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Does a decade of soil organic fertilization promote copper and zinc bioavailability to an epi-endogeic earthworm?

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Abstract

While long-term organic fertilizer (OF) applications tend to decrease copper (Cu) and zinc (Zn) availability in agricultural soils, earthworm bioturbation has been reported to have the opposite effect. Thus, the consequences of OF amendments in earthworm-inhabited soils on Cu and Zn bioavailability to earthworms are still under debate. Here, we assessed the effect of a decade of agronomically realistic OF applications on Cu and Zn availability in earthworm-inhabited soils and the consequences on Cu and Zn bioavailability to earthworms. An epi-endogeic species (*Dichogaster saliens*) was exposed in microcosms to three field-collected soils that had received either no, mineral or organic fertilization for a decade. Dissolved organic matter (DOM) properties (i.e., concentration, aromaticity, and binding properties toward Cu), pH, and Cu and Zn availability (i.e., total concentration and free ionic activity) were determined in the solution of the soil containing earthworms. Cu and Zn bioavailability was assessed by measuring the net accumulation (ng) and concentration of Cu and Zn in earthworms (mg kg^{-1}). Despite soil Cu and Zn contamination induced by a decade of OF applications, organic fertilization induced an increase in soil pH and DOM properties that drove the reduction of Cu and Zn availability in earthworm-inhabited soils, while bioturbation had little effect on soil pH, DOM properties and Cu and Zn availability. Consistently, Cu and Zn bioavailability to earthworms did not increase with OF applications. From an ecotoxicological perspective, our results suggest that agronomically realistic applications of OF for a decade should not pose a risk to earthworms in terms of Cu and Zn net accumulation, but further studies have to be undertaken to understand consequent long-term toxicity after exposure.

Keywords: bioaccumulation, fauna, metal, pore water, potentially toxic element, wastes

1. Introduction

Long-term applications of organic fertilizers (OF) are now ascertained to induce ongoing soil contamination with trace elements (Senesi et al., 1999; Wassenaar et al., 2014). Copper (Cu) and zinc (Zn) are quantitatively the main trace elements added with OF applications. Compared to other trace elements and from a regulatory perspective, the estimated time required to increase the soil concentration from natural background to regulatory thresholds is usually the shortest for Cu and Zn (Belon et al., 2012; Luo et al., 2009; Nicholson et al., 2003). From an ecotoxicological perspective, recent environmental assessments have pointed out that Cu and Zn contribute the most to aquatic and terrestrial ecotoxicity (Michaud et al., 2020; Avadí et al., 2022). These considerations justify deepening our understanding of the main drivers of Cu and Zn fate and effects in OF-amended soils, i.e., the main drivers of the two components of bioavailability: availability (i.e., the soil offers trace elements) and environmental bioavailability (i.e., the uptake of trace elements by organisms) (Harmsen et al., 2005).

Long-term OF applications not only increase Cu and Zn soil contamination but also increase soil pH and organic matter content (Cambier et al., 2014; Vanden Nest et al., 2016; Nobile et al., 2018), two main drivers of soil Cu and Zn availability. Indeed, Laurent et al. (2020) reported that, regardless of the soil type, OF applications for a decade induced an increase in pH and a change in dissolved organic matter (DOM) properties, including concentration, aromaticity and binding properties, thereby mitigating soil Cu and Zn availability despite an increase in soil Cu and Zn contamination. Whether this effect of OF applications also mitigates Cu and Zn environmental bioavailability to soil organisms remains a matter of debate.

Among soil organisms, earthworms are quantitatively very important, as they represent ca. 80 % of the soil living biomass (Yasmin and D'Souza, 2010). The activities of earthworms are known to modify soil physicochemical properties such as pH and DOM (Bertrand et al., 2015; Blouin et al., 2013), thereby acting as a driving force of the increase in trace element availability in bioturbated soils (Sizmur and

Hodson 2009; Sizmur and Richardson 2020). However, among the studies considered by Sizmur and Richardson (2020) in their meta-analysis, very few were conducted in the context of agricultural uncontaminated soil amended with OF. Therefore, the context of contamination brought to the soil via an organic matrix is poorly studied, while organic matter can play an ambivalent role of both ligand for trace elements and substrate for earthworms, raising the question of whether an increase in Cu and Zn availability should be observed in bioturbated, OF-amended soils.

Concerning Cu and Zn bioavailability to soil organisms, uptake of Cu and Zn by earthworms and bioaccumulation in their internal burden have been extensively studied (Nahmani et al. 2007; Richardson et al. 2020). However, to our knowledge, only two studies have been dedicated to the investigation of Cu and Zn bioavailability to earthworms in the specific context of agricultural, OF-amended soils (i.e., Kızılkaya, 2004; Centofanti et al., 2016). These two studies reported an increase in Cu and Zn concentrations in earthworms along with an increase in Cu and Zn contamination in OF-amended soils. However, none of these studies provided mechanistic insight into the relationship between soil Cu and Zn availability and Cu and Zn bioavailability to earthworms. More generally, the causal relationship between availability and bioavailability is not straightforward (Beaumelle et al., 2016).

This relationship between trace element availability and bioavailability to earthworms in soils has been documented by Nahmani et al. (2007) and Richardson et al. (2020), which has fueled the debate on mechanistic insights. They showed that total soil Cu and Zn concentrations were the primary positive explanatory parameters of Cu and Zn bioavailability to earthworms, while negative explanatory parameters were soil organic matter for Cu and pH for Zn. Nevertheless, these authors underlined that these relationships may be partly skewed by the experimental design of the majority of the studies that were conducted with highly contaminated soils (furthermore often spiked under laboratory conditions) and/or a restrictive range of earthworm species and ecological categories (e.g., *Lumbricus terrestris* and *Eisenia fetida*). These authors therefore pointed out the need for more investigations on trace element bioavailability to endogeic or epi-endogeic earthworms in soils with low and chronic contamination, such as agricultural soils historically amended with OF, to derive more generic quantitative relationships between proxies of Cu and Zn availability and proxies of bioavailability to earthworms.

Accordingly, we herein studied the effect of a decade of agronomically realistic OF applications on Cu and Zn availability in earthworm-inhabited soils and their consequences on Cu and Zn bioavailability to earthworms. We selected soil samples from three agricultural field trials that had received either no, mineral or organic fertilization for a decade, which were prone to induce contrasting Cu and Zn contamination levels, pH, and DOM properties (Laurent et al. 2020). Soil samples were incubated in the laboratory with epi-endogeic earthworm species (i.e., *Dichogaster saliens*) collected from one of the field trials and proxies for Cu and Zn availability (i.e., total Cu and Zn concentrations and free ionic activity in soil solutions) and bioavailability (Cu and Zn net accumulation and concentrations in earthworms) were measured to assess relationships.

2. Material and Methods

2.1 Field trial and soil characteristics

The three decadal field trials studied herein were similar to the field trials studied by Laurent et al. (2020), who gave a detailed description of each trial. Briefly, the trials were located on the tropical island of Réunion in the Indian Ocean (55°30'E, 21°05'S) and were conducted on soils belonging to three reference soil groups (hereafter called soil types): Andosol, andic Cambisol, and Arenosol (WRB, 2014). The andosol and arenosol have been under permanent grassland cover since 2004 and plots were either not fertilized or fertilized after every cut (i.e., 4 to 8 times per year) with mineral fertilizer (ca. 10 g N m^{-2} y^{-1}), dairy slurry (ca. 20 to 55 dm^3 m^{-2} y^{-1}), or dairy manure compost (ca. 4 kg m^{-2} y^{-1}). The cambisol has been cultivated with two market garden crop cycles per year since 2004 and plots were fertilized at the beginning of each cycle (i.e., twice a year) with mineral fertilizer (40 g N m^{-2} y^{-1}), pig slurry compost (5 kg m^{-2} y^{-1}) or poultry litter compost (7 kg m^{-2} y^{-1}).

The main OF properties are given in Table S1. The mean annual inputs of Cu and Zn to soil by organic and mineral fertilizers were calculated from the dataset provided by Laurent et al. (2019). They were much higher for OF (i.e., 1 to 12 and 4 to 66 mg kg^{-1} soil for Cu and Zn, respectively) than for mineral fertilizers (i.e., 0.004 to 0.031 and 0.036 to 0.128 mg kg^{-1} soil for Cu and Zn, respectively). The mean annual inputs of Cu and Zn to soil by OF in the three field trials was also higher than those calculated at the French level (Belon et al. 2012).

We subselected 20 out of the 74 soil samples selected by Laurent et al. (2020) from a soil library of these three field trials to encompass the fertilization types and the gradient of Cu and Zn contamination, pH, and organic matter concentration that could influence the Cu and Zn availability in soil and bioavailability to earthworms. The soil properties are given in Table 1. Laurent et al. (2020) underlined that, despite the substantial inputs of Cu and Zn by OF, the Cu and Zn contamination in OF-amended soils was not clearly illustrated by a significant increase in total soil Cu and Zn concentrations. This was attributed to the high natural geochemical background of Réunion soils (Doelsch et al. 2006). Knowing that soilborne Cu and Zn availability is low in Réunion soils (Doelsch et al., 2008), Laurent et al. (2020) showed that the Cu and Zn contamination in OF-amended soils was more clearly illustrated by a significant increase in diethylenetriaminepentaacetic acid (DTPA)-extractable Cu and Zn concentrations.

2.2 Earthworm characteristics, acclimatization, and exposure to soils in microcosms

Sexually mature individuals of *Dichogaster saliens* (Acanthodrilidae) were used for the experiment. *D. saliens* is an epi-endogeic peregrine species commonly found in tropical areas (Huerta and de La Cruz-Mondragon 2006) and notably observed in the cambisol and arenosol field trials. Earthworm individuals were collected by hand-sorting in the topsoil layer of the bottom field margin of the cambisol field trial that received neither OF nor mineral fertilizers. Before exposure to the 20 soil samples, earthworms were acclimatized for 8 days in each of the three soil types that received neither OF nor mineral fertilizers. The three soils were maintained at 85% of the water holding capacity (WHC) at 27 °C in the dark in a climatic chamber.

The 20 soil samples were preincubated for 14 days in the absence of earthworms at 70% of their WHC with a nutrient solution containing (in mM) $Ca(NO_3)_2$ 2, KNO_3 2, $MgSO_4$ 1, and KH_2PO_4 0.05 to mimic an average soil solution composition. Each preincubated soil sample was then subdivided into 5 replicates, packed in microcosms (5.5 cm diameter and 4 cm height) at 0.5, 1, and 1.5 kg dm^{-3} for the andosol, cambisol, and arenosol, respectively, to have a soil layer thickness of 1 cm and finally moistened at 85% of its WHC. Three acclimatized earthworms were then added to each microcosm and exposed to the soil samples for 21 days at 27 °C in the dark in a climatic chamber. The earthworm-to-soil dry mass ratio in each microcosm was chosen to maximize the potential bioinfluence of earthworms on soil properties while collecting enough soil mass to perform the soil solution extraction and analysis. According to Barois et al. (1999) and Huerta and de La Cruz-Mondragon (2006), one individual of small tropical epi-endogeic earthworms can ingest a soil dry mass equivalent to its fresh biomass daily (i.e., ca. 1.3 g of dry soil ingested by three earthworm during the 21 d of the exposure period) and that we needed to have at least 4 g of dry soil to perform soil solution extraction analysis, each microcosm contained 5, 9, and 13 g of dry soil for the andosol, cambisol, and arenosol, respectively. No food source for earthworms was added during the 21 days to avoid any interference between food, earthworm activity and organic fertilization on soil chemistry.

