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Zhang, Lulu; Van Gestel, Cornelis A.M.

published in
Environmental Pollution
2017

DOI (link to publisher)
10.1016/j.envpol.2017.02.070

document version
Publisher's PDF, also known as Version of record

Environmental Pollution 225 (2017) 534–541

Contents lists available at ScienceDirect

Environmental Pollution

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

Toxicokinetics and toxicodynamics of lead in the soil invertebrate *Enchytraeus crypticus*☆

Lulu Zhang, Cornelis A.M. Van Gestel*

Department of Ecological Science, Faculty of Earth and Life Science, Vrije Universiteit, De Boelelaan 1085, 1081 HV Amsterdam, The Netherlands

ARTICLE INFO

Article history:
Received 24 October 2016
Received in revised form
17 February 2017
Accepted 19 February 2017
Available online 17 March 2017

Keywords:
Lead
Toxicokinetics
Toxicodynamics
Enchytraeus crypticus

ABSTRACT

The aim of the present study was to link Pb toxicokinetics to toxicodynamics in *Enchytraeus crypticus*. The enchytraeids were exposed for 14 d to different Pb concentrations (uptake phase) in natural Lufa 2.2 soil, followed by a 14-d elimination phase in clean soil. Pb accumulation and enchytraeid mortality were determined at different time intervals. At each exposure concentration, internal Pb concentration increased with exposure time and achieved equilibrium in approximately 7 d. Median lethal concentration (LC50) based on total Pb concentration in soil decreased with exposure time, but did not reach a steady-state level. Pb toxicity, therefore, showed a delay compared to accumulation in *E. crypticus*. LC50s based on internal Pb concentrations in the surviving animals did reach steady state in approx.14 d, suggesting that linking toxicokinetics to toxicodynamics may reduce the effects of time. This study highlighted that exposure time, as an important factor in metal uptake and toxicity, should be taken into account in ecotoxicological tests for risk assessment.

1. Introduction

With intense industrialization and urbanization, metal pollution has become a significant environmental problem worldwide. In particular, heavy metals in soil have attracted great attention due to their non-biodegradability, posing a potential risk to terrestrial ecosystems. Among heavy metals, lead (Pb) has become widespread in the environment as a result of human activities. Anthropogenic lead primarily originates from industrial processes (i.e. mining, smelting), use (i.e. batteries, pigments, bullets, mineral fertilizer), combustion of fossil fuels, waste incineration, and sewage sludge application. Lead is a nonessential element, having no known physiological functions for animals, plants and microorganisms, and is acknowledged for its high toxicity (Fisher et al., 2006). High Pb concentrations may affect the survival and reproduction of soil invertebrates like earthworms, springtails and enchytraeids (Langdon et al., 2005).

It is commonly acknowledged that the dose determines the toxicity of a chemical. Usually, to assess the environmental risk of a metal in soil, toxicity tests with microorganisms, plants and soil invertebrates are performed with a fixed exposure time to develop dose-effect relationships for selected endpoints. Such traditional ecotoxicological tests relate the resulting toxicity to an external concentration. However, the potential risk of metals in soils depends on their bioavailability rather than on total concentrations. Bioavailability is defined as the fraction of metal in the environment that is available for uptake, leading to adverse effects on organisms (Peijnenburg, 2004). Therefore, to better understand the risk of metals in soil, the relationship between metal toxicity to organisms and its bioavailability in soil should be investigated. Accumulation of metals in organisms could be a good predictor of metal bioavailability in soil, as internal concentrations reflect the actual exposure in the environment. Internal concentrations however, do not only depend on exposure concentration but also on exposure time. Spurgeon and Hopkin (1999) found that internal metal concentrations increased with exposure time until reaching a steady state, when earthworms were exposed to contaminated soils. Mortality of organisms is observed when the internal concentration exceeds a certain threshold (lethal body concentration), which represents a physiological limit (Jager et al., 2011). When the metal uptake rate exceeds the elimination rate, the organism will slowly accumulate the metal, potentially leading to toxic effects in the long run. In such case, no or little toxicity could be observed in the traditional ecotoxicological tests when exposure time is too short to reach equilibrium. He and Van Gestel (2013) found that

☆ This paper has been recommended for acceptance by Prof. W. Wen-Xiong.

* Corresponding author.
E-mail address: kees.van.gestel@vu.nl (C.A.M. Van Gestel).

http://dx.doi.org/10.1016/j.envpol.2017.02.070
0269-7491/

metal toxicity was time-dependent, with median lethal concentration (LC50) for the toxicity of nickel to the enchytraeid *Enchytraeus crypticus* decreasing with time. Time, therefore, should be a vital factor taken into account in toxicity tests.

Van Straalen et al. (2005) also highlighted that instead of body concentration in an organism, metal uptake rate was the superior predictor for bioavailability. Thus, to simulate the time course processes from metal accumulation to causing toxic effects on organisms, simultaneous assessment of toxicokinetics-toxicodynamics might be a better approach to quantify metal toxicity (Ashauer and Escher, 2010). Toxicokinetics translates the external concentration of a metal into an internal metal concentration in an organism over time, including uptake, body distribution, transformation or sequestration and elimination. Toxicodynamics describes the development with time of the toxic action at target sites or active sites, or the resulting subsequent adverse effects at the level of the organism (e.g. effects on survival, reproduction or growth), quantitatively linking the internal metal concentration to toxic effects on organisms. Toxicokinetics-toxicodynamics therefore can provide a mechanistic understanding of the processes of exposure, accumulation, depuration and toxic effects, linking mortality or sublethal endpoints to internal concentration, taking into account the factor of time.

