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Brief Communication

Environmental Toxicology

Copper toxicity thresholds for earthworm *Dendrobaena veneta*: insights from a site with unique monometallic soil contamination

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Abstract

Ecotoxicological studies predominantly rely on artificially contaminated soils and fieldwork on contaminated soils remains scarce. This study focuses on the Kargaly site in the Orenburg region near the southern Urals, where a rare instance of monometallic soil pollution has occurred with copper (Cu). We established Cu toxicity thresholds for *Dendrobaena veneta*, a European nightcrawler, using soils collected along a Cu toxicity gradient (total Cu content of 121–10,200 mg kg^{-1}) in a chernozem (Mollisol) agricultural field. Earthworm survival in the reproduction bioassay was an unreliable predictor of Cu toxicity. However, the number of juveniles in the reproduction bioassay and earthworm avoidance behavior were sensitive indicators of Cu toxicity. While total soil Cu strongly predicted earthworm responses, the effect of soluble (0.01 M $CaCl_2$-extractable) Cu on earthworm responses was not statistically significant. Similarly, the Cu content in earthworm tissues was an unreliable predictor of Cu toxicity in *D. veneta*. The effect concentrations at 25% (EC25) and 50% (EC50) of total soil Cu for earthworms were 177 and 407 mg kg^{-1}, respectively, for the reproduction bioassay, compared with 783 and 1,603 mg kg^{-1}, for earthworm avoidance behavior. This study is among the few that estimate Cu toxicity thresholds for earthworms in real-world contaminated soils rather than artificially spiked ones. This is the first report of the Cu toxicity threshold for the genus *Dendrobaena*, highlighting the novelty of this study.

Keywords: pollution, ecotoxicity, bioavailability, heavy metals, field-contaminated soil

Introduction

Earthworms play a vital role in keeping our soil healthy. They recycle nutrients, improve soil structure, and provide a food source for other important species (Blouin et al., 2013; Edwards & Bohlen, 1996; Fonte et al., 2023). As a result of the earthworms' key-ecological importance, they have been adopted by the International Organization for Standardization (ISO) as indicator taxa for the assessment of soil quality (ISO 11268–2, ISO, 2012).

Current ecotoxicological studies have predominantly focused on artificially contaminated (metal-spiked) soils, limiting our understanding of metal toxicity in natural environments. Real-world contaminated soils, where metal exposure spans longer periods, might offer a more accurate representation of the toxicity effects in nature. Environmental toxicologists have emphasized that prolonged exposure significantly influences toxicity outcomes (Neaman, 2022; Neaman et al., 2020). The disparity between artificially contaminated and anthropogenically contaminated soils is attributed to the fact that metal toxicity depends, among other factors, on the residence time of metals in the soil. The process of transformation of metals in the soil over time is called "aging" (or "ageing"; e.g., Martínez et al., 1999). This time-dependent nature of metal toxicity has not been replicated in artificially metal-spiked soils, resulting in consistently biased conclusions of higher toxicity observed in plants and soil biota in artificially contaminated soils compared to real-world soils contaminated for decades (Santa-Cruz et al., 2021b).

One of the key challenges in determining metal toxicity thresholds in real-world contaminated soils is the presence of multiple metals, which can confound interpretations of results and complicate the attribution of toxic effects to individual metals. Notably, arsenic (As), cadmium (Cd), copper (Cu), lead (Pb), and zinc (Zn) belong to the group of chalcophile elements known for their strong affinity to sulfur. Consequently, their concurrent presence in metal ores is expected and has been documented in previous studies worldwide (Vorobeichik, 2022). Hence, detecting soils polluted predominantly with a single metal presents a unique opportunity to examine the ecotoxicological impacts of that metal on soil organisms and enhance our understanding of instances where multiple metals contaminate a site.

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In recent studies, Dovletyarova et al. (2023, 2024) identified a suitable location for examining Cu ecotoxicity in the Kargaly, Orenburg region, Russia, near the southern Urals (see online supplementary material; Figure 1). The soil samples analyzed by Dovletyarova et al. (2023) demonstrated elevated total Cu concentrations, reaching approximately 10,000 mg kg^{-1}. Importantly, the concentrations of other elements in all soil samples were comparable to those in the background soils (Table 1), highlighting the unique monometallic contamination of this study area. Consequently, this study focused on investigating Cu ecotoxicity at this site, which is characterized by distinct monometallic soil contamination.

Elevated metal concentrations in soil can significantly disrupt soil ecosystems, necessitating the identification of the most sensitive bioassays for early toxicity detection (Broos et al., 2005). In this context, it is noteworthy that soil invertebrates exhibit greater susceptibility to Cu contamination than plants. A single effective concentration value for a specific organism response is insufficient to support significant agricultural or ecological applications. To address this, Checkai et al. (2014) proposed averaging effective concentration values across different species and endpoints. Using this approach, Santa-Cruz et al. (2021a) demonstrated that the average effect concentrations at 50% (EC50) of total Cu in soil for invertebrates in real-world contaminated environments were markedly lower than those for plants, with values of 303 ± 108 and 987 ± 491 mg kg^{-1}, respectively. This underscores the significance of employing earthworms as indicators for early detection of Cu toxicity in soils.

