Quantitative modelling of the response of earthworms to metals

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Chapter IV

Can Commonly Measurable Traits Explain Differences In Metal Accumulation And Toxicity In Earthworm Species?

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Abstract

There is no clear consensus in the literature on the metal accumulation pattern and sensitivity of different earthworm species. In the present study, accumulation and toxicity of Cu, Cd, Ni, and Zn in the earthworms *Lumbricus rubellus* (epigeic), *Aporrectodea longa* (anecic), and *Eisenia fetida* (ultra-epigeic) were determined after 28 days exposure in two soils. Metal accumulation and sensitivity were interpreted using the specific traits of different earthworm species. Results showed that for all four metals tested *L. rubellus* was the most sensitive species, followed by *A. longa* and *E. fetida*. At the same exposure concentration, internal concentrations followed the order: *L. rubellus* > *E. fetida* > *A. longa* for Cu and Ni, *L. rubellus* ≈ *E. fetida* ≈ *A. longa* for Cd, and *L. rubellus* > *A. longa* > *E. fetida* for Zn. Langmuir isotherms were used to model metal accumulation at both nontoxic and toxic exposure concentrations. The Cu, Cd, and Zn concentrations in *E. fetida* generally leveled off at high exposure concentrations but not for the other two species. *A. longa* showed a high capability of regulating internal Ni concentrations. The traits-based approaches suggested that most likely a group of earthworm traits together determined (differences in) metal accumulation and sensitivity. More research is needed in this respect to build up solid relationships between species-specific responses and traits, enabling cross-species extrapolation of accumulation and toxicity data.

4.1 Introduction

Metal contamination in the topsoil usually results from anthropogenic activities such as waste disposal, agricultural inputs, and industrial emissions (Onianwa et al., 2003). Adverse effects of elevated concentrations of metals (e.g., Cu, Cd, Ni, and Zn) on soil functions and on soil-dwelling organisms have been widely recognized (Lock and Janssen, 2001; Santorufo et al., 2012). Earthworms are ecosystem engineers which play a unique role in soil formation and soil fertility (Blouin et al., 2013). They are especially active in the topsoil and continuously exposed to metals through direct contact with soil particles and porewater, providing an early indication of metal pollution and ecosystem disturbance (Nahmani et al., 2007; Sizmur and Hodson, 2009; Spurgeon et al., 2006).

Earthworms are generally classified into three ecotypes according to their ecological strategies: epigeics, endogeics and anecics (Bouché, 1977). The epigeic earthworms (such as *Lumbricus rubellus* and *Dendrobaena octaedra*) live on the surface of the soil in leaf litter. They feed on the surface litter and are found intimately associated with plant roots (Curry and Schmidt, 2007; Domínguez, 2004). The endogeic earthworms (such as *Aporrectodea caliginosa* and *Allolobophora chlorotica*) are mineral soil-dwelling species. They feed predominantly within the mineral soil and rarely venture to the soil surface (Bouché, 1977; Curry and Schmidt, 2007). The anecic earthworms (such as *Aporrectodea longa* and *Lumbricus terrestris*) make permanent vertical burrows in soil. They draw organic matter from the soil surface into their deep, permanent burrows to feed on (Butt, 1993; Domínguez, 2004). The above mentioned earthworm species are commonly found throughout Europe and have been introduced to North America (Hendrix and Bohlen, 2002). The ultra epigeic *Eisenia fetida*, currently used as the standard earthworm in terrestrial ecotoxicology testing (Langdon et al., 2005; Peijnenburg and Vijver, 2009), is cultured for vermicomposting or fishing and is rarely found in natural soils. It inhabits only organic matter-rich locations (e.g., composts and dung heaps) (Edwards and Arancon, 2004). These differences in ecological strategies have consequences for exposure, accumulation and sensitivity to metals. For instance, earthworm traits such as feeding behavior or the soil habitat (e.g., depth at which the earthworm species live) can affect the degree of contact with contaminated soil particles and therefore may cause differences in exposure to metals.

Earthworms have developed physiological mechanisms to adapt to metal exposure. They are able to actively regulate metal bioaccumulation via exclusion or increased elimination (Hopkin, 1989). Spurgeon and Hopkin (1999) showed that equilibrium body concentrations of Cu and Zn in *E. fetida* were reached after fast initial uptake, highlighting a physiological control and an effective excretion of these metals. For Cd and Pb, the excretion was slow or absent. Earthworms have the ability to prevent themselves from toxic effects by sequestration, detoxification, and storage of excess metal (Vijver et al., 2004), resulting in high accumulation levels. Ecophysiological studies showed that metal binding to proteins such as metallothioneins or sequestration in granules (Spurgeon and Hopkin, 1999; Vijver et al., 2004) occurs depending on metal species and earthworm types.

Metal accumulation varied largely between different earthworm species and *E. fetida* is often found to be less sensitive to metals than other species (Nahmani et al., 2007; Spurgeon et al., 2000). Many researchers believe that species-specific differences in ecological strategies and physiological characteristics (detoxification and elimination strategies) might account for the differences in earthworm metal accumulation and sensitivity (Nahmani et al., 2007; Spurgeon et al., 2000; Spurgeon and Hopkin, 1996). In contrast, relatively little variation in metal accumulation between species was also reported (Langdon et al., 2005; Nannoni et al., 2011), although large differences in exposure concentrations were tested.

Based on the existing literature data, it was hypothesized that ecophysiological differences between earthworms will influence metal accumulation patterns and sensitivity, and that species-specific characteristics of earthworms will provide a clue for extrapolation across species. The main objectives of the present study were therefore as follows: (i) to determine the relative sensitivity of three different earthworm species (*L. rubellus, A. longa, and E. fetida*) to Cu, Cd, Ni, and Zn, (ii) to compare differences in accumulation patterns of each metal across different earthworm species, and (iii) to examine whether it is possible to extrapolate ecotoxicological effects from one earthworm species to another species using a traits-based approach.

