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Subcellular localization and compartment-specific toxicokinetics of cadmium, arsenic, and zinc in brandling worm *Eisenia fetida*

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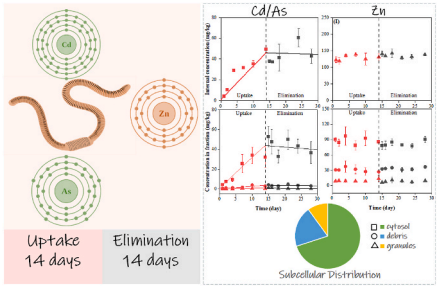
HIGHLIGHTS

- Different kinetics in *E. fetida* were found between non- and essential metal(loid)s.
- A concentration-dependent increase of Cd/As was found in *E. fetida* or subcellular fractions.
- Relatively constant content of Zn was found in earthworm or subcellular compartments.
- Fraction-specific toxicokinetics explained the observed bioaccumulation patterns.
- The level of Cd/As/Zn in subcellular fractions ranked as cytosol > debris > granule.

GRAPHICAL ABSTRACT



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ABSTRACT

Awareness of toxicokinetics at the subcellular level is crucial to deciphering the underlying intoxication processes of metal(loid)s, although this information is often lacking. Here, the toxicokinetics of two non-essential metal(loid)s (Cd and As) and one essential metal (Zn) in both the whole body and subcellular fractions of earthworm (*Eisenia fetida*) were assessed. Earthworms were exposed to natural soils originating from a gradient of metal(loid) pollution for 14 days followed by a 14-day elimination phase in clean soil. Clearly distinct toxicokinetic patterns were found in the earthworms according to the metal(loid) considered. An obvious concentration-dependent increase was observed in earthworms or subcellular compartments where no equilibrium was reached (with slow or no elimination) for Cd and As throughout the experiment. As for Zn, the earthworms were able to retain a steady-state concentration of Zn in its body or each fraction without a clear intake behavior via the dynamic trade-off between uptake and elimination at different pollution levels. These differences in toxicokinetics at the subcellular level supported the observed differences in bioaccumulation patterns and were indicative of the strategy by which non-essential and essential elements are handled by earthworms. Notably, the concentration of Cd and As in subcellular compartments showed the same pattern as for Zn in the order of cellular cytosol > cellular debris > metal-rich granules, which might be associated with the binding of non-essential/essential elements with metallothionein enriched in the cytosol. Our findings enhance the understanding of the underlying mechanisms for metal(loid) accumulation kinetics in earthworms from the perspective of subcellular partitioning, and will be beneficial for accurate risk assessment of Cd, As, and Zn.

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1. Introduction

As a non-renewable natural resource, soil is vital for ecosystems and human society as it not only provides a habitat for most of the Earth's species but also serves as a medium for crop production (Jansson and Hofmockel, 2020). However, anthropogenic activities (e.g. agricultural activities, mining activities, and modern usage) are causing widespread contamination and degradation of soil (Borrelli et al., 2017; Foley et al., 2011). As a major pool for carbon storage, soil contaminated with heavy metals or metalloids, hereafter, referred to as metal(loid)s, might be unable to fulfil the soil carbon cycle, thereby aggravating global climate change (Hou et al., 2020). In addition, worldwide concern over metal (loid) contamination in agricultural soil is rapidly increasing, due to the toxic potency, ubiquity, persistence, and bioavailability for crop uptake of metal(loid)s (Rodríguez Eugenio, 2018). Large quantities of metal (loid)s in agricultural soil pose serious threats to global food safety and security and even damage human health via the food chain (Zhao et al., 2015).

Among metal(loid)s, cadmium (Cd), arsenic (As), and zinc (Zn) are identified as commonly encountered metal(loid) contaminants in agricultural soil globally (Hu et al., 2016; Muehe et al., 2019). In particular, Cd and As accounted for 43% and 17%, respectively, of all soil quality exceedances according to a national soil survey in China (2014). The mean level of Zn was determined to be 4.7 times higher than its secondary environmental quality standard for farmland soil in China (Li et al., 2014). Non-essential trace elements (Cd and As) and essential micronutrients (like Zn) may exert adverse effects on organisms once their body concentrations exceed their respective critical body residue, namely the physiological threshold of a metal(loid) (Han et al., 2022; Palansooriya et al., 2020). The accumulation process of contaminants in organisms is a complex and variable toxicokinetic process which includes uptake, internal distribution, storage, and excretion. Metal accumulation is therefore time-dependent, indirectly causing toxic effects that vary with exposure time (Jager et al., 2011). It is also to be concluded from this summary that results based on acute toxicity assessment often do not fully reflect the long-term exposure risk of contaminants. Exposure time should, therefore, be taken into account in ecotoxicological tests.

Toxicokinetics translate the external exposure concentration of a metal(loid) into an internal body concentration over time. This translation can be made quantitative using a one-compartment model by taking account of both the uptake and the elimination phase due to their simultaneous occurrence during the bioaccumulation process (van Straalen et al., 2005). Given the fact that uptake and elimination processes of metal(loid)s might occur simultaneously in soil organisms, an experimental design including exposure to contaminated soil followed by subsequent transferring to clean soil is desired to obtain a more accurate uptake rate. The uptake rate rather than the internal concentration of a metal(loid)s in an organism is supposed to be a better predictor for bioavailability (van Straalen et al., 2005). Uptake and elimination rates were found to be species-specific (Spurgeon and Hopkin, 1999), metal(loid)-dependent due to the available different detoxification mechanisms (González-Alcaraz et al., 2018), and also affected by the exposure concentration in soils (Giska et al., 2014). Together, differences in a number of factors, such as the organism's properties, the metal(loid) types, as well as the dose and the exposure matrix, might be the reasons for the inconsistent results on the bioaccumulation pattern of Cd, As, or Zn in soil dwelling organisms reported in previous studies (Giska et al., 2014; Gonzalez-Alcaraz and van Gestel, 2016; González-Alcaraz et al., 2018; Kilpi-Koski et al., 2019; Li et al., 2009; Zhang and Van Gestel, 2017; Zhang et al., 2022). From this, it is not surprising that no agreement has been reached on whether uptake-elimination patterns differ between essential and non-essential elements in soil dwelling organisms. Previous study has pointed out that Cu was rapidly taken up and excreted by earthworm *Eisenia andrei* with equilibrium being reached within 1 day, whereas As showed very slow uptake and elimination (Kilpi-Koski et al., 2019). Another study reported that Cu and Zn showed fast uptake and elimination in *Enchytraeus crypticus* with a steady state being reached within 2 day, while Cd and Pb showed a slow linear accumulation and excretion (Zhang et al., 2022). Differently, Zn and As showed similar uptake-elimination patterns in the earthworm *Eisenia Andrei* (Gonzalez-Alcaraz and van Gestel, 2016). These contrasting findings highlight that more research is warranted to concern the dynamic nature of shifts in the uptake of non-essential (Cd and As) and essential (Zn) elements by terrestrial invertebrates and their potential to eliminate these accumulated metal (loid)s over time.

