



Prevention values for copper (low tier approach) in subtropical acidic soils

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Abstract

Copper is a trace element in plants and animals whose importance can be understood due to its role in different essential metabolic processes. Anthropogenic activities such as agriculture and mining are potential sources of pollution due to the emission of copper into the environment. Brazilian legislation ties soil quality to guideline values, among which the Prevention Value indicates the critical environmental limit for trace elements. The aim of this study was to obtain PVs for copper for two subtropical soils (Cambisol and Nitisol), given that the pedological richness was not considered when deriving the PVs contained in the federal normative. Reproduction assays followed ISO guidelines with the earthworm species *Eisenia andrei* and *Perionyx excavatus*, the enchytraeids *Enchytraeus crypticus* and *Enchytraeus bigeminus* and the springtails *Folsomia candida* and *Proisotoma minuta*. Results showed that the sensitivity of the organisms was greater in Cambisol. The most sensitive species were the earthworms, especially *Perionyx excavatus* ($EC_{50} = 67.83$ in Cambisol; $EC_{50} = 264.96$ in Nitisol). The springtails, on the other hand, were the least sensitive to contamination. These findings reinforce the need to include organisms from different ecological groups in ecotoxicological assessments. It was also observed that the PV adopted in federal legislation ($= 60 \text{ mg kg}^{-1}$) is in fact protective for the species and soils we evaluated, since the PVs we obtained based on the EC_{50} were $346.74 \text{ mg kg}^{-1}$ in Nitisol and $134.05 \text{ mg kg}^{-1}$ in Cambisol. It is important to note that our results do not exclude the need for evaluations with other subtropical soils, given the influence of their properties on the toxicity and bioavailability of copper to soil organisms.

Keywords Ecotoxicology · Soil screening values · Trace metals · Soil contamination · Soil bioindicators

Introduction

Among the main contaminants of environmental interest, metals require some attention due to their metabolic involvement, because, although essential, in excess they can result in damage to organisms (Witkowska et al. 2021). Copper

(Cu) is a micronutrient for plants and animals that is naturally present in the soil due to the weathering processes taking place on the parent rock (Kabata-Pendias, 2010). However, the development of human activities, such as mining, agricultural practices and industrial activities (Rehman et al. 2019), has contributed to an increase in Cu concentrations in the environment, which has the potential to undermine the supply of environmental services because of contamination on key species' ecological dynamics.

The group of invertebrates performs ecosystem functions that are extremely important for maintaining life in the soil, with their activities linked to the processes of organic matter and nutrient cycling, soil structuring and bioregulation (Decaëns et al. 2006; Briones 2018; Heděnc et al. 2022). Therefore, it is essential to pay particular attention to the effects of Cu contamination on these organisms to preserve environmental quality. According to Sereni et al. (2021), soil

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fauna in Cu-contaminated sites exhibit morphological and behavioral alterations that can lead to trophic imbalance. Understanding the effects of this contamination has become essential for protecting biodiversity and ecosystem services connected with soil. Although the topic has been extensively researched in northern hemisphere soils (Spurgeon & Hopkin 1996; Lukkari et al. 2005; Kuperman et al. 2006; Maraldo et al. 2006; Amorim & Scott-Fordsmand 2012; Renaud et al. 2020), there are few reports on the effects of Cu in tropical and subtropical soils.

The Brazilian federal legislation, in the form of Resolution 420/2009 of the National Environment Council (CONAMA), ties soil and groundwater quality to guideline values. Among these, the prevention value (PV) refers to the concentrations above which it can be inferred that the soil is polluted by a certain element or substance (Brasil 2009). Due to the great pedological diversity, it is necessary to determine specific PVs to the broadest possible range of tropical and subtropical soil types. This is attributable to the fact that the PVs currently adopted at the national level are those obtained only from soil analysis in the state of São Paulo, making it difficult to identify and manage contaminated areas in other states.

The obtaining procedure of PV is closely linked to analyzing the effects of the exposure on key organisms such as arthropods and oligochaetes. These assessments are fundamental to understanding the effects of contamination and, as a result, to establishing effective prevention and management guidelines. The European Chemicals Agency (ECHA) recommends that such evaluations must be carried out with species representative of the different trophic levels (ECHA 2008). The main methods commonly used for this purpose are described by the International Organization for Standardization (ISO) guidelines and aim to measure the dose–response relationship in parameters such as organism survival (lethality) and reproduction capacities when exposed to contaminated soils (Van Gestel 2012).

Considering this scenario, the aim of the present research was to derive PVs for Cu for two subtropical representative soils of South Brazil through reproduction assessments with the earthworm species *Eisenia andrei* and *Perionyx excavates*, the enchytraeids *Enchytraeus crypticus* and *Enchytraeus bigeminus*, and the springtails *Folsomia candida* and *Proisotoma minuta*. Soil fauna species were chosen due to their well-documented sensitivity to inorganic contaminants and their use in standardized ecotoxicological assessments (Nursita et al. 2005; Kwak et al. 2014; Madani et al. 2015; Simões et al. 2020; Assis et al. 2023). While ecosystems encompass a diverse array of organisms, employing native species requires prior evaluations of their ecological characteristics. Therefore, findings from standardized species are often utilized to elucidate potential impacts on the dynamics of native species. To achieve these objectives, we

hypothesized that (i) the effects of contamination measured on the reproduction of the organisms evaluated vary from soil type, and (ii) the PVs adopted at the national level are not fitting for the subtropical soils tested. The methodology employed was to produce species sensitivity distribution curves (SSDs) and estimate risk concentrations (HC_5 and HC_{50}) based on the effect concentrations (EC_{50}) obtained in the exposure tests.

Materials and methods

Soils sampling and characterization

Two natural soils representative of the subtropical region of Brazil and a tropical artificial soil (TAS) were used in the ecotoxicological assessments: an Cambisol (Cambisol Húmico (Santos et al. 2018)) was collected in Lages, SC [27°48'57"S; 50°21'45"W] and a Nitisol (Nitossolo Vermelho (Santos et al. 2018)) was collected in Concórdia, SC [27°48'71"S; 51°59'34"W]. The soil samples were collected in the surface layer (0–20 cm) in areas under natural, unmanaged vegetation, free from fertilization and the use of agrochemicals. The TAS was made following the proportions proposed by Garcia et al. (2004), i.e. 75% fine sand, 20% kaolinitic clay and 5% dried and sieved coconut fiber. The TAS' pH was corrected to 6.0 ± 0.5 by adding calcium carbonate ($CaCO_3$) (ISO 2012). The characteristics of the soils were determined according to the procedures suggested by EMBRAPA (Teixeira et al. 2017) and are shown in Table 1.

Table 1 Chemical and physical properties of Nitisol, Cambisol, and TAS

Soil properties	Nitisol	Cambisol	TAS
Organic matter ($g\ kg^{-1}$)	42	52	22
CEC ¹	12.5	6.5	1.2
pH ²	5.8	4.7	6.2
Ca ($cmol_c\ dm^{-3}$)	11.7	2.6	2.0
Mg ($cmol_c\ dm^{-3}$)	2.5	1.4	0.9
P ($mg\ dm^{-3}$)	47.1	6.9	11.1
K ($mg\ dm^{-3}$)	162	52	262
Al ($cmol_c\ dm^{-3}$)	0.2	3.0	0.0
Cu ($mg\ dm^{-3}$)	10.6	2.8	0.2
Zn ($mg\ dm^{-3}$)	29.0	3.8	0.7
B ($mg\ dm^{-3}$)	0.65	0.67	1.38
Mn ($mg\ dm^{-3}$)	159	21.1	2.2
Clay ($g\ kg^{-1}$)	543.7	133.7	94.9
Silt ($g\ kg^{-1}$)	272.7	115.7	108.7
Sand ($g\ kg^{-1}$)	183.5	750.5	796.4
Texture	Clay	Sandy Loam	Sandy Loam

¹Cation Exchange Capacity at pH 7.0 ($cmol_c\ dm^{-3}$). ²pH in water

Copper spiking procedure

The soils were spiked with a $\text{Cu}(\text{NO}_3)_2$ solution (Table 2), following a three weeks incubation (Natal-da-Luz et al. 2011). The nominal tested concentrations were based on toxicity data reported in the literature (Spurgeon et al. 1994; Sandifer & Hopkin 1997; Amorin et al. 2005; Lukkari et al. 2005; Domínguez-Crespo et al. 2012; Gomes et al. 2014). Prior to the testing procedure, the moisture content was corrected to 50% of the soil's water holding capacity (WHC) in accordance with the ISO 11268–2 protocol (ISO 2012). After the spiking procedure, soil samples were used in ecotoxicity assays. Soil Cu contents were evaluated through X-ray fluorescence spectrometry (Nascimento Filho 1999), as required by CONAMA Resolution n. 420/2009 (Brasil 2009). The Standard Reference Material was a 2709a San Joaquin Soil.

Test organisms

The earthworms were cultivated in a substrate consisting of equine manure (chemical-free), coconut husk fiber and fine sand (in the proportion of 7:2:1). The pH of the mixture was corrected to 5.7 ± 0.3 range with CaCO_3 . The organisms were fed weekly with oat flakes. The species *Eisenia andrei* was kept at 20 ± 2.0 °C room and the *Perionyx excavatus* species at a 25 ± 2.0 °C. The photoperiod for both was kept in 16 h light. The enchytraeids were grown in TAS (Garcia et al. 2004) according to the ISO 16387 protocol (ISO 2014a). The springtails were cultivated in a substrate composed of plaster, distilled water and activated charcoal (in a ratio of 11:7:1) maintained at a temperature of 20 ± 2.0 °C and at a photoperiod of 16 h light.

Experimental procedures

The assays with the earthworm species *Eisenia andrei* and *Perionyx excavatus* followed the guidelines established in the ISO 11268–2 protocol (ISO 2012). The experimental units ($n=5$) consisted of containers filled with around 500 g

of dry soil and in the presence of ten clitellate individuals weighing between 250 and 600 mg. The organisms were fed weekly with approximately 15 g of horse manure (free from pollutants) and the moisture was kept at 50% of WHC. After 28 days, the adults were removed, and the cocoons and juveniles were incubated for a further 28 days. At the end of the 56 days, the organisms were extracted using a temperature gradient in a laboratory water bath (60°C). The evaluations with enchytraeids were carried out according to the guidelines established in ISO 16387 (ISO 2014a) with adjustments proposed by (Kuperman et al. 2006), and the assay was conducted for four weeks (28 days) for *Enchytraeus crypticus* and three weeks (21 days) for *Enchytraeus bigeminus* (Bandow et al. 2013). The criterion for selecting adult individuals for *Enchytraeus crypticus* was the presence of a clitellum; for *Enchytraeus bigeminus*, organisms were chosen according to their body size (8 to 12 mm), since this species is a fragmented reproducer (Bandow et al. 2013). Ten organisms were placed in containers with 30 g of soil ($n=4$) and fed weekly with around 20 mg of rolled oats and the moisture content was kept at 50% of WHC. At the end of the test, 5 mL of pure alcohol (96% ethanol), 1 mL of rose bengal dye solution (1% w/v) and 80 mL of distilled water were added to each experimental unit for subsequent counting of the organisms. The springtails' assessments lasted 28 days and were carried out according to the procedures listed under ISO standard 11267 (ISO 2014b). Ten organisms with 12 days old were kept in containers with 30 g of soil ($n=5$). Food was added once a week and consisted of around 20 mg of dry biological yeast and the moisture content was kept at 50% of the WHC. At the end of the evaluation period, for the *Folsomia candida* juveniles, each experimental unit was filled with about 80 mL of water and a few drops of black stamp ink (to increase the contrast between the organisms and the surface of the water). After gentle stirring, the number of adult and juvenile individuals was determined using ImageJ 1.54d software (Schneider et al. 2012). To determine the reproduction rate of the *Proisotoma minuta* species, the method used was dry extraction in Berlese-Tüllgren funnels for a period of 24 h with subsequent addition of water and red dye. This adaptation was necessary due to the smaller

Table 2 Tested nominal concentrations of copper for each ecotoxicological assay depending on the soil type

Soil	Organisms and copper nominal concentrations (mg kg^{-1})
Nitisol and TAS Cambisol	Earthworms (<i>Eisenia andrei</i> and <i>Perionyx excavatus</i>) 0, 100, 200, 400, 800, 1200, 1600 and 2000
	0, 50, 100, 200, 300, 500, 700 and 1000
Nitisol Cambisol and TAS	Enchytraeids (<i>Enchytraeus crypticus</i> and <i>Enchytraeus bigeminus</i>) 0, 75, 150, 300, 450, 600, 750 and 1000
	0, 25, 50, 75, 150, 250, 500 and 750
Nitisol, Cambisol and TAS	Springtails (<i>Folsomia candida</i> and <i>Proisotoma minuta</i>) 0, 75, 150, 300, 450, 600, 750 and 1000
	0, 75, 150, 300, 450, 600, 750 and 1000

size and color of the species' exoskeleton (Hopkin 1997; Mendonça et al. 2015). Afterwards, the samples containing the organisms were carefully shaken and the number of individuals determined using ImageJ 1.54d software (Schneider et al. 2012).