Numerous researchers have emphasized the importance of using real-world contaminated soils in ecotoxicological research rather than artificially contaminated soils (review by Nahmani et al., 2007). Nonetheless, the body of scientific literature addressing real-world soil contamination remains limited, with few studies establishing ecotoxicity threshold values for soil metal content (review by Santa-Cruz et al., 2021a). Specifically concerning Cu, only eight studies have identified the estimated Cu toxicity thresholds for earthworms and potworms using real-world contaminated soils (see online supplementary material; Table 1). The paucity of such studies limits the ability to derive generalizable conclusions regarding the impact of Cu contamination on soil biota (Neaman & Yáñez, 2023). Consequently, further investigation of this critical area is both innovative and essential.

Figure 1. This study utilized the earthworm species *Dendrobaena veneta* to evaluate copper (Cu) toxicity in soils from the Kargaly copper deposits in Orenburg, Russia, under controlled experimental conditions.

Eisenia fetida is widely used as a bioindicator organism for the assessment of metal toxicity (review by Nahmani et al., 2007). Although some studies have employed other species, such as *E. andrei*, *Lumbricus rubellus*, *Aporrectodea caliginosa*, and *Ap. tuberculata*, with the majority relying on *E. fetida* (review by Santa-Cruz et al., 2021a). Notably, based on acute toxicity tests, *E. fetida* is less sensitive to Cu than several other species, such as *L. rubellus*, *Ap. longa*, *L. terrestris*, and *Allolobophora chlorotica* (Duque et al., 2023). However, the same study found that the *Ap. caliginosa* and *Ap. rosea* were less sensitive to Cu than those of *E. fetida*. The review by Nahmani et al. (2007) calls for more studies on earthworm species other than *E. fetida* to enhance our understanding of metal toxicity in soil biota. The extensive utilization of *E. fetida* is attributed to its ease of cultivation under laboratory conditions, which renders it a convenient species for assessing metal toxicity. In this context, the earthworm *Dendrobaena veneta* (Rosa, 1886) has garnered attention because it is one of the most commonly used compost earthworms (Podolak et al., 2020). The regions of Turkey and Transcaucasia have been identified as the centers of origin for *D. veneta* (Omodeo & Rota, 1999). Notably, the genus *Dendrobaena* has been employed in only a limited number of studies concerning Cu toxicity in earthworms, all of which were conducted in artificially contaminated soils rather than in natural soil environments (review by Pelosi et al., 2024).

Thus, this study aimed to study Cu toxicity in *D. veneta* under specific conditions of monometallic soil contamination with Cu. The Cu toxicity thresholds obtained for *D. veneta* were compared with those for *E. andrei*, as reported in the study by Neaman et al. (unpublished manuscript) on a different mining site within the extensive Kargaly Cu deposits. Furthermore, this study assessed the effects of various soil Cu pools on earthworm responses.

While the selected study area is particularly significant for Russia, it is crucial to acknowledge that soil pollution represents a global challenge that undermines agricultural sustainability worldwide (review of Santa-Cruz et al., 2021a). A reduction in earthworm population density may result in the long-term degradation of agricultural productivity and soil properties. Earthworms play a vital role in the ecosystem by contributing to processes such as nutrient cycling and soil aggregate formation (Blouin et al., 2013). This underscores the broader significance of our study and extends its implications beyond the local context.

In summary, this study advances the field in three distinct ways:

1. Natural contamination context: It is among the very few investigations that establish Cu-toxicity thresholds for earthworms in soils naturally contaminated by a single metal, rather than in laboratory-spiked substrates.
2. Focus on *Dendrobaena*: It is one of the rare studies to explore Cu toxicity within the genus *Dendrobaena*; prior work on this taxon has been confined to artificially amended soils.
3. First value for *D. veneta*: It delivers the first reported Cu-toxicity threshold for *D. veneta*.

Materials and methods

Choice of study area

To address our research objectives, we selected a specific study area within the extensive Kargaly Cu deposit (López et al., 2003). We focused on an agricultural field with uneven sunflower growth, where chlorotic, stunted plants (see online

Table 1. Total soil contents of elements, soluble copper (Cu; 0.01 M $CaCl_2$-extractable) concentration in the soils under study, and the partition coefficient for Cu (K_d–Cu).

