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Copper toxicity to earthworms: A comprehensive review and meta-analysis

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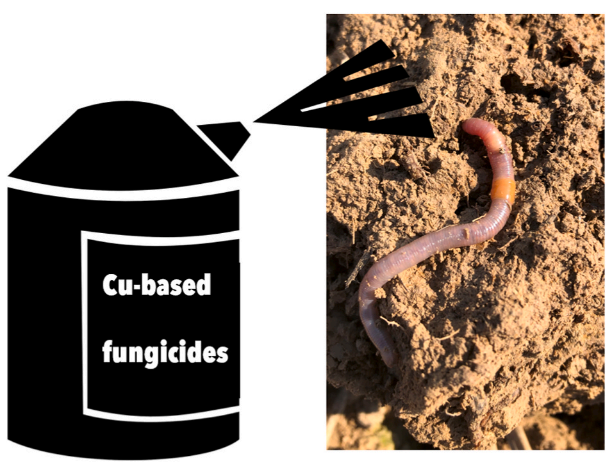
HIGHLIGHTS

- Hormesis occurs for earthworm growth at concentrations below 80 mg Cu kg^{-1} dry soil.
- But effects at the sub-individual level occur at lower concentrations.
- Critical values (LC_{50}, EC_{50} reproduction, and EC_{50} growth) of copper toxicity to earthworms are calculated.
- Earthworms were more sensitive to copper in natural than in artificial soils.
- Many factors influence Cu toxicity, making it difficult to derive thresholds.

GRAPHICAL ABSTRACT



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ABSTRACT

Copper can accumulate in agricultural topsoil through the use of Cu-based fungicides, which may harm soil organisms such as earthworms. This study aimed at reviewing the effects of copper on earthworms at different levels of biological organization, and to determine critical values of copper toxicity to earthworms using a meta-analysis and accounting for lethal and sub-lethal effects and different earthworm species and exposure conditions. Endpoints at the sub-individual level were more sensitive than at higher levels of organization. At the individual level, the most sensitive endpoints were reproduction and growth (hatching success, hatchling growth). Hormetic growth was clearly recognized at copper concentrations less than 80 mg kg^{-1} in dry soil.

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However, effects at the sub-individual level already occurred at lower concentrations. Considering all the exposure conditions, the calculated weighted means were 113 mg Cu kg^{-1} dry soil (95% CI -356; 582) for the LC_{50} (lethal concentration for 50% of the exposed individuals), 94.6 mg Cu kg^{-1} dry soil (95% CI 14.0; 175) for the EC_{50} reproduction, and 144 mg Cu kg^{-1} dry soil (95% CI -12.6; 301) for the EC_{50} growth or weight change. When accounting for the origin of the soil, earthworms were five times more sensitive to copper (LC_{50}) in natural than in artificial soils. The different factors affecting Cu toxicity to earthworms explain the high variability of these values, making it difficult to derive thresholds. However, considering the potential negative effects of copper on earthworms, attention should be given to the more sustainable use of human-contributed copper in agricultural soils.

1. Introduction

In recent decades, agricultural practices have led to strong soil degradation (Davies, 2017), accompanied by a decrease in biodiversity and organic matter (OM) content, and to soil contamination by different chemicals, such as organic pesticides and metals (Hou et al., 2020; Pelosi et al., 2020; Schoffer et al., 2020). Therefore, many calls have been made for an agroecological transition involving switching from conventional to more sustainable food production systems (Sachet et al., 2021). Alternative farming systems such as organic farming have been under study for several years. However, they are still under debate, primarily because copper (Cu) is used as a fungicide, mainly on perennial crops such as vineyards and orchards.

Copper is an oligo-element essential for plants and animals because it is a component of numerous enzymatic substances involved in critical biological processes. In the soil, it is found bound to organic complexes; adsorbed to clays; on oxides and hydroxides of iron, manganese, and aluminium; or contained in the crystalline lattice of minerals (ERSAF, 2007). In soils, copper is not very mobile except under acid conditions (e.g., <4) (Baize, 1997; Ginocchio et al., 2009). It is more prevalent in mafic and intermediate rocks rich in iron-manganese minerals but is present in deficient concentrations in carbonate rocks. Worldwide, the average concentration in soil is approximately 30 mg Cu kg^{-1} (Alloway, 1995), with lower values in sandy soils and higher values in clayey soils. Anthropogenic inputs of Cu in soils are primarily due to agricultural (pesticides, fungicides applied on vines and horticultural crops, zootechnical sewage), industrial (metallurgical activities linked to the production of zinc, lead, and cadmium) and urban (sewage sludge, compost) sources. As Cu is not degraded, it accumulates in soils, leading to elevated concentrations of Cu (Clasen et al., 2021; Karimi et al., 2021; Merrington, 2018; Schoffer et al., 2020), potentially harming soil organisms (Ge et al., 2023; Santa-Cruz et al., 2021a).

Earthworms represent the primary living biomass in soils and can be used as bioindicators of agricultural practices (Paoletti, 1999). They are also key actors in rehabilitating degraded soils because they provide key soil functions (e.g., organic matter (OM) fragmentation, nutrient cycling, soil structure dynamics) and are involved in the provision of essential ecosystem services (Geraskina, 2019; Liu et al., 2019; Zeb et al., 2020). Earthworms are particularly sensitive to copper, and many studies have shown adverse effects at different levels of biological organization (Bart et al., 2017; Paoletti et al., 1998; Uwizeyimana et al., 2017). Karimi et al. (2021), based on two studies performed under laboratory conditions with freshly spiked natural soils (i.e., Bart et al., 2017; Bogomolov et al., 1996), concluded that earthworm biomass was reduced by 15% after the application of 200 kg Cu ha^{-1} $year^{-1}$ (which corresponds to 155 mg Cu kg^{-1} dry soil when considering a penetration depth of 10 cm). Other studies have reported detrimental effects of copper concentrations above 200–250 mg kg^{-1} on earthworm populations in sandy agricultural soils (Klok et al., 2007; Ma, 1988), and Klok et al. (1997) even predicted the extinction of a *Lumbricus rubellus* population at copper concentrations in the range of 200–300 mg kg^{-1} in sandy loam soil. However, lower threshold values were also reported for copper toxicity to earthworms, either in laboratory experiments or through exposure in the field. For instance, van Rhee (1967) reported that earthworms were almost entirely eradicated from orchards at soil copper concentrations above 80 mg kg^{-1}. Similarly, Zhou et al. (2013) reported significant weight loss and reduced cocoon production in *Eisenia fetida* when exposed for 28 days to copper nitrate at 50 mg Cu kg^{-1} in an OECD artificial soil. Different soil types, copper forms and exposure conditions were used in these studies. All these factors may have led to differences in copper bioavailability. Therefore, a comprehensive assessment of the literature regarding these effects could help in better understanding and assessing the implications of agricultural copper use for earthworms.

The decline in earthworm populations observed in copper-treated fields could be due to a decrease in the reproductive rate (Duan et al., 2016; Malecki et al., 1982) and growth rate or potentially also to earthworms avoiding Cu-contaminated zones (Bart et al., 2017; Eijsackers et al., 2005). Moreover, the effects of copper on earthworms are influenced by the soil OM content (e.g., Helling et al., 2000), the Cu form (Arnold et al., 2003), and the sensitivity of different species or ecological categories (Paoletti et al., 1998). Although some information is available on the effects of copper on earthworms, data have never been gathered to determine critical values for different species or ecological categories (e.g., epigeic *vs.* endogeic), for different endpoints (i.e., mortality and sub-lethal endpoints), in different soils (i.e., freshly spiked natural *vs.* artificial soils), or for different Cu-based compounds (i.e., the Cu form).

The present study aimed to review the effects of copper on earthworms at different levels of biological organization and to determine the critical values of copper toxicity to earthworms, considering lethal and sub-lethal effects, different earthworm species and different exposure conditions. For this purpose, a systematic review of the literature was performed. For the quantitative analysis, a meta-analysis of the standard endpoints related to mortality, reproduction, and growth, which are commonly used in chemical risk assessment, was performed. The limitations of the study are also discussed (e.g., field-contaminated *vs.* freshly spiked soils, species tested), and directions for future research are provided.