4.2 Materials and methods

Earthworms

Mature earthworms *L. rubellus* and *A. longa* were collected from an unexploited site (nature reserve) in Leiden, the Netherlands. *E. fetida* was ordered from "Regenwormen Cultivation Farm" in Amsterdam, the Netherlands. Earthworms were kept in a climate room (15 °C, 16 h light and 8 h dark) and fed twice a week with organic-rich food (cow manure). All individuals of *L. rubellus*, *A. longa* and *E. fetida* selected for toxicity tests were adults with a well-developed clitellum. Their fresh weight ranged from 700 to 900 mg, 800 to 1000 mg, and 600 to 800 mg, respectively.

Test soils

Topsoil samples (0-15 cm) were collected at two agricultural sites (soil I and soil II) in the Netherlands. The soils were sieved (< 2 mm) and homogenised before use. Soil pH, organic matter content (OM), texture, cation exchange capacity (CEC) and maximum water-holding capacity were determined following the standard procedures described by Pansu and Gautheyrou (2006). Soil samples were spiked with Cd, Cu, Ni, and Zn in the form of metal acetate (Acros Chemicals; purity 98%). The designed levels of metal concentrations were soil- and metal-specific according to the previous studies (Spurgeon et al., 2000; Qiu et al., 2013). In soil I, except a control there were 9 levels of Cu concentrations (from 50 to 2000 mg/kg), 12 levels of Cd concentrations (from 50 to 3000 mg/kg), 10 levels of Ni concentrations (from 400 to 2800 mg/kg), and 13 levels of Zn concentrations (from 600 to 7000 mg/kg). In soil II, except a control there were 14 levels of Cu concentrations (from 50 to 4000 mg/kg), 14 levels of Cd concentrations (from 100 to 4500 mg/kg), 13 levels of Ni concentrations (from 800 to 7200 mg/kg), and 14 levels of Zn concentrations (from 800 to 10000 mg/kg). After spiking, the soils were subjected to alternated cycles of wetting and drying at 35 °C for two months to enhance acetate mineralization (Qiu et al., 2013).

Toxicity tests

Exposures were performed under controlled conditions at 15 °C for 28 days. Four randomly chosen earthworms were rinsed and put into plastic jars filled with 500 g soil samples. For each treatment, three replicates were used. All soils were maintained at a moisture content equivalent with 80% of the maximum water holding capacity during the experiment. During exposure, the earthworms were fed with cow manure (5g per jar) once a week. After 28 days of exposure, mortality was checked as it is the most frequently used endpoint (also scheduled in OECD guideline) to evaluate metal toxicity in earthworms (Van Gestel and Van Dis, 1988; Peijnenburg et al., 2009). Fresh body weight was measured before and at the end of the experiments. In the control soils, no significant loss of weight was observed after 28 days of exposure. The surviving earthworms were collected, washed and put on wetted filter paper to empty their guts for 48 hours. After gut voiding, earthworms were rinsed again and stored at minus 18 °C for further analysis.

Metal analysis

Soil samples were air-dried and sieved (< 2 mm). Earthworms were freeze dried for 48 hours. The soil and earthworm samples were digested using 1:4 v/v HCl (37% pro analysis, Baker) and HNO_3 (65% pro analysis; Riedel-de-Haen). After digestion, concentrations of Cu, Cd, Ni, and Zn in solution were analyzed by flame atomic absorption spectrophotometry (FAAS, Perkin Elmer AAnalyst 100). The certified reference materials ISE 989 and NRC DOLT-2 were used for analytical quality control. Measured concentrations were within 15% of the certified reference values.

Data analysis and statistics

Assuming that metal concentrations in earthworms would reach saturation at high exposure concentrations (Borgmann et al., 2004; Van Gestel et al., 2011), the relationship between metal concentrations in earthworms and total metal concentrations in soils were fitted by applying a Langmuir isotherm, which reads:

$$C_{\mathrm{internal}} = \frac{C_{\mathrm{max}} \cdot K_{\mathrm{L}} \cdot C_{\mathrm{soil}}}{1 + K_{\mathrm{L}} \cdot C_{\mathrm{soil}}} \qquad (4\text{-}1)$$

where C_{internal} is the metal concentration in earthworms (mg/kg dry body weight); C_{max} is the maximum metal concentration in earthworms (mg/kg dry body weight); C_{soil} is the total metal concentration in soil (mg/kg dry soil); K_{L} is the Langmuir constant (kg/mg). The Langmuir model was used to model metal uptake at all exposure concentrations (both nontoxic and toxic) except where 100% mortality occurred. Parameters estimation was performed by nonlinear regression using Origin 9.0 (Origin Lab, UK).

The metal concentrations causing 50% mortality (LC50) and their 95% confidence intervals were calculated using the trimmed Spearman-Karber method (Hamilton et al., 1977). All the data were statistically analyzed using one way analysis of variance (ANOVA) at significance level of $p < 0.05$ with SPSS 19.0 (IBM, USA). Duncan's test was used to detect significant differences. For all the calculations in the present study, measured concentrations were used unless otherwise stated.

4.3 Results

Soil properties

The selected physicochemical properties of the two unspiked soils are shown in Table 4.1. Total concentrations of Cu, Cd, Ni, and Zn were slightly higher in soil II than in soil I. The two test soils varied in pH, texture, OM, and CEC. The pH_{CaCl2} in soil I and soil II was 6.2 and 5.8, respectively. No significant changes in soil pH were observed after metal spiking and soil incubation for two months (Data not shown). Soil I, consisting of 70.5% sand, was classified as sandy loam. Soil II with 53.2% silt as silt loam.

Table 4.1 Selected physicochemical properties of the unspiked soils used for assessing earthworm-ecotype differences in metal accumulation and sensitivity.