Widely distributed in different soils, Enchytraeids (class Oligochaeta, family Enchytraeidae) play a key role in the functioning of terrestrial ecosystems. As soft-bodied organisms, enchytraeids could be exposed to different stress factors in soil, both via the soil solution and the solid phase. They therefore are recommended as suitable test species in soil ecotoxicology (Didden and Römbke, 2001). Among Enchytraeids, *Enchytraeus crypticus* has been demonstrated to be a useful model species for soil toxicity tests, because of its short generation time, good control performance and wide tolerance to distinct soil properties (e.g. pH, texture, organic matter content) (Castro-Ferreira et al., 2012).

This study investigated the development of Pb bioaccumulation and toxicity with exposure time in *E. crypticus* in a Pb-amended natural soil. Our aims were: (1) determining the kinetics of Pb uptake and elimination in *E. crypticus* at different soil Pb concentrations (toxicokinetics), (2) investigating the development of Pb toxicity with exposure time (toxicodynamics), and (3) linking toxicokinetics (Pb bioaccumulation in time) to toxicodynamics (survival in time).

2. Materials and methods

2.1. Test organism

Enchytraeus crypticus (Enchytraeidae; Oligochaeta; Annelida) has been cultured for several years at the Vrije Universiteit, Amsterdam. The worms were kept on agar prepared with an aqueous soil extract, in a climate room at 16 °C, with 75% relative humidity, and in complete darkness. The animals were fed twice a week with a mixture of oat meal, dried yeast, yolk powder, and fish oil (Castro-Ferreira et al., 2012). Adult *E. crypticus* of approximately 1 cm with white spots in the clitellum region were selected for the tests.

2.2. Test substrates

As a natural standard soil, Lufa 2.2 was chosen to be the test soil, obtained from the LUFA Institute (Landwirtschaftliche Untersuchungs-und Forschungsanstalt) at Speyer, Germany. The soil had a nominal pH-0.01 M $CaCl_2$ of 5.49, 3.5% organic matter, 12% clay and a Cation Exchange Capacity (CEC) of 9.10 cmolc/kg. To obtain nominal concentrations of 0, 100, 200, 400, 800, 1600 and 3200 mg Pb/kg dry soil, soil was spiked by adding aqueous solutions of $Pb(NO_3)_2$ (purity >99.99%; Sigma-Aldrich; USA). For each treatment, the soil was moistened to reach 50% of the maximum water-holding capacity, equaling a soil moisture content of 24% (w/w). The spiked soils were equilibrated for 14 d in a climate room at 20 °C before use in the tests.

2.3. Toxicokinetics and toxicodynamics tests

Pb uptake and elimination kinetics in *E. crypticus* exposed to the different test concentrations were assessed following OECD guideline 317 (OECD, 2010). After 14 d exposure in spiked soils (uptake phase), surviving adults were transferred to clean Lufa 2.2 soil for the 14-d elimination phase. The uptake phase was combined with the assessment of Pb toxicodynamics, and also included observations after 21 days of exposure. So, Pb uptake and toxicity were determined at seven time intervals (1, 2, 4, 7, 10, 14, 21 d), while six sampling times (15, 16, 18, 21, 24, 28 d) were used in the elimination phase for determining internal Pb concentrations. For each treatment and sampling time, ten worms were introduced into a 100 mL glass jar filled with 30 g moist test soil, and 2 mg oatmeal was added for food. The jars were covered with perforated aluminum foils and incubated at 20 °C, 75% relative humidity and 16-h light/8-h dark photoperiod cycle in a climate room. Food and soil moisture content were checked once a week and the water loss was replenished by adding deionized water. At each sampling time, three replicate jars were sampled for each test concentration, survival was determined and surviving adults were collected and transferred to petri dishes (100 mm × 15 mm) with 20 mL ISO solution (ISO, 2004a) for 24 h for gut cleaning. Subsequently, three animals from each replicate were frozen at −20 °C for Pb analysis.

2.4. Chemical analysis

Soil samples were dried at 40 °C for 48 h. To measure total Pb concentrations, soils were digested in a mixture of HNO_3 (65%, Sigma-Aldrich, USA) and HCl (37%, Sigma-Aldrich, USA) (4:1 v/v). Around 130 mg dry soil was mixed with 2 mL of the acid mixture in a tightly closed Teflon container and heated for 7 h in an oven at 140 °C. Total soil concentrations were measured by atomic absorption spectrometry (AAS; AAnalyst 100, Perkin Elmer, Germany). Quality of the analysis was checked by using the certified reference material ISE sample 989 (International Soil-Analytical Exchange), and the measured lead concentrations in the reference material were always within 10% of the certified concentration. The frozen worms were freeze-dried for at least 24 h, weighted individually and digested with 300 μL mixture of HNO_3 (65%; Mallbaker Ultrex Ultra-Pure) and $HClO_4$ (70%; Mallbaker Ultrex Ultra-Pure) (7:1 v/v) in a block heater (TCS Metallblock Thermostat) using a heating ramp ranging from 85 to 180 °C for 2 h. The lead concentrations in worms were measured by graphite furnace AAS (PinAAcle 900Z, Perkin Elmer, Germany). The certified reference material DOLT 4 (Dogfish liver, LGC Standards) was included for quality control and the Pb recoveries were 93.7%–102.2%.