There is still a knowledge gap when it comes to understanding the mechanisms of metal(loid) accumulation in soil organisms. Li et al. (2009) reported that soil organisms immediately sequestered the internalized metals over various subcellular compartments after their entrance into the body. Some compartments have a high affinity for metals, resulting in linear accumulation patterns by excreting them slowly or even not at all, whereas others rapidly release the metals, resulting in an accumulation curve that reaches equilibrium (Vijver et al., 2004). It is likely that different accumulation patterns may lie in these compartments with various types of binding sites. As such, the internal compartmentalization of a metal(loid) in different subcellular fractions within a soil organism allows for a better understanding of its bioaccumulation and potential toxicity instead of the total body load (Vijver et al., 2004; Wallace et al., 2003). Subcellular fractionation is a process that separates the total body concentration of a metal into operationally defined subcellular fractions following a series of differential centrifugations (Wallace et al., 2003). This procedure was used in soil invertebrates to isolate at least three compartments: cellular cytosol (CC), cellular debris (CD), and metal-rich granules (MRGs) (Vijver et al., 2007). Several studies attempted to identify the internal compartmentalization between non-essential and essential metals in different subcellular fractions for terrestrial invertebrates, but no particular pattern was yet shown fully generic. It has been shown that non-essential and essential elements differed in their behavior in the case of detoxification and subcellular compartmentalization for the earthworm *Aporrectodea caliginosa*, in which Zn was mainly found in the CD and its partitioning remained unchanged along the gradient of metal pollution, whereas the portion of CC accounted for most of Cd body burden and its partitioning was modified (Beaumelle et al., 2015). Differently, Wang et al. (2018) found that subcellular partitioning of Cd in earthworm *Metaphire californica* showed the same pattern as Cu with the highest portion in the CC, while the concentration of Pb and Zn were relatively high in the MRGs, in which subcellular distribution of the metal increased with the concentration of the metal in soil. To better understand the subcellular fates of metals and assess their potential biological consequences of accumulated metals, a linkage of the toxicokinetic process with internal metal partitioning should be taken into account. Metal bioaccumulation strategies and their subcellular distribution inside the body are responsible for the uptake and elimination kinetics of metal. Considering the kinetics and dynamics of these processes may gain more insight into bioavailability of metal to soil organism. In this way, such a combination approach is capable of examining metal bioaccumulation and corresponding coping mechanisms in species, allowing for fraction-specific parameter estimations and comparisons in the model. However, these fraction-specific uptake and elimination kinetic data are currently far from sufficient, especially in terrestrial systems (Gimbert et al., 2008; Li et al., 2009; Le et al. 2021, 2022).

In this context, this study investigated the bioaccumulation kinetics of Cd, As, and Zn along a soil concentration gradient in an earthworm (*Eisenia fetida*), at both the organism level and the subcellular level. *E. fetida* was selected as it not only serves as an important component of the soil community and as a good indicator of soil health, but this worm species has also been widely used to estimate bioaccumulation and toxicity of metals (Xiao et al., 2022). We aimed (1) to delineate and examine whether there is a difference in the toxicokinetics of the

non-essential metal(loid)s (Cd and As) and the essential metal (Zn) in *Eisenia fetida* over a wide range of soil metal(loid) concentrations, and (2) to offer first-hand data on the fraction-specific (CC, CD, and MRGs) uptake and elimination of Cd, As, and Zn in the test earthworm after exposure to natural soils originating from a gradient of metal(loid) pollution. The results presented herein will facilitate our understanding of the dynamic accumulation processes and subcellular handing strategies of non-essential (Cd and As) and essential (Zn) elements in earthworms, and ultimately contribute to an accurate assessment of their risk in soils.

2. Materials and methods

2.1. Soil preparation

The natural paddy soil used in the experiment was sampled from Changshu, Jiangsu Province, China. The top ~20 cm layer soil was collected manually and transferred to the laboratory. After being air dried, the soil was sieved through 2 mm mesh, homogenized, and stored at room temperature for further use. Soil physicochemical characteristics (e.g. pH, texture, soil organic carbon) were measured following routine analytical methods. More detailed information is provided in Table S1. The dry soil (~500 g) was spiked with different amounts of $CdCl_2$ (purity >99.95%; Aladdin, Shanghai, China), Na_2HAsO_4 (purity >98%; Aladdin, Shanghai, China), or $ZnCl_2$ (purity >98%; Hushi, Shanghai, China) to obtain a series of soil treatments with low, middle, and high nominal concentrations of Cd (15, 60, and 540 mg/kg dry soil), As (25, 75, and 550 mg/kg dry soil), or Zn (400, 700, and 1000 mg/kg dry soil), respectively. These test concentrations of Cd, As, and Zn were selected according to our preliminary experiment to guarantee the survival rate of the test organism. Besides, they were also within the concentration range found in actual soils (Komnitsas and Modis, 2006; Liao et al., 2005; Pajak and Jasik, 2011). The spiked soil was thoroughly mixed and moisturized by deionized water to reach approximately 60% of the maximum water holding capacity (WHC), and then was equilibrated at 20°C in the dark for a week before use in the tests. Each treatment had three replicates.

2.2. Toxicokinetics experiment

Earthworms *E. fetida* were obtained from a breeding base in Shijiazhuang, Hebei Province, China. Adult earthworms were firstly acclimated to lab conditions for two weeks in natural paddy soil at 25 ± 2°C in complete darkness prior to the experiment.

The uptake and elimination kinetics in *E. fetida* exposed to the different concentrations of Cd, As, and Zn were performed following OECD guideline 317 (OECD, 2010). Controls with non-spiked soil were also included. Specifically, 30 adult earthworms (weight: 390 ± 80 mg) of similar size were put into each black plastic pot containing 500 g spiked soil for 14 days (uptake phase), and then the viable and healthy adults were transferred to non-spiked soils for another 14 days (elimination phase). The earthworms in both phases were not fed to prevent the introduction of new contaminants (Li et al., 2009). Three replicates per treatment/sampling time were used. The tests were run in a growth chamber at 20 ± 2°C and at 80% relative humidity under a light regime of 16/8 h light/dark cycle (~600 lux light intensity). During the experiment, the water content (on the basis of weight loss) was replenished weekly. Two earthworms were sampled from each pot at days 0, 1, 2, 4, 7, 10, and 14 of the uptake phase and at days 15, 16, 18, 21, 24, and 28 of the elimination phase, respectively. After sampling, the animals were carefully rinsed with deionized water, dried with filter paper, weighted, and transferred to plastic petri dishes (100 mm × 15 mm) with wet filter paper for 24 h in the dark for gut cleaning. Subsequently, the collected earthworms were frozen by liquid nitrogen and stored at −80°C for total internal and subcellular metal analysis.

