Effect of metal pollution on genetic variation in natural populations of selected soil invertebrate species with different dispersal potential

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Chapter 1

Toxicokinetics of metals in the earthworm *Lumbricus rubellus* exposed to natural polluted soils – relevance of laboratory tests to the field situation

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Abstract

The aim of this study was to estimate the bioavailability of essential (Zn, Cu) and non-essential metals (Cd, Pb) to the earthworm *Lumbricus rubellus* exposed to soils originating from a gradient of metal pollution in Southern Poland. Metal uptake and elimination kinetics were determined and related to soils properties. Experimental results were compared with tissue metal concentrations observed in earthworms from the studied transect. Cd and Pb were intensively accumulated by the earthworms, with very slow or no elimination. Their uptake rate constants, based on 0.01 M $CaCl_2$-extractable concentrations in the soils, increased with soil pH. Internal concentrations of Cu and Zn were maintained by the earthworms at a stable level, suggesting efficient regulation of these metals by the animals. The estimated uptake and elimination kinetics parameters enabled fairly accurate prediction of metals concentrations reached within a life span of *L. rubellus* in nature.

Keywords

kinetics, metals, bioavailability, *Lumbricus rubellus*

Introduction

Toxicity of chemicals is determined by their bioavailability as organisms respond only to the fraction of chemicals available for uptake from the environment. Therefore, the assessment of bioavailability is crucial for risk assessment. The bioavailability of metals in soil depends on a wide range of factors, including the characteristics of the metal, its concentration, properties of the soil, source and history of pollution as well as the physiology of the organism (Spurgeon and Hopkin 1999b; Van Gestel and Mol 2003; Van Gestel 2008). To estimate bioavailability, a toxicokinetics approach is commonly used (Peijnenburg et al. 1999). It provides information about bioaccumulation and time to reach steady-state tissue concentrations (Van Straalen et al. 2005). It enables prediction of the physiological fate of metals.

The toxicokinetics approach is usually applied in laboratory tests, using standard test conditions (e.g. OECD soil) and cultured test organisms. Standard conditions are important for reproducibility of experiments and comparison between different studies (Nahmani et al. 2007a). On the other hand, by performing toxicokinetics tests at laboratory conditions, heterogeneity found in natural ecosystems is eliminated. Moreover, by using organisms from established cultures, individual variation between tested animals is reduced. This effect could be obscured when using cultures established from field-collected individuals (Spurgeon et al. 2011). Such variation, e.g. in internal metal concentrations, is commonly observed between individual organisms sampled in the field. It is therefore not certain to what extent the values derived from purely laboratory toxicokinetics experiments are indicative of the field situation.

Earthworms, considered to be sentinel species and ecosystem engineers, are of particular concern in ecotoxicological studies. They are commonly used to determine metal toxicity and bioavailability in soil (Hobbelen et al. 2006; Walker et al. 2006; Nahmani et al. 2007a; Diez-Ortiz et al. 2010). According to previous studies (Spurgeon et al. 1994; Spurgeon and Hopkin 1996; Spurgeon et al. 2003; Nahmani et al. 2007b) metal pollution has adverse effects on earthworm life cycle parameters. However, many earthworm species inhabit highly contaminated sites, where they accumulate metals, reaching high internal concentrations (Andre et al. 2010; Tosza et al. 2010). One of the ecological types recognised within earthworms are soil and litter dwelling epigeic earthworms living close to the soil surface. Epigeic earthworms are, thus, exposed to metals accumulated in soil and in the litter layer. As litter and the organic soil layer are often characterized by high accumulation of metals, the real exposure under natural field conditions may be higher than during laboratory tests using only fully homogenised soil.

The aim of this study was to assess bioavailability of Cd, Pb, Cu and Zn to earthworms exposed to metal-polluted natural soils originating from a gradient of pollution in Southern Poland. For this purpose a toxicokinetics approach was applied. The earthworm *Lumbricus rubellus*, a widely distributed epigeic species, which is also present at the studied transect, was used as the test organism. The accumulation of metals in earthworms originating from uncontaminated soils was compared with individuals native to the metal-polluted area. This comparison allowed us to examine to what extent the toxicokinetics-derived data are indicative of the field situation. Toxicokinetics results were used to assess real exposure to metals and to explain the pattern of earthworm distribution observed in the field.

Materials and methods

Soils

Natural soils used in the experiment were collected at five sites (named OL1-OL5; collectively called Olkusz soils) located in a mixed pine forest at increasing distances from the zinc-and-lead smelter 'Bolesław', near Olkusz in Southern Poland (approximately 50°16′-50°19′ N, 19°29′-19°32′ E; see Fig. 1 and Table 1). At each site 10-15 samples were randomly collected from the ~10 cm upper soil layer in an area of approximately 200 m^2. Soil samples were air dried, sieved (2 mm mesh) and homogenized. Forty eight hours before starting the experiment, ground moistened horse dung was added (5% of the dry soil), the soil moisture was adjusted to 50% of the maximum water holding capacity (WHC).

Standard Lufa 2.2 soil was bought from LUFA Speyer (Germany). The main properties of this soil are (mean±SD): pH_{CaCl2} 5.5±0.1, WHC 45.2±5.0%, cation exchange capacity (CEC) 10.0±0.8 cmol kg^{-1}, Total Organic Carbon (TOC) content 1.93±0.20%. During several years of using this soil in our research group, mean total concentrations of metals [mg kg^{-1} dwt.] were determined to be (mean±SD): Cd-0.064±0.042, Pb-18.7±0.36, Cu-4.53±0.473, Zn-21.3±1.17. Mean 0.01 M $CaCl_2$-extractable concentrations were: Cd-0.017±0.005, Pb-0.052±0.000, Cu-0.037±0.000, Zn-0.39±0.191. These concentrations were used in the toxicokinetics modelling. All metal concentrations are expressed on a dry weight (dwt.) basis.

Fig. 1. Location of the Olkusz study sites in the vicinity of the smelter and mine. See Table 1 for approximate distances from the smelter.

Table 1. Physico-chemical properties of the Olkusz soils used in the kinetics experiment with *Lumbricus rubellus* earthworms (mean ± SD) and distances of the study sites from the smelter. OM = Organic Matter; DOC = Dissolved Organic Carbon in the pore water; CEC = Cation Exchange Capacity.

Site/Soil	Distance from the smelter [km]	pH_{CaCl2}	pH_{H2O}	OM [%]	DOC [mg L^{-1}]	CEC [cmol kg^{-1}]
OL1	3.3	5.06 ± 0.06	5.49 ± 0.04	45.1 ± 1.3	73.2 ± 9.0	37.7 ± 0.0
OL2	2.5	4.12 ± 0.03	4.78 ± 0.01	53.5 ± 0.4	119 ± 15	34.4 ± 0.3
OL3	3.9	3.75 ± 0.03	4.40 ± 0.02	41.8 ± 3.1	238 ± 12	23.5 ± 0.4
OL4	5.3	3.46 ± 0.02	4.15 ± 0.05	54.2 ± 2.0	220 ± 7	29.0 ± 0.4
OL5	7.7	4.29 ± 0.01	4.83 ± 0.02	36.3 ± 0.7	178 ± 5	26.3 ± 0.6

Number of samples analyzed: n = 3 for pH, OM and DOC, n = 2 for CEC

Metal kinetics experiment

Earthworms *L. rubellus* were obtained from a commercial earthworm supplier who collected them from an unpolluted site in The Netherlands (Nijkerkerveen). Additionally, we collected some earthworms from the Polish site OL2 – the most polluted site from the transect where *L. rubellus* is present. Only adults with a well-developed clitellum were used. Fresh body weight of an earthworm was equal (mean±SD) 1.40 ± 0.384 g. Prior to the experiment, the earthworms were kept in Lufa 2.2 soil at 15°C for acclimation.