2.5. Data analysis

Assuming that exposure concentration (mg Pb/kg dry soil) is constant, the development of internal concentration with time can be described by a one-compartment model (Crommentuijn et al., 1997):

$$\text{When } t \le tn: \quad C(t) = C_0 \times e^{-k_e \times t} + \left(\frac{k_u}{k_e}\right) \times C_{exp1} \times \left(1 - e^{-k_e * t}\right)$$

$$\text{When } t > tn: \quad C(t) = C_0 \times e^{-k_e \times t} + \left(\frac{k_u}{k_e}\right) \times C_{exp1} \times \left[F_i + (1 - F_i) \times \left(e^{-k_e * (t - t_n)} - e^{-k_e \times t}\right)\right] + \left(\frac{k_u}{k_e}\right) \times C_{exp2} \times \left[1 - (F_i + (1 - F_i) \times e^{-k_e * (t - t_n)})\right] \quad (1)$$

where C(t) is Pb concentration in the worms after t days exposure (mg Pb/kg dry body wt), C_0 the initial or background Pb concentration in the worms (mg Pb/kg dry body wt), k_u the uptake rate constant ($kg_{soil}/kg_{worm}/day$), k_e the elimination rate constant for toxicokinetics (day^{-1}), C_{exp1} the exposure concentration in spiked soil (mg Pb/kg dry soil), C_{exp2} the Pb concentration in clean soil (mg Pb/kg dry soil), t exposure time (days), t_n the time when worms were transferred to clean soil (14 days), and F_i the inert Pb fraction in the body (ranging from 0 to 1). k_u, and k_e and F_i were assumed to be constant and estimated by fitting Equation (1) to all data from each exposure concentration and taking all exposure times, both from the uptake and the elimination phases, together.

Median lethal concentration (LC50) is the estimated effect concentration associated with 50% reduction in survival compared with the control (mg Pb/kg dry soil), calculated with the trimmed Spearman-Karber method (Hamilton et al., 1977). A logistic survival model was used to explain the relationship between survival and exposure time (Crommentuijn et al., 1994):

$$S(t) = \frac{e^{-\mu t}}{1 + \left(\frac{C_{exp1}}{LC50(t)}\right)^b} \quad (2)$$

where S(t) is the survival fraction after t days exposure, C_{exp1} the exposure concentration (mg Pb/kg dry soil), t exposure time (days), LC50(t) the LC50 value after t days exposure (mg Pb/kg dry soil), μ the natural mortality rate (day^{-1}), and b the slope parameter.

It is assumed that mortality only occurs when the body concentration exceeds a certain threshold (lethal body concentration) and that the uptake of Pb in the body is following a first order kinetics model. The estimated LC50s at each time point were used to calculate the ultimate LC50 value (LC50∞), assuming an exponential decrease of the LC50 with time. The ultimate LC50 is (directly or indirectly) related with toxicokinetics, so LC50 value decreases with the rate at which the internal threshold is reached. Since toxicokinetics patterns are mainly determined by the rate at which a metal is eliminated, also the pattern of the LC50-time relationship is determined by an elimination-type rate constant. The relationship between toxicity and time can be described as (Crommentuijn et al., 1994):

$$LC50(t) = \frac{LC50\infty}{1 - e^{-k_d \times t}} \quad (3)$$

where LC50(t) is the LC50 value after t days exposure (mg Pb/kg dry soil), LC50∞ the incipient LC50 value (mg Pb/kg dry soil), k_d the damage rate constant for toxicodynamics (day^{-1}), and t exposure time (days).

The bioaccumulation factor (BAF) in kg_{soil}/kg_{worm} is defined as the ratio of mean concentration in the worms (mg Pb/kg dry body wt) at steady state and the concentration in the soil (mg Pb/kg dry soil) (Belfroid et al., 1996). BAF can also be expressed as the ratio of the uptake (k_u) and the elimination rate constants (k_e) obtained from the toxicokinetics. So, BAF was defined as:

$$BAF = \frac{C_{worm}}{C_{soil}} = \frac{k_u}{k_e} \quad (4)$$

The lethal body concentration (LBC) in mg Pb/kg dry body wt was derived from the LC50∞ as:

$$LBC = LC50\infty \times \frac{k_u}{k_e} \quad (5)$$

The biological half-life for Pb elimination was calculated as:

$$Biological\ t_{\frac{1}{2}} = \frac{ln2}{k_e} \quad (6)$$

Models were fitted using individual internal concentrations. All parameters were estimated by nonlinear regression in SPSS 21.0 based on experimental data.

3. Results

3.1. Soil properties

The measured total Pb concentrations in the test soil were in close agreement with the nominal ones (Table S1, Supplementary material). The measured values were used in all calculations.

3.2. Toxicokinetics

Development with time of internal Pb concentrations in *E. crypticus* exposed to different external concentrations in soil is shown in Fig. 1. The initial Pb concentration in the enchytraeids was 0.10 ± 0.02 mg Pb/kg dry wt. The one-compartment model could not be applied for the highest test concentration (3585 mg/kg dry soil), as all the worms died within 2 d of exposure. Because of the high mortality after 14 d exposure at the second highest Pb concentration (1601 mg/kg dry soil), only data for the uptake phase was available. At each exposure concentration, Pb accumulation in the animals increased with exposure time and reached a steady state after approximately 7 d during the uptake phase. For each exposure time, the body Pb concentration in the animals increased with increasing exposure concentration. The highest Pb concentration in the enchytraeids was 556 mg/kg dry body wt after 1 d of exposure to 3585 mg/kg dry soil of Pb. However, at steady state, the highest internal Pb concentration was 244 mg/kg dry body wt in animals exposed to 1601 mg Pb/kg dry soil. In the elimination phase, body Pb concentration decreased with time and reached a constant level after around 7 d, however, without returning to the initial level.