Sample	Total soil Cu, mg kg^{-1}							Soluble Cu, mg L^{-1}	K_d–Cu, L kg^{-1}
	Cu	As	Cr	Ni	Pb	Cd	Zn		
Contaminated soils									
U-1	8,080	12	55	64	15	1.2	48	0.052	156,587
U-2	6,648	16	55	66	15	1.4	49	0.044	151,100
U-3	7,364	20	59	61	17	1.2	48	0.088	84,061
U-4	5,498	15	61	63	14	1.3	48	0.097	56,684
U-5	10,216	19	60	65	13	1.1	51	0.056	182,433
U-6	5,870	17	62	66	14	1.2	49	0.046	127,329
U-7	6,044	22	61	65	16	1.6	48	0.065	93,268
U-8	2,036	12	55	63	11	0.93	49	0.128	15,876
U-9	2,113	14	58	57	12	0.86	48	0.075	28,246
U-10	1,953	11	60	56	10	0.71	63	0.194	10,072
U-11	2,160	10	64	64	18	0.43	56	0.064	34,012
U-12	1,894	11	65	65	11	0.49	55	0.078	24,432
U-13	1,616	11	67	63	11	0.59	55	0.056	28,708
U-14	1,763	9.3	61	61	11	0.58	53	0.053	33,017
U-15	1,236	11	61	56	11	0.52	51	0.065	19,015
U-16	1,035	8.6	60	55	9.7	0.41	50	0.055	18,912
U-17	948	11	60	53	8.4	0.24	52	0.123	7,718
U-18	813	5.7	57	48	8.7	0.28	47	0.040	20,363
M±SD	3,885 ± 2,887	13 ± 4.0	60 ± 3.1	61 ± 5.0	12 ± 2.7	0.80 ± 0.41	51 ± 3.8	0.077 ± 0.038	60,657 ± 55,582
Control soil (used for setting 100% at the dose–response curve)									
U-control	121	12	59	62	9.2	0.28	53	0.017	6,976
Control soil (used to fill in the second compartment of the container in the avoidance bioassay)									
M-control	27	6.1	50	39	7.2	0.00	40	0.096	281
Background soils in the studied area (Dovletyarova et al., 2023)									
–	59	12	59	61	12	0.43	59	–	–

Note. Total soil contents of elements in background soils in the area under study are also shown (Dovletyarova et al., 2023).

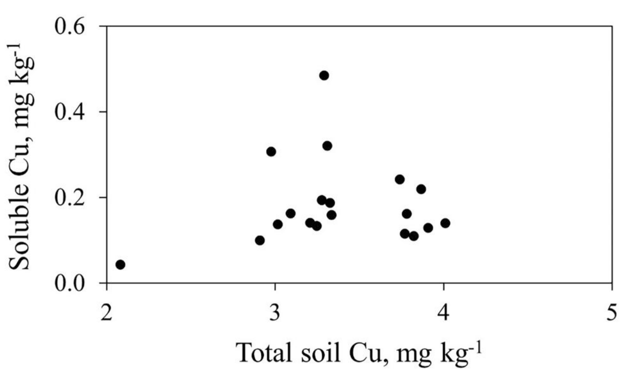

Figure 2. Concentration of soluble (0.01 M $CaCl_2$-extractable) copper (Cu) as a function of total soil Cu content.

supplementary material; Figure 2) suggested Cu toxicity (Marschner, 2003). Copper ore pieces were found on the soil surface, including malachite ($CuCO_3 \cdot Cu(OH)_2$), azurite ($2CuCO_3 \cdot Cu(OH)_2$), crednerite ($CuMnO_2$), chrysocolla ($(Cu, Al)_2H_2Si_2O_5(OH)_4 \cdot nH_2O$), and chalcopyrite ($CuFeS_2$; Neaman et al., unpublished manuscript).

One challenge in establishing metal toxicity thresholds in real-world contaminated soils is finding suitable uncontaminated control soils, where biological responses are considered 100% in dose–response curves. These control samples, which are usually sampled far from the contaminated site, can exhibit different soil physicochemical properties that affect biological responses. For example, the modern Mednogorsk Cu smelter in the Orenburg region impacted a vast area, requiring sampling of control soils approximately 50 km from the smelter (Polyakov et al., 2024). In contrast, historical mining activities in the Kargaly area have a limited spatial impact and are well characterized in archeological research (López et al., 2003). This allows the sampling of control soils within 100 m of the contaminated site, ensuring that soil samples have physicochemical properties that closely match those of the contaminated soils (Dovletyarova et al., 2023).

Study site

The area under study is known for its Cu deposit, where Cu mining and smelting occurred in two periods: the Bronze Age (4th–2nd millennia BC) and the XVII–XX centuries (López et al., 2003). Mining tools (sledgehammer and shovel) found at the site suggest activity during the XVII–XX centuries (Neaman et al., unpublished manuscript).

The study was conducted in a chernozem (Mollisol) agricultural field approximately 51 km northwest of Orenburg, Russia (52°13′53″ N, 55°0′9″ E) near the southern Urals. The site is near the Uranbash village, reflected by the letter "U" in the sample names. According to the farmers, no products were applied for pest or disease management, and weed control was performed mechanically without herbicides.

