Toxicokinetics of Zn and Cd in the earthworm *Eisenia andrei* exposed to metal-contaminated soils under different combinations of air temperature and soil moisture content

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Abstract

This study evaluated how different combinations of air temperature (20 °C and 25 °C) and soil moisture content (50% and 30% of the soil water holding capacity, WHC), reflecting realistic climate change scenarios, affect the bioaccumulation kinetics of Zn and Cd in the earthworm *Eisenia andrei*. Earthworms were exposed for 21 d to two metal-contaminated soils (uptake phase), followed by 21 d incubation in non-contaminated soil (elimination phase). Body Zn and Cd concentrations were checked in time and metal uptake (k_1) and elimination (k_2) rate constants determined; metal bioaccumulation factor (BAF) was calculated as k_1/k_2. Earthworms showed extremely fast uptake and elimination of Zn, regardless of the exposure level. Climate conditions had no major impacts on the bioaccumulation kinetics of Zn, although a tendency towards lower k_1 and k_2 values was observed at 25 °C + 30% WHC. Earthworm Cd concentrations gradually increased with time upon exposure to metal-contaminated soils, especially at 50% WHC, and remained constant or slowly decreased following transfer to non-contaminated soil. Different combinations of air temperature and soil moisture content changed the bioaccumulation kinetics of Cd, leading to higher k_1 and k_2 values for earthworms incubated at 25 °C + 50% WHC and slower Cd kinetics at 25 °C + 30% WHC. This resulted in greater BAFs for Cd at warmer and drier environments which could imply higher toxicity risks but also of transfer of Cd within the food chain under the current global warming perspective.

Keywords: Bioaccumulation; Bioavailability; Climate change; Heavy metals; Mining wastes; Soil invertebrates

1. Introduction

Metal soil contamination by anthropogenic activities (e.g. mining, smelting, agriculture, waste disposal) is an environmental problem worldwide (COM, 2006; FAO and ITPS, 2015; He et al., 2015). Metals exert toxic effects on soil living organisms (van Straalen, 2004; Stankovic et al., 2014), affecting the sustainability of terrestrial ecosystems and, in some cases, human health (Naveed et al., 2014; Zhou et al., 2016; Morgado et al., 2017). Toxicity is known to be related to the metal fraction that can be taken up by organisms and subsequently interact with biological targets (i.e. metal bioavailability; Peijnenburg et al., 2007) rather than to the total metal concentration in the soil. Numerous studies have considered metal body concentrations as estimation of bioavailable fractions (Heikens et al., 2001). However, metal uptake rates are considered better predictors of their bioavailability (van Straalen et al., 2005). Metal uptake and elimination might occur simultaneously in organisms. To cope with this issue, more accurate uptake rates are estimated when toxicokinetics studies include uptake phases (organisms exposed to contaminated soil) followed by elimination phases without uptake (organisms transferred to non-contaminated soil) (van Straalen et al., 2005).

Metal bioavailability depends on multiple factors such as the considered species, the properties of the soil matrix (e.g. pH, organic matter and texture) and exposure time (Heikens et al., 2001; Allen, 2002; Nahmani et al., 2007; Peijnenburg et al., 2007). Climate conditions, especially air temperature and soil moisture content, also play an important role since they can influence the performance of soil organisms as well as the speciation and therefore the bioavailability of the metals present in the system (Holmstrup et al., 2010; Augustsson et al., 2011; González-Alcaraz and van Gestel, 2015). In the actual context of global warming, studies concerning how climate factors

may affect metal bioavailability and thus toxicity to soil organisms are gaining more interest (Løkke et al., 2013; Stahl et al., 2013; Noyes and Lema, 2015). This climatic approach is essential for the future risk assessment of metal-contaminated soils and will help developing adequate remediation strategies (Landis et al., 2013; Rohr et al., 2013).

Earthworms are major components of the soil community (Lavelle and Spain, 2001; Lavelle et al., 2006). They are good bioindicators of soil health and quality and of the biological impact of metal contamination (Spurgeon et al., 2003). Earthworms have been widely used to evaluate metal bioaccumulation (Heikens et al., 2001; Nahmani et al., 2007) although not many studies have been performed considering future climate predictions. A previous work showed that climate conditions differently affected the bioaccumulation of metals in earthworms depending on the element considered, although in that study no elimination phase in non-contaminated soil was considered after metal exposure (González-Alcaraz and van Gestel, 2016b). The present study is a further attempt to better predict metal bioaccumulation in earthworms under future climate change scenarios, considering both uptake and elimination phases. Therefore, the aim was to evaluate if variations in air temperature and soil moisture content affect the uptake and elimination kinetics of Zn and Cd in the earthworm *Eisenia andrei* exposed to a metal-contaminated soil, tested at two dilution rates with non-contaminated soil. To achieve this goal a toxicokinetics approach was followed under different combinations of air temperature (20 °C and 25 °C) and soil moisture content (50% and 30% of the soil water holding capacity, WHC), earthworms being exposed for 21 d to metal-contaminated soils (uptake phase) followed by 21 d incubation in non-contaminated soil (elimination phase). We hypothesize that different climate conditions would lead to changes in metal bioaccumulation kinetics in earthworms.

2. Materials and methods

2.1. Metal-contaminated test soil

An agricultural field located inside the Campo de Cartagena plain, one of the main intensive irrigated agricultural areas in southern Europe (IMIDA, 2005), and in the vicinity of the former mining district of La Unión-Sierra de Cartagena (Murcia, SE Spain; Figure S1, Supplementary material) was selected to collect the test soil. The area is characterized by a Mediterranean semiarid climate with an annual average temperature of ~18 °C, an annual average precipitation of ~250-300 mm (most falling in spring and autumn in form of short intensive rainfall events) and an average evapotranspiration rate of ~850 mm $year^{-1}$. The abandonment of the old tailings has continued leading to the dispersion of great volumes of metal mining wastes via water and/or wind erosion, affecting a wide variety of surrounding ecosystems (Conesa and Jiménez-Cárceles, 2007; Conesa and Schulin, 2010). Numerous studies have pointed at metal contamination problems existing in the area and the urgent need of restoration programs (Jiménez-Cárceles et al., 2008; Párraga-Aguado et al., 2013; Bes et al., 2014; González-Alcaraz and van Gestel, 2016a).

