Toxicokinetics of copper and cadmium in the soil model Enchytraeus crypticus (Oligochaeta)

Santos, Fátima C.F.; van Gestel, Cornelis A.M.; Amorim, Mónica J.B.

published in
Chemosphere
2021

DOI (link to publisher)
10.1016/j.chemosphere.2020.129433

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

Chemosphere 270 (2021) 129433

Contents lists available at ScienceDirect

Chemosphere

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

Toxicokinetics of copper and cadmium in the soil model *Enchytraeus crypticus* (Oligochaeta)

Fátima C.F. Santos [a], Cornelis A.M. van Gestel [b], Mónica J.B. Amorim [a, *]

[a] Department of Biology & CESAM, University of Aveiro, 3810-193, Aveiro, Portugal
[b] Department of Ecological Science. Faculty of Science, Vrije Universiteit Amsterdam De Boelelaan 1085, 1081, HV Amsterdam, the Netherlands

ARTICLE INFO

Article history:
Received 30 September 2020
Received in revised form
18 December 2020
Accepted 21 December 2020
Available online 26 December 2020

Handling Editor: Prof Willie Peijnenburg

Keywords:
Bioaccumulation
Kinetics
Uptake
Elimination
Enchytraeids
Toxicodynamics

ABSTRACT

Toxicokinetics information is key to understanding the underlying intoxication processes, although this is often lacking. Hence, in the present study the toxicokinetics of copper (Cu) and cadmium (Cd) was assessed in the soil invertebrate *Enchytraeus crypticus*. The animals were exposed in LUFA 2.2 natural soil spiked to the estimated EC_{20} for reproduction effects in the Enchytraeid Reproduction Test (ERT), i.e. 80 mg Cu/kg soil Dry Weight (DW) and 20 mg Cd/kg soil DW. Tests followed the OECD guideline 317, including a 14-day uptake phase in spiked soil followed by 14 days elimination in clean soil, with samplings at days 0, 1, 2, 4, 7, 10, and 14. Exposure to Cu showed fast uptake, reaching a steady state after approx. 7 days, whereas for Cd, internal concentration increased and did not reach a clear steady state even after 14 days. When transferred to clean soil, Cu was rapidly eliminated returning to initial levels, while Cd-exposed animals still contained increased residue levels after 14 days. These differences in toxicokinetics have consequences for the toxicity and toxicodynamics and are indicative of the way essential and non-essential elements are handled by enchytraeids, likely also other soil invertebrates. This argues for the relevancy of longer exposure testing for elements like Cd compared to Cu, where phenotypical effects can well occur later at non-tested periods, e.g. after the 21 days' duration of the standard ERT using *E. crypticus*.

1. Introduction

Ecotoxicological data are of key importance to assessing hazards, setting priorities, evaluating risks, and establishing environmental quality criteria for contaminants. Elements like cadmium (Cd) and copper (Cu) can enter the environment via various sources, e.g. metal mining, erosion of geological materials, fossil fuel combustion, mine effluent outfalls, and industrial runoff, etc. (Leung et al., 2017). As a consequence, they pose a hazard to terrestrial ecosystems. Copper is an essential trace element with relevant importance in several cellular processes in organisms, although toxic at high concentrations (Duan et al., 2016). Cadmium is a non-essential element, with a high potential to bioaccumulate in organisms and also to be transferred in food webs (Mortensen et al., 2018; van Gestel et al., 1993). Cd resembles essential elements like calcium, both being divalent cations with similar physicochemical properties, favouring the exchange of these metals in Ca^{2+} binding proteins (Choong et al., 2014). As a consequence, Cd may be taken up by the same pathways as Ca (Gonçalves et al., 2015), causing toxicity by competing and replacing essential elements in metabolic processes (Bicho et al., 2015; Gonçalves et al., 2015; Mortensen et al., 2018).

Standard ecotoxicity tests are performed to assess hazards, exposing the organisms to a range of concentrations for a fixed time period establishing dose-response relationships. This means that the adverse effects observed at the end of a test are related to nominal or measured total soil concentrations (Zhang and Van Gestel, 2017). Because the actual effect is due to the bioavailable fraction (not the total) (Lessard, 2007), one of the best measures of bioavailability is the measurement of bioaccumulation in organisms, resembling the internal exposure concentration (Zhang and Van Gestel, 2017). In the case of soft-bodied organisms, like enchytraeids, with a significant dermal uptake route, bioavailability of contaminants will depend mainly on the sorption equilibrium between the soil solid phase (soil particles) and the pore water (Li et al., 2009).

* Corresponding author.
E-mail address: mjamorim@ua.pt (M.J.B. Amorim).

https://doi.org/10.1016/j.chemosphere.2020.129433
0045-6535/

For a proper assessment of metal bioaccumulation in organisms, assessing toxicokinetics allows for a description of the processes that translate an external concentration into an internal concentration over time, including absorption, distribution, transformation and excretion (Lessard, 2007, Newman, 2019). The internalization of metals can happen through the uptake of ionic metal forms. Once inside the organism, binding to ligands can lead to elimination, detoxification, sequestration and redistribution of the metal. Elimination happens when the metal is lost through (active) excretion (Chan and Rainbow, 1993; White and Rainbow, 1982).