Topsoil samples (0–20 cm) were collected along a transect following a visible Cu toxicity gradient in sunflower plants (see online supplementary material; Figure 2). This sampling strategy follows the expert judgment-sampling approach (Pennock et al., 2008). Sampling began when the most severe plant growth decline was observed (sample U-1) and ended when growth seemed normal (sample U-18). The distance between the sampling points was 1–2 m, with a transect length of approximately 25 m. A total of 18 polluted topsoil samples were collected (Table 1). Additionally, a soil sample collected at approximately 100 m from the mining site (sample U-control) was used as the 100% value in the dose–response curves for earthworm responses.

Table 2. General chemical characteristics of the soils under study.

Sample	pH	EC, μS cm^{-1}	OM, %
Contaminated soils			
U-1	7.6	56	0.78
U-2	7.6	61	0.60
U-3	7.6	65	1.0
U-4	7.6	62	1.2
U-5	7.5	65	0.85
U-6	7.5	56	1.3
U-7	7.6	62	0.64
U-8	7.5	80	1.7
U-9	7.5	63	3.9
U-10	7.5	65	2.1
U-11	7.5	66	2.9
U-12	7.5	72	2.4
U-13	7.5	69	2.1
U-14	7.4	68	1.9
U-15	7.5	64	2.3
U-16	7.5	90	1.9
U-17	7.5	67	2.6
U-18	7.4	41	2.0
M±SD	7.5 ± 0.06	65 ± 9.7	1.8 ± 0.85
Control soil (used for setting 100% at the dose–response curve)			
U-control	7.6	75	3.2
Control soil (used to fill in the second compartment of the container in the avoidance bioassay)			
M-control	7.5	82	5.9

Note. The pH values were measured in 0.01 M $CaCl_2$ extract at a soil/solution ratio of 1/2.5, while electrical conductivity (EC) was measured in water extract at a soil/solution ratio of 1/5. The means and SDs of these values are shown. All the soils under study were calcareous and reacted with HCl. EC = electrical conductivity; OM = organic matter.

Finally, another unpolluted agricultural soil was sampled at the distance of 9 km (52°16′42″ N, 54°48′57″ E), referred to as "M-control" (Tables 1 and 2). In this field, there has been no known pesticide application at the site. Likewise, weeds were mechanically controlled at this field site. This soil was used as the control in the avoidance experiments, as described below.

Soil chemical analysis

Soil samples were air-dried, aggregates were broken in a porcelain mortar, and soils were sieved through a 2-mm sieve. Soluble (and potentially bioavailable) metal fractions in soil are usually evaluated using chemically nonaggressive neutral salts. Kim et al. (2015) noted that 0.01 M $CaCl_2$ and 1 M NH_4NO_3 are commonly used extractants for this purpose. They recommended 0.01 M $CaCl_2$ over 1 M NH_4NO_3 because: (a) its ionic strength closely resembles pore water, (b) its low salt concentration minimizes analytical interference during inductively coupled plasma (ICP) analysis, and (c) Ca^{2+} is more effective than NH^{4+} at displacing divalent metals from exchange sites. We selected 0.01 M $CaCl_2$ solution to estimate the soluble fraction of Cu in the soil. This Cu fraction is referred to as "soluble Cu."

Specifically, a 0.01-M $CaCl_2$ solution was used at a soil/solution ratio of 1/2.5. Cu concentrations were quantified using an ICP optical emission spectrometer (ICP-OES, Agilent 5110, Malaysia). The 0.01-M $CaCl_2$ extract was also used to measure pH. A water extract at a soil/solution ratio of 1/5 was used to measure electrical conductivity. In both cases, the suspension was stirred for 60 min and then filtered through an ashless filter paper. Wet combustion using potassium dichromate and sulfuric acid (Sadzawka et al., 2006) was used to determine soil organic matter content (Table 2).

The same ICP-OES instrument measured the total contents of As, Cd, Cu, Cr, Ni, Pb, and Zn. Soil digestion was performed using a Milestone microwave system (the United States). The following protocol was used (Russian Federal Register FR 1.31.2009.06787): 0.25 g of soil, *aqua regia* (2 mL HNO_3, 6 mL HCl), and 2 mL H_2O_2. Standard Krasnozem and Chernozem reference materials (Ecolan, Russia) were used throughout the analysis. The experimental values were within 100 ± 10% of the certified values for Cu, and 100 ± 20% of the certified values for other trace elements.