Before starting the experiment, the earthworms were rinsed with deionized water, blotted up with filter paper and weighed (Mettler AC 100 Balance). At the start we measured body concentrations of Cd, Cu, Pb and Zn in seven Dutch earthworms originating from an unpolluted site and four earthworms from the site OL2. These concentrations were then considered initial concentrations (C_0). The experiment lasted for 42 days and was divided into an uptake phase and an elimination phase, each 21 days long. For the uptake phase we placed Dutch earthworms individually in 300 ml transparent plastic containers containing an equivalent of 40 g dry soil (corresponding with 78-96 g moist soil). For each soil (OL1-OL5) and each sampling day three replicates were prepared. Earthworms originating from OL2 were exposed only to the soil from this site (soil OL2). Additionally, 20 earthworms (10 Dutch and 10 Polish from site OL2) were kept as a control in containers containing Lufa soil. During the experiment earthworms were maintained in a climate chamber (15°C, 16:8 h light:dark). Every week the test containers were weighed to adjust soil moisture content. At days 1, 2, 4, 7, 10, 15 and 21 of the uptake phase three earthworms were sampled from each soil. After the uptake phase, we transferred all the remaining earthworms, including Polish earthworms native to OL2 site, into Lufa 2.2 soil for the elimination phase. Also at days 1, 2, 4, 7, 10, 15 and 21 of the elimination phase, three replicate earthworms were sampled.

Analytical procedures in toxicokinetics experiment

After sampling, earthworms were rinsed with deionized water, blotted up with filter paper and weighed to check for possible body mass changes. Earthworms were starved individually on moist filter paper in plastic containers with perforated lids for 48 h to ensure soil removal from the gut. The filter papers were changed after 24 and 36 h to minimize coprophagy (Arnold and Hodson 2007). Earthworms were frozen at -20°C, freeze-dried for 48 h and ground to powder using mortars. Approximately 0.1 g of dry tissue was digested in 2 ml of 1:4 destruction mixture of hydrochloric acid (37% p.a., Baker) and nitric acid (65% p.a., Riedel-de Haën). The digestion was performed in Teflon bombs placed for 7 h at 140°C in the oven (Binder).

Soils were analyzed for WHC, pH_{H2O}, pH_{CaCl2}, organic matter content (OM), dissolved organic carbon (DOC) in pore water and CEC. Total, porewater and 0.01 M $CaCl_2$-extractable concentrations of Cd, Pb, Cu and Zn were measured. WHC, pH and $CaCl_2$-extractable concentrations of metals were determined as described elsewhere (Diez-Ortiz et al. 2010; Ardestani and Van Gestel 2013). To estimate OM content we measured percent mass loss on ignition by heating dry soil samples for 6 h at 500°C. For CEC determination we used silver thiourea method (Dohrmann 2006). For porewater extraction we used centrifugation method described by Hobbelen et al. (2004), detailed by Ardestani and Van Gestel (2013). After acidifying with nitric acid, the porewater samples were analyzed for DOC content (HiPerTOC of Thermo, type 2008.122, equipped with ThEuS software package, version 1.4 (0022)). To measure total concentrations of metals in soil we digested ~0.1 g dry soil samples as described for earthworms.

Atomic absorption spectrometry with flame atomization (F-AAS; Perkin Elmer, AAnalyst 100) was used to determine total metal concentrations in soils and earthworms. Extractable soil concentrations and porewater concentrations were measured by AAS with graphite furnace atomization (GF-AAS; Perkin Elmer 5100PC). For the quality assessment we used DOLT 4 (dogfish liver, NRC) certified reference material in case of earthworms. Measured concentrations were within 10% of the certified values. Pb concentration in the reference material was too low (0.16±0.04 $mg kg^{-1}$) to measure it with F-AAS. We did not measure it with GF-AAS as all the earthworms were analyzed with F-AAS. In case of soils ISE 989 (river clay, WEPAL) reference material was used. Measured concentrations were within 12% of the reference values. For all the analyses blanks were included. Correction for blanks was applied only for Pb, as for this metal they showed consistent negative readings corresponding to approximately 2.2 $\mu g\ g^{-1}$ Pb earthworm concentration.

Analytical procedures for earthworms sampled along the Olkusz transect

Earthworms, native to all Olkusz sites, used for the comparison of toxicokinetics results with the field situation were sampled prior to the toxicokinetics experiment and metal concentrations were analyzed in a different laboratory (later mentioned as concentrations in earthworms collected at study sites). Earthworms were prepared for analysis as described above. However, after freeze-drying whole earthworms were digested in boiling nitric acid (Suprapur, Merck), then re-suspended to 5 ml with deionized water and analyzed with F-AAS (Perkin Elmer, AAnalyst 800). Here, the reference material SRM-1577c (bovine liver, NIST) was analyzed with GF-AAS (Perkin Elmer, AAnalyst 800). Measured concentrations were within 10% of the certified value in case of Cd and 20% in case of Pb.

Modelling and statistics

To estimate kinetics parameters, we fitted a one-compartment model (Atkins 1969; Janssen et al. 1991) modified to take into consideration the non-zero exposure concentration during the elimination phase. The model was fitted simultaneously to all data from both the uptake and elimination phases. We performed nonlinear regression fitting of the following equations (uptake phase - Eq. 1, elimination phase - Eq. 2):

$$C_{worm} = C_0 \cdot e^{-k2 \cdot t} + \frac{k1}{k2} \cdot C_{exp1} \cdot (1 - e^{-k2 \cdot t}) \quad (1)$$

$$C_{worm} = C_0 \cdot e^{-k2 \cdot t} + \frac{k1}{k2} \cdot C_{exp1} \cdot \left(e^{-k2 \cdot (t-21)} - e^{-k2 \cdot t}\right) + \frac{k1}{k2} \cdot C_{exp2} \cdot (1 - e^{-k2 \cdot (t-21)}) \quad (2)$$

where:

C_{worm} – internal concentration in the earthworms at sampling time t [μg g^{-1}];

C_0 – initial concentration in the earthworms measured at time t=0 [μg g^{-1}];

k_1 – uptake rate constant [$g_{soil}\, g_{worm}^{-1}\, d^{-1}$];

k_2 – elimination rate constant [d^{-1}];

C_{exp1} – exposure concentration during the uptake phase [μg g^{-1}]; in our experiment – the concentration of a given metal in experimental soil (OL1-OL5);

C_{exp2} – exposure concentration during the elimination phase [μg g^{-1}]; in our experiment - the concentration of a given metal in Lufa 2.2 soil (see above);

t – time [day].

Uptake rate constants were calculated based on the following exposure concentrations (C_{exp1}): total metal concentration in relevant Olkusz soil (k_{1T}, k_{2T}) and $CaCl_2$-extractable concentrations (k_{1C}, k_{2C}). Additionally, influx rates (a_T, a_C) [$\mu g_{metal}\, g_{worm}^{-1}\, d^{-1}$] were calculated as the product of k_1 and C_{exp1}.

Steady state concentrations, based on total metal concentrations sin soil, (C_{TSS}) were calculated as k_1/k_2*C_{exp1} [µg g_{worm}^{-1}].

To determine significance of differences in kinetics parameters (k_1, k_2) between soils, we applied a likelihood ratio test. The impact of soil properties on kinetics parameters was assessed with stepwise regression analysis. For the analyses we used IBM SPSS Statistics 20.

Results

Olkusz soil characteristics

All Olkusz soils were acidic (pH_{H2O} 4.15-5.49, pH_{CaCl2} 3.46-5.06) and rich in organic matter (36.3-54.2% dwt.; Table 1). The soils showed large variation in DOC level (73.2-238 mg L^{-1}), with the lowest value observed in the most polluted soil OL1.

CEC ranged between 23.5 and 37.7 cmol kg^{-1} and was the highest in soil OL1. Total metal concentrations in Olkusz soils covered a broad range of contamination levels and decreased with increasing distance from the smelter (Table 2).

Table 2. Total, porewater and 0.01 M $CaCl_2$-extractable concentrations of Cd, Pb, Cu and Zn (mean ± SD; n = 3) in the Olkusz soils used for the toxicokinetics experiment with *Lumbricus rubellus* earthworms.