2.3 Earthworm measurements

Earthworm mortality was assessed after 7, 14, and 21 days during the exposure period by checking the presence of the three individuals in each microcosm (an absent individual was considered dead). At the end of the exposure period, the activity status (i.e., active or quiescent) of each individual was visually assessed. Quiescence is a status of reduced activity in response to adverse environmental conditions (Koštál, 2006), during which earthworms remain coiled up in the soil. A mortality and quiescence rate (i.e., number of dead or quiescent individuals to three ratio) at the end of the exposure period was calculated for each microcosm. Earthworm biomass was determined by weighing each individual after the acclimatization and exposure periods. Before weighing, each individual was placed for 24 h at 27 °C in the dark on a damp filter paper in a Petri dish to void its gut content.

After weighing, Cu and Zn concentrations were determined on 3 replicates of 2 earthworm individuals collected after acclimatization in each of the three soil types and on pooled individuals exposed in microcosm for 21 days to the 20 soil samples (5 replicates per soil samples). Earthworms were frozen at – 30 °C for 24 h, freeze-dried for 24 h, and finally stored in a freezer before analyzing Cu and Zn concentrations. Freeze-dried earthworms collected in a given microcosm were pooled together and digested at 200 °C and 5.5 MPa in 0.5 ml of HNO_3 (Pico Pure Plus, ChemLAB) in a microwave system (MARSXpress, CEM). The digests were then diluted in 25 ml of ultrapure water. Metal quantification in total earthworms was performed by inductively coupled plasma-mass-spectrometry (ICP-MS) and the reliability of the analysis was assessed by including blanks and replicates of a certified reference material (ERM-BB-422, fish muscle) within the analytical series. We performed tests with either 100 or 10 mg of the certified reference material to encompass the range of Cu and Zn mass effectively accumulated in earthworms after the acclimatization and exposure periods. The recovery percentage was 96% for Cu and Zn with 100 mg of the certified reference material and 77% and 101% for Cu and Zn, respectively, with 10 mg of the certified reference material.

Copper and Zn concentrations in earthworms provide a picture of Cu and Zn bioavailability that can be directly related to toxicity thresholds. However, the exposure of earthworms to Cu and Zn before the exposure period can also impact the Cu and Zn concentrations in earthworms. Accordingly, the average net accumulation (A, ng) of Cu and Zn in one earthworm individual during the exposure period was calculated as a second bioavailability indicator as follows:

$$A = \frac{C_f * m_f}{n_f} - \frac{C_a * m_a}{n_a} \quad (1)$$

where C_a and C_f are the concentration of Cu or Zn measured in pooled earthworms collected after acclimatization (i.e. before the exposure period) and in pooled earthworms collected in each microcosm after the exposure period, respectively, (µg g^{-1}), m_a and m_f are the corresponding earthworm biomasses, (g), and n_a (2) and n_f (between 1 to 3 depending on mortality) are the corresponding number of earthworm individuals pooled for Cu and Zn concentration determinations.

2.4 Soil solution extraction and analysis

After the exposure period, the solution of the 5 replicates of the 20 fresh soil samples was extracted with the nutrient solution used for soil preincubation (see Section 2.2). These soil solutions were considered to be influenced by earthworm bioturbation. The 1:10 soil (dry mass equivalent) to solution mixture was stirred for 2 h and then centrifuged at 1000 g for 10 min, and the supernatant was filtered at 0.22 µm (polyethersulfone filters, Minisart Sartorius). Each soil solution was subdivided and subsamples were stabilized with either 1 mM NaN_3 for the measurement of pH, Cu^{2+} activity, and specific ultraviolet-absorbance (SUVA) at 254 nm or HNO_3 2% (v/v) for the measurement of dissolved organic carbon (DOC) and HNO_3 1% (v/v) for the measurement of total major and trace cation concentrations (Ca, K, Mg, Na, Al, Cd, Co, Cu, Fe, Mn, Ni, Pb, and Zn).

The total concentrations of major and trace cations were determined by ICP–MS (ICAP Q, Thermo Scientific). DOC concentration was determined using a total organic carbon (TOC) analyzer (Schimadzu, TOC-L). SUVA was measured by UV–visible spectrometry (Spectronic Unicam, Helios Beta) to estimate the aromaticity of DOM (Weishaar et al., 2003). The SUVA (l g^{-1} cm^{-1}) was calculated as follows:

$$SUVA = \frac{A_{254}}{b.DOC} \quad (2)$$

where A_{254} is dimensionless, b is the path length (1 cm) and DOC is the DOC concentration (g l^{-1}).

The pH was measured using a combined electrode (Radiometer analytical PHC2051-8). Free Cu activity (i.e., $pCu^{2+} = -\log_{10}\{Cu^{2+}\}$) was measured using a Cu-specific electrode (Thermo Scientific Orion, 9629BNWP) calibrated within the 13.4 and 4.7 range of pCu^{2+} in standard solutions containing imminodiacetic acid as a Cu-complexing compound (R^2 = 0.98, 93% slope efficiency, n = 157).

2.5 Modeling of Cu^{2+} and Zn^{2+} activity in soil solution

Free Cu and Zn activity in each soil solution was predicted using the humic ion-binding model VII included in Windermere Humic Aqueous Model (WHAM, Tipping et al. 2011) as already described by Laurent et al. (2020). We used the comparison between experimental Cu^{2+} measurements and Cu^{2+}WHAM calculations to estimate Zn^{2+} activity and the variability in DOM binding properties.

Free Cu activity was predicted by three approaches and for each the deviation between predicted and measured Cu^{2+} activities was determined by calculating the regression slope and coefficient and the root mean square residual (RMSR). First, the classical approach consisted of predicting Cu^{2+} activity with the WHAM default settings. Sixty-five percent of DOM reacts toward metal cations (*%r-DOM*) as colloidal fulvic acid, as defined in WHAM, and the remaining 35% of DOM is inert. Second, Cu^{2+} activity was predicted by optimizing *%r-DOM* with SUVA as previously done by Laurent et al. (2020). Third, Cu^{2+} activity was predicted by manually optimizing *%r-DOM* and $\log K_{Cu}$ for each replicate within physically meaningful and realistic ranges, i.e., from 35% to 215% for *%r-DOM* and from 1.84 to 2.46 for $\log K_{Cu}$, as previously done by Djae et al. (2017). Finally, Zn^{2+} was predicted on the basis of these Cu^{2+} predictions using the adjusted *%r-DOM,* while $\log K_{Zn}$ remained at the default value of 1.68. As predicted, Zn^{2+} was similar within 0.5 pZn^{2+} unit to Zn^{2+} predicted with WHAM default settings (Fig. S1). The uncertainty in predicted pCu^{2+} resulting from analytical uncertainties regarding the pH and the concentration of total Cu and Zn and DOM was calculated using the Monte Carlo procedure included in WHAM.

2.6 Data processing and analysis

Datasets are accessible via Dataverse (Laurent et al., 2021). Data were log_{10} transformed and analyzed with R packages (R Core Team, 2016). Statistical significance was considered when $p \leq 0.05$. Principal component analysis (PCA) was performed using the FactoMineR and factoextra packages to illustrate the respective effects of soil or fertilization types on the solution chemistry of soils and Cu and Zn availability proxies (i.e., total Cu and Zn concentrations and free ionic activity) in the presence of earthworms. The effect of fertilization types (none, mineral, or organic) on the solution chemistry of soils in the presence of earthworms and on Cu and Zn bioavailability proxies (i.e., net Cu and Zn accumulation and Cu and Zn concentrations in earthworms) to earthworms was assessed for each soil by performing a one-way analysis of variance (ANOVA) and the Tukey honestly significantly different (HSD) post hoc multiple test comparison when conditions of residual normality and homoscedasticity were satisfied, or otherwise the Kruskal-Wallis test and the Dunn post hoc multiple test comparison. For the three soil types considered together, structural equation models (SEMs) using the R package lavaan were built to identify and mathematically characterize the respective contributions of the contamination (DTPA-extractable Cu and Zn concentrations) and the solution chemistry (pH, DOC, and SUVA) of soils to Cu and Zn availability proxies in soils and to bioavailability to earthworms. The indirect effect of the contamination and the solution chemistry on Cu and Zn availability proxies and on bioavailability to earthworms was accounted for when building SEM. The fit of the model was evaluated using three indicators: the comparative fit index (CFI), the root mean square approximation (RMSEA), and the standardized root mean square residual (SRMSR). The adequacy of the fit of the models was reflected by CFI values above 0.95, RMSEA values below 0.05, and SRMR values below 0.08. The variables selected in the model were removed when the path coefficient was not significant (p>0.05).

3. Results

3.1 Effect of the types of soil and type of fertilization on soil solution chemistry and Cu and Zn availability

Soil and fertilization types distinctly influenced the distribution of the dataset on the chemistry and Cu and Zn availability proxies (i.e., total Cu and Zn concentrations and Cu^{2+} and Zn^{2+} activities in soil solutions) in the solution of earthworm-inhabited soils (Fig. 1). The first component (PC1) was determined mainly by Cu and Zn availability proxies, pH, and DOC concentration. The second component (PC2) was determined mainly by SUVA (Fig. 1a). Soil types were dissociated into three clusters and mainly along PC2 (Fig. 1b). Unlike the soil types, the fertilization types were dissociated into two main clusters along PC1 corresponding to mineral and organic fertilization, with the two data points corresponding to the no fertilization treatment in between (Fig. 1c). By performing the PCA on each soil type individually (Fig. S2), the effect of fertilization types on solution chemistry and Cu and Zn availability was even more obvious than it was for the three soil types considered together. These results clearly highlight a distinct effect of OF applications compared to mineral fertilizers or no fertilization on solution chemistry and on Cu and Zn availability in earthworm-inhabited soils, regardless of the soil type.

3.2 Effect of fertilization on soil solution chemistry

Considering all soil and fertilization types, the pH, DOC concentration, SUVA, total Cu and Zn concentrations, and Cu^{2+} and Zn^{2+} activities in the solution of earthworm-inhabited soils ranged from 6.2 to 6.6 pH units, 3 to 37 mg l^{-1} for DOC, 13 to 37 l g^{-1} cm^{-1} for SUVA, 7.5 to 6.6 in pCu_{SS}, 7.1 to 6.1 in pZn_{SS}, 11.6 to 9.3 in pCu^{2+}, and 8.3 to 6.3 in pZn^{2+} (Fig. 2). The pH, DOC concentration, SUVA, and total Cu concentration in the solution of earthworm-inhabited soils increased significantly with OF applications compared to mineral fertilizers and no fertilization, except for SUVA in the cambisol. Conversely, the total Zn concentration and Cu^{2+} and Zn^{2+} activity in the solution of earthworm-inhabited soils decreased significantly with OF applications compared to mineral fertilizers and/or no fertilization, except for the total Zn concentration in the arenosol, for which the decreasing tendency was not significant. Concerning the binding properties of DOM, the site density of DOM (i.e., %*r-DOM*) and the DOM binding affinity toward Cu (i.e., $logK_{Cu}$) significantly increased with OF applications compared to mineral fertilizers and/or no fertilization, except in the case of *%r-DOM* in the cambisol (Fig. 2).

Prediction of Cu^{2+} activity in the solution of earthworm-inhabited soils using the default parameterization of WHAM overestimated the Cu^{2+} activity and fitted the measured Cu^{2+} activities poorly (Fig. 3a). The optimization of *%r-DOM* with SUVA still overestimated Cu^{2+} activity and only slightly improved the fit between predicted and measured Cu^{2+} activities (Fig. 3b). Optimizing *%r-DOM* and $logK_{Cu}$ within a range of physically meaningful values definitely improved the prediction of Cu^{2+} activity for all samples, except one for which the best optimized prediction remained slightly outside the ± 0.5 pCu^{2+} unit range around the measured value (Fig. 3c).

3.3 Bioavailability of copper and zinc to earthworms

A significant mean earthworm biomass loss of 36%, 22%, and 28% was measured during the experiment in the andosol, cambisol, and arenosol, respectively, regardless of the fertilization type (Fig. S3). Earthworm quiescence and mortality occurred, respectively, in 70 and 38% of cases (median values) in the andosol for all fertilization types (Fig. S4a and b). In the cambisol, quiescence and mortality were low and not significantly different between fertilization types (Fig. S4). In the arenosol, the quiescence was significantly higher (median value 70%) in OF-amended soils than in soils with mineral or no fertilization (Fig. S4a). However, mortality was not significantly different among fertilization types (Fig. S4b).