Soil samples were collected (top 20 cm) from three randomly distributed points inside the agricultural field, air dried, sieved through a 2 mm mesh and homogenized before being characterized. No earthworms were found in the agricultural field during soil sampling. The test soil showed clay texture, neutral pH in 0.01M $CaCl_2$ (~7), high electrical conductivity (EC ~3 dS m^{-1}), moderate organic matter content determined as loss on ignition (LOI ~5%), high cation exchange capacity (CEC ~16 $cmol_c$ kg^{-1}) and ~47% of WHC (Table 1). Total metal concentrations were high (Cd ~26 mg kg^{-1}, Cu ~80 mg kg^{-1}, Pb ~8733 mg kg^{-1} and Zn ~8835 mg kg^{-1}; Table1), compared to the

115 geochemical background levels established for the zone (Cd ~0.3 mg kg^{-1}, Cu ~15 mg
116 kg^{-1}, Pb ~9 mg kg^{-1} and Zn ~42 mg kg^{-1}; Hernández Bastida et al., 2005; Martínez-
117 Sánchez and Pérez-Sirvent, 2007; Pérez-Sirvent et al., 2009) and the intervention values
118 set for agricultural soils by the nearby Andalusia Region (Cd ~25 mg kg^{-1}, Cu ~595 mg
119 kg^{-1}, Pb ~275 mg kg^{-1} and Zn ~10,000 mg kg^{-1}; BOJA, 2015). Porewater metal
120 concentrations were ~29 µg L^{-1} for Cd, ~43 µg L^{-1} for Cu, ~67 µg L^{-1} for Pb and ~383
121 µg L^{-1} for Zn (Table 1). Exchangeable metals (extracted with 0.01M $CaCl_2$) showed low
122 concentrations except for Cd (~82 µg kg^{-1}) and Zn (~989 µg kg^{-1}) (Table 1).

123 *2.2. Experimental set-up*

124 *2.2.1. Test species*

125 *Eisenia andrei* Bouché 1972 (Class Oligochaeta, Family Lumbricidae) was cultured
126 at the Vrije Universiteit (Amsterdam, The Netherlands) for >10 years in clean horse
127 manure free of any pharmaceuticals at 20 °C, 75% relative humidity and complete
128 darkness (OECD, 2010). Earthworms were originally obtained from ECT
129 Oekotoxikologie in Flörsheim (Germany) where they were genotyped to confirm their
130 species identity (Römbke et al., 2016).

131 Before starting the toxicokinetics experiment, synchronized sexually mature
132 earthworms (well-developed clitella and ~300-700 mg fresh weight) were transferred to
133 clean soil (Lufa 2.2; Speyer, Germany) and kept for several hours (~6) for acclimation
134 to soil conditions and to replace the gut content of horse manure by soil (Vijver et al.,
135 2005; OECD, 2010). This acclimatization phase was performed in complete darkness at
136 20 °C and 75% relative humidity.

137 *2.2.2. Soil preparation*

The metal-contaminated test soil was mixed with the standard reference soil Lufa 2.2 (Table 1) at ratios (w:w) of 1:1 (50% metal-contaminated soil, hereafter named test soil 1:1) and 1:3 (25% metal-contaminated soil, hereafter named test soil 1:3). Soil mixtures were prepared with dry soils. This dilution approach allowed earthworms to burrow in the soil since the clay texture of the original study soil limited their movement (authors' visual observation from pilot tests performed with the metal-contaminated test soil). To prevent changes in metal availability in the mixing process, the pH (in 0.01M $CaCl_2$) of the Lufa 2.2 soil was adjusted with $CaCO_3$ to approximately 7 (by adding 4 mg $CaCO_3$ g^{-1} dry soil) to mimic the pH of the metal-contaminated test soil (Table 1). The WHC of each soil mixture (~42% for soil 1:1 and ~39% for soil 1:3) was determined using the sandbox method after saturation of the soil with water for 3 h (ISO, 1999).

2.2.3. Toxicokinetics

Toxicokinetics tests with *E. andrei* were performed according to the standardized test guideline OECD 317 (OECD, 2010). The climate conditions recommended by the guideline are 20 °C of air temperature and a soil moisture content of approximately 50% of the soil WHC (standard climate conditions; OECD, 2010). From these standard conditions and in order to recreate future climate predictions for southern parts of Europe (~4 °C of temperature increase and ~10-20% of soil moisture content decrease; Bates et al., 2008; Forzieri et al., 2014), an increase of 5 °C in air temperature and a decrease of 20% in soil WHC were chosen. Toxicokinetics tests were performed for both soil mixtures (soil 1:1 and soil 1:3) under four different climate conditions: 1) 20 °C + 50% WHC (standard climate conditions), 2) 20 °C + 30% WHC, 3) 25 °C + 50% WHC and 4) 25 °C + 30% WHC (climate conditions simulating warming and drier environments).

Toxicokinetics tests consisted of two phases (uptake and elimination), each one lasting 21 d. Before each phase earthworms were rinsed with demineralized water, dried on filter paper and weighed. In the uptake phase earthworms were exposed to both soil mixtures (soil 1:1 and soil 1:3), and then transferred to pH-adjusted Lufa 2.2 soil for the elimination phase. In both phases earthworms were kept individually in 100 mL glass jars containing 30 g of soil previously moistened and 2 g (dry weight) of moistened horse dung for food. Soil moistening was done just before starting the experiment. Tests were run under the different climate conditions established in controlled climate chambers with 75% relative humidity and a 12:12 h light:dark photoperiod (OECD, 2010). Soil moisture content was checked twice a week by weighing the test jars and water loss replenished with demineralized water to keep the initial soil moisture content. At time points 0 (background body metal concentrations), 1, 3, 7, 10, 14 and 21 d during the uptake phase and 22, 24, 28, 31, 35 and 42 d during the elimination phase three earthworms were sacrificed for the determination of the body metal concentrations (three replicates per soil mixture/climate condition/time point). Sampled earthworms were depurated on moist filter paper for 24 h in a petri dish to fully purge their gut content (OECD, 2010), rinsed with demineralized water, dried on filter paper, weighted (to evaluate weight change throughout the experiment) and frozen at -20 °C.