2. Materials and methods

2.1. Literature search

A systematic literature review was conducted in July 2023 in the ISI Web of Knowledge using "All Databases" to find publications addressing earthworms and copper, and the key words "earthworm* or lumbricid* AND copper or cupric" were entered in Topics. The titles and abstracts were used to select articles from among a total of approximately 1220. To deepen the search, starting from the previously selected articles, we identified other authors who had written articles on the subject. We also examined books and other articles from journals of interest. Approximately 100 articles were gathered and used for the qualitative review. A subset of 30 articles was selected for the meta-analysis based on the specific criteria described in the next section. These articles were used to compile the database, resulting in 197 observations (Table S1).

2.2. Article selection

For the review, the selection criteria for articles and the final body of data were as follows:

- Effects of copper studied independently of other contaminants or factors; therefore, studies addressing effects of multiple contaminants or in which too many factors varied simultaneously were excluded.
- Soil treated as the environmental/experimental matrix; thus, studies using exposures to aqueous solutions or on filter paper were excluded.
- Reporting information on the soil total copper concentrations, for homogeneity between studies. Data on bioavailability could have been more informative, but they were not reported systematically.

For the meta-analysis, the following criteria were added:

- Providing data on LC_{50}, which represents the lethal concentration for 50% of the exposed individuals, as well as NOEC (no observed effect concentration), LOEC (lowest observed effect concentration), and EC_{10} or EC_{50} (10% or 50% effect concentration, respectively) (Table S1). A minimum of 3 articles and 10 observations were required to calculate the means and weighted means. As a consequence
  - → LOEC values (3 articles, 6 observations) were excluded.
  - → We used only data at the individual level (Section 3.2), as insufficient data were available on effects at the sub-individual level (e.g., endpoints calculated with molecular outputs, Section 3.1).
  - → For data at the individual level, endpoints related to mortality (17 studies, 87 observations: 85 LC_{50} and 2 NOEC values), reproduction (15 articles, 85 observations: 46 EC_{50}, 31 EC_{10}, and 8 NOEC values) and growth (11 articles, 25 observations: 16 EC50, 4 EC_{10} and 5 NOEC values) were considered. The data of Arnold et al. (2003) for malachite ($Cu_2(OH)_2(CO_3)$) were removed because (i) this Cu form is not comparable to commonly tested copper forms and (ii) extremely high LC_{50} and EC_{50} wt changes (i.e., 8700 and 1241 mg Cu kg^{-1} dry soil, respectively) were found that may be explained by the extremely low solubility of this Cu form.
  - → Data on avoidance were not considered (3 articles, 3 observations).
  - → For each endpoint, a maximum of three moderators were used according to the available data and the criteria mentioned above (a minimum of 3 articles and 10 observations): soil (freshly spiked natural or artificial), earthworms (epigeic or other), and copper form (copper nitrate, chloride or sulphate).
- Use of total Cu nominal doses rather than measured soil concentrations, as the latter were rarely reported in the literature. In only one case (i.e., Spurgeon et al., 2004), we used the measured concentration because the nominal dose was not given.

2.3. Data extraction

To compile the database (Table S1), the following data were extracted from each study: authors, year, soil (artificial or natural), origin of contamination (naturally contaminated or spiked), earthworm genus, species and ecological category, copper compound, endpoint (LC_{50}, EC_{10}, EC_{50}, and NOEC values), and confidence intervals (CIs) or standard deviations (SDs, if available). Each database row was called an observation and referred to a single endpoint value. For NOEC, no data on variance were reported, as it is the concentration at which no statistically significant effect was detected. In Bart et al. (2020), a SD is given as the NEC (no effect concentration) value, which was calibrated in an energy-based model, but this SD value was not considered here.

When the CI was given, the SD was calculated according to:

$$SD = \sqrt{n} * \frac{upperCI - lowerCI}{t_{alpha.df} * 2}$$

with $t_{alpha,df}$ being the T.INV.2T function syntax with the two associated arguments: (i) probability, associated with the Student's t-distribution, here alpha = 0.05 to respect the 95% CI from extracted data, and (ii) the degrees of freedom corresponding to (sample size-1) = (n-1) with which to characterize the distribution. When the number of replicates was not the same between the treatments and the control, the lower number was considered to be conservative. When the number of replicates was not given and the tests were performed according to ISO standards, we considered the number of replicates used was that advised by the corresponding official procedures. When neither the SD nor the 95% CI were given, the largest SD for each endpoint (LC_{50}, $EC_{10,}$ or EC_{50}) in the database was applied to be as conservative as possible.

The experimental duration varied among studies, so we considered all the studies independent of the duration. Data expressed in μmol Cu g^{-1} and were converted to mg Cu kg^{-1} using the molecular mass of copper (i.e., 63.546 g mol^{-1}).

2.4. Meta-analysis

All analyses were performed with R software (R Development Core Team, 2014) using the metafor package (Viechtbauer, 2010) and a multivariate linear model (rma.mv function) to calculate the meta-analysis outputs. Hedge's d metric was used to calculate the normalized mean effect sizes (Hedges and Olkin, 1985). The variance (V) was calculated as follows:

$$V = SD^2$$

Effect sizes in all models were adjusted by weighting them with the inverse of their variance, thus assigning greater importance to studies with robust replication (Koricheva et al., 2013). As no standard deviation are given for the NOEC values, no variance or weight associated with the data can be calculated. The weight for the NOEC values was therefore set to 1 for all observations.

Each model included a random effect linked to the study's identity to address correlated data arising from the same study (Viechtbauer, 2010). Three different Akaike information criterion (AIC) values were calculated for each mean effect size computation, with the three following tested random effects: 'Author' or 'Line' or 'Author + Line'. The AIC strikes a balance between reducing bias with the number of free parameters and achieving parsimony, and lower AIC values indicate a better-fit model. Therefore, for each calculation, the model with the smallest AIC value was chosen for the mean effect size calculation.

To detect publication bias, DOI plots and the LFK index were used (Furuya-Kanamori et al., 2018). This approach is increasingly used due to its higher sensitivity compared to the widely used Egger's regression. Major asymmetries were detected for the EC_{50} ('all endpoints') and LC50 ('all') data, with LFK indices of 5.27 and 5.5, respectively. Therefore, Orwin's failsafe number were calculated, corresponding to the number of null results needed to reduce the observed effect size (default value) by half. In our case, the Orwin's failsafe number for the EC_{50} ('all endpoints') and LC_{50} ('all') data were 62 and 85, respectively.

3. Results

3.1. Sub-individual level

Insufficient data were available on effects at the sub-individual level so this section gives an exhaustive overview of copper effects but no quantitative assessment (i.e. meta-analysis) could be done on sub-individual parameters. Effects of copper at the sub-individual level on earthworms have been studied to identify possible mechanisms of intoxication and biochemical and physiological responses indicative of exposure or effect. Transcriptomics (gene expression) and metabolomics (changes in metabolite profiles) are examples of the first aspect. Examples of the second include enzymes involved in oxidative stress responses, the neutral red retention time (NRRT) as a biomarker for effects on the stability of lysosomes in coelomocytes, and the comet assay to assess DNA damage.

Lumbricus rubellus exposed for 42 days in Kettering loam (pH 7.7, 10% OM) spiked with $CuCl_2$ presented increased expression of the gene encoding the metal-binding protein metallothionein isoform 2 (mt^{-2}) at 159 but not at 51.5 mg Cu kg^{-1} dry soil (corresponding with ~53.4 and ~29.7 mg Cu kg^{-1} dry body weight, respectively) (Galay-Burgos et al., 2003; Spurgeon et al., 2004). The expression of the mitochondrial large ribosomal subunit (l-rRNA), related to energy dynamics, was significantly reduced at 22.2 mg Cu kg^{-1} dry soil but increased at 159 mg Cu kg^{-1} dry soil, indicating that copper affects earthworm metabolic activity through an effect on mitochondrial function. Lysosome-associated glycoprotein (*lgp*) expression, which is indicative of lysosomal function and stability, was significantly reduced at 159 mg Cu kg^{-1} dry soil (Spurgeon et al., 2004). In L. *rubellus* exposed for 21 days to $CuCl_2$ in artificial soil (10% peat, 20% kaolin clay, 70% sand, pH 6.0), the expression of metallothioneins mt^{-1}, mt^{-2}, amine oxidase, and *lgp* was upregulated already at 3.9 and 18.0 mg Cu kg^{-1} dry soil (corresponding to 13.5 and 16.6 mg Cu kg^{-1} dry body weight in earthworms, respectively), but normal or reduced at higher concentrations (Galay-Burgos et al., 2005).