Considering all soil and fertilization types, Cu and Zn net accumulation in earthworms ranged from -10.3 to 30.6 ng for Cu and -384.5 to 309.1 ng for Zn (Fig. 4). Similarly, for all soil and fertilization types, the Cu and Zn concentrations in earthworms ranged from 4.8 to 16.2 mg Cu kg^{-1} and

100.9 to 353 mg Zn kg^{-1}, respectively (Fig. 5). Regardless of the fertilization type, the Cu and Zn concentrations in earthworms after 21 days of exposure to the andosol and the cambisol were higher than the corresponding concentrations before the exposure. For the arenosol, Cu and Zn concentrations were in the same range of magnitude before and after the exposure. In contrast, net Cu and Zn accumulations during the exposure were mainly negative, indicating net Cu and Zn excretion, except for Cu in the cambisol. The accumulation and concentration of Cu and Zn in earthworms were not significantly different between the fertilization types except in the arenosol (Fig. 4 and 5), where the Cu concentration in earthworms was significantly lower in the unfertilized soil (Fig. 5a). Nevertheless, in the arenosol, net Zn accumulation (Fig. 4b) and Zn concentration (Fig. 5b) in earthworms were significantly higher in the soil fertilized with OF and not fertilized than in the soil with mineral fertilization.

3.4 Relationship between copper and zinc availability in soils and bioavailability to earthworms

Using SEM, we showed that the increase in total Cu concentration in the soil solution was well explained (adj-R^2 = 0.8) by the increase in DOC concentration and then by the increase in SUVA and DTPA-extractable Cu concentrations (Fig. 6a). The decrease in Cu^{2+} activity was also adequately explained (adj-R^2 = 0.72) by the increase in DOC concentration and then by the increase in pH and total Cu concentration in the soil solution (Fig. 6a). The indirect effects of DOC concentration, SUVA and DTPA-extractable Cu secondarily contributed to the decrease in Cu^{2+} activity (Fig. 6b). The net Cu accumulation in earthworms was much less well explained (adj-R^2 = 0.32), with a decreasing effect of DOC concentration and an increasing effect of SUVA (Fig. 6a).

The decrease in total Zn concentration in soil solutions was moderately explained (adj-R^2 = 0.31) by the increase in soil solution pH (Fig. 7a). The decrease in predicted Zn^{2+} activity was well explained (adj-R^2 = 0.95) by the decrease in total Zn concentration in soil solutions and the increase in DOC concentration (Fig. 7a). The increase in soil solution pH also contributed indirectly to the decrease in

Zn^{2+} activity (Fig. 7b). The net Zn accumulation in earthworms was moderately explained (adj-R^2 = 0.43) by a negative effect of DTPA-extractable Zn and a positive effect of SUVA (Fig. 7a).

4. Discussion

4.1 Organic fertilization mitigates copper and zinc availability in earthworm-inhabited soils

In the three earthworm-inhabited soils and compared to the mineral or no-fertilization treatments, organic fertilization induced an increase in soil pH and DOM properties (i.e., concentration, aromaticity as seen with SUVA, and binding properties as seen with the optimized *%r-DOM* and $\log K_{Cu}$). Laurent et al. (2020) found similar patterns of soil solution chemistry in the same dry, earthworm-free soils, suggesting that earthworms (i.e., *D. saliens* herein) bioturbation had a low impact on soil pH and DOM properties compared to OF applications. Nevertheless, the pairwise comparison of soil pH and DOM properties between earthworm-inhabited soils (present study) and earthworm-free soils (Laurent et al. 2020) highlighted some differences (Fig. S5). The DOC concentration decreased by ca. 3-fold (Fig. S5b) and conversely SUVA and DOM binding properties increased in earthworm-inhabited soils compared to earthworm-free soils (Fig. S5c, d, and e). Although earthworm bioturbation may partly explain these differences in soil pH and DOM (Sizmur and Hodson 2009), the moistening and incubation (without earthworm) of dry soils for a few weeks has been showed to induce similar differences in soil pH and DOM properties (Amery et al. 2007). In the absence of a control treatment consisting in incubated each soil samples without earthworm, it is not possible to ascertain whether earthworm bioturbation or soil incubation was responsible for the differences in soil pH and DOM properties. Although this distinction should thus be ascertained in a future study, the results presented herein showed that neither soil incubation nor earthworm bioturbation did not change the overall and major effect of organic fertilization on soil pH and DOM properties.

Concerning Cu, organic fertilization induced an increase in soil DTPA-extractable Cu (as a surrogate of soil contamination), SUVA, and DOC concentration that concomitantly induced an increase in total Cu concentration in the solution of earthworm-inhabited soils, with a primary effect of DOC concentration. In contrast, organic fertilization induced an increase in pH and DOC concentration that concomitantly induced a decrease in Cu^{2+} activity in the solution of earthworm-inhabited soils, with again a primary effect of DOC concentration (Fig. 6). These results are fully supported by the literature (notably Amery et al. 2007, 2008; Araujo et al. 2019; Bravin et al. 2012; Cambier et al. 2014; Laurent et al. 2020; Minnich and McBride 1987; Xu et al. 2016) and underline the role of DOM as the main driver of Cu concentration and speciation in the solution of earthworm-inhabited and OF-amended soils. By increasing the soil DOM concentration, aromaticity, and binding properties toward Cu, OF applications seems to have promoted both Cu desorption from the soil solid phase and its complexation by DOM in the soil solution. These two organic fertilization-induced processes were secondarily boosted by the increase in soil Cu contamination and pH. As the free ion in solution is usually considered the main (if not the unique) metal species taken up by soil organisms (Thakali et al. 2006), we conclude that OF applications induced a decrease in Cu availability in the solution of earthworm-inhabited soils.

Concerning Zn, organic fertilization induced an increase in soil pH and DOC concentration, which concomitantly induced a decrease in both total Zn concentration and Zn^{2+} activity in the solution of earthworm-inhabited soils, with a primary effect of pH. These results are supported by the literature (notably Cambier et al. 2014; De Conti et al. 2016; Laurent et al. 2020) and underline the role of pH as the main driver of Zn concentration and speciation in the solution of earthworm-inhabited and OF-amended soils. By increasing soil pH, OF applications primarily promoted Zn sorption from the soil solution to the solid phase, thereby decreasing both the total Zn concentration and Zn^{2+} activity in the soil solutions. By increasing the DOM concentration, OF applications secondarily promoted Zn^{2+} complexation to DOM, thereby decreasing Zn^{2+} activity in soil solutions. The lower effect of DOM on Zn speciation compared to Cu is consistent with the well-known lower affinity of DOM for Zn and the consequent higher percentage of total Zn in solution as Zn^{2+} compared to Cu (Bonten et al. 2008;

Laurent et al. 2020; Fig. S6). We therefore conclude that OF applications induced a decrease in Zn availability in the solution of earthworm-inhabited soils.

As for soil pH and DOM properties, the effect of OF applications on Cu and Zn availability in the solution of earthworm-inhabited soils was very similar to the effect of OF applications described by Laurent et al. (2020) in the same soils but without earthworms, suggesting that earthworms (i.e., *D. saliens* herein) bioturbation had a lower impact on Cu and Zn availability in soil solutions compared to OF applications. Such a result should apparently contradict the positive impact of earthworms on trace element availability in bioturbated soils usually expected according to a recent meta-analysis (Sizmur and Richardson 2020). However, a careful examination of this meta-analysis shows that, under specific experimental conditions as the ones we experienced (i.e., trace element availability determined in a salt solution, soil pH in the range 5.5-6.5, and organic amendments as the source of trace element contamination), the impact should be either not statistically significant or inconclusive due to the low number of studies. Accordingly, OF applications rather than earthworm bioturbation were the main drivers of Cu and Zn availability in our amended soils. This result thus confirms the results already underlined by Laurent et al. (2020), i.e., the organic fertilization induced an increase in soil pH and DOM properties that were able to mitigate Cu and Zn availability in the soils amended with OF for a decade, despite the concomitant and ongoing increase in soil Cu and Zn contamination.

4.2 Organic fertilization does not increase copper and zinc bioavailability to earthworms

For the three investigated soil types, a decade of OF applications neither increased the net Cu and Zn accumulation in earthworms nor their internal concentration compared to no or mineral fertilization (Fig. 4 and 5). To our knowledge, only two studies have previously investigated trace element (notably Cu and Zn) bioavailability to earthworms in uncontaminated agricultural soils amended with OF. Contrary to the results found in our study, Kızılkaya (2004) reported an increase in Cu and Zn concentrations in earthworms (*L. terrestris*) exposed in a pot experiment to an agricultural soil amended with increasing single application rates of sewage sludge. In this study, single application rates ranged from approximately 375 to 6 000 t FW ha^{-1} in a field equivalent (i.e., by considering 20% DM for the sewage sludge, 0.25 m for soil plowing, and 1.2 g cm^{-3} for soil density), which corresponds to Cu an Zn inputs ranging respectively from 5 to 86 and 11 to 174 mg kg^{-1} soil. In agreement with Kızılkaya (2004), Centofanti et al. (2016) reported an increase in Cu and even more in Zn concentrations in *L. terrestris* exposed in a pot experiment to an agricultural soil amended with a single application of composts at a rate of ca. 200 t FW ha^{-1} in a field equivalent. The discrepancy between our results and those of Kızılkaya (2004) and Centofanti et al. (2016) is unlikely to be explained by the distinct ecological categories and genera to which *D. saliens* and *L. terrestris* belong. Indeed, Richardson et al. (2020) explained in their review that no ecological category or earthworm genus exhibits a consistent pattern concerning trace element bioavailability to earthworms. Alternatively, the application rates used by Kızılkaya (2004) and Centofanti et al. (2016) were much higher than an agronomically realistic rate for a single application of any OF. By comparison, OF applications performed in the three field trials we studied were performed under field conditions (not in the laboratory) at much lower and agronomically realistic rates that led to lower annual inputs of Cu and Zn to soils (see 2.1 for rationale). These striking differences between our experimental design and those of Kızılkaya (2004) and Centofanti et al. (2016) could be consistent with our finding of any significant increase in Cu and Zn accumulation and concentration in earthworms exposed to OF-amended soils. Accordingly, our results suggest that OF applications for a decade at agronomically realistic rates should not increase Cu and Zn bioavailability to earthworms.

This conclusion is consistent with the reduction of Cu and Zn availability we observed in OF-amended soils, despite soil Cu and Zn contamination. The link between availability in soils and bioavailability to earthworms was further substantiated by the relatively weak (adj-R^2 = 0.32 and 0.43) but significant correlations found between some availability proxies and the net Cu and Zn accumulation in earthworms (Figs. 5 and 6). The low regression coefficients may be partially explained by the only 3-fold variation range in earthworm Cu and Zn concentrations (5-16 and 100-356 mg kg^{-1} for Cu and Zn, respectively). Regardless of the regression weakness, DOC concentration and SUVA for Cu and DTPA-extractable Zn and SUVA for Zn significantly contributed to the explained percentage of the variance of the net Cu and Zn accumulation in earthworms. Nahmani et al. (2007) and Richardson et al. (2020) stated in their reviews that the primary soil parameter that explains Cu and Zn concentrations and uptake in earthworms was the soil total concentration. They further argued, however, that the effect of soil total concentration was strongly linked to the fact that almost all reviewed data were obtained in highly contaminated soils, either on the field or spiked under laboratory conditions. Considering this statement, our results suggest that in the case of low, diffuse contamination, namely, historically contaminated agricultural soils, total Cu and Zn soil concentrations would not be a strong driver of Cu and Zn bioavailability to earthworms.