Earthworm responses

The earthworm, *D. veneta* utilized in the study (Figure 1), was obtained from a reputable earthworm farm (EcoWorm, Ekaterinburg, Russia), where it was fed with vegetable residues. It is a medium-sized worm with vivid red-violet stripes. The following description was used to identify this species (Vsevolodova-Perel, 1997): prostomium epilobous half closed, first dorsal pore at intersegmental furrow 5/6, setae distant, clitellum starting at segments 26 or 27 and ending at segment 33, and tubercula pubertatis on segments 30–31. However, the taxonomy of the genus *Dendrobaena* is not fully resolved because of the extensive genetic diversity of several species (Shekhovtsov et al., 2024). Thus, we used *D. veneta* for species identification sensu lato, following the recommendations of Szederjesi et al. (2019), however, it is difficult to identify with precision without genetic verification. Thus, developing a verified stock culture will be useful for future research using this species.

For the bioassays, we used adult earthworms with a well-developed clitellum. All earthworm bioassays were conducted in triplicate in a temperature-controlled room at 20 ± 2 °C, with illumination of 400 lx. The earthworm avoidance bioassay was performed according to a standardized protocol (ISO 17512–1, 2008; data are available in Table 2, see online supplementary material). One-liter plastic containers were divided vertically into two equal sections using a separator. One section contained the test soil, while the other contained control soil (M-control) with a low total Cu concentration (27 mg kg^{-1}, Table 1). Both soils were moistened with deionized water to 40% of their water-holding capacities. The separator was removed, and 10 adult earthworms were placed along the dividing line of each container. The earthworms were incubated under constant light for 48 hr and were free to migrate between the test and control soils. This avoidance test assessed the suitability of soil as a habitat for earthworms. After incubation, the separator was reinserted and the earthworms in each section were counted. Earthworms injured during separator reinsertion were counted as 0.5, regardless of their length.

The earthworm reproduction bioassay followed the protocol in ISO 11268–2 (2012; see online supplementary material; Table 2). For each test, 600 g of soil was placed in a 2-L plastic container and moistened with deionized water to 40% of the soil's water-holding capacity, which was maintained throughout the bioassay, with a 12:12-hr light: dark photoperiod. Ten adult earthworms were introduced into each container and fed with fresh and blended banana peel spread on the soil surface.

After 4 weeks, adult earthworms were removed, and their survival was assessed. Although ISO 11268–2 (2012) recommends counting cocoons by hand sorting, it is difficult to nondestructively count cocoons with accuracy at the adult removal stage of earthworm reproduction bioassays (Bart et al., 2018). Therefore, we did not use the number of cocoons as a response variable. Instead, after removing adults, cocoons were left to incubate for four more weeks. The number of hatched juveniles was then counted by hand sorting. However, the weight of hatched juveniles was not recorded.

Adult earthworms removed from the soils after 4 weeks of exposure were transferred to moist filter paper. Their guts were voided for 24 hr, with the filter paper changed every 6 hr (Arnold & Hodson, 2007). The earthworms were dried at 40 °C for 24 hr and digested in a Berghof (Germany) microwave, using the following protocol: 0.1 g of earthworms, 7 mL HNO_3+2 mL H_2O. Atomic absorption spectrometry (AAS 6 Vario, Analytic Jena, Germany) was used for the analysis. A standard reference material (bovine liver, BCR-185R, Community Bureau of Reference, European Commission) was used to obtain excellent experimental environmental values within 100 ± 5% of the certified Cu values.

Statistical analyses

Biological responses were charted based on soluble Cu concentrations, total soil Cu content, and Cu in earthworm tissues. The EC25 and EC50 were calculated using the threshold sigmoid model of the Toxicity Relationship Analysis Program (TRAP), Ver. 1.30a by the United States Environmental Protection Agency (USEPA, 2016).

The soluble Cu concentrations or total soil Cu content served as the dose variable, while earthworm responses (adult earthworm survival in the reproduction test, number of juveniles in the reproduction test, and number of earthworms in the soil under study in the avoidance test) served as the response variable. Similarly, the Cu content in earthworm tissues was used as the dose variable, whereas the aforementioned earthworm responses served as the response variable.

In both the reproduction and avoidance bioassays, the 100% response value was derived from the responses of earthworms exposed to the control soil U-control, which had the lowest total soil Cu content (121 mg kg^{-1}, Table 1).

Results and discussion

Soil characterization

Both the contaminated and control soil samples were calcareous, nonsaline, and had similar pH values and organic matter content (Table 2). The texture was clayey loam, as identified in the field by feel test (Thien, 1979). Thus, the soils exhibited similar physicochemical characteristics, allowing the unbiased use of earthworm responses for the control soil (U-control) as the 100% value in the dose–response curves.

Soils polluted by mining had a high Cu content (up to ∼10,200 mg kg^{-1}; Table 1). Contents of other elements in all soil samples were similar to the control (sample U-Control, Table 1) and background soils (Table 1; Dovletyarova et al., 2023), confirming monometallic pollution. Therefore, the discussion focuses solely on Cu.