2.3. Metal analysis and subcellular partitioning

Total internal concentrations of Cd, As, and Zn were determined on one randomly sampled earthworm. Earthworms were freeze-dried for 48 h, weighed, and stored at −20°C before being digested. Subcellular fractionation was determined on the rest of the collected earthworms of each replicate per treatment. The procedure has been described in detail elsewhere (Beaumelle et al., 2015). Briefly, frozen earthworms were individually weighted and homogenized with a homogenizer in 5 mL Tris-HCl buffer (0.01 M, pH 8.0, ice-cold). As a next step, homogenates were centrifuged at 10,000 *g* for 30 min at 5°C and the supernatant obtained was the cytosolic fraction. The pallet fraction was heated in a water bath at 100°C for 2 min and was hydrolyzed at 70°C for 1 h with 1.5 mL NaOH of 1 M (Merk, Germany). This supernatant contains the cell debris fraction (tissue fragments, cell membranes, and intact cells) and was separated from the pellets (Metals-rich granules, MRGs) after centrifugation at 100,000 *g* for 10 min at 20°C. The whole body and each fraction samples were digested using concentrated nitric acid at 90 °C for 2 h in a graphite digestion instrument (Digi PREP MS, SCP SCIENCE) to measure the total internal and subcellular partitioning of the metal (loid)s in earthworms. Soil samples were air dried, ground, sieved (<0.149 mm), and microwave-digested (Analytic Jena, Germany) in a mixture of HNO_3, HF, and H_2O_2 (3:3:2 v/v/v) at 180°C for 40 min to measure total concentrations of metal(loid)s in soil. Inductively coupled plasma-optical emission spectrometry (ICP-OES; Thermo Fisher Scientific, Germany) was used to analyze total concentrations of metal(loid)s in soils and earthworms and subcellular partitioning of metal(loid)s in earthworms. For the quality assessment, the certified reference material ERM CE278 (mussel tissue, European Commission, Institute for Reference Materials and Measurements) was used in case of earthworms. The elements recoveries in this reference material compared to the certified values were 95.9–102.5%. In case of soil samples, ISE 989 (river clay, International Soil-Analytical Exchange) certified reference material was used, and the measured concentrations were always within 10% of the certified values. Blanks were also included in all analyses.

2.4. Modeling and statistics

Assuming constant exposure concentrations, the development of internal concentrations and subcellular fractions of Cd, As, and Zn in the earthworms over time can be described by a one-compartment model (Crommentuijn et al., 1997), fitting the following equations simultaneously to all data from the uptake (**Equation (1)**) and elimination phases (**Equation (2)**):

$$C(t) = C_0 \times e^{-k_e \times t} + \left(\frac{k_u}{k_e}\right) \times C_{exp\ 1} \times \left(1 - e^{-k_e \times t}\right) (t \le t_n) \quad (1)$$

$$C(t) = C_0 \times e^{-k_e \times t} + \left(\frac{k_u}{k_e}\right) \times C_{exp\ 1} \times \left[F_i + (1\text{–}F_i) \times \left(e^{-k_e \times (t - t_n)} - e^{-k_e \times t}\right)\right] + \left(\frac{k_u}{k_e}\right) \times C_{exp\ 2}$$

$$\times\left[1-\left(F_i+(1-F_i)\times e^{-k_e\times(t-t_n)}\right)\right](t>t_n) \quad (2)$$

where $C(t)$ is the internal or subcellular fraction concentration of the metal(loid)s in the earthworms at time t (mg/kg dry body weight), C_0 the initial internal concentration or the concentration in the subcellular fraction of the studied metal(loid) in the worms at time $t = 0$ (mg/kg dry body weight), k_u the uptake rate constant (kg_{soil}/kg_{worm}/day), k_e the elimination rate constant (day^{-1}), C_{exp1} the actual exposure concentration of the spiked soil (mg/kg dry soil), C_{exp2} the exposure concentration of the clean soil (mg/kg dry soil), t the exposure time (days), t_n the day when earthworms were transferred from spiked soil to clean soil (i.e. 14), and F_i the inert metal(loid) fraction in the earthworm (ranging from 0 to 1).

Models were fitted using total internal and subcellular partitioning of Cd, As, and Zn, respectively. All kinetics parameters were estimated by non-linear regression using JMP 16.0 (SAS institute) based on experimental data.

3. Results

3.1. *E. fetida* performance upon individual Cd, As, and Zn exposure

The properties of the Changshu paddy soil are presented in Table S1. Soil pH measured in water was near neutral (6.31). Soil had a relatively high organic carbon content of 24.9 g/kg. The cation exchange capacity was 10.3 $cmol_c$/kg, while the total nitrogen content was 1.81 g/kg. The background concentrations of As, Cu, and Zn in the soil were 0.34 ± 0.07, 17.9 ± 1.8, and 123 ± 2 mg/kg, respectively (Table S2). The actual concentrations of the metal(loid)s in the low, middle, and high polluted soils were measured to be 17.2 ± 8.4, 61.7 ± 7.0, and 531 ± 7 mg/kg for Cd, 28.5 ± 2.9, 74.3 ± 1.5, and 547 ± 5 mg/kg for As, and 429 ± 39, 664 ± 25, and 953 ± 18 mg/kg for Zn, respectively, which closely agrees with the nominal concentrations (Table S2). During the 28 days of the experiment, the fresh weight of unexposed earthworms ranged from 0.37 ± 0.12 to 0.56 ± 0.17 mg; for Cd-treated earthworms from 0.37 ± 0.11 to 0.62 ± 0.20 mg; for As-treated worms from 0.31 ± 0.04 to 0.64 ± 0.10 mg; and for Zn-treated earthworms from 0.31 ± 0.12 to 0.63 ± 0.26 mg, respectively (Table S3). During the experiment, less than 3 earthworms died in each treatment and mortality was random. At the end of the exposure, the overall survival rate of the treated earthworms was >90% (Table S4), meeting the requirement of validity in the OECD guidelines (OECD, 2016).