DOC concentration in the soil solution contributed negatively to the explained percentage of the variance of the net Cu accumulation in earthworms. This finding agrees with the negative contribution of soil organic matter to Cu concentration and uptake by earthworms highlighted by Nahmani et al. (2007) and Richardson et al. (2020). This finding is also consistent with the primary role that DOM played in the decrease in Cu^{2+} activity in the solution of earthworm-inhabited and OF-amended soils. Accordingly, we conclude that, despite an increase in soil Cu contamination in OF-amended soils, the concomitant increase in DOC concentration contributed to maintaining Cu bioavailability to earthworms at low levels through the reduction of soil Cu availability.

Soil DTPA-extractable Zn contributed negatively to the explained percentage of the variance in the net Zn accumulation in earthworms. At first glance, this finding seems counterintuitive. However, as soil Zn contamination increased in OF-amended soils, as indicated by DTPA-extractable Zn, soil Zn availability decreased, as indicated by the total Zn concentration and Zn^{2+} activity in solution. This decrease in Zn availability in OF-amended soils, which was due mainly to the increase in soil solution pH, likely explained the negative correlation between soil DTPA-extractable Zn and the net Zn accumulation in earthworms. This finding agrees with the negative contribution of soil pH to Zn concentration and uptake in earthworms, as highlighted by Nahmani et al. (2007) and Richardson et al. (2020). Accordingly, we conclude that, despite an increase in soil Zn contamination in OF-amended soils, the concomitant increase in pH contributed to maintaining Zn bioavailability to earthworms at low levels through the reduction of soil Zn availability.

SUVA in the soil solution positively contributed to the explained percentage of the variance in the net Cu and Zn accumulation in earthworms. In our case, the increase in DOM aromaticity in OF-amended soils coincided to the decrease in Cu^{2+} activity in soil solutions. Amery et al. (2008 and 2010) and Welikala et al. (2018) also showed that an increase in DOM aromaticity coincided with a decrease in Cu and, to a lesser extent, Zn lability in soil solutions. From a chemical point of view, the increase in DOM aromaticity observed in OF-amended soils should have reduced soil Cu and Zn availability and consequently contributed to lower Cu and Zn bioavailability to earthworms. From a biological point of view, the positive correlation we found between SUVA and the net Cu and Zn accumulation in earthworms suggests that an increase in DOM aromaticity may increase Cu and Zn bioavailability to earthworms. This contradiction deserves further attention to decipher whether Cu and Zn bound with aromatic DOM are biologically labile for soil organisms such as earthworms.

To explain why Cu and Zn bioavailability to earthworms did not increase in OF-amended soils, physiological regulation of Cu and Zn uptake in earthworms may also be invoked together with the reduction of soil Cu and Zn availability. Indeed, Cu and Zn are essential elements in such a way that their uptake and excretion are actively regulated by earthworms (Spurgeon and Hopkin 1999). Nahmani et al. (2007) estimated the average regression coefficient between soil total concentration and earthworm concentration for essential (Cu and Zn) and nonessential (Cd and Pb) trace elements. This coefficient was notably lower for Cu and Zn than for Cd and Pb, which confirms that the physiological ability of earthworms to regulate their net internal accumulation is higher for Cu and Zn. Cu and Zn concentrations measured in earthworms in our study were in the same range or lower than the Cu and Zn median concentrations reported by Richardson et al. (2020) in their review. Accordingly, the physiological regulation of Cu and Zn taken up by earthworms exposed to agricultural soils moderately contaminated after OF applications may also have contributed to the maintenance of Cu and Zn bioavailability to earthworms (i.e., internal content) at low levels.

Beyond the net Cu and Zn accumulation in earthworms, a toxicological effect on earthworms in relation to their internal Cu and Zn concentrations was looked for. This hypothesis is based on three sets of measurements made during the 21 days of earthworm exposure to soils: (i) the significant decrease in earthworm biomass (Fig. S3), (ii) the occurrence of quiescence and mortality (Fig. S4), and (iii) the internal Cu and Zn concentrations (Fig. 5). According to the literature (e.g., Leveque et al. 2014; Sizmur et al. 2011; Centofanti et al. 2016), a decrease in earthworm biomass is quite common during ex situ microcosm experiments. Sizmur et al. (2011) attributed the decrease in earthworm biomass to the absence of food added to soil, which is necessary to avoid experimental interference (see section 2.2 for rationale). As this biomass decrease was consistent over all fertilization and soil types, it is unlikely that OF applications were responsible for it. Quiescence and mortality occurred only in the andosol and the arenosol and much more frequently in the former. *D. saliens* individuals were quantitatively mainly found and consequently collected in the cambisol. Collected *D. saliens* individuals were thus likely less adapted to inhabit the arenosol and even more so the andosol. In addition, it is well known that the re-humidification of previously air-dried soil is not easy for andosol. Despite the water content of andosol samples was macroscopically controlled as we did for the samples of the two other soil types, the microscopic availability of water in the andosol may have not been optimal for *D. saliens*. Whatever the underlying reason, this poorer adaptation to the arenosol and the andosol did not seem related to OF applications.

Considering the rather low Cu and Zn concentrations measured in earthworms, it is therefore unlikely that any Cu- or Zn-related toxicity could have occurred, nor more particularly in OF-amended soils, as OF applications did not affect Cu and Zn concentrations in earthworms. This result is in line with the fact that earthworms are known to be ubiquitously tolerant to Cu and Zn by sequestering Cu in sulfur-rich metallothionein-like proteins within chloragogenous tissues and Zn in phosphate-rich insoluble granules within calciferous glands (Richardson et al. 2020). Accordingly, OF applications to the three soils for a decade do not seem to have affected earthworms more than no and mineral fertilization, even if the growth and health of earthworms in the soil microcosms was not optimal overall. These results should, however, be completed with measurements of toxicological effects with relevant proxies concerning the energy budget (e.g., protein and lipid content) to account for toxicological bioavailability.

From an ecotoxicological perspective, our results as a whole suggest that a decade of agronomically realistic OF applications should not constitute a risk for earthworms in terms of the bioaccumulation of Cu and Zn. To the best of our knowledge, this study is the first to address this question for agronomically realistic scenarios of historical contamination. Additional studies are needed to extrapolate our conclusion to a larger range of OF and earthworm ecological categories and species. Furthermore, earthworms are well known to be ecological engineers with the ability to modify resources for other key soil organisms, such as plants. Studying plant-earthworm interactions in agricultural OF-amended soils should thus deserve further attention to better understand how these interactions can impact soil Cu and Zn availability as well as their bioavailability to both plants and earthworms.

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Statements and declarations

Compliance with Ethical Standards

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Author Contributions and consent to participate and publish

All authors, namely, C. Laurent, M. N. Bravin, E. Blanchart, O. Crouzet, C. Pelosi, and I. Lamy, contributed to the study conception and design. Material preparation, data collection and analysis were performed by C. Laurent and M. N. Bravin. The first draft of the manuscript was written by C. Laurent and M. N. Bravin and all authors commented on previous versions of the manuscript. All authors consented to participate, then read, and approved the final manuscript. All authors agreed to submit the final manuscript for publication in *Environmental Science and Pollution Research*.

Availability of data and materials

Datasets are accessible via Dataverse (Laurent et al., 2021).

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Figures and tables

Table 1 Soil physical-chemical properties as a function of fertilization types and before exposure to earthworms. Mean values are given with their standard deviation in parentheses.

Soil	Fertilization	*n*[a]	pH-H_2O	Organic C g kg^{-1}	Total Cu mg kg^{-1}	Total Zn mg kg^{-1}	Cu-DTPA mg kg^{-1}	Zn-DTPA mg kg^{-1}
Andosol	No fertilization	1	5.7	115	81	163	1.3	7
	Mineral fertilization	2	5.2 (± 0.4)	111 (± 3)	80 (± 9)	180 (± 11)	1.5 (± 0.3)	8 (± 5)
	Organic fertilization	4	6.2 (± 0.4)	123 (± 6)	83 (± 4)	183 (± 10)	2.3 (0.8)	15 (± 3)
Cambisol	Mineral fertilization	2	5.4 (± 0.2)	34.1 (± 0.1)	80 (± 1)	242 (± 5)	4.5 (± 0.3)	6.0 (± 0.4)
	Organic fertilization	4	6.1 (± 0.6)	36 (± 3)	83 (± 5)	257 (± 14)	7 (± 2)	13 (± 7)
Arenosol	No fertilization	1	6.6	11	74	143	1.5	2
	Mineral fertilization	2	5.8 (± 0.1)	10.2 (± 0.7)	70 (± 2)	136 (± 2)	1.2	1.9 (± 0.2)
	Organic fertilization	4	7.2 (± 0.2)	13.4 (± 2.6)	71 (± 2)	143 (± 6)	1.7 (± 0.4)	7 (± 3)

[a]number of soil samples

Figure 1 Principal component analysis of the solution parameters of earthworm-inhabited soils. The correlation plot (a) shows the contribution of each variable to the two first principal components (PC1, PC2). The solutions of earthworm-inhabited soils (n = 20) were clustered (b) as a function of soil types (andosol, cambisol, and arenosol) or (c) as a function of fertilization types (no fertilization, mineral, and organic). Data points with a larger size in (b) and (c) correspond to the barycenter of each cluster. The quantitative variables considered in the PCA are pH, dissolved organic carbon concentration ($log_{10}DOC$), the specific UV absorbance at 254 nm (log_{10} SUVA), the total concentration of copper and zinc (pCu_{SS} and pZn_{SS}), and the activity of free ionic Cu and Zn (pCu^{2+} and pZn^{2+}) in the earthworm-inhabited soils. Blue dashed arrows represent Cu and Zn concentrations in earthworms (C_{Cu} and C_{Zn}), which were considered herein as supplementary quantitative variables.