Properties	Soil I	Soil II
pH[a]	6.2 ± 0.3	5.8 ± 0.2
Total Cu (mg/kg)	10.6 ± 0.4	26.2 ± 1.7
Total Cd (mg/kg)	0.17 ± 0.05	0.36 ± 0.04
Total Ni (mg/kg)	7.6 ± 0.7	10.9 ± 1.0
Total Zn (mg/kg)	91.0 ± 4.8	132 ± 10.4
Texture[b]	sandy loam	silt loam
Clay (%)	7.0	16.5
Silt (%)	22.5	53.2
Sand (%)	70.5	30.3
OM[c] (%)	5.7 ± 0.2	21.7 ± 1.3
CEC[d] (cmol/kg)	10.1 ± 0.3	38.8 ± 2.2
MWHC[e] (%)	53.5 ± 4.6	69.3 ± 5.7

[a]pH in 0.01 M $CaCl_2$ extract. [b]Determined by the hydrometer method (Pansu and Gautheyrou, 2006). [c]Organic matter content determined by the loss-on-ignition method (Pansu and Gautheyrou, 2006). [d]Cation exchange capacity determined by the ammonium acetate method (Pansu and Gautheyrou, 2006). [e]Maximum water-holding capacity determined by the saturation and gravity drainage method (Pansu and Gautheyrou, 2006).

Metal toxicity

Control earthworm survival was good in all tests, with less than 10% mortality after 28 days of exposure. The LC50 values for the effect of Cu, Cd, Ni, and Zn on the survival of the three earthworm species are listed in Table 4.2. *L. rubellus* was the most sensitive species to all metals, followed by *A. longa* and *E. fetida*. Significant differences in LC50 values between species in each soil were found ($p < 0.05$) (Table 4.2). The maximum differences between the highest and lowest LC50s of Cu, Cd, Ni, and Zn for the three species in soil I were approximately a factor of 18, 18, 3, and 4, respectively, and in soil II 13, 11, 3, and 5, respectively. The difference in species sensitivity was larger for Cu and Cd than for Ni and Zn. Although the LC50s of metals for each species varied between soils, the rankings of species sensitivity (*L. rubellus* > *A. longa* > *E. fetida*) were the same in the two soils which had quite different properties (Table 4.1).

Table 4.2 Median lethal concentrations (LC50s) of Cu, Cd, Ni, and Zn, expressed as total soil concentrations (mg/kg dry soil) and internal concentrations (mg/kg dry body weight), for the earthworms *Lumbricus rubellus*, *Aporrectodea longa*, *and Eisenia fetida* when exposed for 28 days to different metals in the two tested soils.

	LC50s for effect on mortality							
	Total soil Cu	Internal Cu	Total soil Cd	Internal Cd	Total soil Ni	Internal Ni	Total soil Zn	Internal Zn
Soil I								
L. rubellus	72 a	35	108 a	382	557 a	162	1135 a	1056
	(64-80) [*]	(29-40)	(75-156)	(309-472)	(497-624)	(142-185)	(998-1291)	(960-1161)
A. longa	111 b	26	211 b	397	1113 b	N.A.	2504 b	1376
	(80-153)	(22-30)	(173-258)	(357-441)	(911-1359)		(2260-2775)	(1253-1511)
E. fetida	1322 c	79	1932 c (1659-	698	1824 c	221	4121 c	N.A.
	(1033-1692)	(77-81)	2250)	(634-769)	(1655-2010)	(204-239)	(3746-4535)	
Soil II								
L. rubellus	284 a	40	277 a	233	1803 a	238	1532 a	917
	(208-386)	(20-79)	(170-450)	(138-394)	(1476-2203)	(170-334)	(1052-2231)	(695-1211)
A. longa	1942 b	79	1231 b	558	3514 b	N.A.	3828 b	1332
	(1779-2119)	(68-91)	(954-1588)	(485-643)	(3092-3994)		(3588-4083)	(1287-1377)
E. fetida	3717 c	230	2947 c	772	4977 c	N.A.	7264 c	N.A.
	(3678-3756)	(201-258)	(2707-3209)	(722-825)	(4676-5298)		(6677-7902)	

[*] LC50 values (with corresponding 95% confidence intervals in parentheses) marked with the different letters for the three earthworm species within the same soil for each metal are significantly different at $p < 0.05$ by Duncan's test. N.A. = Not applicable

Metal accumulation

Metal concentrations in the earthworms prior to the experiment are shown in Figure 4.1. There were no significant differences in initial internal Cu, Cd and Ni concentration between the earthworms of different species ($p > 0.05$). The Ni concentrations in all three earthworm species were below 0.1 mg/kg dry body weight. The Zn concentration in *E. fetida* (101 mg/kg dry body weight) was significantly lower compared to the other two earthworm species ($p < 0.05$), with 196 and 186 μg Zn/g dry body weight for *L. rubellus* and *A. longa*, respectively.

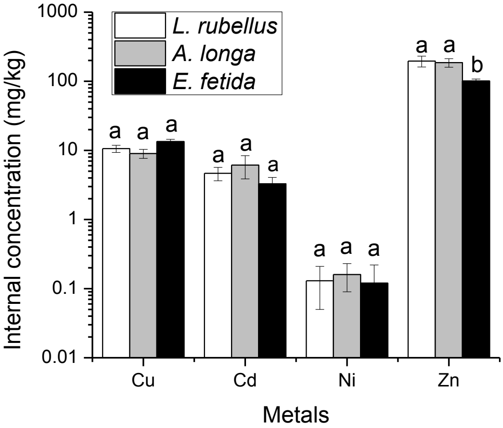

Figure 4.1 Internal metal concentrations in the earthworms *Lumbricus rubellus*, *Aporrectodea longa*, *and Eisenia fetida* before initiation of the accumulation and toxicity testing. Error bars represent the standard errors of the means (*n* = 6). Means marked with the same letter within the same metal are not significantly different at *p* < 0.05 by Duncan's test.