In a 70-day outdoor mesocosm study using Kettering loam soil spiked with $CuCl_2$, metallothioneins mt^{-2} expression in *L. rubellus* significantly increased only at the highest concentration (480 mg Cu kg^{-1} dry soil) at the end of the experiment (Spurgeon et al., 2005). However, a complementary omics approach (transcriptomics and metabolomics) carried out on earthworms from the same experiment revealed alterations in response to sub-lethal copper exposure (Bundy et al., 2008). The expression of genes associated with mitochondrial electron transport was clearly disrupted, suggesting copper-induced mitochondrial dysfunction. Alterations were also observed for genes related to lipid metabolism. Copper induced the expression of the genes encoding metallothionein, general toxic stress genes (hsp70 and hsp40) and genes involved in glutathione metabolism. Copper exposure also caused an alteration in the levels of DNA repair enzymes and enzymes implicated in cell cycle control, probably due to mitochondrial dysfunction, which could increase the generation of reactive oxygen species (ROS). The downregulation of apoptotic regulators was observed, suggesting that Cu causes cellular apoptosis. All these responses indicate plausible modes of cellular disruption, with a NOEC for microarray profiles of 10 mg Cu kg^{-1} dry soil (Bundy et al., 2008).

Yu et al. (2022) analysed both the transcriptomic and metabolomic data of *E. fetida* exposed for 60 days to artificial soil (pH 6.0) spiked with $CuSO_4$ at 0, 68, 169, and 338 mg Cu kg^{-1} dry soil. Mortality was 0, 3.1, 20.0, and 52.5%, and weight loss was 9.8, 21.0, 29.6 and 34.3%, respectively. The differentially enriched genes and metabolites showed dose-related increases compared to the values in the control. Transcriptomic analysis indicated significant effects on genes related to the immune system, energy metabolism, amino acid and lipid metabolism, the antioxidant system, and detoxification processes. Metabolomics indicated effects on amino acid and energy metabolism, confirming the results of the transcriptomic analysis.

A dose-related increased expression of metallothioneins was also detected in *E. fetida* after 28 days of exposure to artificial soil (pH-KCl 7.6) spiked with $CuSO_4$ at 2–20 mg Cu kg^{-1} dry soil, corresponding to ~9.5–16 mg Cu kg^{-1} dry body weight and assuming 20% dry weight. The expression of genes related to oxidative stress (catalase (CAT) and superoxide dismutase (SOD)) or general stress responses (hsp70 and hsp72) was not affected by copper exposure (Unrine et al., 2010). In *E. fetida* exposed for 5, 10, or 15 days to field soil (pH 6.2, 3.6% organic carbon, 14.4% clay) spiked with $CuSO_4$ at 50–400 mg Cu kg^{-1} dry soil, the expression of metallothioneins showed fluctuating patterns of up- and downregulation depending on the concentration and duration of exposure. Other studies have shown variation in the expression of hsp70 (Xiong et al., 2014). Depending on the exposure conditions, hsp70 was significantly reduced at 50 mg Cu kg^{-1} dry soil after 5, 10, and 15 days but was significantly reduced at 100 mg Cu kg^{-1} dry soil after 5 and 10 days, returned to normal after 15 days, decreased considerably after 5 days and significantly increased after 10 and 15 days at 400 mg Cu kg^{-1} dry soil (Xiong et al., 2014). Mincarelli et al. (2019) exposed *Eisenia andrei* for up to 9 days to a clay loam soil (pH 8.26, 37% clay, 1.72% OM) spiked with $CuSO_4$ at 120 mg Cu kg^{-1} dry soil. At this concentration, corresponding to 13.9 and 17.9 mg Cu kg^{-1} dry body weight in the earthworms after 6 and 9 days, respectively, the antimicrobial peptide fetidin, a Toll-like receptor (*tlr*), and metallothioneins were significantly upregulated. No effect was detected on genes involved in oxidative stress responses (CAT, SOD).

In addition to gene expression, the activity of enzymes involved in oxidative stress is useful for assessing the toxicity of copper. In *E. fetida* exposed for 28 days to artificial soil (pH 6.0) spiked with $Cu(NO_3)_2$, the activity of the oxidative stress response enzymes CAT and SOD decreased in a dose-related manner, while the malondialdehyde (MDA) concentration increased in a dose-related manner. The NOECs were <25 mg Cu kg^{-1} dry soil, corresponding to <18.8 mg kg^{-1} dry body weight in the earthworms (Zhou et al., 2013). After *E. fetida* were exposed for 14 days in artificial soil (pH 6.0) spiked with $Cu(NO_3)_2$, the activities of CAT and peroxidase (POD) and the level of glutathione (GSH) significantly increased at 15.9 mg Cu kg^{-1} dry soil, while SOD and total antioxidant capacity (T-AOC) exhibited synergistic effects only at higher concentrations (Zhang et al., 2020). Xiong et al. (2014) exposed *E. fetida* for 5–15 days to field soil (pH 6.2, 3.6% organic carbon, 14.4% clay) spiked with $CuSO_4$ at 50–400 mg Cu kg^{-1} dry soil. Except for glutathione-S-transferase (GST), the activity of all measured enzymes involved in oxidative stress responses (CAT, SOD, and glutathione peroxidase (GPx)) fluctuated with time and exposure concentration and were significant at > 50 mg Cu kg^{-1} dry soil. According to Xiong et al. (2014), variations in responses might be associated with compensatory processes between CAT and GPx. Gautam et al. (2018) reported dose-related effects on the generation of superoxide anions (increase), while nitric oxide, phenoloxidase activity, SOD, CAT, acid and alkaline phosphatase, and total protein exhibited significant decreases in the Indian worm *Metaphire posthuma* exposed for 14 days in field soil (pH 7.6, CEC 27 cmol kg^{-1}) spiked with $CuSO_4$. All the parameters were significantly affected at the lowest exposure level of 40.6 mg Cu kg^{-1} dry soil.

To assess the possible effect of Cu on metabolism, Gibb et al. (1997) applied ^{1}H NMR spectroscopy to *E. andrei* and *L. rubellus* exposed to a forest soil in the laboratory or in outdoor mesocosms in the experiments described by Svendsen and Weeks (1997a and b, respectively). In L. *rubellus*, a dose-related increase in histidine content and an increase in the ratio of histidine to tyrosine were found, while other amino acids were not affected by copper exposure. However, they found no effects of copper on amino acid concentrations in *E. andrei*. Bundy et al. (2008) reported a dose-related effect of copper on the metabolite profiles of *L. rubellus* exposed for 70 days in outdoor mesocosms to Kettering loam soil (pH 7.1, 5% OM; amended with 3% composted bark) spiked with $CuCl_2$. Several metabolite groups responded to copper exposure, e.g., lipophilic amino acids decreased while groups related to membrane stabilization increased at high Cu levels. Moreover, lipid profiles were different in earthworms exposed to copper. The NOEC for changing metabolite patterns was 10 mg Cu kg^{-1} dry soil.

Kwak et al. (2014) exposed *E. andrei* and *Perionyx excavatus* to artificial soil (pH 6.0) spiked with $CuCl_2$ for 7 days to assess the effects on coelomocyte morphology and viability. The NOECs for coelomocyte viability were 100 and < 100 mg Cu kg^{-1} dry soil for *E. andrei* and *P. excavatus*, respectively. Gautam et al. (2018) studied immunological responses and found dose-related reductions in coelomocyte counts and phagocytic activity in the Indian worm *M. posthuma* after 14 days of exposure to field soil (pH 7.6, CEC 27 cmol kg^{-1}) spiked with $CuSO_4$. All the parameters were significantly affected at the lowest exposure level of 40.6 mg Cu kg^{-1} dry soil. Kim et al. (2016) reported a 73–77% reduction in coelomocyte viability in *E. fetida* exposed for 7 days to LUFA 2.2 soil spiked with $CuCl_2$ at concentrations of 100–300 mg Cu kg^{-1} dry soil, corresponding to 19.9–28.5 mg Cu kg^{-1} body weight in earthworms. However, it remains unclear whether the earthworm body concentrations were expressed on a dry or fresh weight basis, making it difficult to compare these results with those reported in other studies. After *E. fetida* were exposed for 14 days to artificial soil (pH 6.0) spiked with $Cu(NO_3)_2$, the total cell apoptotic rate of coelomocytes was positively correlated with the Cu concentration, while the percentage of normal coelomocytes was negatively correlated with the Cu concentration and decreased by 30% at 63.5 mg Cu kg^{-1} dry soil (Zhang et al., 2020). Coelomocytes of *E. andrei* showed significant DNA damage in the comet assay after 9 days of exposure to a clay loam soil (pH 8.26, 37% clay, 1.72% OM) spiked with $CuSO_4$ at 120 mg Cu kg^{-1} dry soil, corresponding to 17.9 mg Cu kg^{-1} dry body weight in the earthworms (Mincarelli et al., 2019).