Data analysis

The ecotoxicological data were subjected to the Shapiro–Wilk normality test ($p > 0.05$) and Bartlett's homogeneity test ($p > 0.05$). The means were then compared using the Dunnett test ($p \leq 0.05$) in order to obtain the No Effect Concentration (NOEC) and the Lowest Effect Concentration (LOEC). The data was then submitted to a non-linear regression analysis to determine the dose of copper corresponding to a 50% effect on the population of organisms (EC_{50}). The EC_{50} values were determined using the model that best fitted the data (Environment Canada 2005). All analyses were carried out using STATISTICA 10.0 software (Statsoft Inc. 2011).

Using ETX 2.0 software (Van Vlaardingen et al. 2004), sensitivity curves were developed for the species tested (SSDs). These distributions illustrate the variability in sensitivity among species to a specific toxicant, allowing us to identify the most and least sensitive organisms. This information is essential for assessing ecological risks and determining safe exposure levels for ecosystems. SSDs are constructed through a cumulative plot of logarithmically transformed NOECs or EC_x against rank-assigned percentiles, to which a statistical distribution is fitted (Wheeler et al. 2002; Fox et al. 2020). To this end, the EC_{50} data were cumulatively grouped into a log-normal distribution. The SSDs were employed to estimate the HC_5 and HC_{50} , with a 95% confidence interval. Since the model assumes a log-normal distribution of the data, log-normality was tested using the Anderson–Darling, Kolmogorov–Smirnov and Cramer von Mises tests ($p < 0.01$). The PVs were obtained after the HC_5 had been calculated considering the Quality Reference Values ($QRV = 93.84 \text{ mg kg}^{-1}$) for soils in the state of Santa Catarina (IMA 2021), according to the equation $PV = QRV + HC_5$.

Results

Ecotoxicological assays

The validation criteria were all met for the species *Eisenia andrei*, *Perionyx excavatus*, *Enchytraeus crypticus*, *Folsomia candida* and *Proisotoma minuta* (ISO 2012, 2014a, b). For the assays with the species *Enchytraeus bigeminus*, the criterion adopted was the occurrence of 25 juveniles in the control groups with a coefficient of variation lower than 50%

(Bandow et al. 2013). The choice of a specific criterion is justified due to the impossibility of identifying clitellate adult individuals as it is a fragmenting species.

The reproduction of the earthworm species *Eisenia andrei* and *Perionyx excavatus* was affected ($p \leq 0.05$) in all the soils that were evaluated (Figure S1). Reductions in the number of *Eisenia andrei* juveniles were observed starting at doses of 50 mg kg^{-1} in Cambisol, 100 mg kg^{-1} in TAS and 200 mg kg^{-1} in Nitisol. For the species *Perionyx excavatus*, the effects were observed at the lowest concentrations evaluated (50 mg kg^{-1} in Cambisol and 100 mg kg^{-1} in TAS and Nitisol). The most significant effect for both species was found in the Cambisol, since the EC_{50} values for this soil (77.52 mg kg^{-1} for *Eisenia andrei* and 67.83 mg kg^{-1} for *Perionyx excavatus*) were lower than those found in Nitisol ($428.01 \text{ mg kg}^{-1}$ for *Eisenia andrei* and $264.96 \text{ mg kg}^{-1}$ for *Perionyx excavatus*) and TAS ($173.81 \text{ mg kg}^{-1}$ for *Eisenia andrei* and $110.46 \text{ mg kg}^{-1}$ for *Perionyx excavatus*). All the enchytraeid species evaluated were also affected ($p \leq 0.05$) by soil contamination with Cu (Figure S2). The reduction in the number of juveniles of *Enchytraeus crypticus* occurred from the concentration of 25 mg kg^{-1} in TAS, 50 mg kg^{-1} in Cambisol and 150 mg kg^{-1} in Nitisol. For the species *Enchytraeus bigeminus*, the effects were verified starting at a concentration of 250 mg kg^{-1} in the TAS, 50 mg kg^{-1} in the Cambisol and 75 mg kg^{-1} in the Nitisol. As observed for the earthworms, the most significant effects for the enchytraeids were also observed in the Cambisol, since the EC_{50} values obtained were lower than those found in the Nitisol and TAS. The springtails *Folsomia candida* and *Proisotoma minuta* also had their reproduction affected ($p \leq 0.05$) in all the soils evaluated (Figure S3). The reduction in the number of juveniles of the species *Folsomia candida* was observed from the concentration of 75 mg kg^{-1} in Nitisol and 300 mg kg^{-1} in Cambisol and TAS. For the *Proisotoma minuta* species evaluations, the significant effects on reproduction occurred from the concentrations of 75 mg kg^{-1} in Cambisol, 150 mg kg^{-1} in Nitisol and 450 mg kg^{-1} in TAS. The NOEC, LOEC and EC_{50} for each assessment are presented in Table 3.

In general, it can also be said that the earthworm species *Perionyx excavatus* is the most sensitive of all the species evaluated, whereas the springtail *Proisotoma minuta* was the least affected by the contamination, independent of soil type.

Chemical evaluations

The data from the analysis of the Cu levels in the natural soils (Table 4) indicated an average percentage recovery (in relation to the nominal dose) of 83.8% in Nitisol and 86% in Cambisol, while in TAS the recovery was around 92%. For this reason, it was decided to employ the nominal doses in the construction of the SSD and the subsequent derivation of the PV.

Table 3 Ecotoxicological endpoints (NOEC, LOEC and EC₅₀) for each evaluated species in Nitisol, Cambisol e TAS. In parenthesis the 95% confidence limits (lower and upper) are presented

	NOEC (mg kg ⁻¹)	LOEC (mg kg ⁻¹)	EC ₅₀ (mg kg ⁻¹)
Nitisol			
<i>Eisenia andrei</i>	100	200	428.01 (300.58–555.44)
<i>Perionyx excavatus</i>	< 100	100	264.96 (184.08–345.15)
<i>Enchytraeus crypticus</i>	75	150	366.61 (328.56–404.67)
<i>Enchytraeus bigeminus</i>	< 75	75	444.71 (303.99–585.44)
<i>Folsomia candida</i>	< 75	75	456.22 (305.94–606.51)
<i>Proisotoma minuta</i>	75	150	636.00 (597.15–674.86)
Cambisol			
<i>Eisenia andrei</i>	< 50	50	77.52 (66.16–88.87)
<i>Perionyx excavatus</i>	< 50	50	67.83 (54.95–80.72)
<i>Enchytraeus crypticus</i>	25	50	142.25 (109.50–175.00)
<i>Enchytraeus bigeminus</i>	25	50	126.77 (56.30–197.24)
<i>Folsomia candida</i>	150	300	255.03 (223.52–286.53)
<i>Proisotoma minuta</i>	< 75	75	513.17 (453.78–572.56)
TAS			
<i>Eisenia andrei</i>	< 100	100	173.81 (148.99–198.63)
<i>Perionyx excavatus</i>	< 100	100	110.46 (73.82–137.10)
<i>Enchytraeus crypticus</i>	< 25	25	171.71 (124.79–218.63)
<i>Enchytraeus bigeminus</i>	150	250	213.70 (181.36–246.03)
<i>Folsomia candida</i>	150	300	333.38 (297.35–369.42)
<i>Proisotoma minuta</i>	300	450	578.74 (512.03–645.45)

Table 4 Nominal concentration (mg kg⁻¹) and chemical measured concentration (mg kg⁻¹) of copper in subtropical natural soils and TAS

Nominal	Real		
	Nitisol	Cambisol	TAS
25		16.7	20.9
50		33.8	41.9
75	48.9	58.3	63.4
100	65.05	77.8	84.5
150	122.8	129.0	136.4
200	170.5	172.0	188.2
250			290.1
300	260.9	261.0	
400	326.5		384.0
450	375.2	429.1	
500		477.5	422.6
600	539.4	551.0	
700		668.5	
750	647.3	716.3	743.4
800	719.2		792.8
1000	889.0	955.0	
1200	1078.8		1185.6
1600	1438.5		1586.1
2000	1798.1		1982.4

Species Sensibility Distribution and prevention values

Once it was possible to assess the EC₅₀ for each of the tested organisms, the SSD were generated and the HC₅ and HC₅₀ were obtained for all the tested soils (Fig. 1). The highest HC₅ values were recorded in Nitisol (252.9 mg kg⁻¹), followed by TAS (76.12 mg kg⁻¹) and Cambisol (40.21 mg kg⁻¹). It can therefore be inferred that Cu contamination is more problematic in Cambisol, since the concentration capable of endangering the organisms is approximately six times lower than in Nitisol.

Finally, based on the Cu QRV (93.84 mg kg⁻¹) published for Santa Catarina state soils (IMA 2021), it was possible to estimate the PV via the HC₅ data (Table 5). We chose not to utilize HC₅₀ for this purpose because we intended to propose PVs that are ecologically safer.

Discussions

The ecotoxicological data gathered from studies conducted in temperate climate soils are extrapolated to tropical and subtropical climate zones. Nevertheless, this decision sometimes proves to be inappropriate since the differences

Fig. 1 Species sensitivity distribution based on the EC_{50} data for the evaluated soils

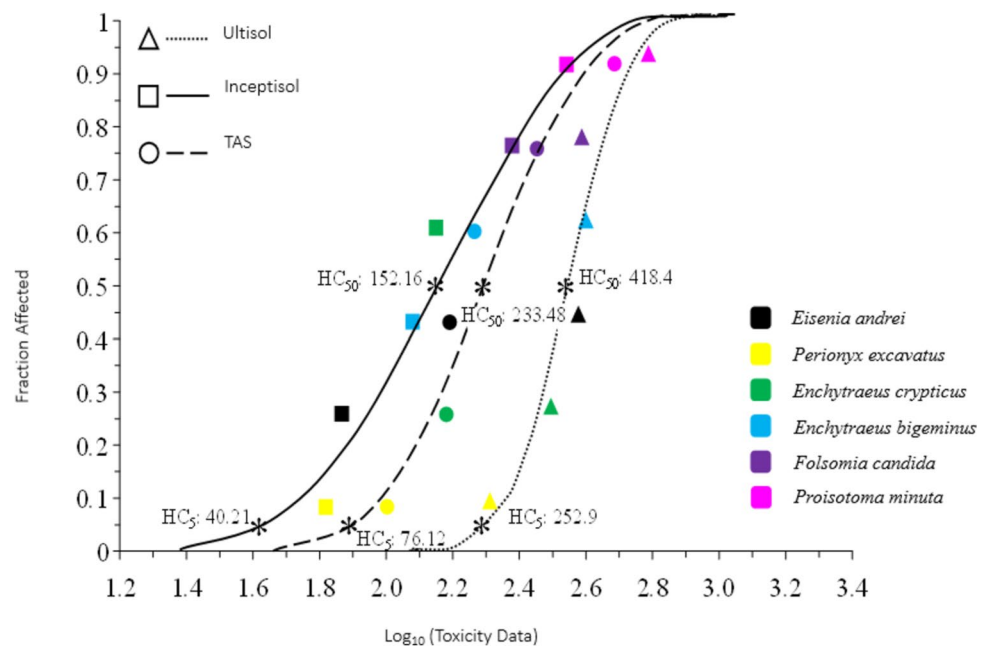


Table 5 Hazardous concentration for 5% of the species and prevention values for Cu based on EC_{50} data for each evaluated soil. LL HC_5 : lowest limit for the calculated HC_5 ($p \leq 0.05$); HC_5 : hazardous concentration for 5% of the species ($p \leq 0.05$) based on the EC_{50} values; UL HC_5 : upper limit for the calculated HC_5 ($p \leq 0.05$); PV: prevention values ($PV = QRV + HC_5$)

Soil	LL HC_5	HC_5	UL HC_5	PV
Nitisol	144.1	252.9	325.40	346.74
Cambisol	9.08	40.21	78.24	134.05
TAS	26.17	81.82	136.24	175.66

in climate conditions originate soils with contrasting characteristics. In this sense, the results presented here tend to overcome this constraint and, in an original way, clarify the Cu dose–effect relationship in natural subtropical Brazilian soils.