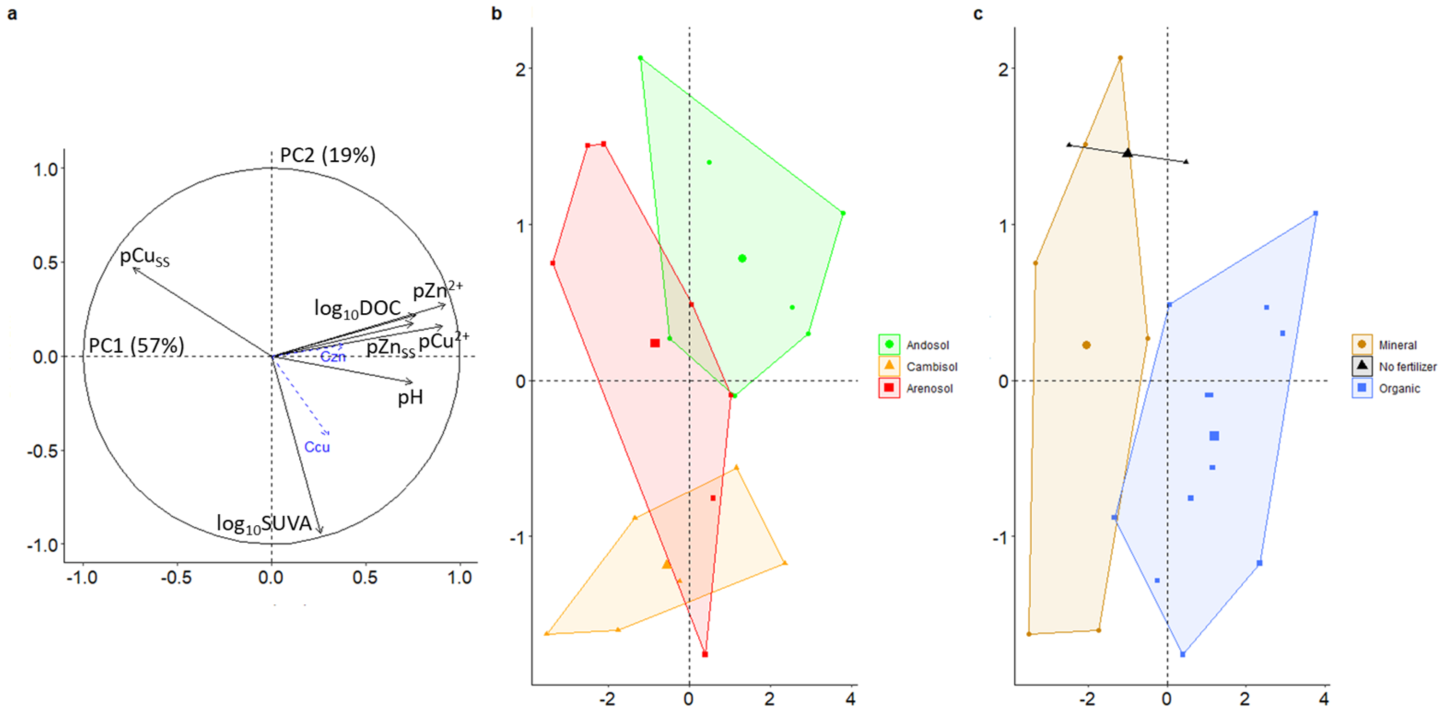


Figure 2 Variation of (a) pH, (b) DOC concentration, (c) specific UV absorbance at 254 nm (SUVA), (d) the percentage of dissolved organic matter reactive toward metal cations (%*r-DOM*), (e) the copper binding constant of DOM ($logK_{Cu}$), (f) total copper concentration (pCu_{SS}), (g) total zinc concentration (pZn_{SS}), (h) the free ionic Cu activity (pCu^{2+}), and (i) the free ionic Zn activity (pZn^{2+}) in the solutions of earthworm-inhabited andosol, cambisol, and arenosol as a function of fertilization types. Different letters indicate significant differences ($p \leq 0.05$). The box and whiskers represent from the bottom to the top the minimum, first quartile, median, third quartile, and maximum values. Where there are outliers, they are represented by data points and the related vertical bars represent 1.5 times the interquartile range above the third quartile or below the first quartile.

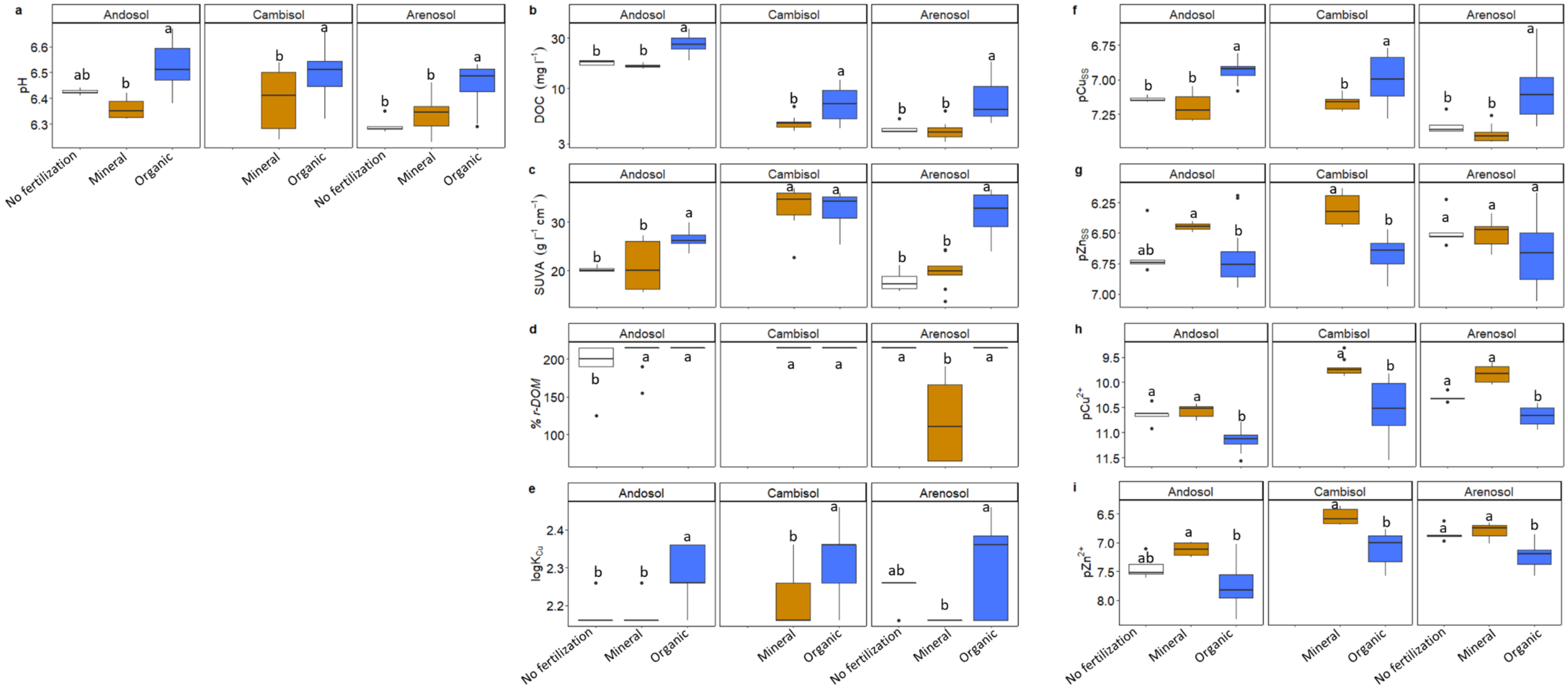


Figure 3 Free copper activity in the solutions of earthworm-inhabited soils (pCu^{2+}) predicted with WHAM as a function of pCu^{2+} measured (n = 99). pCu^{2+} was predicted by (a) using the default parameters of WHAM for the percentage of dissolved organic matter reactive toward metal cations (*%r-DOM*) equal to 65% and the copper binding constant ($logK_{Cu}$) equal to 2.16 for all soil solutions, (b) optimizing the *%r-DOM* with the SUVA measured in soil solutions, or (c) optimizing *%r-DOM* and $logK_{Cu}$ values within a range of physically meaningful values for each batch of five replicates (see the *Material and methods* section for rationale). The blue line, the thin solid line, and the dashed lines represent the regression line, the 1:1 line, and the 1:1 line ± 0.5 pCu^{2+} unit, respectively. Vertical error bars represent the standard deviation of predicted Cu^{2+} activity. The adjusted R-squared (adjR^2), the root mean square residual (RMSR), and the slope of the regressions are given.

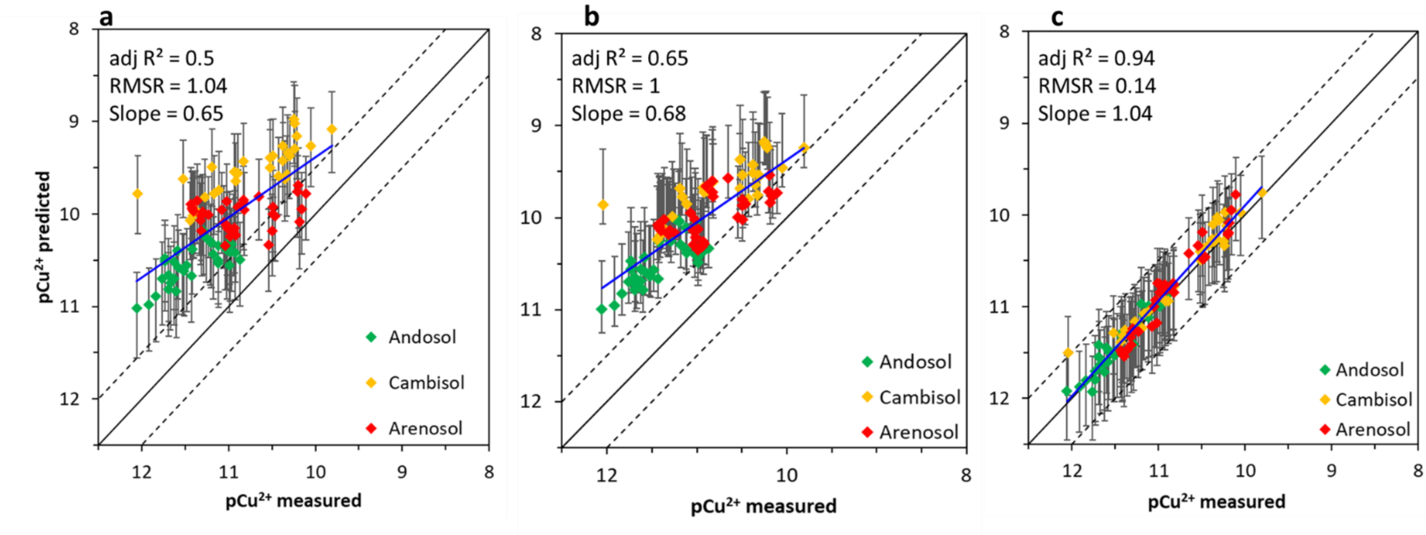


Figure 4 Mean net accumulation of: a) copper (Cu) and b) zinc (Zn) per earthworm individual in the andosol, cambisol and arenosol as a function of the fertilization types (no fertilization, mineral fertilization and organic fertilization). Different letters indicate significant differences ($p \leq 0.05$) among fertilization types. The red dashed lines represent a nil net accumulation. The box and whiskers represent, from bottom to top, the minimum, first quartile, median, third quartile, and maximum values. Where there are outliers, the outliers are represented by data points and the related vertical bars represent 1.5 times the interquartile range above the third quartile or below the first quartile.

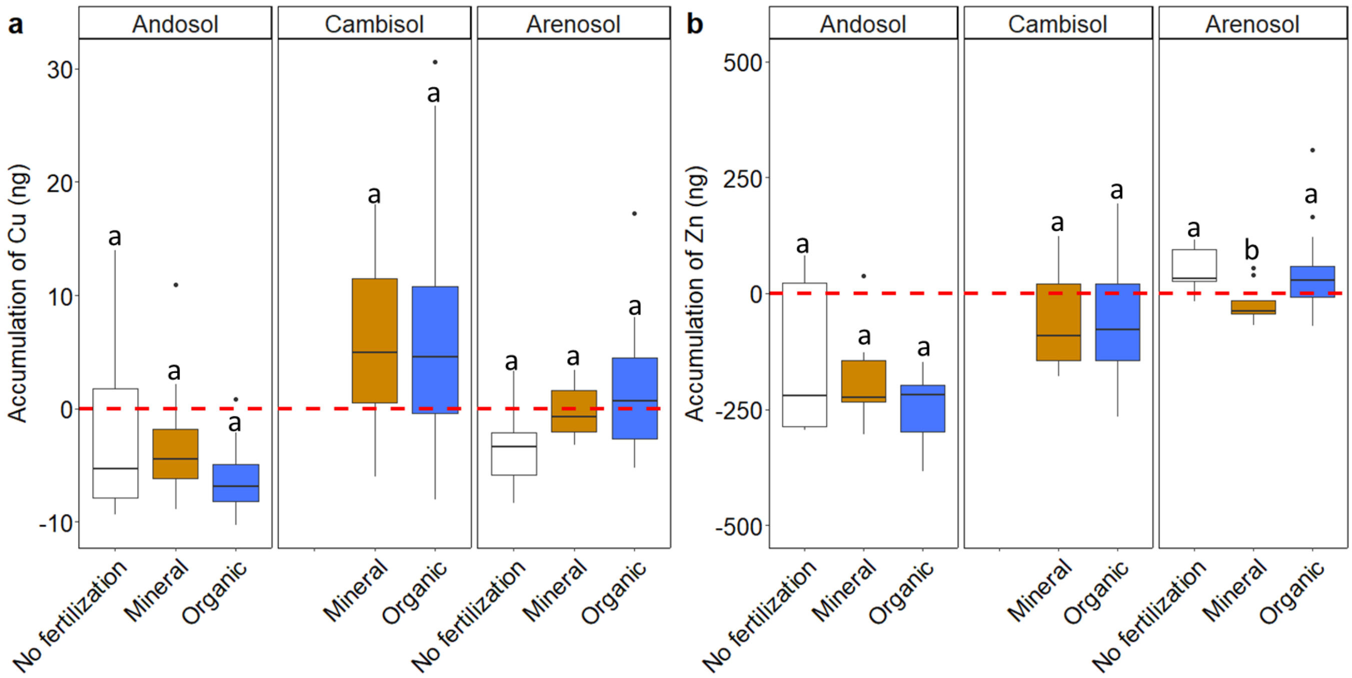


Figure 5 Concentration of copper (Cu, a) and zinc (Zn, b) in earthworms pooled per microcosm in the andosol, the cambisol, and the arenosol as a function of fertilization type (no fertilization, mineral fertilization, and organic fertilization). Different letters indicate significant differences ($p \leq 0.05$). The red dashed lines represent the concentration of Cu or Zn measured in earthworms at the end of the acclimatization period. The box and whiskers represent (from the bottom to the top) the minimum, first quartile, median, third quartile, and maximum values. Where there are outliers, the outliers are represented by data points and the related vertical bars represent 1.5 times the interquartile range above the third quartile or below the first quartile.

