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RESEARCH ARTICLE

Comparative sensitivity of earthworms and microorganisms as bioindicators of copper toxicity at a monometallic contamination site

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Abstract

A. Neaman, F. Tapia-Pizarro, E. V. Kozlova, M. N. Vasilyeva, M. V. Korneykova, A. A. Chaporgina, A. I. Ermakov, E. L. Vorobeichik, D. G. Polyakov, and C. Yáñez. 2025. Comparative sensitivity of earthworms and microorganisms as bioindicators of copper toxicity at a monometallic contamination site. Int. J. Agric. Nat. Resour. 79-91. Ecotoxicological research often relies predominantly on artificially contaminated soils, with studies on real-world contaminated soils remaining scarce. This study focuses on the Kargaly site in the Orenburg region of Russia, a rare instance of monometallic soil pollution by copper (Cu). The similarity of other elements in the soil samples to background levels highlights the uniqueness of this monometallic contamination. We established Cu toxicity thresholds for soil microorganisms and earthworms using soils collected along a Cu toxicity gradient in a chernozem (Mollisol) agricultural field. The total soil Cu predicted earthworm responses as effectively as did 0.01 M $CaCl_2$-extractable Cu. While total soil Cu strongly predicted microbiological responses, 0.01 M $CaCl_2$-extractable Cu was a poor predictor for microorganisms. The effective concentrations of total soil Cu for earthworms at 25% (EC_{25}) and 50% (EC_{50}) were 480 and 1005 mg kg^{-1}, respectively, compared to 4,570 and 7,797 mg kg^{-1} for microorganisms, respectively. Similarly, the EC_{25} and EC_{50} of 0.01 M $CaCl_2$-extractable Cu were 14 and 46 µg L^{-1} for earthworms, respectively, although 0.01 M $CaCl_2$-extractable Cu did not predict microbial toxicity well. Overall, earthworms were more sensitive to Cu than were microorganisms. This study is among the few that estimate Cu toxicity thresholds in real-world contaminated soils rather than artificially spiked soils.

Keywords: pollution; ecotoxicity; bioavailability; heavy metals.

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Highlights

- Copper was the main contaminant at study site
- Derived EC_{25} and EC_{50} for earthworm and microbiological responses
- Total soil Cu concentration strongly predicted biological responses
- Soluble Cu was a poor predictor of microbiological responses
- Earthworms are more sensitive to Cu than microorganisms

Introduction

Current ecotoxicological studies focus predominantly 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, offer a more accurate representation of toxicity effects. Environmental toxicology experts have emphasized that this prolonged exposure, known as "aging", significantly influences toxicity outcomes (Martínez & McBride, 2001). This time-dependent nature of metal toxicity is not replicated in metal-spiked soils, resulting in the toxicity observed in plants and soil biota in artificially contaminated soils being consistently greater than that in real-world soils contaminated for decades (Neaman et al., 2020).

Scientific research on real-world soil contamination remains limited, with few studies establishing ecotoxicity threshold values for soil metal contents (Santa-Cruz et al., 2021). The limited number of studies hinders the ability to draw generalizable conclusions about the impact of copper (Cu) contamination on soil biota. Therefore, further research on this critical topic is both novel and necessary.

It is widely recognized that the total metal content in polluted soil may not be appropriate for forecasting potential toxicity to plants and soil biota. For this reason, several studies have endeavored to estimate the "bioavailable" metal fraction in soil by linking organism responses with various metal pools. However, few studies have established toxicity thresholds for bioavailable Cu pools for earthworms and microorganisms using real-world contaminated soils. We identified only the study of Konečný et al. (2014) on potworms and the studies of Aponte et al. (2021) and Yáñez et al. (2022) on soil microorganisms. Therefore, further research comparing the effects of different soil Cu pools on organismal responses is essential.