Two control sets were performed, one with the original Lufa 2.2 soil (pH in 0.01M $CaCl_2$ ~5.2; Table 1) and another one with the pH-adjusted Lufa 2.2 soil used for soil mixture preparation (pH in 0.01M $CaCl_2$ ~7.0). The first control allowed checking for earthworm performance in non-contaminated soil (OECD, 2010), the second control if soil pH was causing differences in earthworm performance. Control tests were performed under the four climate conditions established following the methodology described above. Earthworm survival, weight change and body metal concentrations

were checked at the end of the uptake (after 21 d) and elimination (after 42 d) phases (six replicates per control soil/climate condition/time point).

2.2.4. Chemical analysis

Frozen earthworms were freeze-dried for 48 h, weighted and digested in 4:1 (v:v) HNO_3 65%:HCl 37% in Teflon bombs heated for 7 h at 140 °C in a destruction oven (Binder). The concentrations of Zn and Cd were measured by flame atomic absorption spectroscopy (Perkin-Elmer AAnalyst 100; detection limit 3 mg L^{-1}). Body metal concentrations are expressed on a dry weight (d.w.) basis. Quality control was checked with the certified reference materials DOLT4 (Dogfish liver, LGCS Standards) and Bovine Liver (BCR-185R); recoveries were 110-117% for Zn and 113-119% for Cd.

2.2.5. Kinetic modelling

For each soil mixture (soil 1:1 and soil 1:3) a first-order one-compartment kinetic model was applied to describe metal uptake and elimination rates in the earthworms. Equations 1 and 2 were used to describe the uptake and elimination phases, respectively:

$$C_t = C_0 + (k_1/k_2) * C_{exp} * (1 - e^{-k2*t}) \quad \text{(Eq. 1)}$$

$$C_t = C_0 + (k_1/k_2) * C_{exp} * (e^{-k2*(t-tc)} - e^{-k2*t}) \quad \text{(Eq. 2)}$$

where C_t = body metal concentration in earthworms (µg g^{-1} d.w.) at time t (d); C_0 = background body metal concentration in earthworms (µg g^{-1} d.w.); k_1 = uptake rate constant (g_{soil} $g^{-1}_{earthworm}$ d^{-1}); k_2 = elimination rate constant (d^{-1}); C_{exp} = total metal concentration in soil calculated from mixture proportion (µg g^{-1} dry soil); t_c = time at which the earthworms were transferred to non-contaminated soil (21 d). Uptake and elimination equations were fitted simultaneously. A growth rate constant (k_g) was

included in the kinetic model to consider changes in earthworm body weight throughout the experiment, but this did not affect k_1 and k_2 values. Results shown therefore are those derived using equations 1 and 2.

A kinetic metal bioaccumulation factor (BAF) in earthworms was calculated as k_1/k_2 (Peijnenburg et al., 1999). Half-life for the elimination of the metals from the earthworms after exposure to the soil mixtures was calculated as $\ln(2)/k_2$.

2.2.6. Statistical analyses

Statistical analyses were performed with IBM SPSS Statistics 22 and differences were considered significant at $p < 0.05$. For each soil mixture (soil 1:1 and soil 1:3), differences in k_1 and k_2 values among the climate conditions tested were evaluated by generalized likelihood ratio tests (Sokal and Rohlf, 1969; van Gestel and Hensbergen, 1997). No statistical analyses could be performed for earthworm fresh weight due to the fact that organisms from the same soil/climate condition/time point were pooled together for cleaning the gut content before being weighed. This made it difficult to distinguish earthworms based on their initial fresh weight. Therefore the data from the different replicates were pooled.

3. Results and discussion

3.1. Earthworm performance under different climate conditions

The validity of the tests performed with *E. andrei* was evaluated according to the following criteria (OECD, 2010): 1) mortality at the end of the test ≤10%; 2) weight loss at the end of the uptake and elimination phases compared to the initial fresh weight for each phase ≤20%. These criteria apply both for controls (original Lufa 2.2 soil and

pH-adjusted Lufa 2.2 soil) and soil mixtures (soil 1:1 and soil 1:3) under standard climate conditions (20 °C + 50% WHC). No mortality was registered in controls and soil 1:3 (25% metal-contaminated soil). In soil 1:1 (50% metal-contaminated soil) one earthworm died (3% mortality). When exposed to standard climate conditions, the earthworms tended to lose weight throughout the experiment (average weight loss at the end of the uptake and elimination phases, respectively): original Lufa 2.2 soil (~13% and ~20%); pH-adjusted Lufa 2.2 soil (~13% and ~16%); soil 1:1 (~8% and ~6%); soil 1:3 (~10% and ~ -2%) (data not shown). Therefore, the validity criteria established by OECD were met.

Earthworm body weight was affected by changing air temperature and soil moisture content compared to the standard climate conditions. In both control soils earthworm weight loss at the end of the uptake and elimination phases was most pronounced at 25 °C. At 20 °C + 30% WHC earthworm weight loss was ~9-11% for the original Lufa 2.2 soil and ~16-19% for the pH-adjusted Lufa 2.2 soil (data not shown). At 25 °C, regardless of the soil moisture content (50% and 30% WHC), earthworm weight loss was ~16-33% for the original Lufa 2.2 soil and ~20-29% for the pH-adjusted Lufa 2.2 soil (data not shown). This trend agrees with a previous study where earthworms showed higher weight loss at 25 °C compared to 20 °C, and no influence was found of the pH of the Lufa 2.2 soil (González-Alcaraz and van Gestel, 2016b). Lima et al. (2011, 2015) also found greater weight loss for *E. andrei* in Lufa 2.2 soil with increasing air temperature (20 °C vs. 26 °C) and no effect of soil moisture content (60%, 40%, 20% and 10% of soil WHC). However, our results do not agree with other studies showing decreasing body weight with lowered soil moisture content in the earthworm species *Eisenia fetida* (Diehl and Williams, 1992) and *Aporrectodea caliginosa* (Holmstrup, 2001).