The assays carried out on natural soils with different chemical properties, as well as on artificial soil, indicated that the organisms were able to respond to the contamination in different ways, with the adverse effects being associated with greater bioavailability of Cu. According to Kabata-Pendias (2010), the bioavailability of trace elements is a result of the soil's adsorptive capacity, which is directly related to CEC, pH and clay content. In fact, Cambisol had lower clay content, CEC, and pH levels when compared to Nitisol. This finding is in line with the results of Duan et al. (2016), who reported that Cu toxicity in *E. fetida* is inversely associated with CEC and pH level. These attributes also tend to result in greater damage to other faunal species, given the dynamics of the contaminant (Natal-da-Luz et al. 2008). Furthermore, despite Cambisol having a lower CEC and clay content, the

organisms were more susceptible to Cu contamination in TAS than in Cambisol. This observation suggests that other factors, such as the presence of Al (which is a toxic element to soil organisms) may influence the organisms vulnerability. These findings confirm the first hypothesis upon which this evaluation was built.

The results of the ecotoxicological assessments showed that Cu was able to reduce the reproduction of all organisms at distinct levels. The species most sensitive to the contaminant was the earthworm *Perionyx excavatus*, followed by the enchytraeid *Enchytraeus crypticus* (for the evaluations in TAS and Nitisol) and the earthworm *Eisenia andrei* (in Cambisol), all oligochaetes with similar trophic habits and forms of exposure to the contaminant. On the other hand, the springtails were less susceptible to Cu exposure. These differences illustrate a scenario that emphasizes the importance of carrying out assessments with organisms from different taxonomic groups, especially when determining critical concentration limits for one or more contaminants in the soil.

In accordance with our findings, Mirmonsef et al. (2017), when assessing the effects of Cu on the reproduction of eight earthworm species collected in a contaminated area, found that the abundance of cocoons decreased as the concentration of the metal increased, thereby indicating a harmful effect on the reproduction of the organisms. Similarly, Maboeta and Fouché (2014), in a study aiming to determine the Cu toxicity in the species *Eisenia andrei*, found a significant reduction in oviposition and cocoon hatching rates. Kwak and An (2021), in a 7-day assessment, were able to identify physiological abnormalities, such as mucous secretion, bleeding and swelling, for doses above 400 and 600 mg of Cu kg^{-1} of dry soil in adults of *Perionyx excavatus* and

Eisenia andrei respectively. Regarding the variation in LOEC which we have verified here between the two earthworm species evaluated, the results suggest that the *Perionyx excavatus* species is more sensitive. This observation corroborates the findings of Spurgeon and Hopkin (1996), who, in an evaluation of ecologically relevant species, concluded that the *Eisenia* genus is the one with the greatest resistance to soil contamination by trace elements. Given the different responses, the importance of using native species in ecotoxicological assessments is therefore emphasized. This is because the use of tropical/subtropical species, such as *Perionyx excavatus* (Silva & van Gestel 2009), can better represent ecological risk analysis in Brazilian soils.

In terms of the damaging effects of Cu on enchytraeids, and in accordance with the results of this evaluation, Maraldo et al. (2006), when assessing the survival of *Enchytraeus crypticus* in Danish soils contaminated with Cu, found a reduction in the survival of this organism at concentrations of 600 mg kg⁻¹. However, there have been few publications to this date on the effect of Cu on the reproduction of enchytraeids in tropical and subtropical soils. Nevertheless, Konečný et al. (2014), in a study assessing the influence of trace elements on *Enchytraeus crypticus*, observed that the reproduction of the animals shows a negative correlation with the concentrations of Co and Cu, the latter being the most environmentally relevant contaminant, with its EC₅₀ determined for the species at 351 mg kg⁻¹. These findings support the need to determine PV for Cu in different soils, given its effects on the reproductive capacity of organisms and the subsequent impact on the ecological functions of important biological groups.

The Cu exposure also had a negative effect on the springtails, although these were the organisms whose results indicate greater resistance to the effects of the metal. According to Renaud et al. (2020), the toxicity of trace elements in *Folsomia candida* tends to be directly related to the solubility of the cations in the soil. In this sense, it should be noted that the changes followed the same pattern as for earthworms and enchytraeids, i.e. the effects in Cambisol were more significant than in the TAS and Nitisol. In contrast to the possibilities here suggested to justify the greater sensibility of the organisms in Cambisol, Sandifer and Hopkin (1996) concluded that there was no clear correlation between the increase in toxicity as a function of the decrease in pH when evaluations were carried out on *Folsomia candida*. However, it should be considered that the assays conducted by the authors were carried out on OECD artificial soil (OECD 1984), where the organic matter content (in the order of 10%) is too high, given that the great majority of tropical soils rarely exceed 5% (Amorim et al. 2005). According to Fountain and Hopkin (2001), although the species feeds on organic matter and is an important fragmenting agent, whose relevance to the provision of ecosystem services is of the

utmost importance, it is highly tolerant to food contamination. Therefore, considering that the Cambisol had the highest levels of organic matter and that the most significant effects were recorded in this soil, it can be assumed that the factor with the greatest impact on Cu toxicity for these species was likely to have been CEC and pH.

Another outstanding aspect of this investigation, and in line with what was observed by Buch et al. (2016) when conducting assessments on the toxicity of Hg in tropical soils, was the greater sensitivity recorded for *Folsomia candida* when compared to *Proisotoma minuta*. In this sense, it should be noted that this difference in responses to environmental stress justifies the need to include more than one species into the same group of organisms to ecotoxicological assessments; this is because, as pointed out by Salmon et al. (2014), springtails tend to show different interspecific responses due to the different habitats and trophic niches they occupy. Moreover, the greater tolerance of certain species may be related to their morphological characteristics, which reflect their ability to absorb, eliminate and immobilize metals (Janssens et al. 2009). Finally, it should be added that the summary choice of bioindicators should be based on their sensitivity to the stressor, considering the mechanisms of toxic action and exposure to the contaminant. Thus, to endorse the use of *Proisotoma minuta* in the current evaluation, it is worth noting that the species shows reasonable sensitivity to Cu (Nursita et al. 2005), despite its lower sensitivity when compared to *Folsomia candida*.

The process of drawing up the SSD made it possible to assess the organisms' sensitivity to Cu by determining risk concentrations (i.e. HC₅). In short, the graphical observation of adverse effects provides a better understanding of the results as it also represents a versatile approach to the development of practices and policies for the prevention, monitoring and mitigation of environmental degradation (Posthuma et al. 2019). Several studies have aimed to develop retrospective and predictive analysis of the effect of contaminants in Ecological Risk Assessment (ERA) and have employed the modeling of such curves, arguing positively for the use of the method (Princz et al. 2017; Posthuma et al. 2019; Bandeira et al. 2021). As stated before, the organisms were more susceptible to contamination in Cambisol, TAS and Nitisol, respectively, implying that the PV differed depending on the soil type. The decision to obtain PV for the TAS was taken in order to clarify which chemical characteristics of the soils, other than their clay content, have the greatest influence on copper toxicity, since Cambisol and TAS have similar textural compositions. The AI content and the greater amount of nutrients seemed to attenuate the effects of contamination in TAS.

The results presented here are pioneering in terms of the construction of the SSD and the concomitant derivation of PVs for subtropical soils. In this sense, it should be noted

that in 2021, the State of Santa Catarina published its first quality reference values (QRV) in compliance with the Brazilian legislation. However, the QRV for Cu in Santa Catarina went beyond the PV present in the federal regulatory framework (Brasil 2009; IMA 2021); therefore, the results presented here tend to improve the ERA and make a unique contribution to the conservation of ecosystems in the Atlantic Forest Biome. The decision to base the PV estimation on the EC₅₀ was driven by the need to safeguard ecosystems without being strictly restrictive, since the PV were estimated according to the HC₅. However, if the amount of protection has to be greater, further assessments should be conducted so that the SSDs are generated with data from higher protective effect concentrations (ECx), such as EC₂₀.

That said, it is worth noting that the results showed that the PV based on the EC₅₀ data for Cu were higher than the stipulated in the CONAMA Resolution (Brasil 2009), thus indicating the effectiveness of the legislation in protecting soil fauna organisms and refuting the second hypothesis here suggested. However, further assessments are required before assuming the efficacy of the national legislation since soils tend to have different capacities for retaining and making Cu bioavailable. The recently published work by Messias et al. (2023) reinforce the discussion of the need to update the PVs for Cu, since the authors found adverse effects at concentrations below the limit established in the federal regulations. In this way, although it can be inferred that the PV set out in the CONAMA Resolution is protective for the soils evaluated, it cannot be concluded beyond the results presented here, given the influence of the chemical characteristics of the soils on the environmental dynamics of Cu.

Conclusions

The results demonstrate that the reproduction of soil organisms, including earthworms, enchytraeids, and springtails, was significantly affected by copper contamination across the different soils evaluated. Responses varied notably among soil types, with Cambisol being the most sensitive, presenting lower EC₅₀ values compared to Nitisol and TAS. This variation in organism responses is likely attributable to soil chemical properties, such as pH and clay content. Additionally, *Perionyx excavatus* proved to be the most sensitive species, while the springtail *Proisotoma minuta* was the least affected, regardless of soil type.

Based on the comparison between the results and current Brazilian legislation, it can be inferred that the preventive guideline values (i.e. PV) for copper are protective for the bioindicators assessed in the soils studied. However, further assessments using additional soil types representative of subtropical climates are needed before it can be conclusively

determined that the PVs established in federal regulations are protective of subtropical terrestrial ecosystems.

It is noteworthy that the data presented here tend to support similar investigations aimed at building an ecotoxicological database, which will enhance our understanding of the effects of trace element pollution on the biotic constituents of various Brazilian ecosystems. This represents a significant step forward in developing public policies focused on environmental conservation, as the evaluated groups of organisms play essential roles in providing the ecosystem services that soil offers.

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Data availability Datasets related to this article can be made available upon request to the corresponding author Osmar Klauberg-Filho (osmar.klauberg@udesc.br).

Declarations

Ethical approval Not applicable.

Consent to participate Not applicable.

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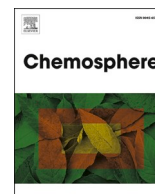
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Ecotoxicity of alkaline residue from the pulp and paper industry to soil faunal organisms

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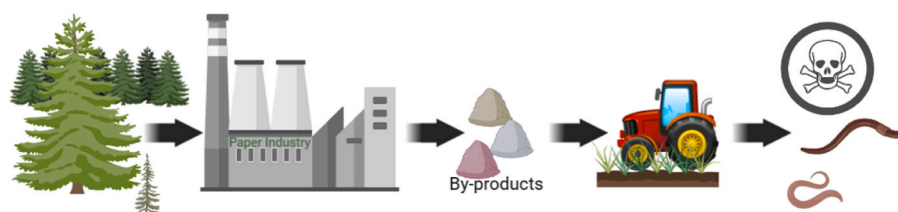
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HIGHLIGHTS

- Dregs are a by-product from the pulp and paper industry that is suggest to be a soil pH corrective.
- Soil organisms are vital to the maintenance of the soil health.
- Dregs are capable of affect the reproduction rates of soils organisms.
- The toxicity of dregs varies according to the soil chemical characteristics.
- Metal levels in the dregs matrix are less of concern than the increases in Na content.

GRAPHICAL ABSTRACT



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ABSTRACT

The increased production and expanding demand for cellulosic products have required companies to provide solutions for the proper disposal of generated residues without compromising soil quality. In this context, the current research aimed at evaluating the ecotoxicological impacts of dregs application in subtropical soils. The experiments were carried out with the earthworm *Eisenia andrei*, the springtail *Folsomia candida*, and the potworm *Enchytraeus crypticus* in two subtropical soils (Neosol and Cambisol) collected in the southern region of Brazil. The considerable differences in texture and organic matter content motivated the choosing of these soils. The reproductive rate of all organisms was influenced, with varying EC_{50} values depending on the soil type. However, more pronounced effects were observed for Neosol. The springtail *F. candida* proved to be the most sensitive to contamination ($EC_{50} = 5.8 \text{ g kg}^{-1}$ for Neosol and 48.5 g kg^{-1} for Cambisol), followed by the earthworm *E. andrei* ($EC_{50} = 62.4 \text{ g kg}^{-1}$ for Neosol and 129.5 g kg^{-1} for Cambisol) and the potworm *E. crypticus* ($EC_{50} = 67.2 \text{ g kg}^{-1}$ for Neosol and 230.4 g kg^{-1} for Cambisol). As a result, while dregs can be used to correct soil acidity, they have been shown to have negative effects on important organisms responsible for functions critical to maintaining ecosystem quality.

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Environmental implication

Paper and cellulose production processes generate a large number of byproducts with various chemical characteristics that can be used to fertilize the soil. Dregs are made by chemically digesting fibrous material in the presence of sodium hydroxide and sodium sulfide. This research looks at how Dregs pollution affects reproduction, which advances the creation of measures to protect soil health. By studying the effects of Dregs pollution on reproduction, researchers can better understand the potential harm these byproducts may pose to soil health. Using this information, measures to reduce the adverse effects of Dregs pollution can then be developed.

1. Introduction

Brazil is the world's second-largest producer of pulp and hardwood pulp (FAO, 2023). Large-scale waste is produced throughout the pulp and paper industry's manufacturing process since, for every 100 t of product, around 50 t of waste is produced (Bellote et al., 1998). In this sense, as the demand for cellulosic products grows, the amount of waste generated has drawn attention in an effort to reduce environmental risks while meeting circular economy demands (Toledo et al., 2015).