Soil	Cd	Pb	Cu	Zn
Total soil concentrations [mg kg^{-1} dwt.]				
OL1	63.2 ± 3.0	3 041 ± 158	67.0 ± 3.1	7 991 ± 536
OL2	49.1 ± 1.1	2 060 ± 37	57.8 ± 0.9	3 960 ± 54
OL3	16.8 ± 3.2	760 ± 123	28.2 ± 4.4	1 142 ± 224
OL4	14.8 ± 0.2	847 ± 38	35.6 ± 2.4	966 ± 22
OL5	12.1 ± 0.7	708 ± 12	27.2 ± 0.2	756 ± 11
Porewater concentrations [µg L^{-1}]				
OL1	24.8 ± 1.1	125 ± 11	41.2 ± 5.7	2993 ± 166
OL2	81.0 ± 3.6	230 ± 19	41.1 ± 1.1	8285 ± 186
OL3	31.1 ± 1.0	392 ± 16	54.8 ± 2.7	2568 ± 98
OL4	22.7 ± 1.0	303 ± 8	46.1 ± 2.3	1565 ± 51
OL5	13.0 ± 0.2	231 ± 16	61.1 ± 21.3	927 ± 16
0.01 M $CaCl_2$-extractable concentrations [mg kg^{-1} dwt.]				
OL1	0.892 ± 0.018	0.553 ± 0.014	0.092 ± 0.018	54.4 ± 1.1
OL2	3.69 ± 0.05	1.98 ± 0.05	0.069 ± 0.002	211 ± 2
OL3	1.87 ± 0.01	1.42 ± 0.03	0.125 ± 0.015	96.0 ± 1.6
OL4	1.98 ± 0.02	1.88 ± 0.02	0.096 ± 0.010	84.3 ± 1.5
OL5	0.688 ± 0.012	0.526 ± 0.015	0.060 ± 0.002	30.8 ± 0.1

Soil OL1 showed the highest total concentrations of all analyzed metals, soil OL5 the lowest. Such a gradient was not observed for porewater or $CaCl_2$-extractable metals concentrations, which were not correlated with the total concentrations in the soils. For all metals, except for Cu, the highest porewater and $CaCl_2$-extractable concentrations were measured in soil OL2.

Metal toxicokinetics in earthworms originating from uncontaminated site

During the experiment nine earthworms died but mortality was random and not related to pollution level. Six earthworms were excluded from further analysis because of an extreme body weight change.

Cd and Pb concentrations in *L. rubellus* increased with time of exposure to all five contaminated soils and did not reach steady state during the uptake phase (Figs. 2 and 3; a-e). The Cd body concentrations after 21 days of exposure were 20.6-46.1 μg g^{-1}. The internal Pb concentrations were higher and reached 75.3-230 μg g^{-1}, depending on the soil. After 21 days of elimination, high body concentrations of Cd and Pb were still observed. The one-compartment model used to calculate kinetics parameters based on total metal concentrations explained 78.3-87.3% and 37.1-71.9% of the total variance in earthworm body concentrations of Cd and Pb, respectively (Table 3).

For Cd, uptake rate constants based on total metal concentrations (k_{1T}) ranged between 0.032 and 0.069 $g_{soil}\, g_{worm}^{-1}\, d^{-1}$ (Table 3). The lowest k_{1T} value was observed in the most polluted soil OL1. It significantly differed from k_{1T} values observed in all other soils (likelihood ratio test, $p<0.05$). For Pb, values of k_{1T} were over five times lower than for Cd and ranged between 0.004 and 0.009 $g_{soil}\, g_{worm}^{-1} d^{-1}$.

The differences between soils in uptake rate constants of Cd and Pb were more pronounced when related to $CaCl_2$-extractable concentrations (Table 4). The k_{1C} of Cd and Pb were the highest in earthworms exposed to OL1 soil. This soil was characterized by the lowest extractability of Cd and Pb but at the same time the highest total metal concentrations (Table 2).

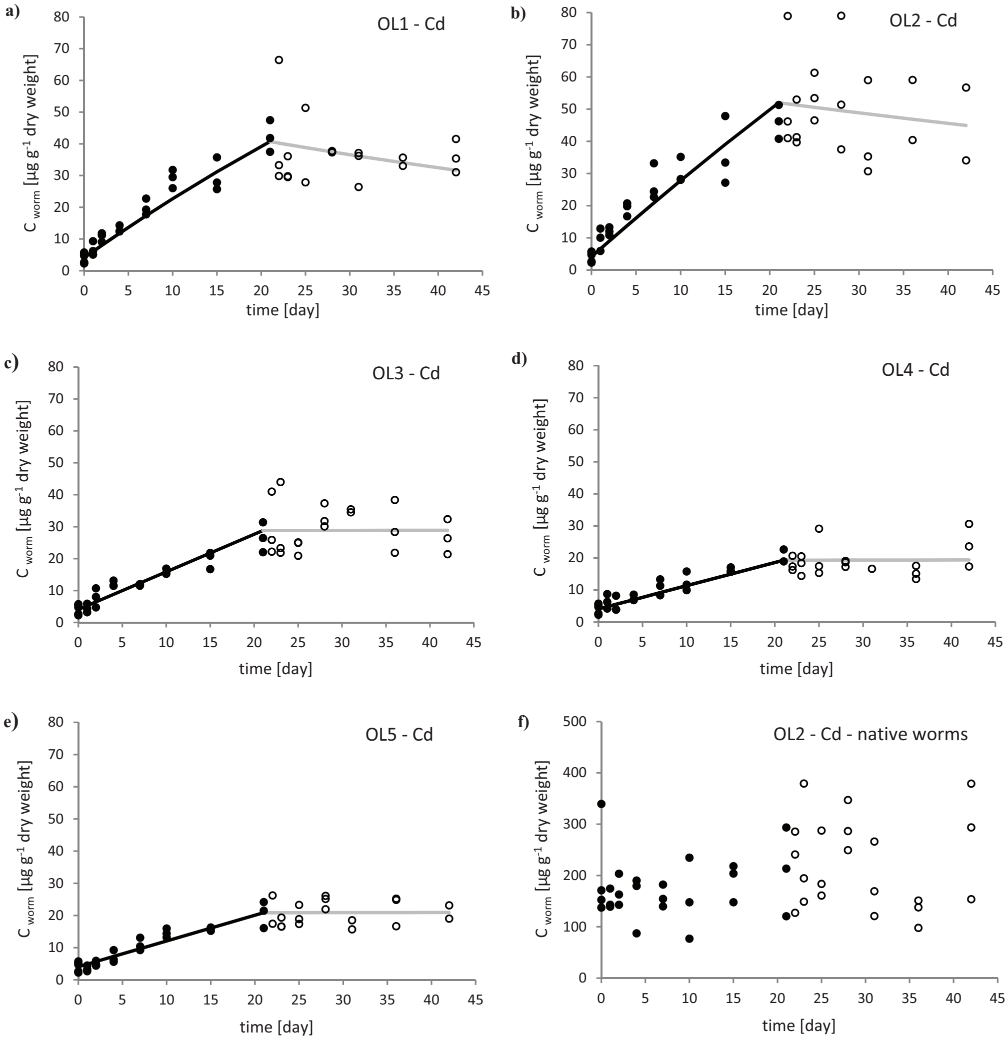

Fig. 2. Uptake and elimination kinetics of cadmium in *Lumbricus rubellus* originating from unpolluted soil and exposed to five natural soils from the gradient of pollution (a-e) and native Polish earthworms from the site OL2 exposed to OL2 soil (f). Dots represent measured internal Cd concentrations (solid dots - uptake phase, open dots - elimination phase). Kinetics curves were estimated according to one-compartment model: black line - the uptake phase (Eq. 1), gray line - the elimination phase (Eq. 2).