When fitting Equation (1) to the data from each exposure concentration separately, the estimated uptake rate constant (k_u) based on total soil concentration ranged between 0.08 and 0.25 $kg_{soil}/kg_{worm}/day$ and the elimination rate constant (k_e) between 0.42 and 1.28 day^{-1} (Table S2). The inert fraction (F_i) was estimated to range between 0 and 0.20 for the four lowest exposure concentrations (114, 202, 391 and 793 mg Pb/kg dry soil; Table S2). When all the data were fitted together, overall uptake rate constant (k_u), elimination rate constant (k_e) and inert fraction (F_i) (± standard error [SE]) were estimated to be 0.14 ± 0.01 $kg_{soil}/kg_{worm}/day$, 0.93 ± 0.11 day^{-1} and 0.14 ± 0.05 (Table S2). The one-compartment model

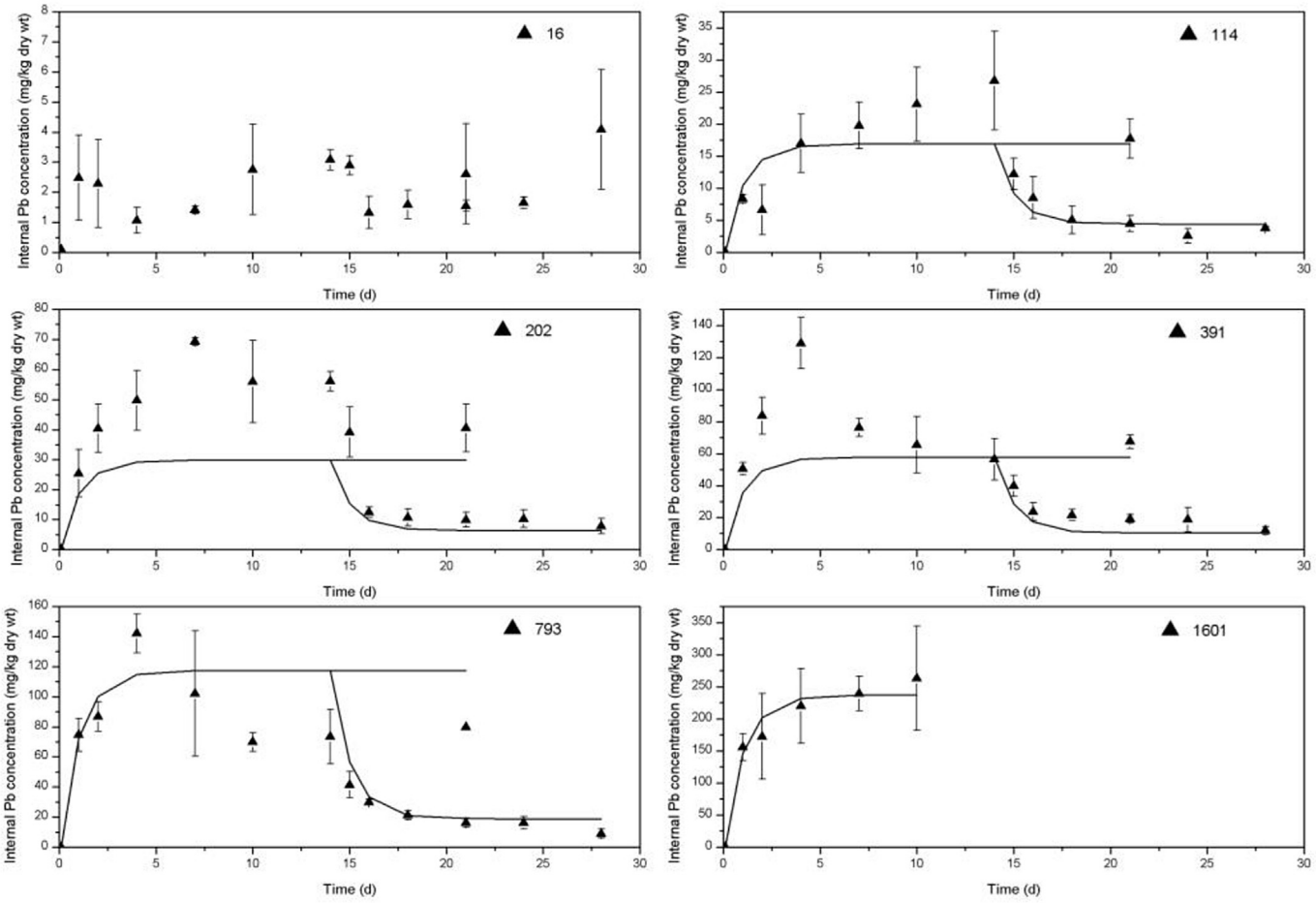

Fig. 1. Development with time of internal Pb concentrations (mg Pb/kg dry body wt) in *Enchytraeus crypticus* when exposed to different Pb concentrations in Lufa 2.2 natural standard soil for a 21-d uptake phase, or for a 14-d uptake phase followed by a 14-d elimination phase in clean soil. Dots represent the average of 3 replicates with standard errors, and lines show the fit of a one-compartment model (Equation (1)) with overall parameters to the data.