Exposure time is known to influence toxicity. For instance, Cd toxicity (reproduction EC_{50}) was higher when enchytraeids were exposed during a 46-day full life cycle (FLC) test compared to the 28-day standard enchytraeid reproduction test (ERT) (Bicho et al., 2015), with EC_{50}s of 5.6 and 35 mg Cd/kg soil DW, respectively. This obviously will also depend on the toxic substance, e.g. Cu toxicity was measured to be similar when exposed via either a 46-day FLC or in a 28-day ERT (Bicho et al., 2017a). A possible reason may be that uptake and elimination occur at a different pace (Spurgeon and Hopkin, 1999; Zhang and Van Gestel, 2017) and also elimination is different, while some organisms can accumulate metals by sequestration to much higher levels before effects occur.

The advantage of the toxicokinetics approach lays precisely in the quantification of the internal concentration during a time series, hence helps explaining toxicity due to constant or varying external concentrations and other environmental variables (Jager and Ashauer, 2018). Therefore, the aim of this study was to investigate the toxicokinetics of the essential Cu and the non-essential Cd and understand if there is a difference in uptake and elimination kinetics in the standard soil species *Enchytraeus crypticus* (OECD, 2010). The animals were exposed in LUFA 2.2 standard soil, and the uptake and elimination were followed over time.

Enchytraeids are one of the most relevant soil organisms given their widespread distribution in different ecosystems and occurrence in large numbers in most soils (Didden and Römbke, 2001). They are saprophagous mesofauna of the litter and upper mineral soil layers, playing an important role in ecological functions like organic matter decomposition and soil bioturbation, improving the small-scale water and air management of soil, especially when population densities are high (Jänsch et al., 2005).

2. Material & methods

2.1. Test organism

Enchytraeus crypticus (Enchytraeidae, Oligochaeta) was kept in cultures in agar media, consisting of Bacti-Agar medium (Oxoid, Agar No. 1) and a sterilized mixture of 4 different salt solutions at final concentrations of 2 mM $CaCl_2 \cdot 2H_2O$, 1 mM $MgSO_4$, 0.08 mM KCl, and 0.75 mM $NaHCO_3$. Cultures were maintained at 19 ± 1 °C with a 16:8-h light: dark photoperiod and fed ground autoclaved oats twice a week.

2.2. Test soil, test substances and spiking procedures

The natural standard LUFA 2.2 soil (Speyer, Germany) was dried at 80 °C for 48 h before use and had the following main characteristics: pH (0.01 M $CaCl_2$) = 5.6, 1.73% organic carbon, cation exchange capacity = 9.8 $cmol_c$/kg, maximum water holding capacity (WHC) = 45.8%, and grain size distribution of 8.3% clay (<0.002 mm), 14.9% silt (0.002–0.05 mm), and 76.8% sand (0.05–2.0 mm).

Test substances Copper (II) chloride dihydrate ($CuCl_2 \cdot 2H_2O$ >99.9% purity, Sigma-Aldrich) and cadmium chloride hemi (pentahydrate) ($CdCl_2.2½H_2O \geq$ 98% purity, Sigma-Aldrich) were used. Soil was spiked by adding $CuCl_2$ and $CdCl_2$ as aqueous solutions to achieve nominal concentrations of 80 mg Cu/kg soil and 20 mg Cd/kg soil (dry weight, DW). These concentrations were selected based on the EC_{20} for effects on the reproduction of *E. crypticus* for Cu (Bicho et al., 2017a) and Cd (Castro-Ferreira et al., 2012) in LUFA 2.2 soil. Soil was moistened to reach 50% of the maximum WHC and equilibrated for 14 days at 20 ± 1 °C prior test start.

2.3. Experimental procedure

The bioaccumulation test followed the OECD guideline 317 (OECD, 2010) including the uptake and elimination kinetics design. In short, 10 adult enchytraeids with well-developed clitellum and of similar size were introduced into each test vessel containing 20 g of moist soil and 15 mg of autoclaved ground oats as food. After 14 days (uptake phase), the surviving animals where transferred to clean soil for an additional 14 days (elimination phase). Five replicates per treatment/sampling day were used. Animals were sampled at 7 plus 6 time points (uptake: 0, 1, 2, 4, 7, 10 and 14 days; elimination: 15, 16, 18, 21, 24, and 28 days) to determine internal concentrations. The tests ran at 20 ± 1 °C and a 16:8-h light: dark photoperiod. During the test, food (15 mg) and water content (based on weight loss) were replenished weekly. Controls with non-spiked soil were included. At each sampling day, the organisms were transferred from soil to ISO water (ISO, 2012) for 12 h, to purge their gut from soil particles. After this, the animals were blotted dry on filter paper; five animals were introduced into cryotubes individually (i.e. 1 organism per tube in 5 cryotubes). All animals were frozen at −20 °C until further analysis. Five replicate soil samples were collected at each sampling day (10 for the control), dried at 40 °C for 48 h and stored at −20 °C until further analysis.

2.4. Chemical analysis

Frozen animals were freeze-dried for 24 h, weighted individually on a microbalance and digested with 300 μL of a 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. The concentrations were measured by graphite furnace atomic absorption spectrometry (AAS; PinAAcle 900Z, Perkin Elmer, Germany). For quality control of the analysis, the certified reference material DOLT 4 was included in the analysis. Mean (±SD; n = 10) cadmium and copper concentrations measured in DOLT 4 were 104.9 ± 7.2% and 98.13 ± 3.6% of the certified values, respectively. Limit of Detection (LOD) for Cu and Cd was 0.003 μg/L.