Influx rates (a_T) ranged between 0.710 and 2.50 $\mu g_{metal}\, g_{worm}^{-1}\, d^{-1}$ for Cd and 4.25-15.2 $\mu g_{metal}\, g_{worm}^{-1}\, d^{-1}$ for Pb (Table 3), and were the highest in earthworms exposed to soils OL1 and OL2. The gradient of influx rates of Cd and Pb was similar to the gradient of total concentrations of these metals in the soils.

Table 3

Kinetics parameters related to total concentrations of Cd and Pb: uptake rate constant (k_{1T}), elimination rate constant (k_{2T}), influx rate (a_T) in *Lumbricus rubellus* exposed to polluted field soils for 21 days followed by an elimination phase in Lufa 2.2 soil (21 days). Kinetics parameters were estimated by simultaneous fitting of the one-compartment model (Eq.1-2) to the uptake and elimination phase data (samples used in regression depending on the soil: n=46-48). The 95% confidence intervals are shown in brackets. Coefficient of determination (R^2) describes variance in earthworms body concentrations of Cd and Pb explained by the fitted model.

Metal	Soil	k_{1T} [$g_{soil}\ g_{worm}^{-1}\ d^{-1}$]	k_{2T} [d^{-1}]	a_T [$\mu g_{metal}\ g_{worm}^{-1}\ d^{-1}$]	R^2
Cd	OL1	0.032 (0.026-0.038)^{a}	0.012 (0.002-0.021)^{a}	2.02 (1.64-2.40)	0.817
	OL2	0.051 (0.040-0.062)^{bc}	0.007 (-0.004-0.019)^{a}	2.50 (1.96-3.04)	0.783
	OL3	0.069 (0.054-0.083)^{b}	-0.001 (-0.010-0.008)*^{a}	1.16 (0.91-1.39)	0.819
	OL4	0.048 (0.036-0.060)^{c}	-0.001 (-0.010-0.008)*^{a}	0.710 (0.533-0.888)	0.783
	OL5	0.065 (0.052-0.077)^{bc}	-0.001 (-0.009-0.007)*^{a}	0.786 (0.629-0.932)	0.873
Pb	OL1	0.005 (0.003-0.007)^{ab}	0.069 (0.036-0.102)^{a}	15.2 (9.12-21.3)	0.371
	OL2	0.004 (0.003-0.005)^{a}	-0.002 (-0.021-0.017)*^{b}	8.24 (6.18-10.3)	0.499
	OL3	0.008 (0.005-0.011)^{b}	0.010 (-0.009-0.028)^{b}	6.08 (3.80-8.36)	0.551
	OL4	0.009 (0.006-0.011)^{c}	-0.002 (-0.016-0.012)*^{b}	7.62 (5.08-9.32)	0.719
	OL5	0.006 (0.005-0.008)^{abc}	0.029 (0.012-0.046)^{ab}	4.25 (3.54-5.66)	0.627

* for modelling kinetics curves k_2 values were set to close to zero (0.000000001) since negative excretion cannot be explained biologically

[a,b]- values marked with different letters significantly differ from each other within a column (likelihood ratio test, $p<0.05$)

During the elimination phase a very slow or even no excretion of Cd and Pb by earthworms was observed (Fig. 1-2; a-e). The elimination rate constants (k_2) were close to zero (Table 3, Table 4). However, a high k_{2T}=0.069 d^{-1} (k_{2C}=0.089 d^{-1}) was found in case of Pb in earthworms exposed to soil OL1. Steady state concentrations (C_{TSS}) were calculated for soil OL1 (Cd, Pb) and OL5 (Pb). Only in these soils elimination rate constants were significantly different from zero. Steady state concentrations of Pb in earthworms [$\mu g\ g_{worm}^{-1}$] were 220.4 and 146.5 for OL1 and OL5, respectively. Steady state concentration of Cd for soil OL1 was 168.5[$\mu g\ g_{worm}^{-1}$].

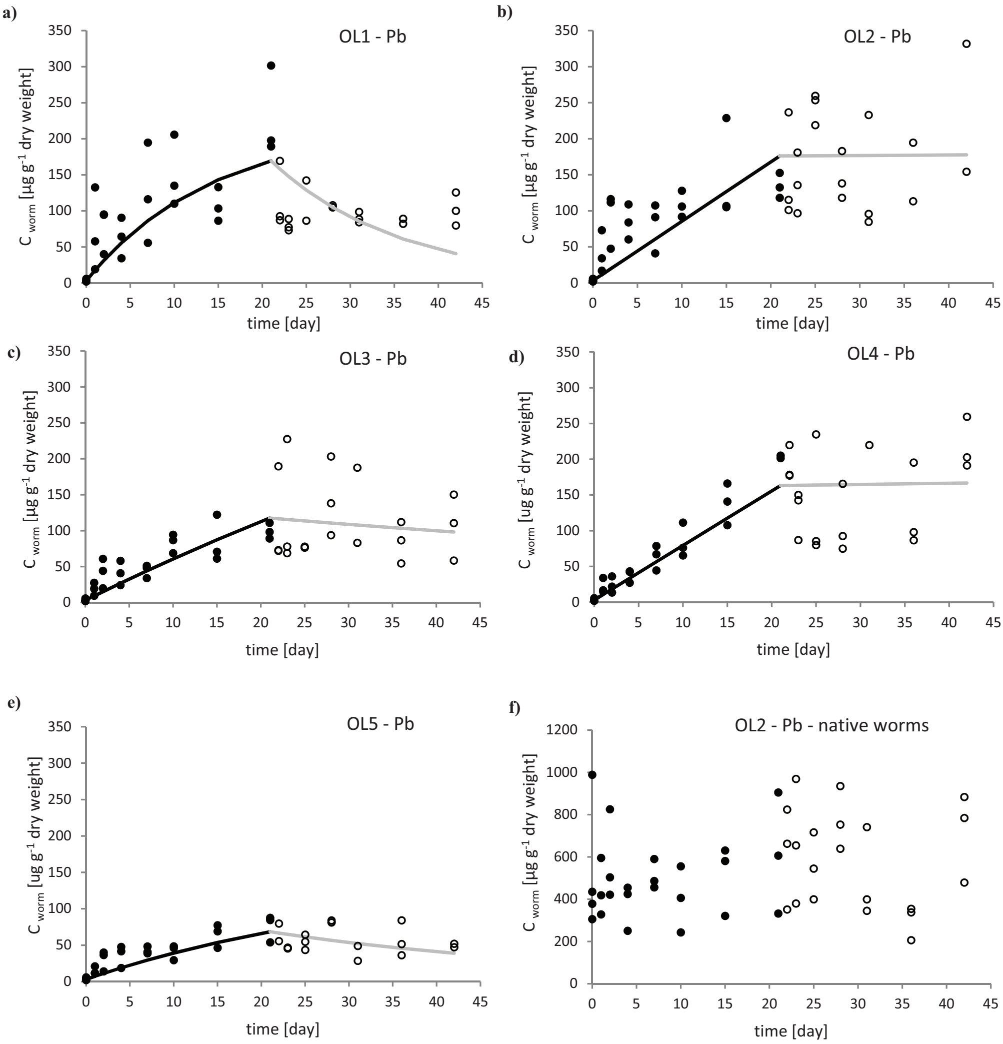

Fig. 3. Uptake and elimination kinetics of lead in *Lumbricus rubellus* originating from unpolluted soil and exposed to five natural soils from the gradient of pollution (a-e) and native Polish earthworms from the site OL2 exposed to OL2 soil (f). Dots represent measured internal Pb concentrations (solid dots - uptake phase, open dots - elimination phase). Kinetics curves were estimated according to one-compartment model: black line - the uptake phase (Eq. 1), gray line - the elimination phase (Eq. 2).

The earthworms showed efficient regulation of the level of essential metals in their body. During 21 days of exposure, body concentrations of Cu and Zn remained at a stable level. However, in earthworms exposed to soil OL1 we observed a small increase of the body concentrations of these metals (Fig. 3-4). In case of both Zn and Cu, the model never explained more than 20% of the total variance in the earthworm body concentrations and in most cases the estimated kinetics parameters were not significantly different from zero (Tables S1-S2). Kinetics curves were not shown for these data.