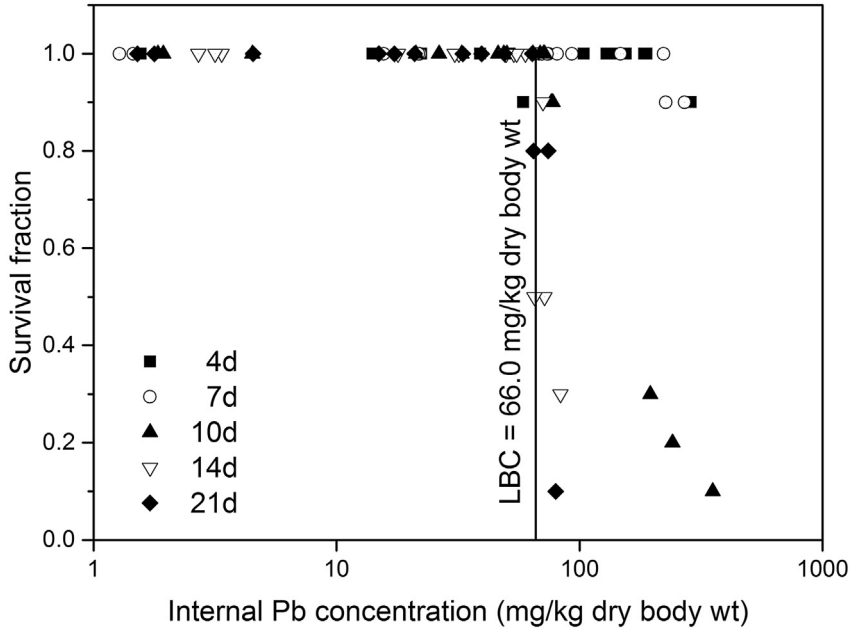

Fig. 2. The relationship between the survival of *Enchytraeus crypticus* and exposure time (d) when exposed to different Pb concentrations (in mg Pb/kg dry soil) in Lufa 2.2 natural standard soil. Dots present the average observed survival fraction with standard errors, lines show the fit of a logistic survival model (Equation (2)) to the data.

described Pb uptake well, with $r^2 = 0.85$, $p < 0.01$. When running a one-compartment model without including the inert fraction, fit was less good but this did not lead to different k_u, k_e and BAF values (Table S2, Fig. S1).

3.3. Toxicodynamics

Enchytraeid survival with exposure time at each Pb concentration is presented in Fig. 2. For the low exposure concentrations (16, 114 and 202 mg Pb/kg dry soil), no mortality was observed during 21 d exposure. For the higher exposure concentrations (391, 793, 1601 and 3585 mg Pb/kg dry soil), survival of the animals decreased with time at each concentration. At each time point, mortality increased with increasing exposure concentration.

Using Equation (2), natural mortality rate μ and slope b (± SE) were estimated to be 0.0005 ± 0.0001 day^{-1} and 7.47 ± 0.32, respectively. The solid line shown in Fig. 2 presents the development with time of the survival fraction of *E. crypticus* when exposed to different Pb concentrations in soil. The model could fit the data accurately, with $r^2 = 0.991$, $p < 0.01$.

The development of LC50 with time for the effects of Pb on the survival of the enchytraeids is shown in Fig. 3. LC50 decreased from 2336 mg Pb/kg dry soil at 4 d to 558 mg Pb/kg dry soil at 21 d and did not reach a steady state within 21 d.

The rate constant for damage increase (k_d) and the incipient median lethal concentration value (LC50 ∞) derived from Equation (3) were 0.046 d^{-1} and 446 mg Pb/kg dry soil, respectively. The solid line in Fig. 3 shows the estimated LC50 value with time. Even though standard errors could not be calculated and few data points were available, the model fitted the data well, with $r^2 = 0.79$, $p < 0.01$.

3.4. Toxicokinetics – toxicodynamics

Using Equation (5), the lethal body concentration (LBC) was calculated to be 66.0 mg Pb/kg dry body wt. Survival of *E. crypticus* as a function of internal Pb concentration after 4, 7, 10, 14 and 21 d exposure is presented in Fig. 4. For each time point, mortality increased with increasing internal Pb concentration, and only occurred when internal concentration exceeded the LBC, however, no clear link could be found between survival and body Pb concentrations. LC50 values on the basis of internal Pb concentrations in the animals could not be calculated for the early exposure times because of the high mortality at the highest test concentration. LC50 values expressed on total Pb concentrations in soil and internal Pb concentrations at different time points are shown in Table 1. The LC50 based on internal Pb concentrations in the enchytraeids seemed to reach steady state after 14 d.

4. Discussion

The combined toxicokinetics-toxicodynamics approach chosen in this study suggests that Pb is rapidly taken up by enchytraeids, with steady state being reached in approx. 7 d, while Pb toxicity develops more slowly with final LC50 not yet being reached after 21 d.