In both soil mixtures (soil 1:1 and soil 1:3) earthworms incubated at 25 °C + 30% WHC reached the highest weight loss values at the end of the uptake phase (~49% and ~44% after 21 d exposure, respectively; Figure S2, Supplementary material), showing a synergistic interaction between metal contamination and warmer and drier conditions (Friis et al., 2004; Holmstrup et al., 2010; González-Alcaraz et al., 2016b). When transferred from metal-contaminated to non-contaminated soil, however, earthworms tended to gain weight, especially those incubated at 25 °C + 30% WHC (weight gain ~22% and ~14% after 21 d in clean soil for organisms earlier exposed to soil 1:1 and soil 1:3, respectively; Figure S2, Supplementary material).

3.2. Metal toxicokinetics in earthworms under different climate conditions

Background body metal concentrations in earthworms were ~100-120 µg g^{-1} d.w. for Zn (Figure 1) and ~2-3 µg g^{-1} d.w. for Cd (Figure 2), normal levels for earthworms from non-contaminated soils (Zn ~90-120 µg g^{-1} d.w. and Cd ~3-6 µg g^{-1} d.w.; Janssen et al., 1997; van Gestel et al., 2002). Similar body metal concentrations were found in earthworms exposed to control soils for 42 d under the different climate conditions tested (Zn ~80-160 µg g^{-1} d.w. and Cd ~2-11 µg g^{-1} d.w.; data not shown). When earthworms were exposed to metal contamination different bioaccumulation patterns were observed for Zn (essential element) and Cd (non-essential element) (Figures 1 and 2).

Body Zn concentrations increased rapidly after few days of exposure to both soil mixtures (soil 1:1 and soil 1:3), reaching a steady state at body Zn concentrations ~240-420 µg g^{-1} d.w. (Figure 1). When transferred to non-contaminated soil, body Zn concentrations rapidly decreased to background levels (~110-140 µg g^{-1} d.w.; Figures 1). This bioaccumulation pattern seems typical for Zn as it has previously been shown

also in other studies (Spurgeon and Hopkin, 1999; Świątek et al., 2017), and may be explained from the presence of efficient regulation mechanisms. Zinc regulation in earthworms occurs via excretion (Spurgeon and Hopkins, 1999), leading to high k_2 values (15-42 fold higher than k_1 values; Table 2) and short half-lives (<1 d; Table 2). Changing air temperature and soil moisture content had no major effects on the bioaccumulation pattern of Zn (Figure 1). This agrees with González-Alcaraz and van Gestel (2016b) who found no impact of climate conditions on Zn bioaccumulation in *E. andrei* exposed for 21 d to metal-contaminated soils of different properties. Despite this, for both soil mixtures (soil 1:1 and soil 1:3), the treatment at 25 °C + 30% showed lower k_1 and k_2 values compared to the other climate conditions tested (Table 2).

Unlike Zn, body Cd concentrations in earthworms tended to increase with exposure time throughout the uptake phase and stayed more or less constant or slowly decreased upon transfer to non-contaminated soil (Figure 2). This is a typical pattern for non-essential elements, with earthworms generally showing very slow or no elimination of Cd (Spurgeon and Hopkins, 1999; Lock and Jansen, 2001; Smith et al., 2010; Giska et al., 2014). Cadmium detoxification in earthworms occurs via its sequestration by metallothioneins (Stürzenbaum et al., 2001, 2004; Conder et al., 2002; Vijver et al., 2006). This agrees with the low k_2 values obtained (1.5-21 fold lower than k_1 values; Table 3). At the end of the uptake phase (21 d of exposure), higher body Cd concentrations were found in earthworms incubated at 50% of the soil WHC, regardless of the air temperature (2.4-3.6 and 1.2-3.1 fold higher in soil 1:1 and soil 1:3, respectively; Figure 2). The treatment at 25 °C + 50% WHC showed the highest k_1 (~0.16 vs. ~0.01-0.06 $g_{soil}\ g^{-1}_{earthworm}\ d^{-1}$ in soil 1:1; ~0.31 vs. ~0.04-0.19 $g_{soil}\ g^{-1}_{earthworm}\ d^{-1}$ in soil 1:3) and k_2 (~0.11 vs. ~0-0.01 d^{-1} in soil 1:1; ~0.12 vs. ~0.002-0.04 d^{-1} in soil 1:3) values (Table 3). This could be related to a higher metabolic activity when

earthworms (poikilothermic organisms) were incubated at higher temperature, enhancing Cd uptake and elimination, which resulted in shorter half-lives (~7 vs. ~75-81 d in soil 1:1; ~6 vs. ~16-377 d in soil 1:3; Table 3). However, this was not the case for earthworms incubated at 25 °C + 30% WHC which showed lower k_1 and k_2 values (Table 3), similar to what happened for Zn bioaccumulation (Table 2). This difference was more marked compared to the treatments moistened at 50% of the soil WHC, especially in soil 1:3 (25% metal-contaminated soil): k_1 values were 5-9 fold lower and k_2 values 24-62 fold lower at 25 °C + 30% WHC (significant, $p<0.05$; Table 3). A warmer and drier environment could have hindered earthworm performance, as shown by the greater weight loss upon exposure to metal-contaminated soils (Figure S2, Supplementary material), slowing down metal uptake and elimination. Therefore, the bioaccumulation pattern of Cd in earthworms changed when changing climate conditions. This agrees with the results of González-Alcaraz and van Gestel (2016b), although they found increasing k_1 and k_2 values at higher air temperature and/or lower soil moisture content. Differences in the properties of the test soils as well as not including an elimination phase in non-contaminated soil in the toxicokinetic study could be responsible of the different results obtained.

