1999) and are sensitive to the contamination (e.g. Abdul Rida and Bouché, 1995). In addition, they are easy to handle in a laboratory exposure, an approach that was selected for this thesis. Finally, bioavailability of metals to earthworms is well-documented in the literature, notably because of the functional importance of this ecosystem engineer (Lavelle, Spain, et al., 2001). Exposure, bioaccumulation and sensitivity to metals vary according to the species considered (Spurgeon et al., 2000; Ernst et al., 2008).

We selected *Aporrectodea caliginosa* (Savigny) as the target species. The choice relied on several criteria. Once the species selected, we further assessed whether its patterns of metal bioaccumulation were different than for other species based on an analysis of bibliographic data.

2.2.1 Selection criteria

There are three ecomorphological groups of earthworms (epigeic, endogeic and anecic). Endogeic earthworms live in the top soil, down to 40 cm depth and feed mostly on soil. This is not the case of epigeic and anecic species, that feed mostly on SOM at the surface of the soil. Endogeic worms are therefore potentially more exposed to metals (Van Vliet et al., 2005; Suthar et al., 2008). We thus chose to focus our research on an endogeic species.

The representativeness of the target species was another determinant criterion. We decided to study an earthworm species that is often found on the field and on a great number of environments contrarily to most ecotoxicological studies that focused on *Eisenia fetida* (Nahmani et al., 2007a), a species that is not found in soils but in compost. *A. caliginosa* is one of the most abundant earthworm species in temperate environments (Pérez-Losada

et al., 2009). In addition, numerous reference data exist on *A. caliginosa*, notably in the case of internal metal concentrations.

The target species had to support well the experimental conditions. Preliminary experiments in our laboratory demonstrated that *A. caliginosa* could be maintained in sieved-soil and in microcosms, while it was proven more difficult for other species (*A. icterica*, *L. terrestris*) (low levels of reproduction and activity (Pelosi, Thénard, Hedde comm. pers.)).

Several species were demonstrated to be more tolerant to metal contamination than other. This is notably the case of the model species in ecotoxicology: *Eisenia fetida* (Spurgeon and Hopkin, 1996b; Langdon et al., 2005). This was further demonstrated recently in the context of pesticides (Pelosi et al., 2013b). In the literature, *A. caliginosa* has been shown sensitive to metal exposure (Klobučar et al., 2011).

2.2.2 Analysis of literature data [1]

The choice of the target species was confirmed based on an analysis of the literature. We compared Cd, Pb and Zn bioaccumulation in different species of earthworms and asked if the patterns of bioaccumulation observed in *A. caliginosa* were specific or could be considered as representative of other species. We collected and analyzed the data of 39 publications reporting metal concentration in earthworms exposed to contaminated soils (the references are given biblio.sp).

Method

Over the 39 studies considered, 17 articles reported data collected *in situ* and 22 papers reported results obtained following laboratory exposure. Among the latters, 6 studies used artificial contamination (soil spiking with prepared metal solutions). Within this corpus of 39 studies, 21 earthworm species were represented. Only 8 different species were represented for the laboratory experiments. There were not several papers reporting data for each of the 21 species, 11 species were discarded from the analysis because the observations were provided by a single paper for each species (*Allolobophora chlorotica*, *Pheretima hupeiensis*, *Aporrectodea trapezoides*, *Bimastos parvus*, *Drawida japonica*, *Eisenia hortensis*, *Lumbricus castaneus*, *Lampito maurii*, *Metaphire guillemi*, *Metaphire posthuma*, *Metaphire tshiliensis*). For each paper, metal concentrations in earthworms and soils were collected. We also reported (1)

[1] Léa Beaumelle, Isabelle Lamy, Mickaël Hedde. Is *Aporrectodea caliginosa* a representative 'real-world' earthworm species for metal bioaccumulation? *Environmental Monitoring and Assessment*, (in prep.)

Table 2.1: Ranges of Cd, Pb and Zn concentrations in soil and earthworms (ew) reported by 39 studies (in mg/kg of dry soil or tissue)

	[Cd]soil	[Pb]soil	[Zn]soil	[Cd]ew	[Pb]ew	[Zn]ew
min	0.09	4.94	9.29	0.30	0.43	10.40
mean	22.42	1772.85	1474.45	72.37	492.42	759.69
median	3.10	263.00	460.00	20.29	36.75	614.00
max	467.00	42700.00	53400.00	1320.00	13033.13	2947.08

if data were acquired following a laboratory or field study, (2) for laboratory experiments, the duration of the exposure and (3) soil physico-chemical characteristics when they were detailled.

We tested the assumption that internal metal concentrations depended on both the metal concentration in the soil and the species, and that the relationship (slope) between accumulation and soil metal content depended on the species. We used ANCOVA to test this assumption for each metal considered. The model assumes a linear increase of earthworm concentration with soil concentration, an effect of the species on internal concentrations (i.e. on average, does earthworm concentrations are different from one species to another?) and an interaction between the effect of soil metal content and the species (i.e. does the slope of the relationship between accumulation and soil metal concentration is the same for all species?). The assumption of the linear model were verified graphically (homoscedasticity, residuals normally-distributed). Moreover, the influence of high leverage observations (Cook's distance > 0.5) on the coefficients of the model was examined by comparing predicted coefficients with and without the high leverage points. In order to test if *A. caliginosa* presented a different bioaccumulation pattern from the other species, the slopes predicted for each species were compared to the slope predicted for *A. caliginosa* using t-test.

We considered only the species for which at least two different articles with different authors' names reported data. In the case of Zn and Pb, 7 species were considered to test the model: *A. caliginosa*, *Dendrobaena octaedra*, *Dendrodrilus rubidus*, *Eisenia andrei*, *E. fetida*, *L. rubellus* and *L. terrestris*. In the case of Cd, *Aporrectodea rosea* and *D. octaedra* were discarded because data were from a single publication.

Zero concentrations values were replaced by the minimum concentrations in the dataset. Data were linearized by logarithm transformation.

Table 2.2: Number of papers reporting data for each species, and number of observations from laboratory (Lab) of field studies (Field)

	Number of studies	Lab	Field
A. caliginosa	11	22	47
A. rosea	2	0	16
D. octaedra	2	0	23
D. rubidus	4	9	7
E. andrei	5	33	0
E. fetida	10	69	12
L. rubellus	15	57	54
L. terrestris	7	13	11

Results, Discussion

Table 2.1 shows the ranges, mean and median values of metal concentrations in soils and earthworms reported by the full corpus of studies considered. Several studies reported extremely high soil metal concentrations (Morgan and Morris, 1982 for Cd, Leveque et al., 2013 for Pb and Nahmani et al., 2007b for Zn). The strong differences between mean and median values for the soil concentrations indicated that such large values were in minority.

Concerning earthworm concentrations, the median values for Cd and Pb were of the same order of magnitude while elevated concentrations were reported for Zn. The differences between mean and median concentrations were higher for non-essential metals (Cd and Pb) than for Zn.

Species considered Table 2.2 shows the number of studies reporting data for each species and indicates the number of observations for field studies and laboratory studies. The species that were the most represented in the corpus were *L. rubellus*, *E. fetida*, *A. caliginosa* et *L. terrestris*. Data for *A. rosea* and *D. octaedra* were exclusively collected in the field, while for *E. andrei* only after laboratory exposure. This means that it is impossible to test for an interaction between the effects of the species and of the origin of the data (field or laboratory). Thus for those species, it is not possible to test if the slope is different because of the species or of the way data were collected.

Effect of earthworm species on metal accumulation with a focus on *A. caliginosa*

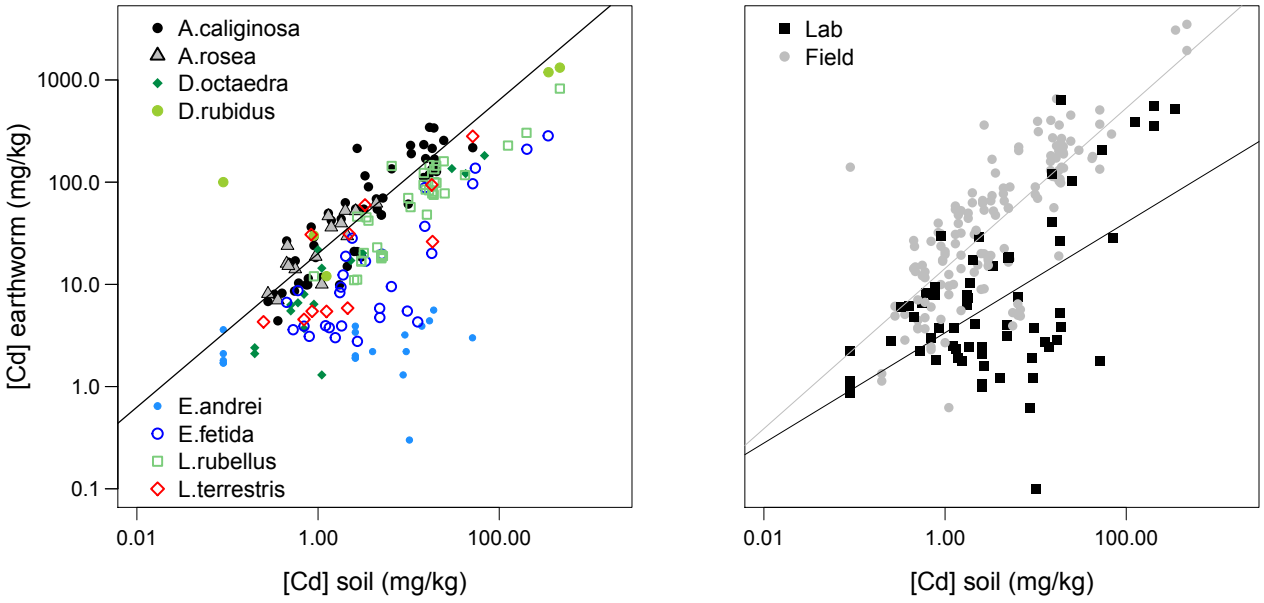


Figure 2.2: **(Left) Cd concentration in 8 earthworm species according to total concentration in soil reported by 23 studies** (on logarithmic scales). Black line is the slope for *A. caliginosa* (ANCOVA model). **(Right) Cd concentration in 21 earthworm species as function of Cd concentration in soil according to laboratory and field experiment (39 studies)**. Lines are the predicted slopes for laboratory exposures (black) and field studies (gray)

Cadmium The ANCOVA significantly explained earthworm Cd concentration and provided a good fit: $F_{12;144} = 72.4$; $p < 0.001$ with $R^2 = 0.85$. Internal concentrations were significantly different according to the species ($F_{5;144} = 67.75$; $p < 0.001$). Such differences can be explained by experimental conditions, notably the origin of the earthworms. Comparing the relationships between internal concentrations and soil metal contents for different species, the interaction was highly significant ($F_{5;144} = 13.98$; $p < 0.001$). Thus internal Cd concentration did not increase similarly for the 6 species considered.

Figure 2.2 shows the increase of internal Cd as a function of soil Cd concentration for the 6 species considered in the model and for 2 species not included in the model (*A. rosea* and *D. octaedra*). The rise of Cd concentrations in earthworms appears similar for 5 species out of 6. Internal Cd concentrations in *E. andrei* were indeed lower and increased less sharply with soil Cd concentrations than for the other species. Only the predicted slopes for *D. rubidus* and *E. andrei* were significantly different from the predicted slope for *A. caliginosa* ($p < 0.05$).

Figure 2.2 shows an observation with high leverage for *D. rubidus* (internal content elevated for a low soil Cd content). A model excluding this observation revealed no significant difference between the accumulation of Cd in *A. caliginosa* and *D. rubidus*. A high leverage observation for *E. andrei* can also be seen on Figure 2.2 (low internal concentration). However this point had no influence over the results of the model.

Cd accumulation by *A. caliginosa* was therefore distinct from *E. andrei*, but not from *D. rubidus*, *E. fetida*, *L. rubellus* and *L. terrestris*. In addition, despite the fact that fewer data were available for *A. rosea* and *D. octaedra*, Figure 2.2 shows similar patterns as for the other species. The analysis of literature data therefore indicates that *A. caliginosa* is an appropriate target species when studying Cd bioavailability to earthworms.

Given the absence of observations for *E. andrei* in field situation, it is not possible to conclude if there is a specific pattern for this species or if the difference is associated to the fact that it was only studied in the laboratory. Figure 2.2 shows the relationship between earthworm and soil Cd concentration according to the origin of the observations (laboratory or field). According to this chart, *E. andrei* is distinct from observations made for other species following laboratory exposure. The results therefore suggest a specific pattern of Cd accumulation in *E. andrei* that should be addressed in future studies. Particularly, observations for higher soil Cd concentrations are needed to conclude.

Lead The model for Pb was highly significant ($F_{14;158} = 35.38$; $p < 0.001$) but less closely adjusted than the Cd model ($R^2 = 0.74$). The effects of the species on earthworm concentrations and the interaction species-soil concentration were highly significant ($F_{6;158} = 10.04$; $p < 0.001$ et $F_{6;158} = 5.25$; $p < 0.001$ respectivement).

Figure 2.3 shows that the earthworm Pb concentrations for the different species were homogeneously distributed.

Comparing the predicted slopes, only the slopes for *L. terrestris* and *E. andrei* were significantly different from the one for *A. caliginosa*. ($t=-2.3$, $p < 0.05$ and $t=-5.05$, $p < 0.001$ respectively). An observation for *L. terrestris* had a strong leverage (low soil Pb content for elevated internal concentration, Fig 2.3). Excluding this observation, the slopes for *L. terrestris* and *A. caliginosa* were not significantly different ($t=-1.33$, $p > 0.1$). Pb accumulation by *A. caliginosa* was thus similar to the other species considered, except *E. andrei*.

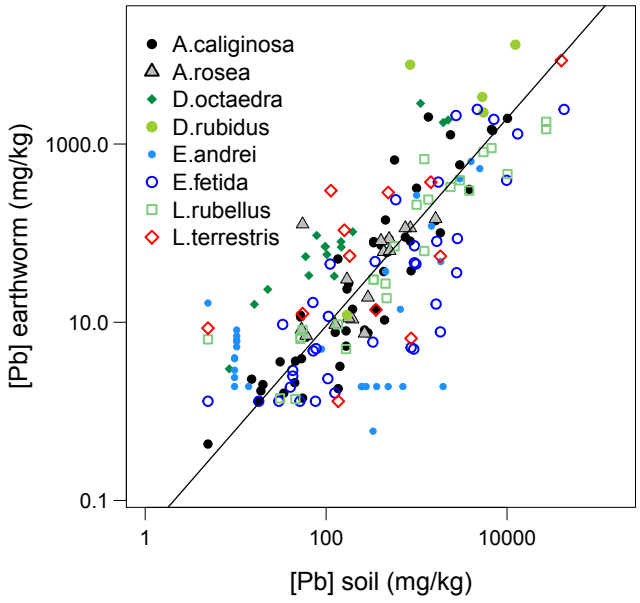


Figure 2.3: **Pb concentration in 8 earthworm species according to total concentration in soil reported by 25 studies** (on logarithmic scales). Solid line: predicted slope for *A. caliginosa* (ANCOVA)

Zinc ANCOVA was highly significant and relatively well adjusted ($F_{14;211} = 44.05$; $p < 0.001$ with $R^2 = 0.73$).

On the contrary to the results for Cd and Pb, the rise of internal Zn concentrations with soil content was similar for all the species considered ($F_{6;211} = 1.65$; $p > 0.1$).

Figure 2.4 illustrates that earthworm Zn concentrations were very different according to the species. It was especially the case for *E. fetida* and *E. andrei* which presented lower internal Zn concentrations than the other species.

E. fetida and *E. andrei* were mostly studied in the laboratory and the apparent effect might be due to the origin of the data. Figure 2.4 shows a strong difference between the observations made on field-collected earthworms and following a laboratory exposure. However, this difference appears mostly related to *E. fetida* and *E. andrei* because laboratory observations for other species are confounded with the observations made in the field. This result therefore suggests specific mechanism of regulation of Zn in *E. fetida* and *E. andrei*, with notably lower internal Zn concentrations than in the other earthworm species considered.

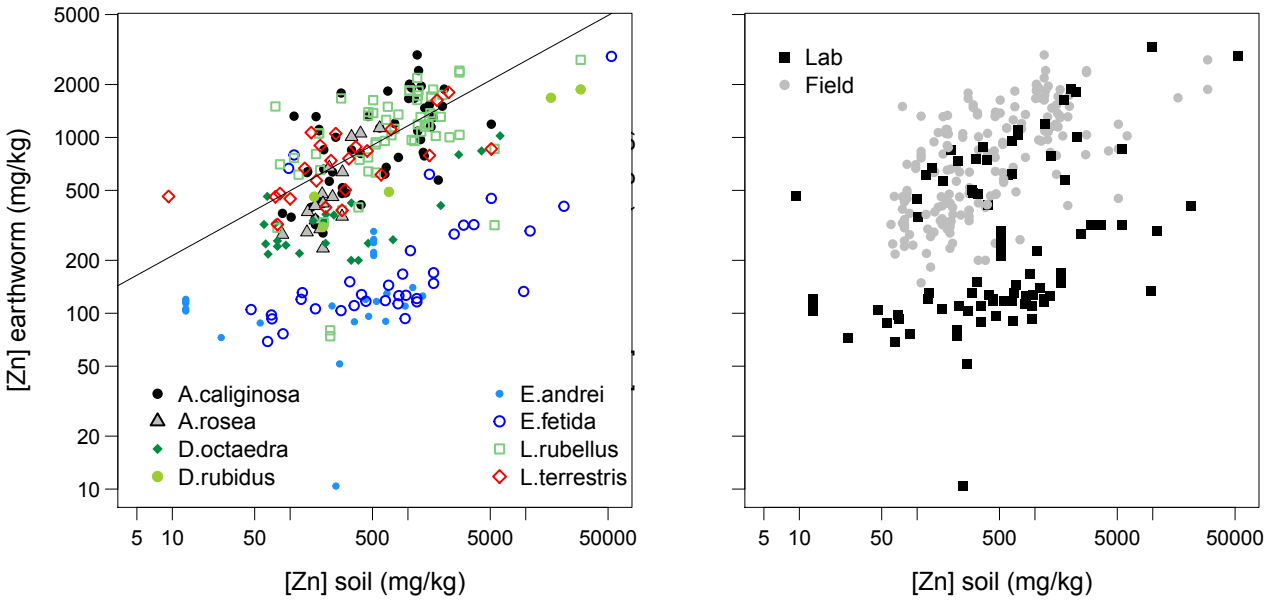


Figure 2.4: **(Left) Zn concentration in 8 earthworm species according to total concentration in soil reported by 27 studies** (on logarithmic scales). Black line is the slope for *A. caliginosa* (ANCOVA model). **(Right) Zn concentration in 21 earthworm species as function of Zn concentration in soil according to laboratory and field experiment (39 studies)**. Lines are the predicted slopes for laboratory exposures (black) and field studies (gray)

2.2.3 Conclusions

In the literature, studies that compared metal bioaccumulation in earthworm species addressed mostly the differences between internal concentrations, and not between the patterns of accumulation (slopes of the relationship between internal and soil concentrations) (e.g. Suthar et al., 2008). The present results are in agreement with Spurgeon et al. (2000). They have shown differences between internal Zn concentrations for 4 earthworm species (intercepts), but comparable rate of increase with total soil Zn content (slopes). The analysis of the literature showed that *A. caliginosa* do not exhibit specific patterns of accumulation of Cd, Pb and Zn compared to other earthworm species commonly found in the field, and even species from other ecomorphological groups and taxonomically far removed (e.g. *L. terrestris*). However, we found distinct patterns from *E. andrei* in the case of Cd and Zn. The results therefore call for vigilance when using this species to study metal bioavailability to earthworms. This analysis therefore strengthens our choice of *A. caliginosa* for the purpose of our study.

Publications considered in the analysis 2.2	
Abdul Rida et al. 1995	Leveque et al. 2013
Andre et al. 2010	Lourenco et al. 2011
Antunes et al. 2008	Lukkari et al. 2004
Asensio et al. 2013	Lukkari et al. 2004b
Becquer et al. 2005	Morgan, Morris 1982
Bernard et al. 2010	Morgan et al. 1998
Berthelot et al. 2008	Morgan et al. 1999
Currie et al. 2005	Morgan et al. 2002
Dai et al. 2004	Nahmani et al. 2007
Demuynck et al. 2007	Nahmani et al. 2009
Galay Burgos et al. 2005	Nannoni et al. 2011
Gaw et al. 2012	Simonsen, Scott-Fordsmand 2004
Hankard et al. 2004	Smith et al. 2010
Hobbelen et al. 2006	Spurgeon et al. 2000
Holmstrup et al. 2011	Suthar et al. 2008
Homa et al. 2003	Svendsen et al. 1997
Ireland, Richards 1977	van Straalen et al. 2001
Langdon et al. 2001	Wang et al. 2009
Langdon et al. 2005	Laszczya et al. 2004
Lee et al. 2009	

2.3 Selection of the series of field-contaminated soils

In order to test the SEM, it was important to have large variations in metal availability (the exogenous latent variable in the model). For the laboratory experiment, we selected the soils in order to have joint variations in both soil metal total concentrations and soil physico-chemical characteristics that influence metal speciation (mainly pH, CEC, soil texture, SOM content). The choice of the studied soils was therefore determinant in our study. We describe in this section the steps and criteria that lead us to the final set of 31 soils.

2.3.1 Criteria

Based on the data acquired for different sites well-known and characterized by our laboratory (projects Ademe, ANR, regional, for example: Bio-indicateurs, Resacor, Epandagri, PhD theses: Nahmani, Fernandez), we constituted gradients of contamination by the metals of interest. We focused on moderately contaminated soils, therefore the soil metal concentrations were far from lethal concentrations reported for *A. caliginosa* (Cd: 540 ppm (Spurgeon et al., 2000), Pb: 2700 ppm (Langdon et al., 2005), Zn: 2000 ppm (Nahmani and Lavelle, 2002)). Our objective was not to study metal toxicity, but bioavailability. Several sites were excluded because contaminated by other pollutants in high quantities (e.g. site Auzon, project Bio-indicateurs).

Soil occupancy was taken into account and only arable plots (crops and pastures) were included. Given the importance of pH, CEC, SOM and clay content in determining metal availability, we selected the soils in order to maximize their ranges. During the process of selection, an effort was made to decorrelate total metal concentrations and pH, CEC, SOM and clay content in order to avoid excessive unbalanced experiment. Figure 2.5 shows for example the relationships between total Cd content and the different physico-chemical properties in the final set of soils. There were no strong correlations between those parameters.

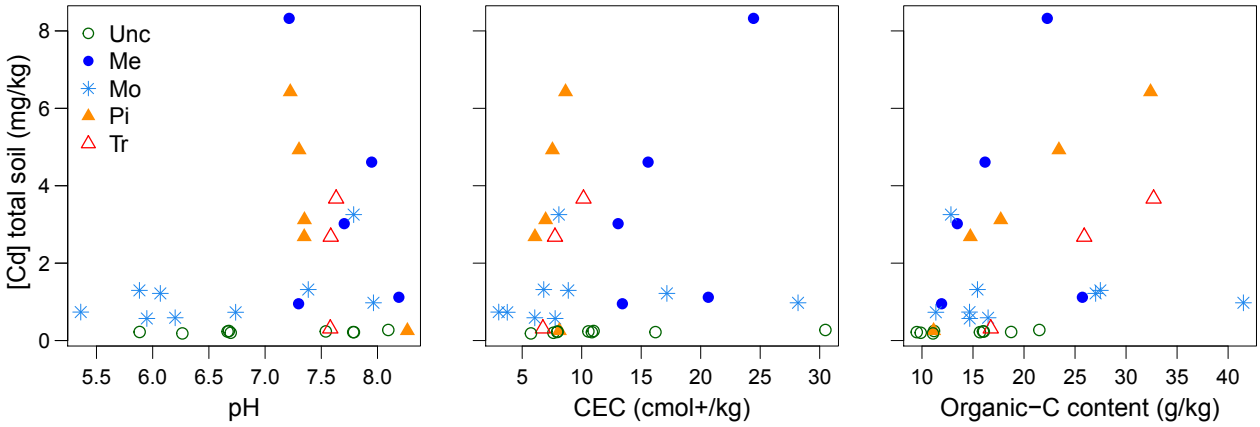


Figure 2.5: Total soil Cd concentration and physico-chemical characteristics of the 31 studied soils. Uncontaminated soils (Unc) are represented in green. Soils from wastewater-contaminated sites are plotted in red/orange: Pierrelaye (Pi) and Triel-sur-Seine (Tr). Soils from smelter-contaminated sites are plotted in blue: Metaleurop (Me) Mortagne-du-Nord (Mo).

2.3.2 Calculation of metal concentration in the soil solution

To definitely make the choice of the soil samples, we further calculated Cd, Pb and Zn concentrations in the soil solution using a semi-mechanistic model that take into account SOM, pH and soil total metal content (Sauvé et al., 2000). The following equations were used:

$$Cd = -0.47 \cdot pH + 1.08 \cdot log_{10}(total) - 0.81 \cdot log_{10}(SOM) + 3.42$$

$$Pb = -0.37 \cdot pH + 0.56 \cdot log_{10}(total) + 1.81$$

$$Zn = -0.55 \cdot pH + 0.94 \cdot log_{10}(total) - 0.34 \cdot log_{10}(SOM) + 3.68$$

where Cd, Pb and Zn indicate logarithm of dissolved metal concentrations in μg/L, total indicate the soil total metal content (in mg/kg) and SOM indicates soil organic matter content (in % of C)

The gradients of metal concentration predicted by the models with the available data from our databases were examined in order to detect potential gaps in the gradient. This

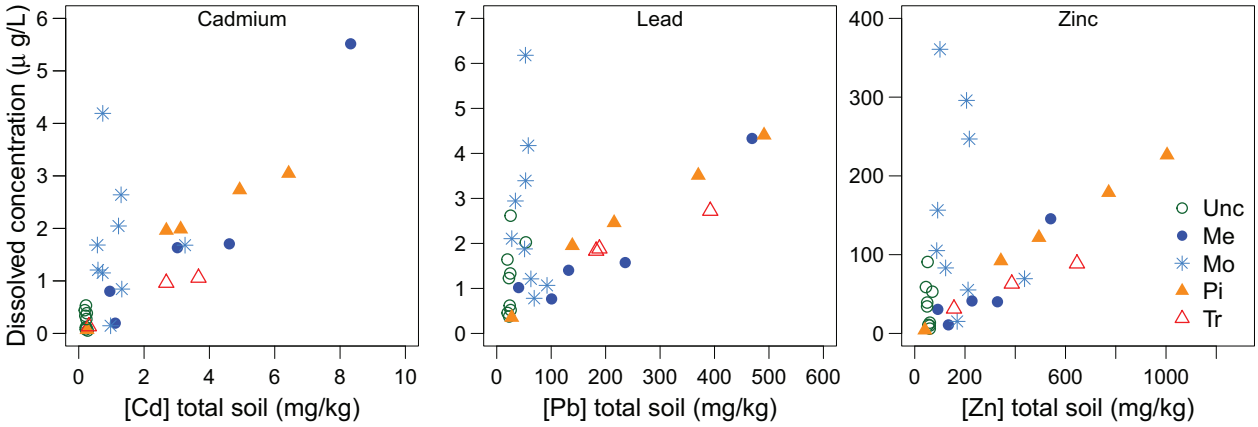


Figure 2.6: Relationship between dissolved Cd, Pb and Zn concentrations (Sauvé et al. 2000) and soil total metal concentrations.

lead us to search for several soils presenting particular characteristics. For example, at this step, two soils from the site Bannost (project NatureParif) were included to increase the number of uncontaminated clay soils.

Figure 2.6 shows dissolved metal concentrations according to total soil content (with the final data acquired for the thesis, that were similar to the initial data from our databases). The selected soils exhibit both high dissolved concentrations for high total soil contents (Metaleurop, Pierrelaye) and high dissolved concentrations for moderate total contents (Mortagne). In the final set of soils, total and available metal concentrations are therefore disconnected.

Moreover, we compared our gradient with the one used by Veltman et al. (2007). The authors calculated earthworm metal concentrations with a mechanistic model (OMEGA). They determined dissolved metal concentrations using the same empirical model that we described previously (Sauvé et al., 2000). That study explored a large range of metal availability as measured by this indicator. Our predicted concentrations cover the same ranges of values (Table 2.3). In that study, most of the records were between 0.4 and 5 μ g/L of Cd, between 1 and 5 μ g/L for Pb and between 50 and 500 μ g/L for Zn. In the study of Veltmann there are thus a few number of observations at low dissolved concentrations. Table 2.3 shows the median dissolved metal concentrations in the set of selected soils. Half of the soils presents concentrations below 1 μ g/L for Cd, 2 μ g/L for Pb and below 60 μ g/L for Zn. The selected soils therefore cover the same range of concentrations with a greater number of observations at low concentrations.

Table 2.3: Ranges of soil solution metal concentrations (μg/L) predicted after Sauvé et al. 2000 for the 31 studied soils compared to the ranges found in Veltman et al. 2007

	Cd	Pb	Zn
minimum	0.1	0.4	4.2
mean	0.7	1.6	52.4
median	1.0	1.8	58.9
maximum	5.5	6.2	360.7
minimum Veltman	0.0	0.4	5.0
maximum Veltman	5.0	10.0	500.0

2.3.3 Final set of 31 soils

Site description

Here is a description of the sites and soils studied. It is part of the Material and Methods section of Beaumelle et al. 2014 (Chap.4, p.81). We report this section here for more clarity.

Soils were sampled in four contaminated and four uncontaminated sites located in France. In each site, several soil samples were taken from different plots. All the plots were arable (crops or pastures), had comparable geological origins (mainly wind-deposited silt, and alluvium) and were noncalcareous.

Two of the contaminated sites (Metaleurop (Me) and Mortagne-du-Nord (Mo)) are located in the North of France near former lead and zinc smelters respectively. They were contaminated by atmospheric deposition of dusts (Van Oort et al., 2002; Bernard et al., 2010). At the Metaleurop site, five soil samples were taken from five arable cropping plots, at different distances from the smelter. At the site of Mortagne-du-Nord, nine soil samples were collected from nine different plots presenting various soil texture (from sandy to loamy), and different land use (crops (Mo3, Mo8 and Mo9) or pastures (Mo1, Mo2, Mo4, Mo5, Mo6, Mo7)).

The two other contaminated sites (Pierrelaye (Pi) and Triel-sur-Seine (Tr)) are located in Paris suburbs and were subjected to raw wastewater spreading for 100 years, leading to the accumulation of large amounts of organic matters and metal pollutants (Lamy et al., 2006). At the Pierrelaye site, soil samples were collected from two arable cropping plots : an unpolluted plot located outside the wastewater irrigated area for which one sample was carried out (Pi1), and a contaminated plot within which four soil samples were collected along a gradient of contamination (Pi2, Pi3, Pi4, Pi5). At the site of Triel, three soil samples were taken from three plots with varying total metal concentrations and land use : one arable

cropping (Tr1), one fallow (Tr2), and one grassland (Tr3).

The four uncontaminated sites had no history of metal contamination. One site named Qualiagro-Feucherolles (Fe) is located 50 km west from Paris on a loamy soil cultivated with a maize/wheat rotation (Houot et al., 2002). The Yvetot site (Yv) is located in Haute Normandie region on a loamy plateau (Hedde et al., 2013). The Bannost site (Ba) is located 30 km south east from Paris on clay loamy soils (Pelosi et al., 2013a). The Closeaux site (Cl) is in the south west Paris suburbs (Versailles) on a silty loam soil. From these sites, a total of nine uncontaminated soil samples was carried out. Three plots receiving different exogenous organic matters (co-compost of green waste and sludge (Fe2), farmyard manure (Fe3) and no amendment (Fe1)) were sampled from Fe site. At the Yvetot site, three soil samples presenting different land use were selected : one arable cropping (Yv1), one permanent pasture established in 1968 (Yv2), and one crop/pasture rotation (Yv3) under crop at the time of the sampling. Two soil samples were collected from two different organic farming plots at the Bannost site. They presented different clay and org-C contents. One sample was carried out from a meadow plot in the Closeaux site. It was considered as our control since the earthworms used in our study were sampled from this plot.

2.4 Choice of experimental conditions of the earthworm exposure

The exposure experiment of *A. caliginosa* to 31 contaminated and uncontaminated soils was conducted to quantify metal bioaccumulation, compartmentalization, and to assess several biomarkers associated to metal contamination (gene expression, enzymatic activities and energy reserves). Experimental conditions in previous studies vary according to the objectives, notably concerning the duration of the exposure and the feeding the earthworms during the experiment. In this thesis, the choice of experimental conditions was based on the analysis of the literature and on preliminary experiment conducted in our laboratory. The choices were further guided by our objectives.

2.4.1 Exposure duration and feeding

The exposure durations the most reported in the literature varied from 14 to 42 days (Conder et al., 2002; Li et al., 2009; Van Gestel et al., 2009; Spurgeon et al., 2004; Nahmani et al., 2007b). The different biomarkers measured in our study can be influenced by a lack of food

if the duration of the exposure is too long. To avoid this phenomenon, a number of studies added food to the experimental devices during the exposure of earthworms (Spurgeon et al., 2004; Ma, 2005). However, adding organic matter in the soil can modify metal speciation and therefore their bioavailability. One of the major goals of this thesis being to relate metal speciation with metal absorption by earthworms, we excluded this procedure.

A 28 days duration was proposed as adequate to assess bioaccumulation of Cd and Pb, and of 40 days for Zn (Nahmani et al., 2007a). Preliminary experiments in our laboratory showed that there was no bioaccumulation of Cd and Zn after 10 days exposure in *A. caliginosa* exposed to field-contaminated soils (Me, Pi sites) (Internships in our laboratory: Rodriguez, 2011; Jarzabeck, 2012). In order to both maximize bioaccumulation and control the influence of diminished food resources on biomarkers (notably energy reserves), we chose an exposure duration of 21 days. Preliminary works showed that *A. caliginosa* was adequately maintained at 12 °C after 21 days without renewing the soil nor adding horse mannure (empirical observations: active individuals, no return to juvenile state, Thénard pers.comm.).

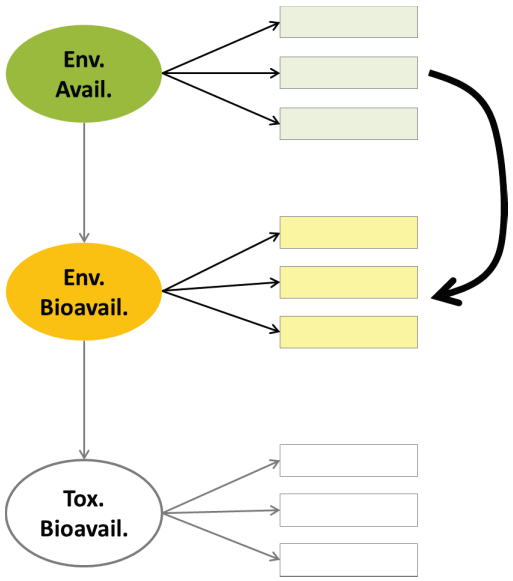
2.4.2 Temperature and moisture

Temperature and moisture level have a strong influence over earthworm physiology, behavior and metal exposure (Eriksen-Hamel and Whalen, 2006). In the literature, various temperatures were used in earthworm exposure to metal contaminated soils (between 10 and 25 °C (e.g. Van Gestel et al., 2009; Steenbergen et al., 2005; Li et al., 2010)). For *Eisenia fetida*, a temperature between 20 and 25 °C is usually used because this compost worm is more active at such temperature. For *A. caliginosa*, 15 °C is the temperature most frequently used (Morgan and Morgan, 1999, e.g.). At the site of origin of the earthworms used in this thesis, the surface soil temperature was monitored once a month during one year from march 2011 to april 2012 (Cheviron pers.comm.). The temperautre at 10 cm depth varied from 0 °C february 2012 to 17.6 °C in june 2011 with a mean of 9.9 °C. In our experiment, earthworms were exposed at 12 °C, a good compromise between the local field reality and what is commonly used in the literature.

Concerning soil water content, most studies used between 60 and 70% of the water holding capacity (WHC) (Smith et al., 2010; Spurgeon and Hopkin, 1996b; Bernard et al., 2010; Spurgeon et al., 2000; Nahmani et al., 2009). Such values are close to the 68% mean WHC observed in the monitoring described above. For our experiment, earthworms were therefore exposed at 60% WHC (which is the most used in the literature).

Preliminary experiments at our laboratory validated those experimental conditions for *A. caliginosa* (healthy and active individuals, Thénard pers.comm.)

Chapter 3



The SEM framework led us to conduct a laboratory exposure experiment that covered a wide panel of soils. It was thus possible to explore the relationships between metal availability and earthworm response over a range of field-contaminated soils. Before going into the SE model, we first investigated the relationships between the manifest variables selected in the conceptual model. In the following chapter, we used the wide panel of soils to determine whether indicators of environmental availability and of environmental bioavailability were individually correlated. The results are presented as a research paper in which the detailed description of soil analyses and biological measurement selected in Chapter 2 can be found in the material and method section.