3.2. Toxicokinetics based on total internal concentration of metal(loid)s

The internal concentrations of the non-essential metal(loid)s (Cd and As) and the essential metal Zn in *E. fetida* after exposure to different external concentrations of the corresponding metal(loid) in soil were measured during 14 days of uptake followed by a 14 days elimination phase, respectively (Fig. 1). The initial Cd, As, and Zn concentrations in the earthworms were measured to be 0.78, 5.75, and 130 mg/kg dry

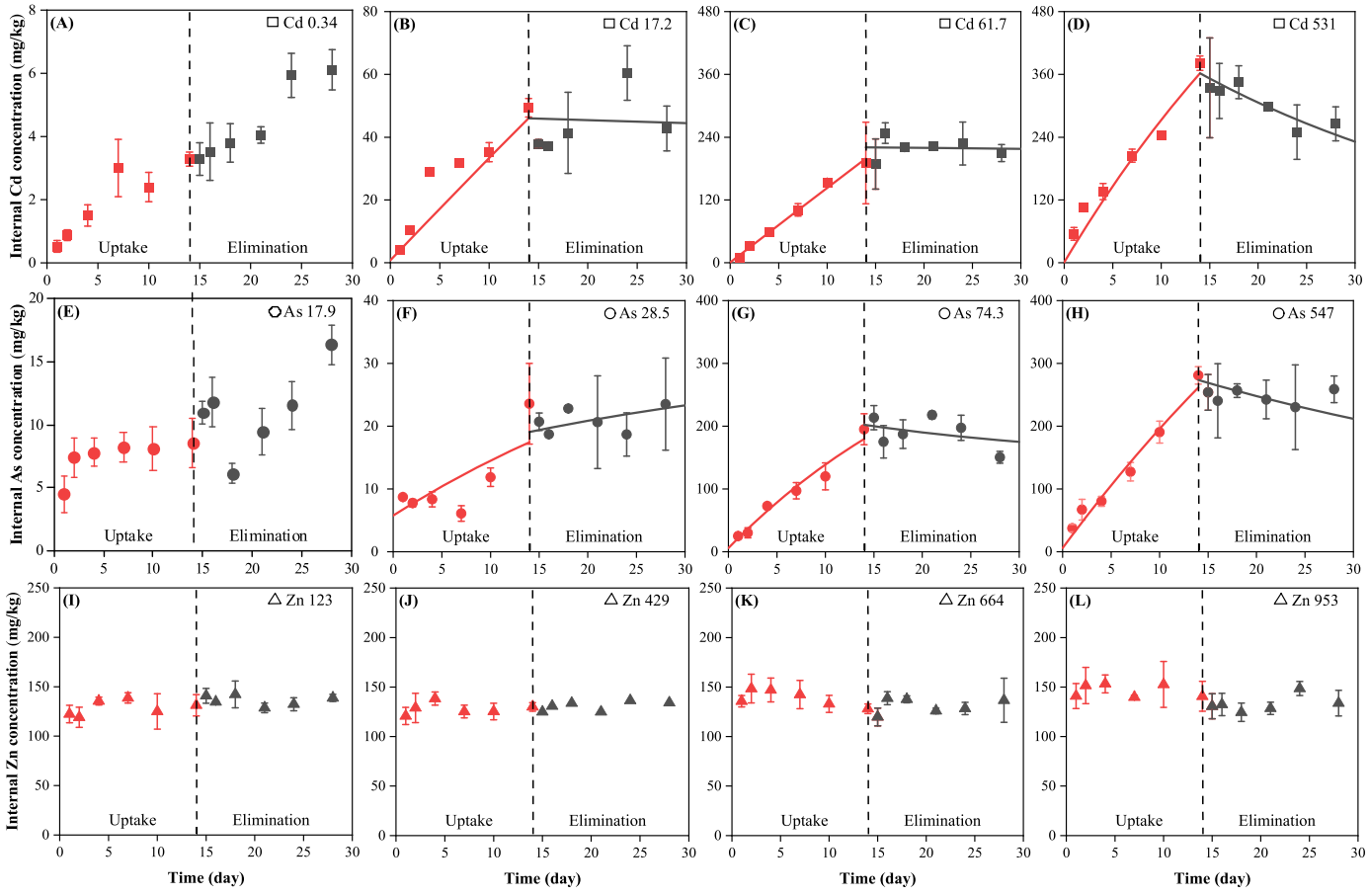

Fig. 1. The internal Cd, As, and Zn concentrations measured in *Eisenia fetida* exposed to Changshu paddy soil amended with different amounts of individual metals during a 14 days uptake phase followed by a 14 days elimination phase, respectively. Each data point represents the mean of 3 replicates with standard errors at each time point (red dots: the uptake phase, grey dots: the elimination phase). Kinetic curves were estimated using a one-compartment model (red line: the uptake phase (**Equation (1)**, grey line: the elimination phase (**Equation (2)**). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

weight, respectively. At each exposure concentration, Cd and As accumulations in *E. fetida* increased with time and did not reach equilibrium during the uptake phase. The Cd and As body concentrations by the end of the uptake phase were 3.36–362 mg/kg and 9.45–262 mg/kg, respectively, as depending on the external exposure concentration. After 14 days of elimination, the final Cd and As concentrations in the animals were way above their initial levels for each exposure concentration. The highest body concentrations in the earthworm reached 6.11, 60.4, 248, and 381 mg/kg for Cd and 16.4, 23.6, 218, and 281 mg/kg for As across the control, low, middle, and high exposure groups, respectively. Unlike Cd and As, body Zn concentrations in earthworms remained at a relatively constant level (internal concentration ranging from 119 to 153 mg/kg) throughout the whole experiment, irrespective of the external exposure concentration and exposure time.

Further, the one-compartment model was used to estimate kinetics parameters of Cd and As in the animals based on actual total soil concentrations of the metal(loid), respectively (Table 1). At each exposure concentration, this model explained 76–97% and 80–96% of the total variation in body concentrations of Cd and As in the animals, respectively. When all the data of individual metal(loid) were fitted together, in total respectively 69% and 63% of the variance in Cd and As accumulation was explained by the one-compartment model. Obviously, this model described the uptake and elimination of Cd and As well. In case of Zn, this model explained about less than 20% of the variation in body Zn concentrations in *E. fetida* and the estimated kinetics parameters were in most cases close to zero (Table S5). As a consequence, kinetics curves were not shown for Zn. For different exposure concentrations, the uptake rate constant (k_u) and the elimination rate constant (k_e) of Cd and As were estimated to be 0.059–0.232 and 0.039–0.217 kg_{soil}/kg_{worm}/day, and 0.001–0.028 and 0.020–0.037 day^{-1}, respectively. The lowest k_u of Cd and As were observed at the highest exposure concentrations. The inert fraction (F_i) of Cd and As were estimated to be 0–0.002 and 0.015–0.088, respectively. On the basis of all the data from each metal (loid), overall the values of k_u, k_e, and F_i of Cd and As were estimated to be 0.086 and 0.036 kg_{soil}/kg_{worm}/day, 0.100 and 0.003 day^{-1}, and 0.499 and 0, respectively. The exposure concentration and the essentiality of the metal(loid)s of concern were therefore responsible for the differences observed in uptake and elimination kinetics within *E. fetida*.