BAF values can be used as indicators of soil metal bioavailability (Fründ et al., 2011) and to predict risks of trophic transfer (Smith et al., 2010); BAF>1 indicates metal accumulation within organisms. For Zn, due to its fast elimination, BAFs were below 1 both in soil 1:1 and soil 1:3 and under the different climate conditions tested (~0.02-0.07 $g_{soil}\ g^{-1}_{earthworm}\ d^{-1}$; Table 2). But for Cd, BAFs were above 1 (~1.50-21.3 $g_{soil}\ g^{-1}_{earthworm}\ d^{-1}$; Table 3), indicating that earthworms concentrated Cd within their body (Smith et al., 2010). BAFs for Cd differed among exposure concentrations and climate conditions. Higher BAFs were found when earthworms were exposed to soil 1:3 (25%

metal-contaminated soil) (Table 3), in agreement with increasing BAFs for metals at lower exposure levels (McGeer et al., 2003). Moreover, in soil 1:3, the treatment at 25 °C + 30% WHC showed the highest BAF value compared to the other climate conditions tested (~21.3 vs. ~3.0-5.3 g_{soil} $g^{-1}_{earthworm}$ d^{-1}; Table 3). Therefore the bioaccumulation potential of Cd in earthworms not only depended on the exposure level but also on the climate conditions, with greater Cd bioaccumulation at warmer and drier environments.

4. Conclusions

The earthworm *E. andrei* rapidly accumulated Zn to a steady state level when exposed to metal-contaminated soils, but also rapidly eliminated Zn to reach background levels upon transfer to non-contaminated soil. This suggests efficient regulation of Zn body concentrations. Air temperature (20 °C and 25 °C) and soil moisture content (50% and 30% of the soil WHC) had no major impacts on the bioaccumulation kinetics of Zn, although a tendency to lower uptake and elimination rates was observed at 25 °C + 30% WHC. On the contrary, different combinations of air temperature and soil moisture content changed the bioaccumulation kinetics of Cd. Earthworms incubated at high soil moisture content had higher body Cd concentrations upon exposure to metal contamination. When high temperature was combined with high soil moisture content earthworms showed faster uptake and elimination rates for Cd. However, when high temperature was combined with low soil moisture content, slower Cd kinetics was found (lower uptake and elimination rates at 25 °C and 30% of the soil WHC). This resulted in higher BAFs for Cd when earthworms were incubated under warmer and drier environments. These findings could not only imply higher toxicity

risks for earthworms in metal-contaminated soils under the actual global warming perspective, but also of transfer/biomagnification of Cd within the food chain. The latter is of major concern if we take into account that earthworms are at the lower levels of most wildlife food chains. Therefore, and considering future climate predictions, more studies concerning the influence of climate factors on metal bioavailability to soil invertebrates are needed to properly predict and manage their potential risks.

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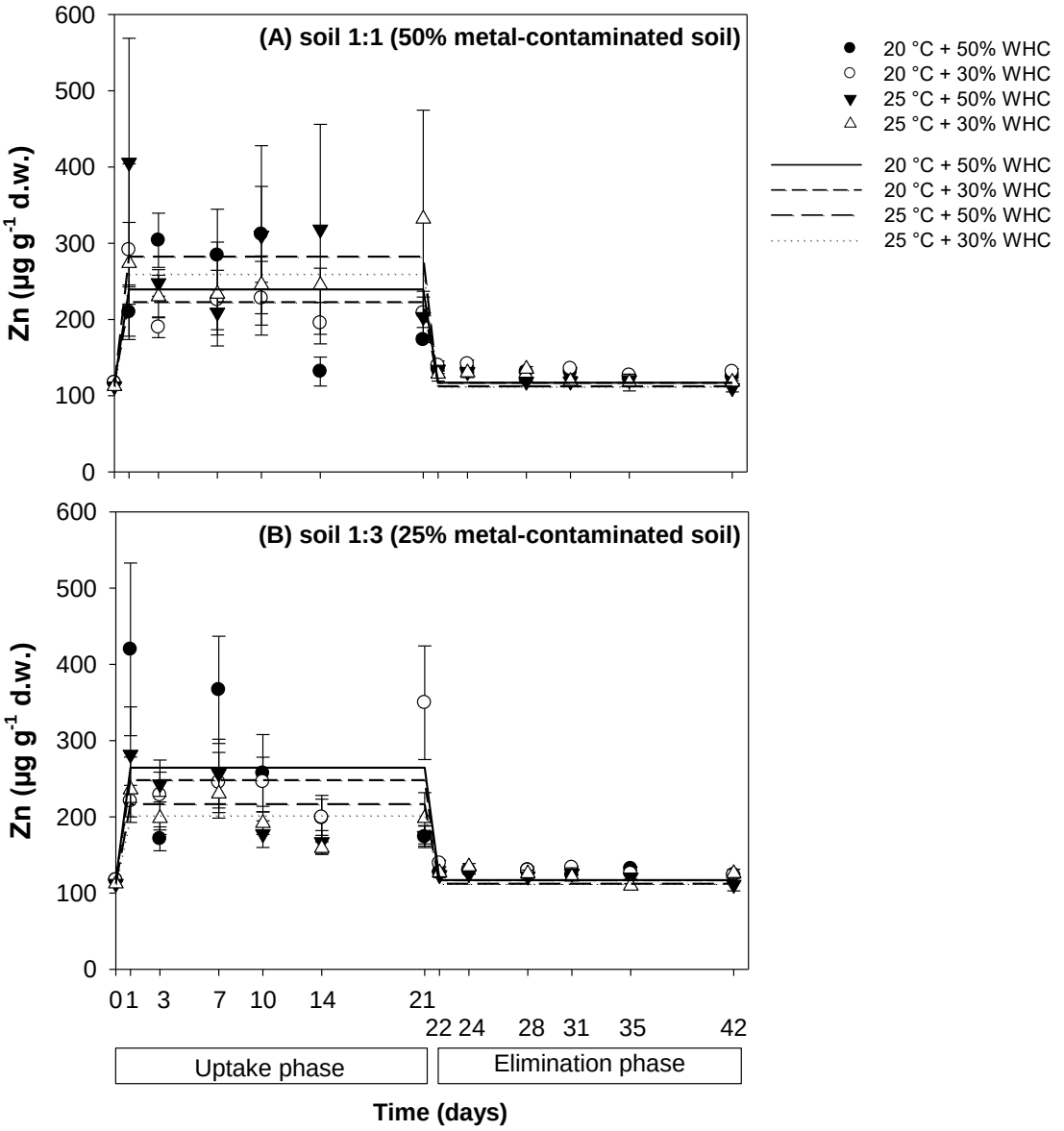