High metal contents in soils can disrupt soil ecosystems, prompting researchers to identify the most sensitive bioassays for detecting early signs of toxicity. However, studies on real-world contaminated soils that compare the sensitivity of different types of organisms as bioindicators of Cu toxicity are lacking. For example, the study of Naveed et al. (2014) demonstrated that earthworms exhibit greater sensitivity to Cu than do bacteria, nematodes, and fungi. These findings underscore the need for additional research to evaluate the sensitivity of diverse organisms as indicators of metal toxicity in anthropogenically contaminated soils. In this study, we compared the relative sensitivity of earthworms and microorganisms as bioindicators of Cu toxicity. Additionally, we examined the effects of different soil Cu pools on organismal responses.

Materials and Methods

Choice of study area

To effectively address our research objectives, it was essential to select a specific study area. One of the key challenges in determining metal toxicity thresholds in real-world contaminated soils lies in the presence of multiple metals, which can confound result interpretation and complicate the attribution of toxic effects to individual metals. Notably, Cu, arsenic (As), cadmium (Cd), lead (Pb), and zinc (Zn) belong to the group of chalcophile elements, known for their strong affinity for sulfur. Consequently, their concurrent presence in Cu ores is expected and has been documented in previous studies worldwide (Polyakov et al., 2024). Hence, detecting soils polluted predominantly with a single metal is valuable for examining the ecotoxicological impacts of that metal on soil organisms.

Recently, Dovletyarova et al. (2023b) identified a suitable area for investigating Cu phytotoxicity at the Kargaly site in the Orenburg region of Russia (Supplementary Figure 1). The soil samples in the Dovletyarova et al. (2023b) study presented high total Cu contents, reaching ~10,000 mg kg^{-1} (here and below, we use commas as thousand separators for values ≥1000). Notably, the contents of other elements in all soil samples were comparable to those in the background soils, underscoring the distinct monometallic contamination in this study area.

Given the extensive area of the Kargaly Cu deposits (Garcia et al., 2010), it was necessary to select a specific site for the study. We focused on an agricultural field displaying uneven sunflower growth (Supplementary Figure 2), where chlorotic, stunted plants suggested Cu toxicity. The proximity to nearby mine tailings (Supplementary Figure 3) supported the likelihood of Cu pollution. The presence of Cu ore, including malachite ($CuCO_3 \cdot Cu(OH)_2$), azurite ($2CuCO_3 \cdot Cu(OH)_2$), and chalcocite (Cu_2S), on the tailings surface further confirmed contamination (Supplementary Figure 4). In the study of Dovletyarova et al. (2024b), Cu toxicity thresholds were established for sunflower. In the present study, we chose to use the same soil set to determine the Cu toxicity thresholds for microorganisms and earthworms, capitalizing on the unique characteristics of this monometallic contamination site.

Another challenge in establishing metal toxicity thresholds in real-world contaminated soils is finding suitable uncontaminated control soils, in which the biological responses are considered 100% in the dose-response curves. These control samples are usually collected far from the contaminated site and thus can exhibit different soil physicochemical properties that can affect biological responses. For example, the modern Mednogorsk Cu smelter in the Orenburg region impacts a vast area, which would require sampling control soils from a distance of approximately 50 km from the smelter (Polyakov et al., 2024). In contrast, historical mining activities in the Kargaly area are known to have a limited spatial impact, which is well characterized in archeological research (Garcia et al., 2010). This allows the sampling of control soils within 100-200 m of the contaminated site, ensuring that the soil samples have physicochemical properties closely matching those of the contaminated soils (Dovletyarova et al., 2023b).

Study site

The study was conducted in a chernozem (Mollisol) agricultural field located approximately 40 km north-northwest of Orenburg, Russia (52° 9' 29" N, 54° 57' 2" E; Supplementary Figure 1). According to the Köppen climate classification (Beck et al., 2018), the study area has a hot-summer humid continental climate (Dfa). The mean annual temperature is 6.1 °C, and the mean annual precipitation is 448 mm (https://en.climate-data.org/asia/russian-federation/orenburg-oblast/orenburg-475).

According to archeological research, the study site was polluted by tailings from Cu mining during the Bronze Age (Bogdanov, 2021; Karpova et al., 2019). According to the information provided by the farmer, no products were applied to the field for pest or disease management. Similarly, no herbicides were used, and weed control was performed mechanically.