4.1. Toxicokinetics

In the present study, internal Pb concentrations in the enchytraeids showed a large variation, especially in the uptake phase. Such large variation was also found in other studies with soil invertebrates, and mainly seems to result from biological variation in animals within the pathways involved in pollutant handling (uptake, metabolism, excretion) (Spurgeon and Hopkin, 1999; Spurgeon et al., 2011; Vijver et al., 2001). *E. crypticus* showed a fast accumulation of Pb, reaching steady state after around 7 d, when exposed to Pb-amended natural soils. Our results are in agreement with Peijnenburg et al. (1999b), who found that *E. crypticus* showed a fast uptake of Pb when exposed to field soils, although it did not reach a steady state in 14 d. Vijver et al. (2001) also reported a fast uptake of Pb in the springtail *Folsomia candida*, reaching steady-state after approx. 3 d exposure in contaminated field soils. The earthworm *Eisenia fetida* however, failed to reach

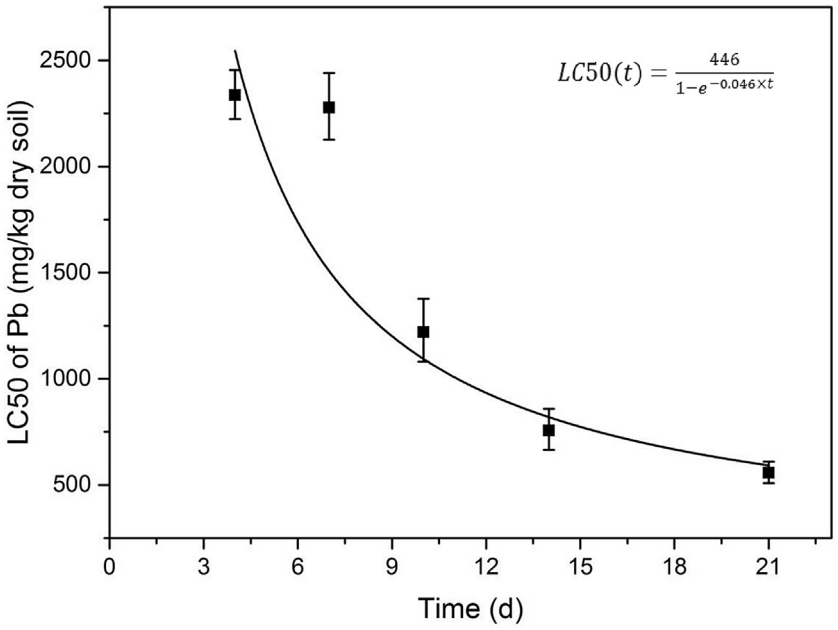

Fig. 3. Development of median lethal concentration (LC50) with time for the toxicity of Pb to *Enchytraeus crypticus* in Lufa 2.2 natural standard soil. Dots present LC50 values with 95% confidence intervals calculated with the trimmed Spearman-Karber method based on measured total Pb concentrations in the test soil, and the line shows the exponential decline of the LC50 according to Equation 3 fitted to the data.

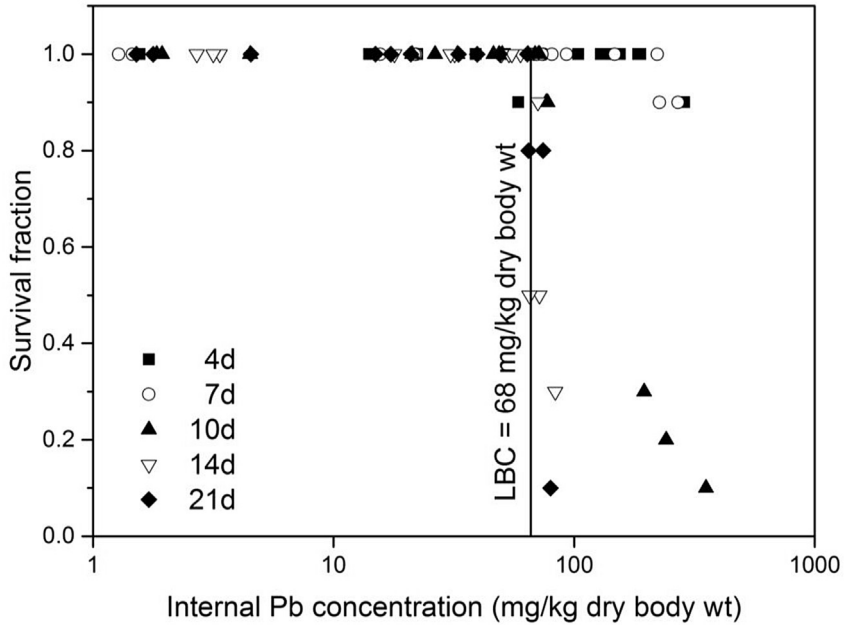

Fig. 4. Relationship between the survival of *Enchytraeus crypticus* and internal Pb concentration measured at different times of exposure to different Pb concentrations in Lufa 2.2 natural standard soil. Dots present the observed data, the vertical line shows the lethal body concentration derived from Equation (5).

Table 1
LC50 values (with corresponding 95% confidence intervals) for the effect of lead on the survival of *Enchytraeus crypticus* at different exposure times in Lufa 2.2 natural soil. LC50 values are based on total Pb concentrations in soil and in the surviving animals.

LC50 based on	Day4	Day7	Day10	Day14	Day21
Total concentration in soil (mg Pb/kg dry soil)	2336 (2336–2455)	2278 (2127–2440)	1220 (1081–1377)	756 (665–859)	558 (509–610)
Internal concentration (mg Pb/kg dry body wt)	>287	>270	161 (148–174)	76.6 (69.1–84.2)	76.4 (75.4–77.4)

equilibrium in body Pb concentrations in 14 d and accumulated Pb slowly in a linear pattern when exposed to Pb-spiked soils (Zhang et al., 2015).