Figure 1. Uptake and elimination kinetics of Zn in the earthworm *Eisenia andrei* exposed to the mixtures of the metal-contaminated test soil with the pH-adjusted Lufa 2.2 soil under the different climate conditions tested: (A) soil 1:1 (50% metal-contaminated soil); (B) soil 1:3 (25% metal-contaminated soil). Uptake and elimination phases lasted 21 d each. Dots represent average body concentrations (on a dry weight basis, d.w.) ± SE (n = 3). Lines represent modelled Zn body concentrations using Eqs. 1 and 2. WHC (water holding capacity).

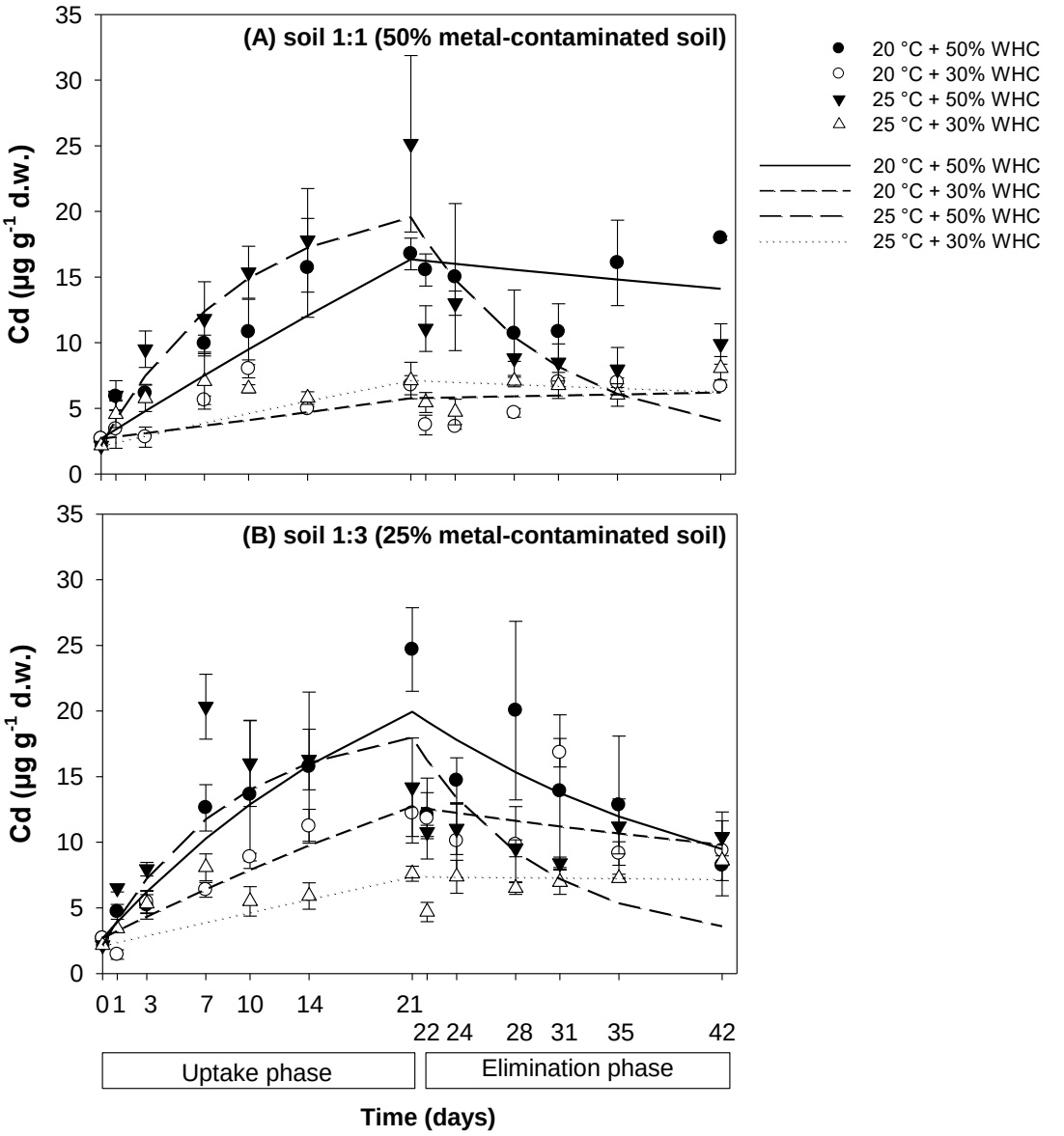

Figure 2. Uptake and elimination kinetics of Cd in the earthworm *Eisenia andrei* exposed to the mixtures of the metal-contaminated test soil with the pH-adjusted Lufa 2.2 soil under the different climate conditions tested: (A) soil 1:1 (50% metal-contaminated soil); (B) soil 1:3 (25% metal-contaminated soil). Uptake and elimination phases lasted 21 d each. Dots represent average body concentrations (on a dry weight basis, d.w.) ± SE (n = 3). Lines represent modelled Cd body concentrations using Eqs. 1 and 2. WHC (water holding capacity).

Table 1. General characterization of the metal-contaminated test soil from SE Spain and the Lufa 2.2 control soil used for the toxicokinetics study with the earthworm *Eisenia andrei* under different combinations of air temperature and soil moisture content. Values are average ± SD (n = 3). EC (electrical conductivity). LOI (total organic matter determined as loss on ignition). CEC (cation exchange capacity). WHC (water holding capacity). d.l. (detection limit).