The concentration of soluble Cu did not follow the trend of the total Cu content in the soil (Figure 2). This can be explained by the different solubilities of the Cu-containing minerals present in the Cu ore at the study site. The solubility products of malachite and chalcopyrite are $K_{sp}=10^{-33}$ and $K_{sp}=10^{-49}$, respectively (Ball & Nordstrom, 1991). Malachite is several orders of magnitude more soluble than chalcopyrite. The solubility products of other minerals present in the Cu ore have intermediate values (Ball & Nordstrom, 1991). Thus, the heterogeneous mineralogical composition of the Cu ore can explain the absence of a relationship between soluble Cu and total Cu in the soils under study (Figure 2). Below, we discuss in detail how copper soil pools determined toxicity.

It is worth noting that the concentrations of soluble Cu in the soils under study were lower than 0.5 mg kg^{-1}. Unfortunately, among the available studies on Cu toxicity in earthworms in real-world contaminated soils worldwide (see online supplementary material; Table 1), none have reported Cu concentrations extracted by chemically nonaggressive neutral salts, hindering direct comparison with our results. Nevertheless, it can be concluded that soluble Cu concentrations in the soils of the area under study were relatively low compared to those in the proximity of the modern Mednogorsk Cu smelter in the Orenburg region (Polyakov et al., 2024). Due to soil acidification by the smelter (soil pH of 5.3 ± 1.3), soluble Cu concentrations in soils in its proximity were two orders of magnitude higher (up to 135 mg kg^{-1}) than in the present study.

Cu soil pools determining toxicity

The survival of adult *D. veneta* earthworms in the reproduction bioassay (Figure 3A–C) is an unreliable predictor of Cu toxicity, being in the range of 83%–100% in all soils under study. In contrast, the numbers of juveniles in the reproduction bioassay (range 0.0–3.0, Figure 3D) and earthworms in the avoidance bioassay (range 0.3–6.3, Figure 3G) were sensitive indicators of Cu toxicity.

The total soil Cu content emerged as a robust predictor of earthworm responses (Figure 3D and G). Conversely, the effect of soluble Cu on earthworm responses was not statistically significant ($p > 0.05$; Figure 3E and H). However, the mechanisms underlying these differences remain unclear. Nonetheless, other extractants may have determined the bioavailable Cu fractions in the study area. These results align with those of other studies (see online supplementary material; Table 1), showing that the total soil Cu content predicts earthworm responses in contaminated soils.

Cu content in earthworm tissues: an unreliable predictor of toxicity

Total soil Cu content was a poor predictor of Cu content in earthworm tissues (Figure 4A), whereas the effect of soluble Cu was not statistically significant (Figure 4B). The Cu content of earthworm tissues was an unreliable predictor of Cu toxicity (Figure 3F and I). This can be explained by the ability of earthworms to actively excrete assimilated Cu (Spurgeon & Hopkin, 1999). Although excretion efficiency decreases with increasing soil Cu content, it leads to an increased Cu content in earthworm tissues in high-Cu soils (Ávila et al., 2009), Cu content in earthworm tissues is an unreliable predictor of Cu toxicity for earthworms exposed to polluted soils (Ávila et al., 2009).

Nevertheless, metal excretion from earthworm tissues requires extra energy, which affects earthworm sexual maturity and juvenile production (Spurgeon & Hopkin, 1996). This mechanism explains the toxic effects of Cu on earthworm reproduction in polluted soil. Thus, earthworms under stress have less energy for reproduction.

Toxicity thresholds: comparison to other studies

As mentioned above, consistently higher metal toxicity was observed in plants and soil biota in artificially contaminated soils compared to real-world soils contaminated for decades (Santa-Cruz et al., 2021b). For this reason, the following discussion only considers real-world contaminated soils. Following Checkai et al. (2014) we averaged the effective concentration values across different species and endpoints, and our results demonstrated that the Cu toxicity thresholds derived for *D. veneta* (Table 3) were higher than those reported in other studies on Cu toxicity in earthworms in real-world contaminated soils (see online supplementary material; Table 1). It should be emphasized that the studies summarized in Table 1 (see online supplementary material) might differ in soil characteristics.