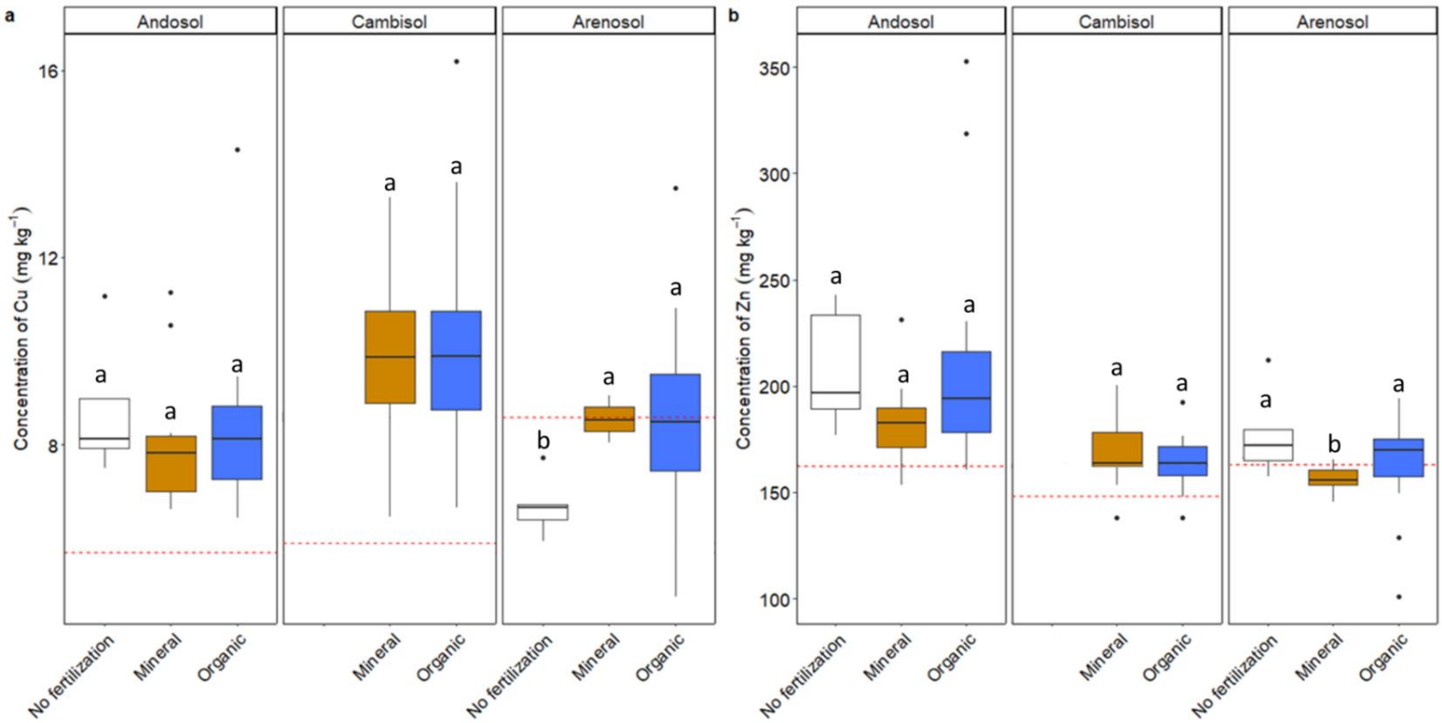


Figure 6 Structural equation model (SEM) (a) explaining the earthworm-inhabited soil availability (total concentration and free ionic activity in soil solution pCu_{SS} and pCu^{2+}) and the earthworm bioavailability (accumulation of Cu, $log_{10}Cu_{Acc}$) of copper as a function of the contamination (DTPA-extractable Cu, $log_{10}Cu_{DTPA}$) and the solution chemistry (pH, dissolved organic carbon concentration, $log_{10}DOC$, and specific UV absorbance at 254 nm, $log_{10}SUVA$) of the three soil types considered together. Path coefficients of significant explicative variables are illustrated (b), with a distinction between their direct and indirect effects.

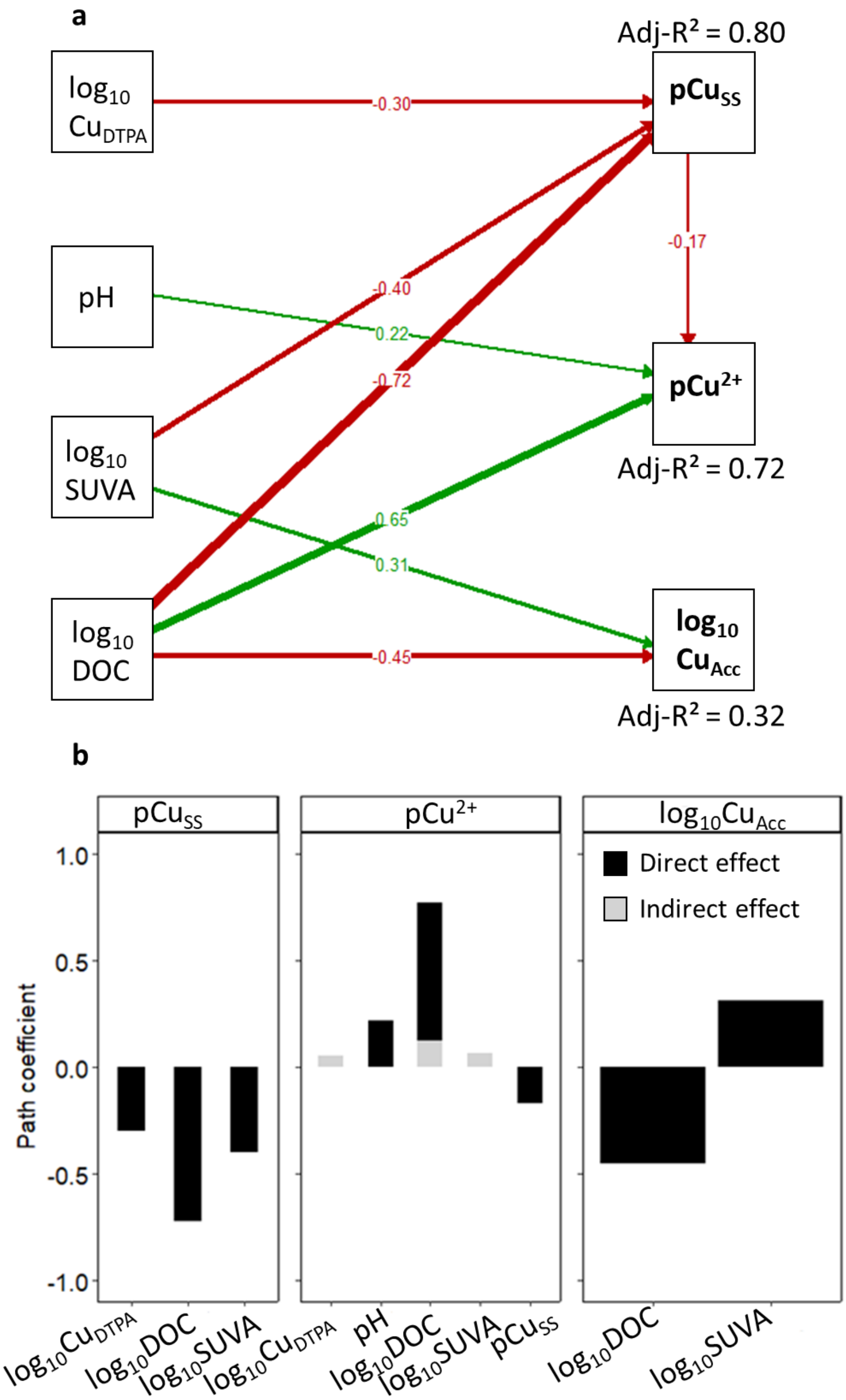
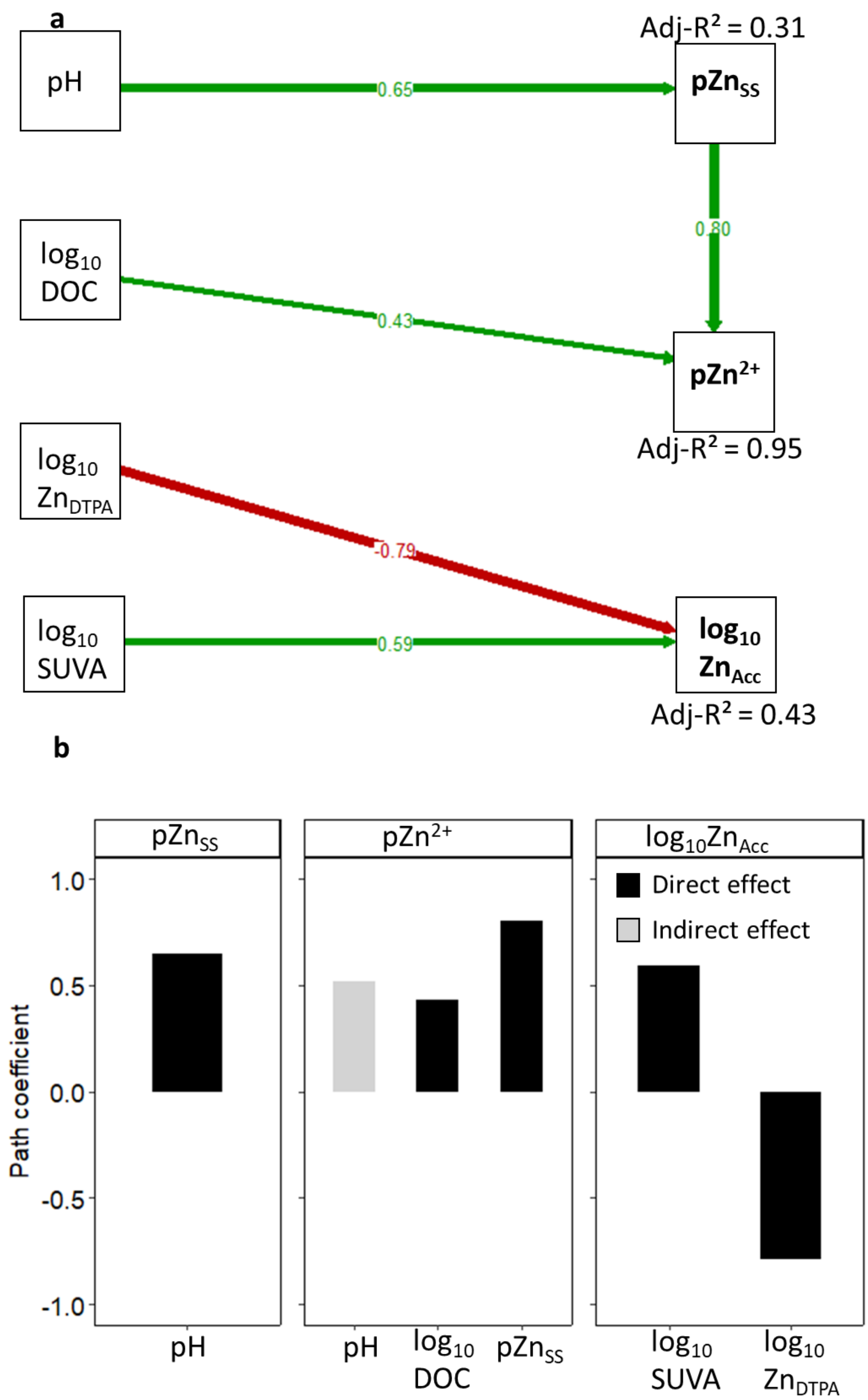


Figure 7 Structural equation model (SEM) (a) explaining the earthworm-inhabited soil availability (total concentration and free ionic activity in soil solution pZn_{SS} and pZn^{2+}) and the earthworm bioavailability (accumulation of Zn, $\log_{10}Zn_{Acc}$) of zinc as a function of the contamination (DTPA-extractable Zn, $\log_{10}Zn_{DTPA}$) and the solution chemistry (pH, dissolved organic carbon concentration, $\log_{10}$DOC, and specific UV absorbance at 254 nm, $\log_{10}$SUVA) of the three soil types considered together. Path coefficients of significant explicative variables are illustrated (b), with a distinction between their direct and indirect effects.