To assess total soil concentrations of Cu and Cd, 130 mg of soil from days 0, 1 and 14 (3 replicates each) in the uptake phase and days 15 and 28 of the elimination phase were digested using 2 ml of a destruction mixture of HNO_3 (65%, Sigma-Aldrich) and HCl (37%, Sigma-Aldrich) (4:1 v/v). Soil and destruction mixture were placed in Teflon containers that were closed tightly and heated at 140 °C for 7 h. After cooling, 8 ml of deionized water was added, and the concentrations were measured by Flame AAS (AAnalyst 100; Perkin Elmer; Germany). Certified reference material ISE sample 989 was included in the analysis; mean (±SD) cadmium and copper concentrations measured in the reference material were 98.1 ± 2.4% and 93.1 ± 1.9% of the certified values, respectively.

For measuring $CaCl_2$-extractable metal concentrations, 25 ml of 0.01 M $CaCl_2$ solution and 5 g of dry soil were shaken for 2 h at 200 rpm. After sedimentation overnight, pH was measured, and the supernatant was filtered over 0.45 μm nylon syringe filters. The

concentrations in the 0.01 M $CaCl_2$ extracts were measured by Flame AAS (AAnalyst 100; Perkin Elmer; Germany).

2.5. Data analysis

Assuming constant exposure concentrations, the uptake and elimination in the animals was modelled using a one-compartment first-order kinetics model (Crommentuijn et al., 1997), fitting Equations (1A) and (1B) simultaneously to data of the uptake and elimination phase:

Uptake phase

$$C_t = C_0 + \frac{K_u}{\left(K_e + K_{growth}\right)} * C_{exp} * \left(1 - e^{-\left(K_e + K_{growth}\right) * t}\right) \quad (1A)$$

Elimination phase

$$C_t = C_0 + \frac{K_u}{\left(K_e + K_{growth}\right)} * C_{exp} * \left(e^{-\left(K_e + K_{growth}\right) * (t_c - t)} - e^{-\left(K_e + K_{growth}\right) * t}\right) \quad (1B)$$

where C_t is the concentration in the enchytraeids after t days of exposure (mg/kg dry body weight), C_0 the background concentration in the enchytraeids (mg/kg dry body weight), K_u the uptake rate constant (kg soil/kg organism/day), K_e the elimination rate constant (day^{-1}), K_{growth} the growth rate (day^{-1}), C_{exp} the exposure concentration (mg/kg dry soil), t the exposure time (days), and t_c the time when the animals were transferred to clean soil.

The uptake and elimination rate constants were calculated using both the total and available ($CaCl_2$-extractable) soil concentrations as the exposure concentration (C_{exp}).

K_{growth}, (based on animal dry weights), was calculated for the whole 28 days of exposure, using an exponential growth model. This gave K_{growth} values of 0.0632 day^{-1} ($R^2 = 0.47$) and 0.0754 day^{-1} ($R^2 = 0.70$), which were used for determining the toxicokinetics of Cu and Cd, respectively.

The bioaccumulation factor (BAF) (kg soil/kg organism), the ratio between the concentration in the organisms (mg/kg dry body weight) and the concentration in the soil (mg/kg dry soil) at the steady state, was calculated as:

$$BAF = \frac{K_u}{K_e} \quad \text{(Eq. 2)}$$

The half-life for elimination of the metals from the enchytraeids was calculated as:

$$DT_{50} = \frac{\ln(2)}{K_e} \quad (3)$$

Individual internal concentrations were used to fit the models. Total measured copper and cadmium concentrations in the soils were used in the calculations, that were run in Excel and SPSS 25.0.

3. Results

3.1. Chemical analysis

The total measured Cu and Cd concentrations (mean ± SE) were in agreement with the nominal ones and amounted 76 ± 1.4 mg Cu/kg dry soil and 16 ± 0.7 mg Cd/kg dry soil. These concentrations were constant over the exposure time period. The non-spiked soil contained 4.0 ± 0.18 mg Cu/kg dry soil and 0.33 ± 0.06 mg Cd/kg dry soil.

$CaCl_2$-extractable concentrations in the spiked soils were 0.8 ± 0.07 mg Cu/kg dry soil and 0.85 ± 0.057 mg Cd/kg dry soil and did not change during the uptake phase period.

3.2. Toxicokinetics

Average survival of the enchytraeids during the uptake and elimination phase was 92% and 80% at the end of the exposure period for Cu and Cd, respectively, fulfilling the validity criteria of ≥80% (OECD, 2010). Results, including the model fit and parameter estimates, are summarized in Fig. 1 and Table 1.

An increase in concentrations in *E. crypticus* with time was observed for both Cu and Cd, with a seemingly larger variation in the uptake phase compared to the elimination phase.

E. crypticus Cu basal level was 17.6 ± 1.1 (AV ± SE) mg Cu/kg dry body weight. Exposure to 80 mg Cu/kg soil DW caused an increase in internal concentrations and a steady state was reached around day 7. When transferred to non-spiked soil elimination occurred fast with Cu concentration returning to the background level within approx. 3 days. An uptake rate constant (K_u) of 0.27 kg soil/kg animal/day and an elimination rate constant (K_e) of 0.98 day^{-1} were estimated. The BAF was 0.27 and the half-life 0.71 days. Based on $CaCl_2$-extractable copper concentrations, K_u was 24.9 kg soil/kg animal/day, and the BAF 25 (Table 1).