Table 4

Kinetics parameters related to 0.01 M $CaCl_2$-extractable concentrations of Cd and Pb: uptake rate constant (k_{1C}), elimination rate constant (k_{2C}), influx rate (a_C) in *Lumbricus rubellus* exposed to polluted field soils for 21 days followed by an elimination phase in Lufa 2.2 soil (21 days). Kinetics parameters were estimated by simultaneous fitting of the one-compartment model (Eq.1-2) to the uptake and elimination phase data (samples used in regression depending on the soil: n=46-48). The 95% confidence intervals are shown in brackets. Coefficient of determination (R^2) describes variance in earthworms body concentrations of Cd and Pb explained by the fitted model.

Metal	Soil	k_{1C} [$g_{soil}\ g_{worm}^{-1}\ d^{-1}$]	k_{2C} [d^{-1}]	a_C [$\mu g_{metal}\ g_{worm}^{-1}\ d^{-1}$]	R^2
Cd	OL1	2.30 (1.87-2.72)[a]	0.013 (0.003-0.023)[a]	2.05 (1.67-2.43)	0.818
	OL2	0.678 (0.532-0.824)[b]	0.008 (-0.004-0.019)[a]	2.50 (1.96-3.04)	0.783
	OL3	0.620 (0.490-0.750)[b]	-0.001 (-0.0010-0.008)[a]	1.16 (0.92-1.40)	0.819
	OL4	0.360 (0.272-0.448)[c]	-0.001 (-0.010-0.008)[a]	0.713 (0.538-0.887)	0.783
	OL5	1.15 (0.925-1.37)[d]	0.000 (-0.008-0.008)[a]	0.791 (0.636-0.943)	0.873
Pb	OL1	32.4 (20.9-43.9)[a]	0.089 (0.050-0.128)[a]	17.9 (11.6-24.3)	0.402
	OL2	3.99 (2.63-5.35)[b]	-0.001 (-0.020-0.018)[bc]	7.90 (5.21-10.6)	0.500
	OL3	4.30 (2.91-5.69)[b]	0.010 (-0.008-0.029)[bc]	6.11 (4.13-8.08)	0.552
	OL4	3.89 (2.86-4.91)[b]	-0.002 (-0.015-0.012)[c]	7.31 (5.38-9.23)	0.719
	OL5	9.20 (6.84-11.6)[c]	0.035 (0.016-0.054)[ab]	4.84 (3.60-6.10)	0.635

[a,b]- values marked with different letters significantly differ from each other within a column (likelihood ratio test, $p<0.05$)

Toxicokinetics in earthworms native to polluted soil

Earthworms collected at the polluted site OL2, exposed again to their native soil, did not show additional uptake of any of the studied metals (Fig. 1-4; f). Concentrations of the metals remained at a stable level during both the uptake and elimination phase, thus kinetics curves were not fitted. Mean metal body concentrations were (μg g^{-1} dwt.; mean±sd): Cd, 196±75; Pb, 539±211; Zn, 1999±626; Cu, 12.6±2.4.

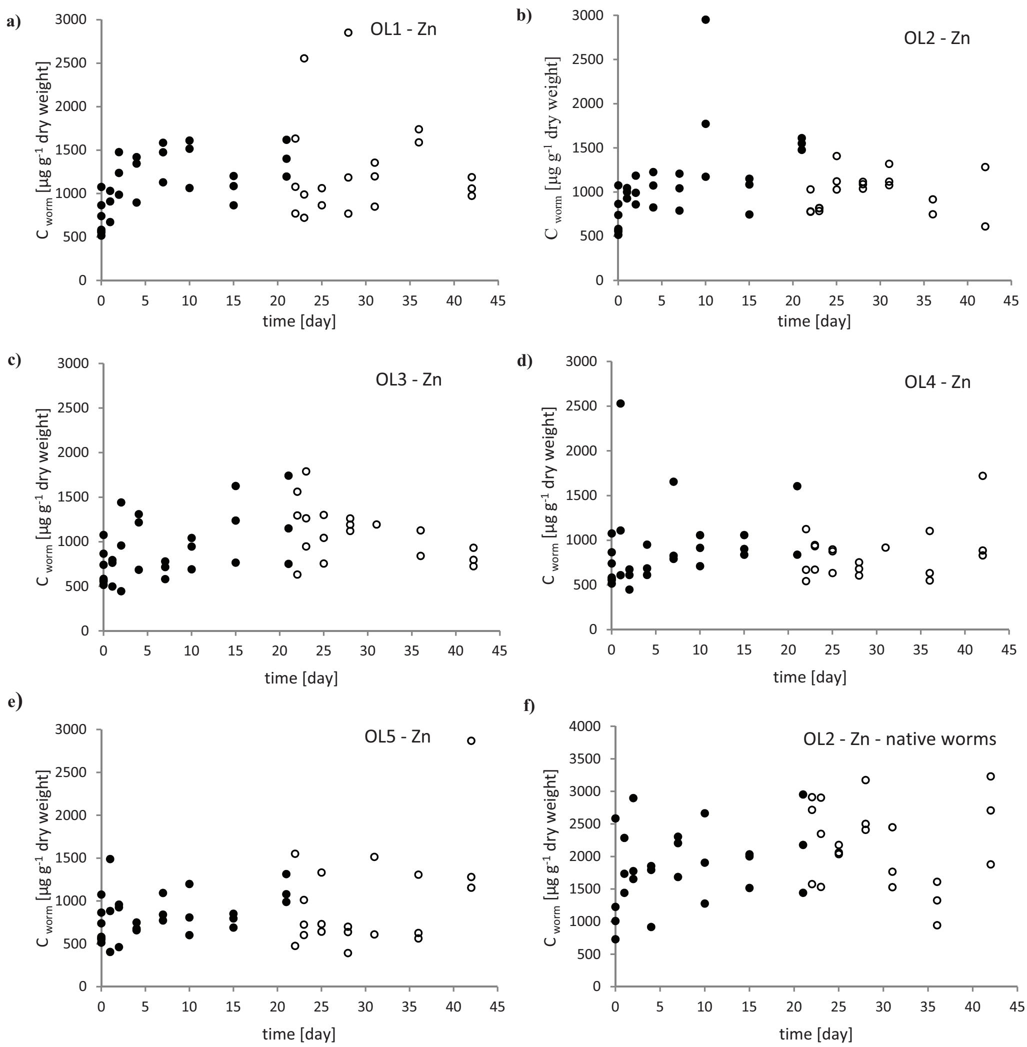

Fig. 4. Zinc concentrations measured in *Lumbricus rubellus* originating from unpolluted soil and exposed to five natural soils from the gradient of pollution (a-e) and native Polish earthworms from the site OL2 exposed to OL2 soil (f). Solid dots represent the uptake phase, open dots - the elimination phase. Kinetics curves were not shown.