Topsoil (0–20 cm) samples were collected along a transect following a visible gradient of Cu toxicity in sunflower plants (Dovletyarova et al., 2024b) (Supplementary Figure 2). This sampling strategy followed the "expert judgment sampling" approach (Pennock et al., 2008). Sampling started where the most severe decline in plant growth was observed (approximately 10 m from the tailings, Sample M4) and ended where plant growth seemed normal (Sample M14). A second transect was sampled, beginning approximately 10 m from the tailings (Sample M15) and ending with Sample M23 (Supplementary Table 1). The distance between sampling points was 1 to 2 m; the transect lengths were approximately 15-20 m. In total, 19 topsoil samples were collected. Additionally, two soils located approximately 200 m from the tailings were sampled as controls (Samples M14c and M23c), along with two samples directly from the tailings (Samples M1 and M2; Supplementary Table 1). We acknowledge that future studies in the Kargaly mining area would benefit from incorporating a larger number of control samples.

Soil chemical analysis

Soil samples were air dried. The soil aggregates were broken in a porcelain mortar. Then, the soils were sieved through a 2-mm sieve. The evaluation of "bioavailable" metal fractions in soil is usually carried out using chemically nonaggressive neutral salts. According to the comprehensive review of Kim et al. (2015), 0.01 M $CaCl_2$ and 1 M NH_4NO_3 are among the most commonly used extractants for this purpose. However, Kim et al. (2015) recommended 0.01 M $CaCl_2$ over 1 M NH_4NO_3 for several reasons: (1) the ionic strength of 0.01 M CaCl2 closely resembles that of pore water, (2) its low salt concentration minimizes analytical interferences during ICP analysis, and (3) Ca^{2+} is more effective than NH_4^+ at displacing divalent metals (such as Cu^{2+}) from exchange sites. We selected 0.01 M CaCl2 to estimate the bioavailable fraction of Cu in the soils. Nevertheless, we recognize the potential applicability of other extractants for determining bioavailable copper fractions in the study area.

Specifically, a 0.01 M $CaCl_2$ solution was used at a soil/solution ratio of 1/2.5. Copper concentrations were quantified using an inductively coupled plasma optical emission spectrometer (Agilent 5110, Malaysia). In the following discussion, this Cu fraction is referred to as "0.01 M $CaCl_2$-extractable Cu". 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 minutes and then filtered through ashless filter paper. Wet combustion using potassium dichromate and sulfuric acid (Sadzawka et al., 2006) was used to determine the soil organic matter (SOM) content (Supplementary Table 2).

The same ICP-OES instrument was used to measure the total contents of As, Cd, Cu, Cr, Ni, Pb, and Zn. Soil digestion was performed with a Milestone (USA) microwave. The following protocol was used (Russian Federal Register FR 1.31.2009.06787): 0.25 g of soil, *aqua regia* (2 mL of HNO_3 and 6 mL of HCl), and 2 mL of 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 within 100 ± 20% of the certified values for other trace elements. The same ICP-OES instrument was used to quantify the Cu concentration in the 0.01 M $CaCl_2$ extract.

Earthworm responses

The earthworm *Eisenia andrei* is widely used as a bioindicator organism for metal toxicity assessment. The ease of culturing *E. andrei* under laboratory conditions makes it a convenient species for metal toxicity testing.

A comprehensive review by Pelosi et al. (2024) of the toxicity of Cu to earthworms in artificially contaminated soils demonstrated that the sensitivities of *E. andrei* and *E. fetida* to Cu are similar. Thus, *E. fetida* can be used as a proxy. In the study by Duque et al. (2023), compared with other earthworm species, *E. fetida* presented intermediate sensitivity to copper. However, additional studies may be needed to further assess its relative sensitivity, for example, using the species sensitivity distributions approach (Posthuma et al., 2001).