Among the main residues from the large-scale production of pulp and paper, green liquor, lime mud, grits, and dregs stand out, the latter being produced in greater amounts than the others (Maciel et al., 2015; Kristanto and Asaloei, 2017). The dregs originate from the chemical digestion of the fibrous material in the presence of sodium hydroxide (NaOH) and sodium sulfide (Na_2S), whose purpose is to promote the delignification of cellulose (Bajpai, 2018). As the dregs are essentially composed of calcium carbonate (CaCO_3) and magnesium (MgCO_3) precipitates, several studies have suggested their use as a soil conditioner (Nurmesniemi et al., 2005; Medeiros et al., 2009; Kristanto and Asaloei, 2017).

However, dregs have metallic elements with high deleterious potential in their constitution, such as lead (Pb), chromium (Cr), and nickel (Ni) (Almeida et al., 2007). Therefore, despite the potential use for the chemical correction of the soil, the application of dregs can increase not only the concentration of essential nutrients (such as Ca, Mg, K, P, and S) but also of metals in concentrations capable of bioaccumulating along the food chain (Nurmesniemi et al., 2005). In addition, the high amounts of sodium (Na) in the dreg's matrix composition can trigger an osmotic imbalance in soil, leading to severe toxic effects on soil organisms (Sá et al., 2021).

Aiming for the protection of ecosystems and the maintenance of soil and water quality, in terms of their ability to support life, the Resolution n° 420/2009 of the Brazilian National Council for the Environment (CONAMA) establishes guidelines for soil preservation according to trigger values (Brasil, 2009). However, there is no specific and detailed legislation regarding the application of residues from the cellulosic industry for agronomic purposes considering the protection of important groups of soil organisms, such as oligochaetes and microarthropods, which perform relevant functions in the soil (Alves et al., 2016; Segat et al., 2020).

Earthworms (*Eisenia andrei*), potworms (*Enchytraeus crypticus*) and springtails (*Folsomia candida*) are excellent bioindicators since they play a key role in providing regulatory ecosystem services and are also highly sensitive to the presence of certain pollutants, such as metals and complex organic matrices (van Gestel et al., 1993; Natal-da-Luz et al., 2011; Maccari et al., 2016; Segat et al., 2020). Although the ecosystem is made up of a wide range of organisms, results with standardized species are widely used as a way to elucidate potential effects on the dynamics of native species. The species *E. andrei* is among the most studied organisms in ecotoxicological assessments, as well as *E. crypticus* and *F. candida* (Natal-da-Luz et al., 2011; Maccari et al., 2016; Segat et al., 2020). Therefore, it is crucial to understand the impending threat to soil health posed by the loss of these organisms capacity for reproduction as

a result of the application of dregs. Nonetheless, few studies have investigated the biological effects of pulp and paper industry by-products on these organisms (Fraser et al., 2009; Song et al., 2018).

An environmental risk assessment with dregs is necessary to evaluate the potential risks associated with its disposal in various environmental scenarios, since soil chemical properties play a critical role in governing the dynamics of heavy metals, which constitutes dreg's matrix in abundance (Almeida et al., 2007). Recent studies have shown that pH significantly influences metal solubility and mobility. Acidic soils, for example, increase the mobility and bioavailability of metal ions, posing higher risks to water contamination (Hou et al., 2020). On the other hand, organic matter contributes to the adsorption and immobilization of metals through complexation, reducing their availability (Zhou et al., 2023). It is essential to emphasize that soils with lower organic matter content are highly susceptible to contamination by several cationic metals due to their strong affinity for the organic fraction (Kabata-Pendias, 2010). This suggests that in regions where soils have this characteristic, extra caution should be taken with organic fertilization and the prolonged use of metal-based pesticides.

In this paper, the ecotoxicological effect of dregs on the reproduction of soil invertebrates (earthworms, enchytraeids, and springtails) was evaluated and effective concentrations determined. The dregs' toxicity and their chemical properties were discussed along with the difference in sensitivity of the test organisms and soil properties. In this sense, this study aimed to assess the potential ecological risk of land application of dregs as soil amendments, using two subtropical soils with contrasting textural and chemical properties. This approach tends to significantly enhance the understanding of contamination processes in subtropical regions, where soils exhibit considerable chemical and physical variability. Thus, considering the above, we hypothesized that (i) dregs have negative effects on the reproduction of studied soil invertebrates; and (ii) the effects vary according to the soil type due to their properties influencing the availability of potentially hazardous chemical elements present on the dregs. Stating that, it is worth noting that this is the first study documenting the effects of dregs on soil organisms in subtropical soils.

2. Material and methods

2.1. Soils and test substance

Two Brazilian subtropical soils were evaluated, a Neosol collected in Lages (29° 00' 19.98" S e 49° 31' 02.84" W) and a Cambisol collected in Araranguá, both municipalities belonging to the Santa Catarina State (Brazil), known to host several subsidiary branches of a pulp and paper industry, (27° 53' 06" S e 50° 24' 57" W). According to the Brazilian Soil Classification, the Neosol is classified as a Neossolo Quartzarênico Órtico típico, and the Cambisol as a Cambissolo Háplico distrófico (Santos et al., 2018). The soils were chosen due to their contrasting chemical properties (such as pH and organic matter content) and texture. The samples were collected at a 0-0,2 m depth. Both soils were air dried sieved ($\phi \leq 2$ mm and manually homogenized. In addition to the clay, silt and sand contents, according to the method proposed by Gee and Bauder (1986), the following chemical parameters (Table 1) were also analyzed: pH (ISO, 2005); electrical conductivity (EC) (ISO, 1994); soil organic matter content (SOM); cation exchange capacity (CEC); and the contents of Al^{3+} , H + Al (Tedesco et al., 1995), Al, Ca, Cr, Cu, Fe, Mg, Ni, Pb, and Zn (USEPA, 1999).

Four subsamples were collected at random points from a batch of dregs from the kraft pulping process, supplied by a pulp and paper industry. The subsamples were air-dried, sieved ($\phi \leq 2$ mm) and homogenized so that they could be used in the tests. The chemical properties of the dregs are shown in Table S1.

Table 1
Chemical and physical characteristics of Neosol and Cambisol.

Attribute	Neosol	Cambisol
pH (KCl)	6.4	5.1
EC (H ₂ O) (dS m ⁻¹) ^a	6.1	4.5
SOM (%) ^b	1.3	3.1
CEC pH 7.0 (cmol _c dm ⁻³) ^c	4.9	20.9
Al ³⁺ (cmol _c dm ⁻³)	0.00	0.22
H + Al (cmol _c dm ⁻³)	1.55	13.75
Al (g kg ⁻¹)	3.44 ± 0.11	37.92 ± 7.75
Ca (g kg ⁻¹)	0.69 ± 0.19	0.53 ± 0.04
Cr (mg kg ⁻¹)	8.53 ± 0.13	32.84 ± 0.13
Cu (mg kg ⁻¹)	nd ^d	88.11 ± 0.65
Fe (g kg ⁻¹)	1.99 ± 0.09	23.24 ± 0.25
K (g kg ⁻¹)	0.18 ± 0.01	0.31 ± 0.04
Mg (g kg ⁻¹)	0.24 ± 0.02	0.65 ± 0.08
Mn (g kg ⁻¹)	117.44 ± 0.18	783.55 ± 0.19
Na (g kg ⁻¹)	2450.01 ± 2.47	349.49 ± 3.33
Ni (mg kg ⁻¹)	nd ^d	22.63 ± 0.47
Pb (mg kg ⁻¹)	7.67 ± 0.28	11.63 ± 0.26
Zn (mg kg ⁻¹)	4.16 ± 0.08	21.18 ± 0.55
Clay (g kg ⁻¹)	40	330
Silt (g kg ⁻¹)	590	230
Sand (g kg ⁻¹)	370	440
Texture	Sandy loam	Clay loam

^a EC: Electrical conductivity in H₂O.

^b SOM: soil organic matter.

^c CEC: cation exchange capacity.

^d nd: not determined.

2.2. Test organisms

The earthworm *E. andrei* was cultivated in a substrate consisting of equine manure, coconut husk fiber and fine sand (in the proportion 70:20:10). The organisms were fed weekly with oat flakes and kept at a temperature of 23 ± 2 °C at a photoperiod of 12 h light and 12 h dark. The potworm *E. crypticus* was cultivated according to the ISO 16387 protocol (ISO, 2014). The springtail *F. candida* was cultivated in a substrate composed of plaster, distilled water and activated charcoal (in the proportion 11:7:1), kept in a controlled environment at a temperature of 20 ± 2 °C and a photoperiod of 12 h light and 12 h dark. All cultures were fed three times a week.

2.3. Experimental procedures

The assays to measure the effects of dregs on the reproduction rates followed the guidelines of the specific protocols for earthworms (ISO, 2012a), enchytraeids (ISO, 2014) and springtails (ISO, 2012b). Assays were performed for both soils Neosol and Cambisol, independently, following a completely randomized design with at least four replications per tested dose.

Earthworms were selected according to their body mass (between 300 and 500 mg) and the presence of a well-developed clitellum. Before the beginning of the tests, the organisms were acclimatized for a period of 24 h in uncontaminated soil, with moisture maintained at about 60% of the water holding capacity (WHC). After this period, 10 adult earthworms were placed in 1 L plastic containers (height: 9 cm; diameter: 14.5 cm), filled with 500 g of soil (n = 4). The organisms were fed weekly with 5 g of uncontaminated dry horse manure. The enchytraeids were also selected according to the presence of clitellum and remained acclimatized for 24 h. The tests were carried out in plastic containers with 120 mL capacity (height: 4.5 cm; diameter: 7.6 cm), filled with 30 g of soil (n = 5) at a moisture of 60% of the WHC. The treatments consisted of the presence of 10 organisms, which were fed weekly with approximately 2 mg of finely ground oats. Assays with springtails consisted of the presence of 10 organisms aged between 10 and 12 days, kept in plastic containers with 120 mL capacity (height: 4.5 cm; diameter: 7.6 cm), filled with 30 g of soil (n = 5) at a moisture of 70% of the WHC. The organisms were fed weekly with approximately 2 mg of dry yeast.

The dregs tested concentrations for all the organisms are presented in Table 2.

2.4. Chemical analysis

For all doses and species evaluated, the EC in H₂O (ISO, 1994) and pH in KCl (ISO, 2005) were measured before and after the tests (Tables S2 and S3). Following soil contamination, three samples of each tested dose were chosen to create a composite sample on which the contents of Al, Ca, Co, Cr, Cu, Mg, Mn, Ni, Pb, Sr, and Zn were determined using the ICP-OES method according to the USEPA 3050B method (USEPA, 1996), and the contents of Na and K were determined using a flame photometer (Tables S4 and S5). The Standard Reference Material (SRM) used was a 2709a San Joaquin Soil.

2.5. Data analysis

The normality and homoscedasticity of variances were evaluated using the Shapiro-Wilk and Bartlett tests, respectively. After confirming the assumptions, the reproduction data were submitted to a variance analysis test (one-way ANOVA) and then compared by Dunnett's test (M < control, p ≤ 0.05) using the Statistica 7.0 software (StatSoft, 2004). The estimated concentration capable of reducing the reproduction of organisms by 50% (EC₅₀) was calculated through non-linear regression using pre-defined mathematical models that best fit (R²) the data (Environment Canada, 2005). The Behrens-Fisher significance test (p ≤ 0.05) was performed to determine whether there is a difference between the two evaluated soils.

3. Results

3.1. Chronic toxicity assay

The average mortality in the control vessels was below 10%, 20%, and 20% for *E. andrei*, *E. crypticus*, and *F. candida*, respectively. The mean number of juveniles was above 30, 25, and 100 individuals to *E. andrei*, *E. crypticus*, and *F. candida*, respectively, with coefficients of variation (CV) below 30% in all the assays. Therefore, the results meet the criteria recommended by ISO 11268-2 (ISO, 2012a), ISO 16387 (ISO, 2014), and ISO 11267 (ISO, 2012b).

For Neosol (p ≤ 0.05), the dregs affected the reproduction of *E. andrei* from the dose of 25 g kg⁻¹ (Fig. 1a) while for Cambisol the effects were observed from the 90 g kg⁻¹ dose (Fig. 1b). The EC₅₀ was 62.4 g kg⁻¹ (43.6–81.3) to Neosol and 129.5 g kg⁻¹ (103.1–155.8) to Cambisol (Table 3).