Calisi et al. (2009) identified five types of coelomocytes in *E. fetida*, of which the granulocytes seemed most responsive to copper exposure because they were significantly enlarged. Similar effects were observed for *Lumbricus terrestris* (Calisi et al., 2011). In both cases, the NRRT, used to assess alterations in the permeability of the lysosomal membrane was significantly reduced while metallothioneins was considerably increased. Since only one concentration of $CuSO_4$ was tested (~55.7 mg Cu kg^{-1} dry soil) in an artificial soil medium, these studies do not allow for assessing possible concentration–response relationships.

Based on the notion that lysosomes are primarily involved in intracellular digestion and also serve a broad range of other physiological functions, Weeks and Svendsen (1996) proposed the use of the NRRT in lysosomes of earthworm coelomocytes as an indicator of chemical exposure-related effects. The authors suggested a threshold value of 40 mg Cu kg^{-1} dry body weight for the earthworms for effects on the NRRT. Their results have been further elaborated and detailed by Svendsen and Weeks (1997a, 1997b; see below). In a review, Svendsen et al. (2004) concluded that the earthworm NRRT is much more sensitive than phenotypic endpoints, with the EC_{75} for the NRRT effects of copper being 30–40 times lower than the LC_{50}. In *Macrochaeta* sp. collected from field soils sprayed several times with the fungicide copper oxychloride ($Cu_2(OH)_3Cl$), the NRRT was significantly reduced in earthworms showing copper concentrations of ~10 mg Cu kg^{-1} dry body weight (Maboeta et al., 2002). Additionally, Reinecke et al. (2002) reported significantly reduced NRRT at similar body concentrations of *E. fetida* exposed for 8 weeks to copper oxychloride in an organic substrate (cattle manure). In an artificial soil spiked with copper oxychloride, the NRRT in *E. fetida* was significantly reduced at the lowest concentration, corresponding to 73.2 mg Cu kg^{-1} dry soil after 7 days of exposure (Maboeta et al., 2004). In *E. andrei* exposed for 28 days to forest soil (96% sand, 4% clay, <1.0% OM, pH 5.6) spiked with $CuCl_2$, the NRRT was already reduced by ~50% at the two lowest measured soil concentrations of 25 and 49 mg Cu kg^{-1} dry soil, corresponding with concentrations in the earthworms of 27 and 40 mg Cu kg^{-1} dry body weight, respectively (Svendsen and Weeks, 1997a). Zhang et al. (2020) reported a significantly reduced NRRT in *E. fetida* exposed for 14 days in artificial soil (pH 6.0) spiked with $Cu(NO_3)_2$ at 15.9 mg Cu kg^{-1} dry soil. Scott-Fordsmand et al. (2000) exposed *E. fetida* for 21 days to a sandy soil, either freshly spiked with $CuCl_2$ or taken from a gradient of historic copper contamination near Hygum, Denmark. The EC_{10} for NRRT effects was 8 mg Cu kg^{-1} dry soil for freshly spiked soil and 69 mg Cu kg^{-1} dry soil for contaminated field soil. In both cases, the NRRT showed a very steep decline with increasing copper concentration in the earthworms, decreasing to almost zero at > 50 mg Cu kg^{-1} dry body weight. Rocco et al. (2011) exposed *E. fetida* for 14 days to sandy soil from Hygum spiked with $CuCl_2$ at 35 and 350 mg Cu kg^{-1} dry soil to assess copper toxicokinetics. The NRRT was significantly reduced after 3 days and returned to normal levels only after 14 days of elimination in clean soil. The NRRT reduction was significant at concentrations >10 mg Cu kg^{-1} dry body weight.

In some cases, the results were more contrasting. After L. *rubellus* was exposed for 42 days in Kettering loam (pH 7.1, 5% OM) spiked with $CuCl_2$, no significant effects on the NRRT were found at < 159 mg Cu kg^{-1} dry soil, corresponding to a concentration of 53.4 mg Cu kg^{-1} dry body weight in the earthworms (Spurgeon et al., 2004). However, in an outdoor mesocosm test using the same Kettering loam soil, the NRRT in *L. rubellus* decreased in a dose-related manner at all test concentrations (10–480 mg Cu kg^{-1} dry soil) after 70 days of exposure (Spurgeon et al., 2005). *L. rubellus* exposed in outdoor mesocosms to sandy forest soil (4% clay, <1.0% OM, pH 5.6) spiked with $CuCl_2$ exhibited a 30% decrease in NRRT at the lowest measured soil concentration (26.0 mg Cu kg^{-1} dry soil), with EC_{50} values of 25, 39 and 38 mg Cu kg^{-1} dry body weight after 17, 40 and 70 days, respectively. After 110 days, the effects were no longer significant at 26.0 and 43.5 mg Cu kg^{-1} dry soil, corresponding to concentrations in the earthworms of 26.8 and 47.1 mg Cu kg^{-1} dry body weight, respectively (Svendsen and Weeks, 1997b).

To summarize, adverse ecotoxicological effects on earthworms at the sub-individual level were found at relatively low copper concentrations (i.e., 40 mg Cu kg^{-1} dry soil and below), depending on the soil properties, earthworm species and additional factors, such as the measured endpoint and duration of exposure. The small number of articles and observations on NOEC, LOEC, and EC_x with endpoints at the sub-individual level (14 observations in total) did not allow calculation of more precise thresholds. Earthworm sub-individual responses were more sensitive to copper-based chemicals than were more traditional endpoints at the individual or population levels. For instance, Bundy et al. (2008) reported that copper disrupted energy metabolism (e.g., through an increase in the transcription of carbohydrate-metabolizing enzymes) in *L. rubellus* at levels lower than those required to reduce reproductive parameters. Similarly, Weeks and Svendsen (1996) observed negative effects on NRRT at body concentrations as low as 20 mg kg^{-1} dry body weight, whereas negative effects at the individual level occurred only at body concentrations in the range of 80–170 mg Cu kg^{-1} dry body weight. Kim et al. (2016) reported a concentration-dependent decrease in the cell viability of the earthworm gut microbial community and proposed the use of the viability of gut microbes as a complementary earthworm biomarker of metal exposure. Sub-individual endpoints can also be seen as proxies for effects at higher levels of biological organization, as shown by the positive linear relationships between the NRRT and several life cycle parameters found by Reinecke et al. (2002). Taken together, these results highlight the difficulties in determining threshold values at the sub-individual level and point to the need to correlate the responses of various biomarkers to assess the health of soil biota.