The *E. andrei* earthworms utilized in the study were obtained from a reputable earthworm farm (Ecoworm, Ekaterinburg, Russia). For the bioassays, we used adult earthworms with a well-developed clitellum and a fresh weight in the range of 0.36-0.52 g. 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 the standardized protocol (ISO 17512-1, 2008), whereas the earthworm reproduction bioassay followed the standardized protocol outlined in ISO 11268-2 (2012). The detailed procedures of these bioassays are described in the Supplementary Material. Although the ISO 11268-2 (2012) protocol recommends counting the number of cocoons at this stage through hand sorting, it is well documented that cocoons are difficult to detect and separate manually (Bart et al., 2018). Therefore, we opted not to use the number of cocoons as a response variable. Instead, after the removal of adult earthworms, the cocoons were left in the soil to incubate for an additional four weeks. At the end of this period, the number of juveniles that hatched from the cocoons was counted via hand sorting.Microbial responses

For microbiological analyses, approximately 100 g of composite soil sample was collected at each sampling point. The samples were stored in the dark at 4 °C (ISO 10381-6, 2009). This temperature was suitable for the selected analyses, as no DNA or RNA assessments were planned. To prevent anaerobic conditions, the samples were stored in loosely tied plastic bags. Microbiological analysis was performed shortly after sampling to minimize potential changes in microbial activity during storage.

In this study, we chose to focus on community-level physiological profiles, basal respiration, and substrate-induced respiration due to the feasibility of conducting these analyses in our laboratory. The detailed procedures of these bioassays are described in the Supplementary Material. However, future studies are needed to examine the utility of other microbiological responses as indicators of Cu toxicity at the site under study.

Statistical analyses

Biological responses were charted as a function of 0.01 M $CaCl_2$-extractable Cu concentrations or total soil Cu contents. The 25% and 50% effective concentrations (EC_{25} and EC_{50}, respectively) were calculated using the REGTOX macro for Microsoft Excel™ (version 7.0.7, © Eric Vindimian, 2001).

The 0.01 M $CaCl_2$-extractable Cu concentration or total soil Cu content was used as the dose variable, whereas the organismal response served as the response variable. The 100% response value was derived from the average responses of the organisms exposed to the control soil samples M14c and M23c, which presented the lowest total soil Cu contents (27 and 26 mg kg^{-1}, respectively; Supplementary Table 1).

We performed backward multiple regressions using 0.01 M $CaCl_2$-extractable Cu as the dependent variable and soil properties (total soil Cu, soil organic matter, and soil pH) as independent variables. This approach followed the model proposed by McBride et al. (1997) to assess controlling metal solubility in contaminated soils:

log 0.01 M $CaCl_2$-extractable Cu = a log total Cu + b log soil organic matter + c pH + d (Equation 1)

where log is the decimal logarithm; a, b, and c are the coefficients; and d is the intercept.

Results and Discussion

Soil characterization

Both the contaminated and control soil samples were nonsaline and calcareous (Supplementary Table 2). The pH values were 7.6 ± 0.14 in the contaminated soils and 7.5 ± 0.55 in the control soils. The organic matter content was 3.2 ± 0.92% and 3.2 ± 0.20% in the contaminated and control soil samples, respectively. The texture of all soils, determined to be clayey loam, was confirmed through manual texture assessment in the field. Thus, the contaminated and control soils presented similar physicochemical characteristics, allowing an unbiased comparison of Cu toxicity between these soils.

The soils polluted with tailings presented high Cu contents (up to approximately 5,500 mg kg^{-1}), whereas the mine tailings presented even higher Cu contents (up to approximately 12,000 mg kg^{-1}; Supplementary Table 1). The contents of other elements in all the tailings and soil samples were comparable to those in the background soils, confirming the monometallic nature of the pollution (Dovletyarova et al., 2023b). Therefore, the following discussion focuses solely on Cu.

With respect to the soil properties controlling Cu solubility (Equation 1), the backward regression algorithm removed pH as a factor. All remaining variables were highly statistically significant ($p<0.001$):

log 0.01 M $CaCl_2$-extractable Cu = –2.8 + 0.56 log total Cu + 1.3 log SOM (R^2=0.89) (Equation 2)

where log is the decimal logarithm, 0.01 M $CaCl_2$-extractable and total Cu are expressed in mg kg^{-1}, and soil organic matter (SOM) is expressed in %. The Pearson correlation between log total Cu and log soil organic matter was not statistically significant ($p>0.05$), allowing these variables to be incorporated into the model without concerns about multicollinearity. Notably, the Pearson correlation between pH and log-transformed soil organic matter was not statistically significant ($p>0.05$), allowing us to examine the effect of soil organic matter without the potential confounding influence of pH.