In the *E. crypticus* assays a Hormesis effect (p ≤ 0.05) was verified for both evaluated soils (Fig. 1c and d). In Neosol, an increase in the number of juveniles was observed at doses of 10 and 20 g kg⁻¹ (compared to the control treatment) with a subsequent decrease from the dose of 40 g kg⁻¹ (Fig. 1c). In Cambisol, there was an increase in the number of juveniles for the 10, 20, 40, and 100 g kg⁻¹ doses when compared to the control treatment, with a negative effect recorded at doses of 200 and 400 g kg⁻¹. The EC₅₀ determined for Neosol was 67.2 g kg⁻¹ (62.1–72.3) and 230.4 g kg⁻¹ (196.9–263.8) for Cambisol (Table 3).

The results obtained for *F. candida* in the Neosol showed a negative

Table 2
Tested concentrations of dregs mixed in each soil.

Soil	Organisms	Dregs tested concentrations (g kg ⁻¹)
Neosol	<i>E. andrei</i>	0; 10; 15; 25; 40; 65; 100
	<i>E. crypticus</i>	0; 10; 20; 40; 60; 100; 200
	<i>F. candida</i>	0; 2.5; 5; 10; 20; 30; 50; 90
Cambisol	<i>E. andrei</i>	0; 10; 20; 30; 50; 90; 150
	<i>E. crypticus</i>	0; 10; 20; 100; 200; 400
	<i>F. candida</i>	0; 10; 20; 40; 80; 150; 300

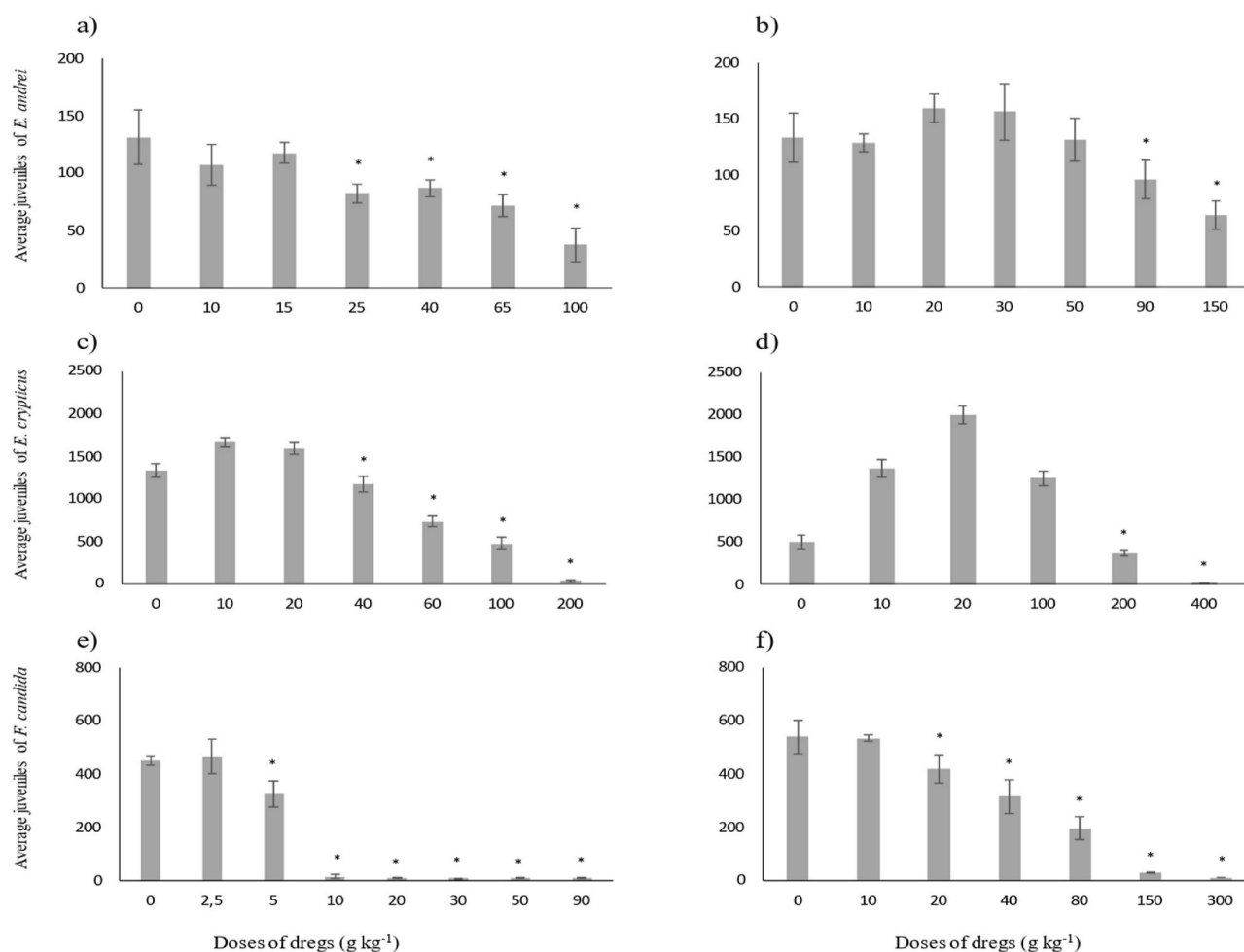


Fig. 1. Mean number of juveniles of *E. andrei* (a), *E. crypticus* (c), and *F. candida* (e) in Neosol and *E. andrei* (b), *E. crypticus* (d), and *F. candida* (f) in Cambisol. (*) indicates a significant difference ($p \leq 0.05$). (—) Standard deviation ($n = 4$). Concentration of 0 represents the control treatment (free from contamination).

Table 3

Ecotoxicological parameters (NOEC, LOEC and EC_{50}) obtained in the acute and chronic toxicity tests in subtropical soils (Neosol and Cambisol).

Test species	Endpoint	Parameter	Concentration of dregs (g kg^{-1})	
			Neosol	Cambisol
<i>E. andrei</i>	56-d reproduction	NOEC	15	50
		LOEC	25	90
		EC_{50}	62.4 (43.6–81.3)	129.5 (103.1–155.8)
<i>E. crypticus</i>	21-d reproduction	NOEC	20	100
		LOEC	40	200
		EC_{50}	67.2 (62.1–72.3)	230.4 (196.9–263.8)
<i>F. candida</i>	28-d reproduction	NOEC	2.5	10
		LOEC	5	20
		EC_{50}	5.8 (5.4–6.2)	48.5 (40.3–56.7)

NOEC – No Effect Concentration; LOEC – Lowest Effect Concentration; EC_{50} – Effect Concentration which reduces by 50% the reproduction of organisms.

effect ($p \leq 0.05$) on reproduction from the 5 g kg^{-1} dose (Fig. 1e). For Cambisol, a decrease in the number of juveniles was observed ($p \leq 0.05$) from the 20 g kg^{-1} dose (Fig. 1f). The EC_{50} was 5.8 g kg^{-1} (5.4–6.2) for Neosol and 48.5 g kg^{-1} (40.3–56.7) for Cambisol (Table 3).

The dregs' toxicity varies ($p \leq 0.05$) between the two evaluated soils

for all the assays performed.

3.2. Chemical evaluations

The addition of increasing concentrations of dregs resulted in an elevation of pH and EC in both soils (Tables S2 and S3). These findings suggest that the metals present in the matrix composition of the dregs (Tables S4 and S5) are possibly found precipitated in the soil.

In the Cambisol there was an evident increase in Na, K, Ca, Cr, Cu, Mg, Mn, Ni, Sr, and Zn contents as the dreg's doses increased. On the other hand, there was a progressive reduction in the Al, Co, and Pb contents (Table S4). For the Neosol, although less evident due to the lower applied concentrations of dregs, there was a progressive increase of most elements as the doses increased, except for Pb and Ba, which remained below the detection limit of the equipment (LDI) for almost all concentrations evaluated (Table S5).

4. Discussions

It is observed that soil contamination by dregs could inhibit the reproduction of all organisms evaluated at low percentages of application, confirming the first. We emphasize the similarity in toxicity levels between *E. andrei* and *E. crypticus*, both oligochaetes with scavenging habits, and the greater susceptibility of *F. candida* to contamination (Table 3). In fact, different responses are expected among different faunal groups (Natal-da-Luz et al., 2004). An example of these variations in sensitivity is that in a study conducted by Fraser, O'halloran and van

den Heuvel (2009), no acute effects (mortality) or reduction in the biomass of *E. fetida* exposed to organic residues from the cellulosic industry were observed; nevertheless, the authors verified a significant reduction in the reproduction of *Enchytraeus albidus*. Moreover, Renaud et al. (2017) found that *F. candida* is a more sensitive bioindicator to organic contamination than *E. crypticus* and *Eisenia* spp., which corroborates the results observed in our evaluations with dregs, despite its inorganic nature. Concerning the implications of the contamination, negative effects on the activity of enzymes involved in the biotransformation process as well as damage to the genetic structure were found for *E. fetida* when exposed to green liquor. (Song et al., 2018). These comparisons shows how by-products from the pulp and paper industry can significantly and negatively impact soil organisms.

The addition of the dregs increased the amount of Na in the soils (Tables S4 and S5), which may have implied harmful consequences on the organisms. This is particularly true given the possibility of an osmotic imbalance in the epithelial cells. Owojori and Reinecke (2009) assessing the effects of NaCl found significant avoidance behavior for the species *E. fetida* at a concentration of 500 mg kg⁻¹, which may suggest a possible saline effect on our results, since the Na concentrations (Tables S4 and S5) in the LOEC determined by us were all higher than the LOEC found by the authors. In our study, we verified that the estimated contents of Na at the lowest doses of dregs with a significant effect for all the evaluated organisms were higher in Neosol than in Cambisol. This is probably why prominent effects were observed in Neosol (Table 3), even though it was not possible to confirm a correlation between the increase in Na content in the soil and the organisms' ability to reproduce. Having stated that we can confirm the second hypothesis on which our study was based.

The greater sensitivity of organisms to the dregs contamination in the Neosol can be attributed to the lower organic matter and clay contents, which are essential for metal retention in soils by enhancing the adsorption and immobilization of contaminants. In the Neosol, this deficiency increases the mobility of metals, raising their bioavailability and toxicity. As Amorim et al. (2005) suggest, soils with low levels of these constituents tend to create more toxic environments for soil organisms when exposed to cationic contaminants. In contrast, soils like Cambisols, richer in clay and organic matter, retain metals more efficiently, restricting their mobility and minimizing harmful effects (Hernandez-Soriano and Jimenez-Lopez, 2015; Hou et al., 2019; Ma et al., 2020). Similarly to what we observed, Maccari et al. (2016) reported pronounced effects in soil with lower levels of CEC and contents of organic matter and clay when analyzing the influence of liquid pig manure on *F. candida* reproduction in subtropical soils, linking the presence of metallic cations to the reported toxicity. Nevertheless, because the highest levels of Co, Cr, Cu, Ni, Pb, Sr, and Zn were found in Cambisol, we might deduce that the metals present in the dregs' matrix (Table S1) are not as concerning as the saline effect. Furthermore, it should be noted that the addition of dregs increased the pH of the soil to levels where metal cations are more likely to precipitate as oxides and hydroxides, which are not bioavailable to the soil organisms (Wan et al., 2024). However, it is crucial to exercise caution in assuming that the metals found in the dregs' matrix are of no concern at all, as some of them have no biological function and are toxic to soil organisms and may pollute water at underground levels. For example, Pb has already been linked to a decrease in *E. crypticus* reproduction (Zhang et al., 2019). Consequently, it is not possible to assume that the metal input via dregs has no negative effect on the environment.

We highlight that due to particularly the difference we found in dregs toxicity between the two evaluated soils, our findings suggest that the chemical and physical characteristics of the soils should be previously considered before proposing the use of dregs for agronomic purposes. It is important to note that the protection of the functions performed by soil fauna organisms is vital to guaranteeing the environmental services provided by the soil, therefore, we emphasize that the lowest of the EC₅₀ verified represents an indication of the maximum capacity of the dregs

that the soil can receive. These EC₅₀ values (5.8 g kg⁻¹ for *F. candida* in Neosol and 48.5 g kg⁻¹ in Cambisol) correspond to agronomic doses of 5.8 and 48.5 t of dregs per hectare (considering only the first 10 cm layer) which are higher than the doses evaluated in a study by Medeiros et al. (2009) that indicates that dregs improved the chemical attributes of an Cambisol. Based on the data presented, we can infer that the agronomic doses being explored are likely significantly lower than the effect concentrations found in this study. However, evaluations with other types of soil are required before it can be stated that dregs do not pose a risk to the environment. Another reason to be cautious is that the input of Na via dregs can also act as a clay dispersant, resulting in soil architecture damage that may affect the organism's activity. However, physical assays were not performed, exposing a gap on which to focus future evaluations. Furthermore, despite not assessing the effects of dregs on plant endpoints, high doses of elements such as Mg, Na, and Ca may trigger nutritional imbalances in crops and eventually impair crop yield in the long term (Sundari and Kanakarni, 2001).