Chapter 3

Changes in the subcellular partitioning of metals in *Aporrectodea caliginosa* along a gradient of metal exposure in 31 field-contaminated soils [1]

[1] Léa Beaumelle, Frédéric Gimbert, Mickaël Hedde, Annie Guérin, Isabelle Lamy. Subcellular partitioning of metals in *Aporrectodea caliginosa* along a gradient of metal exposure in 31 field-contaminated soils *Science of the Total Environment*, (submitted)

Abstract

Subcellular fractionation of metals in organisms was proposed as a better way to characterize metal bioaccumulation. Here we report the impact of a laboratory exposure to a wide range of field-metal contaminated soils on the subcellular partitioning of metals in *Aporrectodea caliginosa*. Field-contaminated soils were chosen to create a gradient of soil metal availability; covering ranges of both soil metal contents and of several soil parameters. Following exposure, Cd, Pb and Zn concentrations were determined in three subcellular compartments: cytosolic, granular and debris fraction. Three distinct proxies of metal availability were investigated: $CaCl_2$-extractable content, dissolved content predicted by a semi-mechanistic model and free ion concentration predicted by a geochemical speciation model. Subcellular partitionings of Cd and Pb were modified along the gradient of metal exposure, while stable Zn partitioning reflected regulation processes. The subcellular distribution of Cd responded more strongly to increasing soil Cd concentrations than the total internal content, when Pb subcellular distribution and total internal content were similarly affected. Free ion concentrations were better descriptors of Cd and Pb subcellular distribution than $CaCl_2$ extractable and dissolved metal concentrations. However, free ion concentrations and soil total metal contents were equivalent descriptors of the subcellular partitioning of Cd and Pb because they were highly correlated. Our results do not support the added value of the three proxies of metal availability compared to soil total metal content in the assessment of metal bioavailability to earthworm following exposure to chronically contaminated soils.

3.1 Introduction

Elucidate the ways edaphic organisms are exposed to and bioaccumulate trace metals is crucial for ecological risk assessment. One of the current scientific challenges is still the understanding of the relationships between external(soil) and internal (organism) metal contents. In the case of earthworms which are extensively used as indicators of soil quality (Pérès et al., 2011), contrasting results were obtained as to which chemical method better assess soil metal pools that can be uptake by earthworms.

Indeed, if internal metal concentration reflects bioaccumulation, the relationships with chemical availability (i.e. soil metal supply) as well as with toxicological bioavailability (i.e. effects on earthworms) are complex (Luoma and Rainbow, 2005). Instead of total body loads, internal compartmentalization of metals in different subcellular fractions allows for a better understanding of metal accumulation in organisms (Wallace et al., 2003; Vijver et al., 2004). Subcellular fractionation is a procedure that separates the total metal body content into operationally defined subcellular compartments following differential centrifugations (Wallace et al., 2003). This procedure was used in terrestrial invertebrates to isolate at least three fractions: metal-rich granules, cytosolic fraction and a fraction consisting of cell membranes, tissue fragments and intact cells (called debris hereafter) (Vijver et al., 2007). The toxic effects of metals on earthworms were suggested to be linked with specific internal pools of metals assumed to be biologically active (Lanno et al., 2004; Vijver et al., 2004). Changes in the subcellular partitioning of metals could thus be interesting indicators of metal bioavailability for earthworms, notably of toxicological bioavailability. As such, they could provide better endpoints than total internal contents to identify pertinent chemical proxies of metal availability to earthworms. The dose-effect relationship between soil metal availability and subcellular partitioning in earthworms was, however, rarely addressed (Vijver et al., 2007). One of the reasons for this could be that the definition of "what is available" for soil-dwelling organisms is not consensual. Earthworms are believed to be exposed mainly to metals from the soil solution (Saxe et al., 2001; Vijver et al., 2003). But the fact that metal accumulation in earthworms was often better correlated to total soil metal content than to chemical availability measurements suggests they are exposed to other pools of metals in soil, like metals bound to organic matters that are ingested.

Several types of extractions are used to assess soil metal availability for living organisms. Chemical extractions quantify operational metal pools in soils. For example, $CaCl_2$ is supposed to extract both dissolved and exchangeable pools of metals (Alloway, 1995). Chemical extractions are extensively used to assess indirectly metal bioavailability for a number of

soil organisms. Besides these operational quantifications, modeling can be used to predict the partitioning of metals in soils. Empirical or semi-mechanistic models describe the solid-solution partitioning based on few input data (total soil metal content, pH and soil organic matter content) (Sauvé et al., 2000). Their output data, i.e. the dissolved soil solution metal concentrations, can be used to predict bioaccumulation in earthworms (Veltman et al., 2007). Geochemical models estimate a theoretical metal speciation provided that all ligands and their corresponding stability constants with metals are known. These models predict rather well Cd, Cu and Zn cations speciation in soils (e.g. Weng et al., 2001; Dijkstra et al., 2004). Pb theoretical speciation was demonstrated to be less accurate, but binding to Mn oxides was often neglected in the calculation (e.g. Groenenberg, 2011), despite their strong binding affinity for Pb (Cancès et al., 2003). Even though geochemical speciation models require extensive input data, they are valuable because the identified metal pools in the soil can be calculated on a mechanistic basis, and notably the free ion concentration in soil solution assumed to impact soil organisms (Steenbergen et al., 2005; Qiu et al., 2014). A number of studies attempted to identify the most pertinent method that predicts bioavailability to earthworm, but no particular assessment was yet shown fully generic.

In this context, the aim of this study was to unravel the relationships between soil metal availability and the subcellular partitioning of metals in earthworms. Because soils are often chronically contaminated with low levels of bioavailable metals, we investigated the robustness of these relationships over a wide panel of field-contaminated soils after a laboratory exposure. Soils were chosen to cover ranges of total metal concentrations as well as soil parameters in order to constitute gradients of metal availability. We tested the three following assumptions (i) metal distribution in subcellular compartments is more consistently related to soil metal availability than total internal earthworm concentrations, (ii) available metals for earthworms are present in the soil solution and are readily extractable, and (iii) geochemical speciation modeling provides a better proxy of metal availability than experimental extractions ($CaCl_2$) and semi-mechanistic calculations (Sauvé et al., 2000).

3.2 Material and Methods

3.2.1 Exposure experiment

Soils

Thirty one soil samples were chosen to achieve a wide gradient of metal availability as described in Chapter 4. Soils were sampled in four contaminated sites, namely Metaleurop (Me) and Mortagne-du-Nord (Mo) (smelter-contaminated), Pierrelaye (Pi) and Triel-sur-Seine (Tr) (wastewater-contaminated), and four uncontaminated sites: Feucherolles (Fe), Yvetot (Yv), Bannost (Ba) Closeaux (Cl) all located in Northern France. Soil samples were collected from March to May 2012. Each sample was a composite of five sub-samples of the top-soil (0-20 cm depth), taken from an area of about 1 m^2. Soil samples were hand sorted to remove soil fauna and plant debris, air-dried, sieved at 2 mm. Table 3.1 gives the ranges of selected soil parameters and Cd, Pb and Zn total contents of the soil samples (detailed information can be found in Chapter 4, p.86).

Exposure experiment

Adult earthworms (*Aporrectodea caliginosa* (Savigny 1826)) were hand sorted from an uncontaminated meadow (Cl site, Versailles, France). They were exposed in microcosms to the 31 soil samples during 21 days. For each of the 31 soil samples, five microcosms were conducted. Each microcosm (1 L glass jar) contained 600 g of air-dried sieved soil moistened to 60% of its water holding capacity. Six earthworms were randomly attributed to each microcosm after 48h voiding on moistened filter paper. Microcosms were placed in the dark at 12 °C for 21 days. No food was added to the soil in order to not disturb initial metal availability. At the end of the exposure, earthworms were removed from the microcosms, depurated for 48 h, weighted and frozen at -80 °C until analysis.

3.2.2 Subcellular fractionation and metal quantification in earthworms

Total internal metal concentrations were quantified on one randomly selected earthworm of each microcosm. Frozen earthworms were freeze-dried for 24 h and their dry weights were recorded. They were individually digested in a concentrated HNO_3 solution (normapur;

Table 3.1: Ranges and median values of soil characteristics and total metal contents over the 31 studied soils

	units	minimum	maximum	median
Clay	g/kg soil	54	476	123
pH		5.4	8.3	7.3
CEC	cmol+/kg soil	3.0	30.5	8.1
organic-C	g/kg	9.5	41.5	16.1
total Cd	mg/kg	0.18	8.32	0.95
total Pb	mg/kg	19.6	491.0	53.5
total Zn	mg/kg	40	1004	134

VWR-Prolabo) in a microwave system (CEM, MarsXpress, Matthews, NC, USA). The digests were then diluted to 25 mL with ultrapure water (miliQ water, Milipore, 18 Mohm). Subcellular fractionation was performed on one randomly selected earthworm of each microcosm. The procedure was performed according to Wallace et al. (2003) and Gimbert et al., 2008. Briefly, frozen earthworms were individually homogenized with an ultraTurrax (IKA T10) in 5 mL of 0.01 M Tris-HCl, pH 7.5 buffer (Sigma-Aldrich). An aliquot of 1 mL of the homogenate was used to quantify the total initial metal concentrations before fractionation. Homogenates were centrifuged at 10 000 g for 30 min at 4 °C. The pellet fractions were put in a 100 °C water bath for 2 minutes, afterwards 4 mL of 0.5 M NaOH (Merck, Darmstadt, Germany) were added and the fractions were hydrolyzed for 1 h at 70 °C. After a centrifugation at 10 000 g for 10 min at 20 °C, the supernatants (debris) were separated from the pellets (granules). Pellets were resuspended in 0.5 mL of HNO_3. Blanks were conducted using 5 mL buffer to insure the absence of contamination during the homogenization and fractionation procedures. All fractions were stored at -20 °C before being digested with 3.5 mL of HNO_3 in a final volume of 50 mL adjusted with ultrapure water. Metal quantification in total earthworms and subcellular fractions was performed by inductively coupled plasma mass spectroscopy by the 'Laboratoire d'Analyse des Sols' (INRA, Arras, France) applying standardized methods and quality assurance procedures. The reliability of the analysis was assessed with blanks, spiked solutions and standard reference materials (TORT-2, lobster hepatopancreas, and DOLT-4, dogfish liver [National Research Council of Canada, Ottawa, ON]). Measured concentrations in reference materials were within 20% of the certified values. Furthermore we excluded the data for individuals that were visually assessed full of soil at the weighing step. It concerned 13 and 7 individuals respectively for total internal concentrations and subcellular fractions contents.

Table 3.2: Selected soil parameters used to calculate Cd, Pb and Zn speciation in 31 soils. Soils are ordered from top to bottom according to sampling sites and from lowest to highest contamination levels. FA: fulvic acid, HA: humic acid, DOC: dissolved organic carbon, SUVA: specific UV-absorbance

	pH	FA/HA	DOC (mg/L)	SUVA (l/g/cm)	Ferrihydrite (g/kg)	Goethite (g/kg)	MnO_2 (g/kg)
Cl	6.68	0.75	30.06	28.70	5.26	2.77	0.37
Yv1	6.26	0.91	29.92	21.90	6.93	4.4	0.95
Yv2	5.88	0.83	56.72	18.20			
Yv3	6.67	0.99	48.64	20.70			
Fe1	6.69	0.75	25.40	26.70	5.57	4.02	0.52
Fe2	7.79	1.25	39.54	35.20			
Fe3	7.54	0.56	42.11	31.20			
Ba1	7.78	0.70	16.79	29.90	3.49	9.39	0.7
Ba2	8.10	1.34	36.64	22.30			
Mo1	5.95	1.07	42.95	22.00	4.2	2.32	0.45
Mo2	6.20	0.60	37.27	25.60			
Mo3	6.74	0.54	21.12	34.00			
Mo4	5.36	0.74	28.68	27.00			
Mo5	7.96	1.02	69.13	16.60			
Mo6	6.07	0.61	71.23	25.90			
Mo7	5.88	0.74	45.55	22.00			
Mo8	7.38	0.36	25.33	31.50			
Mo9	7.79	0.48	17.74	32.00			
Me1	7.30	0.45	25.57	29.10	2.9	2.75	0.53
Me2	8.19	0.67	25.90	26.60			
Me3	7.70	1.02	26.48	34.40			
Me4	7.95	0.47	25.79	25.80			
Me5	7.21	1.18	29.28	29.50			
Tr1	7.58	0.42	17.60	37.90	3.37	2.94	0.39
Tr2	7.58	0.52	17.65	33.60			
Tr3	7.63	0.32	19.33	34.80			
Pi1	8.27	0.52	18.31	38.50	4.29	1.84	0.18
Pi2	7.35	0.96	15.74	37.90			
Pi3	7.35	1.10	22.54	32.30			
Pi4	7.30	0.45	19.93	32.50			
Pi5	7.22	0.74	22.20	28.50			

3.2.3 Soil metal availability determinations

On each of the 31 soil samples, soil metal availability was assessed following three ways, so that the available pools of metals were assimilated to:

1. exchangeable pools of metals with a neutral salt, quantified using a chemical extraction with 0.01 M $CaCl_2$ (w:v of 1:10) carried out according to Houba et al., 1990;

2. dissolved metal species in solution using the semi-mechanistic equations proposed by Sauvé et al., 2000. The metal-specific models were used to calculate total metal contents in soil solution using soil pH, soil organic matter (SOM) contents and total soil

metal concentrations as explaining variables;

3. free metal species in solution estimated by geochemical speciation modeling using the software package Visual Minteq 3.0 Gustafsson (2011). Parametric background was made for each of the 31 soil samples in order to account for their differences in soil nature and constituents. Input data included total reactive metal contents, expressed as 0.05 M EDTA extractable metal concentrations (Bonten et al., 2008) soil solution composition (after batch-solution extractions in a 1:2 w:v ratio with water), and concentration of organic and mineral components. Clay was not included in the model as its contribution to metal speciation could be neglected (Cancès et al., 2003). Two solid surfaces were considered: SOM and metal (hydr)oxides, either amorphous and/or crystalline. Amorphous iron oxides were modeled as hydrous ferric oxide (HFO) using the two site diffuse double layer of Dzombak and Morel, 1990 with a specific surface area of 600 m^2/g (Dzombak and Morel, 1990; Dijkstra et al., 2004), and crystalline iron oxides were modeled as goethite using the Charge Distributed Multi-Site Complexation model (CD-MUSIC, Hiemstra and Van Riemsdijk, 1999) with the model parameters reported by Weng et al. (2001) and a specific surface area set to 100 m^2/g (Weng et al., 2001; Dijkstra et al., 2004). The amount of goethite was estimated as the difference between Fe extractable contents in the two following extractions: dithionite-citrate-bicarbonate (DCB) and oxalate (see below), applying a conversion factor of 1.6 (Lumsdon, 2004). For the amorphous iron oxides we used the Fe oxalate extractable content and the conversion factor of 1.7 (Lumsdon, 2004). Metal binding to hydrous Mn oxides (HMO) was calculated using the two site diffuse double layer model with a specific surface of 200 m^2/g as proposed by (Tonkin et al., 2004). Similarly to iron oxides, the Mn contents in oxalate extractions were used to calculate the content in manganese oxides using a factor of 1.6 (Amery et al., 2008). NICA-Donnan model was used to describe metal ion binding to both solid and dissolved organic matter (DOM) (Kinniburgh et al., 1999). The carbon content of organic matter was set to 50% (Pribyl, 2010). For organic matter in the solid phase, reactive SOM was calculated as 50% of total SOM (Cancès et al., 2003; Schröder et al., 2005). In order to take into account the various nature of the organic matters in the series of 31 soils, reactive SOM was further described as a mixture of humic and fulvic acids (HA and FA) using the measured FA/HA ratio (see below). The recommended model parameters for FA and HA were used (Milne et al., 2001; Milne et al., 2003). For organic matter in the liquid phase, the simulation of the various nature of DOM in the soil series was made taking into account the aromaticity of dissolved organic carbon (DOC). The percentage of reactive DOM (ascribed to fulvic acids) was calculated as a function of DOM with a "specific UV-absorbance" SUVA-index determined for each soil solutions as described in Amery et al.,

2008 and Cornu et al., 2011. Table 3.2 reports the input data used for organic and mineral components in the geochemical model.

We verified that the mean charge balance indicated no or little deviation from electroneutrality for most of the soils (median charge balance: 8%). The calculated charge balance was above 20% for only 4 out of 31 soil samples. For two soil samples, this difference was above 30% (namely Cl and Mo9 soil samples). The results were still analyzed and the influence these two soils had in the results was carefully accounted for. We verified that the predicted dissolved and exchangeable concentrations were consistent with the observed $CaCl_2$ extractable metal concentrations (see supplementary information).

Soil analysis

Soil particle size determination was carried out according to NF X31-107, 2003, soil pH determination according to NF ISO 10390, 2005 and soil cation exchange capacity (CEC) according to NF X31-130, 1999. Total organic C and N assessments were performed according to NF ISO 10694, 1995 and NF ISO 13878, 1998. Total metal contents were determined according to NF X31-147, 1996 (tri-acid HF - HCl - HNO_3 digestion). FA and HA contents were determined after extraction from the bulk soil samples using the procedure of Holtzclaw et al., 1976 slightly modified. Each air-dried sieved (2 mm) soil sample was first extracted with 0.5 M NaOH during 16 h using a soil:solution ratio of 1:4 (w:v). The mixture was centrifuged at 17 000 g for 10 min to separate the extracted soluble humic substances (HS) from the solid matrix. The HS were then acidified to pH 1 with 6 M HCl, left standing 3 h to allow complete precipitation of HA, and centrifuged at 17 000 g for 10 min to isolate the FA in the supernatant from the HA in the pellets. Soluble organic C was then monitored in a sub-sample of HS and in FA solutions by high temperature catalytic combustion followed by CO_2 measurement (Shimadzu TOC-VCSN). The FA/HA ratio was determined using HA concentration calculated as the difference between HS and FA organic C concentrations. Dithionite-citrate-bicarbonate (DCB) extraction of Fe was performed according to Mehra, Jackson, et al., 1960. Oxalate extractions of Fe and Mn were performed according to Schwertmann, 1964. Fe and Mn were quantified in the solutions by flame atomic absorption spectrophotometry (AAS) (Varian SpectrAA 220). Soil solution was extracted with water in a mass soil:water ratio of 1:2, after a 30 min shake and centrifugation for 10 min at 17 000 g. pH was measured on the supernatants using a calomel combined electrode (Hach Lange). Dissolved inorganic carbon (DIC) and total carbon were quantified using a TOC analyzer (Shimadzu TOC-VCSN). Cations (Ca^{2+}, Mg^{2+}, Na^+, K^+, NH_4^+) were quantified using flame AAS (Varian SpectrAA 220) and anions (Cl^-, NO_3^-, SO_4^{2-}, and HPO_4^{2-}) using capillary elec-

trophoresis (Beckman Coulter, P/ACE^{TM} MDQ) after filtration of the supernatants through 0.2 µm cellulose membrane.

3.2.4 Data Analysis

We used linear mixed effect models to test the effect of soil metal contamination and availability on metal concentrations and proportions in each subcellular fraction. Soil contamination or availability were considered as fixed effects. Soil was modeled as a random effect on the intercept in order to take into account the fact that the 5 earthworms exposed to the same soil would exhibit more similarity than with other earthworms. The independent variables were non-binomial percentages and were log-transformed when necessary (when model residuals were not normally distributed on a qq plot). Metal concentrations (total and available) were log-transformed to linearize the relationships. The significance was tested with Likelihood ratio (LR) tests comparing a null model with the random effect to a full model with the random effect and the metal concentration. For the LR test, models were estimated with maximum likelihood (ML) instead of restricted maximum likelihood (REML) estimation used to estimate the coefficients (Bates et al., 2012; Bolker et al., 2009). The models were compared based on Akaike information criterion (AIC).

Statistical analysis were performed using R software version 3.1.0 (R Core Team, 2014), and 'lme4' package (Bates et al., 2012).

3.3 Results and Discussion

3.3.1 Metal availability

Figure 3.1 shows the three proxies of Cd, Pb and Zn availability and their relationship to soil total concentrations. These three expressions of metal availability exhibited globally the same pattern, i.e. they increased with increasing soil total metal contents (Fig. 3.1). But differences appeared for soils with low metal contents (from Mo site); $CaCl_2$ extractable pools and dissolved concentrations predicted after Sauvé et al., 2000 were independent of total soil contents, and could present higher concentrations than in soils with high total metal contents (Fig. 3.1). This pattern is explained by the low pH of several of these soils (namely Mo1, Mo4, Mo6, Mo7 Table 3.2). The soil Mo4, which exhibited the lowest pH of the dataset exhibited the second highest $CaCl_2$ extractable Pb content, while $CaCl_2$ generally extracted small amounts of Pb in the other soils. $CaCl_2$ extracted small amounts of Pb in several

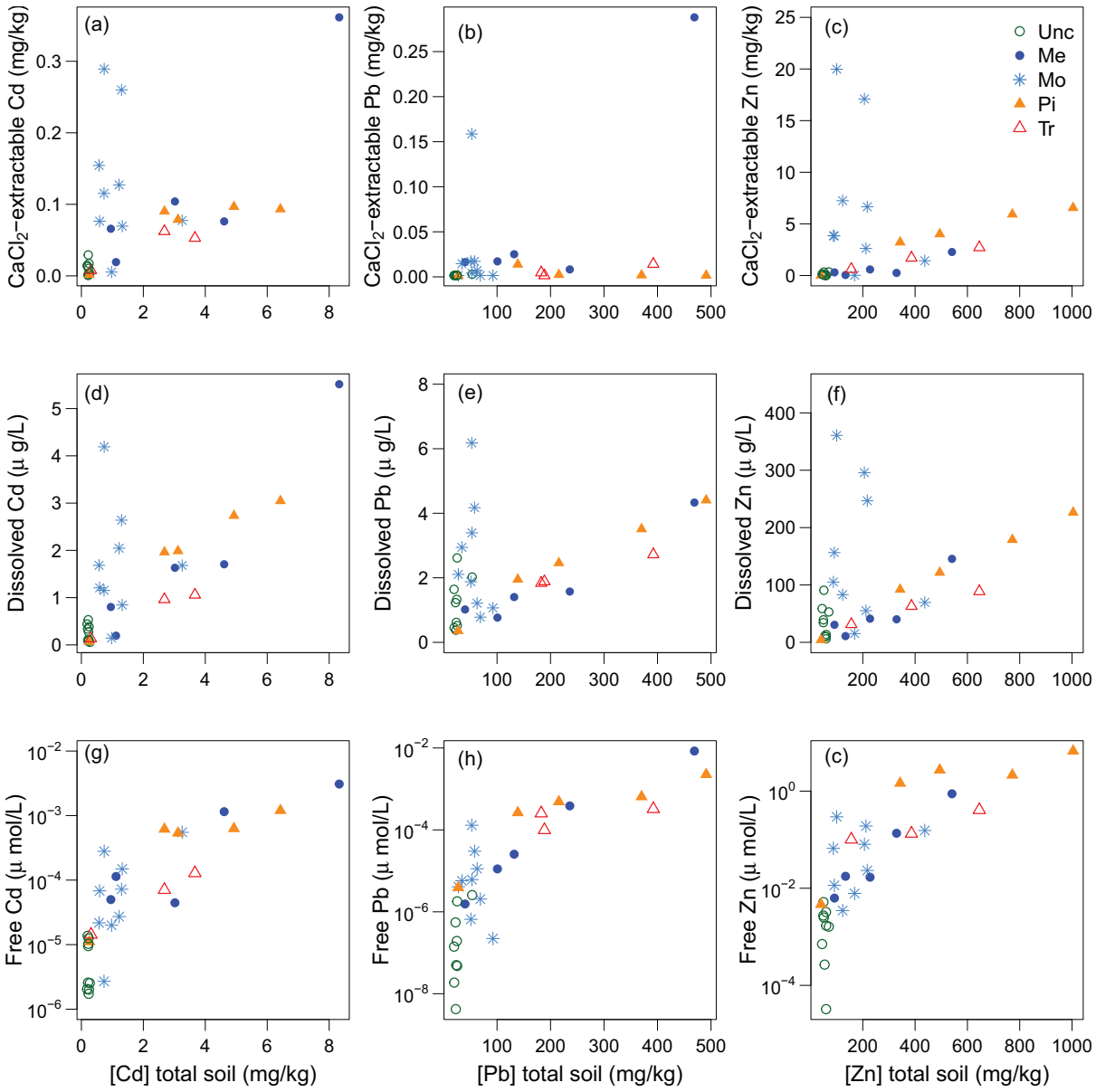


Figure 3.1: Relationships between three measurements of availability and total metal concentrations in soil for Cd (left panels), Pb (center panels) and Zn (right panels). $CaCl_2$ extractable metals (a, b, c), dissolved concentrations were obtained following the equations of Sauvé et al 2000 (c, d, e), and free ions concentrations were determined using a multisurface mechanistic model of speciation (f, g, h, on a log-scale). Symbols differentiate the sites of sampling; Unc: uncontaminated soils (from Cl, Yv, Ba and Fe sites)

wastewater-contaminated soils (Pi4, Pi5 and Tr3). The highest $CaCl_2$ extractable Pb content was found for a soil with the same total metal content but from a smelter-contaminated site

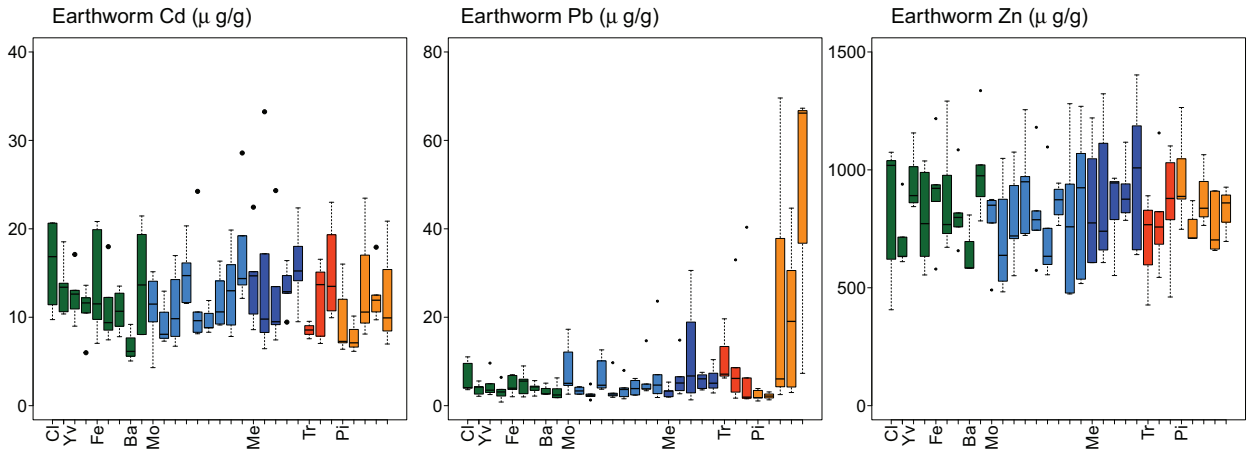


Figure 3.2: Inter-individual variability of total internal metal concentrations in earthworms exposed to the 31 soil samples. Concentrations in μg/g of dry weight. From left to right, data are ordered according to the sampling site and within sites from lowest to highest contamination levels

(Me5). Those differences are certainly related to the organic-rich nature of soils from Pi and Tr sites. It is noteworthy that the calculated dissolved Pb content does not exhibit the same pattern, most likely because SOM is not taken into account in the calculation for Pb (Sauvé et al., 2000).

Ranges of free ion concentrations were found in soils with low total metal contents, notably in soils from Mo site. Nevertheless, free ion concentrations were more strongly correlated to the total soil content than $CaCl_2$ extractable and dissolved concentrations. The partitioning of dissolved metals between free ions, organically bound, and inorganically bound as predicted by geochemical modeling showed that most dissolved metals were bound to DOM (99% of dissolved Cd and Pb and 77% for Zn, on average over the 31 soil samples). Less than 1% of dissolved Cd and Pb were present as free ions, while free Zn represented 22% of all dissolved species. Zn was also present as inorganic-complexed forms (about 1%). The contribution of the different surfaces to the sorption of metals showed that Cd and Zn were mainly adsorbed on SOM, while Pb was mostly bound to Mn oxides. Significant proportions of Pb and Zn were bound to iron oxides, while this was not the case for Cd. More detailed informations can be found in supplementary information.

3.3.2 Internal metal concentrations in earthworms

Metal concentrations in earthworms exposed to the 31 soil samples are shown Figure 3.2. Different patterns were observed according to the metal. Internal concentrations of Zn in earthworms appeared stable from one soil to the other despite the wide gradient of Zn availability (Fig. 3.2). In soils containing less than 150 μg/g of Zn, Becquer et al., 2005; Spurgeon et al., 2000 and Nannoni et al., 2011 reported from 350 to 1320 μg/g of Zn in *A. caliginosa* and we report from 407 to 1337 μg/g for the same range of soil Zn concentrations. The assumption of an active regulation of internal Zn concentration, is thus confirmed by our findings (Spurgeon and Hopkin, 1999).

Internal Cd contents exhibited higher inter-individual variability than inter-soil variability. Figure 3.2 shows elevated individual values (outliers) for earthworms exposed to several smelter-contaminated soils, but no clear pattern could be distinguished. In the literature, internal Cd concentrations in *A. caliginosa* exposed or living in soils containing less than 1 μg/g total Cd ranged from 4 to 36 μg/g consistently with the range reported here (4-24 μg/g) (Nannoni et al., 2011; Gaw et al., 2012; Łaszczyca et al., 2004; Morgan and Morgan, 1998; Morgan and Morgan, 1999). Several studies showed that earthworms can accumulate high amounts of Cd after short term exposure to high soil Cd concentrations (e.g. Marino and Morgan, 1999; Giska et al., 2014). The present results indicate a lower bioaccumulation potential in lowly and chronically contaminated soils (Spurgeon and Hopkin, 1995).

Pb internal concentrations were highly elevated in earthworms exposed to several wastewater-contaminated soils. Earthworms also exhibited higher concentrations after exposure to Mo1 and Mo4, two soils with low soil total Pb content but high $CaCl_2$-extractable Pb. Internal Pb concentrations reported here ranged from 0.8 to 17 μg/g after exposure to soils with less than 80 μg/g total Pb, close to the range found in the literature for the same species: from 0.4 to 12 μg/g (Dai et al., 2004; Becquer et al., 2005; Nannoni et al., 2011; Gaw et al., 2012). The variability of Pb internal concentrations was extremely high in earthworms exposed to Pi soils (e.g. in Pi5: from 7 to 67 μg/g). Earthworms exposed to the same soil reacted very differently to Pb exposure highlighting the individual-specific response of Pb accumulation that can be attributed to differences in biotic factors (e.g. age, biomass, behavior).

3.3.3 Metal distribution in three subcellular fractions

Table 3.3 shows the global partitioning of Cd, Pb and Zn in the three subcellular fractions considered. The detailed concentrations are given in supplementary information. For Cd

Table 3.3: Distribution and concentration of Cd, Pb and Zn in three subcellular fractions (cytosol, debris and granular) in earthworms exposed to 31 soil samples. Values are the mean (standard deviations) of n=144-148 individuals. Distributions: in percentages of initial homogenate concentrations. Concentrations: in μg/g of fresh weight.

	% Cd	% Pb	% Zn	Cd (μg/g)	Pb (μg/g)	Zn (μg/g)
Cytosol	82.3 (9.8)	16.9 (8.7)	25.7 (5.8)	1.09 (0.50)	0.09 (0.07)	18.81 (5.48)
Debris	20.5 (7.7)	62.8 (17.7)	92.1 (17.5)	0.27 (0.13)	0.39 (0.44)	68.37 (19.70)
Granules	2.2 (1.7)	42.7 (24.5)	18.7 (9.0)	0.03 (0.03)	0.41 (0.87)	18.65 (9.01)

and Pb, recoveries of initial (homogenate) concentrations were close to 100% but a higher recovery was found for Zn (on average 105%, 122% and 136% respectively for Cd, Pb and Zn). Recovery of initial concentrations following subcellular fractionation of metals in earthworms are seldom reported. The present results indicate no significant loss of metals during the fractionation procedure.

Cd was mainly retrieved from the cytosolic fraction consistently with previous findings (Li et al., 2008; Vijver et al., 2006). Our results are different from Vijver et al. (2006) who showed equivalent percentages of Cd between debris and granular fractions, while we found higher percentages of Cd in the debris fraction, consistently with Li et al. (2008). Pb partitioning in this experiment was close to what has been reported by Li et al., 2008 (15% Pb in the granular fraction, 40% in the debris fraction and 35% in the cytosolic fraction). Jones et al., 2009 found higher concentrations of Pb in the cytosolic fraction compared to the granular fraction while we found the opposite. In addition, Vijver et al. (2006) showed very small percentages of Pb in the cytosol, while we found significant proportions of Pb in this fraction (on average 16.91% (n=142)). Finally, Zn was mainly found in the debris fraction, with lower percentages in the two other fractions. Vijver et al. (2006) found Zn more evenly distributed between the three fractions.

Overall, the results are consistent with previous reports, with differences that may be explained by (i) the species (*Eisenia fetida* Li et al., 2008), (ii) the site of sampling (earthworms collected from field contaminated soils (Vijver et al., 2006) while our earthworms were field collected from an unpolluted plot) and (iii) by the fact that we calculated metal partitioning based on the concentrations of metals in the homogenate and not by summing the concentrations in the different fractions.

Table 3.4: Relationship between subcellular distribution of Cd and Pb in three subcellular compartments and total soil concentrations. Results of linear mixed effects models: Likelihood ratio tests (χ^2 and p-value associated) and predicted slope and standard error (s.e.) associated

Metal	Internal pool	Transformation	χ^2	p	slope	s.e.
Cd	Total internal	log	3.90	(0.05)	0.05	0.03
Cd	Cytosol	_	12.30	***	-3.14	0.84
Cd	Debris	log	16.60	***	0.17	0.04
Cd	Granules	log	14.80	***	0.25	0.06
Pb	Total internal	log	16.60	***	0.03	0.06
Pb	Cytosol	log	0.10		n.s.	n.s.
Pb	Debris	_	2.50		n.s.	n.s.
Pb	Granules	log	8.90	**	0.17	0.05

3.3.4 Relationship between subcellular partitioning and soil total metal concentrations

We tested if the distribution of metals in the three subcellular compartments changed as a function of soil metal contamination. Table 3.4 gives the results of the statistical models. Zn was considered in the analysis but the results are not reported afterwards. No relationship was found between Zn concentrations in earthworm or in subcellular fractions and soil total Zn contents. Overall, the regulation of internal Zn highlighted previously was also reflected by its stable subcellular partitioning in this study.

Cd body burden increased significantly but weakly with total soil Cd (Table 3.4). This was consistent with the patterns observed on figure 3.2. On the contrary, Cd subcellular distribution was highly and significantly affected by soil total Cd content. The proportion in the cytosol decreased with soil total Cd, while Cd concentration in this fraction was unchanged as related to soil total content (χ^2=0.12, p>0.05). The proportions of internal Cd in the debris and granular fractions increased with total soil content (Table 3.4), and Cd concentrations in these two fractions also increased with the soil content (χ^2=22, p<0.001 , χ^2=17, p<0.001, respectively for Cd concentration in the debris and granular fractions).

Figure 3.3 illustrates the greater variations in the proportions of Cd in the cytosol and debris compared to the variations in the granular fraction (note the different scale). This fraction accounted for only a small portion of Cd body burden (Table 3.3) but higher proportions were found in earthworms exposed to Cd contaminated soils.

The results show that the subcellular partitioning of Cd was more strongly affected by

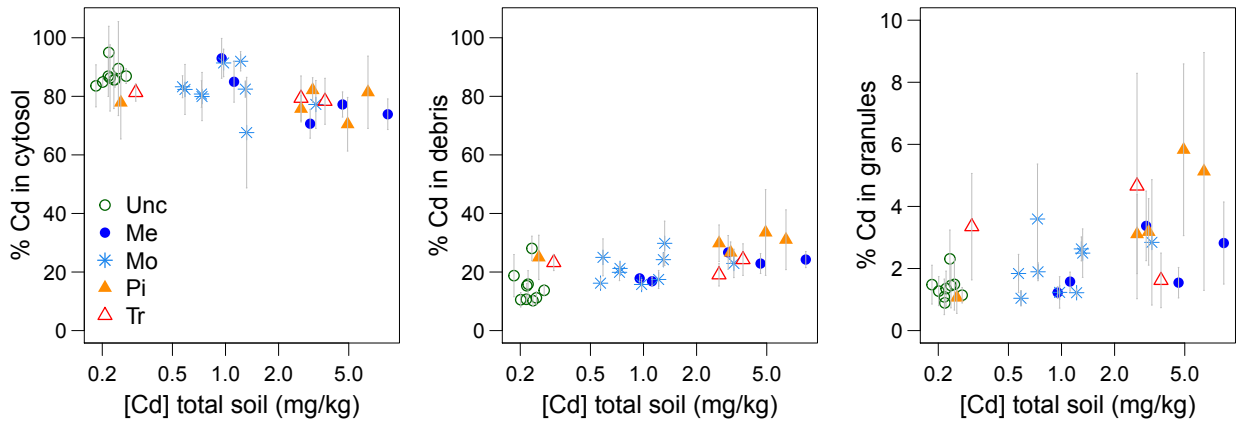


Figure 3.3: Modification of Cd partitioning in three subcellular compartments of earthworms as related to total Cd content in soil. Each point is the mean percentage of initial homogenate concentration over 5 individuals. Error bars indicate standard deviations. Legend: Site codification, Unc: uncontaminated soils (from Cl, Yv, Fe, Ba sites)

Cd exposure than the total body burden. The insoluble (debris+granules) fraction of Cd increased more rapidly than the cytosolic fraction that account for most of internal Cd concentrations (Table 3.3). This certainly explains the weak increase of total internal concentrations with soil Cd. In the literature, Cd in the cytosol was mainly associated to a heat-stable protein fraction containing metallothioneins that chelate Cd (Conder et al., 2002). The fact that Cd concentration in the cytosol was stable indicates that the internal pool of Cd bound to MT proteins was saturated. It is likely that MT proteins were not yet produced in sufficient quantities to chelate all internalized Cd, and that the remaining internalized Cd accumulated in the insoluble fraction.