3.2. Individual level

Several endpoints, such as avoidance (and other behavioural endpoints), survival, reproduction, and growth (or weight change), are traditionally used at the individual level. A few studies have reported the avoidance of copper-contaminated soils by earthworms (e.g., Bart et al., 2017; Eijsackers et al., 2005). Van Zwieten et al. (2004) reported that 90% of *E. fetida* individuals avoided a natural soil contaminated with 553 mg Cu kg^{-1} dry soil. They also showed that earthworms preferred non-contaminated control soils over soils with 34 mg Cu kg^{-1} dry soil. The soil penetration rate of *E. andrei* individuals was halved when exposed to $CuCl_2$ at a concentration of 200 mg kg^{-1} dry soil (Kim et al., 2016). At this concentration, abnormalities such as fragmentation, thinning, swelling, bleeding, and mucous secretion were visible. Loureiro et al. (2005) reported an EC_{50} avoidance of 181 mg Cu kg^{-1} dry soil (95% CI 169; 195) for *E. andrei* exposed to $CuSO_4$ in a LUFA 2.2 soil (pH 5.0), while Xing et al. (2017) reported half this value (i.e., 93.4 mg Cu kg^{-1} dry soil, 95% CI 89.0; 97.9) for *E. fetida* exposed to $Cu(NO_3)_2$ in an

OECD artificial soil (pH 6.0). The EC_{50} value calculated by Bart et al. (2017) for *Aporrectodea caliginosa* exposed to copper oxychloride in a natural soil (pH 7.5) was lower (i.e., 51.2 mg kg^{-1} dry soil; 95% CI 46.0; 77.5). Reinecke et al. (2002) reported a reduced feeding rate for *E. fetida* at the highest exposure concentration used in their study (i.e., 330 mg Cu kg^{-1} dry soil utilizing the fungicide Viricopt® containing 850 g kg^{-1} of copper oxychloride). The authors explained that earthworms tend to avoid copper, leading to detrimental effects on growth and maturation. However, additional studies are needed on avoidance, a reliable ecotoxicological endpoint (Alcívar et al., 2021).

Most studies have reported no mortality of earthworms due to copper exposure, except at high concentrations. However, from the collected literature data, we calculated a mean LC_{50} (17 studies, 85 observations) of 550 mg Cu kg^{-1} dry soil (min–max 32.4–3717) (Table 1; Table S1), which does not account for the uncertainty in the available LC_{50} data (i.e., variance). When accounting for this uncertainty by calculating the weighted mean, the LC_{50} value for the whole dataset was 113 mg Cu kg^{-1} dry soil (95% CI -356; 582) (Table 1; Fig. 1), which underlines the high variability in the values due to different experimental conditions. We distinguished between freshly spiked natural test soils and artificial soils and found a 5-fold greater weighted mean LC_{50} for artificial soils (Table 1; Fig. 1), indicating a greater copper sensitivity of earthworm survival in natural soils than in artificial soils. The high variability in natural soils is probably due to the diversity of the tested natural soils, while artificial soils are standardized. No striking difference was observed between the different earthworm ecological categories, both of which were not significantly different from 0 (weighted mean LC_{50} 124 mg Cu kg^{-1} dry soil with 95% CI -453; 701 for epigeics and 84.5 with 95% CI -473; 642 for other earthworms) (Table 1; Fig. 1). Copper sulphate and nitrate copper had similar LC_{50} values, but the variability was greater for the sulphate form (Fig. 1; Table S1). The reduced amount of data for copper chloride (4 studies, 5 observations) did not allow the calculation of a weighted mean.

Regarding the effects of copper on earthworm reproduction, cocoon production was reported to be one of the most sensitive parameters at the individual level (Duan et al., 2016; Reinecke et al., 2002; Spurgeon and Hopkin, 1996; Spurgeon et al., 1994), with significant adverse effects of copper from 50 to 100 mg Cu kg^{-1} dry soil (EHC, 1998; Ma, 1984, 1988; Owojori et al., 2010; Reinecke et al., 2002; Spurgeon et al., 2004). Bart et al. (2019) reported that *A. caliginosa* cocoon production was halved and hatching time was prolonged (by 5 days) at the highest tested copper concentration (233 mg Cu kg^{-1} dry soil) compared to that of the control, but no effects were found on the mean weight of the cocoons produced. Here, we calculated the mean EC_{10} and EC_{50} values of 86.1 and 183 mg Cu kg^{-1} dry soil, respectively (Table 1). The weighted means for EC_{10} and EC_{50} reproduction were 61.1 (95% CI 13.4; 109) and 94.6 mg Cu kg^{-1} dry soil (95% CI 14.0; 175), respectively (Table 1; Fig. 2). These values are close to the NOEC values reported for the effect of copper on the reproduction of *Dendrobaena rubida*, *L. rubellus* and *A. caliginosa* (i.e., 122, 30 and 50 mg Cu kg^{-1} dry soil, respectively) (Bengtsson et al., 1986; Ma, 1982). The weighted mean NOEC calculated using values from the overall dataset (8 studies, 15 observations, including mortality, reproduction and growth data; see Table S1) was 188 mg Cu kg^{-1} dry soil (95% CI 73.0; 303) (Table 1), which is higher but still coherent with previous values. Similarly, with a modelling approach (i.e., description of hatching success as a linear function of exposure concentration above a certain threshold), Jager and Klok (2010) considered a threshold for effects on *Dendrobaena octaedra* hatching of 151 Cu kg^{-1} dry soil and a tolerance concentration of 77.7

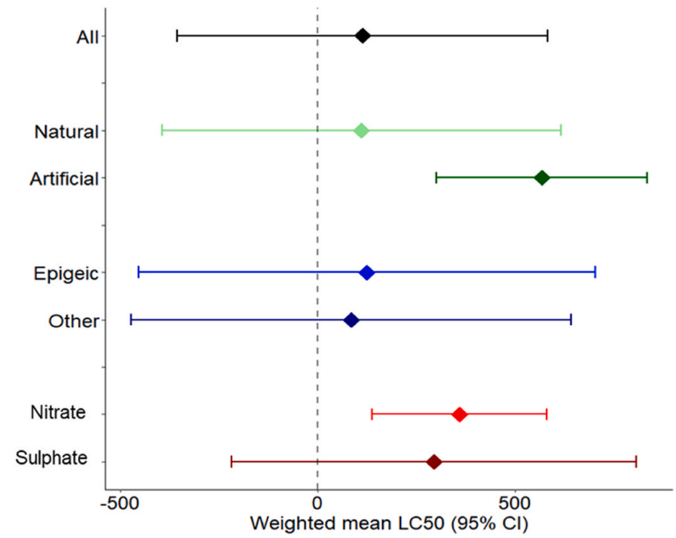

Fig. 1. Weighted mean LC_{50} values [95% CI] derived for the toxicity of copper to earthworms according to three different categories: the soil (freshly spiked natural or artificial soil), the ecological category of earthworms (epigeic or other), and the copper form (copper nitrate or sulphate).

Table 1
Mean (min–max) and weighted means with 95% confidence intervals (CIs) for different endpoints (LC_{50}, NOEC, EC_{10}, and EC_{50}) and moderators (soil, earthworms and copper form) of copper toxicity to earthworms calculated from the data included in the collected database (Table S1). "All endpoints" for NOEC means that mortality, reproduction and growth are considered. "All endpoints" for EC_{10} and EC_{50} means that reproduction and growth are considered.

Endpoint Moderator	Category	Number of studies	Number of observations	Mean	Min	Max	Weighted mean	95% CI
LC_{50} (i.e., mortality)	All	17	85	550	32.4	3717	113	−356; 582
Soil	Natural	11	76	546	32.4	3717	110	−393; 615
	Artificial	6	9	583	325	883	567	300; 834
Earthworms	Epigeic	16	56	638	32.4	3717	124	−453; 701
	Other	3	29	380	39.5	1942	84.5	−473; 642
Copper form	Nitrate	8	21	488	145	836	359	138; 580
	Sulphate	5	39	469	200	1256	294	−218; 806
NOEC (all endpoints)		8	15	188	29.0	725	188	73.0; 303
EC_{10} (all endpoints)		6	35	95.0	10.0	428	80.8	22.3; 139
EC_{10} reproduction		6	31	86.1	10.0	270	61.1	13.4; 109
EC_{50} (all endpoints)		18	62	198	27.7	716	112	−3.51; 227
EC_{50} reproduction		13	46	183	27.7	716	94.6	14.0; 175
Copper form	Chloride	5	12	150	51.0	344	105	51.7; 158
	Sulphate	5	21	192	27.7	517	118	42.2; 194
EC_{50} growth		7	16	239	81.8	601	144	−12.6; 301