It is important to highlight the positive coefficient for soil organic matter in Equation 2. It is well established that Cu concentrations in soil solutions decrease as the pH increases to ~7 but increase when the pH exceeds this threshold (McBride et al., 1997). This occurs because the concentration of dissolved organic carbon in the soil solution increases at pH values above ~7, which subsequently increases the concentration of Cu due to the formation of dissolved organic complexes.

Determining the toxicity of copper in soil pools

Earthworm survival in the reproduction bioassay (Figures 1a and 1b) and average well color development in the MicroResp™ technique (Figures 2e and 2f) were unreliable predictors of Cu toxicity. In contrast, other earthworm responses, such as the number of juveniles in the reproduction bioassay and the number of earthworms in the avoidance bioassay, along with microbiological responses (basal and substrate-induced respiration), were found to be sensitive indicators of Cu toxicity.

The total soil Cu content emerged as a robust predictor of both earthworm (Figures 1a, 1c, and 1e) and microbiological (Figures 2a, 2c, and 2e) responses. Conversely, 0.01 M $CaCl_2$-extractable Cu poorly predicted the microbiological response (Figure 2b, 2d, and 2f) but effectively predicted the earthworm response (Figure 1b, 1d, and 1f). The exact underlying mechanisms driving these differences remain unclear.

These results are consistent with the findings of Konečný et al. (2014) and Yáñez et al. (2022), who demonstrated that the total soil Cu content could predict potworm and microbiological responses as effectively as extractable Cu, i.e., extracted by 0.05 M EDTA in the study of Konečný et al. (2014) or 0.1 M KNO_3 in the study of Yáñez et

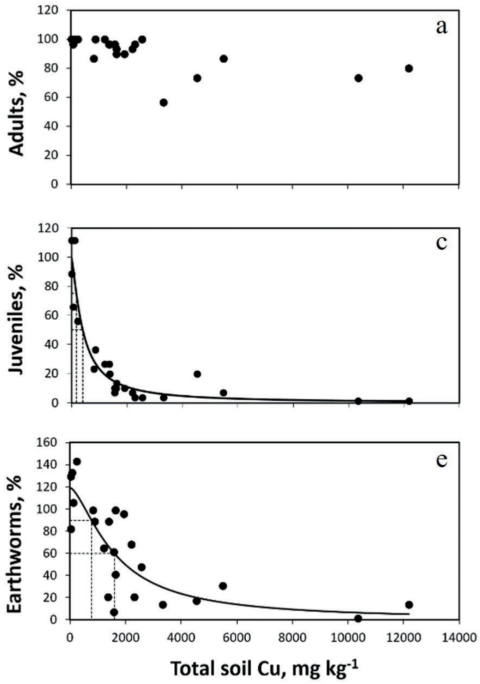

Figure 1. Earthworm responses to total soil copper and 0.01 M $CaCl_2$-extractable copper concentrations. In the panels without dose-response curves, the effect of the soil copper pool on earthworm responses was not statistically significant (p>0.05). Panels with dose-response curves display the effective concentrations at 25% (EC_{25}) and 50% (EC_{50}). a, b: adult earthworm survival (reproduction test); c, d: the number of juveniles (reproduction test); e, f: the number of earthworms in the soil under study (avoidance test).