Total internal Pb content and Pb proportion in the granular fraction increased significantly with total soil concentration (Table 3.4). There was however no effect of the soil content on the proportions in cytosol and debris fractions. Pb concentrations in the three fractions rose significantly with soil total Pb content (χ^2 = 7, 12 and 8 for cytosol, debris and granules respectively, $p<0.01$). This means that Pb concentrations in the cytosol and debris fraction increased proportionally to Pb body burden with soil concentration while Pb concentration in the granules increased more strongly than the body burden with the soil content. The subcellular partitioning of Pb was thus partially modified after a short term exposure to field-contaminated soils. The results showed that with increasing soil Pb content, earthworms bioaccumulated Pb, and more internal Pb was partitioned into the granular fraction. In this compartment, Pb is assumed to be bound to metal-rich granules that play a key role in detoxification processes (Vijver et al., 2004). Therefore our results highlight this

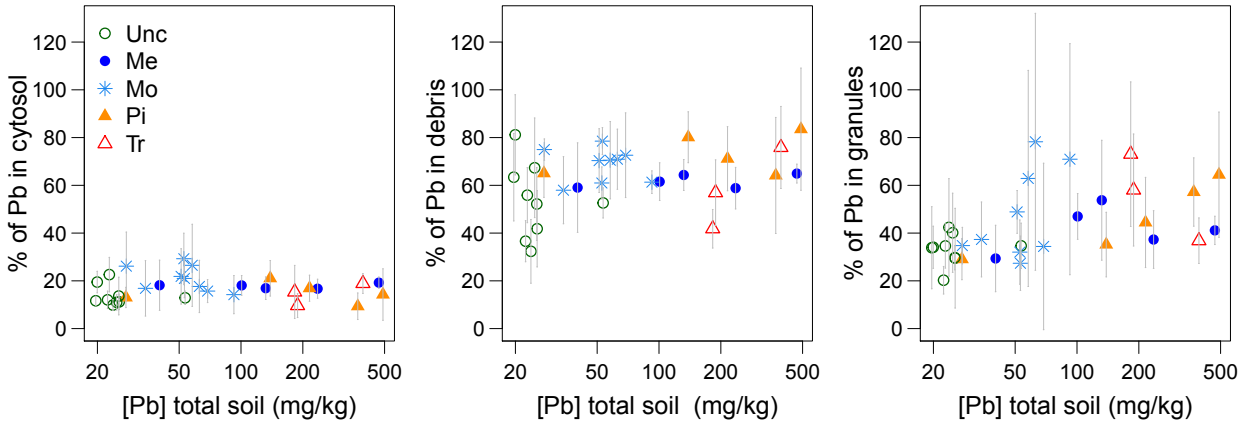


Figure 3.4: Variations in Pb partitioning in three subcellular compartments are not fully explained by total Pb concentration in soil (on a log-scale). Each point represents the mean percentage of total Pb concentration in earthworm for 5 individuals. Error bars indicate standard deviations. Legend: Site codification, Unc. for uncontaminated soils (Cl, Yv, Fe, Ba sites)

fraction has a short-term storage capacity of Pb.

Figure 3.4 illustrates that the variations in Pb subcellular partitioning were not fully explained by the soil concentration. The high variations of Pb proportion in the debris fraction observed in uncontaminated soils may reflect the influence of soil properties. Pb proportion in the cytosol was higher in Mo2, Mo4 and Mo7 soils contaminated with low levels of total Pb but low pH (Table 3.2) and higher available metal contents (Fig. 3.1). In addition we found high inter-individual variability, especially for the granules fraction (see error bars Fig. 3.4). These high variations are probably associated to the individual-specific patterns of Pb body burdens observed previously (Fig. 3.2).

3.3.5 Relationship between subcellular partitioning and soil metal availability

The relationship between the subcellular partitioning of Cd and Pb and three proxies of metal availability were tested. For each fraction and each metal, four models were compared: with $CaCl_2$-extractable, Sauvé-predicted dissolved metal concentration, free ion concentrations, and the model with total concentrations reported table 3.4. The relative fit of the models was evaluated based on the AIC criterion.

The results are given in table 3.5. All chemical measurements of Cd availability were significantly related to the proportions of Cd in the three subcellular compartments. How-

Table 3.5: Relationship between Cd and Pb partitioning in three subcellular compartments and soil metal availability assessed by $CaCl_2$ extraction, semi-mechanistic model of dissolved metals (diss.) and mechanistic model of free ion concentrations (free). Results of linear mixed effects models: AIC total refers to the model with total soil concentration as explaining variable (Table 2.4), likelihood ratio tests (χ^2) and predicted slope (standard error). AIC total refers *** : $p < 0.001$, ** : $p < 0.01$, * : $p < 0.05$

Metal	Internal pool	AIC $CaCl_2$	AIC diss.	AIC free	AIC total	χ^2 $CaCl_2$	slope $CaCl_2$	χ^2 diss.	slope diss	χ^2 free	slope free
Cd	Cytosol	1073	1073	1071	1069	8.69**	-1.91 (0.62)	8.07**	-2.4 (0.82)	11.31***	-1.72 (0.48)
Cd	Debris	38	35	28	31	9.75**	0.1 (0.03)	12.86***	0.14 (0.04)	20.95***	0.11 (0.02)
Cd	Granules	247	245	249	244	12.45***	0.16 (0.04)	13.31***	0.22 (0.06)	10.44**	0.12 (0.04)
Pb	Cytosol	221	n.s.	n.s.	n.s.	14.57***	0.13 (0.03)	0.85	n.s.	0.18	n.s.
Pb	Debris	n.s.	n.s.	n.s.	n.s.	0.64	n.s.	2.43	n.s.	3.27	n.s.
Pb	Granules	n.s.	n.s.	255	250	0.52	n.s.	2.06	n.s.	5.35*	0.04 (0.02)

ever, based on the AIC criterion, better fits were obtained with soil total contents (lowest AIC values). Indeed, even if the model explaining Cd proportion in the debris by free ion concentration had a lower AIC than the model with total Cd content, a difference of less than two units of AIC is not considered to be to be substantial (Burnham and Anderson, 2002). The relationships between the subcellular partitioning and Cd availability could be indirect through the correlation between chemical measurements of availability (and particularly free ion content) and soil total Cd concentrations (Fig. 3.1).

Although the proportion of Pb in the cytosol was not affected by the level of total contamination, there was a significant increase with $CaCl_2$-extractable Pb (Table 3.5). Predicted free ion concentrations were only related significantly to the proportion of Pb in the granular fraction but AIC indicated similar fit with soil total Pb as explaining variable. Pb proportion in the debris fraction was not related to any chemical measurements. The relationship between $CaCl_2$-extractable Pb and the proportion of Pb in the cytosolic fraction is consistent with our previous findings that energy reserves in earthworms exposed to the same 31 soils decreased with $CaCl_2$-extractable Pb (Beaumelle et al., 2014). Indeed, Pb in the cytosol is considered to be indicative of toxic pressure (Jones et al., 2009). The present results indicate an opposition between bioaccumulation parameters (body burdens, granules fraction) related to soil total Pb in soil and the supposed toxic fraction of Pb (cytosol) that is related to Pb availability as assessed by $CaCl_2$ extraction.

Between three proxies of metal availability, free ion concentration was in general a best descriptor of Cd and Pb subcellular partitioning. However, compared to soil total metal content, free ion concentration was equivalent in the ability to predict Cd and Pb subcellular partitioning. This striking result was due to the strong correlation between soil total and free ion concentrations found in this study (correlation coefficients: Cd 0.87, Pb 0.65, Zn 0.8). Iwasaki et al., 2013 reported similar results in aquatic environment. But to our knowledge,

such a strong relationship was never highlighted in previous soil surveys. Whether this is due to our range of field-contaminated soils (arable soils chronically contaminated) or if this is a more general pattern remain to be addressed.

Overall, the present results showed that modifications of Cd and Pb subcellular partitioning were more related to total soil concentrations than to available concentrations. This is consistent with the conclusions of previous field studies that used body burden as an endpoint (e.g. Hobbelen et al., 2006). If earthworms are exposed mainly to metals in the soil solution, the question arises why bioaccumulation and changes in the subcellular partitioning are more related to the contamination level than to independent measurements of this available pool. We believe that earthworms are exposed to multiple metal pools in the soil because they feed on it. This explains the recurrent correlations between biological measurements and total metal concentrations. Total metal concentrations are therefore an appropriate proxy of 'available' metals for earthworms, at least in agricultural soils that are not heavily contaminated. In the case of earthworms, the definition of availability must be refined and include other metal pools than the ones present in the soil solution. It is noteworthy that the SOM-bound metal concentrations predicted by the geochemical model could not be used to test this assumption in the present study. Indeed for the three metals, we found strong correlations between predicted SOM-bound metal and soil total metal content (correlation coefficients were >0.9, data not shown). It was thus impossible to disentangle the effects from total, SOM-bound or free ion concentrations on Cd, Pb and Zn bioaccumulation and subcellular partitionings.

On the other hand, total concentrations do not reflect the effect of metals on earthworms. Such an impact depend on various factors. In this study we show that individual factors (e.g. behavior, age) that we did not control in the experiment had a strong influence over bioaccumulation and compartmentalization of Cd, Pb and Zn in earthworms. In addition, abiotic factors, especially soil parameters determined the subcellular partitioning and body burdens (e.g. the relationship between $CaCl_2$ extractable Pb and cytosolic Pb proportion can be due to a pH effect). When assessing metal bioavailability for earthworms, it might thus not be a priority to assess metals in the soil solution, but rather to have a strong knowledge on soil physico-chemical characteristics (Pauget et al., 2012).

3.4 Conclusions

This study demonstrated that Cd and Pb subcellular partitionings were modified in earthworms after exposure to field-contaminated soils. Changes in Cd partitioning provided more

sensitive assessments of metal bioavailability than the total body burden, while changes in Pb subcellular distribution were concomitant to an increase in total internal concentration. The added value of Pb subcellular partitioning may mostly arise when considering toxicological effects and trophic transfers.

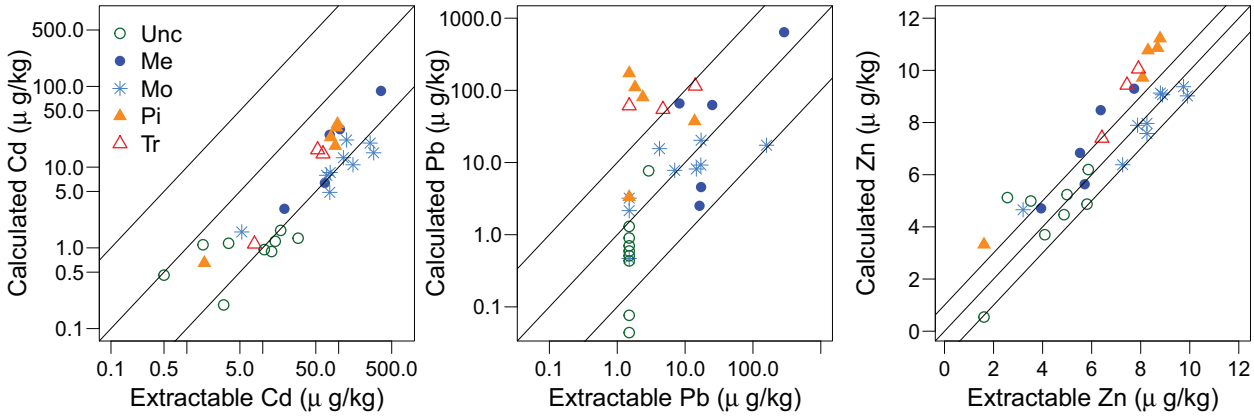


Figure 3.5: Calculated (dissolved + exchangeable) and 0.01 M $CaCl_2$-extractable metal concentrations in the 31 soil samples (logarithmic scales). Legend: Site codification, Unc. for uncontaminated soils. Black lines indicate the perfect fit (center) and variation of ten-order magnitude.

Supplementary Information

3.4.1 Geochemical model results

Calculated 'dissolved + exchangeable' Cd were about 10 times lower than $CaCl_2$ contents (Fig. 3.5). However, they increased linearly with the observed extractable concentrations. The calculated and observed Pb contents were of the same order of magnitude, but they were not linearly related. $CaCl_2$-extractable contents were under the quantification limit of 3 μ g/kg for several soils, in which the model predicted a range of concentrations (Fig. 3.5): (i) Soils from Pi and Tr sites (wasterwater-contaminated, in orange and red Fig. 3.5) exhibited higher calculated than $CaCl_2$-extractable concentrations of Pb. In those organic-rich soils, $CaCl_2$ may not extract all dissolved metals, and the model might overestimate dissolved or exchangeable concentrations. (ii) Uncontaminated soils (in green) had low dissolved Pb concentrations consistently with what is expected for these samples. There was a much closer association between Zn calculated and observed concentrations (Fig.3.5) that reinforced the quality of the geochemical model results.

Overall, the results of the geochemical model were consistent with $CaCl_2$ measurements of metal availability in the 31 soil samples. It is noteworthy that this coherence was achieved without adjusting the parameters to improve charge balance or model fit. As $CaCl_2$ extracts metal pools operationally defined, a comparison with modelled data does not constitute a validation of the model. To validate the model, measurements of free ion activities or

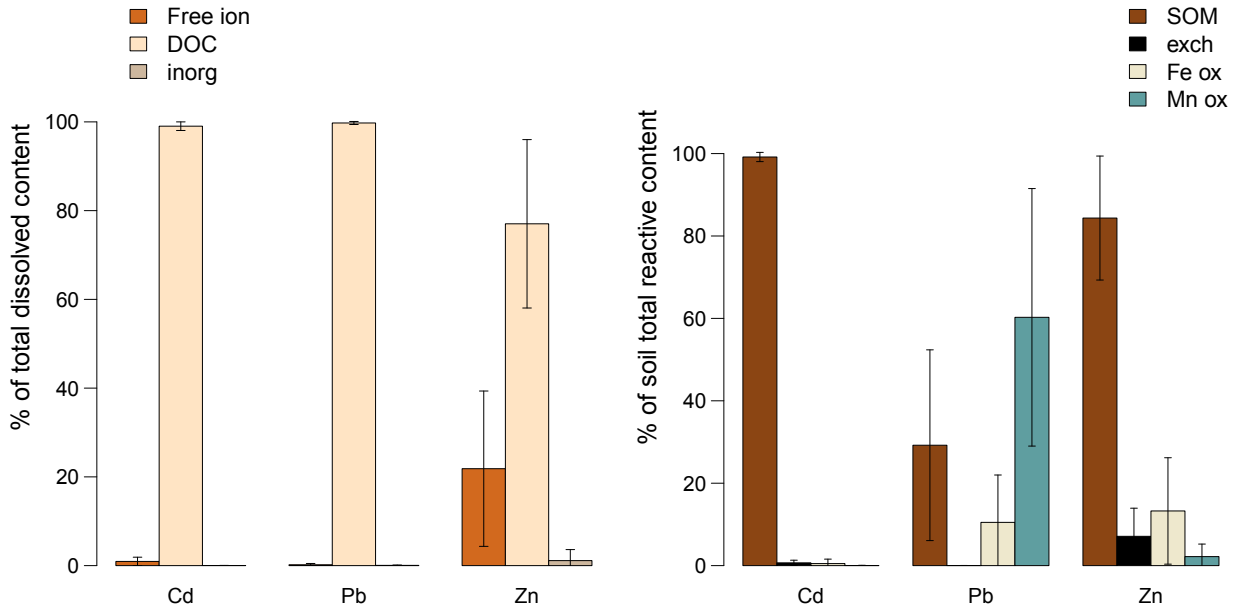


Figure 3.6: **Speciation of Cd, Pb and Zn in soil solution (left) and on solid surfaces (right) predicted by a geochemical model for 31 soil samples.** (Left) Distribution among free ion, bound to DOC and inorganic (inorg) species as percentages of the total dissolved concentrations (predicted by the geochemical model). (Right) Distribution of metals bound to SOM, exchangeable (exch) and bound to Fe and Mn oxides (Fe ox, Mn ox) as percentages of the total reactive concentration (EDTA-extractable)

solution metal contents are generally carried out. However, the consistency of the observed $CaCl_2$ extractable concentrations and predicted dissolved-exchangeable concentrations made it possible to use the modelled data as proxies of metal availability and to confront them with internal metal concentrations in earthworms.

3.4.2 Metal concentrations in three subcellular fractions

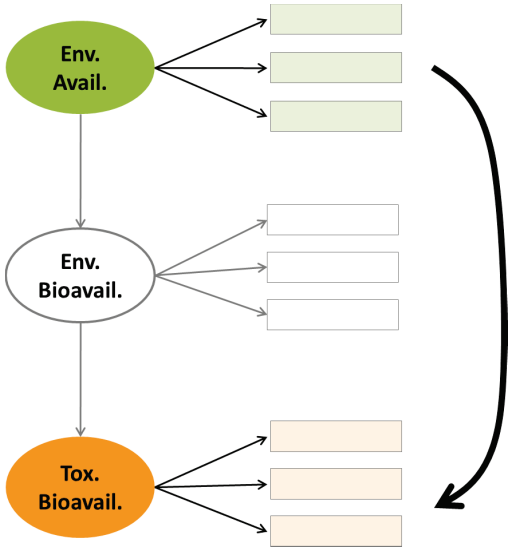
Table 3.6 gives the mean concentrations of Cd, Pb and Zn in 3 subcellular fractions of earthworms exposed to the 31 soil samples. Concentrations of Cd in the cytosol were similar between earthworms exposed to different soils, while concentrations in the debris and granular fractions were higher in earthworms exposed to contaminated soils. The concentrations of Pb in the granular and debris fractions followed similar trends as the internal global concentrations: they were higher in earthworms exposed to wastewater-contaminated soils (from Pi and Tr sites) and to a lesser extent in earthworms exposed to smelter-contaminated soils (from Me and Mo sites). In the cytosol, Pb concentrations were higher in earthworms

Table 3.6: Concentrations of metals in three subcellular fractions of earthworms (μg/g fresh weight)

	Cd cytosol	Cd debris	Cd granules	Pb cytosol	Pb debris	Pb granules	Zn cytosol	Zn debris	Zn granules
Cl	1.40 (0.15)	0.16 (0.03)	0.02 (0.00)	0.06 (0.01)	0.28 (0.07)	0.18 (0.03)	23.8 (4.4)	76.2 (14.1)	13.9 (8.0)
Yv1	0.85 (0.49)	0.18 (0.07)	0.01 (0.00)	0.04 (0.02)	0.18 (0.10)	0.10 (0.05)	17.0 (2.3)	54.2 (6.5)	9.3 (4.0)
Yv2	1.21 (0.86)	0.23 (0.09)	0.02 (0.01)	0.06 (0.03)	0.22 (0.17)	0.12 (0.11)	19.5 (10.8)	52.9 (27.2)	10.9 (11.9)
Yv3	0.94 (0.29)	0.31 (0.04)	0.03 (0.01)	0.04 (0.06)	0.27 (0.10)	0.24 (0.06)	16.1 (7.5)	59.9 (29.9)	10.1 (16.2)
Fe1	1.19 (0.55)	0.15 (0.06)	0.01 (0.01)	0.08 (0.02)	0.27 (0.17)	0.14 (0.14)	23.7 (3.5)	72.2 (15.7)	18.6 (5.8)
Fe2	1.26 (0.87)	0.16 (0.11)	0.02 (0.01)	0.07 (0.07)	0.21 (0.33)	0.13 (0.15)	22.5 (8.5)	85.0 (14.5)	15.0 (11.9)
Fe3	1.22 (0.42)	0.14 (0.13)	0.02 (0.03)	0.06 (0.03)	0.22 (0.21)	0.26 (0.30)	22.8 (4.8)	64.2 (8.9)	12.2 (8.4)
Ba1	1.03 (0.28)	0.17 (0.05)	0.01 (0.01)	0.06 (0.01)	0.24 (0.05)	0.10 (0.01)	17.6 (6.8)	60.5 (27.0)	9.7 (10.4)
Ba2	0.70 (0.57)	0.11 (0.16)	0.01 (0.01)	0.05 (0.05)	0.13 (0.20)	0.07 (0.23)	20.7 (5.0)	56.7 (23.3)	13.7 (7.7)
Mo1	1.66 (0.21)	0.31 (0.05)	0.03 (0.01)	0.10 (0.07)	0.40 (0.30)	0.30 (0.34)	18.5 (2.9)	85.9 (25.7)	12.0 (4.7)
Mo2	0.92 (0.30)	0.33 (0.06)	0.01 (0.01)	0.11 (0.03)	0.31 (0.07)	0.14 (0.05)	17.1 (2.1)	64.2 (15.1)	10.2 (3.8)
Mo3	1.05 (0.14)	0.25 (0.07)	0.05 (0.01)	0.06 (0.07)	0.21 (0.09)	0.15 (0.09)	19.9 (3.8)	57.1 (13.7)	18.2 (8.5)
Mo4	1.22 (0.31)	0.33 (0.04)	0.03 (0.00)	0.13 (0.02)	0.28 (0.12)	0.15 (0.31)	20.9 (3.0)	75.0 (19.7)	16.1 (6.5)
Mo5	1.06 (0.28)	0.18 (0.04)	0.01 (0.00)	0.07 (0.02)	0.32 (0.07)	0.21 (0.04)	21.1 (4.0)	79.8 (9.2)	16.0 (10.8)
Mo6	1.34 (0.55)	0.25 (0.16)	0.02 (0.02)	0.06 (0.10)	0.25 (0.25)	0.09 (0.33)	24.8 (3.2)	83.5 (10.3)	18.9 (4.1)
Mo7	1.27 (0.47)	0.37 (0.03)	0.04 (0.00)	0.14 (0.09)	0.41 (0.16)	0.38 (0.05)	15.6 (7.0)	61.5 (8.5)	11.6 (3.4)
Mo8	0.71 (0.97)	0.29 (0.15)	0.02 (0.02)	0.09 (0.05)	0.37 (0.27)	0.53 (0.27)	14.5 (7.2)	67.3 (45.1)	23.6 (4.6)
Mo9	1.18 (0.34)	0.34 (0.07)	0.04 (0.03)	0.10 (0.03)	0.58 (0.45)	0.88 (1.05)	17.4 (3.3)	62.7 (14.6)	14.7 (10.0)
Me1	0.95 (0.37)	0.18 (0.07)	0.01 (0.03)	0.05 (0.02)	0.18 (0.09)	0.10 (0.06)	17.0 (8.5)	56.9 (12.2)	9.4 (8.1)
Me2	1.08 (0.19)	0.22 (0.06)	0.02 (0.01)	0.10 (0.05)	0.36 (0.15)	0.33 (0.69)	17.6 (6.2)	77.8 (18.6)	11.8 (19.1)
Me3	1.09 (0.28)	0.39 (0.05)	0.05 (0.02)	0.06 (0.05)	0.27 (0.16)	0.27 (0.20)	14.3 (5.5)	57.0 (3.7)	19.5 (2.8)
Me4	1.11 (0.27)	0.33 (0.06)	0.02 (0.01)	0.08 (0.01)	0.29 (0.11)	0.22 (0.04)	15.9 (2.4)	66.3 (13.2)	11.2 (7.0)
Me5	1.05 (0.97)	0.34 (0.32)	0.04 (0.06)	0.10 (0.07)	0.33 (0.87)	0.20 (1.37)	15.4 (4.5)	60.5 (28.2)	10.5 (7.5)
Tr1	0.67 (0.34)	0.19 (0.08)	0.03 (0.03)	0.30 (0.05)	1.19 (0.96)	2.64 (1.99)	21.5 (2.0)	65.2 (5.7)	13.7 (3.4)
Tr2	1.44 (0.35)	0.33 (0.11)	0.07 (0.01)	0.17 (0.01)	1.22 (0.10)	1.89 (0.07)	18.6 (1.5)	66.9 (8.6)	17.2 (17.2)
Tr3	0.99 (0.59)	0.31 (0.09)	0.02 (0.04)	0.09 (0.08)	0.37 (0.90)	0.11 (2.03)	16.6 (3.3)	67.5 (15.6)	11.2 (6.5)
Pi1	1.03 (0.27)	0.32 (0.10)	0.01 (0.01)	0.04 (0.08)	0.23 (0.33)	0.11 (0.04)	17.3 (2.9)	81.0 (9.9)	22.9 (2.9)
Pi2	0.66 (0.04)	0.24 (0.04)	0.03 (0.01)	0.04 (0.10)	0.20 (0.86)	0.08 (2.47)	17.8 (5.0)	68.6 (17.8)	13.5 (5.5)
Pi3	0.96 (0.28)	0.30 (0.06)	0.04 (0.00)	0.08 (0.01)	0.34 (0.05)	0.26 (0.03)	21.3 (3.7)	75.8 (13.7)	11.3 (4.6)
Pi4	0.81 (0.61)	0.34 (0.12)	0.06 (0.01)	0.08 (0.04)	0.85 (0.08)	1.29 (0.03)	19.4 (6.4)	76.3 (22.1)	13.2 (7.4)
Pi5	1.55 (0.13)	0.56 (0.04)	0.09 (0.01)	0.14 (0.03)	1.24 (0.17)	1.40 (0.34)	17.1 (5.0)	77.7 (22.7)	17.6 (5.8)

exposed to soils from Tr, Mo and Pi sites. Finally, Zn concentrations in the three fractions followed the same trend as the global internal concentrations: they were similar in earthworms exposed to the 31 soils.

Chapter 4



The previous chapter highlighted that metal partitioning in subcellular compartments was modified by metal exposure. These modifications were more closely related to total metal concentration in soil than to the manifest variables of environmental availability considered. These results are consistent with a general pattern in the literature showing that total internal contents in earthworms are better related to total metal concentration in soil than to metal availability assessments. In the following chapter, we jump a step in the causal diagram and investigate the relationships between observed variables of availability and of toxicological bioavailability.

Chapter 4

Is there a relationship between earthworm energy reserves and metal availability after exposure to field-contaminated soils?[1]

Abstract

Generic biomarkers are needed to assess environmental risks in metal polluted soils. We assessed the strength of the relationship between earthworms energy reserves and metal availability under conditions of cocktail of metals at low doses and large range of soil parameters. *Aporrectodea caliginosa* was exposed in laboratory to a panel of soils differing in Cd, Pb and Zn total and available ($CaCl_2$ and EDTA-extractable) concentrations, and in soil texture, pH, CEC and organic-C. Glycogen, protein and lipid contents were recorded in exposed worms. Glycogen contents were not linked to the explaining variables considered. Variable selection identified $CaCl_2$ extractable metals concentrations and soil texture as the main factors affecting protein and lipid contents. The results showed opposite effects of Pb and Zn, high inter-individual variability of biomarkers and weak relationships with easily extractable metals. Our results support the lack of genericity of energy reserves in earthworms exposed to field-contaminated soils.

[1]Léa Beaumelle, Isabelle Lamy, Nathalie Cheviron, Mickaël Hedde. (2014) Is there a relationship between earthworm energy reserves and metal availability after exposure to field-contaminated soils? *Environmental Pollution* 191, 182-189

4.1 Introduction

In soil, assessing environmental risks associated with metallic trace elements is still challenging. Both sensitive and generic indicators of metal bioavailability are needed and despite the great number and variety of candidates, their potential as risk assessment tools has to be questionned. This is especially the case for soil organisms like earthworms, even though metal bioavailability was extensively studied (Nahmani et al., 2007a).

Biomarkers are assumed to be direct measurements of metal bioavailability (Lanno et al., 2004). The decrease of energy reserves (carbohydrates, proteins, lipids) has been proposed as a biomarker of metal stress (Scott-Fordsmand and Weeks, 2000), but the subject was rarely addressed in the case of earthworms and metal contamination. For other soil organisms, several works reported that energy reserves were affected by metal contamination (Weeks et al., 2004; Amorim et al., 2012) while other studies have shown the opposite (Schill and Köhler, 2004; Bindesbøl et al., 2005; Bednarska et al., 2013). These previous surveys considered total metal concentrations and not metal availability.

Weak and stronger metal extractions have been used to describe earthworm internal metal concentrations, but with variable results (e.g. Vijver et al., 2005; Ernst et al., 2008). No particular chemical extraction was proven to be generic to mimic metal bioavailability for earthworms: neither soil solution concentrations (Nahmani et al., 2009; Veltman et al., 2007), nor weak salt extractions (Bernard et al., 2010; Gaw et al., 2012), nor extractions using chelating agents (Ernst et al., 2008; Daoust et al., 2006). Yet $CaCl_2$ and ethylene diamine tetraacetic acid (EDTA) are extensively used to measure soil metal availability (e.g. Fritsch et al., 2011). Their pertinence to indicate metal bioavailability for earthworms must be confirmed. In order to determine if chemical extraction can mimic bioavailability, a mathematical relationship between the extractable metal content and a biological measurement of bioavailability is needed (Harmsen, 2007; ISO 17402, 2008). If a number of studies correlated chemical extractions with internal concentrations in earthworms, few attempted to link metal availability measurement with earthworm biomarkers.

In complex systems such as soils, several factors are expected to modulate biomarkers responses to metal availability. Although soils are often moderately contaminated, soil organisms energy reserves have been mainly studied in the case of high levels of metal contamination and their response to low doses of metals is not known yet. In aquatic organisms, significant increase of energy reserves at low doses of xenobiotics and decrease at higher doses have been reported (De Coen and Janssen, 2003; Smolders et al., 2003). Low doses of available metals could thus exert hormetic-like effects on earthworm energy reserves. Besides, *in*

situ, soils are very often contaminated with cocktails of metals. Earthworms regulate metals either by sequestration (Cd, Pb) or excretion (Zn, Cu) (Spurgeon and Hopkin, 1999). Holmstrup et al. (2011) have recently suggested that the ways earthworms coped with internal metals are associated to different energy demands. A complex response of energy reserves in multi-contaminated soils can therefore be expected. Finally, it is likely that the energy status of soil organisms is influenced by other factors in the environment, notably soil characteristics (Amorim et al., 2012). Soil texture and organic carbon content have been shown to influence earthworm biomass (Nahmani et al., 2007b), which could be associated with a change in energy allocation.

In the end, the effects of low doses, multiple contamination and soil characteristics could prevent the use of earthworm energy reserves as generic indicators in risk assessment. In soil, these factors intervene concomitantly, and the response of the entire system can be different from what is observed when considering separately its different parts (Beketov and Liess, 2012). Therefore, it is important to study the system as a whole. In this work, we determined whether the relationship between metal availability and earthworm energy reserves was strong enough to be found after exposure to a wide panel of field soils. The studied soils were chosen to exhibit jointly low doses of metals present in cocktails and various soil parameters.

4.2 Materials and Methods

4.2.1 Sites description

The design of the experiment included 31 soil samples chosen to achieve a wide array of soil characteristics and levels of metal contamination. They were sampled in four contaminated and four uncontaminated sites located in France. In each site, several soil samples were taken from different plots. All the plots were arable (crops or pastures), had comparable geological origins (mainly wind-deposited silt, and alluvium) and were noncalcareous.

Two of the contaminated sites (Metaleurop (Me) and Mortagne-du-Nord (Mo)) are located in the North of France near former lead and zinc smelters respectively. They were contaminated by atmospheric deposition of dusts (Van Oort et al., 2002; Bernard et al., 2010). At the Metaleurop site, five soil samples were taken from five arable cropping plots, at different distances from the smelter. At the site of Mortagne-du-Nord, nine soil samples were collected from nine different plots presenting various soil texture (from sandy to loamy),

and different land use (crops (Mo3, Mo8 and Mo9) or pastures (Mo1, Mo2, Mo4, Mo5, Mo6, Mo7)).

The two other contaminated sites (Pierrelaye (Pi) and Triel (Tr)) are located in Paris suburbs and were subjected to raw wastewater spreading for 100 years, leading to the accumulation of large amounts of organic matters and metal pollutants (Lamy et al., 2006). At the Pierrelaye site, soil samples were collected from two arable cropping plots: an unpolluted plot located outside the wastewater irrigated area for which one sample was carried out (Pi1), and a contaminated plot within which four soil samples were collected along a gradient of contamination (Pi2, Pi3, Pi4, Pi5). At the site of Triel, three soil samples were taken from three plots with varying total metal concentrations and land use: one arable cropping (Tr1), one fallow (Tr2), and one grassland (Tr3).

The four uncontaminated sites had no history of metal contamination. One site named Qualiagro-Feucherolles (Fe) is located 50 km west from Paris on a loamy soil cultivated with a maize/wheat rotation (Houot et al., 2002). The Yvetot site (Yv) is located in Haute Normandie region on a loamy plateau (Hedde et al., 2013). The Bannost site (Ba) is located 30 km south east from Paris on clay loamy soils (Pelosi et al., 2013a). The Closeaux site (Cl) is in the south west Paris suburbs (Versailles) on a silty loam soil. From these sites, a total of nine uncontaminated soil samples was carried out. Three plots receiving different exogenous organic matters (co-compost of green waste and sludge (Fe2), farmyard manure (Fe3) and no amendment (Fe1)) were sampled from Fe site. At the Yvetot site, three soil samples presenting different land use were selected: one arable cropping (Yv1), one permanent pasture established in 1968 (Yv2), and one crop/pasture rotation (Yv3) under crop at the time of the sampling. Two soil samples were collected from two different organic farming plots at the Bannost site. They presented different clay and org-C contents. One sample was carried out from a meadow plot in the Closeaux site. It was considered as our control since the earthworms used in our study were sampled from this plot.

4.2.2 Soil sampling and chemical extractions

Soil samples were collected from March to May 2012. Each sample was a composite of five subsamples of the top-soil (0-20 cm depth), taken from an area of about 1 m^2. Soil samples were hand sorted to remove soil fauna, plant material and debris, air-dried, homogenized and sieved at 2 mm. Afterwards, samples were quartered and five representative subsamples were isolated. Soil analyses were conducted on each subsample. Soil particle size determination was carried out according to NF X31-107, soil pH determination according to NF ISO 10390

and soil cation exchange capacity (CEC) according to NF X31-130. Total organic C and N assessments were performed according to NF ISO 10694 and NF ISO 13878. Total Zn, Pb, Cu, and Cd according to NF X31-147 (tri-acid HF+HCl+HNO3 digestion). Soil metal availability was assessed using two extracting reagents: a neutral salt, calcium chloride ($CaCl_2$) and an organic chelating agent, the ethylene diamine tetraacetic acid (EDTA). 0.01 M-$CaCl_2$ extraction was carried out according to Houba et al. (1990), with a ratio mass:volume of 1:10. 0.05 M-EDTA extraction was performed according to Quevauviller (1997) (BCR method) at pH 7, with a ratio mass:volume of 1:10. Total, $CaCl_2$ and EDTA-extractable metal concentrations in solution were obtained from inductively coupled plasma mass spectroscopy. The quantification limits were 1 $\mu g.kg^{-1}$ for Cd, 3 $\mu g.kg^{-1}$ for Pb, 10 $\mu g.kg^{-1}$ for Zn. Quantification limits (LQ) were determined according to NF T90-210 slightly adapted, taken into account first the analysis of ten blanks and then the analysis of solutions whose metal contents were close to the first approximation of LQ, in order to make adjustment. All analyses were made by the Laboratoire d'Analyse des Sols (INRA, Arras, France) applying standardized methods and quality assurance procedures.

4.2.3 Exposure of earthworms

Adult earthworms of the species *Aporrectodea caliginosa* (Savigny 1826) were hand sorted during spring 2012 from an uncontaminated meadow of the Closeaux site. They were maintained in the laboratory in their soil of origin that had been subjected to the same experimental procedure than the other soil samples (air dried, sieved to < 2 mm). Earthworm biomass was 0.334 ± 0.085 g fresh weight (mean $\pm$ standard deviation, n=155).

For each of the 31 soil samples, five microcosms were conducted using the five soil subsamples previously separated for soil analyses. Water holding capacity (WHC) was measured as the maximum quantity of deionised water retained by the soil sample before observing percolation on a cotton placed underneath in a funnel. One week before the onset of the experiment, 600 g of air-dried sieved soil were placed in 1 L glass jars, moistened to 60% of their WHC using deionised water and placed in the dark at 12 °C. 48 h prior to exposure, earthworms were gently washed in tap water and placed in Petri dishes containing moistened filter paper in order to void their gut. Petri dishes were placed in the dark at 12 °C. Filter paper was cleaned and re-moistened every 12 h. Once voided, earthworms were weighted before being randomly assigned to each microcosm. One microcosm contained 6 individuals. Microcosms were placed in the dark at 12 °C for 21 days. No food was added to the soil in order to not disturb initial metal availability. An exposure duration of 21 days was chosen

as a compromise between what is recommended to study metal accumulation in earthworms (28 days, Nahmani et al. (2007a)) and to avoid that earthworms lack of food (14 days, Rault et al. (2008)). Soil moisture was checked regularly by weighting the microcosms. At the end of exposure, earthworms were removed from the microcosms and were gently washed in tap water before being weighted and placed at -80 °C until further analysis.