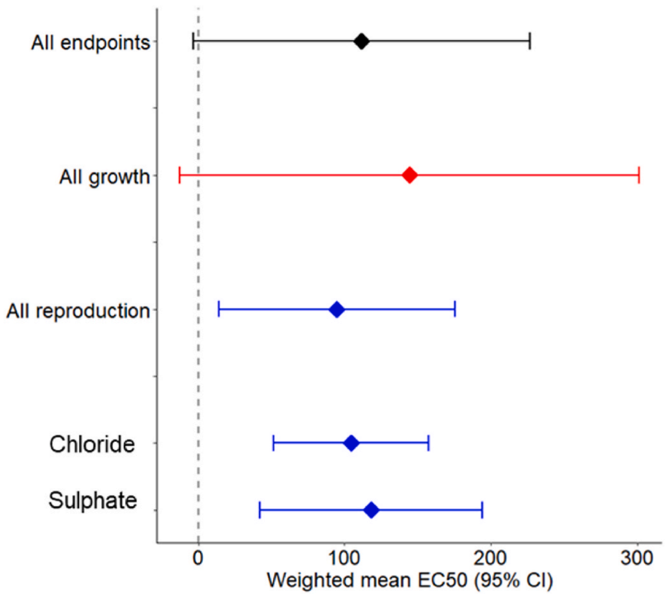

Fig. 2. Weighted mean EC_{50} values (and 95% CIs) derived for the toxicity of copper to earthworms considering all the EC_{50} data (in black), growth data only (in red), and reproduction data only (in blue) in the copper form category (copper chloride or sulphate). For the other categories (soil and earthworms), the data were insufficient for calculating a weighted mean (Table S1). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Cu kg^{-1} dry soil. Kula and Larink (1997) found a threshold for the effect of copper chloride on cocoon production of between 10 and 32 mg Cu kg^{-1} dry soil, depending on the soil type (artificial soil of LUFA 2.2) and the species (*E. fetida* or *E. andrei*).

Other parameters are rarely used to assess the effects of copper and other contaminants on earthworm reproduction, but they could be even more sensitive than cocoon production is. For instance, already at 15.9 mg Cu kg^{-1} dry soil, Helling et al. (2000) reported negative effects of copper on the total number of hatchlings, mean cocoon weight, mean number of hatchlings per cocoon, maximum number of hatchlings per cocoon, and number of hatchlings per total number of cocoons produced by *E. fetida*. For the cocoon incubation time in the same study, effects were observed starting at 39.5 mg kg^{-1} dry soil. Similarly, Reinecke et al. (2002) tested copper oxychloride in a cattle manure substrate. They reported that at all exposure concentrations greater than 3.3 mg kg^{-1} dry soil (measured effective concentration: 8.9 mg Cu kg^{-1} dry soil), the hatching success of *E. fetida* was significantly lower than that of the control (4 mg Cu kg^{-1} dry soil). Gao et al. (2019) explained that hatching success after 56 days of exposure was more sensitive to Cu exposure than the other reproductive parameters, showing a significant decrease starting at 80 mg Cu kg^{-1} dry soil, while effects on the number of cocoons per worm (*E. fetida*) or the biomass of the juveniles occurred starting at 120 mg Cu kg^{-1} dry soil.

Interestingly, Bengtsson et al. (1986) reported that Cu concentrations of 100 mg kg^{-1} dry soil were beneficial for cocoon production in *D. rubida*. This could be due to hormesis (i.e., the stimulatory effect caused by low levels of potentially toxic agents; Stebbing, 1982), which has been reported several times in studies assessing growth or biomass changes in earthworms exposed to copper (Haghparast et al., 2013; Jager and Klok, 2010; Mirmonsef et al., 2017; Reinecke et al., 2002; Scott-Fordsmand et al., 2022; Spurgeon et al., 2005; van Gestel et al., 1989). Van Gestel et al. (1991) showed that Cu at 10 and 18 mg kg^{-1} dry soil stimulated earthworm growth and sexual development. Similarly, Svendsen and Weeks (1997a) suggested a reasonably uniform weight gain at exposures of as much as 80 mg Cu kg^{-1} dry soil and weight loss at higher concentrations. Spurgeon et al. (2004) exposed the earthworm *L. rubellus* to clay loam soil spiked with copper and also observed growth stimulation at lower copper concentrations (10 and 40 mg kg^{-1} dry soil). Bindesbøl et al. (2007) and Jager and Klok (2010) suggested that 80 mg Cu kg^{-1} dry soil was optimal for population growth. This could be explained by copper being an essential element, but it is unlikely that soil levels are limiting due to geochemical backgrounds (Jager and Klok, 2010). Thus, the primary assumption is that low copper doses reduce the negative impact of fungi, hence reducing the presence of possible earthworm parasites and pathogens (Gunnarsson and Rundgren, 1986).

Negative effects on earthworm growth occur above a concentration of approximately 80 mg Cu kg^{-1} dry soil (Bogomolov et al., 1996; Clasen et al., 2021; Helling et al., 2000; Reinecke et al., 2002). According to Maboeta et al. (2004), the threshold for short-term effects of copper oxychloride on the biomass of adult *Eisenia* spp. possibly lies between 80 and 122 mg Cu kg^{-1} dry soil. In the study of Helling et al. (2000), the threshold for the impact of copper oxychloride on the growth rate of *E. fetida* was 8.92 mg Cu kg^{-1} dry soil. These authors reported a negative effect on maturation time at 39.5 mg Cu kg^{-1} dry soil, with only 93.3% of the individuals reaching maturity at the end of the experiment. Here, we calculated a mean EC_{50} growth or weight change of 239 mg Cu kg^{-1} dry soil (min–max 81.8; 601) and a weighted mean for the same endpoint of 144 mg Cu kg^{-1} dry soil (95% CI -12.6; 301) (Table 1). The lowest NOEC for growth reported in the literature was 56 mg Cu kg^{-1} dry soil for juvenile *E. andrei* exposed to copper chloride (van Gestel et al., 1991). This is in accordance with the findings of Martin (1986), who reported zero growth at 106 mg Cu kg^{-1} dry soil. Moreover, biomass losses in mature *A. caliginosa* and *E. fetida* were reported at 50 mg Cu kg^{-1} dry soil by Bart et al. (2017) and Zhou et al. (2013), respectively. Bart et al. (2020) also recorded the total absence of growth in *A. caliginosa* exposed for 56 days at 233 mg Cu kg^{-1} dry soil for juveniles and at 77.5 mg Cu kg^{-1} dry soil for new hatchlings. Scott-Fordsmand et al. (2022) also reported that *E. fetida* juveniles were particularly susceptible during the first 28 days of post-hatching development, probably due to rapid Cu uptake via the dermis in immature specimens. Finally, based on data acquired at the individual level, Spurgeon et al. (2003) calculated the *L. rubellus* population growth rate, which was negative at 640 mg Cu kg^{-1} dry soil. They reported severe effects on the population growth rate above a toxic threshold of 160 mg Cu kg^{-1} dry soil, sufficient to cause population decline and, ultimately, extinction.

3.3. Population and community levels

The effects of copper at the population and community levels are mainly studied under field conditions. Nielsen (1951) studied the effects of an accidental spill of copper sulphate in grassland and reported that earthworm populations were reduced by half at a concentration of 150 mg Cu kg^{-1} in the surface soil, with almost total elimination occurring at 260 mg Cu kg^{-1} dry soil. In line with these observations, Van Rhee (1967) reported a gradual decline in earthworm populations in orchard soil with 85 mg Cu kg^{-1} dry soil. Paoletti et al. (1998) and Paoletti (1999) reported a consistent decrease in earthworm numbers when copper concentrations (from Bordeaux mixture applications) in field soils (orchards in Italy under different practices and fruit crops) ranged between 100 and 150 mg kg^{-1} dry soil (depth not specified). They reported that *Allolobophora chlorotica* was the species most affected by copper, and this species disappeared when the copper concentration was greater than 175 mg kg^{-1} dry soil. However, they also found that some species, including *Aporrectodea rosea* and several epigeic species, such as *Lumbricus castaneus* and *L. rubellus*, could tolerate high levels of copper. Thus, copper changes the species composition, favouring the most resistant species and decreasing the most sensitive ones. Maboeta et al. (2002) reported a quasi-systematic decrease in the abundance and biomass of the earthworm *Microchaetus* sp. (giant anecic from Africa) in copper-contaminated plots compared with those in control plots without added copper (30–60 mg Cu kg^{-1} dry soil), according to the period (from −35% in June to −69% in October 1999). They also recorded a decrease in the NRRT (i.e., an indicator of cellular integrity) when the concentration in soil sprayed with copper oxychloride was approximately 60 mg Cu kg^{-1} dry soil. Taken together, these findings indicate that the NRRT can be used as an early warning signal of stress resulting from the use of copper-based agrochemicals and is indicative of long-term effects observed at the population level (Maboeta et al., 2002). According to Naveed et al. (2014), earthworms were more sensitive to Cu than bacteria, nematodes, and fungi were. Their abundance sharply decreased with increasing Cu concentration (approximately 175 mg kg^{-1} dry soil). The ecological structure of the earthworm community was also strongly impacted by the Cu concentration, with endogeic and anecic earthworms disappearing and epigeic species increasing with increasing Cu concentration. The same phenomenon was also reported by Mirmonsef et al. (2017). The probable reason for this difference in sensitivity between species is that epigeic earthworms live in the litter layer on the soil surface and are not necessarily exposed to the Cu present in the soil. In contrast, endogeic and anecic species are geophagous, digging horizontal and vertical burrows, and are highly exposed to elevated soil Cu concentrations. For example, the epigeic *D. octaedra* was the only earthworm species observed in highly contaminated areas with 2228 mg kg^{-1} dry soil of total Cu (Holmstrup and Hornum, 2012). Its regulatory capacity (uptake and excretion) for metals can explain the ability of *D. octaedra* to survive even in highly metal-contaminated areas (Fisker et al., 2013). Belotti (1998) compared the densities of two endogeic earthworm species (*A. caliginosa* and *A. rosea*) in several abandoned vineyards and established a critical total copper content of 33 mg kg^{-1} dry soil, above which these earthworm species could become detrimentally affected.