For Cd, basal concentration level was 0.56 ± 0.15 (AV ± SE) mg Cd/kg dry body weight. Exposure to 20 mg Cd/kg soil DW caused an increase with time but not reaching a clear steady state up to 14 days, where the highest concentration of 7.33 mg Cd/kg dry body weight was measured. Elimination occurred at a slower rate and was incomplete (ca. 50%) after 14 days. The K_u for Cd based on the total soil concentrations was 0.032 kg soil/kg animal/day, the K_e was 0 day^{-1}. Since K_e was zero, it is not possible to calculate a bioaccumulation factor (BAF) at steady state or a half-life for Cd. When ignoring growth of the animals, K_u was 0.028 kg soil/kg animal/day and K_e was 0.058 day^{-1}. The fact that K_e was lower than the K_{growth} of 0.075 day^{-1} indicates that the decrease of Cd concentrations in the animals during the elimination phase was due to a growth dilution. When based on $CaCl_2$-extractable concentrations, K_u was 0.62 kg soil/kg animal/day (Table 1).

4. Discussion

4.1. Toxicokinetics

Copper and cadmium showed clearly distinct toxicokinetic patterns in *E. crypticus* with Cu being regulated and showing faster kinetics as opposed to Cd where no equilibrium was reached after 14 days with a much slower elimination.

Studies with the earthworms *Eisenia andrei* and *Eisenia fetida* report a similar pattern of accumulation for Cu, reaching a steady state concentration and being regulated after a few days of exposure and also eliminating fast, returning to initial levels within 2–3 days (Kilpi-Koski et al., 2019; Spurgeon and Hopkin, 1999). The inability to regulate internal concentrations of Cd has been supported by other studies, e.g. for *E. crypticus* when exposed at different temperatures and to field-collected soil (Cedergreen et al., 2013; Peijnenburg et al., 1999b), and for the earthworms *E. fetida* (Lock and Janssen, 2001; Nahmani et al., 2009; Spurgeon and Hopkin, 1999), *E. andrei* (Kilpi-Koski et al., 2019; Peijnenburg et al., 1999a) and *Lumbricus rubellus* (Giska et al., 2014).

The observed larger variation in terms of internal concentrations of copper and cadmium during the uptake phase has also been reported in other studies with metals in soil invertebrates (Zhang et al., 2019; Zhang and Van Gestel, 2017). Such variation may be related with heterogeneous distribution of the metal in soil and also the biological variation in animal responses (Spurgeon

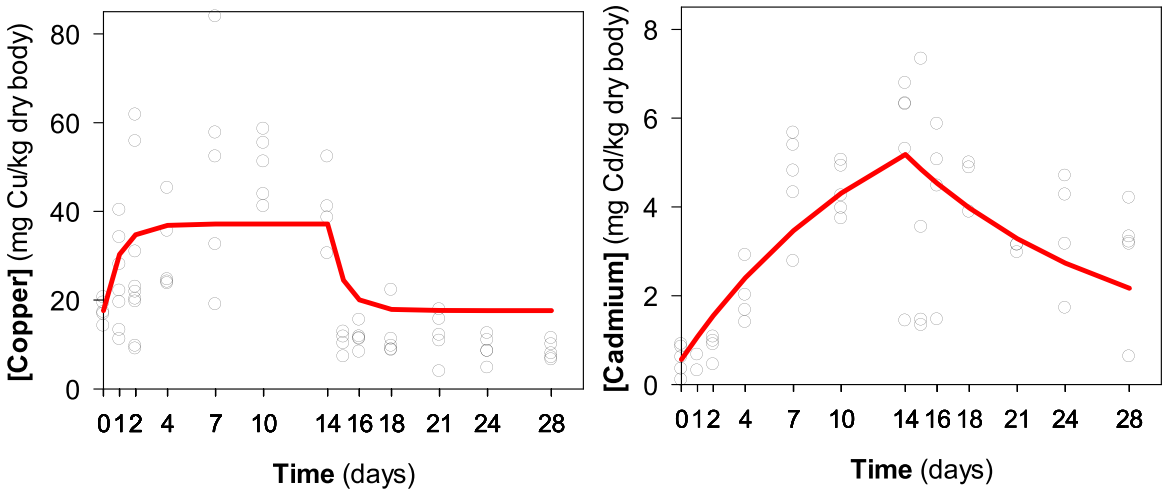

Fig. 1. Uptake and elimination of Cu (left) and Cd (right) in *Enchytraeus crypticus* exposed for 14 days in LUFA 2.2 soil spiked with 80 mg Cu/kg soil DW or 20 mg Cd/kg soil DW followed by 14 days elimination in non-spiked soil. Red lines represent the fit to the data of a first order one-compartment model (Eq. (1)), based on total soil concentrations and taking into account the growth rate of the animals during the 28-day test period. Each dot represents a replicate individual enchytraeid. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 1
Toxicokinetic model parameters estimated for the uptake and elimination of copper and cadmium in *Enchytraeus crypticus* when exposed in LUFA 2.2 natural soil. K_u: uptake rate constant, K_e: elimination rate constant, C_0: background concentration in the animals, brackets [] show the 95% confidence intervals, C_{exp}: metal exposure concentration in spiked soil, K_{growth}: growth rate of the animals during the test, BAF: bioaccumulation factor, DT_{50}: time needed to eliminate 50%. Values were calculated relating concentration in the enchytraeids to total and 0.01 M $CaCl_2$-extractable concentrations in the soil.