Although a fast excretion of Pb in *E. crypticus* was observed in this study, a portion of Pb remained in the body when the animals were transferred to clean soil after 14 d exposure. This might be explained by the Pb detoxification mechanism in *E. crypticus*. Giska et al. (2014) found that earthworms *Lumbricus rubellus* excreted Pb only very slowly and most Pb taken up could not be eliminated, probably due to Pb detoxification by sequestration. In general, Pb accumulation and elimination patterns are species dependent.

The one-compartment model fitted the data well (Fig. 1), showing that this model was accurate in describing the uptake and excretion of Pb in *E. crypticus*. Variations of k_u and k_e were observed when internal concentrations for different exposure concentrations were fitted separately by the one-compartment model, which also has been reported by other authors (Peijnenburg et al., 1999a; Spurgeon and Hopkin, 1999). The k_us based on total Pb concentration in soil calculated in this study (0.08–0.25 kg_{soil}/kg_{worm}/day) were comparable to the values of 0.0–0.3 kg_{soil}/kg_{worm}/day reported by Peijnenburg et al. (1999b) for *E. crypticus* exposed in different soil types. Giska et al. (2014) found much lower uptake rate constants of 0.004–0.009 kg_{soil}/kg_{worm}/day when exposing *L. rubellus* to field-contaminated soils containing 708–3041 mg Pb/kg dry soil. Compared to the LUFA 2.2 soil used in this study, their test soils had much higher organic matter contents (36.3–54.2% OM) and cation exchange capacity (CEC: 23.5–37.7 cmolc/kg), and consequently Pb bioavailability was much lower. The uptake rate constants therefore depend not only on the test organism but also on soil properties affecting metal bioavailability (Crommentuijn et al., 1997).

The k_u increased with increasing exposure concentration, peaking at 0.25 kg_{soil}/kg_{worm}/day, and then decreased at the higher exposure concentrations (Table S2). This trend was also observed in *E. crypticus* exposed to Ni and Ag (He and Van Gestel, 2013; Topuz and Van Gestel, 2015). The decline of k_u might be explained by the fact that the availability of metal transporters for Pb ions passing through membranes is limited at high exposure concentrations, leading to interference with the physiological functions of the organism (Li et al., 2009). Thus, no clear relationship was found between uptake rate constant and total Pb concentration in soil. This result is consistent with the finding of Peijnenburg et al. (1999b), who found that uptake rate constant based on total soil concentration was more related to soil properties (CEC and OM content) rather than to exposure concentration, when *E. crypticus* was exposed to Pb contaminated soils with different properties.

The calculated k_e (0.42–1.28 day^{-1}), on the basis of internal concentration, seems much higher than the values reported by Giska et al. (2014) for *L. rubellus* (0.010–0.089 day^{-1}). *E. fetida* showed Pb elimination rate constants ranging from 0.02 to 1.15 day^{-1}, when exposed to Pb contaminated soils with different properties (Spurgeon and Hopkin, 1999). The elimination rate constant, therefore, seems to be organism-specific (Crommentuijn et al., 1994). The analysis of elimination patterns may give information on the possible detoxification mechanisms in the organisms. The high excretion rate of Pb in *E. crypticus* found in this study suggests that the main detoxification pathway for Pb is elimination, which is different from earthworms in which sequestration within inorganic matrices or binding to organic ligands seems the dominating mechanism (Spurgeon and Hopkin, 1999). Besides, the high

k_e values also ensured that a steady-state level could be achieved during the uptake phase of the test (Nahmani et al., 2009).

The high k_e value, however, did not lead to complete elimination of all Pb from the enchytraeids. The inert fraction F_i estimated in this study, ranging from 0 to 0.20, demonstrates the presence of a storage detoxification system for Pb in *E. crypticus*. This low inert fraction suggests that Pb storage is limited. C_{sc} (storage capacity, defined as the product of C_{ss} (steady-state internal concentration) and F_i) increased with increasing exposure concentration and but leveled off at approximately 16 mg Pb/kg dry body wt (Table S2). This value is much lower than the ones found in earthworms. For *E. fetida*, for instance, an F_i value of approximately 0.7 was reported upon exposure to Pb-amended soils for 14 d followed by transfer to Pb-unamended soils for a 14 d elimination period (Zhang et al., 2015). For *L. rubellus*, 60–100% of internal Pb concentration remained after 21 d exposure in field contaminated soils and a 21-d elimination period in clean soil (Giska et al., 2014).

4.2. Toxicodynamics

In this study, the LC50 based on total Pb concentrations in soil decreased with exposure time from 2336 mg Pb/kg dry soil at 4 d to 558 mg Pb/kg dry soil at 21 d. The 21 d-LC50 value is similar to the 21d-LC50s of 543–779 mg Pb/kg dry soil found in our previous study (Zhang and Van Gestel, 2017). These results are partly in agreement with Langdon et al. (2005), who found that LC50 values for *E. andrei*, *L. rubellus* and *Aporrectodea caliginosa* decreased from 7063, 4778 and 4728 mg Pb/kg dry soil at 7 d to 5511, 2940 and 2982 mg Pb /kg dry soil at 28 d, respectively. Davies et al. (2003) observed a constant LC50 value for *E. fetida* from 7 d to 28 d, when exposed to Pb-spiked OECD artificial soil. Generally, Pb toxicity was not only species dependent but also time dependent. In this study, the Pb toxicity (LC50) to *E. crypticus* did not reach a steady state within 21 d. The estimated LC50∞ value based on total soil Pb concentrations was 446 mg Pb/kg dry soil, which is lower than the 21-d LC50 of 558 mg Pb/kg dry soil. A steady state for Pb toxicity (LC50) to *L. rubellus*, *A. caliginosa* and *E. fetida* was reported to be reached within 21 d exposure, but for *E. andrei* LC50 still was decreasing after 28 d (Davies et al., 2003; Langdon et al., 2005). Nowadays, ecotoxicity tests are performed with specific standardized exposure times (OECD, 1984; ISO, 2004b), which is not always sufficient for organisms and chemicals that need longer time to reach the ultimate LC50 value. In such cases, the use of a standardized test duration may result in an underestimation of toxicity.