Parameter	**Test soil**	**Lufa 2.2 soil**
pH 0.01M $CaCl_2$[a]	7.01 ± 0.05	5.21 ± 0.04
EC (dS m^{-1})[b]	2.95 ± 0.09	0.10 ± 0.002
LOI (%)[c]	5.30 ± 0.10	3.12 ± 0.05
CEC ($cmol_c$ kg^{-1})[d]	16.3 ± 0.6	7.8 ± 1.9
WHC (%)[e]	46.5 ± 0.5	44.4 ± 0.7
Texture[f]	Clay	Sandy loam
Porewater metals[g]		
Cd (µg L^{-1})	28.7 ± 2.1	<d.l. (3)
Cu (µg L^{-1})	43.3 ± 1.2	59.0 ± 17.1
Pb (µg L^{-1})	67.3 ± 13.1	32.3 ± 17.5
Zn (µg L^{-1})	383 ± 37	16.0 ± 17.5
0.01M $CaCl_2$-extractable metals[h]		
Cd (µg kg^{-1})	81.6 ± 2.9	<d.l. (15)
Cu (µg kg^{-1})	<d.l. (30)	<d.l. (30)
Pb (µg kg^{-1})	<d.l. (225)	<d.l. (225)
Zn (µg kg^{-1})	989 ± 87	246 ± 3
Total metals[i]		
Cd (mg kg^{-1})	25.6 ± 0.1	<d.l. (0.2)
Cu (mg kg^{-1})	80.3 ± 4.4	3.1 ± 0.1
Pb (mg kg^{-1})	8733 ± 2479	15.0 ± 2.1
Zn (mg kg^{-1})	8835 ± 96	23.6 ± 2.5

a: 1:5 (w:v) soil:0.01M $CaCl_2$ suspensions after 2 h shaking at 200 rpm.

b: 1:5 (w:v) soil:H_2O suspensions after 2 h shaking at 200 rpm.

c: combustion following a heating ramp from 200 °C to 500 °C for 8 h.

d: saturation of soil exchange complex with 1M CH_3COONa pH 8.2 and displacement of adsorbed sodium with 1M CH_3COONH_4 pH 7.0 (Chapman, 1965). Sodium concentration determination by flame AAS.

e: sandbox method after soil saturation with water for 3 h (ISO, 1999).

f: laser grain size HELOS-QUIXEL analyzer (Konert and Vandenberghe, 1997).

g: soil saturation with water at 100% WHC for 7 d, centrifugation for 45 min at 2000 rcf over a 0.45 µm membrane filter and metal concentrations determined by flame AAS.

h: metal concentrations determined in 0.01M $CaCl_2$ extracts by atomic absorption spectroscopy (AAS; Perkin-Elmer Analyst 100).

i: acid digestion in 4:1(v:v) HNO_3 65%:HCl 37% at 140 °C for 7 h. Metal concentrations determined by flame AAS.

Table 2. Uptake rate constant (k_1), elimination rate constant (k_2), bioaccumulation factor (BAF) and half-life for the bioaccumulation of Zn in the earthworm *Eisenia andrei* exposed to the mixtures of the metal-contaminated test soil with the pH-adjusted Lufa 2.2 soil under the different climate conditions tested: soil 1:1 (50% metal-contaminated soil) and soil 1:3 (25% metal-contaminated soil). No 95% confidence intervals could be calculated for the k_1 and k_2 values. WHC (water holding capacity).

Contaminated soil	Climate condition	k_1 ($g_{soil}\ g^{-1}_{earthworm}\ d^{-1}$)	k_2 (d^{-1})	BAF ($g_{soil}\ g^{-1}_{earthworm}\ d^{-1}$)	Half-life (d)
1:1	20 °C + 50% WHC	0.47	16.9	0.03	0.04
	20 °C + 30% WHC	0.37	14.0	0.02	0.05
	25 °C + 50% WHC	0.50	13.0	0.04	0.05
	25 °C + 30% WHC	0.37	11.1	0.03	0.06
1:3	20 °C + 50% WHC	1.00	15.0	0.07	0.05
	20 °C + 30% WHC	0.86	14.4	0.06	0.05
	25 °C + 50% WHC	0.61	12.9	0.05	0.05
	25 °C + 30% WHC	0.51	12.6	0.04	0.06

Table 3. Uptake rate constant (k_1), elimination rate constant (k_2), bioaccumulation factor (BAF) and half-life for the bioaccumulation of Cd in the earthworm *Eisenia andrei* exposed to the mixtures of the metal-contaminated test soil with the pH-adjusted Lufa 2.2 soil under the different climate conditions tested: soil 1:1 (50% metal-contaminated soil) and soil 1:3 (25% metal-contaminated soil). 95% confidence intervals are given in between brackets. For each percentage of contaminated soil, different letters at the same column indicate significant differences among climate conditions (likelihood ratio test, $p<0.05$). WHC (water holding capacity).

Contaminated soil	Climate condition	k_1 ($g_{soil}\ g^{-1}_{earthworm}\ d^{-1}$)	k_2 (d^{-1})	BAF ($g_{soil}\ g^{-1}_{earthworm}\ d^{-1}$)	Half-life (d)
1:1	20 °C + 50% WHC	0.055 b (0.035 – 0.074)	0.009 b (0*– 0.029)	6.42	81.3
	20 °C + 30% WHC	0.011 c (0.004 – 0.017)	0*	–	–
	25 °C + 50% WHC	0.159 a (0.103 – 0.216)	0.106 a (0.062 – 0.150)	1.50	6.5
	25 °C + 30% WHC	0.020 c (0.011 – 0.030)	0.009 b (0* – 0.036)	2.19	75.2
1:3	20 °C + 50% WHC	0.194 a (0.113 – 0.276)	0.044 ac (0.012 – 0.077)	4.38	15.6
	20 °C + 30% WHC	0.087 b (0.057 – 0.117)	0.016 b (0* – 0.037)	5.30	42.3
	25 °C + 50% WHC	0.306 a (0.183 – 0.431)	0.115 a (0.061 – 0.169)	3.02	6.1
	25 °C + 30% WHC	0.039 c (0.023 – 0.055)	0.002 bc (0* – 0.024)	21.3	377

*: Each zero comes from a negative k_2 value generated by the mathematical model (Eqs. 1 and 2). It means that there was no elimination.