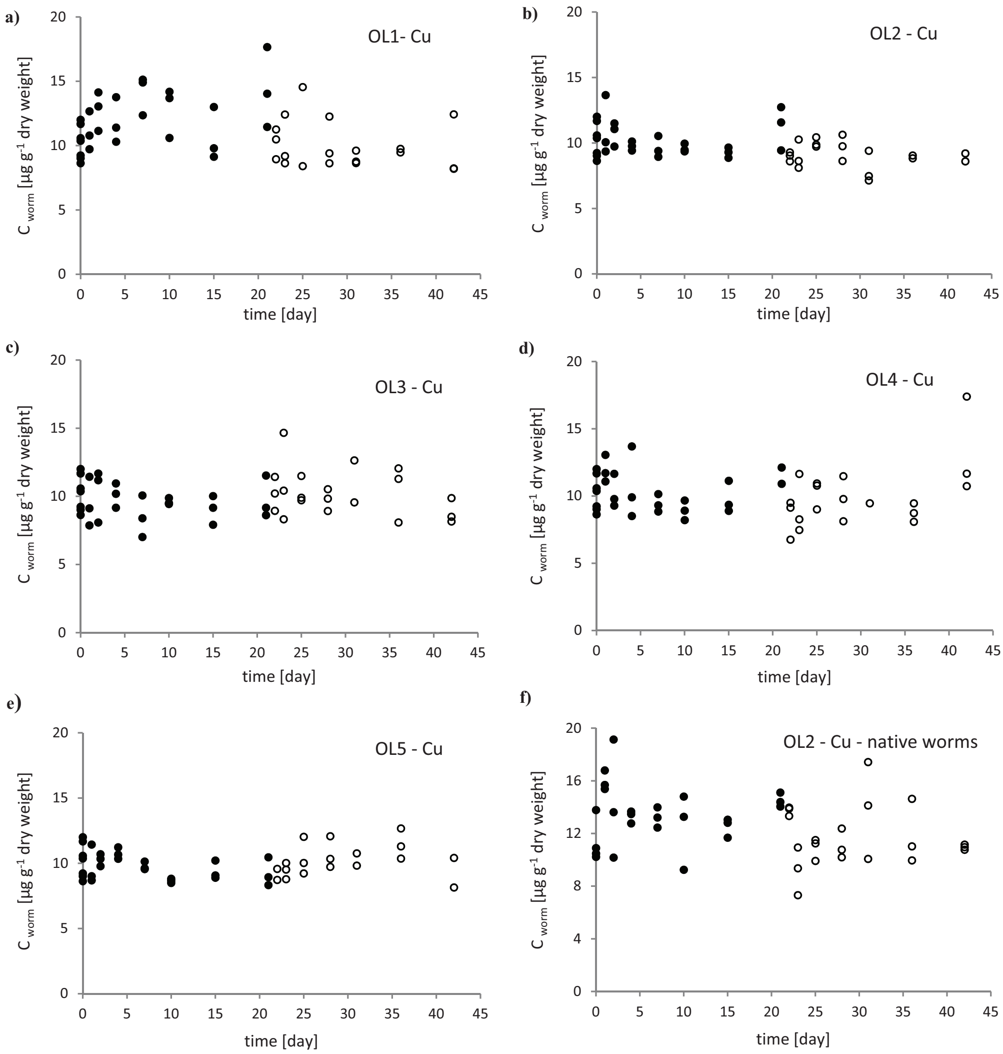

Fig. 5. Copper concentrations measured in *Lumbricus rubellus* originating from unpolluted soil and exposed to five natural soils from the gradient of pollution (a-e) and native Polish earthworms from the site OL2 exposed to OL2 soil (f). Solid dots represent the uptake phase, open dots - the elimination phase. Kinetics curves were not shown.

Metal toxicokinetics as a function of soil properties

CEC and pH were the only soil properties significantly influencing the metal uptake rate constants, with pH explaining the highest percentage of the k_{1C} variance. The estimated regression models explained over 93% of the variation in the k_{1C} for Cd (Eq. 3) and over 80% of the variation in the k_{1C} for Pb (Eq. 4).

$$k_{1C[Cd]} = 1.22\ pH_{CaCl2} - 4.02\ (p=0.008;\ R^2=0.931) \quad (3)$$

$$\log k_{1C[Pb]} = 0.560\ pH_{CaCl2} - 1.48\ (p=0.024;\ R^2=0.809) \quad (4)$$

Over 83% of the variation in the uptake rate constants of Cd based on total soil concentrations (k_{1T}) was explained by CEC (Eq. 5).

$$k_{1T[Cd]} = 0.123 - 0.002\ CEC\ (p=0.030,\ R^2=0.836) \quad (5)$$

The variation in the uptake rate constants of Pb based on total concentration in soil (k_{1T}) could not be explained by any of analyzed soil properties. Elimination rate constants were not influenced by soil characteristics.

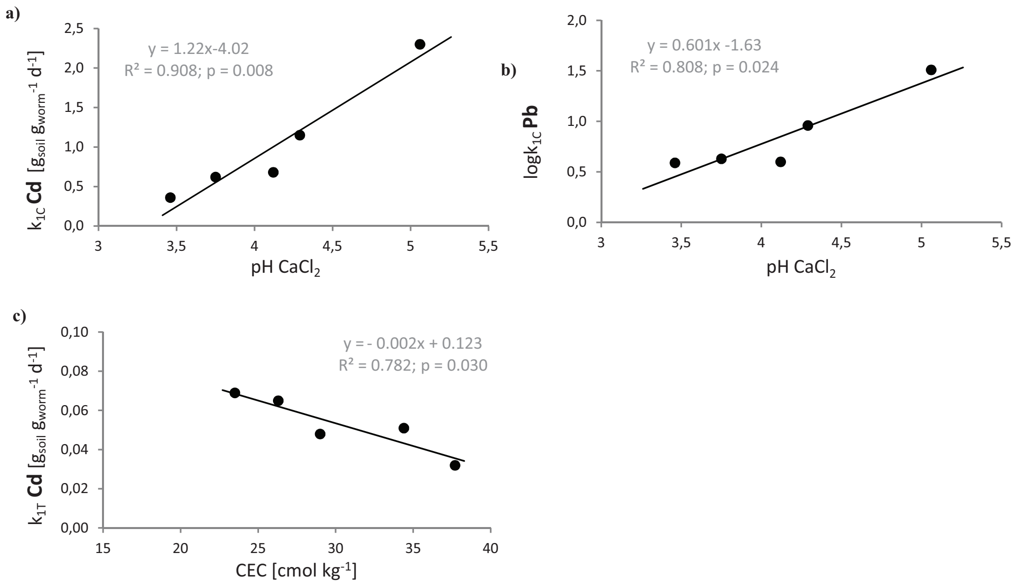

Fig. 6. Relationships between uptake rate constants and soil properties (pH_{CaCl2}-a,b, CEC-c). See Figure 1 for locations of the study sites, Table 1 for soil properties and Tables 2-3 for kinetics parameters. Only relationships found to be statistically significant are shown. Regression equations, adjusted R^2 and p-values are reported.

Use of toxicokinetics to predict metal body concentrations reached by earthworms in the field

When comparing earthworms sampled at studied transect with individuals sampled at the end of the uptake phase (Table 5), we see that 21 days of exposure usually was not enough to reach the concentrations measured in earthworms collected at study sites. Concentrations of Cd in earthworms sampled at the end of the uptake phase in all soils (except for OL5; t-test, $p=0.053$) were significantly lower than concentrations measured in earthworms from the field (t-test, $p<0.05$). The same pattern was observed in soils OL1 and OL2 for Pb. In contrast to the experimental specimens, earthworms from the study sites showed higher variation in measured tissue Cd and Pb concentrations. Nevertheless, accumulation of metals with the kinetics parameters derived from the toxicokinetics experiment could result in Cd and Pb concentrations observed in the individuals sampled at respective polluted sites. According to these estimations, the average field concentrations could be reached by the earthworms at the latest after approx. 90 days of exposure to polluted soil for Pb, and after 164 days for Cd (Table 5).

Table 5. Comparison of the concentrations of Cd and Pb in *Lumbricus rubellus* collected at the study sites (OL2-OL5) with the concentrations in experimental earthworms sampled after 21 days of exposure to polluted field soils (OL2–OL5) and the estimation of approximate time needed to reach the concentrations observed in earthworms native to respective polluted sites assuming kinetics parameters derived from the experiment. Negative k_2 values were set to close to zero (0.000000001). Values in brackets represent minimum and maximum concentrations. Number of analyzed earthworms: n=3-6.