The damage rate constant (k_d) is related to the toxicodynamics of Pb as it is based on the development of the toxic effect (mortality) with time. In the present study k_d therefore reflected the decrease of the internal effective Pb fraction, which is the actual concentration leading to toxic effects. A large number of studies determined metal elimination based on the decrease of the internal concentration in soil invertebrates, but only few data is available that relate metal elimination to toxicity. For *E. crypticus* survival based elimination rate constants of 0.070 d^{-1} for Ni and 0.324 d^{-1} for Ag have been reported (He and Van Gestel, 2013; Topuz and Van Gestel, 2015), which are higher than the value of 0.046 d^{-1} for Pb in this study. This finding suggests that *E. crypticus* has a lower capacity to eliminate the toxic fraction of Pb compared to Ni and Ag.

4.3. Toxicokinetics – toxicodynamics

The elimination rate constants k_e and the damage rate constant k_d were estimated on the basis of the development with time of internal concentrations (toxicokinetics) and toxic effects (mortality; toxicodynamics), respectively. On the basis of k_e and k_d, the biological half-life of Pb was calculated to be 0.75 d and 15.0 d, respectively, indicating that toxicokinetics were much faster than toxicodynamics. This suggests that Pb toxicity (toxicodynamics) was delayed compared to Pb accumulation (toxicokinetics). This might due to the state "damage" in toxicodynamics (Jager et al., 2011), which is an integrative state combining all kinds of biochemical and physiological processes involved in toxicity. The internal metal concentration distributed over the different organs and tissues in the organism changes with exposure time, leading to damage first, and it takes some time before one vital target organ or tissue fails, leading to toxic effects (Van Straalen et al., 2005). That is also the reason why time-course internal concentration could not explain time-course toxic effects. He and Van Gestel (2013) also found a slight delay of Ni toxicity in *E. crypticus*, whereas Ag was eliminated from the body at more or less that same rate as the occurrence of mortality of *E. crypticus*, suggesting a good agreement of the development of toxic effects and Ag accumulation (Topuz and Van Gestel, 2015). In this study, the C_{ss} values (up to 244 mg Pb/kg dry body wt) increased with increasing exposure concentration and were comparable to the values (up to 248 mg Pb/kg dry body wt) for *E. crypticus* reported by Peijnenburg et al. (1999b). The higher values, especially those at the higher exposure concentrations, however, do exceed the LBC, confirming the delay in the onset of mortality compared to Pb uptake.

When relating toxicokinetics (time-course internal concentration) to toxicodynamics (time-course survival), an LC50 based on internal concentrations ($LC50_{inter}$) could be calculated. The $LC50_{inter}$ is supposed to be a better parameter to describe metal toxicity than the LC50 based on external concentration, since it is independent of exposure time (He and Van Gestel, 2013; Topuz and Van Gestel, 2015). In this study, $LC50_{inter}$, however, decreased with time and reached equilibrium only after 14 d, which also might be due to the delay in toxicity compared to Pb bioaccumulation. Thus, in the present study only when $LC50_{inter}$ reached steady state, internal Pb concentration in *E. crypticus* could predict Pb toxicity well. The lethal body concentration of 68 mg Pb/kg dry body wt, estimated from the ultimate median lethal concentration value (LC50 ∞) and the BAF, was only slightly lower than the ultimate $LC50_{inter}$ value (76 mg Pb/kg dry body wt). This suggests that the results from toxicokinetics and toxicodynamics were in a good agreement.

5. Conclusions

In the present study, a combined toxicokinetics-toxicodynamics approach was used to assess the time-course bioaccumulation and toxic effects of Pb in *E. crypticus* in a natural soil. Pb bioaccumulation and toxicity in *E. crypticus* were dependent not only on exposure concentration, but also on exposure time. Internal Pb concentration reached equilibrium after about 7 d exposure, whereas toxicity (LC50) did not reach steady state within 21 d. Pb toxicity in *E. crypticus* therefore seems delayed compared to Pb bioaccumulation. LC50 values based on internal Pb concentrations in the enchytraeids at exposure times longer than 14 d might provide a better measure of Pb toxicity than values based on external concentration, as effects of time could be taken into account by linking toxicokinetics and toxicodynamics. The results of this study highlight the need for caution when ecotoxicological tests with a specific exposure time are used to predict metal toxicity for risk assessment or provide safety thresholds for metals in the environment.

Acknowledgements

The Ph.D.-scholarship of Lulu Zhang was provided by the China Scholarship Council (CSC) under number 201406380110. We thank Rudo A. Verweij for his assistance in various phases of the work.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.envpol.2017.02.070

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