Supplementary information

Table S1 Characteristics of organic fertilizers applied on the three field trials.

		Andosol and arenosol		Cambisol	
	Unit, dry mass	Dairy slurry	Dairy manure	Pig slurry compost	Poultry litter compost
Dry matter	%	9.1	46	52	47
pH-H_2O		7.5	7.5	7.6	7.1
Organic C	g kg^{-1}	890	232	298	333
Total Cu	mg kg^{-1}	50	96	180	109
Total Zn	mg kg^{-1}	259	383	464	319

Figure S1 Free zinc activity in the earthworm-inhabited soils (pZn^{2+}) predicted with WHAM with same optimization of *%r-DOM* as pCu^{2+} prediction (see Section 2.5 for rationale) as a function of pZn^{2+} predicted with WHAM default parameterization (n = 97). The blue line, the thin solid line, and the dashed lines represent the regression line, the 1:1 line, and the 1:1 line ± 0.5 pZn^{2+} unit, respectively. The adjusted R-squared (adjR^2), the root mean square residual (RMSR), and the slope of the regressions are given.

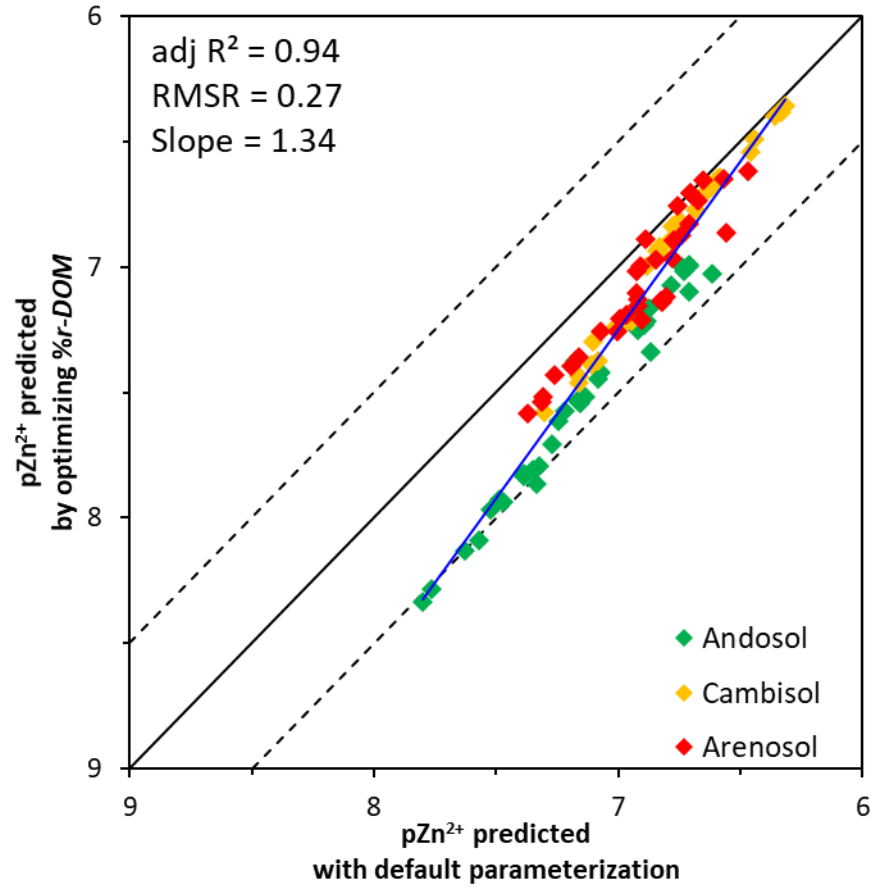


Figure S2 Principal component analysis of the solution parameters of earthworm-inhabited soils: (a and b) the andosol (n = 7), (c and d) the cambisol (n = 6), and (e and f) the arenosol (n =7). The correlation plots (a, c, and e) show the contribution of each variable to the two first components (PC1, PC2). The solutions of earthworm-inhabited soils were clustered (b, d, and f) as a function of fertilization type (no fertilization, mineral fertilization, and organic fertilization). Data points with a larger size in (b), (d) and (f) correspond to the barycenter of each cluster. The quantitative variables considered in the PCA are pH, dissolved organic carbon concentration ($\log_{10}$DOC), the specific UV absorbance at 254 nm ($\log_{10}$ SUVA), the total concentration of copper and zinc (pCu_{SS} and pZn_{SS}), and the activity of free ionic Cu and Zn (pCu^{2+} and pZn^{2+}) in earthworm-inhabited soils. Blue dashed arrows represent Cu and Zn concentrations in earthworms (C_{Cu} and C_{Zn}), which were considered herein as supplementary quantitative variables.

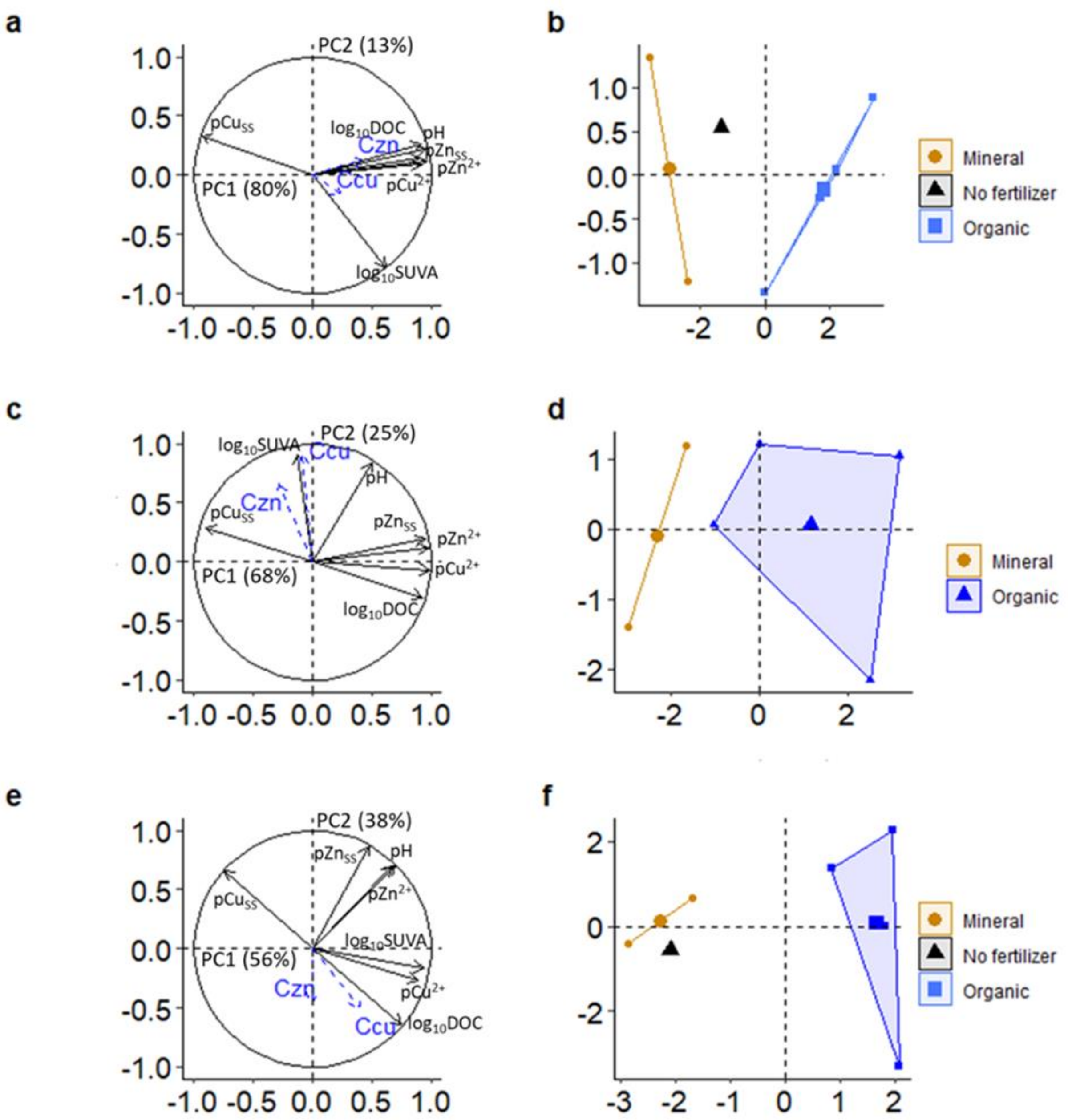


Figure S3 Earthworm individual fresh biomass at the beginning (dark colors) and at the end of the 21 days of exposure (light colors) to the andosol, the cambisol, and the arenosol as a function of fertilization types (no fertilization, mineral, and organic). Different letters indicate significant differences ($p \leq 0.05$). The box and whiskers represent from the bottom to the top the minimum, first quartile, median, third quartile, and maximum values. Where there are outliers, they are represented by data points and the related vertical bars represent 1.5 times the interquartile range above the third quartile or below the first quartile.

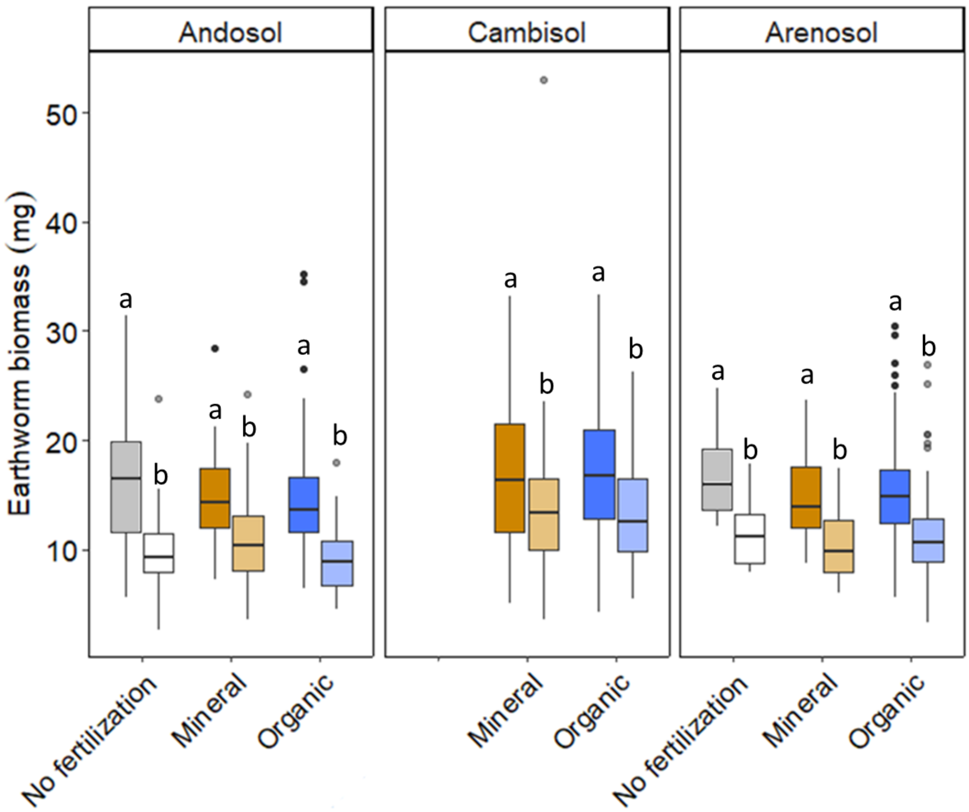


Figure S4 Rate of earthworms in quiescence (a) and mortality (b) at the end of the 21 days of exposure to the andosol, the cambisol, and the arenosol as a function of fertilization types (no fertilization, mineral fertilization, and organic fertilization). Different letters indicate significant differences ($p \leq 0.05$). The box and whiskers represent (from the bottom to the top) the minimum, first quartile, median, third quartile, and maximum values. Where there are outliers, the outliers are represented by data points and the related vertical bars represent 1.5 times the interquartile range above the third quartile or below the first quartile.