Parameters	Cu		Cd	
	Total	$CaCl_2$	Total	$CaCl_2$
K_u (kg soil/kg animal/day)	0.27 [0.03–0.50]	24.9 [3.15–47]	0.032 [0.023 –0.041]	0.62 [0.45–0.80]
K_e (day^{-1})	0.98 [-0.006–1.9]	0.98 [-0.006–1.9]	0	0
C_0 (mg/kg dry body wt) (AV ± SE)	17.6 ± 1.1	17.6 ± 1.1	0.56 ± 0.15	0.56 ± 0.15
K_{growth} (day^{-1})	0.063		0.075	
C_{exp} (mg/kg dry soil)	76.1	0.82	16.7	0.85
BAF	0.27	25	–	–
DT_{50} (days)	0.71	0.71	–	–

et al., 2011). Differences in gut voidance may possibly have contributed to the variation in the internal metal concentrations in the animals. But according to our experience, a period of 8–10 h is sufficient for the enchytraeids to remove all soil from their intestines. Because of this and since all animals were treated in the same way, we don't expect this factor to be of great relevance. In the case of Cu, however, the fast elimination, with a half-life of 0.71 days, may have affected the results. This fast elimination theoretically may have resulted in a loss of about 40% of the Cu from the animals during the 12-h gut voidance period in ISO water.

Table S1 summarizes available literature on the toxicokinetics of Cu and Cd in oligochaetes (enchytraeids and earthworms), showing results of studies performed at different exposure conditions, times of exposure, temperature, main characteristics of the soil, soil properties, etc.

Metal availability depends on soil properties (e.g., pH, CEC, OM, clay content among the most relevant) but also on the metal, its concentration, kind of pollution and history as well as the organism's physiology (Giska et al., 2014; Nahmani et al., 2009). (Peijnenburg et al. (1999a) and González-Alcaraz and Van Gestel (2016) reported similar values as ours for the uptake and elimination rate constants of Cd in *E. andrei* when exposed to contaminated field soils for 63 and 21 days, respectively. Kilpi-Koski et al. (2019) exposed *E. andrei* to medium and high field-contaminated soils in Finland and reported that even though the total soil concentrations were high (791 and 642 mg/kg dry soil), the $CaCl_2$-extractable concentrations (representing the available fraction) were much lower (4.58 and 2.22 mg/kg dry soil for the medium and high contaminated soils, respectively), hence arguing for the lower uptake rate constants compared to our study. The elimination rate constants however, were higher than in our study, which probably was due to the differences between test species.

A study by Nahmani et al. (2009), exposing *E. fetida* to soils from 8 metalliferous sites reported uptake rate constants ranging between 0.032 and 0.0191 kg soil/kg animal/day for Cu and elimination rate constants between 0.13 and 0.623 day^{-1}. These values are higher than the ones for *E. crypticus* in the present study. For Cd, the uptake rate constant in *E. fetida* ranged between 0.22 and 4.92 kg soil/kg animal/day and the elimination rate constant between 0 and 4.79 day^{-1}. This means that the earthworms were not able to eliminate Cd taken up from some soils, while the high elimination rate of Cd taken up from other soils is contrary to the literature generally reporting little or no excretion for this element (Spurgeon and Hopkin, 1999).

The difference between Cu and Cd kinetics is likely related with differences in element handling by the enchytraeids. The kinetics for Cu are faster than those for Cd. Being an essential element, the internal Cu concentration is regulated by conserved mechanisms,

as also shown for earthworms and enchytraeids (reported for Cu in L. *rubellus* (Giska et al., 2014), *E. andrei* (Kilpi-Koski et al., 2019) and *E. fetida* (Nahmani et al., 2009)). There are several possible mechanisms, e.g. for zinc (Zn), also essential, the shrimp *Palaemon elegans* increases the elimination to match the uptake rate since they do not control the internalization of zinc through their body surface (White and Rainbow, 1982). Another mechanism is to actively restrict the internalization as done by the crab *Carcinus maenas*; further, when the environmental concentrations get extremely high, the internal body concentrations do increase but excess zinc is mainly stored in the hepatopancreas and in the exoskeleton (Chan and Rainbow, 1993). In *E. crypticus*, the regulation of Cu seems to function by increasing the elimination rate to match the uptake rate, as shown by the steady state achieved in ca. 7 days, followed by the fast excretion when the animals were transferred to clean soil. A proteomics and metabolomics study in *E. crypticus* (Maria et al., 2018a, 2018b) showed that exposure to Cu activated conserved mechanisms after a short exposure period (7 days), allowing detoxification (via active excretion), these being shut down later (14 days). It was also observed that the activated mechanisms used constitutive genes, and Cu is taken up by the cell via membrane transporters (Gomes et al., 2018b). Additionally, when studying the effect of multigenerational exposure to $CuCl_2$ (4 generations in spiked soil plus 2 generations in clean soil) in *E. crypticus* (Bicho et al., 2017b), increased tolerance to Cu was observed after a few generations, i.e. Cu was less toxic at F2 and F3 than at F1 when considering effects on reproduction. One hypothesis is that tolerant organisms can take up more Cu by increasing their storage capacity and reach a new Cu homeostasis state. This hypothesis would also explain why organisms performed worse when transferred to clean soil, i.e., suffering from Cu deficiency (Bicho et al., 2017b).