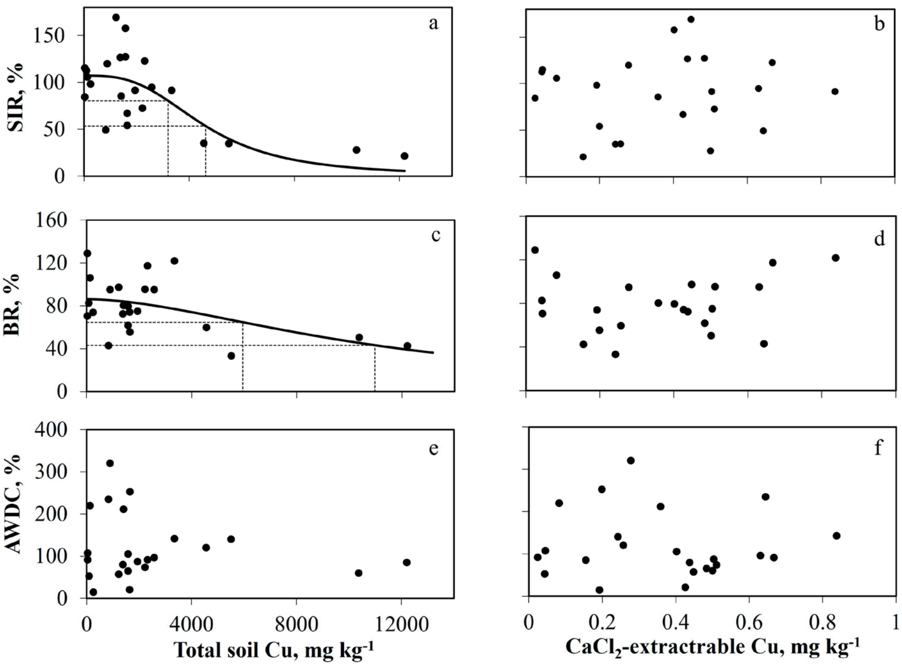

Figure 2. Microbiological responses to total soil copper and $CaCl_2$-extractable copper in soil extracts. For panels without dose-response curves, the effect of the soil copper pool on microbiological responses was not statistically significant (p>0.05). Panels with dose-response curves display the effective concentrations at 25% (EC_{25}) and 50% (EC_{50}). a, b: substrate-induced respiration (SIR); c, d: basal respiration (BR); e, f: average well color development (AWCD).

al. (2022). However, it is important to note that in the soil set used, simple linear regression between log 0.01 M $CaCl_2$-extractable Cu and log total Cu yielded an R^2 of 0.59 ($p<0.001$). This correlation between 0.01 M $CaCl_2$-extractable and total Cu may explain why both Cu pools effectively predicted ecotoxicity. These findings underscore the need for further research using soil sets where 0.01 M $CaCl_2$-extractable Cu concentrations are weakly correlated with the total soil Cu content.

Cu ecotoxicity thresholds: comparison with other studies

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. With this approach, the Cu toxicity thresholds derived in this study for earthworms and microorganisms (Table 1) were found to be one order of magnitude greater than those reported in other studies (Table 2). These discrepancies are explored in greater detail below.

Under alkaline conditions, the Cu ores present in the studied soils are expected to exhibit low solubility. The partition coefficient (K_d) serves as an indicator of metal solubility in contaminated soils. This coefficient is defined as the ratio between the total Cu content in the soil (expressed in mg kg^{-1}) and the concentration of extractable Cu (expressed in mg L^{-1}). Notably, higher K_d-Cu values correspond to lower solubilities of Cu-containing phases. Dovletyarova et al. (2024b) demonstrated that the partition coefficient of Cu is a reliable predictor of Cu phytotoxicity for sunflowers across different sites.

The studied tailing-impacted soils presented a mean K_d-Cu value of ~11,500 L kg^{-1}, indicating very low solubility of Cu-containing phases. In contrast, the studies by Arthur et al. (2012) and Yáñez et al. (2022) reported K_d-Cu values that were an order of magnitude lower than those reported in the present study (Supplementary Table 7), suggesting higher solubility of Cu-containing phases in their soils. This difference in solubility may explain why the EC_{50} values for total soil Cu affecting microorganisms in the studies by Arthur et al. (2012) and Yáñez et al. (2022) were also an order of magnitude lower than those determined in the present study (Supplementary Table 7). This indicates greater Cu toxicity for microorganisms in the soils studied by Arthur et al. (2012) and Yáñez et al. (2022) than in the soils of the present study.