Cu

Accumulation of Cu in earthworms at nontoxic (no mortality is seen) and toxic (mortality is seen) exposure concentrations is shown in Figure 4.2. At low exposure levels (nontoxic concentrations), Cu concentrations in the earthworms increased almost linearly with increasing total soil concentrations. At the same exposure level, internal Cu concentrations followed the order: *L. rubellus* > *E. fetida*> *A. longa*. Cu concentrations in *E. fetida* did clearly level off with increasing exposure concentrations, reaching ca. 80 mg/kg dry body weight in soil I and ca. 240 mg/kg in soil II. For the other two species, no leveling off with concentrations of internal Cu was observed.

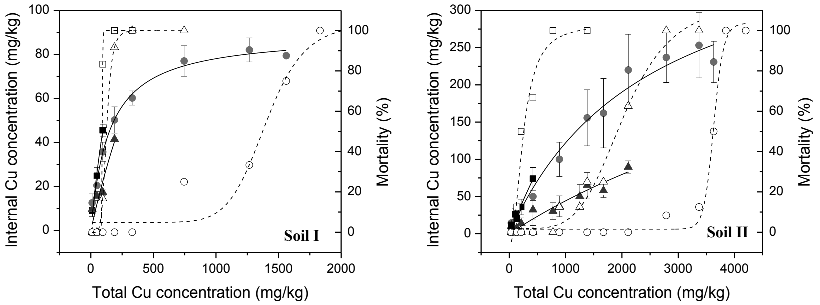

Figure 4.2 Relationships between internal Cu concentration (left Y-axis) in earthworms and earthworm mortality (right Y-axis) on one hand and total Cu concentration in the two soils tested on the other hand, following exposure for 28 days. Solid symbols indicate Cu accumulation in *Lumbricus rubellus* (■), *Aporrectodea longa* (▲), and *Eisenia fetida* (●). Open symbols indicate Cu toxicity in *L. rubellus* (□), *A. longa* (Δ), and *E. fetida* (○). Solid lines are Langmuir model fits (equation 4-1). Dashed lines are logistic toxicity model fits.

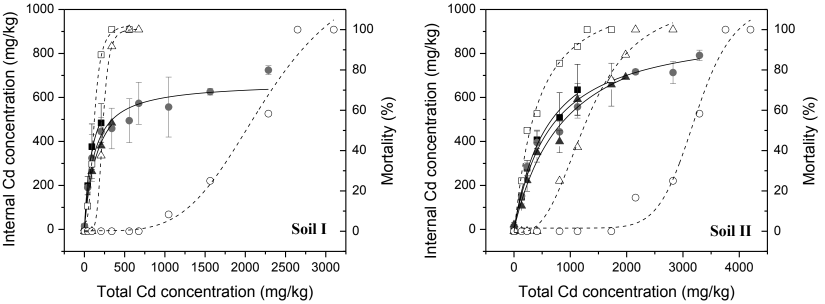

Figure 4.3 Relationships between internal Cd concentration (left Y-axis) in earthworms and earthworm mortality (right Y-axis) on one hand and total Cd concentration in the two soils tested on the other hand, following exposure for 28 days. Solid symbols indicate Cd accumulation in *Lumbricus rubellus* (■), *Aporrectodea longa* (▲), and *Eisenia fetida* (●). Open symbols indicate Cd toxicity in *L. rubellus* (□), *A. longa* (Δ), and *E. fetida* (○). Solid lines are Langmuir model fits (equation 4-1). Dashed lines are logistic toxicity model fits.

The Langmuir model fitted the Cu accumulation data well, with $R^2 > 0.9$ for all species in the two soils (Table 4.3). The maximum body concentration ($C_{\max}$) of Cu for each species differed in each soil. When relating Cu toxicity to internal concentrations, poor model (logistic) fits were obtained with R^2 of 0.49, 0.56 and 0.70 in soil I and 0.62, 0.75, 0.20 in soil II for *L. rubellus*, *A. longa*, and *E. fetida*, respectively. LC50s of Cu based on internal concentrations are listed in Table 4.2. For each species in the different soils, LC50 values based on internal concentrations were much closer than that based on total soil concentrations.

Table 4.3 Langmuir isotherm parameters relating internal metal concentrations in the earthworms *Lumbricus rubellus*, *Aporrectodea longa*, and *Eisenia fetida* to total metal concentration in soil, after 28 days exposure.

		Soil I			Soil II		
		L. rubellus	*A. longa*	*E. fetida*	*L. rubellus*	*A. longa*	*E. fetida*
Cu							
	$C_{\max}$[a]	167 (79)[d]	147 (86)	89 (3)	717 (138)	394 (98)	431 (60)
	$\log K_L$[b]	-2.41	-2.71	-2.17	-3.57	-3.89	-3.49
	R^2_{adj}[c]	0.979	0.906	0.986	0.955	0.936	0.971
Cd							
	$C_{\max}$	712 (71)	705 (53)	661 (31)	946 (97)	1008 (107)	930 (55)
	$\log K_L$	-1.97	-2.21	-2.01	-2.81	-3.07	-2.88
	R^2_{adj}	0.994	0.997	0.924	0.993	0.935	0.952
Ni							
	$C_{\max}$	390559[e](-)[f]	81 (28)	405 (61)	476060[e] (-)	87 (36)	290854[e] (-)
	$\log K_L$	-7.12	-3.03	-3.12	-7.61	-3.49	-6.69
	R^2_{adj}	0.975	0.938	0.919	0.883	0.711	0.766
Zn							
	$C_{\max}$	1961 (348)	2597 (761)	818 (278)	2428 (360)	2704 (707)	530 (151)
	$\log K_L$	-3.00	-3.43	-3.71	-3.55	-3.59	-3.48
	R^2_{adj}	0.937	0.830	0.833	0.915	0.878	0.774

[a]$C_{\max}$ = maximum internal concentration (mg/kg dry body weight). [b]K_L = Langmuir constant (kg/mg). [c]Determination coefficients adjusted for the degrees of freedom. [d]Standard error are given in the brackets. [e]Far outside the range of concentrations measured in earthworms and Langmuir isotherm had linear shape. [f]Very wide standard errors.