On the other hand, Cd is known to compete with Ca, disturbing its homeostasis and disrupting embryo development, as observed at 16 mg Cd/kg soil (Gonçalves et al., 2015). Gomes et al. (2018a), confirmed that when *E. crypticus* were exposed to Cd, gene regulation mechanisms were activated to synthesize more Ca channel proteins. Further, besides the extracellular competition, Cd competes intracellularly, which causes a reduction in Ca efflux and potentiates Cd embryotoxicity. This could as well explain the increased toxicity observed when testing Cd in a Full Life Cycle test (Bicho et al., 2015), i.e., exposing *E. crypticus* from cocoon stage instead of adults: embryos are more sensitive to Cd than adults, and hence it is not only the additional exposure time compared to the ERT (46 and 28 days, respectively) that accounts for the differences in toxicity obtained, but also a difference in the sensitivity of different life stages. In the present study adults were exposed to 20 mg Cd/kg soil and survived even when excretion was negligible, hence we also learned that large amounts can be taken up before effects can be observed.

4.2. Consequences for toxicodynamics

The present toxicokinetics data for Cu and Cd are well in agreement with the previous results in terms of survival and reproduction effects, i.e. Cu uptake was faster and *E. crypticus* regulated internal concentrations to reach homeostasis shortly after exposure, hence longer term exposure to lower soil concentrations did not increase effects (Bicho et al., 2017b). For Cd, uptake was slower not reaching equilibrium within 14 days of exposure and apparently not regulating Cd, hence toxicity was higher (compared to e.g. Cu) (Castro-Ferreira et al., 2012) and also increased with longer exposure time (ERT compared to FLC) (Bicho et al., 2015). Bioaccumulation studies allowed to understand the key turning point in time at which a chemical reaches a critical level leading to adverse effects on the animals.

The exposure concentrations (EC_{20} for the effects on the reproduction for Cu (Bicho et al., 2017a) and Cd (Castro-Ferreira et al., 2012)) are expected to cause adverse phenotypic effects due to the internal concentration exceeding a critical level (Jager et al., 2011; Lanno et al., 2004). Posthuma et al. (1997) exposed *E. crypticus* for 4 weeks to an artificial soil spiked with Cu and found EC_{50} and EC_{25} values based on internal body concentrations of 43.9 and 30.1 mg/kg dry body weight, with an EC_{50} based on total soil concentrations of 477 mg/kg. The maximum and steady state internal concentrations of 83.9 and 37.2 mg Cu/kg dry body weight found in this study (Fig. 1) exceed the EC_{50} and EC_{25}, respectively reported by Posthuma et al. (1997), suggesting that already at 80 mg Cu/kg dry LUFA 2.2 soil some effects on reproduction are to be expected. Lock and Janssen (2001) exposed the earthworm *E. fetida* for 28 days in a standard artificial soil spiked with cadmium and found a 50% reduction in cocoon production at 577 mg Cd/kg dry body weight in the animals. This is much higher than the highest concentration found in *E. crypticus* of 7.33 mg Cd/kg dry weight at day 14 (Fig. 1). This suggests effect may not be likely at the Cd exposure level used in this study. It should however be noted that no equilibrium was reached after 14 days and that it may not be excluded that enchytraeids have a different way of handling cadmium than earthworms and therefore may have different critical body concentrations. However, no critical body concentrations were found for Cd in enchytraeids.

5. Conclusions

Toxicokinetics of Cu and Cd in *E. crypticus* could be described by a one-compartment model. Copper showed fast uptake and elimination kinetics reaching a steady state after ca. 7 days, with *E. crypticus* regulating its body concentration. For cadmium no steady state was reached up to 14 days, and virtually no elimination occurred, hence the enchytraeids were not able to regulate Cd body concentration. The results support the observed chronic effects, namely reproduction. Further, the findings are in line with known mechanisms of metal regulation in invertebrates for Cu and Cd. This argues for the relevancy of longer term exposure testing, particularly for elements like Cd, where phenotypical effects can well occur later, beyond the test duration applied in the current test guidelines.

CRediT author statement

F.C.F. Santos: Methodology, Investigation, Writing - original draft. **C.A.M. van Gestel**: Supervision, Conceptualization, Data curation, Resources, Writing - review & editing. **M.J.B. Amorim**: Supervision, Conceptualization, Data curation, Resources, Funding acquisition, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

Thanks are due to FCT/MCTES for the financial support (UIDP/50017/2020 + UIDB/50017/2020), through national funds, and FCT via a PhD grant to Fátima da Conceição Ferreira dos Santos (SFRH/BD/118294/2016). The authors would like to thank Rudo Verweij for his technical support in the experimental work.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2020.129433.