3.3. Toxicokinetics based on subcellular partitioning of metal(loid)s

The relative distribution of Cd, As, and Zn over the three subcellular fractions-including cellular cytosol (CC), cellular debris (CD), and metal-rich granules (MRGs) in *Eisenia fetida* during the exposure to different metal(loid) concentrations in soils is depicted in Fig. 2. The percentages reported were obtained by relating the metal(loid) concentration retrieved from each subcellular fraction to the total level of metal(loid) in the earthworms at each exposure time. In non-exposed earthworms, the subcellular partitioning with time showed inconsistent patterns where the proportion of accumulation in CC, CD, and MRGs were 55.4–86.8%, 8.7–34.8%, and 2.5–9.8% for Cd, 27.3–63.6%, 14.3–51.8%, and 7.24–50.0% for As, and 64.9–72.0%, 21.3–27.9%, and 5.30–10.7% for Zn, respectively. In exposed earthworms, the concentration of Cd and As in subcellular fractions showed the same pattern as Zn in the order of CC > CD > MRGs, irrespective of the exposure time and concentration. To be specific, the distribution of Cd with time in the CC ranged from 76.1 to 91.2%, while the distribution of CD was 6.52–20.5% and that of the MRGs was 0.87–5.3%. The proportion of As ranged from 45.4 to 81.9% in the CC, 15.8–39.6% in the CD and 2.17–19.6% in the MRGs. For Zn, its proportion accounted for 56.3–72.4% in the CC, followed by CD (23.1–34.1%) and MRGs (0.60–18.2%).

In addition, the accumulation patterns of Cd, As, and Zn in the three subcellular fractions (CC, CD, and MRGs) of the earthworms are shown in Fig. 3. Generally, the Cd and As contents in subcellular fractions increased with total soil concentrations of Cd and As. Differently, no relationship between Zn concentrations in any of the subcellular fractions and total Zn levels in soils was found. At each exposure concentration, uptake of non-essential metal(loid)s (Cd and As) and no uptake of essential metal (Zn) by *E. fetida* were seen in different subcellular fractions, which is agreement with the observations based on total internal concentrations in earthworms. The one-compartment model was subsequently fitted to exposure data to obtain the estimated k_u and k_e of Cd and As in each subcellular fraction, and this model explained 43–96% and 39–97% of the total variation in subcellular fractions of Cd and As, respectively (Table 2). From the model fit, the values of k_u and k_e of Cd for CC, CD, and MRGs were estimated to be 0.0056–0.0349, 0.0008–0.0051, and 0.0001–0.0008 kg_{soil}/kg_{worm}/day and 0.0078–0.0195, 0.0107–0.0420, and 0.0276–0.0523 day^{-1}, respectively. For As, the k_u and k_e for CC, CD, and MRGs were estimated to be 0.0048–0.0182, 0.0010–0.0063, and 0.0001–0.0046 kg_{soil}/kg_{worm}/day and 0.0005–0.0385, 0.0004–0.0119, and 0.0044–0.1527 day^{-1}, respectively. For Zn, this model only explained 0.1–29% of the total variance in the subcellular fractions of Zn in earthworms and most of the kinetics parameters were estimated to be around zero (Table S6). Kinetics curves were therefore not shown for these data.

Table 1
Toxicokinetics parameters related to total concentrations of Cd and As: uptake rate constant (k_u), elimination rate constant (k_e), inert fraction (F_i) in *Eisenia fetida* exposed to different metal concentrations in Changshu paddy soil (C_w) for a 14 days uptake phase followed by a 14 days elimination phase, respectively. The coefficient of determination (R^2) describes the variance in internal metal concentrations explained by the one-compartment model (**Equations (1) and (2)**).

Metal	C_w	k_u	k_e	F_i	R^2
	(mg/kg dry soil)	(kg_{soil}/kg_{worm}/day)	(day^{-1})	–	–
Cd	17.2	0.193	0.004	0.000	0.76
	61.7	0.232	0.001	0.002	0.97
	531	0.059	0.028	0.000	0.97
	Overall	0.086	0.100	0.499	0.69
As	28.5	0.040	0.025	0.050	0.80
	74.3	0.217	0.037	0.088	0.94
	547	0.039	0.020	0.015	0.96
	Overall	0.036	0.003	0.000	0.63

4. Discussion

4.1. Uptake and elimination kinetics related to internal metal(loid) concentrations in E. fetida

The present study showed clearly distinct toxicokinetic patterns for non-essential metal(loid)s (Cd and As) and essential metal (Zn) in *E. fetida*. Uptake of Cd/As by *E. fetida* and efficient regulation of Zn in the body were observed. This is in agreement with the findings of Giska et al. (2014) who reported that non-essential metals (Cd and Pb) were intensively accumulated by the earthworm *L. rubellus* while essential metals (Zn and Cu) were maintained at a stable level in their body. This might be explained by biological variation in the worms within the processes of uptake, metabolism, and elimination related to non-essential and essential metal(loid)s handling (Spurgeon et al., 2011). In general, for essential elements, an internal pool is needed to meet the metabolic requirements in organisms. The internal concentrations of essential metals in organisms are often regulated at a constant level, where uptake balances elimination (Ardestani et al., 2014). Our findings showed that the earthworms remained a steady-state concentration of Zn in their body without a clear intake behavior (Fig. 1). This could be explained by detoxification of Zn in the body via the dynamic trade-off between uptake and elimination (Spurgeon and Hopkin, 1999). Regulation the internal body concentration of the essential metal Zn by earthworms (*E. andrei* and *E. fetida*) exposed to artificial soils (with Zn of 56–1000 mg/kg) and field soils has been reported also by van Gestel

Fig. 2. Relative distribution of Cd, As, and Zn in different subcellular fractions: cellular cytosol (CC), cellular debris (CD), and metal-rich granules (MRGs), of the earthworm *Eisenia fetida* exposed to Changshu paddy soil amended with different amounts of individual metal at intervals over time for a 14 days uptake phase followed by a 14 days elimination phase, respectively. Values display the mean of 3 replicates at each time point.

et al. (1993) and Lock and Janssen (2001). The efficient regulation of Zn in soil organisms can be explained by actively increasing k_u and/or by decreasing k_e (Hobbelen et al., 2006; Lock and Janssen, 2001; Veltman et al., 2007). González-Alcaraz et al. (2018) reported that body Zn concentrations increased rapidly reaching a steady state (~240–420 μg/g) during the uptake phase and rapidly decreased to initial levels (~110–140 μg/g) during the eliminate phase, which differs from our results.