4.2.4 Biochemical measurements

Glycogen, protein and lipid contents were measured on individual earthworms. For a given soil sample, five replicates were carried out ; one individual was randomly selected among the 6 earthworms contained in each microcosm. Frozen earthworms were individually homogenized using an ultra-turrax (IKA T10, at 15000 rpm) in 5 mL of ice-cold phosphate buffer 100 mM, pH 7.2, 1 mM EDTA. All biochemical measurements were carried out in duplicate for each individual.

Glycogen was quantified in the tissue homogenates according to Holmstrup et al. (2011). 250 μL of tissue homogenates were placed in 0.5 M NaOH at 80 °C for 3 h and 100 μL of the extraction mixtures were incubated for 2h at 37 °C with 50 μL of 10 mg.mL^{-1} amyloglucosidase (*Aspergillus niger*, Sigma-Aldrich) in 850 μL of 0.25 M acetate buffer pH 4.4. Glucose was quantified using hexokinase reagent (Sigma-Aldrich) by measuring absorbance at 340 nm. Glycogen contents were calculated relative to a glycogen standard curve (Rabbit liver glycogen, Sigma-Aldrich).

Total soluble protein contents were determined using a bicinchoninic assay kit (Smith et al., 1985) and bovine serum albumine as a standard (Sigma-Aldrich).

Lipids were extracted following Folch et al. (1957). Tissue homogenates (500 μL) were stored at -20 °C in 1.25 mL methanol until analysis. Chloroform was added to achieve a methanol/chloroform ratio of 2/1 (v/v). After centrifugation (5 min, 1700 g), lipids were separated from the water-soluble material by adding 1 volume chloroform followed by 1 volume of miliQ water (Milipore, 18 MΩ) to achieve a volume ratio of 1/1/0.3. After a second centrifugation (5 min, 1700 g), the chloroform layer was evaporated using nitrogen flux. The extraction mixtures were incubated with 5 mL of sulfuric acid in boiling water for 10 min, cooled down on ice for 5 min, and 200 μL of each sample were mixed with 3 mL of phosphoric acid-vanillin reagent freshly prepared following Knight et al. (1972). After an incubation of 15 min at 37 °C, optical density at 525 nm was read and lipid contents were calculated relative to olive oil standards (Sigma-Aldrich).

4.2.5 Statistics

For each microcosm, weight loss (ΔW) was calculated as % of initial weight based on the mean weight of the 6 individuals exposed to the same microcosm before and after exposure.

Values below the quantification limits were transformed as halves of the limits. Data were linearized when necessary using log-transformations. In order to verify that the experimental conditions did not affect earthworm energy status, Wilcoxon tests were used to compare energy reserves in earthworms before and after being exposed to their soil of origin (Cl). Spearman correlations were performed to study the relationships between the different variables.

Stepwise variable selection was used to identify the main parameters affecting earthworms energy reserves. We assumed that the biomarkers were determined by a great number of factors (including some that we did not measured). The purpose of this analysis was to investigate significant multivariate relationships with metal availability and soil characteristics. The selected regression models were not used as predictive models. Therefore, we were more interested in the significance of the models (F and p values) than in them having a close fit (R^2). In the studied soil samples, the major metal contaminants were Cd, Pb and Zn. The explaining variables considered were the total, $CaCl_2$ and EDTA-extractable concentrations of Cd, Pb and Zn, and selected soil characteristics (clay, silt and sand contents, org-C, C/N, pH, CEC). The best multiple regression models were selected based on Akaike information criterion (AIC). To verify the assumptions of linear models, residuals were checked graphically for homoscedasticity and normality. We also verified that the selected explaining variables were not strongly correlated (ρ >0.7). All statistical analyses were performed using R software (R development Core Team 2011).

4.3 Results

4.3.1 Soil characteristics and metal availability

Table 4.1 gives selected soil characteristics and total metal concentrations. It shows that the 31 soil samples covered a range of texture, org-C content, pH and CEC and of total metal concentrations. Except Me3 that was loamy, soil samples from Metaleurop site were silty, with one soil sample exhibiting higher clay contents (Me5). Me5 was also one of the most contaminated soil sample under study (Table 4.1). Soil samples from the Mortagne site presented low levels of total metal concentrations (Table 4.1). They exhibited a textural

Table 4.1: Soil characteristics, total and extractable concentrations of selected metals in the 31 soil samples. The first two letters of the samples names indicate the site of sampling (see the text for the codification of the sites). Values are the means of five measurements. Values in parentheses are the standard deviations. Values below the quantification limits were set as halves of the limits.

	Clay (%)		Silt (%)		Sand (%)		org-C (g.kg^{-1})		pH		CEC (cmol+.kg^{-1})		Cd (mg.kg^{-1})		Pb (mg.kg^{-1})		Zn (mg.kg^{-1})		Cd $CaCl_2$ (μg.kg^{-1})		Pb $CaCl_2$ (μg.kg^{-1})		Zn $CaCl_2$ (μg.kg^{-1})		Cd EDTA (mg.kg^{-1})		Pb EDTA (mg.kg^{-1})		Zn EDTA (mg.kg^{-1})	
Cl	16.8	(0.3)	61.2	(0.6)	22.1	(0.6)	11.2	(0.4)	6.7	(0.1)	11.0	(0.1)	0.25	(0.01)	53.5	(5.0)	70.1	(6.7)	17.2	(0.3)	2.9	(1.4)	351.4	(3.5)	0.17	(0.00)	18.6	(0.5)	8.1	(0.0)
Yv1	12.3	(0.2)	68.7	(0.4)	19.0	(0.4)	11.1	(0.0)	6.3	(0.1)	5.7	(0.2)	0.18	(0.01)	19.6	(2.2)	44.6	(0.3)	14.6	(0.4)	1.5	(0.0)	147.6	(1.1)	0.11	(0.00)	3.3	(0.1)	2.8	(0.0)
Yv2	16.1	(0.3)	63.8	(0.3)	20.1	(0.5)	18.7	(0.2)	5.9	(0.0)	7.9	(0.3)	0.22	(0.00)	25.4	(4.1)	51.2	(0.3)	29.2	(0.2)	1.5	(0.0)	333.6	(5.8)	0.14	(0.00)	6.0	(0.3)	2.8	(0.0)
Yv3	14.0	(0.3)	66.1	(0.3)	19.9	(0.5)	16.1	(0.2)	6.7	(0.0)	8.0	(0.3)	0.23	(0.00)	24.8	(0.5)	49.3	(0.3)	10.5	(0.4)	1.5	(0.0)	59.7	(2.8)	0.16	(0.00)	6.5	(0.1)	3.1	(0.1)
Fe1	14.5	(0.1)	79.1	(0.3)	6.4	(0.3)	9.9	(0.1)	6.7	(0.0)	7.6	(0.1)	0.20	(0.00)	22.4	(0.2)	49.5	(0.7)	13.1	(0.6)	1.5	(0.0)	130.8	(12.8)	0.13	(0.01)	5.4	(0.2)	3.9	(0.1)
Fe2	15.6	(0.1)	76.3	(0.7)	8.1	(0.7)	15.7	(0.3)	7.8	(0.0)	10.9	(0.2)	0.22	(0.01)	25.5	(0.7)	59.6	(1.5)	1.6	(0.1)	1.5	(0.0)	13.0	(0.4)	0.16	(0.05)	7.9	(0.0)	8.7	(0.1)
Fe3	15.4	(0.2)	77.6	(1.0)	7.0	(1.0)	16.0	(0.2)	7.5	(0.0)	10.6	(0.1)	0.24	(0.01)	23.8	(0.6)	60.0	(0.7)	3.5	(0.2)	1.5	(0.0)	33.7	(1.8)	0.16	(0.00)	7.9	(0.3)	9.7	(0.2)
Ba1	24.2	(0.4)	70.8	(0.3)	5.0	(0.3)	9.5	(0.1)	7.8	(0.0)	16.2	(0.0)	0.22	(0.00)	20.0	(1.4)	54.2	(1.6)	0.5	(0.0)	1.5	(0.0)	5.0	(0.0)	0.11	(0.00)	4.6	(0.1)	1.3	(0.1)
Ba2	47.6	(0.6)	43.7	(0.2)	8.7	(0.7)	21.5	(0.4)	8.1	(0.1)	30.5	(0.4)	0.27	(0.01)	22.9	(0.2)	59.7	(1.1)	3.1	(0.3)	1.5	(0.0)	5.0	(0.0)	0.09	(0.00)	3.1	(0.1)	1.5	(0.1)
Mo1	11.8	(0.6)	25.5	(1.4)	62.8	(2.0)	14.7	(0.6)	5.9	(0.1)	7.8	(0.3)	0.57	(0.01)	34.3	(0.8)	91.2	(1.8)	154.4	(5.3)	14.6	(1.1)	3842.0	(132.0)	0.49	(0.01)	13.7	(0.5)	14.0	(0.5)
Mo2	7.9	(0.4)	27.6	(0.7)	64.5	(0.7)	16.5	(0.3)	6.2	(0.0)	6.1	(0.2)	0.59	(0.01)	27.7	(0.7)	87.6	(3.4)	76.4	(1.5)	1.5	(0.0)	3848.0	(70.5)	0.45	(0.01)	9.2	(0.4)	26.5	(0.4)
Mo3	5.4	(0.4)	16.2	(0.4)	78.4	(0.3)	11.4	(0.6)	6.7	(0.1)	3.7	(0.1)	0.73	(0.01)	51.1	(2.0)	123.0	(2.9)	115.4	(1.5)	17.1	(0.7)	7244.0	(89.9)	0.56	(0.02)	18.9	(0.5)	47.4	(1.4)
Mo4	6.4	(0.1)	20.9	(0.5)	72.7	(0.5)	14.7	(0.2)	5.4	(0.0)	3.0	(0.1)	0.74	(0.02)	52.7	(0.7)	100.4	(1.5)	289.2	(2.7)	158.6	(7.1)	19980.0	(535.7)	0.55	(0.01)	20.5	(0.1)	35.4	(0.4)
Mo5	27.2	(0.4)	54.2	(0.3)	18.7	(0.3)	41.5	(1.4)	8.0	(0.0)	28.2	(0.2)	0.98	(0.02)	68.8	(2.5)	168.8	(2.0)	5.3	(7.0)	1.5	(0.0)	24.6	(6.2)	0.65	(0.01)	24.3	(0.6)	20.7	(0.4)
Mo6	22.4	(0.3)	35.8	(0.4)	41.8	(0.3)	27.0	(0.4)	6.1	(0.0)	17.2	(0.2)	1.22	(0.02)	53.0	(1.1)	217.2	(2.6)	127.2	(4.3)	4.2	(2.1)	6654.0	(113.7)	0.92	(0.01)	18.5	(0.3)	52.1	(0.6)
Mo7	11.6	(0.1)	25.9	(0.9)	62.5	(0.8)	27.5	(0.6)	5.9	(0.0)	8.9	(0.1)	1.30	(0.01)	57.9	(3.0)	206.2	(2.4)	260.0	(8.0)	17.2	(0.5)	17100.0	(308.2)	1.03	(0.03)	26.0	(0.6)	63.4	(1.5)
Mo8	8.6	(0.3)	19.5	(0.5)	71.9	(0.6)	15.4	(1.0)	7.4	(0.1)	6.8	(0.2)	1.32	(0.03)	62.7	(5.8)	212.2	(5.2)	69.5	(1.1)	7.0	(0.6)	2624.0	(118.9)	1.07	(0.01)	30.7	(0.1)	88.8	(0.8)
Mo9	10.9	(0.4)	37.9	(1.0)	51.3	(1.2)	12.8	(0.7)	7.8	(0.0)	8.1	(0.3)	3.25	(0.06)	92.4	(8.3)	437.0	(5.9)	77.4	(1.7)	1.5	(0.0)	1428.0	(32.7)	2.32	(0.04)	40.0	(0.5)	159.6	(0.9)
Me1	18.7	(0.2)	54.1	(0.7)	27.2	(0.6)	11.9	(0.3)	7.3	(0.0)	13.4	(0.1)	0.95	(0.01)	40.2	(0.6)	91.7	(1.5)	65.9	(2.2)	16.3	(1.5)	303.6	(16.7)	0.68	(0.01)	20.2	(1.1)	11.8	(0.2)
Me2	23.5	(0.4)	71.0	(0.5)	5.5	(0.7)	25.7	(0.9)	8.2	(0.0)	20.6	(0.1)	1.12	(0.01)	100.8	(43.1)	134.0	(1.6)	19.3	(1.2)	17.2	(1.5)	51.4	(1.2)	0.92	(0.01)	42.6	(2.3)	16.9	(0.3)
Me3	18.6	(0.4)	30.0	(0.5)	51.4	(0.8)	13.5	(0.1)	7.7	(0.0)	13.1	(0.2)	3.02	(0.09)	132.0	(1.6)	227.8	(2.8)	104.0	(1.4)	25.1	(0.8)	583.6	(10.3)	2.38	(0.05)	93.7	(0.6)	57.0	(0.8)
Me4	19.0	(0.4)	57.3	(0.3)	23.7	(0.3)	16.2	(0.4)	7.9	(0.0)	15.6	(0.1)	4.61	(0.10)	236.0	(25.8)	329.2	(4.3)	76.2	(1.2)	8.2	(0.6)	250.8	(5.9)	3.68	(0.08)	178.6	(1.8)	74.1	(0.9)
Me5	30.9	(0.4)	53.2	(0.3)	15.9	(0.2)	22.3	(0.4)	7.2	(0.0)	24.4	(0.1)	8.32	(0.08)	469.0	(4.8)	541.0	(17.8)	361.4	(10.5)	288.0	(29.9)	2278.0	(87.0)	7.12	(0.10)	394.2	(6.1)	117.6	(1.7)
Tr1	6.3	(0.4)	8.7	(0.5)	85.0	(0.8)	16.7	(0.8)	7.6	(0.0)	6.7	(0.2)	0.31	(0.01)	182.6	(53.5)	156.2	(4.1)	7.8	(0.2)	4.7	(0.6)	612.0	(7.3)	0.26	(0.01)	91.3	(4.6)	52.2	(4.0)
Tr2	6.9	(0.6)	7.9	(0.4)	85.3	(0.8)	25.9	(1.6)	7.6	(0.0)	7.8	(0.2)	2.68	(0.10)	188.6	(32.8)	386.2	(11.6)	62.2	(0.6)	1.5	(0.0)	1704.0	(19.5)	2.14	(0.07)	123.6	(29.9)	176.2	(4.7)
Tr3	6.0	(0.3)	6.2	(0.7)	87.7	(0.9)	32.7	(1.6)	7.6	(0.0)	10.2	(0.2)	3.67	(0.26)	392.2	(79.5)	645.6	(53.0)	52.9	(1.0)	14.1	(1.0)	2714.0	(38.5)	2.61	(0.05)	191.4	(7.0)	335.0	(6.2)
Pi1	9.2	(0.3)	8.2	(0.5)	82.6	(0.8)	11.1	(0.4)	8.3	(0.0)	8.1	(0.2)	0.25	(0.00)	27.5	(8.7)	40.4	(0.4)	1.7	(0.1)	1.5	(0.0)	5.0	(0.0)	0.16	(0.00)	12.2	(0.4)	6.9	(0.2)
Pi2	6.1	(0.2)	5.6	(0.4)	88.3	(0.4)	14.7	(0.6)	7.3	(0.0)	6.1	(0.1)	2.68	(0.09)	138.8	(13.6)	342.8	(4.0)	90.2	(5.2)	13.8	(5.8)	3216.0	(342.6)	1.96	(0.01)	83.2	(1.6)	162.0	(3.1)
Pi3	7.2	(0.3)	8.9	(0.5)	84.0	(0.6)	17.7	(0.8)	7.3	(0.0)	7.0	(0.2)	3.12	(0.06)	215.6	(61.4)	494.2	(7.8)	78.6	(1.3)	2.4	(1.3)	4012.0	(16.4)	2.40	(0.02)	126.6	(1.5)	279.6	(1.5)
Pi4	7.2	(0.3)	10.5	(0.7)	82.3	(0.7)	23.4	(0.8)	7.3	(0.0)	7.5	(0.2)	4.93	(0.02)	370.2	(39.8)	771.8	(12.5)	96.6	(3.1)	1.8	(0.7)	5926.0	(95.0)	3.46	(0.09)	203.8	(14.4)	430.4	(12.8)
Pi5	7.4	(0.3)	13.4	(0.4)	79.3	(0.5)	32.4	(1.4)	7.2	(0.0)	8.6	(0.2)	6.43	(0.18)	491.0	(21.8)	1004.2	(13.4)	93.3	(2.9)	1.5	(0.0)	6558.0	(76.6)	4.09	(0.08)	271.0	(3.2)	568.8	(8.9)

gradient from sandy to loamy, with more fine sand particles (50 μm to 200 μm) than coarse ones (200 μm to 2000 μm). For Pierrelaye and Triel sites, the soil samples were sandy-textured and exhibited more coarse sand particles than fine ones, as opposed to the sandy soil samples of the Mortagne site. Their total metal concentrations are among the highest of the studied soils (Pi4, Pi5 and Tr3 in Table 4.1). The uncontaminated soil samples were mainly silty, except Pi1 that was sandy and Ba2 that was the most clayey soil sample under study (Table 4.1).

$CaCl_2$ and EDTA-extractable amounts of metals in each soil sample are reported in table 4.1. Over all soil samples, the average $CaCl_2$ extraction yield was 6.8 ± 8.7% of total Cd, 0.02 ± 0.05% of Pb and 1.77 ± 3.9% of Zn. EDTA extracted 71.9 ± 10.5% of total Cd, 43.3 ± 16.9% of Pb and 25.71 ± 17.1% of Zn.

Concentrations of Cd, Pb and Zn extracted by $CaCl_2$ were significantly correlated with total concentrations (ρ=0.65, 0.45 and 0.60 for Cd, Pb and Zn respectively, $p<0.05$), although the coefficients were far lower than the ones for EDTA (ρ >0.95 for all metals considered). EDTA-extractable concentrations of Cd, Pb and Zn were all strongly correlated with each other (ρ >0.9, $p<0.01$). $CaCl_2$-extractable Cd was highly correlated with $CaCl_2$-extractable Zn (ρ=0.88, $p<0.001$), and to a lesser extent with $CaCl_2$-extractable Pb (ρ=0.67, $p<0.001$). The correlation between extractable Zn and Pb was lower (ρ= 0.50, $p<0.001$).

4.3.2 Experimental conditions and weight loss

No mortality occurred during the experiment. Weight loss ranged from −5.3 to +13.4% and was on average 4.6 ± 4.2%. Earthworms lost the more weight after being exposed to Ba2 and Pi4 soil samples. On average, earthworms exposed to their soil of origin (Cl) gain about 2% of their initial weight (mean weight loss: −2.1 ± 7.5%).

4.3.3 Glycogen contents

The mean glycogen content in earthworms was 4.9 ± 1.4 mg glycogen.g^{-1} fresh weight (FW), ranging from 2.3 to 8.2 mg.g^{-1} FW (Table 4.2). The lowest glycogen contents were observed in earthworms exposed to Tr1 and Pi5 samples. Comparing earthworms glycogen contents before and after exposure to the control sample (Cl) revealed no significant differences ($p>0.05$). Before exposure, earthworms exhibited 6.2 ± 4.0 mg.g^{-1} FW, while 5.5 ± 3.4 mg.g^{-1} FW after exposure to Cl soil. Glycogen contents in the earthworms exposed to the same soil sam-

ple were highly variable. The coefficients of variation (CV) calculated for each soil sample on the basis of the 5 replicates were on average 45 ± 16%.

The stepwise approach failed to give a satisfactory model explaining variations in glycogen contents. The main factors affecting glycogen contents variations were neither total, $CaCl_2$, EDTA metal concentrations, nor the soil characteristics considered.

4.3.4 Protein contents

The average protein content in earthworms was 57.3 ± 9 mg.g^{-1} FW, ranging from 36.7 to 75.2 mg.g^{-1} FW for earthworms exposed to Tr3 and Pi5 respectively (Table 4.2). Protein contents were similar (p>0.05) in earthworms before (61.4 ± 6.1 mg.g^{-1} FW) and after exposure to the control soil sample (54.9 ± 8.7 mg.g^{-1} FW). Across the 5 replicates, the CV of protein contents were 15 ± 7%.

Stepwise variable selection identified three main parameters affecting protein contents.

$$Protein = log(Zn_{CaCl_2}) \cdot 2.9 - log(Pb_{CaCl_2}) \cdot 3.5 + log(Clay) \cdot 5.7 + 16.9 \qquad (4.1)$$

The model obtained is shown in equation (4.1), with $F_{4;27} = 4.52$; p=0.011, and $R^2 = 0.33$. Neither Cd, org-C, pH nor CEC were involved in the model. Protein contents were significantly related to easily extractable Pb and Zn. Figure 4.1 shows the univariate relationships between protein contents and $CaCl_2$-extractable Pb and Zn. Protein contents significantly increased with $CaCl_2$-extractable Zn contents and decreased with $CaCl_2$-extractable Pb. In the stepwise approach, the first variable selected to explain protein contents was $CaCl_2$-extractable Zn. The variable selection also indicated that protein contents were positively linked with soil clay contents.

4.3.5 Lipid contents

Lipid contents in earthworms were 8.9 ± 1.5 mg.g^{-1} FW, ranging from 6.7 to 11.9 mg.g^{-1} FW. The lowest lipid contents (< 7 mg.g^{-1} FW) were found for earthworms exposed to Me5 and Mo8 samples (Table 4.2). Lipid contents in earthworms exposed to their soil of origin were not significantly different from lipid contents recorded in earthworm before exposure (p> 0.05; 10.9 ± 1.4 mg.g^{-1} FW and 10 ± 3.1 mg.g^{-1} FW respectively). The CV of lipid contents were 15 ± 4%.

Table 4.2: Glycogen, protein and lipid contents in earthworms after exposure to the 31 soil samples. The first two letters of the samples names indicate the site of sampling (see the text for the codification of the sites). Values are the means of five measurements. Values in parentheses are the standard deviations.

	Glycogen contents ($mg.g^{-1}$ FW)	Protein contents ($mg.g^{-1}$ FW)	Lipid contents ($mg.g^{-1}$ FW)
Cl	5.5 (3.4)	54.9 (8.7)	10.9 (1.4)
Yv1	3.8 (2.5)	46.3 (13.5)	8.8 (0.8)
Yv2	5.2 (1.9)	61.0 (10.1)	11.3 (1.5)
Yv3	4.6 (2.6)	53.0 (10.6)	9.5 (1.4)
Fe1	5.1 (1.9)	55.1 (14.8)	8.8 (1.7)
Fe2	4.9 (1.0)	55.5 (5.8)	10.7 (1.8)
Fe3	4.6 (1.9)	61.1 (12.0)	9.9 (1.2)
Ba1	5.2 (2.0)	54.7 (6.5)	9.1 (1.4)
Ba2	8.2 (2.7)	51.8 (7.9)	7.1 (0.5)
Mo1	5.5 (2.7)	58.0 (3.2)	9.9 (2.3)
Mo2	5.7 (2.5)	62.8 (9.7)	9.8 (1.4)
Mo3	6.0 (3.1)	63.7 (6.8)	7.6 (0.8)
Mo4	5.0 (2.2)	53.5 (7.1)	8.3 (0.9)
Mo5	3.2 (1.7)	62.7 (2.1)	8.4 (2.0)
Mo6	3.5 (1.8)	67.4 (12.4)	10.6 (1.5)
Mo7	3.1 (0.9)	72.4 (5.2)	8.0 (1.0)
Mo8	5.2 (2.4)	41.3 (9.9)	6.7 (1.0)
Mo9	3.3 (2.9)	58.5 (8.1)	10.3 (1.0)
Me1	6.1 (1.4)	47.1 (9.0)	8.2 (0.9)
Me2	6.4 (2.1)	59.6 (11.4)	11.9 (2.2)
Me3	7.0 (2.7)	52.3 (11.3)	9.9 (2.6)
Me4	5.8 (2.3)	64.0 (6.4)	8.7 (1.6)
Me5	5.9 (2.4)	48.6 (4.5)	6.7 (1.2)
Tr1	2.3 (0.9)	61.2 (6.0)	6.9 (1.2)
Tr2	5.0 (0.8)	54.2 (1.8)	9.6 (1.9)
Tr3	6.8 (2.0)	36.7 (5.7)	6.8 (1.2)
Pi1	3.8 (2.8)	45.6 (12.2)	7.0 (1.3)
Pi2	3.0 (1.1)	59.9 (3.5)	6.8 (0.7)
Pi3	3.6 (2.7)	66.4 (10.1)	7.9 (1.2)
Pi4	4.7 (2.2)	73.1 (8.5)	9.3 (1.1)
Pi5	2.6 (1.7)	75.2 (5.3)	10.5 (1.4)

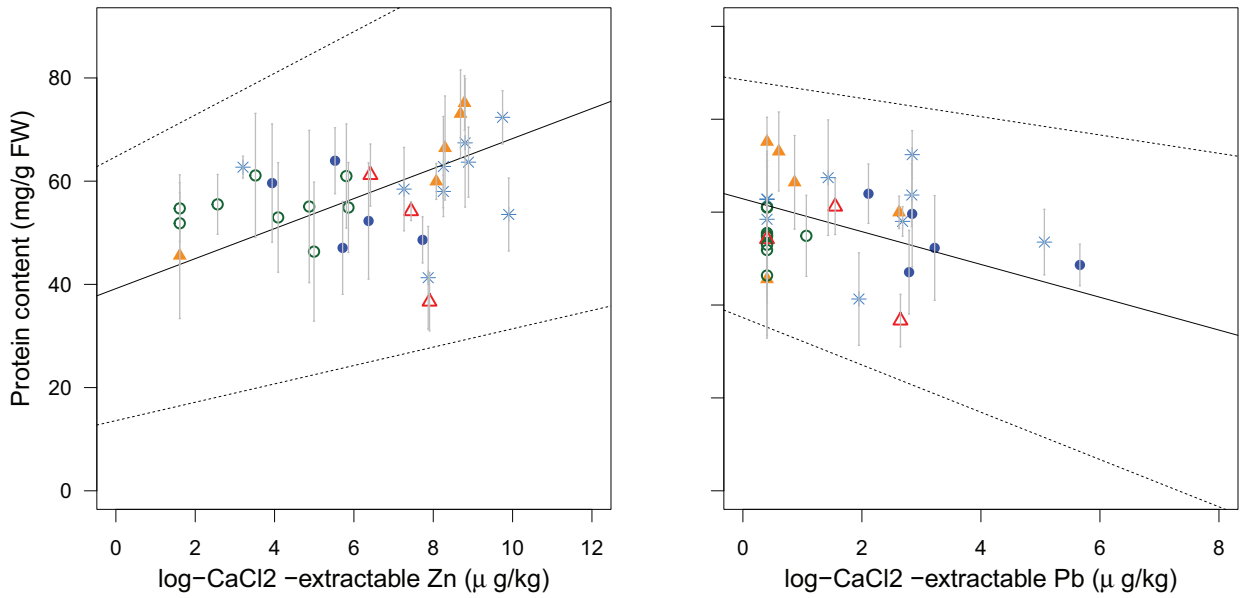


Figure 4.1: Relationships between protein contents and $CaCl_2$-extractable Zn (left) and Pb (right). Each point represents the mean of five replicates. Error bars indicate standard deviations. The solid line is the slope and the dashed lines are the 80% confident intervals predicted by the multiple regression model (4.1).

$$log(Lipid) = log(Silt) \cdot 0.13 - log(Pb_{CaCl_2}) \cdot 0.06 + log(Zn_{CaCl_2}) \cdot 0.03 + 1.29 \qquad (4.2)$$

The model obtained from stepwise variable selection is given in equation 4.2, with $F_{4;27} = 7.66$; p=0.0007, and $R^2 = 0.46$. Lipid contents were significantly and positively linked with $CaCl_2$-extractable Zn, and negatively linked with $CaCl_2$-extractable Pb. Moreover, lipid contents significantly increased with silt content, that was the first variable selected by the stepwise approach. The univariate relationship with silt is reported in figure 4.2. Generally, for the less silty soil samples, lipid contents were lower than in soils with higher silt contents. A number of points diverged from this relatioship, related either to clayey soils either to contaminated soils. But according to the model 4.2, these divergences were rather explained by extractable metals.

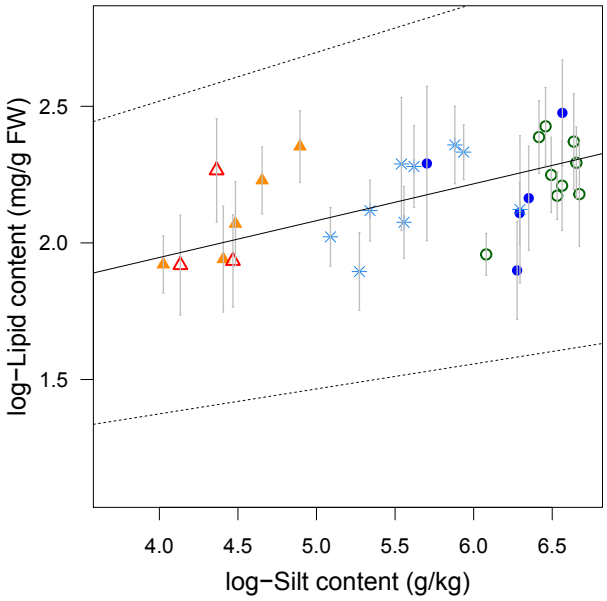


Figure 4.2: Relationship between log-lipid and log-silt contents. Each point represents the mean of five replicates. Error bars indicate standard deviations. The solid line is the slope and the dashed lines are the 80% confident intervals predicted by the multiple regression model (4.2).

4.4 Discussion

Relationship between energy reserves and low doses of available metals

To our knowledge, no study reported simultaneously *A. caliginosa* glycogen, protein and lipid contents in metal polluted soils. In the case of earthworms that were not exposed to contaminated soils, the energy reserves contents observed are close to our ranges. Protein contents around 50 mg.g^{-1} FW have been reported in *A. caliginosa* (Rault et al., 2007; Schreck et al., 2008) which is near our mean value of 57.34 mg.g^{-1} FW. In our study, glycogen contents were, on average, 4.87 mg.g^{-1} FW, which is of the same order of magnitude as the values reported by Holmstrup and Overgaard (2007) for *A. caliginosa* (5-6 mg.g^{-1} FW considering a dry weight/fresh weight ratio of 0.16 (data not reported here)). For lipid contents, Albro et al. (1992) observed around 1.2% FW in *Lumbricus terrestris* close to our mean value of 0.89%. Moreover, energy reserves levels were not significantly different before and after exposure to the control soil (Cl) and no significant weight loss was observed. These results show that the experimental conditions did not affect earthworms energy status as

measured after 21 days of exposure.

This study demonstrated that glycogen, protein and lipid contents did not respond the same way to low doses of available metals. Glycogen contents were not related to Cd, Pb or Zn availability assessed by either $CaCl_2$ or EDTA extractions. They presented a high inter-individual variability compared to protein and lipid contents, which certainly participate to explain this lack of response. Contrasting results were observed for protein and lipid reserves; they were significantly related to low doses of $CaCl_2$ extractable metals. Glycogen is considered to be rapidly mobilized when organisms face an energy demand, while protein and lipids are considered as long-term reserves (Levesque et al., 2002).

Given the lack of datas on earthworm energy reserves in response to metal contamination, we chose to maximize the number of soils and doses considered in this experiment and to focus on the general state of earthworm energy reserve at one point in time. It is noteworthy that the time variation of these energy pools might also be an interesting biomarker. In our experiment, glycogen contents might have changed during the first days of exposure. It may thus be a transient biomarker, and its time variation needs to be addressed. This study provides insights to define the sampling dates for kinetic studies. The results suggest that after 21 days, it is possible to observe differences in protein and lipids contents, even at low doses of available metals. This is not the case for glycogen contents.

Energy reserves response to different pools of available metals

This study included a wide range of metal availability assessed by two common proxies: $CaCl_2$ and EDTA extractions. Among all metal concentrations considered, proteins and lipids were only related to $CaCl_2$ extractions. This result suggests that earthworm energy reserves respond to easily extractable metals but not to more strongly bound ones. It must be pointed out that the regressions may include the effect of Cd through the Zn and Pb parameters because we found high correlations of $CaCl_2$ extractable Cd with Zn and Pb. In highly contaminated soils, earthworms biological responses (growth, reproduction) were not significantly related to metal availability assessed by $CaCl_2$ extractions (Arnold et al., 2003; Smith et al., 2010). However, other authors demonstrated the opposite (Daoust et al., 2006; Owojori et al., 2010). The correlation coefficients values ranged from 0.3 to 0.6, which is quite in agreement with the weak relationships reported herein. Our results therefore extend these previous observations to the case of biomarkers responses to lower levels of contamination (*e.g.* about 4 times lower Pb contents in our survey compared to the values of Smith et al. (2010)).

It was assumed that EDTA extractions could mimic global earthworm exposure, including dietary intake. The results did not agree with this statement. Using another chelating agents with properties close to EDTA (DTPA: diethylene triamine pentaacetic acid), Owojori et al. (2010) observed that the extractable metal contents were related to earthworm biomass, survival and reproduction. Daoust et al. (2006), however, found the opposite. The pool of EDTA-extractable metals might encompass too many metal species to represent metal bioavailability for earthworms. Indeed, in the present study, EDTA-extractable metals concentrations were highly correlated with total concentrations and the levels of metals extractability were elevated.

Opposite effects of Zn and Pb on earthworm energy reserves

The results further showed that *A. caliginosa* energy reserves levels responded differently to available Pb and Zn. It is consistent with Holmstrup et al. (2011) who concluded that the different mechanisms of metal regulation (Cd-Pb *versus* Cu-Zn) in earthworms are associated with different energy demands. However Holmstrup et al. (2011) found excretion of Zn to be more energy (glycogen) demanding than sequestration of Pb. In our conditions, protein and lipid contents tended to decrease with available Pb contents and to increase with available Zn. It raises new questions about the effect of metals on energy reserves in earthworms, particularly in the case of moderate levels of contamination.

Several mechanisms can be invoked to explain the opposite effects of Zn and Pb found herein. In earthworms, Pb is known to induce oxidative stress that ultimately damages proteins and lipids (Labrot et al., 1996; Maity et al., 2008). Besides, energy cost of detoxifying Pb has been demonstrated (Ireland and Richards, 1977). Concerning Zn, the active regulation of internal Zn levels by excretion (Spurgeon and Hopkin, 1999) could be achieved by increased protein synthesis. In addition, several studies have shown that at low doses, Zn has a protective role when interacting with other contaminants because of its antioxidant properties (Valko et al., 2005; Mansour and Mossa, 2009; Prasanthi et al., 2010; Cherif et al., 2011). Yet, it was never demonstrated in the case of earthworms. Whether Zn and Pb effects on energy reserves are mediated through oxidative stress mechanisms or through energy demanding regulation processes thus remain to be addressed.

Given these results, it appears that multi-contamination affects the response of *A. caliginosa* energy reserves to metal availability. In realistic situations of soils contaminated by cocktails of metals, there is every reason to believe that the response of energy reserves will be difficult to interpret. The presence of different metals having opposite effects explain, at

least partly, previous observations of similar levels of soil invertebrates energy reserves in contaminated and uncontaminated soils (e.g. Bednarska et al., 2013). In the end, our results demonstrate the importance of taking into account the separate effects of different metals when questioning the genericity of biomarkers. Such an approach can however be difficult to carry out because the common origin of the different metals lead to correlations between their concentrations (*e.g.* Cd in our study).

Influence of soil texture on earthworm energy reserves

Soil texture appeared as the only soil characteristic explaining *A. caliginosa* protein and lipid contents. Soil texture is known to determine metal availability (Sauvé, 2002). Several studies reported decreased effects of metals on earthworms in clayey soils (Criel et al., 2008; Leveque et al., 2013). In the regression models explaining protein and lipid levels, the soil texture parameters were included in addition to available metal concentrations. As $CaCl_2$-extractable metal concentrations integrate the effect of soil texture on metal availability, this result means that soil texture exerted a direct effect on earthworm energy status. Even though glycogen contents were not related to any of the soil parameters considered, the lowest glycogen contents were found in earthworms exposed to sandy soils (Tr1, Pi5). Besides, low protein contents were also recorded in earthworms exposed to sandy soils (Tr3) and lipid contents were low after exposure to sandy (Mo8) and clayey (Me5) soils. Sandy and clayey are both constraining soil textures for earthworms. Negative effects of clay (Vandecasteele et al., 2004) and sand (Nahmani et al., 2007b) on earthworms biomass have been reported. The fact that weight losses in our experiment were greater after exposure to sandy (Pi4) and clayey (Ba2) soil samples is consistent with these results. Clay could be associated with mechanical constraints on earthworms burrowing capacity, or with a decreased food or water availability (Owojori et al., 2010). Sand on the other hand, can irritate earthworms skin (Lee et al., 1985; Spurgeon and Hopkin, 1996b) or gut. On the basis of these findings, soil texture has more impact on earthworm energy reserves than organic matter quantity and quality, pH or CEC over a short time exposure.