Highlights

1. Climate change simulated by higher air temperature and lower soil moisture content.

2. Zn toxicokinetics in *Eisenia andrei* not affected by climate conditions.

3. Faster Cd kinetics in earthworms at higher air temperature and soil moisture content.

4. Cd kinetics at higher air temperature slowed down with decreasing soil moisture.

5. Higher Cd-BAFs in earthworms incubated under warmer and drier conditions.

SUPPLEMENTARY MATERIAL

Toxicokinetics of Zn and Cd in the earthworm *Eisenia andrei* exposed to metal-contaminated soils under different combinations of air temperature and soil moisture content

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Supplementary material summary: 1 cover page and 2 figures.

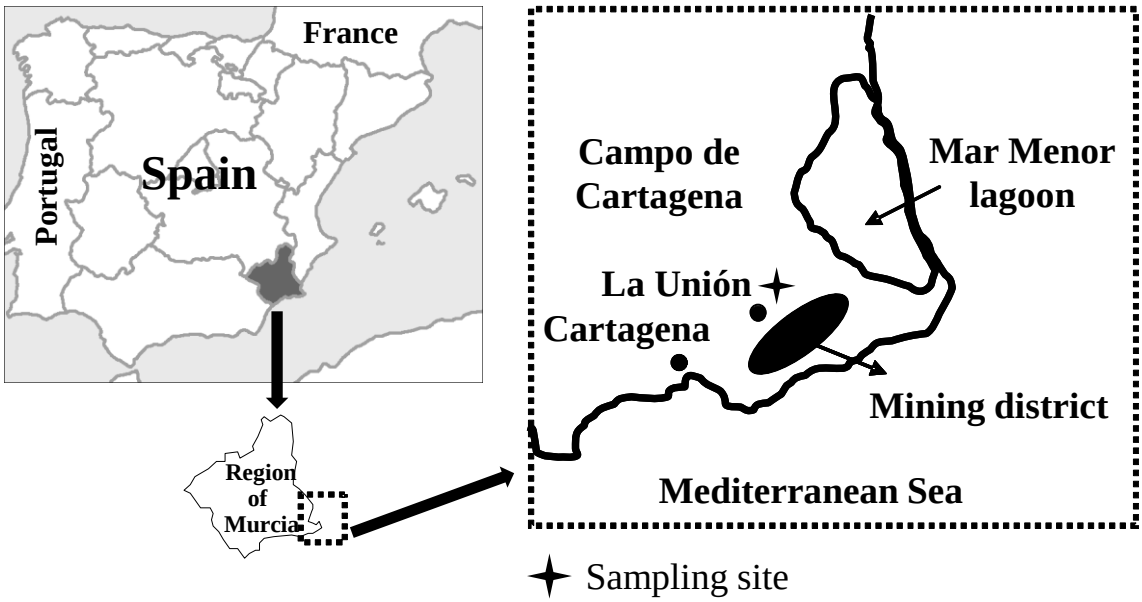

Figure S1. Location map of the sampling site of the agricultural metal-contaminated test soil in the Murcia Region (SE Spain).

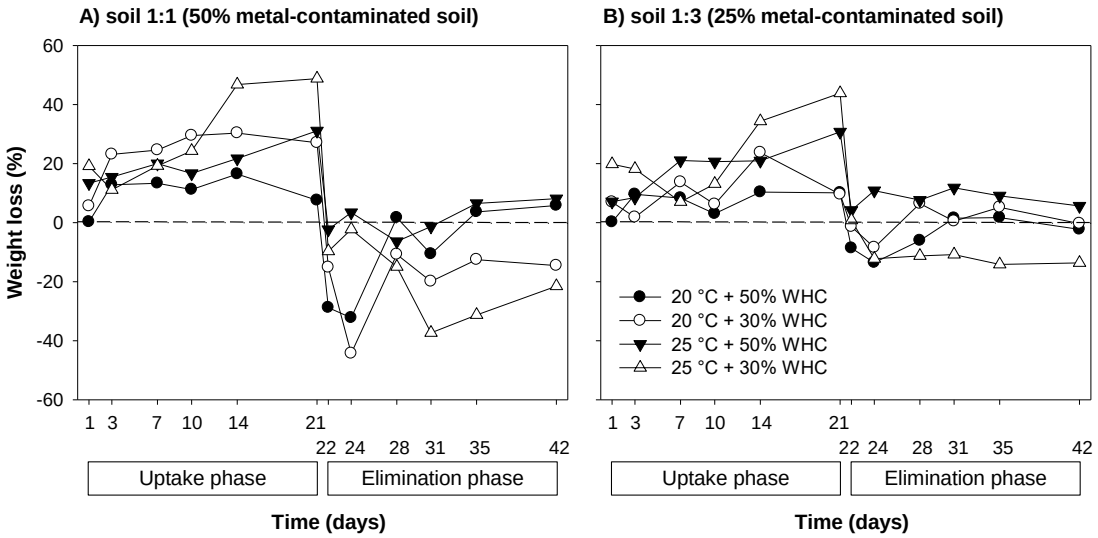

Figure S2. Weight loss of earthworms *Eisenia andrei* exposed to the mixtures of the metal-contaminated test soil with the pH-adjusted Lufa 2.2 soil under the different climate conditions tested: (A) soil 1:1 (50% metal-contaminated soil); (B) soil 1:3 (25 % metal-contaminated soil). Values are percentages compared to initial fresh weight at the start of the uptake (for time points 1, 3, 7, 10, 14 and 21 d) and elimination (for time points 22, 24, 28, 31, 35 and 42) phase. Positive values indicate loss of fresh weight and negative values weight gain. WHC (water holding capacity).