Body Cd and As concentrations continuously increased during the uptake phase and no equilibrium was reached. The eliminations were slow and residual concentrations remained high after the elimination phase (Fig. 1). This rapid increase in body concentrations in the earthworms indicated that Cd and As were not regulated by the animals. Also in other studies, internal Cd concentration in enchytraeids, springtails, and earthworms increased linearly with exposure time without reaching a clear steady state after 14–28 days exposure (Cedergreen et al., 2013; Giska et al., 2014; Santos et al., 2021; Vijver et al., 2001; Zhang et al., 2022). Lee and Kim (2013) also reported that the As body burden of *E. fetida* increased with increasing exposure concentration within a 28-day exposure period and did not reach steady-state in most of the treatments, whereas no elimination was found after another 28 days of exposure. Such continuous uptake pattern was also found in Pb accumulated by soil invertebrates, where equilibrium was approached requiring 7 day in enchytraeids (Zhang and Van Gestel, 2017) and 42 day in earthworms (Spurgeon et al., 2011). As such, exposure time could be a crucial factor affecting the internal concentrations of contaminants in soil animals. Thus, longer exposure periods might be required for assessing the risk of slowly accumulating non-essential metal(loid)s. Unlike our results, Kilpi-Koski et al. (2019) found that no uptake of As was observed in *E. andrei* upon exposure to the low (10.1 ± 5.5 mg As/kg) contaminated field soil, which may be due to fact that the total As concentration in low contaminated soil were lower than the lowest concentration of As used in our study (28.5 ± 2.9 mg As/kg). Very slow uptake and elimination were seen in the medium and high (850–2810 mg As/kg) contaminated field soils where steady-state was not reached by the end of the 21-day exposure, which are consistent with our findings. The accumulation of As could be explained by sequestration of As in worm tissues in the form of As-thiol complexes, leading to lack of elimination (Morgan et al., 1994). When the exposure concentration of non-essential elements is low, earthworms might have the ability to limit their accumulation via the dynamic trade-off between uptake and elimination with no uptake pattern being seen. With the increase of exposure concentration, an increase of internal body concentrations will occur, as the regulation ability of organisms to excrete non-essential elements is limited (Cedergreen et al., 2017). For non-essential elements, organisms have a different strategy either by excreting them from the excess pool or storing them in a non-toxic form in order to survive (Ardestani et al., 2014).

In this study, the k_u of Cd ranged between 0.059 and 0.232 kg_{soil}/kg_{worm}/day. These values fell within the reported range (0.022–4.92 kg_{soil}/kg_{worm}/day) in *E. fetida* exposed to field-contaminated soils by Nahmani et al. (2009). In comparison to a previous study yielding Cd uptake rate constants for *L. rubellus* in natural polluted soils (0.032–0.069 kg_{soil}/kg_{worm}/day), these values were relatively high (Giska et al., 2014). The k_u of As ranged between 0.039 and 0.217 kg_{soil}/kg_{worm}/day, which was higher than the reported values for *E. andrei* exposed to different contaminated Hartola field soils (0.011 and 0.0065 kg_{soil}/kg_{worm}/day) (Kilpi-Koski et al., 2019) and different Dutch field soils (0.0046 kg_{soil}/kg_{worm}/day) (Peijnenburg et al., 1999). The uptake of metal(loid)s in soil invertebrates seems dependent both on the