References

Bicho, R.C., Santos, F.C.F., Gonçalves, M.F.M., Soares, A.M.V.M., Amorim, M.J.B., 2015. Enchytraeid Reproduction TestPLUS: hatching, growth and full life cycle test—an optional multi-endpoint test with *Enchytraeus crypticus*. Ecotoxicology 24, 1053–1063.

Bicho, R.C., Santos, F.C.F., Scott-Fordsmand, J.J., Amorim, M.J.B., 2017a. Effects of copper oxide nanomaterials (CuONMs) are life stage dependent – full life cycle in *Enchytraeus crypticus*. Environ. Pollut. 224, 117–124.

Bicho, R.C., Santos, F.C.F., Scott-Fordsmand, J.J., Amorim, M.J.B., 2017b. Multigenerational effects of copper nanomaterials (CuONMs) are different of those of CuCl2: exposure in the soil invertebrate *Enchytraeus crypticus*. Sci. Rep. 7, 8457.

Castro-Ferreira, M.P., Roelofs, D., van Gestel, C.A.M., Verweij, R.A., Soares, A.M.V.M., Amorim, M.J.B., 2012. *Enchytraeus crypticus* as model species in soil ecotoxicology. Chemosphere 87, 1222–1227.

Cedergreen, N., Nørhave, N.J., Nielsen, K., Johansson, H.K.L., Marcussen, H., Svendsen, C., Spurgeon, D.J., 2013. Low temperatures enhance the toxicity of copper and cadmium to *Enchytraeus crypticus* through different mechanisms. Environ. Toxicol. Chem. 32, 2274–2283.

Chan, H.M., Rainbow, P.S., 1993. On the excretion of zinc by the shore crab *carcinus maenas* (L.). Ophelia 38, 31–45.

Choong, G., Liu, Y., Templeton, D.M., 2014. Interplay of calcium and cadmium in mediating cadmium toxicity. Chem. Biol. Interact. 211, 54–65.

Crommentuijn, T., Doornekamp, A., Van Gestel, C.A.M., 1997. Bioavailability and ecological effects of cadmium on *Folsomia candida* (Willem) in an artificial soil substrate as influenced by pH and organic matter. Appl. Soil Ecol. 5, 261e271.

Didden, W., Römbke, J., 2001. Enchytraeids as indicator organisms for chemical stress in terrestrial ecosystems. Ecotoxicol. Environ. Saf. 50, 25–43.

Duan, X., Xu, M., Zhou, Y., Yan, Z., Du, Y., Zhang, L., Zhang, C., Bai, L., Nie, J., Chen, G., Li, F., 2016. Effects of soil properties on copper toxicity to earthworm *Eisenia fetida* in 15 Chinese soils. Chemosphere 145, 185–192.

Giska, I., van Gestel, C.A.M., Skip, B., Laskowski, R., 2014. Toxicokinetics of metals in the earthworm *Lumbricus rubellus* exposed to natural polluted soils – relevance of laboratory tests to the field situation. Environ. Pollut. 190, 123–132.

Gomes, S.I.L., Gonçalves, M.F.M., Bicho, R.C., Roca, C.P., Soares, A.M.V.M., Scott-Fordsmand, J.J., Amorim, M.J.B., 2018a. High-throughput gene expression in soil invertebrate embryos – mechanisms of Cd toxicity in *Enchytraeus crypticus*. Chemosphere 212, 87–94.

Gomes, S.I.L., Roca, C.P., Pegoraro, N., Trindade, T., Scott-Fordsmand, J.J., Amorim, M.J.B., 2018b. High-throughput tool to discriminate effects of NMs (Cu-NPs, Cu-nanowires, CuNO 3 , and Cu salt aged): transcriptomics in *Enchytraeus crypticus*. Nanotoxicology 12, 325–340.

Gonçalves, M.F.M., Bicho, R.C., Rêma, A., Soares, A.M.V.M., Faustino, A.M.R., Amorim, M.J.B., 2015. Development of an embryotoxicity test for Enchytraeus crypticus – The effect of Cd. Chemosphere 139, 386–392. https://doi.org/10.1016/j.chemosphere.2015.07.021.

González-Alcaraz, M.N., Van Gestel, C.A.M., 2016. Metal/metalloid (As, Cd and Zn) bioaccumulation in the earthworm *Eisenia andrei* under different scenarios of climate change. Environ. Pollut. 215, 178–186.

ISO, 2012. Determination of the Inhibition of the Mobility of *Daphnia magna* Straus (Cladocera, Crustacea) - Acute Toxicity Test. International Organization for Standardization, Geneva, Swizerland. ISO 6341.

Jager, T., Albert, C., Preuss, T.G., Ashauer, R., 2011. General unified threshold model of survival - a toxicokinetic-toxicodynamic framework for ecotoxicology. Environ. Sci. Technol. 45, 2529–2540.

Jager, T., Ashauer, R., 2018. How to evaluate the quality of toxicokinetic-toxicodynamic models in the context of environmental risk assessment. Integrated Environ. Assess. Manag. 14, 604–614.

Jänsch, S., Amorim, M.J., Römbke, J., 2005. Identification of the ecological requirements of important terrestrial ecotoxicological test species. Environ. Rev. 13, 51–83.

Kilpi-Koski, J., Penttinen, O.-P., Väisänen, A.O., van Gestel, C.A.M., 2019. An uptake and elimination kinetics approach to assess the bioavailability of chromium, copper, and arsenic to earthworms (*Eisenia andrei*) in contaminated field soils. Environ. Sci. Pollut. Res. 26, 15095–15104.