Table 1. Effective concentrations at 25% and 50% (EC_{25} and EC_{50}) of total soil Cu and 0.01 M $CaCl_2$-extractable Cu for earthworm bioassays and microbiological responses. The 95% confidence intervals are shown in parentheses. The soluble (0.01 M $CaCl_2$-extractable) Cu concentrations are expressed in mg kg^{-1} and µg L^{-1} for ease of comparison with those used in future studies. Soluble Cu was a poor predictor of Cu toxicity for microorganisms. The raw data are available in Supplementary Table 3. Here and below, we used commas as thousand separators for values ≥1000.

Variables	EC_{25}	EC_{50}	EC_{25}	EC_{50}
	Reproduction test		Avoidance test	
Total soil Cu, mg kg^{-1}	177 (102-288)	407 (292-553)	783 (356-1143)	1,603 (1,112-2,024)
Soluble Cu, mg kg^{-1}	0.05 (0.02-0.09)	0.10 (0.05-0.15)	0.02 (0.004-0.25)	0.13 (0.04-0.50)
Soluble Cu, µg L^{-1}	20 (8-36)	40 (20-60)	8.0 (1.6-100)	52 (16-200)
	Substrate-induced respiration		Basal respiration	
Total soil Cu, mg kg^{-1}	3,198 (2,277-4,480)	4,619 (3,608-6,172)	5,942 (2,764-10,374)	10,975 (7,029-22,288)

Table 2. Summary of studies reporting effective concentrations (ECs) of Cu for earthworms and microorganisms. The values are presented as the means ± standard deviations, with medians in parentheses. For the EC_{10} values, the differences between organisms were not statistically significant (Dunn test, p>0.05). For the EC_{25} values, there were insufficient data to compare different groups. For the EC_{50} values, different letters indicate statistically significant differences between different groups on the basis of the Dunn test, p<0.05. "Extractable Cu" refers to the Cu pool extracted by 0.05 M EDTA in the study by Konečný et al. (2014) for earthworms or 0.1 M KNO_3 in the study by Yáñez et al. (2022) for microorganisms. The raw data can be found in Supplementary Tables 4-6.

Group of organisms	EC for total soil Cu, mg kg^{-1}			EC_{50} for extractable Cu, mg kg^{-1}	EC for extractable Cu, µg L^{-1}		
	EC_{10}	EC_{25}	EC_{50}		EC_{10}	EC_{25}	EC_{50}
Earthworms	123 ± 26 (110)	115	293 ± 96 (335) A	100	-	-	-
Microorganisms	515 ± 562 (170)	193–212	1,145 ± 941 (532) B	139	108	172	248

Although Supplementary Table 7 contains only three data points, it validates our arguments and underscores the need for future research in this area.

Importantly, in the study of Arthur et al. (2012), soils were contaminated by Cu due to the activities of a wood treatment factory, whereas in the study of Yáñez et al. (2022), soils were contaminated with Cu from mining activities. Despite these differing contamination sources, both studies reported similar K_d-Cu values and EC_{50} values for total soil Cu (Supplementary Table 7). This consistency suggests the need for further research to evaluate the potential of the Cu partition coefficient as a reliable predictor of Cu ecotoxicity in real-world Cu-contaminated soils.

Unfortunately, among the available studies on Cu toxicity in earthworms in real-world contaminated soils (Supplementary Table 4), only the study by Konečný et al. (2014) reported extractable Cu concentrations. This limitation prevents a comprehensive examination of the relationship between K_d-Cu values and EC_{50} values across studies. Therefore, we encourage colleagues in the field of soil ecotoxicology to include extractable metal concentrations in their research, facilitating a deeper understanding of the factors influencing metal ecotoxicity in real-world metal-contaminated soils.