Under natural field conditions, long-term studies (i.e., several months or years) allow us to highlight phenomena that are not readily observable in the laboratory, such as delayed effects, possibly due to variations in environmental conditions that could determine the biogeochemical routes of contaminants in polluted soils. For instance, Amossé et al. (2020) found no effect on earthworm communities exposed to copper oxychloride at 40 kg Cu ha^{-1} (equal to 31 mg Cu kg^{-1} dry soil when considering a penetration depth of 10 cm) six months after the last application. Nevertheless, the species diversity (Shannon diversity index) decreased after 12 months, mainly due to effects on the anecic *L. terrestris* and the endogeic *A. caliginosa*. Maboeta et al. (2002) also reported a delayed effect for *A. caliginosa* exposed to the fungicide copper oxychloride in a grassland soil in South Africa. Biomass and abundance drastically decreased compared to those of the control (−65 and −57%, respectively) six months after spraying had stopped. Van Zwieten et al. (2004) investigated earthworm communities in two avocado orchards (New South Wales, Australia) and reported a negative relationship between earthworm density and long-term soil copper contamination. They found 155 and less than 1 earthworm per square metre in soils with 29 and 269 mg Cu kg^{-1} dry soil, respectively. Using microcosms implanted outdoors in a prepared grassland area, Owojori and Reinecke (2010) did not find any significant effect on *A. caliginosa* 28 days after the application of 95 mg Cu kg^{-1} dry soil (copper oxychloride, Efekto Virikop®, calculated from the data reported in the article). These studies highlight that the effects of copper on soil biota at the population and community levels are sometimes not immediate but can appear after several months of application and accumulation in the soil. These effects on earthworm populations and communities may have negative consequences for soil properties and functioning. For instance, Eijsackers et al. (2005) reported that copper oxychloride had a negative impact on the earthworm burrowing rate in a vineyard treated with a copper pesticide compared to that in an adjacent grassland. They also highlighted an inverse relationship between earthworm burrowing activity and soil bulk density that could also be related to the copper content.

4. Discussion

We showed that copper can affect earthworms at different levels of biological organization, with endpoints at the lowest biological organization level (e.g., lysosomal damage) being the most sensitive. We also calculated the weighted means for different endpoints: mortality (LC_{50} of 113 mg Cu kg^{-1} dry soil), reproduction (EC_{50} of 94.6 mg Cu kg^{-1} dry soil), and growth or weight change (EC_{50} of 144 mg Cu kg^{-1} dry soil). These "traditional" endpoints were used in the meta-analysis because they were available in the literature. In most cases, they are calculated with measured effects on adult individuals, but more sensitive endpoints and stages of development could be used in ecotoxicological studies and risk assessment procedures, such as juvenile growth (Bart et al., 2020; Helling et al., 2000; Scott-Fordsmand et al., 2022) or biomarkers at the sub-individual level (e.g., NRRT (Svendsen et al., 2004)). For instance, Bindesbøl et al. (2007) showed that juvenile survival was affected at 200 mg Cu kg^{-1} dry soil, while this concentration did not affect adult survival. Moreover, 80% of the observations used epigeic composting worms (i.e., *E. fetida*, *E. andrei* or *Dendrodrilus rubidus*) to assess the effects of copper on earthworms (see Table S1). Although the weighted mean LC_{50} of epigeic earthworms (also including *L. rubellus*) was not different from that of other species (i.e., including endogeic and anecic species), Pelosi et al. (2013) highlighted in a meta-analysis that earthworm species found in natural soils are more sensitive to chemicals than model composting species are.

In addition to the intrinsic tolerance of organisms, copper toxicity in terrestrial organisms can be influenced by other factors, which can explain the high variability of the calculated weighted means for the different endpoints. This makes it difficult to derive thresholds from available data. Multiple stresses (e.g., copper, drought or cold) can lead to a decrease in LOEC and NOEC values (Friis et al., 2004; Holmstrup et al., 1998; Orrego et al., 2020). For example, Bindesbøl et al. (2005, 2009) showed that temperatures below −2 °C aggravated the effect of copper on *D. octaedra*. Furthermore, soil type and properties such as pH, OM content, and clay content (Bengtsson et al., 1986; Ma, 1984) also impact earthworm exposure and ecotoxicological effects due to their influence on metal bioavailability, as has been shown for other terrestrial organisms such as plants (e.g., Ginocchio et al., 2006, 2009). For instance, Owojori et al. (2010) reported no effect on the weight gain of *E. fetida* at 640 mg Cu kg^{-1} dry soil in an artificial soil (pH 6.0) with 40% clay, but negative effects occurred at the same concentration in soils with lower clay contents. Similarly, the LC_{50} decreased as the clay content decreased. Ma (1988) concluded that in acidic (~pH 5.0) agricultural sandy soils with 4–6% OM, detrimental effects on earthworm populations were expected above 50 mg kg^{-1} of total Cu, with a drastic decline above 100 mg Cu kg^{-1} dry soil. He proposed liming to raise the soil pH or adding an organic amendment to increase the soil OM content to reduce Cu mobility and bioavailability in soils and thus alleviate Cu toxicity to earthworms. Here, we distinguished between freshly spiked natural and artificial soils and showed that the LC_{50} was lower in natural soils, although it was highly variable. This could be due to the above-mentioned parameters (e.g., OM content, pH) that influence copper bioavailability and, as a consequence, effects on earthworms.

In nature, soils are usually contaminated with a mixture of compounds. Deciphering the effect of copper in multi-contaminated soils is difficult (even using regressions such as those applied by, e.g., Klok et al., 2007), as the presence of other contaminants may either increase the sensitivity of organisms to copper or protect them against copper toxicity. Moreover, assessing the effects of copper in these field-contaminated soils would be more relevant because metals are affected by 'ageing' (Wang et al., 2018), a lengthy process in which metal bioavailability/toxicity depends on the residence time of the metal in the soil (Nahmani et al., 2007; Spurgeon and Hopkin, 1995; Zeng et al., 2017). McBride and Cai (2015) demonstrated that the decrease in bioavailability associated with ageing occurs due to the reduction in the exchangeable fraction of Cu during the first years of residence in the soil, leading to differences in speciation in soils. This result is supported by Guo et al. (2011), who reported that the exchangeable and carbonate-bound forms of Cu increased over time, whereas the amounts of Cu bound to Fe–Mn oxides and OM exhibited the opposite pattern. These findings suggest that there was variability in the different Cu fractions and that their toxicity decreased as the ageing process progressed. Zeng et al. (2017) reported that the decrease in Cu bioavailability in the soil due to ageing occurs initially rapidly (fast process) and then continues to decrease, albeit at a slower pace (slow process). This rapid process is primarily caused by the precipitation/nucleation of Cu within OM, where the soil pH and OM content can influence these processes individually (Ma et al., 2006). The slow process occurs through the diffusion of Cu ions into small and medium pores on soil surfaces, with temperature playing a crucial role. This ageing effect can explain why the literature described a difference in ecotoxicity between field-contaminated and freshly spiked soils (McBride and Cai, 2015). For example, Santa-Cruz et al. (2021b) reported greater metal toxicity in spiked soils than in field-contaminated soils for different organisms (e.g., plants, invertebrates, and microorganisms). In our corpus, information about ageing was scarce, and only two studies specifically addressed copper contamination and earthworms in field-contaminated soils according to our criteria. Therefore, no statistical analysis was possible.