Lanno, R., Wells, J., Conder, J., Bradham, K., Basta, N., 2004. The bioavailability of chemicals in soil for earthworms. Ecotoxicol. Environ. Saf. 57, 39–47.

Lessard, D.R., 2007. Framework for Metals Risk Assessment. Washington, DC 20460.

Leung, H.M., Duzgoren-Aydin, N.S., Au, C.K., Krupanidhi, S., Fung, K.Y., Cheung, K.C., Wong, Y.K., Peng, X.L., Ye, Z.H., Yung, K.K.L., Tsui, M.T.K., 2017. Monitoring and assessment of heavy metal contamination in a constructed wetland in Shaoguan (Guangdong Province, China): bioaccumulation of Pb, Zn, Cu and Cd in aquatic and terrestrial components. Environ. Sci. Pollut. Res. 24, 9079–9088.

Li, L.-Z., Zhou, D.-M., Wang, P., Allen, H.E., Sauvé, S., 2009. Predicting Cd partitioning in spiked soils and bioaccumulation in the earthworm *Eisenia fetida*. Appl. Soil Ecol. 42, 118–123.

Lock, K., Janssen, C.R., 2001. Zinc and cadmium body burdens in terrestrial oligochaetes: use and significance in environmental risk assessment. Environ. Toxicol. Chem. 20, 2067–2072.

Maria, V.L., Licha, D., Ranninger, C., Scott-Fordsmand, J.J., Huber, C.G., Amorim, M.J.B., 2018a. The *Enchytraeus crypticus* stress metabolome – CuO NM case study. Nanotoxicology 12, 766–780.

Maria, V.L., Licha, D., Scott-Fordsmand, J.J., Huber, C.G., Amorim, M.J.B., 2018b. The proteome of *Enchytraeus crypticus*—exposure to CuO nanomaterial and CuCl2—in pursue of a mechanistic interpretation. Proteomics 18, 1–6.

Mortensen, L.H., Rønn, R., Vestergård, M., 2018. Bioaccumulation of cadmium in soil organisms – with focus on wood ash application. Ecotoxicol. Environ. Saf. 156, 452–462.

Nahmani, J., Hodson, M.E., Devin, S., Vijver, M.G., 2009. Uptake kinetics of metals by the earthworm *Eisenia fetida* exposed to field-contaminated soils. Environ. Pollut. 157, 2622–2628.

Newman, M.C., 2019. Fundamentals of Ecotoxicology, Fundamentals of Ecotoxicology. CRC Press, fifth ed. CRC Press, Boca Raton. 2020.

OECD, 2010. Test No. 317: Bioaccumulation in Terrestrial Oligochaetes, Organisation for Economic Co-operation and Development, OECD Guidelines for the Testing of Chemicals, Section 3. OECD, Paris, France.

Peijnenburg, W.J.G.M., Baerselman, R., de Groot, A.C., Jager, T., Posthuma, L., Van Veen, R.P.M., 1999a. Relating environmental availability to bioavailability: soil-type-dependent metal accumulation in the oligochaete *Eisenia andrei*. Ecotoxicol. Environ. Saf. 44, 294–310.

Peijnenburg, W.J.G.M., Posthuma, L., Zweers, P.G.P.C., Baerselman, R., de Groot, A.C., Van Veen, R.P.M., Jager, T., 1999b. Prediction of metal bioavailability in Dutch field soils for the oligochaete *Enchytraeus crypticus*. Ecotoxicol. Environ. Saf. 43, 170–186.

Posthuma, L., Baerselman, R., Van Veen, R.P.M., Dirven-Van Breemen, E.M., 1997. Single and joint toxic effects of copper and zinc on reproduction of *Enchytraeus crypticus* in relation to sorption of metals in soils. Ecotoxicol. Environ. Saf. 38, 108–121.

Spurgeon, D.J., Hopkin, S.P., 1999. Comparisons of metal accumulation and excretion kinetics in earthworms (*Eisenia fetida*) exposed to contaminated field and laboratory soils. Appl. Soil Ecol. 11, 227–243.

Spurgeon, D.J., Lister, L., Kille, P., Pereira, M.G., Wright, J., Svendsen, C., 2011. Toxicokinetic studies reveal variability in earthworm pollutant handling. Pedobiologia 54, S217–S222.

van Gestel, C.A.M., Dirven-van Breemen, E.M., Baerselman, R., 1993. Accumulation and elimination of cadmium, chromium and zinc and effects on growth and reproduction in *Eisenia andrei* (Oligochaeta, Annelida). Sci. Total Environ. 134, 585–597.

White, S., Rainbow, P., 1982. Regulation and accumulation of copper, zinc and cadmium by the shrimp *Palaemon elegans*. Mar. Ecol. Prog. Ser. 8, 95–101.

Zhang, L., Van Gestel, C.A.M., 2017. Toxicokinetics and toxicodynamics of lead in the soil invertebrate *Enchytraeus crypticus*. Environ. Pollut. 225, 534–541.

Zhang, L., Verweij, R.A., Van Gestel, C.A.M., 2019. Effect of soil properties on Pb bioavailability and toxicity to the soil invertebrate *Enchytraeus crypticus*. Chemosphere 217, 9–17.