Regarding the ecological risk analysis, the pioneering of the present research, concerning ecotoxicological assessments in subtropical soils with alkaline residue from the pulp and paper industry, tends to help understand the implications of using dregs as soil corrective and fertilizer. Finally, we would like to draw attention to the fact that, whereas the world is moving toward a fully circular economy, it is not always possible to apply industrial by-products into the soil for agricultural uses without previous chemical treatment. This means that although dregs have improved soil pH and are capable of providing certain important nutrients to plant crops, their effects on soil organisms should not be neglected since the ecosystem operates with interdependencies and interferences in key-species dynamics tend to be harmful to soil health.

5. Conclusions

The application of dregs in subtropical soils was able to reduce the reproductive rate of all evaluated soil invertebrates. The highest sensitivity was recorded for the springtail *F. candida*, followed by *E. andrei* and *E. crypticus*. Different effects regarding the toxicity of dregs were also observed depending on the soil evaluated, leading us to infer that soil properties can attenuate the effects of contamination. The most pronounced effects were observed in the Neosol, possibly due to its lower organic matter and clay content. However, although metal concentrations were higher in the Cambisol, the Na content appeared to be the main factor responsible for the ecotoxicological effects evaluated.

To summarize, despite its use as a soil pH corrective, dregs may pose a risk to soil ecosystems, as important species could potentially become endangered due to its application, particularly as a result of increased Na concentration. However, further evaluations with other agriculturally relevant soil types are necessary to improve our understanding of dregs toxicity in soils.

CRedit authorship contribution statement

Josieli Pietro Biasi: Writing – original draft, Investigation, Formal analysis. **Thiago Ramos Freitas:** Writing – original draft, Visualization, Validation, Formal analysis. **Eduardo Oliveira da Silva Lunardi:** Writing – original draft, Visualization. **Luís Carlos Iúñez de Oliveira Filho:** Writing – review & editing, Visualization, Validation, Supervision. **Mari Lucia Campos:** Writing – review & editing, Supervision, Funding acquisition. **Osmar Klauber-Filho:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Dilmar Baretta:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

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 will be made available on request.

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Appendix A. Supplementary data

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Copper prevention values in Brazilian subtropical soils

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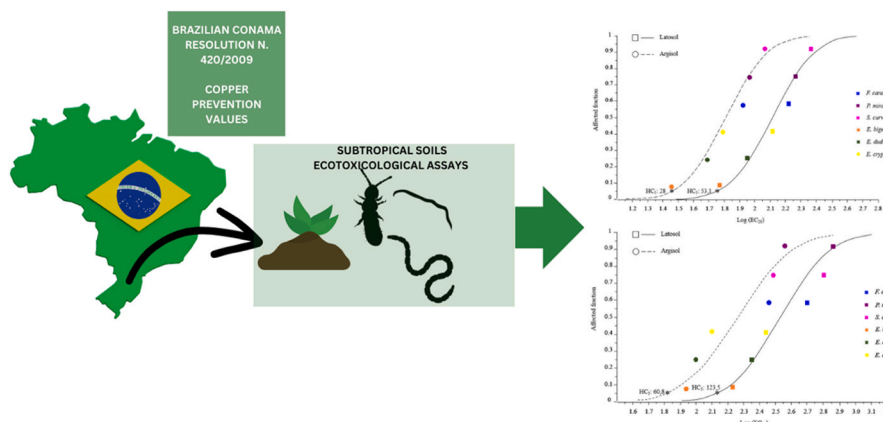
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HIGHLIGHTS

- The Brazilian National Environmental Council Resolution 420/2009 establishes soil quality guideline values for copper.
- Soil organisms are vital to the maintenance of the soil health.
- Copper contamination was capable of affect the reproduction rates of soils organisms.
- The toxicity of copper varies depending on the chemical characteristics of the soil.
- The Brazilian Prevention Value adopted for copper is suitable for protecting the organisms we evaluated.

GRAPHICAL ABSTRACT



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ABSTRACT

The Brazilian National Environmental Council (CONAMA) Resolution 420/2009 establishes soil quality guideline values for copper, as the prevention value (PV). It defines the maximum concentrations of chemical substances permitted in the soil. Thus, the aim of the present research was to evaluate the effectiveness of the copper PV adopted at the national level by conducting exposure assays with enchytraeids (*Enchytraeus crypticus*, *Enchytraeus bigeminus* and *Enchytraeus dudichi*) and springtails (*Folsomia candida*, *Proisotoma minuta* and *Sinella curviseta*) in two Brazilian subtropical soils: a Latosol and an Argisol. Results revealed that copper reduces the reproductive capacity of all organisms as its concentration increases in the evaluated soils. Argisol presented the lowest effective concentration of 20% (EC₂₀) and 50% (EC₅₀) in species reproduction for all organisms evaluated. The most sensitive organism was the enchytraeid *E. bigeminus*, with EC₂₀ values of 58 mg kg⁻¹ in Latosol and 30 mg kg⁻¹ in Argisol, and EC₅₀ values of 155 mg kg⁻¹ in Latosol and 91 mg kg⁻¹ in Argisol. Among the springtails, *S. curviseta* exhibited the highest EC₂₀, with values of 230 mg kg⁻¹ in Latosol and 136 mg kg⁻¹ in Argisol. Conversely, the highest values of EC₅₀ were recorded for the species *P. minuta* (526 mg kg⁻¹ in Latosol and 415 mg kg⁻¹ in Argisol). Based on the species sensitivity distribution (SSD) approach, the PVs calculated were 172 mg kg⁻¹ for Latosol and 106 mg kg⁻¹ for Argisol, derived from EC₅₀ data. For EC₂₀ data, the PV values calculated

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were 158 mg kg⁻¹ in Latosol and 100 mg kg⁻¹ in Argisol. By comparing the PV with the Brazilian adopted PV for copper (60 mg kg⁻¹), it was observed the efficacy of the CONAMA Resolution.

1. Introduction

The accumulation of metals in soil due to anthropogenic activities poses significant risks to both human health and the environment. These risks arise via exposure through food consumption, skin contact, and inhalation of contaminated particles (Nriagu and Pacyna, 1988; Hooda, 2010). While some metals are essential for biological processes, their excessive concentration can cause harmful effects, disrupting ecosystems and increasing health and environmental risks (Cannas et al., 2020; Poggere et al., 2023). Recognizing these risks and understanding the complex interactions of metals in soil systems is essential for implementing more effective environmental management strategies (Ahmad et al., 2021).

Copper is of particular concern due to its widespread use in agriculture and other human activities, such as the application of animal waste as fertilizer, pesticide application, mining, and fossil fuel combustion (Xiong et al., 2010; Segat et al., 2019; Pesce et al., 2024; Cassella et al., 2007). Its low mobility in soil results in accumulation in surface layers, particularly in organic matter, severely impacting soil biodiversity and disrupting ecosystem services (Strawn and Baker, 2007; Kabata-Pendias, 2010; Sereni et al., 2022).

In Brazil, the National Environmental Council (CONAMA) Resolution 420/2009 sets Prevention Values (PVs) for metals in soil, aimed at protecting soil-dwelling organisms and identifying areas at risk of contamination. The concept of pollution aligns closely with the PV definition, which refers to the introduction of substances at levels capable of harming soil health and ecosystem integrity (Brasil, 2009). This overlap underscores the importance of PVs as preventive tools to mitigate the impacts of contamination. However, the PVs established in CONAMA Resolution 420/2009 are based solely on phytotoxicity tests and fail to account for the diversity of soil types in Brazil, which vary in factors such as texture, composition, and pH – each of which influences the behavior and toxicity of copper (Alloway, 1995; Messias et al., 2023).

As environmental services also result from the functions performed by soil fauna, the PVs should consider the toxic effects of copper on soil animals, rather than relying solely on phytotoxicity data. It is important to note that soil fauna plays a crucial role in maintaining ecosystem functionality, with activities linked to nutrient cycling and organic matter decomposition (Decaëns et al., 2006; Briones, 2018; Hedénec et al., 2022). These functions are vital for sustaining agricultural productivity and ensuring soil health. Thus, by protecting soil biodiversity, we also safeguard human food security.

In this context, several soil fauna bioindicators have been extensively utilized in ecotoxicological assessments, particularly in countries with well-established frameworks to the establishment of soil guiding values, such as those within the European Union (ECHA, 2008), the United Kingdom (Environment Agency, 2022), and the United States (US EPA, 2005). The species recommended for the ecotoxicological assessment are described in standards from the International Organization for Standardization (ISO), which include oligochaetes and arthropods, such as enchytraeids and springtails (ISO, 2014a, 2014b). Additionally, the European Food Safety Authority (EFSA, 2017) recommends the use of alternative non-standardized species to enhance the accuracy of toxicity result interpretations.

Although ecotoxicological assays allow for the establishment of metrics to assess the effects of environmental contamination on several bioindicators, the PVs can only be obtained by considering ecological aspects and employing statistical validation and extrapolation procedures (ECHA, 2008). One plausible approach in this case is the construction of species sensitivity distributions (SSDs) by considering the set

of responses to contamination and the subsequent calculation of hazardous concentrations (HC_x). These HC_x represent a percentage level of protection for the evaluated species, generally relative to the lower fifth percentile (HC₅).

Therefore, the aim of the present research was to evaluate the effectiveness of the copper PV adopted at Brazilian national level and to propose a more suitable PV by conducting exposure assays with soil fauna bioindicators in two Brazilian subtropical soils: a Latosol and an Argisol. To this end, the effects of contamination were assessed on the reproduction of the enchytraeid species *Enchytraeus crypticus*, *Enchytraeus bigeminus*, and *Enchytraeus dudichi*, as well as on the springtail species *Folsomia candida*, *Proisotoma minuta*, and *Sinella curviseta*. The hypotheses evaluated were that (i) the effects of contamination vary according to the chemical characteristics of the evaluated soils, and (ii) the copper PV established in CONAMA Resolution 420/2009 does not provide adequate protection for the assessed organisms.

2. Materials and methods

2.1. Soils sampling and characterization

Two natural soils were used in the ecotoxicological assessments: a dystrophic Red-Yellow Argisol (native forest: 28°23'24.0"S, 49°22'6.9"W) developed from the weathering of sedimentary rocks (sandstone and siltstone), and a dystrophic Red Latossol (native forest: 27°22'33.6"S, 51°05'27.6"W) derived from basaltic rocks. The soils were selected due to their contrasting clay contents. The samples were collected in the surface layer (0–0.2 m) in areas under natural vegetation, air-dried, sieved through a 2 mm mesh, manually homogenized, and maintained at room temperature until the assays were conducted.

The chemical and textural attributes of the soils (Table 1) were obtained from the assessment of a composite sample (n = 3) using the methods described in the Soil Analysis Methods Manual of the Brazilian Agricultural Research Corporation (Teixeira et al., 2017), while the total concentrations of copper were determined using method 3050B (US EPA, 2007).

2.2. Test organisms and experimental procedures

The springtail and enchytraeid cultures were maintained at a temperature of 20 ± 2 °C with a photoperiod of 16 h of light, in accordance with the recommendations of ISO standards for chronic assays (ISO,

Table 1
Chemical and physical properties of the Argisol and Latosol.

Soil properties	Latosol	Argisol
pH in water	5.53	5.32
pH in KCl	4.34	4.07
Ca (cmol _c kg ⁻¹)	9.16	0.15
Mg (cmol _c kg ⁻¹)	2.44	0.29
K (cmol _c kg ⁻¹)	1.04	0.09
Na (cmol _c kg ⁻¹)	0.23	0.25
Al (cmol _c kg ⁻¹)	4.69	8.84
H + Al (cmol _c kg ⁻¹)	9.97	5.55
CEC ^a (cmol _c kg ⁻¹)	22.84	6.33
P (mg kg ⁻¹)	6.14	3.56
TOC (g kg ⁻¹)	18.9	9.05
Cu (mg kg ⁻¹)	155	8
Sand (g kg ⁻¹)	39	649
Clay (g kg ⁻¹)	621	201
Silt (g kg ⁻¹)	340	150

^a Cation Exchange Capacity at pH 7.

2014a; ISO, 2014b). The substrate for culturing the enchytraeids was a tropical artificial soil (TAS), consisting of a mixture of 75% fine industrial sand, 20% kaolinitic clay, and 5% coconut coir dust (Garcia et al., 2004). The pH of the TAS was adjusted to 6.0 ± 0.5 by adding CaCO_3 . The springtails were cultured in a substrate composed of a mixture of plaster, distilled water and activated charcoal in a ratio of 11:7:1. The enchytraeids were fed twice a week with properly autoclaved fine oat flakes to prevent contamination of the cultures by mites or other external agents. Meanwhile, the springtails received about 20 mg of dry yeast as food. Only healthy adult individuals, exhibiting normal behavior and no visible signs of damage to their morphological structures, were selected for the ecotoxicological assays.