Cd

Internal Cd concentrations in the three earthworm species generally increased with increasing exposure concentrations in the two soils (Figure 4.3). At any given exposure concentration within the range of 0-500 mg Cd/kg in soil I and 0-1500 mg Cd/kg in soil II, no statistically significant differences in internal Cd concentrations of all three earthworms species were observed ($p > 0.05$) (Figure 4.3). Similar accumulation patterns were observed for each species in different soils. Internal Cd concentrations in *E. fetida* levelled off at approximately 700 mg/kg dry body weight with increasing total Cd concentrations in the two soils. There were no leveling off with concentrations of internal Cd in *L. rubellus* and *A. longa*.

There were no significant differences in $C_{\max}$ values of Cd for the three earthworm species in each soil ($p > 0.05$). However, $C_{\max}$ values of Cd for each earthworm species differed in the two soils (Table 4.3). LC50 values of Cd expressed as internal concentrations are shown in Table 4.2. Large differences in LC50 values between soils indicated that internal concentration failed to explain the variance in Cd toxicity in the two soils selected.

Ni

At the same exposure level, internal Ni concentrations ranged as follows: *L. rubellus* > *E. fetida*> *A. longa* (Figure 4.4). *A. longa* was found to regulate its internal Ni to a fairly low level (ca. 50 mg/kg dry body weight) at different exposure concentrations. For *L. rubellus* and *E. fetida*, regulation of internal Ni was not observed. For these two species 100% mortality occurred when internal Ni exceeded 250 mg/kg dry body weight in soil I and 300 mg/kg dry body weight in soil II.

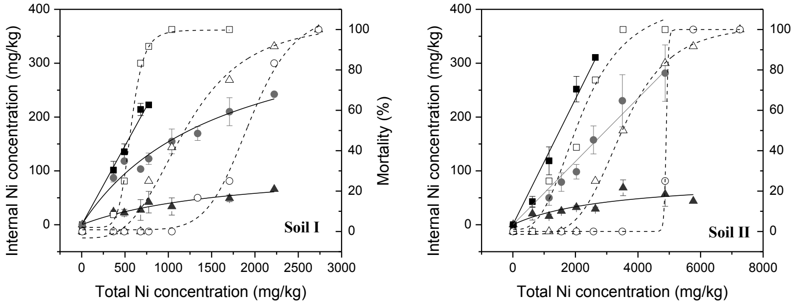

Figure 4.4 Relationships between internal Ni concentration (left Y-axis) in earthworms and earthworm mortality (right Y-axis) on one hand and total Ni concentration in the two soils tested on the other hand, following exposure for 28 days. Solid symbols indicate Ni accumulation in *Lumbricus rubellus* (■), *Aporrectodea longa* (▲), and *Eisenia fetida* (●). Open symbols indicate Ni toxicity in *L. rubellus* (□), *A. longa* (Δ), and *E. fetida* (○). Solid lines are Langmuir model fits (equation 4-1). Dashed lines are logistic toxicity model fits.

The $C_{\max}$ values of Ni for *A. longa* were similar in the two soils tested (Table 4.3). Ni concentrations in *L. rubellus* and *E. fetida* increased almost linearly with increasing total Ni concentrations, resulting in a linear Langmuir curve, with unrealistically high values of $C_{\max}$. This shows the Langmuir model was not an optimal model for fitting the accumulation data in these cases. LC50 values of Ni based on internal concentration are listed in Table 4.2. For *A. longa*, the calculation of LC50 based on internal Ni concentrations was not possible because of the relatively constant amount of internal Ni at different exposure concentrations.

Zn

At the same exposure level, internal Zn concentrations followed the order: *L. rubellus* > *A. longa* > *E. fetida* (Figure 4.5). A fairly constant Zn concentration (200-400 mg/kg dry body weight) was found in *E. fetida* in both soils spiked with different levels of Zn, indicating that Zn was highly regulated by *E. fetida*. For the other two earthworms, no obvious regulation of Zn was shown and accumulation patterns of Zn were similar. When internal Zn concentration exceeded 1500 mg/kg dry body weight, both *L. rubellus* and *A. longa* died.

The C_{max} values of Zn and corresponding $\log K_L$ values for the three earthworm species are given in Table 4.3. The Langmuir model provided good fits for Zn accumulation in the different earthworm species as reflected by the high R^2 values given in Table 4.3. LC50 values of Zn for *L. rubellus*, expressed as internal concentrations, were similar in the two soils. This was also the case for *A. longa* (Table 4.2). At different exposure concentrations, *E. fetida* maintained Zn at a constant level. It therefore was not possible to calculate LC50 values based on internal concentrations for *E. fetida*.

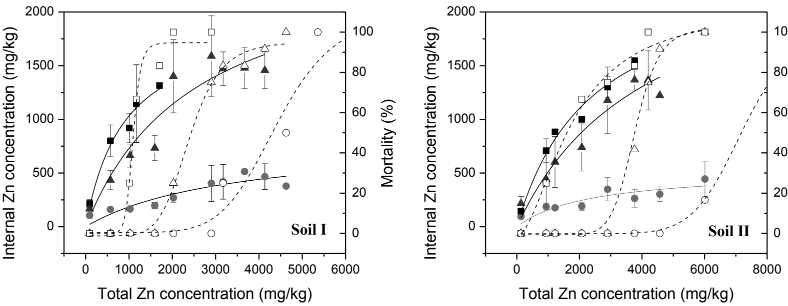

Figure 4.5 Relationships between internal Zn concentration (left Y-axis) in earthworms and earthworm mortality (right Y-axis) on one hand and total Zn concentration in the two soils tested on the other hand, following exposure for 28 days. Solid symbols indicate Zn accumulation in *Lumbricus rubellus* (■), *Aporrectodea longa* (▲), and *Eisenia fetida* (●). Open symbols indicate Zn toxicity in *L. rubellus* (□), *A. longa* (Δ), and *E. fetida* (○). Solid lines are Langmuir model fits (equation 4-1). Dashed lines are logistic toxicity model fits.