The results clearly support the conclusion that other factors than the availability of metals and soil characteristics influence glycogen, protein and lipid contents in *A. caliginosa*. Indeed, the regression models explained less than 50% of energy reserves contents variations. Other contaminants not accounted for in this report might have affected earthworms energy reserves. Moreover, a high inter-individual variability was observed consistently with the results from previous reports (Bednarska et al., 2013; Holmstrup et al., 2011). This variability certainly

explains the weakness of the relationships. Therefore, other determinants of energy levels in earthworms remain to be identified, particularly individual factors and the effect of other contaminants (e.g pesticides, PAHs, PCBs, that were not quantified in this study).

4.5 Conclusion

In this study, our objective was to focus on the effects of low dose, multi-contamination and soil characteristics on earthworm energy response to metal availability. At low doses and despite the influence of multi-contamination and soil characteristics, it was possible to find a link between earthworm energy reserves (protein and lipid) and metal availability. Easily extractable metals were even the main factors explaining biomarkers responses. Thus, over a short time exposure, metal availability do affect earthworm energy reserves (protein and lipid). However, the weakness of the relationships, the fact that different metals exhibited opposite effects and the high inter-individual variability clearly illustrate the lack of genericity of energy reserve response in moderately and multi-contaminated soils. Many other parameters are involved and affect the relationship, in particular the complex effects of different metals and of soil texture highlighted by our findings. We are convinced that searching for suitable indicators of metal bioavailability will need integrated approaches that question their genericity. Such approaches not only provide interesting results for risk assessment purpose, but also bring insights on the behavior of indicators in the interesting case of diffuse rather than massive pollutions.

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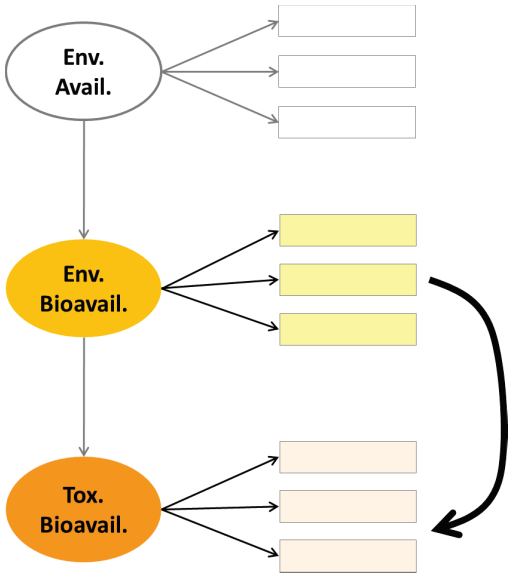
Chapter 5



In the previous chapter, biomarkers were related to soil metal availability but to a low extent in our panel of field soils. In the following chapter we go one step further in the causal diagram and investigate the relationship between biological responses and internal metal concentrations. Our set of soils permitted to confront the strength of this relationship to combined low metal availability, multiple metals and ranges of soil characteristics. We questioned if a specific internal compartment better explained earthworm response in such conditions.

Chapter 5

Relationships between metal compartmentalization and biomarkers in earthworms exposed to field-contaminated soils [1]

5.1 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 where 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 (MT)) (Stürzenbaum et al., 2004; Wallace and Lopez, 1996). Both processes decrease metal availability in the organism.

Subcellular fractionation procedures were proposed to evaluate internal metal compartmentalization in organisms (Wallace et al., 2003). Using serial centrifugations, up to five internal metal fractions can be separated. The soluble or cytosolic fraction combines three compartments containing: (i) microsomes and organelles, (ii) heat denatured proteins (both

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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 MT proteins thus constituting a detoxified fraction (Conder et al., 2002). Jones et al., 2009 showed that Pb was not sequestered by metal binding proteins in this fraction, concluded that the soluble Pb fraction could be indicative of Pb toxicity in earthworm. The insoluble fraction is further separated in a compartment of tissue, membranes and cellular debris (called debris hereafter) and a compartment containing the MRG. The debris fraction is the more operational (Wallace et al., 2003; Jones et al., 2009) but was also proposed to be associated to the organism metabolic needs because most of the body burden of essential elements (Zn and Cu) is retrieved from this fraction in earthworms (Vijver et al., 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).

Partitioning tissue metal concentration into subcellular compartments reflecting 'toxicologically available' pools may be good descriptors of the effects of metals, notably in earthworms (Vijver et al., 2004). Using subcellular fractionation procedures, several authors addressed the kinetics of metal accumulation in different earthworm subcellular compartments (Huang et al., 2009; Vijver et al., 2006; Conder et al., 2002). However, the relationships between internal compartmentalization of metals and biological endpoints such as biomarkers were rarely addressed (Colacevich et al., 2011; Jones et al., 2009).

The use of several of biomarkers to determine the effects of metals on organisms is generally recommended in order to reflect the multidimensionality of the stress response (Berthelot et al., 2009; Van Straalen, 2003). Individual biomarkers may respond better 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 (Chap.4 p. 80). As earthworms use different accumulation strategies according to the metal, the relationships between biomarkers response and internal accumulation in multi-contaminated soils could be complex. It is not certain that a particular subcellular fraction can be indicative of earthworm stress response to metal exposure.

The aim of this study was to assess the relationship between biomarkers in earthworm and Cd, Pb and Zn concentrations in three subcellular fractions in an attempt to identify potential metal fractions indicative of a toxic pressure. Biomarkers representing distinct

processes by which metals can exert their effects on earthworms were considered: (i) the expression of a gene coding a metallothionein protein (MT), previously demonstrated to increase with Cd exposure (Bernard et al., 2010; Spurgeon et al., 2004), (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 as a manifestation of the energy cost associated to metal exposure (Holmstrup et al., 2011). Biomarkers were further combined in an integrated biomarker index (IBR) proposed by Colacevich et al., 2011; Asensio et al., 2013 to seek for a global assessment of earthworms response to internal metal concentrations in three subcellular fractions.

5.2 Methods

After an exposure experiment of *A. caliginosa* (Savigny 1826) to 31 field contaminated and uncontaminated soils described (Chap.2 p.26, Chap.4 p.80), Cd, Pb and Zn concentrations in earthworms and in three subcellular fractions were quantified (Chap.3 p.55). Energy reserves (protein, lipid and glycogen contents) were measured and the results are presented in Chap.4. Here we present the methods used to quantify MT expression and GST and CAT activities.

5.2.1 Enzymes activities

To quantify CAT and GST activities, frozen earthworms were individually homogenized using an ultra-turrax (IKA T10, at 15000 rpm) in 5 mL of ice-cold phosphate buffer 100 mM, pH 7.2, 1 mM EDTA. Tissue homogenates were centrifuged at 9000 g for 30 min at 4 °C and enzymes activities and protein contents were recorded in the supernatants, immediately after homogenization since a decrease of activities had been observed when supernatants were stored at -80 °C. Measurements were carried out in duplicate for each individual. Protein contents were determined as described by (Smith et al., 1985), using the bicinchoninic acid method (BCA Protein assay kit BCA-1, Sigma-Aldrich) and bovine serum albumin as a standard. CAT activity was measured according to Claiborne, 1985. The rate of hydrogen peroxide reduction at 240 nm (ϵ = 43,6 M-1.cm-1) was monitored during 1 min at 25 °C. A 20 μl aliquot of each sample was mixed with 1.98 ml of 100 mM phosphate buffer (pH 7.2, 1 mM EDTA). The reaction was initiated by the addition of 500 μl of 10 mM H_2O_2. Measurements were carried out in triplicate for each sample. The CAT activity was corrected for nonspecific

activity and expressed as μmol of H_2O_2 consumed per min per gram of proteins. GST activity was assayed as described by Habig et al., 1974. The substrate used was 1-chloro-2,4-dinitrobenzene (CDNB). The appearance of reduced gluthation (GSH)-CDNB complex was followed at 340 nm (ϵ = 9,6 mM-1.cm-1) for 5 min at 37 °C, after an initial 1 min incubation step. Samples were diluted 1:5 (v:v) with the homogenization buffer. A 5 μl aliquot of each dilute sample was mixed with the assay buffer to achieve the following final conditions: 100 mM phosphate buffer, 2 mM GSH (Sigma-Aldrich-G4251) and 1 mM CDNB (Sigma-Aldrich-237329) in a final volume of 255 μl. Blanks (without sample) and negative control (without GSH) were carried out to insure measurement quality. The final GST activity was corrected for nonspecific reaction and was expressed as μmol of GSH-CDNB complex produced per min per gram of proteins.

5.2.2 MT expression

MT expression was assessed by real-time quantitative PCR (qPCR). In order to select two final reference genes, the expression of four potential candidates were quantified as well: ribosomal protein S13, ubiquitin, actin and catalase.

RNA extraction

Expression analyses were conducted on total RNA extracted from the whole body of individual worms using Trizol reagent (Molecular Research Center, Inc, Cincinnati, USAa) according to manufacturer's instructions. RNA purity and integrity controls and reverse transcription were conducted as previously described Brulle et al., 2006. RNA concentrations were quantified using Nanodrop (Thermo Scientific).

Isolation of the sequences of interest

For *A. caliginosa*, the sequences of the selected effectors were unknown. The effectors were thus cloned and sequenced. Cloning of the effectors was performed using cDNA. cDNA synthesis was conducted on total RNA pooled from 5 individuals randomly picked among the total 163 individuals. Reverse Transcription PCR (RT-PCR) reactions were conducted with 1.5 μg of cDNA using the RevertAid First Strand cDNA Synthesis Kit (ThermoScientific) following the manufacturer instructions. Random hexamer primers were used. In order to isolate the selected effectors sequences, PCR were conducted on cDNA using degenerate sense and antisense primers. The primers were determined according to Brulle et al. 2006.

Table 5.1: Sequences of the primers used to quantify MT, S13, Ubiquitin, Actin and Catalase gene expression

gene	Forward	Reverse
MT	5'- GAACCAGCTGCGCACTTG-3'	5'-TGAATGCCCACCAAACTGC-3'
S13	5'- GGTATTTCCCAATCCGCTCT-3'	5'-GAGATCCTCTGGGATGGTTG-3'
Ubiquitin	5'- GGTGGAATGCCTTCCTTG-3'	5'-GTGAAGACCCTGACGGGAAA-3'
Actin	5'- ACCACTGGTATCGTGC-3'	5'- GCTCGAAGTCGAGAGC-3'
Catalase	5'-CGAACAAGGAGAAGCTGTGT-3'	5'-TGGCCAGATCTTTGTCAGGT-3'

Briefly, protein sequences of the selected effectors in other invertebrate species were collected from NCBI. Alignements were done using MULTALIN. And primers were determined using CODEHOP software. PCR reactions were done with GoTaq Flexi DNA Polymerase (Promega) using the following cycling conditions: 2 min at 95 °C, 10 cycles : 0.5 min at 95 °C, 0.5 min at 65 °C, decrease of 1 °C per cycle, 1 min at 72 °C, then 29 cycles : 0.5 min 95 °C, 0.5min 60 °C, 1min 72 °C, followed by 5 min at 75 °C. Expected size products were resolved on 1% (w/v) agarose gel stained by ethidium bromide and subsequently cloned into the pGEM-T Vector (Promega). The recombinant clones were purified using Wizard Plus SV Miniprep DNA Purified system kit (Promega). Five clones were sequenced in one direction (forward) by Genoscreen (Lille, France). Two clones were sequenced for ubiquitin.

The sequences obtained were used to design specific primers for quantitative PCR. Specific primers were edited with Primer3 software. Primers used are reported in Table 5.1.

Quantitative PCR (qPCR)

qPCR reactions were performed in duplicates on cDNA generated from individual worms using MESA Blue qPCR MasterMix Plus for SYBR assay No ROX (Eurogentec) and LightCycler 480 (Roche). cDNA samples were diluted 50 times in DEPC water. Each reaction was conducted as follows: 2 μl of diluted cDNA, 10 μl of 2x reaction buffer (containing dNTP, Meteor Taq DNA polymerase, MgCl2, SYBR Green I, Blue dye and stabilizers), 2 μl of each primer (initial concentration 100 mM) and 4 μl of DEPC water. The PCR conditions were: one step of activation of MeteorTaq (5 min at 95 °C) followed by amplification and quantification step repeated 40 times (3 seconds at 95 °C, 30 seconds at 60 °C and 10 seconds at 72 °C), melting curve step (15 seconds at 95 °C, 1 min at 60 °C, heating range 0.1 °C per second to 95 °C and continuous fluorescence measurement). Melting curves were analyzed to insure the quality of amplifications (single peaks). Crossing points (CP; number of cycles at which the noise band intersects the fluorescent curve) were determined using the "Fit Point

Method" of the LightCycler 480 Software 1.5.0.39 (Roche). Noise bands were set for each gene as the fluoresence level for which amplification curves were all parallels. Amplification efficiencies (E) of each gene was determined with relative standard curves generated from serial dilutions (1:10, 1:20, 1:50, 1:100, 1:1000, 1:10000) of a cDNA sample constituted from a pool of 10 analyzed cDNA randomly picked among the 160 individuals. Standard curves were based on CP values and log10 values of cDNA dilution. They were highly significant ($p<0.05$) with R2 values > 0.99 for all genes. For each gene, E was calculated from the given slope of the standard curve according to $E = 10^{-1/slope}$. They ranged from 1.93 to 2.14. Levels of expression were calculated according to E^{CP}.

Normalization of gene expression

Identification of reference genes was conducted following the method described by Brulle et al., 2014. CP values for catalase were stable. This gene was included in the selection process in order to determine if it could serve or not as a reference gene. Two algorithms (NormFinder (Andersen et al., 2004) and geNorm (Vandesompele et al., 2002)) were used to rank the four potential reference genes according to their expression stability. NormFinder stability value is based on the combination of intra and inter-group variations. As our experiment is not based on "treatment approach", we segregated the datasets (i) according to the 8 sites under study and (ii) we arbitrarily defined groups of soils according to Cd availability measured by CaCl2 extractions (<40 µg/kg, between 40 and 90 µg/kg, and > 90 µg/kg). NormFinder was used with each set of groups. The results ranked the genes as follows: Ubiquitin > Actin > S13 > Catalase, Ubiquitin being the more stable. geNorm stability value is based on the principle that the ratio of two ideal reference genes is equal in all samples. It performs stepwise removal of the least stable gene according to the pairwise variation of each gene with all the others (M-value). The gene with the highest M value is removed at each step. According to this algorithm : the best reference genes in our experiment were : S13 = Ubiquitin > Catalase > Actin. As different ranking results were obtained, we discarded the least stable genes according to the two algorithms, namely catalase (for NormFinder) and actin (for geNorm). S13 and ubiquitin were thus finally selected as appropriate reference genes. MT relative expression level was calculated as the ratio of MT expression level over the geometric mean of S13 and ubiquitin expression levels and is given in arbitrary units (AU).

Barcoding

As *A. caliginosa* is considered as a complex of species (Pérès et al., 2011), we verified that gene expression variations were not due to the presence of different species by sequencing the COXI mitochondrial gene for every single individual on which qPCR was conducted. Amplification of COXI gene was conducted on the cDNA used for qPCR reactions. The following conditions were used: 2 min at 94 °C, 45 cycles consisting of 30 seconds at 94 °C, 30 seconds at 50 °C and 1 min at 72 °C, and a final step of 5 min at 72 °C. GoTaq Flexi DNA polymerase (Promega) was used. In a final volume of 50 μl, PCR reactions were conducted as follows: 4 μl of cDNA template, 10 μl of buffer, 27.8 μl of water (DEPC), 1 μl of each primer (initial concentration 10 mM), 1 μl of 10 mM dNTP mix, 5 μL of 25 mM MgCl2 and 0.2 μl of 5 U/μl Taq. The 163 sequences of COXI mitochondrial gene were sequenced by Genoscreen (Lille, France). A phylogenetic tree was performed using COXI sequences from other *Aporrectodea* species were collected from the the Consortium for the Barcode of Life . *A. rosea, A. giardi, A. longa, and A. icterica* were used as outgroups. The phylogenetic tree was performed using *Phylogeny.fr* software (Dereeper et al., 2008). MUSCLE alignments are processed (Edgar, 2004), followed by a Gblocks curation step (Castresana, 2000). The PhyML algorithm is used to estimate the phylogeny based on maximum likelihood estimation (Guindon et al., 2010). TreeDyn is finally used to compute the phylogeny tree.

The results showed that 152 individuals belong to one single species (*A. caliginosa*) and segregate in two lineages of *A. caliginosa* previously identified by Pérès et al., 2011 (L2 and L3). 100 individuals were identified as *A. caliginosa* L2, 52 individuals as L3. For 9 individuals, it was not possible to determine the genotype. Using ANOVA, we tested if the lineage affected MT expression. The results showed no significant effect ($F_{2,145}$=0.56, p>0.05). We therefore concluded that the discrepancies observed in MT expression from one worm to the other were not linked to the genetic lineage of the individuals.

5.2.3 Data analysis

The relationships between Cd, Pb and Zn internal concentrations and biomarkers were assessed based on the mean values calculated for each soil (n=5). The data were log-transformed when necessary to meet linearity and normality. Using Pearson's correlations, we assessed if biomarkers were more correlated to metal concentrations in total earthworms or in the subcellular fractions. We further assumed that biomarkers responded to multiple internal metals. Variable selection was conducted to identify which metal subcellular concentrations better explained the biomarkers variations.

An integrated biomarker response index (IBR) was calculated according to Broeg and Lehtonen, 2006. The mean values of each biomarker for each soil were standardized to mean of 0 and a variance of 1. The lowest values were set to zero by adding the value obtain for each station to the absolute value of the minimum value in the dataset. Star plot areas were calculated using the obtained values B_i according to:

$$IBR = [(B_1.B_2)/2] + [(B_2.B_3)/2] + \ldots + [(B_{n-1}.B_n)/2] + [(B_n.B_1)/2]$$

The IBR value was divided by the number of biomarkers (n=6). We used the inverse values of lipid and glycogen contents to calculate the IBR because there decrease is supposed to indicate a stress, while the opposite was expected for the other biomarkers.

5.3 Results and Discussion

5.3.1 Biomarkers in earthworms after exposure to the 31 soils

Before the onset of the exposure experiment (T0), earthworms exhibited MT relative expression level of 0.11 ± 0.05 (mean ± standard deviation (n=8), in arbitrary units (AU)). After exposure to the 31 soils, MT expression ranged from 0.04 to 0.31 with a mean value of 0.13 ± 0.06 (n=31). The mean values for each soil are reported Table 5.2. The highest MT expression were observed in earthworms exposed to contaminated soils irrespective to the total soil metal concentrations (Table 4.1, p.86, Chap.4).

At T0, earthworms exhibited a mean CAT activity of 0.17 ± 0.04, and a GST activity of 0.37 ± 0.08 (n=9). CAT and GST activities after exposure to the 31 soils are presented in Table 5.2. The activities were lower after the exposure experiment than at T0. CAT activity was stable in earthworms exposed to different soils (Table 5.2). GST activity was higher in earthworms exposed to Me and Mo soils (smelter-contaminated) and in the most contaminated soil of Pi site (wasterwater-contaminated). However, in uncontaminated soils (such as Fe and Yv), high GST activities were recorded, indicating that GST was affected by other factors than soil metal contamination.

Lipid, glycogen and protein contents are reported in (Table 4.2, p.89, Chap.4). Lipid contents ranged from 6.7 to 11.9 mg/g fresh weight (FW) with a mean value of 8.9 ± 1.5 mg/g FW. The mean glycogen content was 4.9 ± 1.4 mg glycogen/g FW, ranging from 2.3

Table 5.2: MT relative expression levels, GST and CAT activities in earthworms after exposure to the 31 soil samples. The first two letters of the samples names indicate the site of sampling (see the text for the codification of the sites). Values are the means of five measurements. Values in parentheses are the standard deviations. For the soil Ba2, GST and CAT activities were recorded on a single individual (NA). (CAT U: μ mol H_2O_2 min^{-1}, GST U: μ mol GSH-CDNB min^{-1})

	MT expression (arbitrary units)		GST activity (U/g proteins)		CAT activity (U/(g proteins)	
Cl	0.11	(0.03)	0.22	(0.04)	0.13	(0.02)
Yv1	0.09	(0.04)	0.28	(0.05)	0.15	(0.03)
Yv2	0.06	(0.02)	0.26	(0.02)	0.16	(0.04)
Yv3	0.09	(0.04)	0.21	(0.04)	0.13	(0.03)
Fe1	0.06	(0.02)	0.20	(0.01)	0.11	(0.02)
Fe2	0.05	(0.04)	0.28	(0.05)	0.15	(0.02)
Fe3	0.06	(0.02)	0.28	(0.05)	0.13	(0.02)
Ba1	0.04	(0.01)	0.26	(NA)	0.13	(NA)
Ba2	0.09	(0.08)	0.21	(0.02)	0.17	(0.03)
Mo1	0.14	(0.04)	0.27	(0.05)	0.14	(0.05)
Mo2	0.12	(0.05)	0.31	(0.04)	0.15	(0.03)
Mo3	0.14	(0.04)	0.23	(0.07)	0.15	(0.02)
Mo4	0.23	(0.09)	0.25	(0.02)	0.16	(0.02)
Mo5	0.08	(0.02)	0.26	(0.01)	0.16	(0.01)
Mo6	0.08	(0.03)	0.26	(0.05)	0.16	(0.01)
Mo7	0.12	(0.04)	0.28	(0.02)	0.15	(0.02)
Mo8	0.13	(0.07)	0.25	(0.06)	0.15	(0.02)
Mo9	0.12	(0.06)	0.29	(0.07)	0.15	(0.05)
Me1	0.15	(0.18)	0.27	(0.06)	0.14	(0.04)
Me2	0.14	(0.08)	0.28	(0.01)	0.14	(0.02)
Me3	0.24	(0.24)	0.29	(0.06)	0.16	(0.02)
Me4	0.15	(0.07)	0.24	(0.02)	0.13	(0.01)
Me5	0.14	(0.04)	0.26	(0.02)	0.16	(0.01)
Tr1	0.12	(0.04)	0.28	(0.07)	0.13	(0.02)
Tr2	0.15	(0.09)	0.26	(0.03)	0.16	(0.02)
Tr3	0.15	(0.06)	0.25	(0.04)	0.17	(0.02)
Pi1	0.11	(0.05)	0.21	(0.03)	0.14	(0.01)
Pi2	0.10	(0.04)	0.23	(0.01)	0.12	(0.04)
Pi3	0.26	(0.15)	0.23	(0.07)	0.14	(0.03)
Pi4	0.23	(0.07)	0.23	(0.05)	0.14	(0.02)
Pi5	0.31	(0.16)	0.34	(0.04)	0.14	(0.01)

Table 5.3: Correlations between Cd, Pb and Zn concentrations in three subcellular fractions (cytosol (cyto), debris (deb) and granules (gran)) and in total earthworms (int). Pearson's correlations on log-transformed data. **: $p < 0.01$, ** $p < 0.05$, : $p < 0.1$

	Cd cyto	Cd deb	Cd gran	Cd int	Pb cyto	Pb deb	Pb gran	Pb int	Zn cyto	Zn deb	Zn gran
Cd cyto											
Cd deb	0.15										
Cd gran	0.24	0.74**									
Cd int	0.21	0.12	0.21								
Pb cyto	0.18	0.38*	0.50**	0.07							
Pb deb	0.18	0.52**	0.70**	0.04	0.81**						
Pb gran	0.09	0.45*	0.73**	0.09	0.74**	0.93**					
Pb int	0.24	0.48**	0.67**	0.34 :	0.56**	0.73**	0.68**				
Zn cyto	0.20	-0.62**	-0.27	-0.07	0.01	-0.09	-0.11	-0.03			
Zn deb	0.36*	0.11	0.08	-0.05	0.21	0.26	0.14	0.29	0.42*		
Zn gran	0.11	0.26	0.31 :	0.01	0.11	0.18	0.21	0.11	0.16	0.39*	
Zn int	0.18	0.00	-0.01	0.63**	-0.11	-0.24	-0.19	0.02	-0.01	0.02	0.14

to 8.2 mg/g FW. The average protein content was 57.3 ± 9 mg/g FW, ranging from 36.7 to 75.2 mg/g FW .

5.3.2 Metal concentrations in earthworms and in three subcellular fractions

Metal concentrations after exposure are reported in Table 3.6 (p.75, Chap.3). The cytosolic fractions contained 1.1 ± 0.2 μg Cd/g FW, 0.1 ± 0.1 μg Pb/g FW, and 18.8 ± 2.9 μg Zn/g FW. The debris fractions contained 0.3 ± 0.11 μg Cd/g FW, 0.39 ± 0.3 μg Pb/g FW, and 68.3 ± 9.7 μg Zn/g FW. The granules fractions contained 0.03 ± 0.02 μg Cd/g FW, 0.4 ± 0.6 μg Pb/g FW, and 14.1 ± 3.9 μg Zn/g FW.

Table 5.3 shows the correlations between Cd, Pb and Zn concentrations in the three subcellular fractions and in total earthworms. There were significant relationships between Cd and Pb internal concentrations, especially between the concentrations in the granules and debris fractions.

5.3.3 Relationship between biomarkers and internal metal pools

MT expression increased with total internal Cd and Cd concentrations in the debris and granules fractions (Table 5.4). There was no relationship with Zn concentrations. We found significant correlations with total internal Pb and to a lower extent with Pb concentrations in the three subcellular fractions. MT is not known to be induced by Pb (Kägi, 1991; Morgan et

Table 5.4: Relationships between MT expression, catalase (CAT) and glutathione-s-transferase (GST) activities and Cd, Pb and Zn concentrations in three subcellular fractions (cytosol (cyto), debris (deb) and granules (gran)) and in total earthworms (int). Pearson's correlations on log-transformed variables are reported. **: $p < 0.01$, ** $p < 0.05$, : $p < 0.1$

	MT	CAT	GST	Lip	Prot	Gly
Cd cyto	0.03	0.06	0.28	0.63**	0.03	0.26
Cd deb	0.70**	0.25	0.27	-0.08	-0.18	0.14
Cd gran	0.69**	0.14	0.25	0.05	-0.24	0.37*
Cd int	0.40*	0.31 :	-0.07	0.08	0.33 :	-0.22
Pb cyto	0.40*	0.08	0.41*	-0.02	-0.30 :	0.29
Pb deb	0.48**	0.01	0.37*	0.10	-0.45*	0.36*
Pb gran	0.44*	-0.04	0.35 :	0.12	-0.41*	0.36*
Pb int	0.67**	0.15	0.31 :	0.20	-0.19	0.31 :
Zn cyto	-0.35 :	-0.21	-0.25	0.25	-0.17	0.29
Zn deb	0.13	-0.14	-0.06	0.20	-0.27	0.24
Zn gran	0.15	0.07	-0.17	-0.11	-0.15	-0.02
Zn int	0.14	0.17	-0.31 :	-0.10	0.31 :	-0.16

al., 2004). Those relationships might be explained by the fact that Cd and Pb concentrations in the debris and granules were correlated (Table 5.3).

Variable selection identified debris and granules Cd concentrations as the best predictors of MT expression ($F_{2;28}$ =17.4, 2, 28, p < 0.001, R2=55%).

Figure 5.1 illustrates that Cd concentrations in the debris and granules better explained MT expression than the global internal concentration. The highest MT expression levels remain unexplained by total Cd concentrations, while they are related to the highest Cd concentrations in the debris and granules fractions. Figure 5.1 further shows that it is not possible to distinguish if MT was more related to Cd concentration in the debris fraction or in the granules, both being correlated (Table 5.3). Variable selection indicated that both concentrations significantly affected MT expression.

Our results therefore suggest that MT expression responds to increasing concentrations of Cd in the insoluble fraction (debris+granules). Conder et al., 2002 concluded that the insoluble fraction was indicative of Cd toxic pressure. Other authors consider that the debris fraction is operational and cannot be assigned to a toxic or detoxified internal pool (Wallace et al., 2003; Jones et al., 2009). In the present study, Cd concentrations in debris and granules increased with soil Cd availability while cytosolic fraction concentration remained stable. Thus the insoluble fraction was indicative of both Cd uptake and MT expression.

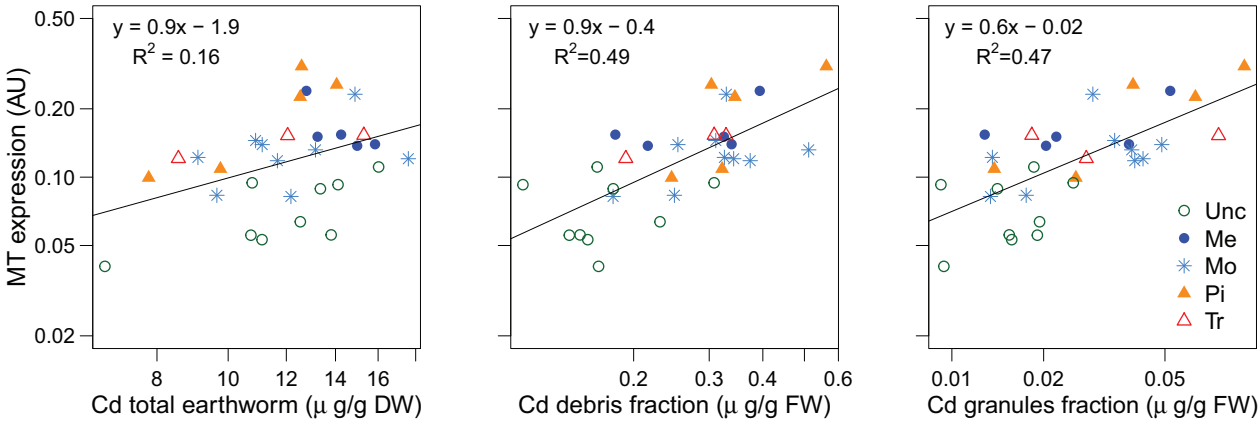


Figure 5.1: MT expression levels (arbitrary units: AU), was better related to Cd concentrations in debris fraction (center panel) and in granular fraction (right panel) than in total earthworm (left panel) on logarithmic scales. Total internal content normalized by the dry weight (DW). Cd concentrations in the subcellular fractions normalized by the fresh weight (FW). Each point is the mean of 5 individuals. Colors indicate different sites with uncontaminated soils (Unc) from the Cl, Ba, Fe and Yv sites. The linear model equation, R^2 and slope (black line) are reported.

5.3.4 GST and CAT activities

There were no significant correlation between CAT activities and Cd, Pb and Zn concentrations in earthworms and in subcellular fractions (Table 5.4). Our results show that characterizing more precisely metal subcellular distribution does not make it possible to explain variations in CAT activity. This is in agreement with previous findings that CAT activity in earthworms is lowly sensitive to metal exposure (Berthelot et al., 2009).

GST activity was correlated to Pb concentrations in the three subcellular fractions (Table 5.4). The relationship with total internal Pb concentration was only significant at $\alpha = 0.1$. As it was observed for MT expression, GST activity was better related to Pb concentrations in two subcellular fractions than with the total internal content.

Variable selection identified Cd, Pb and Zn concentrations in the cytosol as the best predictors of GST activity ($F_{3;27}$ =4, p = 0.02, R2=31%). The effects of Cd and Zn were however merely significant (p<0.1).

The final relationship with cytosolic Pb concentration is shown figure 5.2. Jones et al., 2009 suggested that the cytosolic fraction of Pb represents a toxicologically active fraction. Our results are in agreement with this asumption. Despite the fact that Pb concentrations in the three subcellular fractions were correlated, the response of GST activity was better

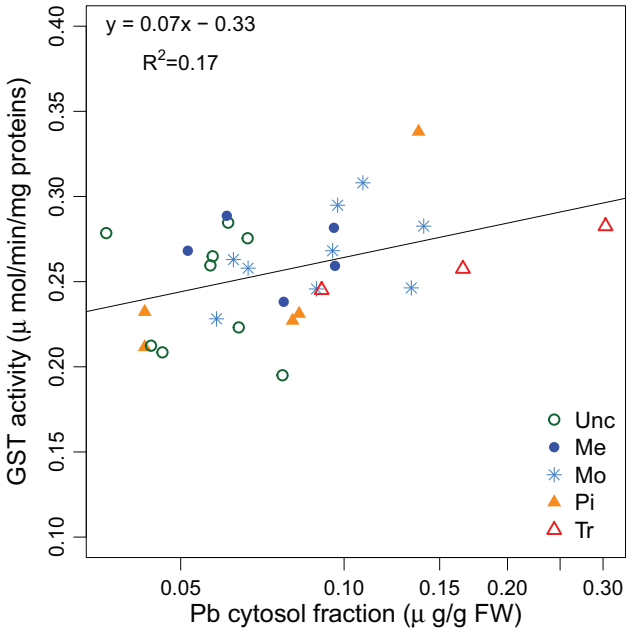


Figure 5.2: GST activity as related to Pb concentration in the cytosol on a logarithmic scale. Each point represents the mean value of 5 individuals. Colors indicate different sites with uncontaminated soils (uncontam) from the Cl, Ba, Fe and Yv sites. The line represents the linear model (bivariate) the relationship, with the model equation and R^2 associated.

explained by the cytosolic concentration of Pb than by debris, granules or total internal concentration. However the low R^2 value of the selected model, together with the various GST activities observed for a given Pb concentration depicted on figure 5.2 shows that GST was mainly influenced by other determinants than metals.

5.3.5 Energy reserves

Lipid contents were correlated to Cd concentration in the cytosol. Protein and glycogen contents were correlated to Pb concentration in the debris and granules fractions, and proteins were correlated to Cd concentration in the granules.

Variable selection indicated that only Cd concentration in the cytosol was significantly related to lipid content ($F_{1;29}$ =19.3, $p < 0.001$, R2=40%). The relationship was positive. This result was surprising given the fact that we observed stable concentration of Cd in the cytosol over the 31 soils (Chap.3 p.67), and because we expected lipid content to decrease with internal metal concentration. This result may suggest that Cd partitioning in the soluble fraction was influenced by biotic parameters.

Concerning protein, variable selection identified Cd concentration in the granules and Zn concentration in the cytosol as the best predictors ($F_{2;28}$ =6, $p < 0.05$, R2=30%). Both coefficients were positive. We observed that Cd concentration in the granules was correlated with Pb concentrations in debris, granules and total earthworms, therefore the relationship with granular Cd concentration might include the effect of Pb. In the case of glycogen contents, variable selection identified Pb concentration in the debris as the best explaining variable ($F_{1;29}$ =7.2, $p < 0.05$, R2=20%).

The results indicated that protein and glycogen contents responded to increased Cd and Pb concentrations in the insoluble fractions rather than to cytosolic or total internal contents. The decrease of glycogen concentration with increasing Pb in the insoluble fraction is consistent with the asumption of an energy cost associated with metal exposure. The increase of protein contents with Cd and/or Pb concentrations in the insoluble fraction can be explained by an increase in protein synthesis to cope with internal metals. An asumption consistent with the elevated MT expression levels and increased GST activity observed in this experiment. Such increased protein contents with contaminant exposure were reported in previous studies (Schreck et al., 2008).

5.3.6 Integrated biomarker response

We finally calculated an index of integrated biomarker response (IBR) in order to give comprehensive information on the biomarkers response. The index ranged from 0.4 (Fe1) to 4.9 (Pi5) with a mean value of 1.8 ± 1.

Table 5.5 shows that the index was correlated significantly with the biomarkers that responded significantly to internal metal concentrations (i.e. there was no correlation with CAT activity and lipid content). The IBR was correlated to Cd concentrations in the insoluble fractions, and with Pb concentrations in the three subcellular fractions and with the total Pb concentration.

Variable selection identified Cd concentration in the granules fraction, and Pb concentration in the cytosol as the best predictors of the IBR ($F_{4;26}$ =4.7, $p < 0.001$, R2=42%).

The relationships between the IBR and the concentrations of granular Cd and cytosolic Pb are shown on figure 5.3. Using a similar approach with Hg contaminated soils, (Colacevich et al., 2011) concluded that the cytosolic concentration of Hg in *L. terrestris* muscle could be considered as a toxic fraction of Hg because it was correlated to the IBR. In our experiment, several metal concentrations in different fractions were correlated to the IBR. Cd concentration in the granules may have been selected as the best predictor for IBR because

Table 5.5: Correlations between the integrated biomarker response index (IBR), individual biomarkers, and Cd, Pb and Zn concentrations in three subcellular fractions (cytosol (cyto), debris (deb) and granules (gran). Pearson's correlations on log-transformed data.

	IBR
MT	0.62**
CAT	0.20
GST	0.51**
Lip	-0.07
Prot	-0.61**
Gly	0.60**
Cd cyto	0.04
Cd deb	0.51**
Cd gran	0.62**
Cd int	-0.05
Pb cyto	0.56**
Pb deb	0.61**
Pb gran	0.56**
Pb int	0.59**
Zn cyto	-0.11
Zn deb	0.11
Zn gran	0.19
Zn int	-0.12

it integrates insoluble Cd and Pb concentrations in total earthworms and in three subcellular fractions.

The most soluble internal pools of metals were thus not systematically the best predictors of biomarkers response. The cytosolic and debris fraction combine several metal pools indicative of both toxicity and detoxification. In addition, some biomarkers are sensitive to metal sequestration (e.g. glycogen) rather than to the presence of metabollicaly active metals. Nevertheless, our results show that for a number of biomarkers, the subcellular fractionation provided better predictors than the total internal contents hence encouraging its use when assessing toxicological bioavailability of metals to earthworms.