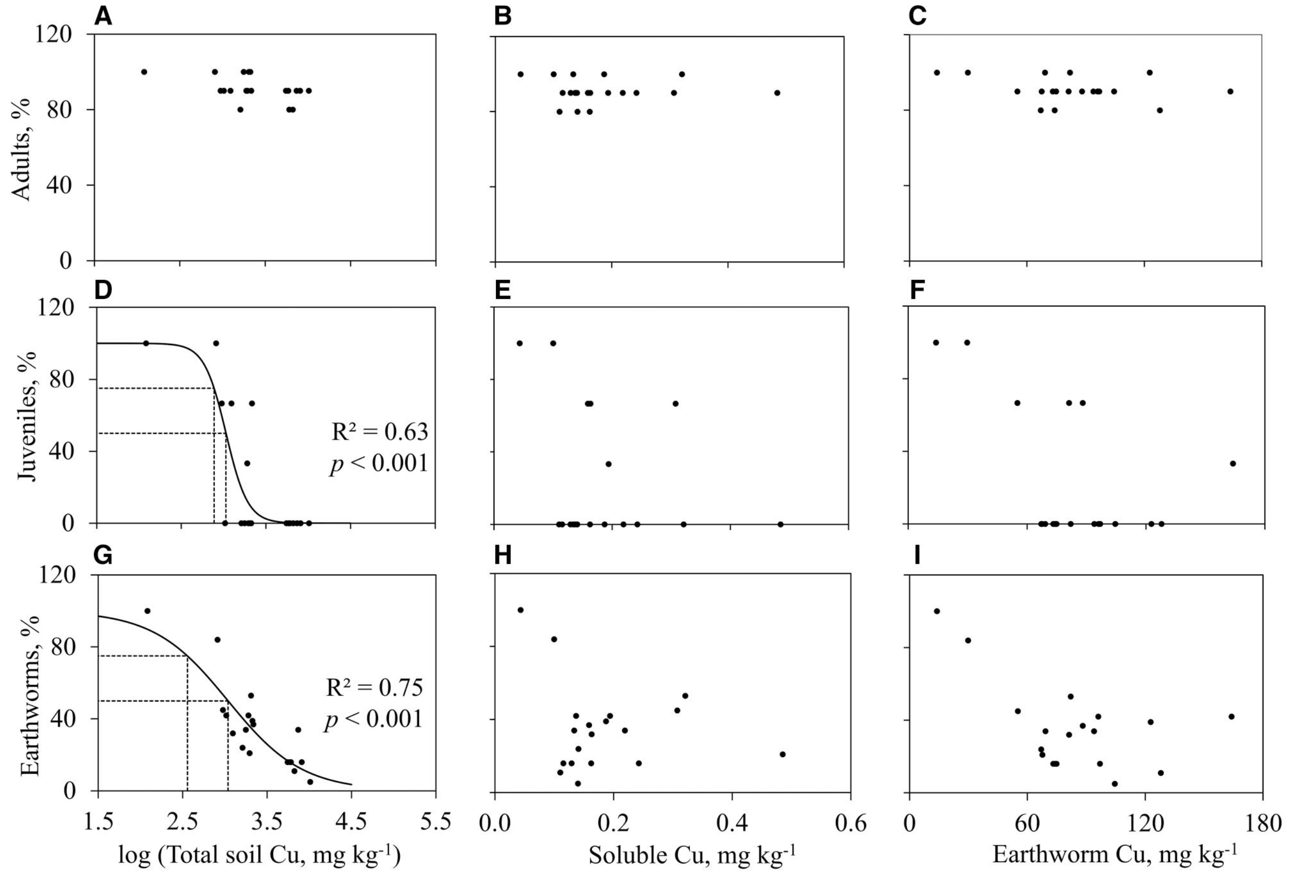

Figure 3. Earthworm responses to log total soil Cu, soluble (0.01 M $CaCl_2$-extractable) Cu, and Cu content in earthworms' tissues. In the panels without dose-response curves, the effects were not statistically significant ($p > 0.05$). Panels with dose-response curves display the effect concentrations at 25% (EC25) and 50% (EC50). a, b, c: adult earthworm survival (reproduction test); d, e, f: the number of juveniles (reproduction test); g, h, i: the number of earthworms in the soil under study (avoidance test).

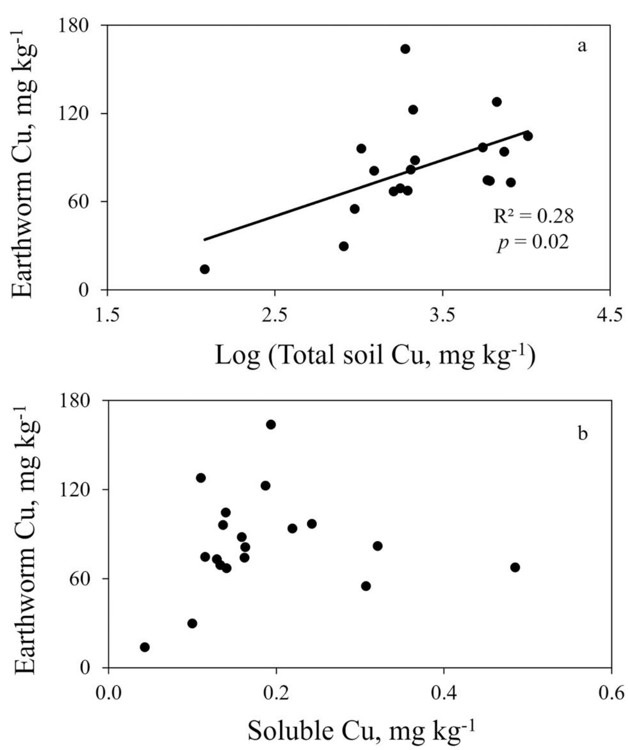

Figure 4. Impact of (a) log total soil Cu ($R^2 = 0.28$, $p = 0.02$) and (b) soluble soil (Cu; $p > 0.05$) on Cu content in the tissues of earthworms exposed to soils under study.