Soil	Concentrations in earthworms collected at study sites [$\mu g\ g^{-1}$]		Concentrations in earthworms sampled at the end of the uptake phase [$\mu g\ g^{-1}$]		Time to reach concentrations observed in native earthworms [days]	
	Cd	Pb	Cd	Pb	Cd	Pb
OL2	244±120*	743±234*	46.1±5.3	134±17	162	90
	(145-440)	(559-1167)	(40.8-51.3)	(118-153)	(73-∞)	(68-141)
OL3	197±42*	1016±434*	26.6±4.7	99.5±10.9	164	∞
	(171-246)	(715-1513)	(22.0-31.4)	(89.2-111)	(142-206)	(∞)
OL4	80.7±33.0*	209±211	20.8±2.6	203±3	106	27
	(61.8-139)	(76.3-584)	(18.9-22.7)	(202-205)	(80-186)	(9-76)
OL5	70.2±35.7	71.9±75.4	20.6±4.1	75.3±18.6	83	23
	(39.6-119)	(30.8-225)	(16.1-24.2)	(53.9-87.6)	(45-144)	(7-∞)

* significant differences in metal concentrations between earthworms collected at study sites and earthworms sampled at the end of the uptake phase (t-test; $p<0.05$)

Discussion

Toxicokinetics of metals in Lumbricus rubellus

The present study showed uptake of non-essential metals (Cd and Pb) by *L. rubellus* and very efficient regulation of body concentrations of essential metals (Cu and Zn), what is in agreement with earlier studies (e.g., Spurgeon and Hopkin 1999b).

Uptake rate constants of Cd based on total soil concentrations ranged between 0.032 and 0.069 $g_{soil}\ g_{worm}^{-1}\ d^{-1}$. In comparison to previous studies with different earthworm species (Vijver et al. 2005; Smith et al. 2010) these values were rather low. Nahmani et al. (2009) however, reported a very broad range of Cd uptake rate constants (0.022-4.92 $g_{soil}\ g_{worm}^{-1}\ d^{-1}$) in *Eisenia fetida*, depending on the soil type. Uptake rate constants of Pb based on total soil concentrations ranged between 0.004 and 0.009 $g_{soil}\ g_{worm}^{-1}\ d^{-1}$, and were within the range (0.001-0.023 $g_{soil}\ g_{worm}^{-1}\ d^{-1}$) reported by Nahmani et al. (2009).

Both Cd and Pb did not achieve steady state levels in the earthworms during the uptake phase. This is in agreement with Spurgeon and Hopkin (1999b) who found that after 21 days of exposure to polluted soils internal concentrations of Cd and Pb in *E. fetida* were still increasing. Such continuous uptake of Cd and Pb was observed also in *L. rubellus* kept for 90 days in natural polluted soils (Mariño and Morgan 1999). Cd and Pb usually have very slow or no elimination, which results in a linear uptake pattern. This could be explained by detoxification of these metals by sequestration. Even when exposure stops, the earthworm body concentrations could stay constant if the metals are bound to organic ligands or sequestrated within inorganic matrices (Spurgeon and Hopkin 1999b). It is known that in earthworms the chloragogenous tissue, which is rich in phosphate, calcium and sulphur, is responsible for the storage of metals and other elements (Morgan and Morgan 1990). Metallothionein-like proteins also take part in metal detoxification and have a high affinity for binding Cd (Stürzenbaum et al. 2004). However, the absence of additional uptake in native earthworms exposed again to polluted soil (Fig. 1-2; f) suggests that in the field Cd and Pb reached steady state level.

Although higher uptake rate constant is indicative of higher bioavailability it cannot be used for the risk assessment irrespective of metal concentration in soil. Internal metal concentration is the result of all the uptake and elimination rate constants and exposure concentration. The influx rate

[$\mu g_{metal}\ g_{worm}^{-1}\ d^{-1}$] of Cd and Pb, calculated as the product of uptake rate constant and exposure concentration, seems to be a better estimator of real exposure and organism burden.

In this study earthworms did not show a clear uptake of Cu and Zn. This could be explained by detoxification by rapid excretion. Earthworms physiologically control body concentrations of essential elements (Spurgeon and Hopkin 1999b). In contrary to the present results, fast initial accumulation of essential metals and equilibrium reached within a few days of exposure are reported in some other toxicokinetics studies with earthworms (Peijnenburg et al. 1999; Spurgeon and Hopkin 1999b; Vijver et al. 2005). However, Mariño and Morgan (1999) observed very slow or no accumulation of Cu and Zn in *L. rubellus* exposed to natural polluted soils during the first 20 days of exposure, while faster uptake was reported during 20-90 days of exposure to the same soils. Slight uptake of Cu and Zn observed at the beginning of the exposure to the most polluted soil OL1 (Fig. 3-4, a) is consistent with the most commonly reported pattern. It suggests that total concentration is more important when predicting accumulation of Cu and Zn by earthworms as porewater and $CaCl_2$-extractable concentrations of Cu and Zn in soil OL1 were lower than in the other soils. It seems that regulation of essential metals is efficient until a threshold influx. This would explain why Nahmani et al. (2007b) observed a broad range of internal Cu (11.3-28.7 $\mu g\ g^{-1}$) and Zn (106-2890 $\mu g\ g^{-1}$) concentrations in *E. fetida* exposed for 42 days to polluted soils with a wide range of total concentrations. Possibly at higher exposure levels strict and efficient regulation of the body concentrations costs too much energy (Van Gestel et al. 1993). This may also happen in case of long-term exposure to lower concentrations in the field or during longer laboratory experiments as suggested by Nahmani et al. (2009). Measured internal Cu and Zn concentrations could be affected by 48 h depuration. As these metals are usually rapidly removed from the organism, we may lose information about real tissue concentrations which may be higher than reported ones.

Bioavailability of metals and soil properties

From previous studies it is known that bioavailability of metals depends on many factors, including various soil properties (Spurgeon and Hopkin 1999b; Van Gestel and Mol 2003; Van Gestel 2008). In this study we showed that in *L. rubellus* uptake rate constants of Cd and Pb based on 0.01 M $CaCl_2$-

extractable concentrations were significantly related tohigher at higher soil pH_{CaCl2} (Eq. 3-4). Such significant positive relation of uptake rate constants of Cd and Pb with soil pH was previously reported by Peijnenburg et al. (1999) for the earthworm *Eisenia andrei*. According to the literature, the availability of Cd and Pb measured only by chemical methods is higher at lower pH (Hobbelen et al. 2006). The opposite relation we observed may be explained by biotic ligand model. At higher pH the competition between H^+ and Cd^{2+} ions to bind to biotic ligand sites on the earthworm is reduced. Additionally, at higher pH more cations are bound to soil particles. Depending on the affinity of different cations to binding sites on soil particles, the relative availability of cations in soil solution may be changed Therefore, metal uptake from the soil solution may increase with pH (Di Toro et al. 2001; Ardestani and Van Gestel 2013). This pattern cannot be seen when using only chemical methods to assess availability of metals.

We found that the uptake rate constant of Cd based on total concentrations in soil was significantly lower at higher CEC (Eq. 5). This is in agreement with other studies showing that an increase in CEC decreases metal availability in soil (Haghiri 1974; Domínguez et al. 2009).

Toxicokinetics results and the field situation

Populations of *L. rubellus* have been reported to display high genetic diversity (King et al. 2008; Andre et al. 2010), which may result in different responses to pollution depending on the genotype. Indeed, a common pattern observed in earthworms sampled from contaminated sites is high variability in internal concentrations of metals. Spurgeon et al. (2011) concluded that such high inter-individual variation in pollutant handling is a characteristic of earthworms under many exposure scenarios, including both natural field exposure and laboratory experiments. However, it is still not known if this variation has a genetic basis. The degree of genetic variation may increase within geographical distance. Therefore, we have to take this into consideration when comparing the Dutch earthworms used in the toxicokinetics experiment with the Polish specimens native to polluted sites.

In this study we showed that standard toxicokinetics experiments with the uptake phase lasting for 21-28 days are too short to imitate the situation in the field as equilibrium concentrations in the earthworms were not reached. However, this may depend on the metal and its concentration in the soil. In case of Pb in the less polluted soils OL4 and OL5, after 21 days of exposure the earthworms reached body concentrations similar to those observed in specimens sampled at polluted sites (Table 5).