5.4 Conclusions

The present study shows that biomarkers may respond to low levels of metal exposure in earthworms. However the relationship is noisy and may not be detected if a small number

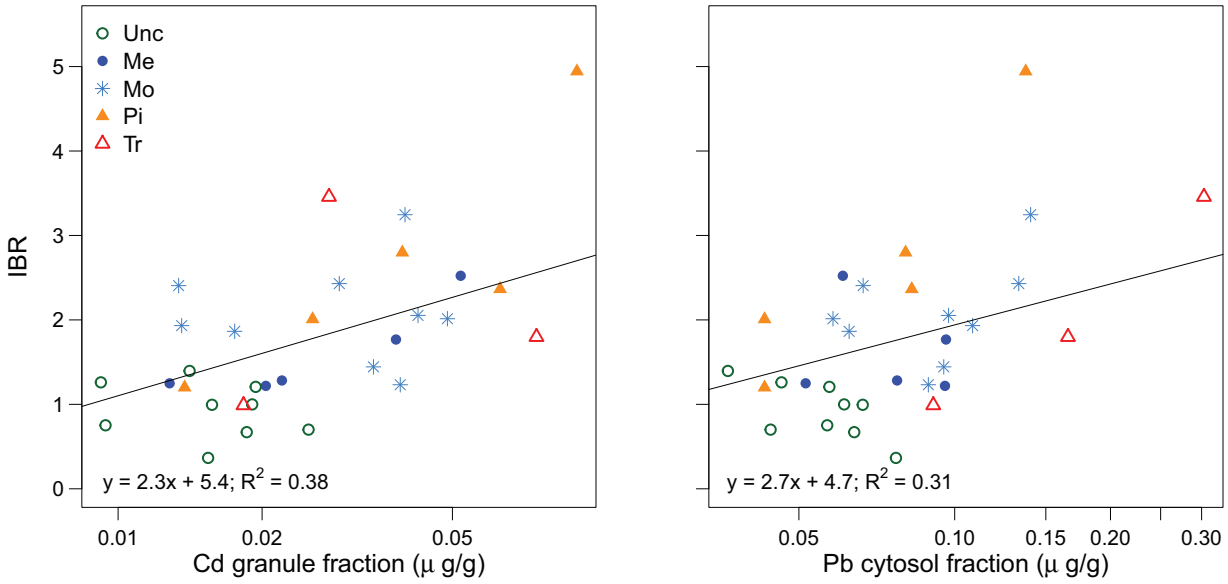
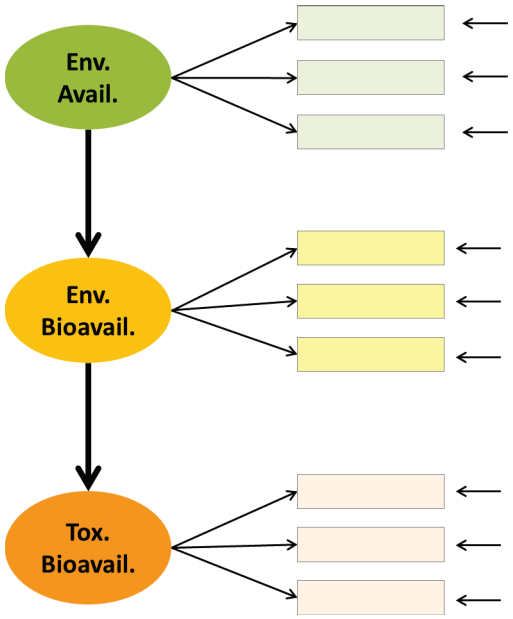


Figure 5.3: Index of integrated biomarker response (IBR, no units) related to Cd concentration in the granular fraction (left panel) and to Pb concentration in the cytosolic fraction (right panel). Each point is the mean value of 5 individuals. Lines indicate the slope predicted by linear (bivariate) models, with equations and associated R^2

of observations are made or if the focus of the analysis is on comparing mean responses between several treatments. Relating internal metal concentrations in subcellular fractions with biomarkers was not facilitated by the correlations between metal concentrations: between a given metal in different fractions, and between different metals. Our results indicated that metal concentrations in both insoluble and soluble fractions explained biomarkers response depending on the metal and on the biomarker considered.

Chapter 6



The previous chapters explored the relationships between the single manifest variables of each step of bioavailability. In the following chapter, single indicators are used to reflect three latent variables, each representing a step of bioavailability. The relationships are now investigated at the scale of these theoretical constructs and no longer between individual measurements.

We have seen previously that geochemical modeling predictions were highly correlated to the total metal content in soil. This was also the case of the predicted concentration of metals bound to organic matters, as pointed out in chapter 3 (p.69). In the SEM of bioavailability, total soil metal concentration was thus considered as an integrative measurement of the strongly bound metal pools, and was used in place of the chemical assessments of these pools in the SEM.

Chapter 6

A structural equation model of soil metal bioavailability to earthworm: confronting the causal theory to observations following a laboratory exposure to field-contaminated soils [1]

6.1 Introduction

The concept of bioavailability has reached a clarified and structural definition that is now widely accepted by the scientists (Peijnenburg et al., 1997; Lanno et al., 2004). A framework to study bioavailability based on this definition has even been normalized (Harmsen, 2007; ISO 17402, 2008). Bioavailability is described as a three-step process in which (i) environmental availability (ea) designates the physico-chemical processes governing the desorption of a contaminant, (ii) environmental bioavailability (eb) reflects uptake processes of the contaminant by the organism and (iii) toxicological bioavailability (tb) represents the processes by which a fraction of internal metals leads to toxicological effects.

In the case of soil metal contamination, different measurements or indicators of bioavailability exist (Peijnenburg et al., 2007; ISO 17402, 2008). Chemical measurements (extrac-

[1] Léa Beaumelle, Denis Vile, Isabelle Lamy, Franck Vandenbulcke, Frédéric Gimbert, Mickaël Hedde. A structural equation model of soil metal bioavailability to earthworm: confronting the causal theory to observations following a laboratory exposure to field-contaminated soils *Environmental Pollution*, (in prep.)

tions, speciation models) reflect (ea) and biological measurements (internal concentrations, biomarkers, toxicological endpoints) reflect (eb) or (tb). A number of confounding factors (soil properties, biological processes, behavior, etc.) can affect the indicators of (ea), (eb) and (tb) and their relationships. The causal relationships implied by the definition of bioavailability therefore remain to be addressed, notably in the context of field-contaminated soils.

In situ, soils are often moderately or lowly contaminated. In addition, they exhibit contrasted physico-chemical properties that can influence both metal speciation and the organism physiology and behavior. Field-contaminated soils are also frequently contaminated by multiple contaminants. In order to identify pertinent and generic indicators of metal bioavailability, it is crucial to address the strength of the relationships between (ea), (eb) and (tb) in such a context, especially for soil dwelling organisms that are exposed to metals in soil and are affected by soil properties.

Earthworms are well known bio-indicators of soil quality, notably because of their functional importance (Pérès et al., 2011). It remains unclear whether earthworms are mainly exposed to metals in the soil solution ('actual' available (Harmsen, 2007)) or to other sources including metals bound to soil organic matters that earthworms digest. Several studies demonstrated that earthworms are mainly exposed to metals present in the soil solution (Saxe et al., 2001; Vijver et al., 2003; Scott-Fordsmand et al., 2004). However no chemical indicator was proven generic to mimic metal bioavailability for earthworms, and soil total metal concentrations were often better descriptors of internal concentrations in earthworms (Hobbelen et al., 2006). Internal metal concentrations have been criticized as indicators of bioavailability because of their complexity (Luoma and Rainbow, 2005). They are the result of mechanisms of both absorption and excretion, and their relationship with toxicity are complex (Vijver et al., 2004). But at the same time, they are considered as good indicators of metal exposure (Lanno et al., 2004; Luoma and Rainbow, 2005). Within a structural definition of bioavailability, the position of internal metal content is unclear: in ISO 17402, 2008, bioaccumulation is a measurement of toxicological bioavailability, while Lanno et al., 2004 proposed to consider bioaccumulation as intermediary between environmental bioavailability and toxicological bioavailability. In earthworms, biomarkers are used as early-warning signals of metal exposure. If several biomarkers were shown to respond to metal contamination, it is unclear if their response is generic in the case of field-contaminated soils that often combine multiple contamination and low doses, and in which confounding factors such as the soil characteristics, may modulate the effects of metals.

In this study, we argue that a graphical modeling approach, structural equation modeling (SEM), can be used to identify the most pertinent indicators of bioavailability. SEM has

been widely used in social sciences, and is now more and more used in ecology. It is a multivariate analysis that can represent structural relations and theoretical variables (Grace et al., 2012). SEM provides a way to confront a causal theory such as the three-step bioavailability concept to empirical observations. Theoretical entities (latent or construct variables) can be integrated in SEM as unobserved variables reflected by several indirect observed measurements (manifest variables or indicators) (Grace et al., 2010). In the three-step definition of bioavailability, each step is an unobserved variable for which several indirect measurements exist. In the model, latent are the underlying cause of covariation between their manifest variables so that the correlation between manifest variables reinforces the validity of the construct instead of leading to biased coefficients such as in multiple regression. In the three-step definition of bioavailability, there is an underlying causal connection between (ea), (eb) and (tb) that can be represented in SEM. Instead of relating individual observed variables to each other, causal relationships in SEM are assumed between theoretical constructs that are reflected by several observed variables. In SE models, causal relationships are reflected by the covariances between the manifest variables. A model-implied covariance matrix is predicted based on an *a priori* model. The causal relationships are tested by comparing the structure of covariances predicted by a causal model to the observed structure of covariances (Shipley, 2002).

In this study, we tested a SE model of bioavailability of metals for earthworms using the results of a laboratory exposure to a wide panel of field-contaminated soils. The model was used to assess the relevance of several indicators frequently used in the literature, by confronting them to the conceptual causal framework. We assumed that the most pertinent indicators must give an illustration of the widely accepted three-step definition of bioavailability in a range of soils moderately contaminated by multiple metals and presenting various soil characteristics.

6.2 Model description

The *a priori* structural model was constructed based on the three-step definition of bioavailability (Harmsen, 2007; ISO 17402, 2008) and is depicted Figure 6.1. Each step was modeled by a latent variable and there is a flow of positive causation from (ea) to (eb) and from (eb) to (tb): the more metals are available in the soil, the more they enter the organism, and an effect can only be observed if metals have entered the body. The measurement model represents the relationships between latent variables and their manifest variables.

(ea) Latent variables are not additive combination of observed variables, so that it is not

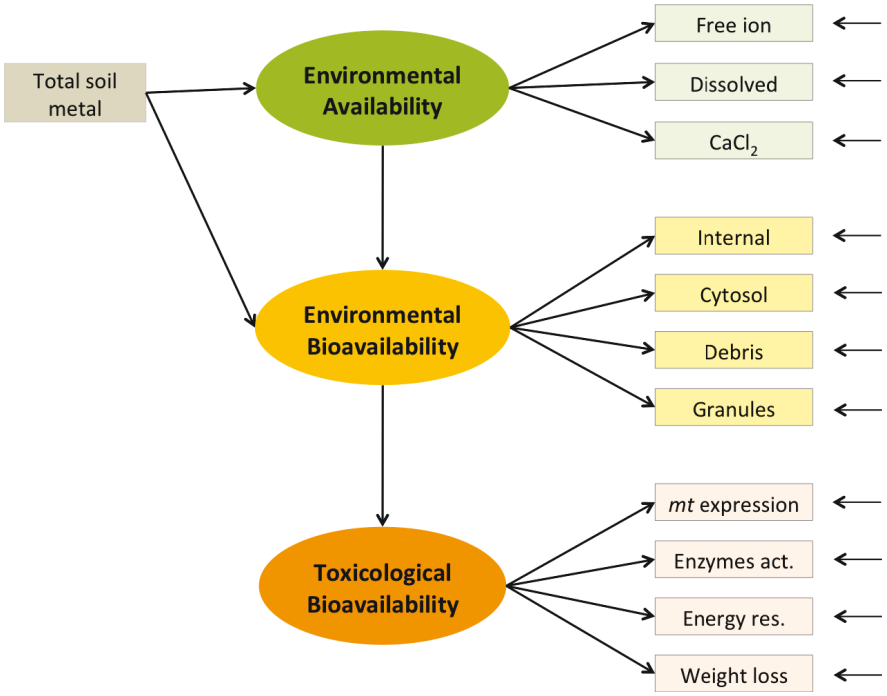


Figure 6.1: A structural equation model (SEM) of metal bioavailability for earthworms. Latent variables are shown in ellipses, observed variables are shown in rectangles. Unidirectional arrows indicate cause-effect relationships. Manifest variables of environmental availability (Env. Avail.) were metal concentration obtained after $CaCl_2$ extraction, semi-mechanistic model of dissolved concentration and geochemical model of free ion content. Environmental bioavailability (Env. Bioavail.) was reflected by metal concentration in total earthworm (internal) and in three subcellular fractions (cytosol, debris and granules). Toxicological bioavailability (Tox. Bioavail.) was indirectly measured by biomarkers from different levels of biological organization; expression of the gene coding metallothionein protein (MT expression), energy reserves, GST and CAT activities (Enzymes act.) and weight loss.

possible to consider (ea) in the SE model as the sum of all available fractions (Grace and Bollen, 2008). In the SEM model, the latent variable "availability" was thus defined as the 'actual' available metal pools (present in the soil solution and easily extractable, (Harmsen, 2007; ISO 17402, 2008)), with the assumption that there was a common process of desorption that lead to observe metals in the soil solution and as free ion in the solution. Three commonly used measurements of 'actual' available metal were chosen as manifest variables of (ea): $CaCl_2$ extraction, dissolved concentrations predicted by an empirical model (Sauvé et al., 2000), and free ion concentrations predicted by a geochemical model of speciation. In order to test whether earthworms are exposed only to the 'actual' available metals or to other sources of metals in soil, soil total metal concentration was included in the model. We assumed that

the total concentration affects directly (ea) because available metals concentrations depend on the level of total contamination. In addition, we further considered a direct effect of soil total concentration on (eb) in order to test if the relationship between (eb) and total concentrations could be explained solely by an indirect effect through (ea) or not and to quantify their relative effects.

(eb) We defined environmental bioavailability as the uptake processes leading to the observed concentrations of metals in earthworms and in three subcellular fractions (cytosol, cellular debris and granules). Internal metal concentrations alone are insufficient to accurately reflect (eb). Metal concentrations in subcellular fractions were proposed as interesting tools to assess metal accumulation in earthworms (Vijver et al., 2004). Subcellular partitioning can separate three subcellular fractions: a cytosolic fraction, a fraction containing tissue, intact cells and membranes (debris) and a granular fraction containing metal-rich granules that are supposed to play a key role in detoxification by sequestration (Wallace et al., 2003). We assumed that the underlying cause for the joint variation in total internal concentrations and in the three subcellular fractions was the uptake processes. In other words, we considered that the increased concentrations in total worm and subcellular fractions were the manifestation of the uptake processes.

(tb) toxicological bioavailability was defined as the fraction of internal metal leading to an effect on several biomarkers reflecting different processes by which metals exert their effects. The expression of the metallothionein (MT) gene is known to increase in earthworms following metal exposure, especially Cd exposure (Bernard et al., 2010). Several enzymes involved in the response to oxidative stress were shown to respond to metal contamination. Two enzymes were often used in the literature: catalase (CAT) and glutathion-S-transferase (GST) (Łaszczyca et al., 2004; Maity et al., 2008). Furthermore, energy reserves (lipid, glycogen and protein contents) are assumed to decrease following metal exposure because of an energy cost of dealing with metals (Holmstrup et al., 2011). Although protein contents may increase as a result of increased protein synthesis to cope with contaminant exposure (Schreck et al., 2008). Finally, metal exposure can impair growth and lead to significant weight loss (Smith et al., 2010). We considered earthworm weight loss as reflecting toxicological bioavailability.

The model assumes linear relationships between the variables. Although it is a rather simplistic and strong assumption, we believe it is appropriate in a primary approach such as the one proposed in this study. The pertinent indicators are therefore defined as linearly related to the other variables in the SEM model.

Testing this model, three main questions were addressed: First, if the indicators reflected the latent variables (the measurement model), second, if the causal structure between latent

Table 6.1: Ranges of selected soil properties over the 31 soil samples under study

	minimum	maximum	median
pH	5.36	8.27	7.35
CEC (cmol/kg)	3.03	30.48	8.12
organic C (g/kg)	9.49	41.50	16.06
Clay (g/kg)	54.20	476.00	123.20

variables was consistent with the data (the structural model) and third, if earthworms were mainly exposed to 'actual' available metals or to other sources by testing the direct effect of total metal concentrations on (eb). The model was tested for Cd, Zn and Pb separately in a primary approach.

6.3 Methods

6.3.1 Experimental section

Studied soils

The experiment has been described elsewhere (Beaumelle et al., 2014) (Chap.4, p.80). Briefly, 31 soils were chosen to achieve a wide gradient of metal availability. The soils were sampled from 8 different sites of the North of France. Four sites had no previous history of metal contamination, two sites were contaminated by deposition of dusts following industrial activities and two sites were contaminated by wastewater spreading. Soil characterization was reported previously. The ranges of pH, CEC, organic C and clay contents are given Table 6.1.

Exposure experiment

Adult earthworms of the species *Aporrectodea caliginosa* (Savigny 1826) were sampled from an unpolluted plot. They were exposed during 21 days in microcosms. Five replicate microcosms were conducted for each soil. Each microcosm contained 6 individuals and 600 g of air-dried soil, sieved to 2 mm. Earthworms had their gut cleared during two days on moistened filter paper before and after the experiment (except the worms for which energy reserves and enzymatic activities were analyzed).

Soil analyses

Soil analyses were described previously (Chap. 4, p.80; Chap.3, p.55 and Chap.2, p.26). For the SE model, we quantified several soil metal pools experimentally and theoretically: (i) total concentrations were quantified following tri-acid digestion (Chap.4), (ii) 0.01 M $CaCl_2$ extractions were performed according to Houba et al., 1990, (iii) dissolved metal contents were calculated following Sauvé et al., 2000 (the equations included pH, organic matter content and total metal concentration) and (iv) free ion concentrations were obtained using a multisurface speciation model. A detailed description can be found in Chap.3, p.59. Input data were EDTA extractable metal concentrations, pH, soil organic matter (SOM) and dissolved organic matter (DOM) characteristics, metal (hydr)oxides contents and the concentrations of cations and anions in the soil solution.

Metal quantification in earthworms

Total internal concentrations were quantified following nitric acid digestion as described in Chapter 3. Subcellular fractionation was conducted to separate three subcellular fractions following serial centrifugations: cytosol (supernatant of the first centrifugation step), debris (supernatant of the second centrifugation step) and granules (pellet of the second centrifugation step). The complete description of the procedure is given in Chapter 3 (p.57). Metal were quantified in digested solutions by inductively coupled plasma mass spectroscopy at the 'Laboratoire d'Analyse des Sols' (INRA, Arras, France).

Biomarkers

MT expression was measured by real-time quantitative PCR (qPCR) as described in Chap.5 (p.102). RNA were extracted from whole individuals, cDNA was obtained after retro-transcription. qPCR was conducted on cDNA using primers designed to be specific of the MT gene of *A. caliginosa*. Two reference genes were used to normalize MT expression: S13 and ubiquitin. CAT and GST activities were quantified following Claiborne, 1985 and Habig et al., 1974 respectively (see Chap.5 p.101). Energy reserves were measured as described in Chapter 4 (p.81). Lipid contents were quantified following Folch et al., 1957 and Knight et al., 1972, glycogen contents following Holmstrup et al., 2011 and protein contents after Smith et al., 1985.

Table 6.2: Untransformed data characteristics: units, ranges, minimum and maximum values, means and standard deviations (sd). For units of biological indicators: DW stands for dry weight and FW for fresh weight.

	units	min	max	median	sd
Cd total	mg/kg soil	0.18	8.32	0.95	2.06
Cd $CaCl_2$	μg/kg soil	0.50	361.40	65.86	87.85
Cd free ion	μg/kg soil	0.00	0.69	0.01	0.14
Cd dissolved	μg/L soil solution	0.05	5.52	0.96	1.31
Pb total	mg/kg soil	19.64	491.00	53.46	136.99
Pb $CaCl_2$	μg/kg soil	1.50	288.00	2.38	57.07
Pb free ion	μg/kg soil	0.00	3.49	0.00	0.64
Pb dissolved	μg/L soil solution	0.36	6.18	1.85	1.40
Zn total	mg/kg soil	40	1004	134	241
Zn $CaCl_2$	μg/kg soil	5	19980	612	4743
Zn free ion	μg/kg soil	0	875	2	173
Zn dissolved	μg/L soil solution	4	361	59	90
Cd internal	mg/g DW	6.795	17.590	12.538	2.524
Cd cytosol	mg/g FW	0.659	1.657	1.059	0.248
Cd debris	mg/g FW	0.110	0.562	0.303	0.105
Cd granules	mg/g FW	0.009	0.091	0.022	0.020
Pb internal	mg/g DW	2.200	46.919	5.046	8.883
Pb cytosol	mg/g FW	0.036	0.303	0.077	0.051
Pb debris	mg/g FW	0.133	1.237	0.280	0.304
Pb granules	mg/g FW	0.075	2.640	0.204	0.596
Zn internal	mg/g DW	658.40	1000.43	832.07	86.73
Zn cytosol	mg/g FW	14.32	24.81	17.80	2.86
Zn debris	mg/g FW	52.92	85.89	66.90	9.67
Zn granules	mg/g FW	9.29	23.61	13.54	3.88
MT	arbitrary unit	0.040	0.308	0.121	0.064
Lipid	mg/g FW	6.65	11.89	8.83	1.51
Protein	mg/g FW	36.66	75.15	58.01	8.98
Glycogen	mg/g FW	2.30	8.16	5.03	1.39
GST	U/mg proteins	0.195	0.338	0.259	0.032
CAT	U/mg proteins	0.108	0.170	0.148	0.015
Weight loss	% of initial weight	− 15.6	4.5	−5.5	4.6

Data analysis

Data were the means of 5 observations (replicated microcosms) for each soil. Table 6.2 gives the characteristics of the untransformed variables for the three metals. Except weight loss that was arcsin transformed, all variables were log-transformed for the analysis in order to

achieve linearity and normality. Bivariate correlations (Pearson) were used to assess the bivariate relationships between single observed variables.

In SEM, the graphical model Figure 6.1 is translated into structural equations. The parameters to be estimated are path coefficients (arrows 6.1) and error variances of latent and observed variables. Path coefficients give the magnitude of the effect of one variable on the other, holding constant all the other variables. Standardized path coefficients are useful to compare path coefficients, because they do not depend on the scale of the variables. Error variances indicate the effects of external factors not included in the model. (Shipley, 2002; Pugesek and Tomer, 2003).

Generally in SEM, parameters are estimated using a maximum likelihood method. The estimation method requires multivariate normality of endogenous variables (Shipley, 2002), although when the emphasis of the model is on linkages rather than predictions such as in this study, Grace et al., 2012 indicated that linear Gaussian approximation was often justifiable. The assumption of multivariate normality of endogenous variables was checked with Mardia's coefficient. Transformed data did not show significant skew but significant kurtosis was detected in the case of the Cd dataset. We used maximum likelihood estimation with robust standard errors and a Satorra-Bentler scaled test statistic as it is effective in the cases of non-normal data and moderate sample size (Rosseel, 2012).

The goodness-of-fit of the model against the data was evaluated by a Satorra-Bentler scaled chi-square statistic. Basically, the observed covariance structure is compared to the covariance structure predicted by the model. The null hypothesis of the test is that the two covariance matrices are not different, the model is thus rejected if the p-value of the test is < 0.05. The fit of the model was further assessed by three indicators of fit: the root mean square error approximation (RMSEA), the standardized root mean square residual (SRMR), and the comparative fit index (CFI). Good model fits are reflected by values below 0.05, 0.08 and above 0.95 respectively for RMSEA, SRMR and CFI respectively (Pugesek and Tomer, 2003).

We first used confirmatory factor analysis (CFA) to test the measurement model for each metal, *i.e.*the relationships between the latent variables and their indicators. CFA is identical to SEM except that the relationships between latent variables are correlations and not causal relationships. In the CFA, we did not include the effects of soil total metal concentration, only the three latent variables related by correlations and their manifest variables (Fig. 6.1). The selection of the final measurement model was conducted by removing the manifest variables for which the paths were nonsignificant ($p>0.05$) and by considering only measurement models that were consistent with the data (according to the χ^2 and p-value of the whole

Table 6.3: Correlations between individual biomarkers. Pearson's correlation coefficients on log-transformed values (arcsin-transformed for weight loss). '**' p-value < 0.01, '*' p-value < 0.05 and ':' p-value < 0.1

	MT	Lipids	Proteins	Glycogen	GST	CAT
Lipids	−0.13					
Proteins	0.12	0.42*				
Glycogen	−0.01	0.06	−0.45*			
GST	0.16	0.40*	0.27	−0.27		
CAT	0.29	−0.05	−0.22	0.27	0.30	
Weight.loss	0.25	−0.23	0.03	0.01	−0.06	0.25

model).

The final measurement models were used to test the SEM including the structural relationships and the effect of soil total metal concentration on (ea) and (eb) (Fig. 6.1). The models were respecified based on (i) the residual covariances (*i.e.*observed - predicted covariance), (ii) the modification indices (or Lagrange multipliers). The modification indices estimate how the χ^2 test statistic of a model would improve if a particular parameter were added. We considered adding a given path if the modification index was above 4 and if it was scientifically supported (Pugesek and Tomer, 2003). During the re-specification process, we kept model complexity minimal and avoided to 'over adjust' the model to the dataset by extending data analysis.

The models were tested using the R-package lavaan (Rosseel, 2012), (R Core Team, 2014).

6.4 Results and discussion

6.4.1 SEM for Cd bioavailability

Tables 6.3 and 6.4 gives the correlations between the variables in the SE model of Cd bioavailability. All soil Cd concentrations were highly and significantly correlated. Among Cd internal contents, only the concentrations in the debris and granules were correlated significantly. Biomarkers were not correlated to Cd concentrations in the soil or in earthworms, with the exception of MT expression. Contrarily to the soil and earthworm Cd concentrations, we found only a few significant correlations between biomarkers (e.g. between lipid and protein but not between lipid and glycogen contents Table 6.3).

Table 6.4: Correlations between manifest variables in the SEM of Cd bioavailability. Pearson's correlation coefficients on log-transformed variables '**' p-value < 0.01, '*' p-value < 0.05 and ':' p-value < 0.1

	Total.Cd	CaCl2.Cd	Diss.Cd	Free.Cd	Internal.Cd	Cytosol.Cd	Debris.Cd	Granules.Cd
Total.Cd								
CaCl2.Cd	0.69 **							
Diss.Cd	0.75 **	0.95 **						
Free.Cd	0.88 **	0.65 **	0.74 **					
Internal.Cd	0.32 :	0.37*	0.27	0.25				
Cytosol.Cd	0.04	0.17	0.21	−0.04	0.21			
Debris.Cd	0.68 **	0.67 **	0.72 **	0.70 **	0.12	0.15		
Granules.Cd	0.66 **	0.65 **	0.66 **	0.54 **	0.21	0.24	0.74 **	
MT	0.71 **	0.69 **	0.66 **	0.64 **	0.40*	0.03	0.70 **	0.69 **
Lipids	−0.16	−0.07	−0.04	−0.18	0.08	0.63 **	−0.08	0.05
Proteins	0.17	0.19	0.23	0.17	−0.24	0.26	0.08	0.34 :
Glycogen	−0.07	0.01	−0.07	−0.17	0.33 :	0.03	−0.18	−0.24
GST	0.23	0.20	0.22	0.18	−0.07	0.28	0.27	0.25
CAT	0.27	0.26	0.20	0.06	0.31 :	0.06	0.25	0.14
Weight.loss	0.20	0.11	0.07	0.20	0.01	−0.36*	0.18	0.04

The CFA was used to identify the measurement model (which manifest variables for each latent). Several manifest variables were excluded because paths were nonsignificant. The analysis confirmed that (ea) was reflected by dissolved, $CaCl_2$-extractable and free Cd concentrations. For (eb), only Cd concentration in the debris and granules were identified as manifest variables. Total internal content, and Cd concentration in the cytosol were not retained as pertinent manifest variables for (eb). The latent (tb) was only reflected by MT expression. The results of the CFA were in agreement with the patterns of multivariate correlation (Table 6.4).

Using the results of the CFA, the initial SEM (Fig. 6.1) was re-specified. Since Cd affected only one biomarker significantly, we replaced (tb) by a latent variable representing the induction of the MT gene. The latent variable was assumed perfectly measured by the observed MT expression level by fixing the measurement error variance of MT to 0. The model was not consistent with the data (Scaled χ^2=45.04, df=12, p= 0). The p-value indicated that observed and predicted covariances were significantly different.

There was an elevated residual covariance between free ion concentration and soil total content: the model underestimated the observed correlation. Thus this correlation was not fully explained by an indirect effect of total Cd concentration on the free ion concentration through (ea) as assumed in the model. In addition, the error variance of the free Cd concentration was elevated compared to dissolved and $CaCl_2$-extractable concentrations. Finally, modification indices revealed that adding a residual correlation between $CaCl_2$-extractable

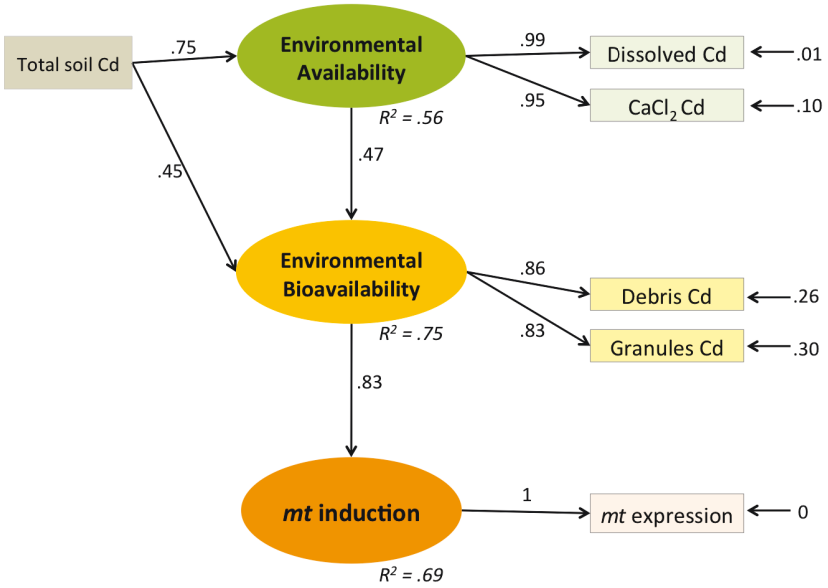


Figure 6.2: Final SEM model of Cd bioavailability describing the relationships between Cd contamination (soil total content), 'actual' availability, uptake by earthworms and induction of the MT gene. Standardized path coefficients are shown on edges (path coefficients) and represent partial regression coefficients. Standardized error variances are shown at the right of the arrows pointing to the observed variables and indicate the proportion of variance of the observed variables unexplained by its latent factor.

and dissolved Cd concentrations would improve model fit, and thus the relationship between the two manifest variables was not fully captured by the latent variable (ea) with the free ion concentration as a manifest variable. We removed the free ion concentration from the model based on these results.

The final model provided a good fit of the data (Scaled χ^2=6.48, df=7, p= 0.49). Figure 6.2 shows the final SEM and the standardized path coefficients. The unstandardized estimates are given in supplementary information. Indices of approximate fit further indicated the good correspondence between the observed and predicted covariance structures (RMSEA = 0, CFI = 1, SRMR = 0.02).

In this study, the conceptual definition of Cd bioavailability was supported by empirical observations made over a range of field-contaminated soils. The results showed that the increase of Cd concentrations in earthworms and subsequent effect on MT expression depended not only on readily available Cd, but also on the total soil concentration. The standardized coefficients indicated that the effects of total and available Cd on (eb) were of the same order of magnitude (Fig. 6.2). The assumption that earthworms are mainly exposed to metals

from the soil solution is therefore not confirmed by our findings.

In our conditions, several indicators were consistent with the causal structure proposed. $CaCl_2$ extraction and Sauvé empirical model provided consistent indirect measurements for a fraction of Cd available for earthworms. The free Cd concentration predicted by the speciation model was however highly correlated with the total soil concentration. This was surprising given the wide range of soils considered. In our conditions, this indicator was not pertinent to indicate readily available Cd and rather reflected the contamination level, along with total soil concentration. To our knowledge, no study reported the correlation between total metal soil content and predicted concentrations in the soil solution on such a range of agricultural soils with low levels of metal contamination. However, in aquatic system Iwasaki et al., 2013 showed high correlation between the input concentrations in the model (*i.e.*total dissolved) and the output concentrations (*i.e.*free ion activity). The results therefore indicate that future work is necessary to determine if this is a general feature of speciation models or if it is due to the panel of soils considered (arable soils with low to moderate levels of contamination).

Cd concentrations in the granules fraction and in the debris fraction jointly indicated the latent (eb) affecting the expression of the MT gene. This was in agreement with Conder et al., 2002 who showed the insoluble (debris and granules) fraction to be related to toxicological effects of Cd on *Eisenia fetida*. On the contrary, total internal and cytosol Cd concentrations did not covary with chemical indicators or with MT expression. Their levels were quite stable despite the wide gradient of Cd availability. This may be due to the short duration of the exposure and the low levels of Cd bioavailability (Spurgeon and Hopkin, 1995). The results therefore highlight Cd concentrations in the insoluble fraction as a sensitive endpoint of Cd exposure over a short-term exposure to field-contaminated soils.

MT expression was the only biomarker affected in a consistent manner by Cd and was not correlated to the other biomarkers. Translating the conceptual definition of bioavailability within a SEM framework, we found that it is not reasonable to assume a common toxicological portion of internal Cd jointly affects several sub-individual biomarkers in our range of field soils. The available Cd levels in soils were low to moderate and the biomarkers may be jointly affected in a consistent manner only after a certain threshold concentration. Furthermore, sub-individual biomarkers are sensitive to a number of confounding factors, and to multiple metals. Non-linear relationships and more complex networks of relationships (notably including soil parameters) might be more appropriate to account for biomarkers response. MT expression was the only endpoint consistent with the structural model. It has been suggested that MT expression responded to other factors than metals in field-contaminated soils (Pérès

Table 6.5: Correlations between the manifest variables of the SEM of Zn bioavailability. Pearson's correlation coefficients on log-transformed variables. '**' p-value < 0.01, '*' p-value < 0.05, and ':' p-value < 0.1. Correlations between biomarkers are reported Table 3

	Total.Zn	CaCl2.Zn	Diss.Zn	Free.Zn	Cytosol.Zn	Debris.Zn	Granules.Zn	Internal.Zn
Total.Zn								
CaCl2.Zn	0.63 **							
Diss.Zn	0.54 **	0.94 **						
Free.Zn	0.86 **	0.73 **	0.65 **					
Cytosol.Zn	−0.28	−0.14	−0.09	−0.25				
Debris.Zn	0.17	0.07	0.05	0.31 :	0.42*			
Granules.Zn	0.11	0.09	−0.05	0.12	0.16	0.39*		
Internal.Zn	0.01	−0.16	−0.15	0.00	−0.01	0.02	0.14	
MT	0.67 **	0.64 **	0.52 **	0.69 **	−0.35 :	0.13	0.15	0.14
Lipids	−0.16	−0.09	0.03	−0.18	0.25	0.20	−0.11	−0.10
Proteins	0.24	0.28	0.33 :	0.25	0.32 :	0.23	−0.02	−0.15
Glycogen	−0.23	−0.19	−0.22	−0.38*	−0.17	−0.27	−0.15	0.31 :
GST	0.25	0.24	0.23	0.23	−0.25	−0.06	−0.17	−0.31 :
CAT	0.24	0.21	0.19	0.05	−0.21	−0.14	0.07	0.17
Weight.loss	0.20	0.09	0.02	0.09	−0.11	−0.01	0.15	0.02

et al., 2011). The results demonstrate that the effect of Cd (ea) and (eb) surpassed the potential effects of such confounding factors. The fact that the model explained high amounts of MT variations (R^2 0.7) suggests the sensitivity and the specificity of this biomarker for Cd in a context of moderately and field-contaminated soils.

6.4.2 SEM for Zn bioavailability

Table 6.5 shows the correlations between the variables involved in the SEM of Zn bioavailability. The internal Zn concentrations were not related to the soil Zn concentrations considered. Biomarkers were also poorly related to soil Zn concentrations. There was no consistent pattern in the correlations between earthworm Zn concentrations (e.g. cytosol concentrations were correlated to the debris concentration but not to the concentration in the granules, while debris and granules concentrations were correlated). Biomarkers were weakly related to Zn concentrations in earthworms.