Comparative sensitivity of earthworm and microbiological responses

The gross averaging of effective concentrations proposed by Checkai et al. (2014) overlooks the concept of a hierarchical cascade of biological responses to stressors, where resistance to metal-induced stress varies with the level of biological organization (Santa-Cruz et al., 2021). Typically, lower levels of organization (molecular, cellular, and individual) are more sensitive to stress than are higher levels (population and community). The review by Dovletyarova et al. (2023a) highlighted a decrease in metal toxicity thresholds with increasing levels of biological organization, following the pattern molecular < cellular < individual. Therefore, averaging the effective concentration values for response parameters at different levels of biological organization may misrepresent the true assessment of metal toxicity.

Most studies on Cu toxicity for earthworms in real-world contaminated soils (Supplementary Table 4) focus exclusively on individual-level responses. These limitations result in insufficient data for analyzing Cu toxicity across different levels of biological organization. In contrast, studies on Cu toxicity for microorganisms in real-world contaminated soils typically examine responses at the community level of biological organization. As a result, it is challenging to directly compare the sensitivity of earthworms and microorganisms to Cu within the same level of biological organization.

Nevertheless, the results from the available studies (Table 2) indicate that earthworms are more sensitive to Cu than are microorganisms, as shown by the mean EC_{50} values for soil total metal contents obtained across different studies (Dunn test, $p<0.05$). The findings of this study (Table 1) similarly demonstrate that earthworms exhibit greater sensitivity to Cu than do microorganisms. This conclusion can be partly explained by the fact that the available data on earthworm responses focus predominantly on the individual level of biological organization, whereas microorganism responses are assessed at the community level. As previously discussed, individual-level responses are generally more sensitive to metal toxicity than are community-level responses.

In summary, earthworm responses may provide more robust indicators of Cu toxicity compared to microbiological responses. This study aligns with previous research (e.g., Schoffer et al., 2024), confirming that earthworm responses are excellent bioindicators of Cu toxicity in contaminated soils. In contrast, microbiological responses appeared to be less reliable indicators of Cu ecotoxicity in the soils under study (Figure 2), echoing the conclusions of other studies on soils contaminated by Cu mining activities (Dovletyarova et al., 2024a; Yáñez et al., 2022).

Study limitations and future research directions

This study provides valuable insights into the long-term effects of Cu on earthworms and soil microorganisms, but its scope is limited to a single agricultural field. Given the expansive area of the Kargaly Cu deposits (~1500 km^2) (Garcia et al., 2010), future studies should extend to other sites within the region that are similarly affected by monometallic Cu contamination. This research serves as a foundational step toward deeper exploration of Cu ecotoxicity across the broader Kargaly deposit area.

Soil properties such as the soil organic matter content, clay content, cation exchange capacity, and pH can affect Cu ecotoxicity. However, the present study does not address these effects, as it was conducted in a single agricultural field with homogeneous soil properties. Therefore, it would be valuable to investigate other sites within the Kargaly deposit area that exhibit contrasting soil physicochemical properties to better understand the impacts of these properties on Cu ecotoxicity.

Conclusions

The Kargaly site in the Orenburg region of Russia is a rare case of monometallic Cu soil pollution. This study provides key insights by establishing Cu toxicity thresholds for earthworms and soil microorganisms in soils from this site. The total soil Cu content predicted earthworm responses as effectively as the 0.01 M $CaCl_2$-extractable Cu fraction did. Moreover, the total soil Cu content was a reliable predictor of microbiological responses, whereas 0.01 M $CaCl_2$-extractable Cu had no statistically significant relationship with these responses. Importantly, earthworms demonstrated greater sensitivity to Cu than did microorganisms, which aligns with the findings of previous studies and reinforces the robustness of these findings.

Due to the restricted availability of reports on metal toxicity thresholds in real-world polluted soils, future studies using soils predominantly polluted by a single metal could enhance our comprehension of metal toxicity for microorganisms and earthworms under varying edaphoclimatic conditions. This study contributes to that requirement by focusing on Cu toxicity thresholds in mining polluted soils rather than artificially spiked soils. The combined data on soil invertebrate and microbial responses provide valuable insights, underscoring the necessity for further ecotoxicological research in environments dominated by monometallic pollution.

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Contributions

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Data availability

The data are available in the Supplementary Material: https://zenodo.org/uploads/15694016

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