4.4 Discussion

Nontoxic and toxic exposure concentrations

Earlier studies on metal accumulation in earthworms mainly focused on relatively low, nontoxic exposure concentrations (Hobbelen et al., 2006; Li et al., 2009; Santorufo et al., 2012). In the present study, Cu, Cd, Ni and Zn uptake by three different earthworm species was investigated at broad concentration ranges, even at levels causing toxicity. From the metal accumulation patterns in each species (Figure 4.2-4.5), it can clearly be seen whether the bioaccumulation was affected by toxic effects or not. Generally, within the range of nontoxic exposure levels, internal metal concentrations of the three earthworm species increased linearly with increasing exposure concentrations. At toxic exposure concentrations, internal metal concentration either leveled off or had a tendency to level off. Therefore, the Langmuir isotherm was chosen to relate internal metal concentrations in the earthworms with (non)toxic exposure concentrations in soil. Contrary to the Freundlich isotherm or linear regression, which just gives a mathematical description of the relation between body concentration and soil concentration, the Langmuir isotherm does have some mechanistic meaning (Borgmann et al., 2004). It provides an indication of the maximum level that may be reached in the earthworms, while it also gives a measure of the uptake affinity (indicated by

K_L) (Van Gestel et al., 2011). The relative high logK_L values of Cd compared to that of Cu, Ni and Zn in the present study indicated a high uptake affinity of the three earthworm species for Cd. The frequent observation of a high concentration plateau for metals in all three species suggested that internal sequestration or detoxification mechanism might be involved (Vijver et al., 2004). In some cases the Langmuir isotherm had an almost linear shape (e.g., *L. rubellus* for Ni). This suggested that earthworms did not possess an active excretion mechanism that may assist in eliminating excess metal from the body (Van Gestel et al., 2011). In this case, metal accumulation reflected the level of external exposure. When relating toxicity to internal concentrations in the surviving earthworms, the obtained LC50 values between soils for each species were much closer to each other than those based on total soil concentrations. This implied that external exposure might not be the main factor but some internal processes (toxicological bioavailability) driving metal toxicity, which is in agreement with previous studies of Peijnenburg et al. (1999) and Vijver et al. (2004). Although internal concentrations did represent the bioavailability much better than total soil concentrations, the internal concentrations cannot explain all toxicity variations between soils especially for those cases where internal metal concentrations of earthworms started to level off when exposure was approaching toxic levels. In those cases, earthworm traits might give more explanatory power.

Traits involved in metal accumulation and toxicity

Traits-based approach has a long history in evolutionary biology, facilitating mechanistic prediction on performance across environmental gradients (Webb et al., 2010). Previously, the results of earthworm ecotoxicological tests were often interpreted from the perspective of bioavailability and toxicity of metals (Hobbelen et al., 2006; Lukkari et al., 2005; Nannoni et al., 2011; Peijnenburg et al., 1999). Only a few studies dealt with the effects of ecophysiological traits of earthworms (e.g., Morgan and Morgan, 1999). However, recently characteristics of organisms received more attention in ecotoxicology, and showed to have explaining power in interpretation of organism vulnerability (sensitivity) to chemicals (Baird et al., 2008).

Following trait-based approaches, organisms are classified in terms of physiological, morphological or life-history traits (Webb et al., 2010). Rubach et al. (2012) have proven experimentally that there are quantitative links between organismal traits and toxicokinetic endpoints following exposure to pesticides. In the present study, the focus was only on the traits that likely might explain the differences as found in the earthworm accumulation and sensitivity to metals (Table 4.4). It should be noted that the traits given in Table 4.4 represent a non-conclusive list and other traits might also be of relevance. For now, there is no clear standard about the selection of traits (De Lange et al., 2013). The earthworms species selected in the present study all belong to the same family (Lumbricidae), but differ in their genus (Pop and Pop, 2004). Within commonly applied ecotoxicity tests with earthworms, *E. fetida* is often used being a representative species for the Lumbricidae group (Spurgeon et al., 2000; OECD, 2004). Measuring the accumulation and toxicity related traits might assist in extrapolating the results over species. Additionally it might give answers on why one species is more sensible to metals than another species, because two species of the same genus may show large differences in sensitivity. Hence it has been argued that taxonomy is not an inherently informative indicator for prospective effect assessment of metals (Rubach et al., 2010). Uptake and sensitivity related organism characteristics were either measured or collected from literature and linked to the observed accumulation patterns and toxicological responses as seen in our study (see Table 4.4). These selected traits are assumed to make the species react to metal stress differently from each other. Some of the earthworm traits were quantifiable, such as time to maturity, cocoon production, body weight, and length, and others were qualitative, such as calcium glands activity, immune system, food choice and mobility. Traits like cocoon production and respiration rate were included as they are related to the energy flow. Respiration is a measure of the part of the food intake which is converted to heat and hence represents the energy loss from the system (Terhivuo, 1989). More energy spent on reproduction and respiration indicates that less energy can be spent on detoxification strategies (Holmstrup et al., 2011). Food preference behavior was selected as it was suggested that the difference in dietary uptake was an important factor in contributing to the observed differences in metal accumulation between *L. rubellus* and *A. caliginosa*, although other factors are also contributory (Morgan and Morgan, 1999). Since *E. fetida* is artificially cultured for vermicomposting (Domínguez, 2004), it is assumed to be the species preferring the organic-rich food most. Doube et al. (1997) studied the food preference behavior of *A. longa* and *L. rubellus*, and found that after 7 days of exposure, the percentage of food (cow dung) removed by *L. rubellus* was much higher than that by *A. longa* in a cow dung and soil system. Therefore the following order of food choice (organic rich) was estimated: *E. fetida* > *L. rubellus* > *A. longa*. Transporters in membrane (ion channels and transport proteins) of earthworms were estimated to be similar across species based on the findings on aquatic organisms (Niyogi and Wood, 2004). As earthworms have permeable skin and high exposure via the porewater of soils (70-100% for metals) (Vijver et al., 2003), it was assumed that the terrestrial earthworms can be compared to aquatic organisms respecting their uptake. Moreover, general binding ligands and transporters (such as calcium and sodium transporters) in the membrane are inherent to every living cell. Therefore, the extrapolation among different (even aquatic) organisms appears to be valid.