For Zn it was not possible to find a consistent measurement model, most likely due to the absence of consistent correlations between the internal Zn concentrations considered. The results reflected the well-known regulation of Zn in earthworms (Spurgeon and Hopkin, 1999; Giska et al., 2014). It was assumed that subcellular fractionation would allow for a better description of (eb) than global body loads. The results demonstrate that Zn concentrations

Table 6.6: Correlations between the manifest variables of the SEM of Pb bioavailability. Pearson's correlation coefficients on log-transformed variables. '**' p-value < 0.01, '*' p-value < 0.05, and ':' p-value < 0.1. Correlations between biomarkers are reported Table 3

	Total.Pb	CaCl2.Pb	Diss.Pb	Free.Pb	Cytosol.Pb	Debris.Pb	Granules.Pb	Internal.Pb
Total.Pb								
CaCl2.Pb	0.36*							
Diss.Pb	0.52 **	0.47 **						
Free.Pb	0.87 **	0.50 **	0.71 **					
Cytosol.Pb	0.50 **	0.22	0.47 **	0.54 **				
Debris.Pb	0.64 **	−0.08	0.41*	0.57 **	0.81 **			
Granules.Pb	0.54 **	−0.11	0.26	0.43*	0.74 **	0.93 **		
Internal.Pb	0.70 **	0.00	0.46 **	0.59 **	0.56 **	0.73 **	0.68 **	
MT	0.70 **	0.42*	0.57 **	0.72 **	0.40*	0.48 **	0.44*	0.67 **
Lipids	−0.21	−0.35 :	0.01	−0.26	−0.02	0.10	0.12	0.20
Proteins	0.16	−0.20	0.30	0.17	0.27	0.33 :	0.34 :	0.26
Glycogen	−0.18	0.26	−0.20	−0.24	−0.30 :	−0.45*	−0.41*	−0.19
GST	0.18	0.05	0.20	0.14	0.41*	0.37*	0.35 :	0.31 :
CAT	0.16	0.22	0.18	0.08	0.08	0.01	−0.04	0.15
Weight.loss	0.09	−0.04	−0.04	0.01	−0.09	0.06	0.08	0.05

in three subcellular fractions also reflected regulation processes.

Our results show that in the case of an essential element, Zn, the causal definition of bioavailability is not verified by observations made after a laboratory exposure to a panel of soils using a SE model. This study highlights the difficulty of assessing Zn bioavailability in field-contaminated soils because as an essential element, Zn is regulated and may exert positive effects on biomarkers at moderate doses.

6.4.3 SEM for Pb bioavailability

Table 6.6 shows the bivariate correlations between the observed variables involved in the SEM model of Pb bioavailability. Soil and earthworms Pb concentrations were correlated. $CaCl_2$-extractable Pb concentration was however less strongly correlated to the other soil Pb concentrations, and there was no significant relationships with earthworm Pb concentrations. Internal Pb concentrations were correlated to several biomarkers: MT expression, protein contents, GST activity and glycogen contents. Lipid were more related to $CaCl_2$ extractable Pb.

We assumed that the correlation between soil Pb and MT expression was mediated through an indirect effect of Cd because MT is not known to respond to Pb exposure (Kägi, 1991; Morgan et al., 2004). MT expression was thus not included in the model. Based

on the CFA, (ea) was reflected only by dissolved and free Pb contents. $CaCl_2$-extractable Pb was excluded from the model, not because the path from (ea) to $CaCl_2$-extractable Pb was not significant, but because when included, the measurement model was not consistent with the data (p-value of the whole model was < 0.05). (eb) was reflected by all the internal Pb concentrations considered except Pb concentration in the debris fraction, for which the model predicted negative error variance. This was due to the strong collinearity between Pb concentrations in the debris and granules fraction (Table 6.6) and we therefore excluded Pb concentration in the debris fraction from the final measurement model. Finally, three biomarkers reflected (tb): GST activity, protein and glycogen contents. For the other biomarkers, factor loadings (paths from latent to manifest variables) were nonsignificant.

The conceptual SEM was tested with the selected measurement model. When soil total metal concentration was included in the SEM, the error variance of free Pb content was negative, which was attributed to the strong collinearity between those two variables (Table 6.6). Free Pb content was therefore not retained as a manifest variable of (ea) in the final measurement model.

The SEM was consistent with the data (Scaled χ^2=20.72, df=19, p= 0.35). However, the path from (ea) to (eb) was not significant. Removing this path, the SEM provided a good fit (Scaled χ^2=19.18, df=18, p= 0.38). Indices of approximate fit further showed that the model was consistent with the data: RMSEA = 0.05, CFI = 0.98, SRMR = 0.07. There was no further modification suggested by modification indices.

The final model with standardized coefficients is shown Figure 6.3. The unstandardized estimates are given in supplementary information. The SEM showed that increased concentrations of Pb in earthworms were related to soil total Pb concentration, but not to (ea) as defined herein. SEM confirmed the assumption that earthworms are exposed to multiple Pb species in the soil and not only the 'actual' available Pb pools.

In the model, there was no direct relationship between (ea) and (eb), and the relationship was indirect, through the direct effect of soil total Pb concentration. We found that three commonly used measurements of 'actual' available Pb were not pertinent indicators within the three-step causal definition of bioavailability. This may be due to the fact that the manifest variable of (ea) was calculated from a semi-mechanistic model that is known to predict less accurately Pb than Cd and Zn speciation (Sauvé et al., 2000). In this study, $CaCl_2$ extraction was not pertinent to reflect Pb availability within the SEM framework because of its low power to extract Pb that resulted in several values below the quantification limit in uncontaminated soils. However, the results suggested that $CaCl_2$-extractable Pb was correlated to several internal Pb concentrations and to several biomarkers (Table 6.3). Consistently with the

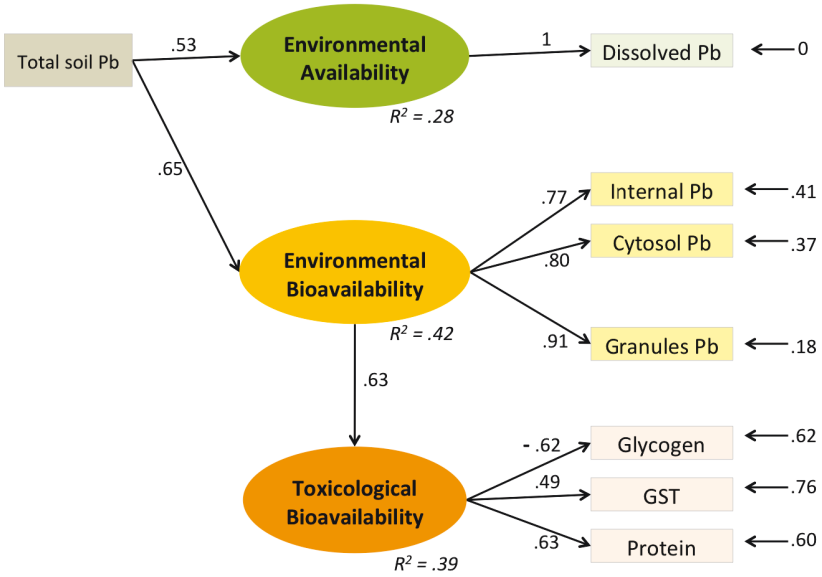


Figure 6.3: Final SEM of Pb bioavailability describing the relationships between Cd contamination (soil total content), 'actual' availability, uptake by earthworms and toxicological bioavailability. Standardized path coefficients are shown on edges (path coefficients) and represent partial regression coefficients. Standardized error variances are shown on the right of the arrows pointing to the observed variables and indicate the proportion of variance of the observed variables unexplained by its latent factor.

previous findings for Cd, free Pb concentration was not a pertinent indicator of (ea) within the SEM framework due to its high correlation with soil total concentration. This does not mean that free Pb is not available, but rather that soil total concentration integrated its influence over (eb) and (tb) in the model.

The SEM model further demonstrated the joint variations of GST activity, protein contents and glycogen reserves under the influence of increased Pb concentrations in earthworms. In the model, (tb) was reflected by (i) increased GST activity, which can be explained by an oxidative stress associated with the presence of internal Pb (ref), (ii) increased protein contents, that have been suggested to reflect increased protein synthesis in response to contaminant (Schreck et al., 2008) and (iii) by decreased glycogen contents, consistently with the energy cost of dealing with Pb demonstrated in other studies (Holmstrup et al., 2011). It should not be neglected that the model explained small amounts of the observed variations in the biomarkers(R^2: 0.4, 0.4, 0.2 for protein, glycogen contents and GST activity respectively). Biomarkers were thus affected by other parameters. We suggest that the effects of multiple metals and of soil parameters (notably soil texture) are the main factors that would improve future SE model of metal bioavailability to earthworm (Beaumelle et al., 2014).

6.5 Conclusions

We proposed SEM as a tool to express explicitly the structural implications of the definition of bioavailability and to confront the theory to empirical observations. The results demonstrated that for two non-essential metals, several of the most commonly used measurements of bioavailability for earthworms were consistent with the causal structure using a laboratory exposure to moderately and multi-contaminated soils that exhibited ranges of different soil physico-chemical parameters. Hence, despite the low doses of metal considered and the ranges of soil characteristics covered by our panel of soils, the three-step causal relationships were found robust. The results suggested several pertinent indicators, especially metal concentrations in subcellular fractions, and MT expression, the latter being highly specific of Cd contamination (although we found a correlation with internal Pb content that rises the question whether Pb can induce MT expression in *A. caliginosa*) and not strongly affected by soil parameters. The models for the non-essential metals suggested that earthworms are rather exposed to multiple metal species than to the sole species found in soil solution. For Cd, the chemical methods chosen to reflect (ea) were pertinent to estimate Cd bioavailability because they verified the causal framework. For Pb, none of the chemical methods verified the causal framework and other methods to assess (ea) need to be identified. However, the fact that soil total metal concentrations had a direct effect on (eb) in both models gives rise to questions on the utility of chemical assessment of 'actual' available metals in the assessment of metal bioavailability for earthworm in agricultural field-contaminated soils.

Supplementary

Table 6.7: Parameter estimates and associated standard errors of the SEM model for Cd bioavailability. Operators indicate the nature of the relationship where =˜ stands for the loading of an indicator on its latent variable, ˜ stands for a causal relationship from on latent to the other, and ˜˜ stands for the error variances

Variable	Operator	Variable	Parameter	Standard Error
Availability	=˜	CaCl2 Cd	1.036	0.134
Availability	=˜	Dissolved Cd	0.855	0.102
Env. bioavail.	=˜	Debris Cd	0.165	0.027
Env. bioavail.	=˜	Granules Cd	0.245	0.049
Tox.bioavail.	=˜	MT	0.267	0.032
Availability	˜	Total Cd	0.951	0.154
Env. bioavail.	˜	Availability	0.625	0.289
Env. bioavail.	˜	Total Cd	0.759	0.246
Tox.bioavail.	˜	Env. bioavail.	0.741	0.204
MT	˜˜	MT	0	0
Availability	˜˜	Availability	1	0
Env. bioavail.	˜˜	Env. bioavail.	1	0
Tox.bioavail.	˜˜	Tox.bioavail.	1	0
CaCl2 Cd	˜˜	CaCl2 Cd	0.261	0.11
Dissolved Cd	˜˜	Dissolved Cd	0.017	0.077
Debris Cd	˜˜	Debris Cd	0.038	0.014
Granules Cd	˜˜	Granules Cd	0.105	0.033
Total Cd	˜˜	Total Cd	1.436	0

Table 6.8: Parameter estimates and associated standard errors of the SEM model for Pb bioavailability. Operators indicate the nature of the relationship where =˜ stands for the loading of an indicator on its latent variable, ˜ stands for a causal relationship from one latent to the other, and ˜˜ stands for the error variances

Variable	Operator	Variable	Parameter	Standard Error
Env. avail.	=˜	Dissolved Pb	0.634	0.069
Env. bioavail.	=˜	Cytosol Pb	0.272	0.074
Env. bioavail.	=˜	Granules Pb	0.652	0.126
Env. bioavail.	=˜	Internal Pb	0.404	0.077
Tox. bioavail.	=˜	Protein	0.079	0.028
Tox. bioavail.	=˜	Glycogen	-0.145	0.056
Tox. bioavail.	=˜	GST	0.047	0.019
Env. avail.	˜	Total Pb	0.619	0.158
Env. bioavail.	˜	Total Pb	0.846	0.26
Tox. bioavail.	˜	Env. bioavail.	0.608	0.267
Env. avail.	˜˜	Env. avail.	1	0
Env. bioavail.	˜˜	Env. bioavail.	1	0
Tox. bioavail.	˜˜	Tox. bioavail.	1	0
Dissolved Pb	˜˜	Dissolved Pb	0	0
Cytosol Pb	˜˜	Cytosol Pb	0.075	0.026
Granules Pb	˜˜	Granules Pb	0.161	0.086
Internal Pb	˜˜	Internal Pb	0.199	0.066
Protein	˜˜	Protein	0.015	0.005
Glycogen	˜˜	Glycogen	0.055	0.02
GST	˜˜	GST	0.011	0.003
Env. avail.	˜˜	Tox. bioavail.	0.327	0.206

Discussion & Perspectives

Discussion & Perspectives

In this thesis, the causal structure of bioavailability was explicitly addressed within a graphical model. The conceptual model was the starting point for the settlement of a laboratory exposure experiment designed specifically to test a SEM of metal bioavailability to earthworm. Chemical and biological methods that had proved reliable in the literature were selected to specify the measurement model (Chap.1, p.12, Chap.2, p.27). A wide panel of field soils was selected to create a realistic gradient of metal exposure (Chap.2, p.44). We chose a target earthworm species, *A. caliginosa*, commonly found in temperate agroecosystems. Based on a comprehensive analysis of literature data, we verified that metal bioaccumulation in *A. caliginosa* was representative of several other common species (Chap.2, p.35). Before confronting the conceptual SEM to our empirical observations (Chap.6, p.117), the relationships between single observed variables were evaluated (Chap.3 p.55, Chap.4, p.80, Chap.5, p.99). In the present chapter, we will discuss how the two approaches complemented one another to achieve a better understanding of metal bioavailability to earthworm in field-contaminated soils.

The starting point of our discussion is to highlight how the SEM framework led to address the robustness and strength of chemical and biological assessments of bioavailability. We then discuss the interests and disadvantages of modeling each step of bioavailability as a latent variable, before presenting a hypothetical causal network that summarizes the results. Finally, we indicate several future directions and propose the SEM framework as a means to improve our understanding of the complex notion of bioavailability.

Strength and robustness of the relationships between indicators of metal bioavailability to earthworm

Terrestrial ecotoxicology mostly established dose-effect relationships following experiments designed to minimize natural variability. Such an approach can highlight the existence of

mechanisms in principle, but whether if such mechanisms are still prevalent at a broader scale of observation remains unclear (Chapman, 2002).

In our opinion, the first strength of the SEM framework is its contribution to the change in paradigm from a bottom-up approach, which seeks to simplify the degree of complexity found in natural soils, to a top-down approach, which views the system as a whole network of interacting components. More than a statistical tool, SEM is a scientific framework (Grace et al., 2008). The requirements and assumptions of the model led us to design an experiment based on a wide panel of soils. This experimental design made it possible to explore the strength and robustness of the relationships between chemical and biological indicators of bioavailability at a broader scale of observation than generally considered in bottom-up experiments. In this thesis we focused on increasing the complexity of the soil gradient characteristics, we studied a real-world earthworm species, and we kept a traditional approach by conducting a laboratory exposure on a single species. At this modest yet higher scale of observation, several patterns observed in bottom-up experiments were conserved, highlighting the robustness of the dose-effect relationship to confounding factors.

Overall, we found consistent patterns between soil and earthworm metal concentrations. But the relationships were less clear between biomarkers and metal contents (in both soil and earthworm). Three main reasons explain this outcome. First, the results reflected the notion of a hierarchical cascade in biological responses to metal exposure (Spurgeon et al., 2005). Indeed, MT expression appeared as the most sensitive biomarker, while at the cellular level, biomarkers were partially affected (energy reserves and GST activity) or not affected (CAT activity), and at the individual level, biomarkers were unaffected (no significant weight loss and no mortality). The lack of sensitivity of biomarkers at the cellular and individual level to low doses of bioavailable metals is thus a primary explanation for our results. Secondly, the results from Chapter 4 suggested that several different metals could affect biomarkers in multi-contaminated soils. Our results thus pointed to the lack of specificity of biomarkers at cellular and individual levels compared to the high specificity of MT expression for Cd exposure. Finally, biomarkers were affected in a complex manner by soil parameters, most probably through the influence of the soil environment on earthworm physiology and behavior.

The fact that biomarkers were weakly related to metal concentrations in soil and earthworms was not a surprise given the previous findings in the literature detailed in Chapter 1 (p.17). The striking result was that several biomarkers were still related to metal exposure despite low doses, multiple metals and contrasted soil properties covered by our series of soils. Defining the strength of a relationship as the degree to which the dependent variable

change in response to the explaining variable, our findings reveal biomarkers response to metal exposure is weak. But defining the robustness of a relationship as the degree to which this relation is conserved despite the influence of external factors; our findings highlight that biomarkers response to metal exposure is robust. This result have implications for the use of biomarkers in risk assessment of metals in soil that have to be considered and further investigated.

An equally important challenge that emerged in our experiment when relating indicators of bioavailability was the high variability of response from one individual to the other. Metal concentrations and biomarker responses are often measured on pools of several individual earthworms to drown out inter-individual variability. In this thesis, we performed all biological measurements on individual worms in order to appreciate the variability between individuals exposed to the same soil sample. This highlighted the individual-specific response of earthworm to metal exposure, regarding Pb bioaccumulation (Chap.3 p.64), but also MT expression (Chap.5, p.110) and other biomarkers (Chap.4, p.90). The question arises whether there is a connection between variability in internal metal contents and in biomarkers response, and if using variance instead of mean values of biological parameters could provide interesting assessments within the framework of bioavailability.

Latent variables in SEM representing each step of bioavailability

Single measurements of metal bioavailability are often criticized as to their ability to properly reflect the three steps of bioavailability. The imperfection of observed variables to reflect a particular theoretical construct is an important source of measurement error (Pugesek and Tomer, 2003). In a traditional statistical approach such as linear regression, it is assumed that measurements are made without error and violation of this assumption can bias the predictions. Measurement models in SEM address and quantify the imperfection of single observed variables to reflect theoretical variables. These features were particularly interesting in relation to the complex definition of bioavailability and the great number of possible methods to quantify it. SEM permitted to address more general relationships between three steps of bioavailability less likely influenced by undesirable errors than single measurements of each of these steps.

The fact that latent variables have causal effects on their manifest variables in SEM has strong implications, and requires paying attention to the way constructs are defined and interpreted (Shipley, 2002). We showed that in the case of availability, the definition provided in ISO 17402, 2008 was hardly adaptable to such causal assumptions. It was thus necessary

to clarify the concept of availability in order to apprehend it as a latent variable. In the case of environmental bioavailability, the measurement model provided an adequate vision of the position of internal metal concentrations in the three-step bioavailability concept. Indeed, in the model total internal content was viewed as a consequence of metal uptake, having no influence on toxicological bioavailability, in agreement with the general acceptation (Luoma and Rainbow, 2005). The influence of external factors (for example physiology, age, biomass) was explicitly taken into account and the latent toxicological bioavailability was regressed on environmental bioavailability without the influence of these external factors. Finally, the measurement model of toxicological bioavailability provided a comprehensive view of the response of biomarkers to metal exposure, that was difficult to achieve considering individually each biomarker.

Latent variable are not the only way to address unobserved variables in SEM. We have seen in chapter 5 that indices such as the IBR could also combine the influences of several observed variables (Berthelot et al., 2009). IBR provided a comprehensive assessment of toxicological bioavailability that dampen the drawback associated to the weak response of biomarkers in chronically contaminated soils. Besides, we have seen in Chapter 2 that composite variables can be modeled in SEM. Environmental availability could be better reflected by a composite than by a latent variable. However, this will need further investigation notably (1) identifying measurements of the 'potentially' available metal pools that could be integrated in the measurement model and (2) dealing with the collinearity between metal concentrations in soils that can biased a measurement model with composite variable (Grace and Bollen, 2008).

The structural model of bioavailability was created to address relationships between latent variables and not between single observed variables. Paired relationships between single observed variables were complementary to this approach providing more precise relationships. This was notably the case when we addressed the effect of internal compartmentalization of metals on biomarkers response (Chap.5, p.99) and when we attempted to identify a particular chemical method best reflecting bioavailability (Chap.3, p.55, Chap.4, p.80). Furthermore, the analysis of the relationships between single observed variables allowed to explore the influence of soil parameters and of multiple metals on biological response (Chap.4). In the SE model, the influences of soil parameters and multiple metals were integrated in the error variables but not explicitly distinguished from the effect of other sources of error. Both the approaches thus provided a complementary view of the bioavailability concept.

Structural implications of the three-step definition of bioavailability

The three-step definition of bioavailability not only implies three unobserved variables, but also a hierarchical relationship in which the effect of available metals in soil can only occur if the organism absorbs the contaminant. Such a definition has encouraged research on bioavailability to study the three steps simultaneously (Spurgeon et al., 2006; Berthelot et al., 2009). SEM was capable of articulating these three steps together in a causal network, providing a means to quantify the relative influences of total metal and available metal concentrations in soil on environmental bioavailability and subsequently on toxicological bioavailability.

The term 'availability' is often used to designate metal concentrations in the soil solution based on the assumption that the organism is mainly exposed to this soil compartment. In Chapters 3, 4 and 6 we tested the assumption that the 'actual' available fraction could estimate bioavailability. Overall available metals were related to metal concentrations in earthworms (Chap.3, p.70), to biomarker responses (Chap.4, p.80) and they verified the SEM model of Cd bioavailability (Chap.6, p.117). However, total metal content in soil was generally a better predictor of metal concentration in earthworm (Chap.3), consistently with several surveys over gradients of natural soils (Chap.1, p.14). Moreover, SEM showed that total metal concentration in soil directly affected environmental bioavailability of both Cd and Pb in a model including the effect of the 'actual' available pool. Therefore our results highlight that endogeic earthworms are exposed to multiple metal species and that the quantification of the 'actual' available pools is not sufficient to estimate bioavailability to earthworms. Soil ingestion may render available a fraction of strongly bound metals (bioaccessibility designates this fraction available by oral uptake route). Moreover, we showed that in chronically contaminated soils, total metal concentration in soil might be available for uptake by earthworms, or at least represents a pertinent proxy for the available pools.

This conclusion appears in contradiction with the results found in Chapter 4 and with the notion that risk is not related to the total contamination level. An explanation is illustrated in a conceptual model depicted on Figure 6.4. Earthworms are exposed to multiple metal species in field-contaminated soils by both dermal and oral uptake routes. Among the chemical assessments presented herein, total soil metal content was a good descriptor because it provided an integrative proxy of the multiple available metal pools. The total available ('actual' and 'potentially' available) metal fraction is the result of the combined influences of soil total contamination level and soil parameters that determine metal speciation. As a result, chemical assessments of metal availability integrate both these effects (e.g. $CaCl_2$ extraction, Sauvé-calculations).

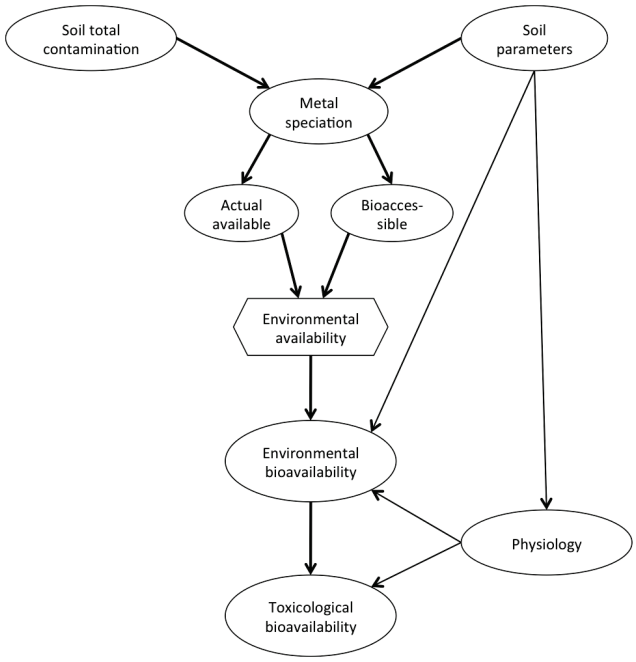


Figure 6.4: Causal diagram of bioavailablity. Soil parameters exert a complex set of influences on each step of bioavailability. Environmental availability is viewed as a composite variable combining the actual available and bioaccessible fractions of metals.

Soil parameters are not only determinant for availability, they also affect directly metal uptake. Indeed, as stated in the biotic ligand model, the competition of cations for binding to the biotic ligand plays a direct effect on metal uptake by the organism (Paquin et al., 2002). Soil parameters further influence directly earthworm physiology (Chap.1, p.17). The complex set of indirect effects of soil physico-chemical characteristics on biomarker responses can explain that the biomarkers response was more strongly related to a chemical proxy of availability than to total metal concentration in soil in Chapter 4 (p.80). Chemical assessments of availability are indeed integrative of soil contamination level and soil properties that both influence indirectly biomarker response. One of the main perspective of this work is thus to integrate the effect of soil properties in the SEM of bioavailability in order to quantify the relative importance of total metal concentration in soil, metal availability and soil parameters in the conceptual model.

Perspectives

In SEM, a common approach is to first develop a core model that can be sophisticated/refined afterwards. This thesis can be viewed as the first step of such an approach. The core model

was developed based on an *a priori* knowledge of the processes involved in bioavailability. This set of hypothesis was verified against our experimental dataset (Chap.6, p.117). Several perspectives to improve and refine this model were highlighted by the analysis of the relationships between single indicators (Chap.3, p.55, Chap.4, p.80 and Chap.5, p.99).

One of the main features of graphical modeling is its capacity to disentangle the influences of different factors. In the context of multiple contamination, bottom-up approaches address the mechanisms of action of metal mixtures but usually not address complexity that arises from interactions with the environmental context in field-contaminated soils. SEM could thus complement bottom-up approaches by unraveling the effects of each contaminant in mixtures in network models integrating the effects of confounding factors on bioavailability. The inclusion of soil parameters in the model is also a main perspective in light of our findings. Future SEM of metal bioavailability could integrate the assumptions of the biotic ligand model but also the direct effect of the soil on earthworm physiology that certainly modulates the uptake of metals and the biological response associated.

Such complicated models would require very large datasets. Three solutions to overcome this issue can be proposed: (1) to use path analysis to model the complex networks with single indicator variables, with the disadvantage of relying on imperfect assessments of each bioavailability step, (2) to model latent variables reflected by concentrations of several metals in the model, with the drawback of lacking a mechanistic understanding of the individual effects of each metal and (3) to use other statistical methods in the SEM framework that can manage small datasets and non-linearity, such as Bayesian statistics and partial least square analysis that are emerging in the SEM literature (Grace et al., 2012; Haenlein and Kaplan, 2004). Although they are not yet fully implemented in SEM softwares, it may soon be the case because of their flexibility.

While complexity is generally viewed as confusing, graphical models such as SEM offer comprehensive and synthetic frameworks to address complex set of hypothesis about systems. The seldom use of graphical models in ecotoxicology is certainly related to the dominant bottom-up approach in this field (Beketov and Liess, 2012). Graphical models can participate to the paradigm change in ecotoxicology from a bottom-up to a top-down approach because they can handle natural ecosystems complexity. It is our hope that this thesis will contribute to the establishment of graphical models as a central scientific framework in ecotoxicology.

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Modélisation graphique de la biodisponibilité des métaux pour le ver de terre

Les pages qui suivent constituent un résumé étendu de la thèse en français. Nous reprenons ici comment a été construit le modèle par équations structurelles et les résultats principaux de chaque chapitre sont décrits. Pour la bibliographie et les méthodes détaillées nous renvoyons aux sections dédiées du manuscrit de thèse.

Modélisation graphique de la biodisponibilité des métaux pour le ver de terre

Introduction

La contamination diffuse des sols par des éléments en trace métalliques (ETM) est un enjeu environnemental important. Dans le cadre de l'évaluation du risque lié à cette contamination, il est nécessaire de prendre en compte la capacité du contaminant à interagir avec le vivant dans un environnement donné : la biodisponibilité. L'évaluation de la biodisponibilité fait encore débat dans la communauté scientifique. Cette problématique se pose avec d'autant plus d'acuité dans les sols, qui sont des milieux très hétérogènes. Les sols sont composés de différentes phases interagissant plus ou moins avec les métaux. Selon la nature du sol, les contaminants peuvent donc se présenter sous des formes chimiques diverses et dans des proportions variables.

Une question importante est de savoir quelles sont les formes chimiques préférentiellement absorbées par les organismes vivant dans les sols contaminés. La littérature montre que les plantes et les animaux absorbent surtout les métaux en solution (Peijnenburg et al., 2007; Veltman et al., 2007). Cependant, pour certains invertébrés comme les vers de terre, les teneurs en éléments trace dans l'eau du sol seules ne suffisent pas à expliquer les quantités de métaux absorbées (Peijnenburg and Jager, 2003). Pour relier l'exposition à l'absorption, il serait donc intéressant de prendre en compte simultanément différents indicateurs chimiques de métaux disponibles dans le sol. Connaître les formes chimiques prises en charge par les organismes du sol ne suffit pas cependant à évaluer les risques. Les risques sont aussi associés aux effets des métaux et non uniquement à l'assimilation en elle-même. En effet, les organismes sont capables de réguler, de stocker et de détoxifier les contaminants (Vijver

et al., 2004). Il est donc important de pouvoir relier la présence effective de contaminants sous des formes 'bioactives' dans l'organisme à la manifestation biologique des effets de ces polluants.

L'objectif principal du projet de thèse était d'étudier la biodisponibilité de manière intégrée, en tenant compte des relations de causalité existant entre les trois composantes qui la constituent: (1) La disponibilité environnementale (dispo. env.), reflète la part des contaminants potentiellement accessible pour un organisme, celle qui peut potentiellement être absorbée, (2) la biodisponibilité environnementale (biodispo. env.) qui renseigne des processus d'absorption des contaminants dans l'organisme, (3) la biodisponibilité toxicologique (biodispo. tox.) qui reflète la part des contaminants qui conduit à des effets, et qui se manifeste par ces réponses biologiques à la contamination.

Les modèles graphiques tel que le modèle SEM (modèle par équations structurelles) apparaissent très pertinents dans le contexte de cette définition causale de biodisponibilité. Ils permettent en effet de représenter des relations structurées et des variables théoriques.

Dans cette thèse, on présente un modèle SEM de biodisponibilité des métaux pour le ver de terre. L'objectif était de confronter le modèle théorique à des observations couvrant un large panel de sols contaminés *in situ*, et de participer ainsi à l'identification de jeux d'indicateurs chimiques et biologiques appropriés pour l'évaluation des risques environnementaux liés aux métaux dans les sols.

Chapitre 2. Conception du schéma expérimental

Un modèle par équations structurelles de la biodisponibilité

La modélisation par équations structurelles (SEM) peut permettre de tester des liens de causalité entre des variables, et surtout de traiter des variables latentes, c'est-à-dire des variables que l'on ne peut pas mesurer directement, mais qui sont décrites par plusieurs variables mesurées.

Les trois maillons de cette chaîne de causalité sont reliés entre eux par des relations de cause à effet, symbolisées par des flèches unidirectionnelles (Figure 6.5). Les relations causales entre les trois composantes de la biodisponibilité représentent le modèle structurel. Chaque composante est décrite par plusieurs indicateurs mesurés. Les liens entre les variables latentes et les indicateurs sont également des liens de causalité, et représentent le modèle de mesure.

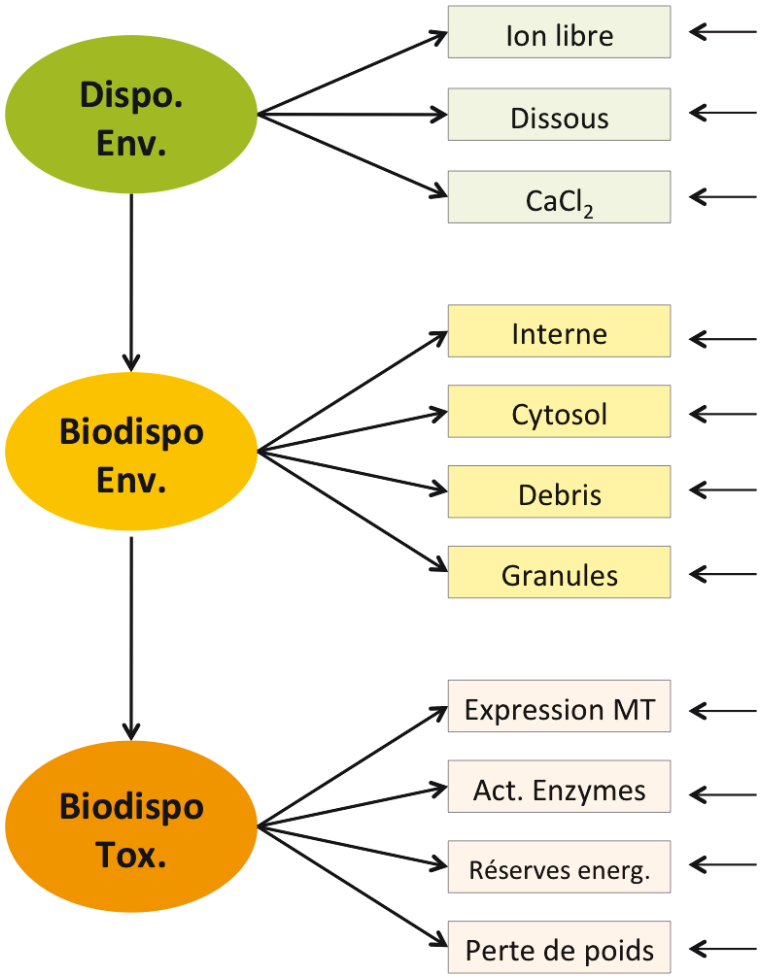

Figure 6.5: Un modèle par équations structurelles de biodisponibilité des métaux pour le ver de terre. Les variables latentes sont illustrées par des ellipses et les variables observées par des rectangles. Les flèches unidirectionnelles indiquent les relations de cause à effet. Dans cette étude, on considère que la disponibilité environnementale est reflétée par les concentrations de différents pools de métaux: ions libres, dissous et extraits au $CaCl_2$. La biodisponibilité environnementale est reflétée par les concentrations en métaux dans les vers de terre (teneurs internes totales (interne), et dans trois fractions subcellulaires: cytosol, débris cellulaires et granules). La biodisponibilité toxicologique est mesurée à travers différents biomarqueurs (expression du gène de la MT impliquée dans la détoxification des métaux, activités d'enzymes impliquées dans la réponse au stress oxydant, réserves énergétiques et perte de biomasse).

Modèle de mesure

Dans le modèle par équations structurelles présenté Figure 6.5, les trois étapes de la biodisponibilité sont reflétées par différentes variables observées ou manifestes (c'est le modèle de mesure). Les variables manifestes ont été choisies d'après une revue de la bibliographie.

La littérature montre que la voie d'absorption majoritaire des ETM chez les vers de terre est l'absorption cutanée (Vijver et al., 2003). Les métaux disponibles sont donc des métaux présents dans la solution du sol ou bien faiblement adsorbés aux constituants du sol. Un grand nombre d'études montre que les teneurs internes dans les vers sont corrélées à des métaux extraits par des sels ($CaCl_2$, $Ca(NO_3)_2$, e.g. Gaw et al., 2012, Lee et al., 2009). Cependant, les vers de terre ingèrent du sol et pourraient aussi être exposés à des formes de métaux plus fortement adsorbés. Cette hypothèse est confirmée par plusieurs travaux montrant que les teneurs totales en métaux dans le sol sont mieux corrélées aux teneurs internes que les teneurs extractibles par des extractants faibles (Kamitani, Kaneko 2007, van Vliet et al., 2005). Des travaux récents ont de plus démontré l'importance de l'absorption par voie digestive en réalisant une extraction simulant le contenu enzymatique du tube digestif d'un ver de terre (Ma et al., 2009). Les extractions par des sels apparaissent donc insuffisantes pour renseigner complètement les formes de métaux disponibles pour les vers de terre.

Pour renseigner la disponibilité environnementale, deux extractions ont été sélectionnées : l'extraction $CaCl_2$, supposée refléter la fraction des métaux en solution et échangeables, et l'extraction EDTA, supposée extraire les métaux liés fortement aux constituants du sol et notamment aux matières organiques. De plus, deux modèles de spéciation ont été sélectionnés : un modèle semi-mécanistique pour calculer la concentration en métaux dans la solution du sol (Sauvé et al., 2000) et un modèle géochimique plus complexe qui tient compte de différentes surfaces solides et de la compétition avec les cations en solution pour calculer la concentration en ions libres dans la solution du sol.

La biodisponibilité environnementale est le maillon central de la chaîne de causalité disponibilité absorption impact. L'hypothèse généralement admise est que les teneurs internes totales en métaux renseignent l'absorption et permettent à la fois de déterminer les formes disponibles dans les sols et de confirmer que la réponse biologique mesurée est liée à l'exposition aux métaux. Cependant, les teneurs internes totales représentent l'accumulation des métaux à un temps t et ne permettent pas de tenir compte des processus de régulation, de séquestration par lesquels les organismes se protègent. Les teneurs internes sont-elles suffisantes pour jouer ce rôle de maillon clé de la chaîne causale ? La question se pose particulièrement vis-à-vis des biomarqueurs. Pour confirmer la pertinence des biomarqueurs,

il pourrait être intéressant de les mettre en relation avec les pools internes de métaux responsables de la toxicité. L'approche par fractionnement subcellulaire permet de séparer par centrifugation différentes fractions opérationnelles d'ETM qui sont supposées représenter des pools de métaux ayant des fonctions différentes au sein de l'organisme (Gimbert et al., 2008, Li et al., 2008). Cette méthode permet de séparer au moins trois fractions subcellulaires : (i) des granules enrichies en métaux (fraction de métaux supposés détoxifiés), (ii) une fraction de débris cellulaires (fraction interprétée dans la littérature soit comme un pool toxique soit comme une fraction trop hétérogène pour être interprétée comme toxique ou non), et (iii) une fraction cytosol total (fraction supposée détoxifiée du Cd car lié à des protéines, mais toxique du Pb, car n'ayant pas de ligand connu dans cette fraction (Jones et al., 2009)). Aucune étude n'a encore confirmé les fonctions de ces différents pools de métaux en les mettant en relation avec la manifestation d'une réponse toxique chez les vers de terre.