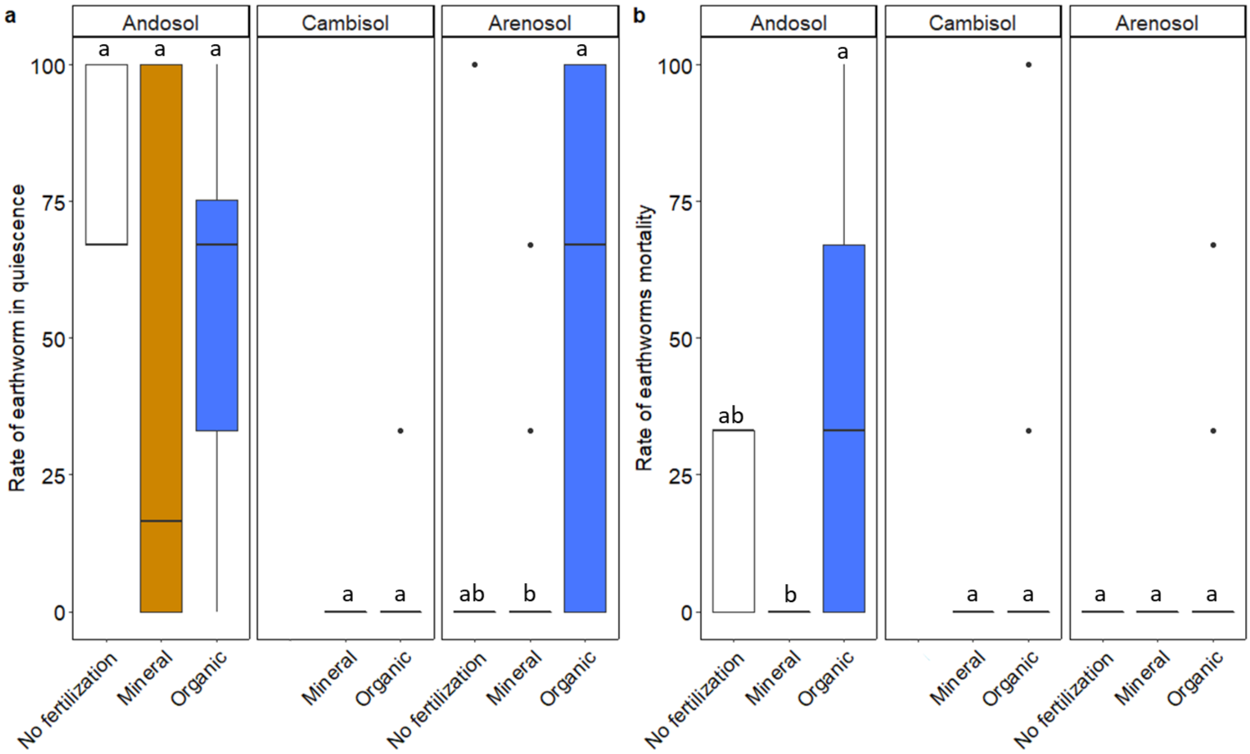


Figure S5 Mean values of: (a) pH, (b) dissolved organic carbon (DOC) concentration, and (c) specific UV absorbance at 254 nm (SUVA), (d) the optimized percentage of dissolved organic matter reactive toward metal cations (*%r-DOM*), (e) the optimized copper binding constant ($logK_{Cu}$), (f) total copper (pCu_{SS}) and (g) zinc concentration (pZn_{SS}), (h) measured free ionic copper (pCu^{2+}), and (i) the predicted free ionic zinc (pZn^{2+}) activity in the solution of earthworm-inhabited soils as a function of the same parameters in the solution of the earthworm-free soils for the andosol, the cambisol, and the arenosol. Data for earthworm-free soils were taken from Laurent et al. (2020). Vertical error bars represent the standard deviation. The thin line represents the 1:1 line.

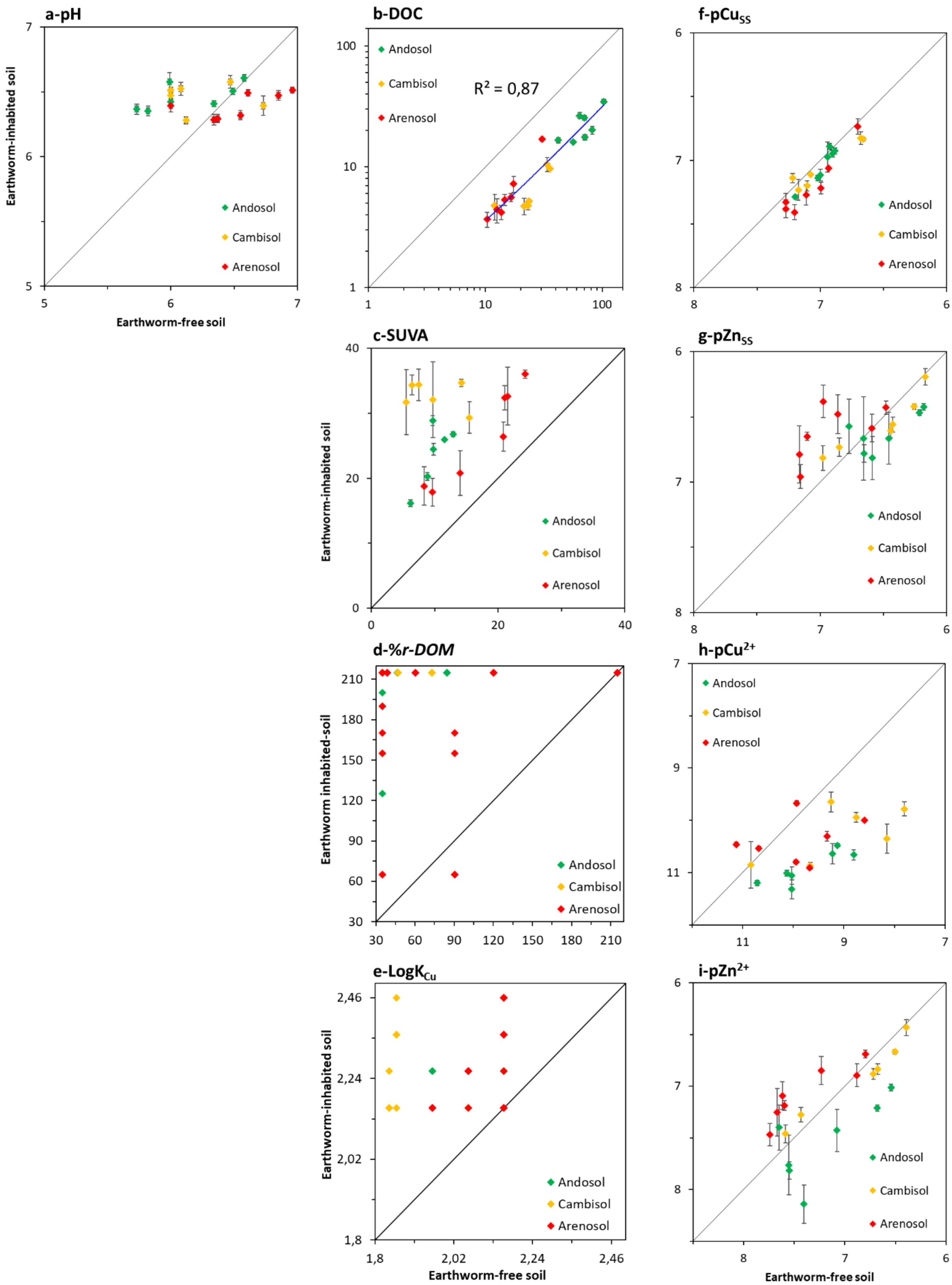
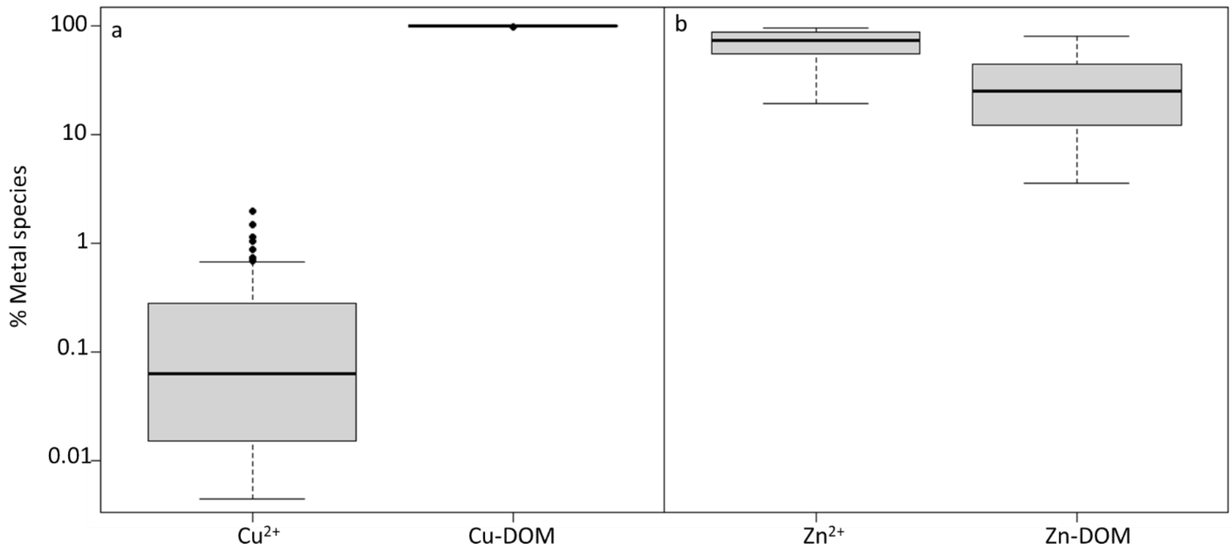


Figure S6 Predicted proportion of: (a) free ionic Cu (Cu^{2+}) and Cu bound to DOM (Cu-DOM) and (b) free ionic Zn (Zn^{2+}) and Zn bound to DOM (Zn-DOM) in the solution of the three earthworm-inhabited soils. The scale of the y-axis is expressed as log.



SI Reference

Laurent C, Bravin MN, Crouzet O et al. (2020) Increased soil pH and dissolved organic matter after a decade of organic fertilizer application mitigates copper and zinc availability despite contamination. Science of The Total Environment 709:135927. https://doi.org/10.1016/j.scitotenv.2019.135927