Table 4.4 Differences in the traits probably affecting metal accumulation and toxicity in the earthworm species *Lumbricus rubellus*, *Aporrectodea longa*, and *Eisenia fetida*.

Type of traits	Traits	Quantitive/Qualitative differences between species			Sources
		L. rubellus	*A. longa*	*E. fetida*	
Life-history	time to maturity (months)	6-7	4	2-3	Butt, 1993; Edwards and Bohlen, 1996
	cocoon production (n/year)	106	18.8	> 200	Edwards and Bohlen, 1996; OECD, 2004
Ecological	habitat (depth) (cm)	0-5	> 10	5-10	Bouché, 1977
	organic rich (food choice)	*E. fetida* > *L. rubellus* > *A. longa*			Doube et al., 1997; Domínguez, 2004
	mobility	good	limited	reasonable	Edwards and Bohlen, 1996
Physiological	calcium glands activity	*L. rubellus* and *E. fetida* > *A. longa*			Piearce, 1972; Spurgeon and Hopkin, 1996
	transporters in membrane	*L. rubellus* ≈ *A. longa* ≈ *E. fetida*			Estimated based on the aquatic organisms (Niyogi and Wood, 2004)
	respiration rate	*L. rubellus* and *E. fetida* > *A. longa*			Uvarov and Scheu, 2004
	mucus production	*E. fetida* > *L. rubellus* and *A. longa*			Zhang et al., 2009
	Immune-competent cells	*E. fetida* > *L. rubellus* and *A. longa*			Plytyce et al., 2011a; 2011b
Morphological	length (mm)	60-90	90-140	70-110	present study
	weight (mg)	700-900	800-1000	600-800	present study
	surface area/ mass	*L. rubellus* > *E. fetida* > *A. longa*			Spurgeon and Hopkin, 1996; Spurgeon et al., 2000

Do earthworm traits provide a clue for predicting metal toxicity?

In the present study, species sensitivity to Cd, Cu, Zn and Ni decreased in the following order: *L. rubellus* > *A. longa* > *E. fetida*. Langdon et al. (2005) reported that both *L. rubellus* and *A. caliginosa* were more sensitive to Pb than *E. andrei*. Spurgeon et al. (2000) found that *L. rubellus* and *A. caliginosa* were more sensitive to Zn than *L. terrestris* and *E. fetida.* Based on the traits theory, differences in sensitivity between species might be explained as follows. The epigeic earthworm *E. fetida* (or *E. andrei*) feeds almost entirely on the soil surface on organic matter (cow manure in the present study), whereas the endogeic *A. caliginosa* and anecic *A. longa* would be more exposed to metals via the soil porewater as they live and feed in the soil (Langdon et al., 2005). Some authors suggested that the differences in sensitivity are partially attributed to the activity of the calciferous glands in earthworms as calcium is involved in the sequestration and elimination of many metals through the chlorogogenous tissue (Piearce, 1972; Morgan and Morgan, 1988; Spurgeon and Hopkin, 1996; Spurgeon et al., 2000). *E. fetida*, which has active calciferous glands, was least sensitive, while *A. caliginosa and A. longa* having less active or inactive calciferous glands (Piearce, 1972; Spurgeon et al., 2000), were more sensitive. *L. rubellus* was shown to be more sensitive than the other species despite that it has active calciferous glands. The relatively small size of *L. rubellus* with a large ratio of surface area to mass (potential high uptake rate) probably serves as an explanation, as some studies have shown that the rate of uptake, not the concentration itself, was the superior predictor of metal toxicity (e.g., Van Straalen et al., 2005). Besides, the least sensitive earthworm species *E. fetida* has higher immune ability and mucus production compared to the other species. Plytycz et al. (2011a; 2011b) reported that earthworms have immune systems displaying striking inter-species differences. For instance, eleocytes (one type of immune-competent cells) are very seldom present in species belong to *Lumbricus* and *Aporrectodea* genera, but are common in species such as *E. fetida*. Overall, ranking of species sensitivity cannot be quantitively linked to one specific trait. It is most likely that each species possesses different trait combinations to cope with metal stress (De Lange et al., 2013).

Do earthworm traits provide a clue for predicting metal accumulation?