Les biomarqueurs sont des changements biologiques (le plus souvent au niveau subindividuel) dûs à la contamination. Ils sont de plus en plus utilisés pour renseigner la biodisponibilité des contaminants. Mais peu de biomarqueurs ont été corrélés aux teneurs internes en métaux chez les vers de terre. Leur pertinence pour évaluer la biodisponibilité reste donc à confirmer. Il est généralement admis que la mesure d'un unique biomarqueur ne permet pas de renseigner le stress car celui-ci est multidimensionnel (van Straalen 2003). Les approches "multi-biomarqueurs", dans lesquelles une batterie de biomarqueurs de différents niveaux d'organisation biologique sont mesurés, s'avèrent plus appropriées pour renseigner le stress subi par les organismes (Spurgeon et al., 2005). Parmi eux, l'expression du gène de la métallothionéine (MT), protéine impliquée dans la détoxification du Cd notamment a souvent été montrée comme un biomarqueur pertinent de l'exposition des vers au Cd (Spurgeon et al., 2004, van Gestel et al., 2009). Les activités d'enzymes impliquées dans la réponse au stress oxydant sont très souvent utilisées comme biomarqueurs. Plusieurs travaux ont mis en évidence un effet de la contamination métallique sur ces activités enzymatiques (Catalase (CAT), Glutathione-S-transférase (GST), etc.). Mais aucune étude n'a encore établi de corrélation entre teneurs internes en métaux et modification de l'activité de ces enzymes. Ces enzymes répondraient aux contaminants de manière hormétique, c'est-à-dire que la réponse à forte dose serait inverse à celle des faibles doses (par exemple la GST serait augmentée à forte dose et inhibée à faible dose (Laszczyca et al., 2004)). Les réserves énergétiques (lipides, glycogène, protéines) sont également des biomarqueurs de stress plus récemment utilisés. Les organismes mobiliseraient leurs réserves énergétiques pour lutter contre les contaminants (Holmstrup et al., 2011). Cette différence d'allocation énergétique pourrait conduire à diminuer la croissance et la reproduction. Des travaux portant sur les daphnies ou les gastéropodes

ont montré que les réserves énergétiques pourraient répondre à la contamination métallique, et que leur réponse pouvait être reliée à des effets au niveau populationnel (Moolman et al., 2007, De Coen and Janssen 2010). La réponse des réserves énergétiques des vers de terre aux ETM n'a été que plus rarement étudiée.

Modèle biologique

Les vers de terre sont particulièrement intéressants pour étudier la biodisponibilité. Ils sont exposés aux ETM car ils vivent dans la matrice du sol, ils accumulent les ETM (e.g. Spurgeon, Hopkin 1999), et sont sensibles à la contamination (e.g. Abdul, Rida 1995). De plus, il est possible de les utiliser dans le cadre d'expériences en conditions contrôlées, approche qui a été retenue dans ce travail de thèse. Enfin, biodisponibilité et impact sur les vers de terre sont bien documentés dans la littérature scientifique notamment du fait de l'importance fonctionnelle de ces ingénieurs des écosystèmes (Lavelle, Spain 2001).

L'exposition, la bioaccumulation et la sensibilité aux ETM varient en fonction de l'espèce étudiée (Spurgeon and Hopkin 1996, Ernst et al., 2008). Parmi les trois groupes écomorphologiques de vers de terre (épigés, endogés, anéciques), nous avons choisi de travailler sur une espèce endogée. En effet, les endogés consomment plus de sol que les épigés et les anéciques, ils sont donc potentiellement plus exposés aux ETM (van Vliet et al., 2005). Certaines espèces sont plus résistantes à la contamination métallique que d'autres. Par exemple, le modèle courant et standardisé en écotoxicologie des sols, *Eisenia fetida*, serait moins sensible que d'autres vers de terre (Spurgeon and Hopkin 1996). L'espèce *Aporrectodea caliginosa* (Savigny 1826) est apparue la plus intéressante car elle remplit les trois critères précédents (endogée, accumulatrice et sensible). De plus, c'est une espèce abondante et largement répartie.

Au début de la thèse, une analyse bibliographique a été menée sur la bioaccumulation des ETM dans différentes espèces de vers de terre. Les données issues de 39 articles ont été récoltées. Dans cette analyse, on a montré que l'espèce *A. caliginosa* ne présentait pas de pattern spécifique d'accumulation du Cd, du Pb et du Zn en la comparant à 6 autres espèces (*Eisenia fetida, Eisenia andrei, Lumbricus terrestis, Lumbricus rubbellus, Dendrodrilus rubidus, Dendrobaena octaedra*). En revanche, les espèces modèles en écotoxicologie (notamment *E. andrei*) peuvent présenter des différences par rapport à *A. caliginosa*.

La figure 6.6 montre les résultats de cette analyse bibliographique obtenus pour le Zn. Les pentes des relations entre concentrations dans les animaux et dans les sols sont similaires pour l'ensemble des espèces (modèle ANCOVA). Par contre notre analyse montre que les espèces

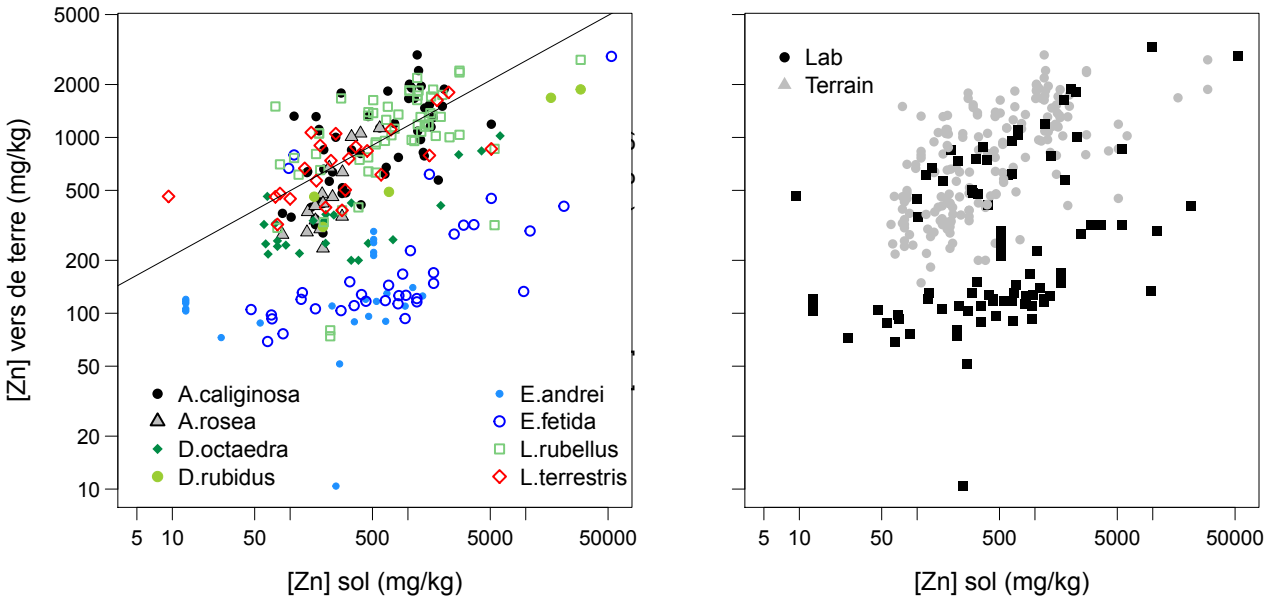

Figure 6.6: **(Gauche) Concentration en Zn dans les vers de terre (8 espèces) en fonction de la concentration en Zn dans le sol telles que rapportées par 27 publications** (sur des échelles logarithmiques). La ligne noire représente la pente prédite pour l'espèce *A. caliginosa* (modèle ANCOVA). **(Droite) Concentration en Zn dans les vers de terre (21 espèces) par rapport à la concentration dans le sol et en fonction de l'origine des observations (exposition au laboratoire (Lab) ou sur le terrain (Terrain)) pour 39 études.** Les lignes représentent les pentes prédites pour les observations de laboratoire (noir) et de terrain (gris)

modèles en écotoxicologie (*E. fetida, E. andrei*) ont globalement des concentrations internes en Zn plus faibles que d'autres espèces qui vivent réellement dans le sol. La figure de droite (Fig. 6.6) montre que ce pattern n'est pas relié au fait que les deux espèces modèles soient principalement étudiées au laboratoire, puisqu'on observe un certain nombre d'observations recueillies au laboratoire pour d'autres espèces qui sont plus proches du nuage de points des observations de terrain.

Cette analyse bibliographique a conforté notre choix de l'espèce *A. caliginosa* pour mener l'expérience en conditions controlées permattant de tester le modèle SEM. Parallèlement, la sélection du gradient de sols a également fait l'objet d'une attention particulière.

Création d'un gradient de disponibilité

Le design expérimental comportait 31 échantillons de sol choisis pour atteindre une grande gamme de caractéristiques physico-chimiques (texture, pH, CEC, C-organique) et de niveaux de contamination en métaux (Cd, Pb, Zn). Ils ont été prélevés au sein de 4 sites contaminés et 4 sites non contaminés. Pour chaque site, plusieurs échantillons de sols ont été prélevés au niveau de différentes parcelles. Toutes les parcelles étudiées étaient des terres arables (cultures ou prairies).

Deux sites contaminés (Metaleurop et Mortagne-du-Nord) sont localisés dans la région Nord à proximité d'anciennes fonderies de Pb et Zn. Au niveau de ces sites, la contamination provient de dépôts de poussières contaminées. Sur le site Metaleurop, 5 échantillons ont été prélevés dans 5 parcelles sous culture, à différentes distances de l'ancienne fonderie. Sur le site Mortagne, 9 échantillons ont été prélevés. Ils présentent différentes textures (de sableux à limoneux) et différents usage des sols (cultures ou prairies).

Les deux autres sites contaminés (Pierrelaye et Triel-sur-Seine) sont localisés dans la banlieue parisienne et ont été soumis à l'épandage des eaux usées brutes de Paris pendant 100 ans. Sur le site de Pierrelaye, les échantillons ont été collectés dans deux parcelles cultivées : un échantillon de sol a été pris dans une parcelle non contaminée située en dehors de la zone d'épandage, et 4 échantillons ont été collectés dans une parcelle contaminée, en suivant un gradient de contamination. Au niveau du site de Triel, 3 échantillons de sol ont été prélevés. Ils présentent différentes teneurs totales en métaux et différentes occupations des sols (une culture, une prairie et une jachère).

Les quatre sites non contaminés n'ont pas d'histoire connue de contamination métallique. Les sites choisis sont nommés Feucherolles (site expérimental Qualiagro, situé dans les Yvelines), Yvetot (Haute Normandie), Bannost (Seine et Marne), et Closeaux (Yvelines). Sur le site de Feucherolles, 3 échantillons ayant reçu différents épandages de matières organiques ont été prélevés. Sur le site d'Yvetot, 3 échantillons ont été prélevés dans des parcelles avec différents modes d'occupation (prairie permanente ou grande culture). Sur le site de Bannost, 2 échantillons ont été prélevés sur deux différentes parcelles sous agriculture biologique.

L'ensemble des sols sélectionné a permis d'explorer des gammes de caractéristiques physico-chimiques ainsi que de teneurs totales en métaux. Le tableau 6.9 montre l'étendue des valeurs prises par le pH, le taux de matières organiques (MOS), le taux d'argile et la CEC. Ces variations de caractéristiques déterminantes pour la biodisponibilité ont permis de faire varier au maximum la disponibilité environnementale.

Table 6.9: Etendues des caractéristiques physico-chimiques et des teneurs totales en métaux couvertes par les 31 sols étudiés

	units	minimum	maximum	mediane
Argile	g/kg sol	54	476	123
pH		5.4	8.3	7.3
CEC	cmol+/kg sol	3.0	30.5	8.1
C-organique	g/kg sol	9.5	41.5	16.1
Cd total	mg/kg sol	0.18	8.32	0.95
Pb total	mg/kg sol	19.6	491.0	53.5
Zn total	mg/kg sol	40	1004	134

Conditions d'exposition des vers de terre aux 31 sols

Les vers de terre adultes (*A. caliginosa*) ont été collectés sur une prairie (La Cage) située à Versailles. Après la collecte, les individus ont été maintenus dans leur sol d'origine (sol dit des Closeaux) à 12 °C et dans l'obscurité. Le sol avait au préalable été tamisé à 2 mm et maintenu à 60% de la capacité de rétention en eau. Pour chaque échantillon de sol, 5 microcosmes de 600 g de sol sec ont été mis en place. Deux jours avant le début de l'exposition, les vers ont été mis à jeûner pendant 48 heures dans des boîtes de Pétri contenant du papier filtre humidifié. Les papiers filtres étaient nettoyés deux fois par jour. Les vers ont été attribués par tirage au sort à chaque microcosme (à raison de 6 vers par microcosme). Ils ont été exposés pendant 21 jours dans les microcosmes maintenus dans l'obscurité à 12 °C et à 60% de la capacité de rétention en eau. L'humidité a été vérifiée régulièrement (1 fois par semaine). A la fin de l'exposition, chaque individu a été attribué à une mesure biologique par tirage aléatoire.

Chapitre 3 Relations entre compartimentation des métaux dans le ver de terre et spéciation des métaux dans le sol

Les organismes sont capables de se protéger des effets néfastes des métaux. Ils utilisent notamment des stratégies de compartimentation qui permettent de partitioner les métaux absorbés dans des tissus ou des compartiments subcellulaires précis. Au niveau subcellulaire, deux mécanismes principaux de séquestration des métaux sont identifiés: (1) la liaison à des protéines chaperones telles que la métallothionéine et (2) la séquestration dans des granules insolubles extracellulaires (Stürzenbaum et al., 2004).

Cette compartimentation subcellulaire peut être évaluée par des techniques de fraction-

nement qui font intervenir différentes étapes de centrifugations des tissus animaux étudiés. Cette procédure a été mise en oeuvre dans le cas des vers de terre mais rarement après exposition à un vaste panel de sols (Vijver et al., 2007).

Dans ce chapitre, nous avons exploré les relations entre la compartimentation des métaux dans les vers et la spéciation (expérimentale et théorique) des métaux dans les sols en faisant l'hypothèse que la modification de la distribution des métaux dans trois fractions subcellulaires pouvait être un indicateur intéressant de la biodisponibilité.

Résultats principaux

On a pu montrer dans ce chapitre que le fractionnement subcellulaire du Cd répondait plus fortement à la contamination du sol que la teneur interne totale. Ce résultat était lié au fait que plus de 80% du Cd interne se trouve dans la fraction cytosolique et que cette fraction n'a pas vu sa concentration augmenter en fonction de la teneur totale en Cd dans le sol. Pour le Pb en revanche, le fractionnement subcellulaire a été modifié de manière concomitante aux teneur internes totales. On a montré notamment que la fraction granules, fraction supposée détoxifiée du Pb, présentait une capacité à court terme de stockage du Pb dans des vers de terre exposés à des sols modérément contaminés. Le fractionnement subcellulaire du Zn a confirmé que cet élément essentiel était régulé très finement par les vers de terre. Ce résultat était connu pour les teneurs internes totales, mais ce chapitre montre que cette régulation est également reflétée par des niveaux stables de Zn dans trois fractions subcellulaires.

Le fractionnement subcellulaire du Cd et du Pb était plus fortement relié aux teneurs totales en métaux dans le sol qu'à trois mesures de disponibilité environnementale. Parmi ces trois mesures, nous avions notamment considéré la concentration en ion libre, calculée par un modèle thermodynamique de spéciation. Un des résultats principaux de ce chapitre était que cette concentration en ion libre était fortement corrélée à la teneur totale en métaux dans le sol. Ainsi il n'était pas possible de déterminer quelle mesure en particulier (teneur totale ou teneur en ion libre) était plus fortement liée aux concentrations dans les animaux.

Conclusions

Les résultats du chapitre 3 suggèrent que le fractionnement subcellulaire du Cd est un indicateur potentiellement plus sensible à l'exposition métallique que la bioaccumulation totale. Ce n'est pas le cas pour Pb ni Zn, pour lesquels la compartimentation interne pourrait s'avérer un outil intéressant uniquement lorsque l'on s'intéresse à expliquer les effet des métaux sur

les organismes. Le chapitre 3 a permis de plus de confirmer le fait que les teneurs internes en métaux dans les vers de terre étaient plus fortement liées aux teneurs totales en métaux dans le sol. Ces résultats suggèrent donc que les mesures chimiques de disponibilité n'apportent pas d'informations supplémentaires pour expliquer les concentrations en métaux dans les vers par rapport aux teneurs totales.

Chapitre 4. Relations entre disponibilité environnementale et biodisponibilité toxicologique

Les biomarqueurs sont supposés être des mesures directes de la biodisponibilité (Lanno et al., 2004) et devraient donc répondre plus fortement à la disponibilité environnementale qu'à la teneur totale en métaux dans le sol. Pourtant, les relations entre biomarqueurs subindividuels et disponibilité chimique des métaux est rarement évaluée. La diminution des réserves énergétiques (sucres, protéines, lipides) a été proposée comme un biomarqueur de stress lié à l'exposition aux métaux. En effet, l'exposition métallique pourrait accroitre les dépenses énergétiques du fait de la nécessité pour les organismes de réguler et détoxifier les métaux (Scott-Fordsmand and Weeks, 2000). Le sujet a rarement été étudié dans le cas de vers de terre exposés aux métaux dans le sol.

Dans les systèmes complexes que sont les sols, plusieurs facteurs pourraient moduler la réponse des biomarqueurs à la disponibilité des éléments en trace. Il n'est pas certain que les réserves énergétiques soient modifiée par de faibles niveaux de contamination, ce qui est souvent le cas des sols contaminés *in situ*. De nombreuses études en milieu aquatique montrent que la réponse des réserves énergétiques à faible dose pourrait être hormétique (augmentation à faible dose puis une diminution à plus forte dose). En outre, les sols contaminés *in situ* sont très souvent contaminés par des mélanges de métaux. Holmstrup et al. (2011) ont récemment suggéré que des demandes énergétiques différentes pouvaient être associées aux différent métaux. Enfin, l'état des réserves énergétiques des organismes du sol pourrait être influencée par d'autres facteurs de l'environnement, notamment les caractéristiques du sol (Amorim et al., 2012). La texture du sol et la teneur en carbone organique sont des paramètres déterminants de l'abondance et de la croissance des vers de terre.

Ainsi dans les sols contaminés *in situ*, la réponse des réserves énergétiques des vers de terre à la disponibilité des métaux pourrait être complexe. Dans le sol, ces facteurs (faible dose, multi-contamination et différents paramètres physico-chimiques) interviennent simultanément et la réponse du système pris dans son entier pourrait être différente de celle

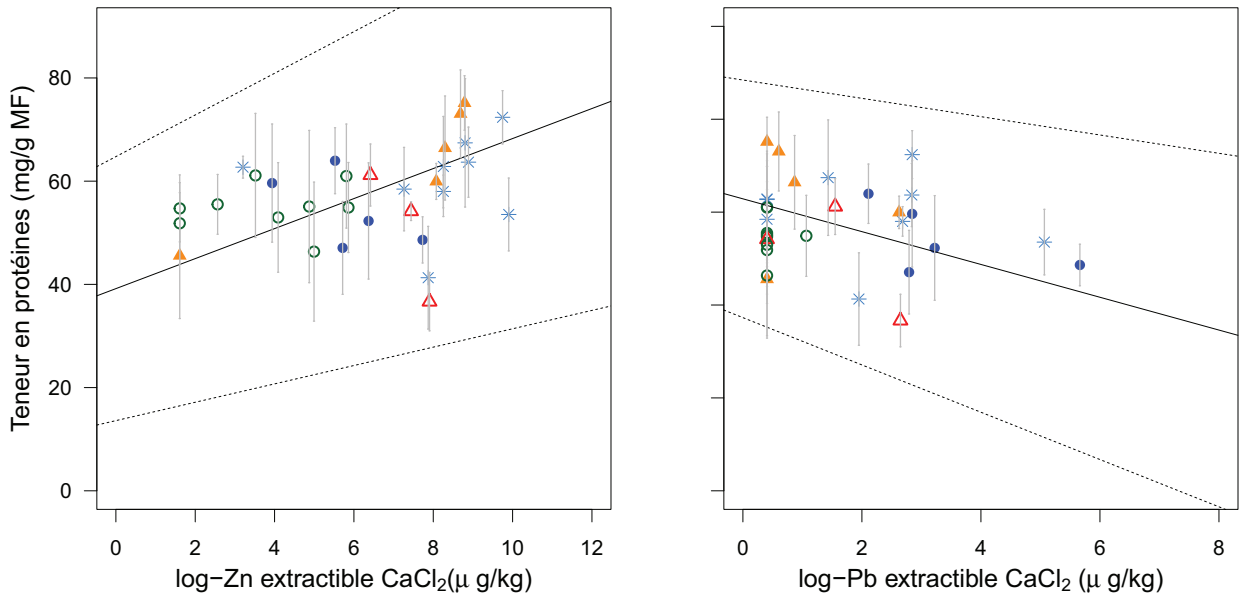

Figure 6.7: Relation entre teneur en protéines dans les vers de terre et teneur en Zn (à gauche) et en Pb (à droite) extractibles au $CaCl_2$. Chaque point représente la moyenne de 5 réplicats. Les barres d'erreur indiquent les écart types. La ligne pleine représente la pente prédite par une régression linéaire multiple, les lignes pointillées représentent l'intervalle de confiance à 80%.

observée en considérant un à un l'effet de chacun de ces facteurs. Dans ce chapitre, nous avons cherché à déterminer si la relation entre disponibilité environnementale et réserves énergétiques était suffisamment robuste pour être observée après exposition des vers à un vaste panel de sols contaminés *in situ*.

Principaux résultats

Ce chapitre a montré que les concentrations en glycogène, en protéines et en lipides dans les vers de terre répondaient différemment à de faibles doses de métaux disponibles après exposition à 31 sols. Aucune relation significative n'a été trouvée entre concentration en glycogène et concentration en métaux extractibles ($CaCl_2$, EDTA, teneur totale) alors que les teneurs en proteines et en lipides étaient affectées. Leur variations étaient principalement expliquées par la teneur en Zn et en Pb extractibles au $CaCl_2$.

Les résultats ont par ailleurs montré des effets opposés du Zn et du Pb sur les teneurs en protéines et en lipides. En effet, les teneurs en protéines et en lipides dans les vers étaient reliées positivement à la teneur en Zn extractible $CaCl_2$, et négativement à la teneur en Pb

extractible au $CaCl_2$. Ce résultat est illustré figure 6.7 pour les protéines. Cette figure montre que la relation entre réserves énergétiques et disponibilité des métaux était de faible intensité, comme on pouvait s'y attendre compte tenu des faibles niveaux de contamination considérés. La multi-contamination des sols a donc eu un impact sur les réserves énergétiques des vers exposés, avec différents métaux ayant des effets opposés sur les biomarqueurs considérés. Ces résultats démontrent qu'il est important de tenir compte de l'effet de chaque métal séparément dans l'analyse de la réponse des biomarqueurs à la disponibilité environnementale.

Enfin, les résultats ont permis de montrer que les réserves énergétiques étaient principalement affectées par la texture du sol. Le taux de limons dans le sol était corrélé positivement aux teneurs en lipides dans les vers, et la teneur en argile était reliée négativement à la teneur en protéines. La texture du sol est un paramètre déterminant de la disponibilité des métaux, mais influence également directement les vers de terre. En effet, des textures déséquilibrée (sols très sableux ou très argileux) peuvent être défavorables pour les vers de terre et conduire à de plus grandes dépenses énergétiques (Lavelle, 1988).

Conclusions

Dans ce chapitre, on a fait le lien entre les deux éléments les plus éloignés de la chaîne de causalité décrivant la biodisponibilité. En tenant compte des effets séparés des différents métaux et des caractéristiques des sols, il a été possible de mettre en évidence une relation significative entre biomarqueurs et disponibilité environnementale. Cependant, les résultats suggèrent une faible généricité des réserves énergétiques en tant qu'indicateur de la biodisponibilité des métaux. D'autres biomarqueurs, notamment à des niveaux d'organisation plus faibles comme celui du gène, pourraient être plus sensibles dans le contexte de sols contaminés *in situ*.

Chapitre 5. Relation entre compartimentation des métaux dans les vers de terre et biomarqueurs d'exposition

La concentration des contaminants dans certaines fractions subcellulaires est supposée mieux refléter les risques encourus par les organismes. En effet, la bioaccumulation totale des métaux ne suffit pas à indiquer les effets des métaux sur les organismes puisque ceux-ci sont capables de compartimenter les contaminants sous forme inerte. Dans ce chapitre on a cherché à savoir si l'on pouvait mieux expliquer la réponse de différents biomarqueurs d'exposition

par les concentrations en métaux dans trois fractions subcellulaires. Le large panel de sols considéré a de plus permis de confronter l'intensité de cette réponse dans des situations de faible dose et de contamination multi-éléments.

Résultats principaux

Cette approche a montré que selon les biomarqueurs considérés et selon le métal, les biomarqueurs pouvaient répondre à la fois à la fraction soluble (cytosol) et insoluble (débris + granules) des métaux. L'expression du gène de la MT était le biomarqueur le plus fortement corrélé. Ce biomarqueur a plutôt répondu au Cd dans la fraction insoluble. L'activité de la glutathione-s-transférase (GST) était quant à elle plus fortement liée au Pb dans la fraction soluble.

Un indice intégré de la réponse des biomarqueurs (IBR) a été calculé d'après (Colacevich et al., 2011). Cet indice donne une évaluation intégrée de la réponse des différents biomarqueurs et s'est montré plus sensible aux concentrations en Cd et en Pb considérées que les biomarqueurs pris individuellement.

Dans l'ensemble plusieurs biomarqueurs ont répondu aux faibles niveaux de disponibilité environnementale considérés dans notre étude. Ces réponses de faible intensité ont pu être détectées car nous avons considéré un grand nombre de sols, alors que cela peut être difficile si l'on compare un petit nombre de traitements.

Chapitre 6. Test du modèle par équations structurelles de biodisponibilité des métaux pour le ver de terre

Le concept de biodisponibilité a désormais une définition claire et structurée élaborée dans la récente décennie (Peijnenburg et al., 1997; Lanno et al., 2004; Harmsen, 2007). De plus en plus d'études prennent en compte simultanément les trois étapes de la biodisponibilité (Berthelot et al., 2009). Cependant, la prise en compte des relations structurelles entre ces trois étapes est rendue difficile par le fait que les méthodes statistiques les plus courantes ne permettent pas de rendre compte de relations de causalité.

De plus, dans le cas de la biodisponibilité des métaux dans les sols, de nombreux facteurs confondants peuvent intervenir et moduler l'intensité des relations entre indicateurs chimiques et biologiques de biodisponibilité. Les chapitres précédents ont montré par exemple que les

biomarqueurs étaient affectés par des facteurs biologiques (variabilité inter-individuelle) et physico-chimiques (effet des paramètres du sol). On a vu également que ces biomarqueurs pouvaient avoir une réponse de faible intensité, en lien avec le faible niveau de contamination souvent caractéristique des sols agricoles contaminés *in situ*. Enfin, les biomarqueurs peuvent être affectés par différents métaux dans les sols multi-contaminés.

Afin de pouvoir identifier des indicateurs pertinents et génériques de la biodisponibilité, il est important de confirmer si les méthodes couramment utilisées pour évaluer la biodisponibilité vérifient les relations de cause à effet entre ces trois étapes de biodisponibilité.

Dans ce dernier chapitre, le modèle SEM décrit précédemment a été confronté aux données acquises au cours de la thèse.

Principaux résultats

Le modèle SEM de biodisponibilité a été vérifié pour les deux éléments non-essentiels considérés dans la thèse (Cd et Pb). Pour le Zn, le modèle a conforté les résultats précédents : les concentrations en métaux dans les vers n'ayant pas présenté d'augmentation significative avec la disponibilité. Il n'y avait donc pas de chaîne de causalité entre disponibilité, biodisponibilité environnementale et biodisponibilité toxicologique pour le Zn dans notre étude. Cela montre que pour les éléments essentiels, d'autres indicateurs que des concentrations de métaux à un temps donné doivent être recherchés pour évaluer la biodisponibilité (par exemple des mesures d'accumulation en cinétique).

Le modèle SEM pour le Cd est représenté Figure 6.8. Nous avons montré que parmi les biomarqueurs considérés, seule l'expression de la MT vérifiait le modèle de mesure. Nos résultats confirment la pertinence de cet indicateur pour renseigner la biodisponibilité du Cd. Ils montrent de plus que ce biomarqueur est robuste aux facteurs confondants que sont les paramètres physico-chimiques du sol et la multi-contamination.

Les résultats ont mis en évidence que l'unique prise en compte des fractions directement disponibles du Cd (dans la solution du sol ou facilement extractible) ne suffisait pas à expliquer la relation entre biodisponibilité environnementale et teneur totale en Cd dans le sol. En effet dans le modèle, la teneur totale avait un effet direct sur la variable latente décrivant la biodisponibilité environnementale. Cela montre que les fractions de métaux disponibles pour les vers ne sont pas uniquement les fractions solubles ou facilement extractibles, mais également des formes plus fortement adsorbées aux constituants du sol.

Pour le Pb, le modèle SEM de biodisponibilité présentait également un bon ajustement

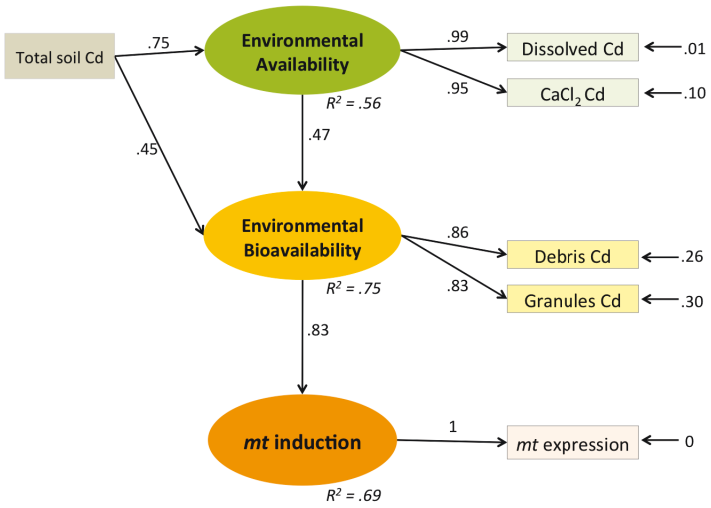

Figure 6.8: Modèle SEM de biodisponibilité du Cd pour le ver de terre. La disponibilité environnementale est reflétée par les concentrations de Cd dissous prédit par un modèle semi-mécaniste et de Cd extractible au $CaCl_2$. La biodisponibilité environnementale est reflétée par les concentrations en métaux dans deux fractions subcellulaires: débris cellulaires et granules). La biodisponibilité toxicologique est reflétée par un seul biomarqueur: l'expression de la MT et représente donc l'induction de la MT.

aux données. Des indicateurs différents de ceux identifiés pour le Cd se sont révélés pertinents par rapport à la définition causale de biodisponibilité (voir Figure p.133). Les résultats ont montré que, comme pour le Cd, la biodisponibilité environnementale du Pb était influencée directement par la teneur totale en Pb dans le sol.

Conclusions et Perspectives

Dans cette étude, nous avons montré l'intérêt de l'approche SEM pour aborder la nature structurelle de la biodisponibilité. Dans notre gradient de sols, plusieurs méthodes souvent utilisées pour mesurer la biodisponibilité étaient reliées de manière cohérente avec le modèle causal. Ces résultats renforcent donc leur pertinence dans le cadre de l'évaluation du risque environnemental lié aux métaux dans les sols. Notamment, la teneur totale en métaux dans le sol pourrait représenter une mesure pertinente de la disponibilité pour les vers de terre lorsque l'on s'intéresse à des sols agricoles contaminés historiquement.

Un intérêt majeur du modèle est de pouvoir représenter des variables théoriques qui sont reflétées par plusieurs mesures. Cela a permis de s'affranchir des imperfections de chacune des variables observées pour modéliser chaque étape de la biodisponibilité. De plus, ce modèle

présente l'avantage de rendre compte de manière synthétique des relations entre disponibilité et biodisponibilité. Ce type de modèle nécessite cependant un grand nombre d'observations. Dans le cadre de cette thèse, nous nous sommes focalisés sur les relations causales entre les trois maillons de la chaîne de causalité qui constituent la biodisponibilité, et nous avons montré que ces relations pouvaient être robustes.

Perspectives

Une des perspectives majeure serait d'intégrer l'effet des paramètres physico-chimiques dans le modèle. Celui-ci peut intervenir à plusieurs niveaux. Tout d'abord les paramètres du sol modulent la disponibilité environnementale. D'autre part, ils affectent la biodisponibilité environnementale de manière directe selon les hypothèses du modèle du ligand biotique. Dans ce modèle, la compétition entre les ions métalliques libres et d'autres cations pour se fixer au site d'action toxique est prise en compte pour prédire la toxicité des métaux pour les organismes. Enfin les paramètres physico-chimiques affectent la physiologie et le comportement des organismes des sols tels que les vers de terre. Cette influence affecte à la fois la biodisponibilité environnementale et toxicologique. Une seconde perspective majeure de ce travail serait d'intégrer les effets de différents métaux afin de mieux prédire la biodisponibilité toxicologique. Le modèle SEM est finalement un excellent outil pour identifier des mesures chimiques et biologiques pertinentes et robustes dans les sols contaminés *in situ*. Il offre un nouveau cadre d'analyse pertinent pour étudier les interactions complexes qui existent entre métaux, sol et organismes du sol.

Résumé

Evaluer le risque environnemental que représente la contamination du sol par des éléments en trace métalliques est un enjeu important. Lorsque l'on cherche à faire le lien entre l'exposition aux métaux et ses impacts sur un organisme, il est nécessaire de prendre en compte la biodisponibilité du contaminant plutôt que sa teneur totale dans le sol. Cependant, la biodisponibilité est un concept dont il est difficile de rendre compte par une unique mesure chimique ou biologique. Dans la littérature, les indicateurs les plus utilisés ne sont en fait pas hiérarchisés, apparaissent non génériques, et leur analyse peut être faussée par des facteurs confondants. La biodisponibilité peut être décrite comme un processus à trois étapes : (i) la disponibilité des métaux dans le sol, (ii) leur absorption par l'organisme et (iii) les effets des métaux sur l'organisme. La modélisation graphique (tel que le SEM: modèle par équations structurelles) représente un cadre d'analyse pertinent pour tester les hypothèses de causalité qui sous-tendent cette définition et à terme parvenir à identifier des jeux d'indicateurs pertinents pour mesurer la biodisponibilité. Dans cette thèse, un modèle SEM décrivant la biodisponibilité des métaux pour le ver de terre a été développé. Pour le tester, nous avons réalisé une expérience d'exposition de vers de terre en conditions contrôlées. L'espèce de ver *Aporrectodea caliginosa* a été choisie car elle est fréquemment trouvée dans les sols tempérés. Nous avons de plus vérifié sa représentativité quant à la bioaccumulation des métaux en la comparant à d'autres espèces à partir des données de la littérature. Un large panel de sols contaminés *in situ* et non contaminés a été choisi afin de créer un gradient d'exposition réaliste aux métaux (Cd, Pb et Zn). La disponibilité des métaux dans le sol a été mesurée par des méthodes expérimentales (extractions) et théoriques (modélisation). Les métaux absorbés par les animaux ont été quantifiés après exposition dans les vers entiers et dans trois fractions subcellulaires. Enfin des biomarqueurs de différents niveaux d'organisation biologique ont permis d'évaluer les effets des métaux sur les vers de terre. Nos résultats montrent que les relations entre indicateurs considérés individuellement étaient dépendantes de l'indicateur et du métal considéré. De plus, nous avons montré les difficultés qui émergent lorsque l'on cherche à relier indicateurs chimiques et biologiques de biodisponibilité dans des sols modérément contaminés par plusieurs métaux. Enfin, le modèle SEM a été confronté aux données et a permis d'identifier des jeux d'indicateurs chimiques et biologiques qui vérifiaient les hypothèses d'une chaîne de causalité entre disponibilité, absorption et effets, et ce dans le contexte de sols faiblement contaminés *in situ*. Cette étude montre la pertinence de l'approche SEM qui permet d'aller au-delà de mesures uniques représentant imparfaitement le concept de biodisponibilité et de fournir un cadre d'analyse synthétique pour évaluer la biodisponibilité dans un contexte environnemental réaliste.