Unfortunately, the limited number of studies (eight in total) does not allow us to draw any generalizations and necessitates more research.

However, it should be emphasized that the Cu toxicity thresholds derived for *D. veneta* were of the same order of magnitude as those obtained for *E. andrei* in the study by Neaman et al. (unpublished manuscript) at another mining site in an extensive area of the Kargaly Cu deposits (Table 3). These observations can be explained as follows:

Under alkaline conditions, the Cu ores present in the studied soils were expected to have low solubility. The partition coefficient (K_d) indicates the metal solubility in contaminated soils, defined as the ratio between the total Cu content in the soil (mg kg^{-1}) and the concentration of extractable Cu (mg L^{-1}). Notably, higher K_d–Cu values correspond to the lower solubility of the Cu-containing phases. The studied mining-impacted soils exhibited a mean K_d–Cu value of approximately 60,600 L kg^{-1}, indicating the very low solubility of Cu-containing phases (Sauvé et al., 2000). Similarly, Neaman et al. (unpublished manuscript) found that mining-impacted soils at another Kargaly Cu deposit site had a mean K_d–Cu value of approximately 13,000 L kg^{-1}, indicating very low Cu-containing phase solubility.

To date, the published studies assessing copper toxicity to earthworms in real-world contaminated soils worldwide (see online supplementary material; Table 1) have not reported soil-solution partition coefficients (K_d–Cu). We, therefore, urge the soil-ecotoxicology community to routinely determine and publish metal soil-solution partition coefficients, because this will

Table 3. Effect concentrations at 25% and 50% (EC25 and EC50) of total soil copper (Cu) for reproduction and avoidance tests in *Eisenia andrei* (Neaman et al., unpublished manuscript) and *Dendrobaena veneta* (this study).

Earthworms	Reproduction test		Avoidance test	
	EC25	EC50	EC25	EC50
Eisenia andrei	177 (102–288)	407 (292–553)	783 (356–1,143)	1,603 (1,112–2,024)
Dendrobaena veneta	771 (483–1,230)	1,066 (804–1,414)	359 (163–791)	1,090 (746–1,593)

Note. The confidence intervals are presented in parentheses. Both studies were conducted in the Kargaly Cu deposit.

substantially advance our understanding of the variables governing metal ecotoxicity in real-world soils.

Conclusions and future research needs

The Kargaly site in Russia's Orenburg region is a rare case of monometallic Cu soil pollution. This study established Cu toxicity thresholds for *D. veneta*, with total soil Cu content reliably predicting earthworm responses, unlike soluble Cu. This highlights the long-term effects of Cu on earthworms but is limited to one agricultural field. Given Kargaly's expansive Cu deposits (~1,500 km^2; López et al., 2003), future studies should explore other similarly affected sites. This research represents a fundamental step toward a broader exploration of Cu ecotoxicity in Kargaly. Soil properties, such as organic matter, clay content, cation exchange capacity, and pH, affect Cu ecotoxicity (Broos et al., 2007; Rooney et al., 2006), but this study did not address these effects. Exploring Kargaly sites with varied soil properties could enhance our understanding of their impacts on Cu ecotoxicity. Future studies on soils polluted by a single metal could improve comprehension of metal toxicity for earthworms under diverse edapho-climatic conditions.

As mentioned above, the extensive utilization of *Eisenia* earthworms in ecotoxicological research is attributed to its ease of cultivation under laboratory conditions. This study suggests that *D. veneta* is another convenient earthworm species for assessing metal toxicity in contaminated soils. Future studies should directly compare the sensitivity to metals of *Eisenia* species and *D. veneta*.

Supplementary material

Supplementary material is available online at *Environmental Toxicology and Chemistry*.

Data availability

The data underlying this article are available in the article and in its online supplementary material.

Author contributions

Elvira Dovletyarova (Conceptualization, Funding acquisition, Project administration, Resources), Felipe Tapia-Pizarro (Data curation, Formal analysis, Visualization), Alexander Ermakov (Investigation, Methodology), Dina V. Nesterkova (Investigation, Methodology), Evgenii Vorobeichik (Conceptualization, Resources, Supervision, Validation, Writing—review & editing), Dmitry Polyakov (Investigation, Methodology, Resources), and Carolina Yáñez (Software, Validation, Writing—original draft), and Alexander Neaman (Conceptualization, Investigation, Methodology, Project administration, Supervision, Visualization, Writing—original draft, Writing—review & editing).

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Conflicts of interest

None declared.

Ethical statement

Russian legislation indicates that approval from an Institutional Animal Care and Use Committee is not required for research on earthworms.

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