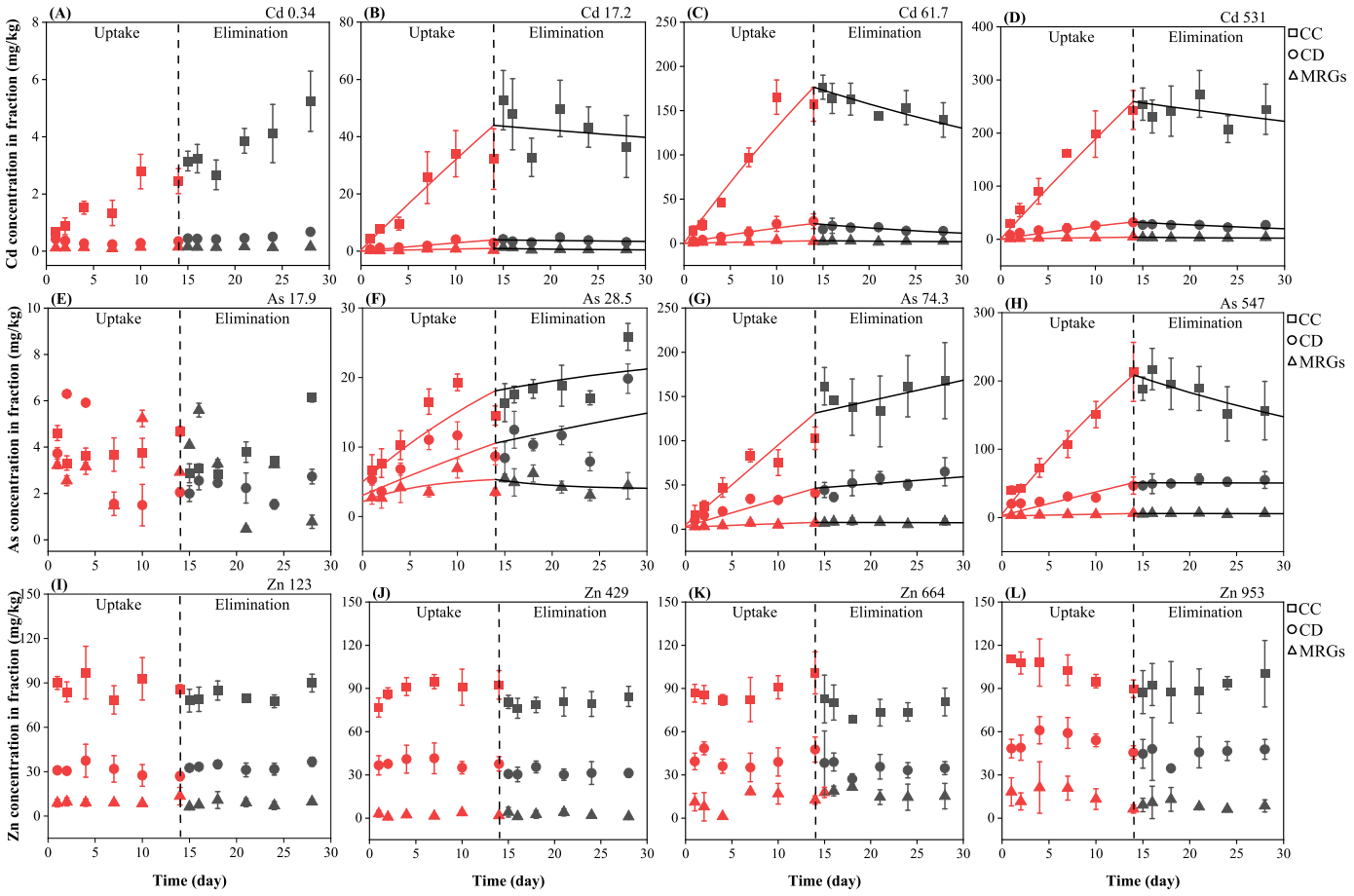

Fig. 3. Subcellular distribution of Cd, As, and Zn in *Eisenia fetida* exposed to Changshu paddy soil amended with different amounts of individual metal at intervals over time for a 14 days uptake phase followed by a 14 days elimination phase, respectively. CC (□) = cellular cytosol, CD (○) = cellular debris, and MRGs (△) = metal-rich granules. Each data point represents the mean of 3 replicates with standard errors at each time point (red dots: the uptake phase, grey dots: the elimination phase). Kinetic curves were estimated using a one-compartment model (red line: the uptake phase (**Equation (1)**, grey line: the elimination phase (**Equation (2)**). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 2
Toxicokinetics parameters related to subcellular partitioning of Cd and As: uptake rate constant (k_u) and elimination rate constant (k_e) in *Eisenia fetida* exposed to different metal concentrations of Cd and As in Changshu paddy soil (C_w) for a 14 days uptake phase followed by a 14 days elimination phase, respectively. The coefficient of determination (R^2) describes the variance in different subcellular fractions explained by the one-compartment model (**Equations (1) and (2)**).

Metal	C_w (mg/kg dry soil)	Cellular cytosol			Cellular debris			Metal-rich granules		
		k_u	k_e	R^2	k_u	k_e	R^2	k_u	k_e	R^2
		(kg_{soil}/kg_{worm}/day)	(day^{-1})	–	(kg_{soil}/kg_{worm}/day)	(day^{-1})	–	(kg_{soil}/kg_{worm}/day)	(day^{-1})	–
Cd	17.2	0.0285	0.0078	0.85	0.0026	0.0107	0.76	0.0008	0.0523	0.43
	61.7	0.0349	0.0195	0.95	0.0051	0.0420	0.87	0.0006	0.0276	0.50
	531	0.0056	0.0097	0.96	0.0008	0.0305	0.93	0.0001	0.0414	0.55
As	28.5	0.0073	0.0385	0.74	0.0032	0.0119	0.49	0.0046	0.1527	0.39
	74.3	0.0182	0.0005	0.92	0.0063	0.0004	0.88	0.0010	0.0211	0.72
	547	0.0048	0.0253	0.97	0.0010	0.0041	0.89	0.0001	0.0044	0.77

test organism and the soil properties (Crommentuijn et al., 1997). The behavior and physiology of the organism have a great influence on bioaccumulation of metal(loid)s as each animal own a specific strategy to prevent damage (Rainbow, 2002). Soil properties such as soil pH, cation exchange capacity, organic matter, and clay content are important factors affecting metal availability and as a consequence bioaccumulation kinetics of metals in soil organisms (Nahmani et al., 2009; Van Gestel and Mol, 2003). With increasing exposure concentrations, the k_u of Cd and As increased first and then decreased. This trend was also found for other non-essential metals (e.g. Pb, Ni, and Ag) accumulated by *E. crypticus* (He and van Gestel, 2013; Topuz and van Gestel, 2015; Zhang and Van Gestel, 2017). Such decline of k_u might be due to the limited availability of metal(loid) transporters for passing through membranes at high exposure concentrations (Li et al., 2009). No clear relationship was therefore determined between the uptake rate constant of metal(loid) and total metal(loid) concentration in soil. This is consistent with the finding of previous studies (Peijnenburg et al., 1999; Zhang and Van Gestel, 2017).

The analysis of elimination patterns offers useful clues for the possible detoxification mechanisms in the animals. The slow elimination of Cd and As in *E. fetida* was observed in our study with the values of k_e ranging from 0.001 to 0.028 day^{-1} for Cd and from 0.020 to 0.037 day^{-1}

for As, respectively. These slow elimination kinetics are supported by Zhang et al. (2022) who reported that the estimated k_e for Cd in *E. crypticus* exposed to metal-contaminated field soils ranged from 0.010 to 0.023 and Kilpi-Koski et al. (2019) who found that the k_e for As in *E. andrei* exposed to contaminated Hartola field soils were 0.0062 and 0.012 day^{-1}, respectively. Slow or no elimination of Cd and As in earthworms was verified to be a typical response pattern of earthworms to exposure to non-essential metals (Giska et al., 2014; González-Alcaraz et al., 2018; Lee and Kim, 2013). The low value of k_e of Cd and As in *E. fetida* found in our study indicates that their main detoxification pathway might be sequestration within inorganic matrices or binding to organic ligands rather than excretion (Spurgeon and Hopkin, 1999). Additionally, the low k_e also implied that the earthworms failed to reach equilibrium with regard to body Cd and As concentrations during the uptake phase. The overall inert fraction F_i of Cd was estimated to be 0.499, demonstrating the presence of a storage detoxification system for Cd in *E. fetida*. This value is lower than the value of F_i of Pb reported for *E. fetida* (~0.7) by Zhang et al. (2015) and higher than the one for *E. crypticus* (0–0.2) by Zhang and Van Gestel (2017). It should be noted that the estimated value of F_i were not reliable as the internal concentration of metal(loid)s did not reach equilibrium during the elimination phase, which agrees with the case reported by Zhang et al. (2022).