We found that kinetics parameters derived from the laboratory toxicokinetics experiments were relevant of the field situation. Accumulation of Cd and Pb with estimated uptake and elimination rate constants could result in their body concentrations measured in earthworms sampled in the field at respective polluted sites (Table 5). However, this was not a case for Pb in OL3 soil. Concentrations of Pb observed in individuals collected from OL3 site were relatively high. It is known from other studies done in the same area (Giska, unpublished data) that this site is characterized by mitochondrial haplotypes (mtATP6) absent in the other populations. It still remains to be determined whether this genetic difference could explain for the difference in Pb accumulation.

In the comparison of toxicokinetics results with data obtained from the field collected earthworms, we did not include soil OL1 as *L. rubellus* was not present there. The absence of *L. rubellus* can be explained either by the extremely high metal concentrations at this site or the drought stress. High influx rates of Cd (a_T=2.02 $\mu g_{metal}\, g_{worm}^{-1}\, d^{-1}$) and Pb ($a_T$=15.2 $\mu g_{metal}\, g_{worm}^{-1}\, d^{-1}$), together with exposure to Zn and Cu could be detrimental to earthworms populations, leading to local extinction of *L. rubellus* or simply avoidance of such contaminated soil. From field observations we know that this site is characterized by extreme water depletion resulting in relatively low soil moisture contents compared with the other sites from the transect. Apart of that, soil properties at OL1 site (Table 1) seem suitable for *L. rubellus*.

It is worth noting that field observations showed decreased density of *L. rubellus* with decreasing pollution level. Site OL2 is characterized by the highest abundance of this species, site OL5 by the lowest. Site OL3 is exceptional because earthworms are very hard to collect there, although we were able to collect single individuals. From previous research it is known that earthworm distribution in the Olkusz transect is patchy (Tosza et al. 2010), which could be related to heterogeneity of the area. Thus, we cannot assume that pollution alone is the major factor shaping *L. rubellus* abundance near Olkusz. Similar patterns of higher density and biomass of earthworms at polluted sites in comparison to reference sites were found by Klok and Thissen (2009). In their research an additional factor, bird predation, played an important role in controlling earthworm populations. When comparing or extrapolating laboratory results to the field situation we have to be aware of all possible factors that may influence earthworm populations in nature.

Conclusions

In this study we applied a toxicokinetics approach to estimate the bioavailability of metals (Cd, Pb, Cu, Zn) to the earthworm *L. rubellus* in soils from a gradient of pollution. To simulate more natural conditions than in usual laboratory ecotoxicological tests, we used earthworms collected in the field and exposed them to natural polluted soils. We found that earthworms intensively accumulated Cd and Pb, almost without elimination. Uptake kinetics parameters of these metals, when related to $CaCl_2$-extractable concentrations, were explained by soil pH. Uptake kinetics based on total soil concentrations were not related to soil properties. *L. rubellus* was able to regulate body concentrations of essential metals (Cu, Zn). We showed that the gradient of bioavailability, based on influx rate [$\mu g_{metal}\ g_{worm}^{-1}\ d^{-1}$] calculated as the product of uptake rate constant and exposure concentration, was similar to the gradient of total metal concentrations in soils. When using the experimentally-derived kinetics parameters it was possible to estimate the time necessary to reach the internal Pb and Cd concentrations observed in earthworms sampled in the field at the studied sites. This shows that toxicokinetics-derived data are predictive of metal accumulation levels in earthworms in the field.

Acknowledgments

We would like to thank Sebastian Żmudzki for his help with sampling earthworms in the field and Rudo Verweij for his technical assistance during laboratory work. This study was performed in the frame of the "Environmental Stress, Population Viability and Adaptation" project (MPD/2009-3/5) whilst I. Giska had a research studentship at VU University in Amsterdam.

Appendix 1

Supplementary materials to Chapter 1

Table S1. Kinetics parameters related to total concentrations of Cu and Zn: uptake rate constant (k_{1T}), elimination rate constant (k_{2T}), influx rate (a_T) in *Lumbricus rubellus* earthworms exposed to polluted field soils for 21 days followed by an elimination phase in Lufa 2.2 soil (21 days). Kinetics parameters were estimated by simultaneous fitting of the one-compartment model (Eq.1 and Eq.2) to the uptake and elimination phase data. The 95% confidence intervals are shown in brackets. Coefficient of determination (R^2) describes variance in earthworms body concentrations of Cu and Zn explained by the fitted model.

Metal	Site	k_{1T} [$g_{soil}\ g_{worm}^{-1}\ d^{-1}$]	k_{2T} [d^{-1}]	a_T [$\mu g_{metal}\ g_{worm}^{-1}\ d^{-1}$]	R^2
Cu	OL1	0.005 (0.002-0.008)	0.021 (0.006-0.035)	0.335 (0.134-0.536)	0.055
	OL2	0.001 (-0.001-0.002)	0.006 (-0.002-0.014)	-	0.176
	OL3	0.000 (-0.005-0.005)	0.001 (-0.009-0.012)	-	-
	OL4	-0.005 (-0.009- -0.001)	-0.014 (-0.025- -0.003)	-	0.120
	OL5	-0.004 (-0.008-0.000)	-0.008 (-0.016-0.000)	-	0.067
Zn	OL1	0.006 (0.002-0.010)	0.011 (-0.009-0.031)	47.9 (16.0-80.0)	0.041
	OL2	0.015 (0.006-0.024)	0.029 (0.005-0.052)	59.4 (23.8-95.0)	0.015
	OL3	0.025 (-0.002-0.052)	0.000 (-0.021-0.022)	-	0.190
	OL4	0.010 (-0.019-0.039)	0.000 (-0.025-0.025)	-	-
	OL5	0.009 (-0.021-0.039)	-0.003 (-0.024-0.017)	-	0.035

Table S2. Kinetics parameters related to 0.01 M $CaCl_2$-extractable concentrations of Cu and Zn: uptake rate constant (k_{1C}), elimination rate constant (k_{2C}), influx rate (a_C) in *Lumbricus rubellus* earthworms exposed to polluted field soils for 21 days followed by an elimination phase in Lufa 2.2 soil (21 days). Kinetics parameters were estimated by simultaneous fitting of the one-compartment model (Eq.1 and Eq.2) to the uptake and elimination phase data. The 95% confidence intervals are shown in brackets. Coefficient of determination (R^2) describes variance in earthworms body concentrations of Cu and Zn explained by the fitted model.

Metal	Site	k_{1C} [$g_{soil}\ g_{worm}^{-1}\ d^{-1}$]	k_{2C} [d^{-1}]	a_C [$\mu g_{metal}\ g_{worm}^{-1}\ d^{-1}$]	R^2
Cu	OL1	7.48 (2.76-12.2)	0.050 (0.018-0.081)	0.688 (0.254-1.12)	0.089
	OL2	1.15 (-2.34-4.64)	0.011 (-0.011-0.032)	-	0.177
	OL3	0.060 (-1.30-1.42)	0.002 (-0.012-0.015)	-	-
	OL4	-2.80 (-4.80- -0.793)	-0.022 (-0.039- -0.006)	-	0.124
	OL5	-3.55 (-7.17-0.074)	-0.018 (-0.038-0.001)	-	0.056
Zn	OL1	0.881 (0.293-1.47)	0.011 (-0.009-0.031)	47.9 (15.9-80.0)	0.041
	OL2	0.283 (0.119-0.448)	0.028 (0.005-0.052)	59.7 (25.1-94.5)	0.015
	OL3	0.294 (-0.023-0.610)	0.000 (-0.021-0.021)	-	0.190
	OL4	0.114 (-0.215-0.443)	0.000 (-0.025-0.024)	-	-
	OL5	0.222 (-0.501-0.945)	-0.003 (-0.024-0.017)	-	0.035