The exposure time is also an essential factor when studying the effects of copper on earthworms, as in general, the uptake and elimination kinetics of crucial elements such as Cu are fast. According to Kilpi-Koski et al. (2019), Cu accumulates rapidly in *E. andrei*, and steady-state (i.e., state of a system in which the conditions do not change over time) concentrations were reached after 1 day of exposure, probably due to active regulation of body concentrations by earthworms. In contrast, as described in Sections 3.1 and 3.3, toxic effects on earthworms can take longer than one month to occur, and long-term monitoring of the effects of copper should thus be considered. To have a sufficiently large dataset, we considered all studies, regardless of the exposure time. We could have excluded, e.g., studies with less than 28 days of exposure to the LC_{50}, but this represented 80 observations (i.e., more than 90% of the dataset).

The effects of copper on earthworms also depend on the copper form. Arnold et al. (2003) proposed the following classification for copper forms in terms of their toxicity to *E. fetida*: nitrate > sulphide > carbonate, which is explained by the solubility of these salts. We found similar weighted mean LC_{50} values for the nitrate and sulphate forms of copper. This finding is in line with Malecki et al. (1982), who exposed *E. fetida* individuals to six different copper salts for eight weeks and found significant effects on cocoon production for both copper nitrate and copper sulphate at 100 mg Cu kg^{-1} dry soil. Copper chloride reduced growth at 500 mg Cu kg^{-1} dry soil, and the other tested salts affected growth and reproduction at copper concentrations >1000 mg Cu kg^{-1} dry soil. The least toxic was the poorly soluble copper oxide, which significantly affected growth and reproduction at > 20,000 mg Cu kg^{-1} dry soil. The extremely high LC_{50} and EC_{50} for the toxicity of malachite ($Cu_2(OH)_2(CO_3)$) to *E. fetida* reported by Arnold et al. (2003) may be explained by the extremely low solubility of this Cu form.

A stimulatory effect of copper at low levels (i.e., hormesis) has been reported several times for earthworms (see Section 3.2). Some studies have suggested that a certain minimum level of Cu is needed for some organisms. For molluscs, White and Rainbow (1985) estimated that a minimum concentration of approximately 26.3 mg Cu kg^{-1} dry weight would be required for metabolizing tissue considering the Cu requirements of different enzymes. A greater amount of Cu is needed if an organism uses Cu-containing haemocyanin for respiration (White and Rainbow, 1985). This value has been compared with Cu levels found in different organisms in Luoma and Rainbow (2008), who reported body concentrations in aquatic crustaceans from clean environments varying between 15 and 232 mg Cu kg^{-1} dry body weight. This may suggest that a concentration of less than 15 mg kg^{-1} dry body weight may lead to Cu deficiency. Notably, the margins between the deficiency and toxicity limits are insignificant. Considering the effects at the sub-individual level (see Section 3.1), starting at concentrations above 10 mg kg^{-1} dry body weight and being obvious at 20 mg kg^{-1} dry body weight, the margin is probably minimal for earthworms and copper.

Finally, the choice was made in the meta-analysis to use nominal concentrations rather than measured soil or internal concentrations, as the latter two are not always given in scientific studies. In metal risk assessments, 'added risk' is sometimes used to account for the fact that all soils contain a certain background level of metal (Struijs et al., 1997). From that perspective, it might be helpful in future studies to express effects based on total (measured) and added (nominal) concentrations. In contrast, if a test soil has a considerable background level of Cu, a small amount of Cu added may already have a significant effect, leading to low effect concentrations when expressed on nominal rather than measured total concentrations. Therefore, it may be slightly risky to combine data from studies with measured and nominal concentrations. An alternative approach could be to speak of added concentrations and consider the background level to have a negligible contribution to the effect. Another approach would be to consider earthworm internal concentrations of copper to be linked to effects (e.g., Eijsackers et al., 2005). *Octolasion cyaneum* was found to regulate tissue copper concentrations at approximately 40–100 mg kg^{-1} dry body weight when exposed to soil with concentrations up to 400 mg Cu kg^{-1} dry soil (Streit and Jäggy, 1982). Laboratory studies have indicated that tissue copper concentrations above 100–120 mg kg^{-1} dry body weight for several species (*Eisenia* spp., *L. rubellus*, *D. octaedra* and *O. cyaneum*) are associated with severe reductions in growth, reproduction and survival (Bindesbøl et al., 2007; Mirmonsef et al., 2017; Scott-Fordsmand et al., 2000; Spurgeon et al., 2004; Svendsen and Weeks, 1997a,b). Similarly, according to Ma (2004), adult *L. rubellus* exhibit an apparent physiological tolerance range of body Cu concentrations of 8–60 mg kg^{-1} dry body weight. The approximate internal thresholds were 30–40 and 60 mg Cu kg^{-1} dry body weight for adverse effects on sublethal endpoints and survival, respectively (Bogomolov et al., 1996; Ma, 2004). In Duan et al. (2016), for *E. fetida*, the tissue Cu-based EC_{50} reproduction ranged between 15.5 and 62.5 mg Cu kg^{-1} dry body weight depending on the soils tested (15 Chinese soils).

To summarize, further studies on Cu effects on earthworms and other soil invertebrates are needed and should consider, in addition to traditional endpoints (e.g. reproduction of adult individuals), sensitive indicators such as juvenile growth. Avoidance behaviour can also provide useful information on the effects of copper applications in the field. Moreover, natural soils and earthworm species that inhabit natural soils should be used in studies as they are representative of fields where Cu-based fungicides can be applied. Multiple stress should also be considered in studies, in particular climatic parameters, especially in the actual context of climate change. Measuring soil concentrations in addition to the nominal dose would allow to better determine the exposure conditions of the tested organisms. Finally, we drastically miss field studies on medium or long-terms, i.e. several years, to assess the effects of copper (avoiding multi-contamination situations) on earthworms and other soil invertebrates under real natural conditions.

5. Conclusion

On the basis of a systematic review of the literature and a meta-analysis, we determined the copper concentrations at which the survival, reproduction and growth of earthworms are affected by 50%. Although mainly based on laboratory studies, as field-contaminated soils are rarely contaminated with only copper, this study provides an overview of the ecotoxicological effects of copper on earthworms at different levels of biological organization. Despite a hormesis effect at low concentrations, strong harmful effects have also been reported at concentrations lower than 40 mg Cu kg^{-1} dry soil. Nevertheless, it is worth remembering that earthworm sensitivity to copper can vary according to earthworm developmental stage, species and endpoints but also according to factors such as copper form, temperature, and soil type and properties, among other factors, which increase the difficulty of threshold calculation. Understanding these complexities is essential for accurate risk assessment and environmental management processes. In light of this review, further research should be conducted on the effects of copper on earthworm communities and earthworm-mediated functions under naturally contaminated conditions. Moreover, considering the negative effects that copper may have on earthworms and potentially on soil biodiversity in general, it is imperative that regulators focus their attention on a more sustainable use of Cu-based pesticides in agricultural areas.

CRediT authorship contribution statement

C. Pelosi: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Data curation, Conceptualization. **F. Gavinelli:** Writing – review & editing, Investigation, Data curation. **L. Petit-dit-Grezeriat:** Writing – review & editing, Methodology, Formal analysis. **C. Serbource:** Writing – review & editing, Methodology, Formal analysis. **J.T. Schoffer:** Writing – review & editing, Investigation, Data curation. **R. Ginocchio:** Writing – review & editing, Validation. **C. Yáñez:** Writing – review & editing, Validation. **G. Concheri:** Writing – review & editing, Validation. **M. Rault:** Writing – review & editing, Validation. **C.A.M. van Gestel:** Writing – review & editing, Writing – original draft, Validation, Investigation, Data curation.

Declaration of competing interest

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

Data availability

Data are in the Table S1

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Appendix A. Supplementary data

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