Reproduction assessments were conducted following the methodologies outlined in ISO standards for chronic testing with springtails and enchytraeids (ISO, 2014a; ISO, 2014b). Soils were contaminated with increasing concentrations of copper, distinct for each evaluated faunal group (Table 2), through the addition of saline solutions prepared with deionized water and CuCl_2 . The doses were chosen based on the PV (60 mg kg^{-1} ; Brasil, 2009) and literature data (Posthuma et al., 1997; Lock and Janssen, 2002; Amorim and Scott-Fordsmand, 2012; Messias et al., 2023). Dilutions for each dose were prepared using volumetric flasks, corresponding to approximately 50% (g/g dry soil) of the water holding capacity (WHC) of each soil, and the mixtures (soil + solution) were manually homogenized in plastic bags. Control treatments included the addition of only deionized water until 50% of the WHC of the soils was reached. The contamination procedure was conducted individually for each assessment based on the bioindicator species and soil type.

The plastic bags containing the contaminated soils and controls were sealed and kept closed for 7 days to allow chemical equilibrium of the contaminant with the solution. This adaptation of the methodology was proposed by Fendorf et al. (2004).

The assays with enchytraeids involved adding approximately 20 g of dry soil weight into cylindrical glass containers (5 cm in diameter and 8 cm in height) with perforated lids, to which approximately 10 mg of fine oats were also added weekly to ensure that the organisms' feeding was not interrupted. Ten individuals were selected according to specific criteria for each species, namely, only adults of *E. crypticus* with a well-defined clitellum (ISO, 2014b); and adults individuals of the species *E. dudichi* and *E. bigeminus*, which are non-clitellated, with a length ranging from 0.8 to 12 mm (Bandow et al., 2013). To meet the validation criteria for the assays, all treatments included five replicates ($n = 5$). Soil moisture was adjusted weekly by adding deionized water, while the photoperiod and temperature were maintained under the same cultivation conditions (16 h of light; $20^\circ\text{C} \pm 2$). At the end of the 21-day experimental period, 5 mL of absolute alcohol (96% ethanol) was added to preserve the samples, along with 1 mL of rose bengal dye solution (1% w/v) and approximately 50 mL of distilled water. After a period of 72 h the samples were washed on a 125 μm mesh sieve and placed in trays for the subsequent counting of the organisms.

The assays with springtails were conducted by adding 30 g of dry soil mass into cylindrical plastic containers (7.5 cm in diameter and 5 cm in height) with properly perforated lids to allow air exchange with the external environment. The organisms were fed weekly with approximately 10 mg of granulated yeast, and moisture was maintained at about 50% of the WHC. For the evaluations with the species *F. candida* and

P. minuta, 10 individuals aged between 10 and 12 days were added to each experimental unit. For the species *S. curviseta*, 20 individuals were inoculated into the experimental units, following the adaptation proposed by Bandow et al. (2013). The assays lasted for 28 days, and at the end of the experimental period, distilled water and dark stamp ink were added to the experimental units for the assays with *F. candida*. Although the same procedure was followed for *P. minuta* and *S. curviseta*, no dye was added at the end of the assays, as the exoskeleton of these species has some coloration. Subsequently, photographs of the experimental units were taken, and the organisms (adults and juveniles) were identified using ImageJ 1.54d software (Schneider et al., 2012). All treatments included five replicates ($n = 5$).

2.3. Data analysis

The ecotoxicological data were subjected to the Shapiro-Wilk normality test ($p > 0.05$) and Bartlett's homogeneity test ($p > 0.05$). Means were compared using Dunnett's test ($p \leq 0.05$), and outliers were identified and removed from the statistical evaluation using Grubbs' test ($p \leq 0.05$). Data was then submitted to a non-linear regression analysis, employing exponential, logistic or Gompertz models (Environment Canada, 2007), to determine the copper doses corresponding to a 20% and 50% effect on species reproduction (EC_{20} and EC_{50}). The EC values were determined using the model that best fitted the data. The results of EC_{20} and EC_{50} were subjected to the Behrens-Fisher significance test ($p \leq 0.05$) (generalized likelihood ratio test) to determine whether there were differences in EC values among the evaluated soils. All analyses were carried out using STATISTICA 10.0 (Statsoft Inc., 2011).

2.4. SSDs and copper prevention values

Using ETX 2.0 software (Van Vlaardingen et al., 2004) the EC_{20} and EC_{50} were grouped, individually, into a log-normal distribution to construct the SSD with a 95% confidence interval. The log-normality of the data was evaluated using the Anderson-Darling ($p > 0.01$) and the Kolmogorov-Smirnov ($p > 0.01$) tests.

It is important to note that the SSD are mathematical models grounded in statistical distributions, and as such, the quantity and the quality of data is a significant variable in establishing more feasible protection levels (Messias et al., 2023). According to Del Signore et al. (2016), the European Union recommends using a minimum of five species for the construction of an SSD.

As a result, the predicted no-effect concentrations (PNECs) were determined using a restrictive assessment factor (AF) of 5 (Environment Canada, 2007). The following equation describe how the PNEC were calculated.

$$\text{PNEC} = \text{HC}_5/\text{AF}$$

Finally, the Quality Reference Values (QRVs), corresponding to the natural copper contents in the soils, were added to the PNEC to obtain the PVs. This procedure is crucial, as the calculation of PVs is based on the added risk concept, which refers to the concentration of the element that exceeds the natural background level and poses a potential risk to the dynamics of the assessed species.

QRVs were established by the local environmental agency (IMA, 2021) through normalization of chemical data across a wide range of soils. The concentrations established are $146.92 \text{ mg kg}^{-1}$ for soils with clay content equal to or greater than 59%, and 93.84 mg kg^{-1} for soils with clay content below 59%. The following equation outlines how we calculated the PVs for copper.

$$\text{PV} = \text{QRV} + \text{PNEC}$$

Table 2

Tested nominal concentrations of copper for each evaluated species.

Test species	Copper nominal concentrations (mg kg^{-1})
<i>E. crypticus</i>	0, 25, 50, 75, 150, 300 and 600
<i>E. bigeminus</i>	
<i>E. dudichi</i>	
<i>F. candida</i>	0, 60, 120, 240, 480, 720 and 1080
<i>P. minuta</i>	
<i>S. curviseta</i>	

3. Results

3.1. Ecotoxicological assays

The average mortality in the control treatments was below 20% for all assessments performed. The average number of juveniles exceeded 25 and 100 individuals for the tests with enchytraeids and springtails, respectively, with a coefficient of variation (CV) not exceeding 30% (Table S1). Thus, all validation criteria outlined in the ecotoxicological test standardization guidelines for springtails and enchytraeids were met (ISO, 2014a; ISO, 2014b).

For tests with the fragmenting species (*E. bigeminus* and *E. dudichi*), where adults could not be identified at the end of the experimental period, the validation criteria were consistent with those reported by Bandow et al. (2013).

Springtail reproduction was affected by copper ($p \leq 0.05$) in both evaluated soils (Fig. 1). A reduction in juveniles of *F. candida* was observed at doses of 120 mg kg⁻¹ in the Argisol and 60 mg kg⁻¹ in the Latosol. For the species *P. minuta* and *S. curviseta*, the observed effects occurred at a dose of 120 mg kg⁻¹ in both soils. Overall, the most pronounced contamination effects for all evaluated springtail species were observed in the Argisol, where the EC₂₀ and EC₅₀ values were lower than those observed in the Latosol (Table 3).

Regarding the sensitivity of springtails, based on the EC₂₀ results presented here for both evaluated soils, it can be inferred that the species most affected by contamination was *F. candida*, followed by *P. minuta* and *curviseta*, respectively. However, when determining the EC₅₀, a different outcome emerged; that is, the organisms showed a reduction in

Table 3

Ecotoxicological endpoints (mg kg⁻¹) for each evaluated species in Latosol and Argisol. Parentheses indicate the confidence interval (lower and upper limits) ($p \leq 0.05$).

Test Species	Latosol	Argisol
EC₂₀		
<i>F. candida</i>	163 (114–212) ^e	95 (54–137) ^{lo}
<i>P. minuta</i>	180 (123–237) ^{lo}	107 (42–171) ^{lo}
<i>S. curviseta</i>	230 (127–334) ^g	136 (87–187) ^g
<i>E. bigeminus</i> ^a	58 (36–80) ^g	30 (16–44) ^g
<i>E. dudichi</i> ^a	89 (62–116) ^g	53 (42–65) ^{lo}
<i>E. crypticus</i> ^a	127 (93–161) ^g	67 (47–87) ^g
EC₅₀		
<i>F. candida</i>	385 (304–466) ^e	333 (255–410) ^{lo}
<i>P. minuta</i>	526 (445–606) ^{lo}	415 (288–542) ^{lo}
<i>S. curviseta</i>	470 (371–569) ^g	350 (289–410) ^g
<i>E. bigeminus</i> ^a	155 (126–184) ^g	91 (70–112) ^g
<i>E. dudichi</i> ^a	198 (169–227) ^g	105 (91–119) ^{lo}
<i>E. crypticus</i> ^a	232 (203–261) ^g	136 (116–157) ^g

^a Reflect significant differences (calculated using the Behrens-Fisher generalized likelihood ratio test ($p \leq 0.05$)) between the EC obtained in different soils for the same species. Superscript letters indicate the regression model used: e for exponential; lo for logistic; and g for Gompertz.

reproduction in the following order: *F. candida* > *S. curviseta* > *P. minuta*.

All species of enchytraeids were also affected ($p \leq 0.05$) by soil contamination with copper (Fig. 2). The recorded effects began at a dose of 75 mg kg⁻¹ for the species *E. crypticus* in both soils. For the species *E. bigeminus*, a significant reduction was observed at doses of 50 mg kg⁻¹ in the Argisol and 75 mg kg⁻¹ in the Latosol. The impacts on the

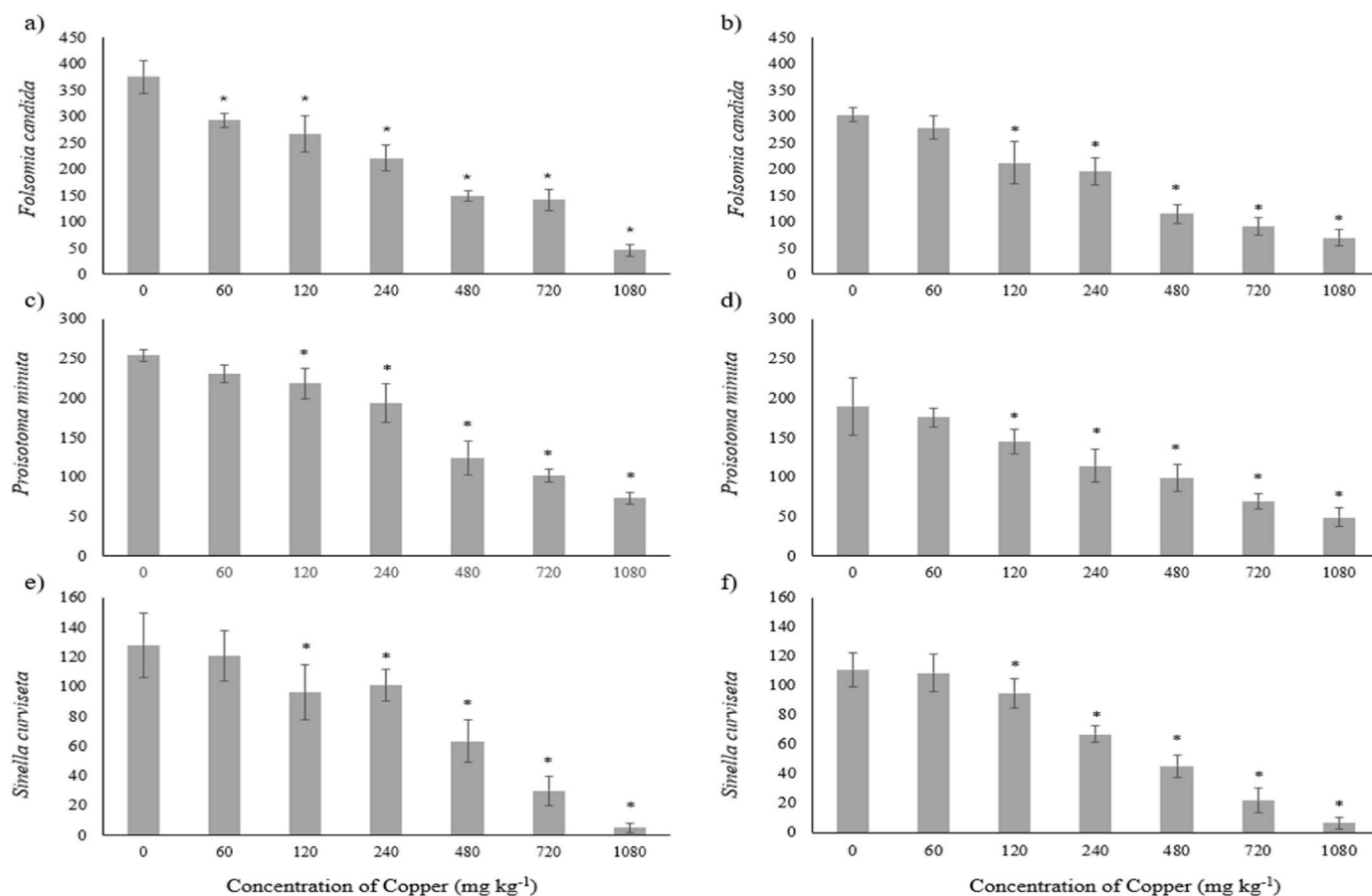


Fig. 1. Average number of juvenile of *Folsomia candida*, *Proisotoma minuta*, and *Sinella curviseta* in Latosol (a, c, e) and in Argisol (b, d, f) contaminated with different copper concentrations.