4.2. Uptake and elimination kinetics related to subcellular partitioning of metal(loid)s in E. fetida

In the procedure that we used, the earthworm was separated into CC (mainly containing microsomes, metallothioneins (MT), heat-stable proteins (HSP), and denatured proteins), CD (cell membrane and tissues), and MRGs (insoluble fractions). A clear exposure concentration-dependent increase of the internal concentrations of the non-essential metal(loid)s (Cd and As) and a relatively constant concentration of essential metal (Zn) in the whole earthworm body or in its subcellular fractions were observed upon different exposure concentrations throughout the experiment. This agrees with the results reported by Beaumelle et al. (2015). In non-spiked soils, the relative distribution of As in different subcellular fraction fluctuated with time. The binding of As among the different subcellular fractions might be unbiased in non-exposed earthworms (Vijver et al., 2006). In spiked soils, the three elements showed similar distribution patterns in the earthworms with the highest amount of Cd, As, and Zn accumulated in the CC, followed by the CD and MRGs per exposure concentration. Our results are consistent with previous findings that Cd was mainly retrieved from the CC (Beaumelle et al., 2015; Li et al., 2008). The fact that Cd is tightly bound by MT in the CC as toxicologically unavailable form has been widely reported in aquatic (Stürzenbaum et al., 2001) and terrestrial organisms (Xiao et al., 2022). Similarly, As is also sequestered due to formation of As-MT complexes which result in the accumulation of Cd in the CC (Langdon et al., 2005). Wang et al. (2018) found that Cd in the earthworm *Metaphire californica* decreased in the order of CC (54.9%) > CD (31.6%) > MRGs (13.5%) while Zn in subcellular fractions ranked as MRGs (42.0%) > CD (36.0%) > CC (22.0%), which is not entirely consistent with our results. Andre et al. (2009) indicated that Zn preferred to accumulate in the MRGs in the non-soluble form and this was a crucial way for *L. rubellus* to detoxify metals. Vijver et al. (2006) found that Zn was more evenly distributed among the three fractions within earthworms. Differing element distributions may be explained by the species, exposure concentration, and the duration of exposure (Bednarska and Swiatek, 2016).

Generally, the metal could be taken up by the organism. This does however not necessarily mean that metal uptake will bring hazards, as metals can be detoxified and sequestered into subcellular compartments, even when the metals are accumulated in the organisms at high levels (Vijver et al., 2004). Hence, metal(loid)s partitioning in different subcellular fractions within the animals can provide valuable information for the assessment of the accumulation capability of metal(loid)s in organisms dwelling in highly contaminated areas (Beaumelle et al. 2015, 2017). The results showed that the CC accounted for most of the internal concentrations of Cd, As, and Zn, followed by CD and MRGs, regardless of the gradient of metal(loid) exposure. It is thus likely that the three elements are commonly accumulated by the earthworms in the form of ions, leading to high concentrations in the CC. The microsomes, MT, HSP, and other substances in the CC were found to be more sensitive to combinations of metals, such as MT binding with Cd which causes an increased accumulation rate of Cd (Lévêque et al., 2015; Liang et al., 2011). In this study, the estimated k_u and k_e of Cd for CC were 0.0056–0.0349 $kg_{soil}/kg_{worm}/day$ and 0.0078–0.0195 day^{-1}, respectively. Our results were lower than the values reported by Li et al. (2009) for *E. fetida* (0.13 $kg_{soil}/kg_{worm}/day$ and 0.051 day^{-1}). This is probably due to the soil used in this study with a much higher concentration of Cd (17.2–531 mg/kg) than the reported one (1.16 mg/kg). It is therefore not surprising to see an uptake pattern that is shaped by the low elimination from this compartment.

Compounds present in the CD have a high lipophilicity and are capable of efficiently binding with hydrophobic chemicals (Yu and Lanno, 2010). The MRGs includes organelles with physiological functions, and possesses low levels of metals as a consequence of detoxification processes. The values of k_e of Cd from the CD and MRGs fractions were 0.0107–0.0420 day^{-1} and 0.0276–0.0523 day^{-1}, respectively. The higher value of k_e as compared to the case of CC implies the presence of easily removable Cd forms in the CD and MRGs. This agrees with the findings of Li et al. (2009) and Conder et al. (2002). Similar to Cd, the low estimated value of k_e of As from each subcellular fraction was responsible for the observed uptake pattern of As in the CC, CD and MRGs. The presence of Cd and As in the MRGs would be expected to be related to biological impairment as granule has various membrane-bound enzymes for protein synthesis and transport (Vijver et al., 2004). The extremely low concentration of non-essential metal (loid)s in this compartment might be the reason for the observed lack of poisoning symptoms in earthworms throughout the experiment. Andre et al. (2009) reported that metals could be absorbed or stored in MRGs by binding to proteins or other ligands without biological activity. The granules and heat shock proteins own the capacity to biologically detoxify metals (Wallace and Lopez, 1996), and the microsomal and denatured proteins in the CC are considered to be sensitive to metals (Wallace et al., 2003). Another study showed that the MRGs and CC might be responsible for immobilization of metals and the CD appeared to be a pool of metabolic detoxification for metals (Vijver et al., 2006). All these fraction ascriptions relied however only on uptake and elimination kinetics rather than on toxicological responses (Huang et al., 2009). In view of the high percentages of Cd, As, and Zn in the CC, this fraction was considered as a main pool for their sequestration in this study. However, more research is in demand to examine which fraction is associated with the occurrence of toxic effects.

5. Conclusions

Our results showed clearly distinct toxicokinetic patterns for non-essential metal(loid)s (Cd and As) and essential metal (Zn) in *E. fetida*. As for the non-essential metal(loid)s, an obvious concentration-dependent increase of was observed in earthworms or subcellular compartments where no equilibrium was reached due to slow or even complete lack of elimination throughout the experiment. Differently, the essential element Zn was efficiently regulated by *E. fetida* with a stable level in body or in each fraction at different exposure levels. This could be explained by detoxification of Zn in the body via the dynamic trade-off between uptake and elimination. Interestingly, non-essential and essential metal(loid)s showed similar distribution patterns regardless of the external exposure concentration. Cellular cytosol (CC) accounted for most of the internal concentrations of Cd, As, and Zn, followed by cellular debris and the metal-rich granules. In view of the high percentages of Cd, As, and Zn in the CC, this fraction was considered as a

main pool for the sequestration of these metal(loids). The dynamics of Cd, As, and Zn in the CC therefore contributed most to the observed kinetics of the body burdens of these metal(loids). The results provide original data on the fraction-specific uptake and elimination of Cd, As, and Zn in *E. fetida* over a wide range of pollution levels, and thus deepen our understanding of the mechanisms underlying the bioaccumulation kinetics of non-essential (Cd and As) and essential (Zn) elements in earthworms, in turn allows for a more precise assessment of the risk of these metal(loids) in soils.

Author contribution statement

Xupeng Wang: Data curation, Writing – original draft, **Bing Gong**: Methodology, Writing – original draft, **Erkai He**: Visualization, Writing – review & editing, Funding acquisition, **Willie J.G.M. Peijnenburg**: Writing – review & editing, **Hao Qiu**: Conceptualization, Supervision, Writing – review & editing, Funding acquisition

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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2022.136482.

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