The amount of metal accumulated in earthworms at the same exposure concentration followed the order: *L. rubellus* > *E. fetida* > *A. longa* for Cu and Ni, *L. rubellus* ≈ *E. fetida* ≈ *A. longa* for Cd, and *L. rubellus* > *A. longa* > *E. fetida* for Zn. Assuming the traits theory applies in our cases, a set of traits can be related to the metal accumulation ranking in the earthworm species tested (Table 4.4). In addition to good mobility (Edwards and Bohlen, 1996), *L. rubellus* has larger surface area/mass ratio (thus higher potential uptake rate as suggested by Spurgeon et al. (2000)) than the other two species. This may cause *L. rubellus* to be more exposed to metals, resulting in higher internal metal concentrations compared to the other species. The relatively low accumulation of metals in *A. longa* might be explained by its limited mobility (which was also observed in the present study) and thus less exposure as this species was inclined to stay in their deep burrows (Emmerling and Strunk, 2012). As the traits listed in Table 4.4 differed largely between the different species, it was surprising that the three earthworm species accumulated similar amounts of Cd at the same exposure concentrations. This might be attributed to the high uptake affinity of earthworms for Cd (as reflected by logK_L). Traits that showed to be similar over all species were transporters in membrane (i.e., ion channels and transport proteins), which determines uptake affinity for different metals (Niyogi and Wood, 2004). Implementing the traits theory for quantitatively explaining all the observed accumulation patterns of different earthworm species was shown to be difficult.

Earthworms did not appear to accumulate Cu at high concentrations even when exposed to heavily contaminated soils. This is in agreement with the findings by Nannoni et al. (2011), and is probably caused by the relatively high intrinsic earthworm toxicity of Cu. Although Cu concentrations in the three earthworm species were almost the same before initiation of the accumulation and toxicity testing, internal Cu concentrations after 28 days were always higher in *L. rubellus* than in *E. fetida* and *A. longa* at the same exposure levels. Internal Cd concentrations were identical for the three species at approximately 500 mg Cd /kg in soil I and 1000 mg Cd/kg in soil II. At higher soil concentrations, the internal Cd concentrations in *E. fetida* strongly leveled off while 100% mortality of *L. rubellus* and *A. longa* occurred. This suggests that *E. fetida* is less sensitive to Cd than *L. rubellus* and *A. longa* not because it can tolerate more Cd inside the body but because it is able to more effectively prevent uptake of Cd from soil. Langdon et al. (2005) reported no significant differences in internal Pb levels in *L. rubellus*, *A. caliginosa* and *E. andrei* exposed to same soil Pb concentrations. They claimed that the relatively small size of the soil container likely prevented the traits of different earthworms manifesting itself. Nannoni et al. (2011) found similar internal concentrations of Cu, Cd, Zn, Pb, As, and Sb in the endogeic earthworms *A. rosea* and *A. caliginosa* collected from 15 contaminated sites. They suggested that earthworm species belonging to the same ecophysiological group have a similar tendency to accumulate metals. However, it has been argued that two species that are strongly taxonomically related do not necessarily respond similar to metals and that the taxonomic distance can only give a hint (Rubach et al., 2010). Internal Ni concentration was regulated at a fairly low level by *A. longa*, which was different from *L. rubellus* and *E. fetida* who did not show this pattern, as reflected by the linear Langmuir curves. This pattern was Ni-specific. There are comparatively few data on Ni accumulation in *A. longa*. Yan et al. (2011) reported that internal Ni concentrations ranged from 109 to 286 mg/kg dry body weight in *E. fetida* exposed for 28 days to the highest Ni concentrations (1000 mg/kg) spiked into a range of Chinese soils. Ni concentrations in *E. fetida* in the present study were at the same level. Generally, the maximum level of Ni that may be reached in all the three earthworm species was lower than that of Cd and Zn. This could be due to the mode of action of Ni. This metal is known to cause skin irritation, which may lead to damage to the epithelium of both the gut and the body wall (Muñoz and Costa, 2012). As a consequence, uptake of the metal may be hampered by its damaging effect to the membranes that are responsible for its uptake. Internal Zn levels in *E. fetida* were maintained at a constant level (ca. 250 ug/g dry body weight) at different exposure concentrations. This finding is in agreement with earlier observations showing that the body concentration of Zn in *E. fetida* is regulated at a fairly constant level, regardless of the Zn concentration of the soil (Lock and Janssen, 2001; Lukkari et al., 2005). This may be explained by the rapid Zn elimination in *E. fetida* (Spurgeon and Hopkin, 1999). *L. rubellus* and *A. longa* showed to have accumulation patterns that could be described by the Langmuir isotherm. When the internal Zn concentration of these two species exceeded 1500 mg/kg dry body weight, 100% mortality occurred regardless of the soil in which the earthworms were exposed. Previous studies reported that Zn concentrations in *L. rubellus* ranged from 394 to 3873 mg/kg dry body weight (Morgan and Morgan, 1988). At such high internal exposure levels, earthworms must either store Zn in nontoxic forms (e.g., binding to specific metal-binding proteins, and storage in granules) (Vijver et al., 2004) or suffer from Zn toxicity (Hobbelen et al., 2006).

Earthworms have different capacities to accumulate metals. Generally, the accumulation patterns of the different metals were not straightforwardly related to the different earthworm species or one specific trait. Empirical traits-based approaches face the challenge of how to make a priori selection of traits to be included for analysis and quantitatively integrating these traits.

4.5 Conclusions

From this study, it can be concluded that metal accumulation patterns and sensitivity differed between earthworm species. Variations in metal accumulation and sensitivity of species might result from differences in ecological strategies and from physiological differences in detoxification and elimination strategies. At the same exposure concentration, internal Cu and Ni concentrations were highest in *L. rubellus*, intermediate for *E. fetida*, and lowest in *A. longa*. For internal Zn concentrations, the order was *L. rubellus* > *A. longa* > *E. fetida*. There were no significant differences in internal Cd concentrations between species. Species sensitivity to all metals followed the same order: *L. rubellus* > *A. longa* > *E. fetida*. In the context of risk assessment, the results obtained with *E. fetida* should be interpreted with caution taking into account the low sensitivity of this species. Although traits-based approach showed to be powerful in explaining evolutionary processes, and is promising in ecotoxicology, quantitative interpretation of our results with traits was difficult. Based on our findings, likely more than one trait is involved in the processes of metal accumulation and toxicity in earthworms. More research is needed on these aspects (e.g., identifying new traits), enabling ecologically-sound extrapolation of ecotoxicological data across species of earthworms.