* Indicate statistically significant difference ($p \leq 0.05$) by the Dunnett test. (&boxhd) Indicates the standard deviation. Concentration 0 represents control treatment (free from contamination).

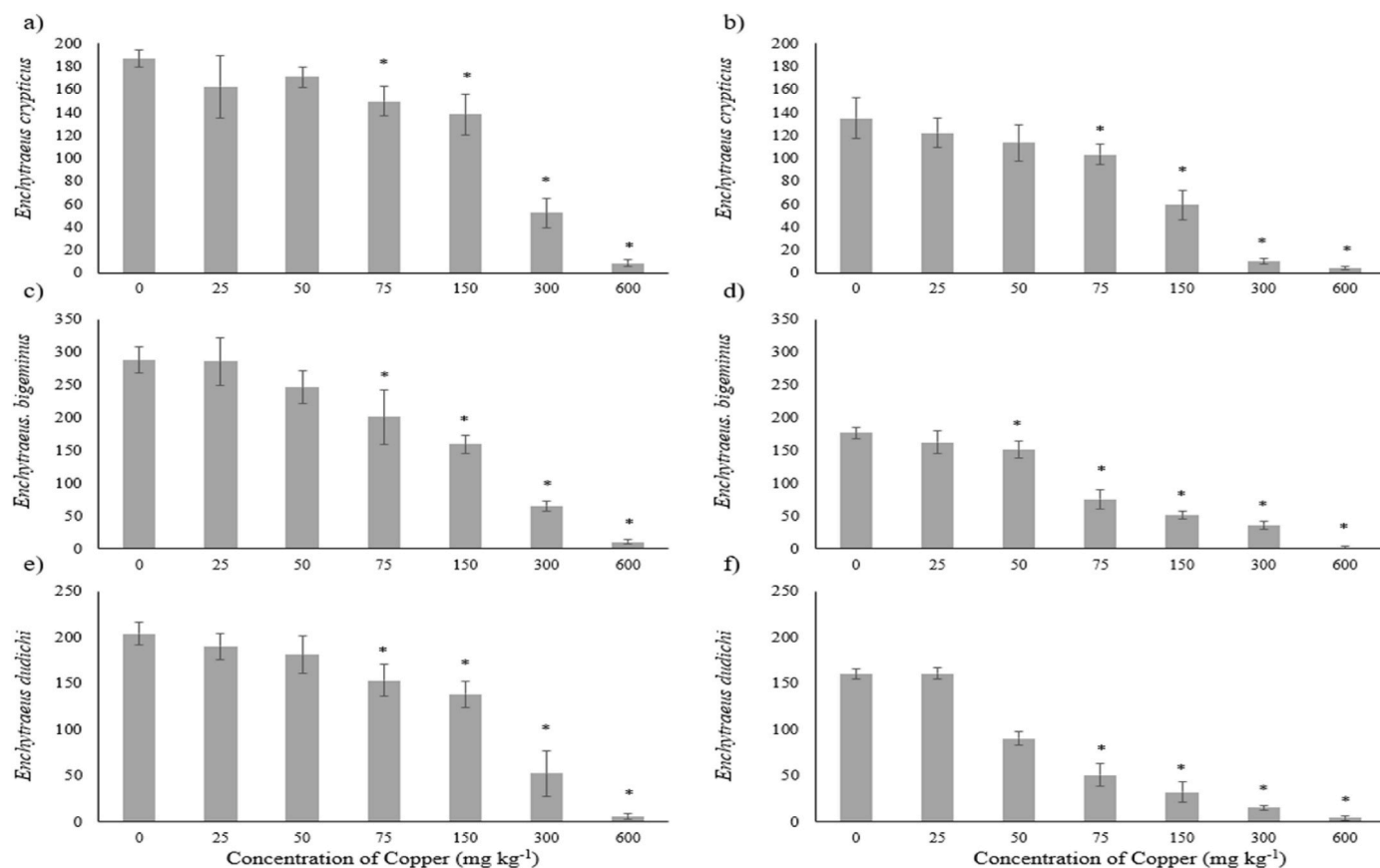


Fig. 2. Average number of juvenile of *Enchytraeus crypticus*, *Enchytraeus bigeminus*, and *Enchytraeus dudichi* in Latosol (a, c, e) and in Argisol (b, d, f) contaminated with different copper concentrations.

* Indicate statistically significant difference ($p \leq 0.05$) by the Dunnett test. (—) Indicates the standard deviation. Concentration 0 represents control treatment (free from contamination).

reproduction of the species *E. dudichi* were observed, for both evaluated soils, starting at a dose of 75 mg kg⁻¹. As with the springtails, the most pronounced effects for the enchytraeids were also observed in the Argisol, since the EC₂₀ and EC₅₀ values were lower than those found in the Latosol. Regarding the enchytraeids sensitivity, the fragmenting species *E. bigeminus* was the most severely impacted by contamination in both evaluated soils, followed by *E. dudichi* and *E. crypticus*. This trend was observed in both the EC₂₀ and EC₅₀ results.

It was found through the Behrens-Fisher statistical test ($p \leq 0.05$) that the enchytraeids were more sensitive to contamination in the Argisol. Springtails exhibited a similar pattern to enchytraeids, however, no significant differences in sensitivity were observed between the assessed soils (Table 3).

The SSDs derived based on the EC₂₀ and EC₅₀ for all evaluated species in both Latosol and Argisol are presented in Figures S1 and S2, respectively. The PV are presented in Table 4.

Table 4

HC₅, PNEC and PV (mg kg⁻¹) as function of the AF (=5) and the ecotoxicological endpoint for Latosol and Argisol.

Soil	Ecotoxicological endpoint	HC ₅	PNEC	PV
Latosol	EC ₂₀	53	11 (4–17)	158
	EC ₅₀	123	25 (9–38)	172
Argisol	EC ₂₀	28	6 (2–9)	100
	EC ₅₀	61	12 (3–22)	106

QRV for copper is 146.9 mg kg⁻¹ for soils with clay content equal to or greater than 59% (such as the Latosol) and 93.8 mg kg⁻¹ for soils with clay content less than 59% (such as the Argisol) (IMA, 2021). In parentheses, the lower and upper limits of the confidence interval for the PNEC value.

4. Discussion

The assessments revealed a significant reduction in the reproductive capacity of all organisms evaluated due to copper contamination. Among them, enchytraeids, particularly the fragmenting species *E. bigeminus* and *E. dudichi*, proved to be the most sensitive. Although these species are not standardized, ISO guidelines (ISO, 2014b) recommend the genus *Enchytraeus* for ecotoxicological evaluations. This recommendation is supported by the fact that organisms capable of asexual reproduction, like some species of the genus *Enchytraeus*, offer an advantage in ecotoxicological studies due to their genetic uniformity, which leads to more consistent and precise contamination responses (Weltje, 2003; Bandow et al., 2013). However, it is important to note that while genetic uniformity can reduce variability within laboratory strains, the sensitivities of asexually reproducing species may vary significantly in the field and across geographic regions (Fisker et al., 2011).

In addition to the higher sensitivity of fragmenting species, it is noteworthy that the effect concentrations estimated in this study (i.e., EC₅₀ and EC₂₀) were significantly lower than those reported by other researchers in evaluations conducted with temperate climate soils. For instance, Amorim and Scott-Fordsmand (2012) determined an EC₅₀ value of 251 mg kg⁻¹ and an EC₂₀ value of 137 mg kg⁻¹ while studying the effects of CuCl₂ contamination on the reproductive capacity of *E. albidus*. Similarly, Maraldo et al. (2009) reported an EC₅₀ value of 341 mg kg⁻¹ in their study on copper contamination in Danish soils and its impact on the reproductive capacity of *E. crypticus*. These discrepancies between the results presented here and those found in the literature can be attributed to differences in soil properties (Kabata-Pendias, 2010). In

this context, Bandow et al. (2013) suggest that the fragmenting species *E. bigeminus* is more suitable for evaluations in warm-climate soils, thus justifying its use as a bioindicator in ecotoxicological testing.

Enchytraeids exhibited greater sensitivity in assessments conducted in Argisol, likely due to the higher bioavailability of copper. According to Amorim et al. (2005), soils with low organic matter and clay content tend to create a more toxic environment for enchytraeids when exposed to cationic contaminants. Supporting this observation, Oliveira et al., (2024) identified more pronounced effects in subtropical soils with lower pH and organic matter contents. Similarly, although the evaluated Latosol exhibited a natural copper content eighteen times higher than that in Argisol, this characteristic does not appear to have directly influenced the toxicity to enchytraeids when comparing the effects between the soils. This may be attributed to the stability of copper within the structure of the clay minerals and the greater adsorptive capacity of Latosol (Tiecher et al., 2013).

The sensitivity of springtail species to contamination effects on reproduction was consistent in both Latosol and Argisol assessments. *F. candida* had the lowest EC₅₀ value, followed by *S. curviseta* and *P. minuta*. Although only *F. candida* is recommended for ecotoxicological assessments according to ISO 11267 (ISO, 2014a), the Organisation for Economic Co-operation and Development (OECD) advocates for the inclusion of *P. minuta* and *S. curviseta* in ecotoxicological evaluations (OECD, 2016).

Therefore, it was observed that springtails were the organisms exhibiting greater resistance to the harmful effects of the contaminant. Renaud et al. (2020) found that the toxicity of trace elements in *F. candida* is directly related to the solubility of cations in the soil. However, although the bioavailability of copper may be higher in Argisol, no significant differences were observed between the soils when comparing the EC₂₀ and EC₅₀ values. Sandifer and Hopkin (1996) argue that there is no clear correlation between increased toxicity and lower pH values when assessments are conducted with this species. As a result, by comparing the effects between the two soil types, we can partially confirm the first hypothesis, as enchytraeids exhibited distinct and significant effects between the evaluated soils, while springtails did not.

In comparison to the results presented here for *F. candida*, Sandifer and Hopkin (1996) reported an EC₅₀ value of 700 mg kg⁻¹ in artificial soil. Similarly, Amorim et al. (2005) determined an EC₅₀ of 794 mg kg⁻¹ in assessments involving European soils. For the species *P. minuta*, Nursita et al. (2005) obtained an EC₅₀ of 696 mg kg⁻¹ in evaluations conducted with Australian soils. Lastly, concerning the results for the springtail *S. curviseta*, Xu et al. (2009) identified an EC₅₀ value of 442 mg kg⁻¹ in Chinese soil. All these findings diverge from the results presented here for subtropical soil assessments. Variations in effect concentrations recorded among organisms of the same species arise from the influence of soil chemical attributes on contaminant dynamics. Consequently, it is not reasonable to compare the effects between soils from different countries for legal purposes. According to Marques et al. (2022), the primary factor influencing copper bioavailability in soil is the parent material. Soils derived from basalt, such as the Latosol evaluated in this study, exhibit a greater capacity to retain cationic elements compared to soils formed from sandstones.

Although no significant differences were observed in the reproductive rates of springtails among the evaluated soils, it is essential to highlight that soils with lower organic matter content are highly susceptible to copper contamination due to the element's strong affinity for the organic fraction (Kabata-Pendias, 2010). This suggests that in regions where such soils predominate, extra care should be taken regarding organic fertilization and the prolonged use of copper-based pesticides. Frequent applications may lead to the saturation of functional groups and subsequent interactions with lower-energy adsorption sites, thereby increasing the concentration of this element in the soil solution (Couto et al., 2014; Biasi et al., 2024). Furthermore, the formation of chelates with soluble organic ligands tends to enhance copper mobility within the soil profile, resulting in a higher likelihood of

groundwater contamination, particularly in sandy and well-drained soils (Marques et al., 2022).

When determining the PNEC and the corresponding PV, it is important to highlight two possible approaches: a deterministic, which derives the guiding value based on effect concentrations or on the no observed effect concentrations of the most sensitive organism to the contaminant; and a stochastic, which aims to determine a probabilistic concentration of concern, typically associated with the fifth percentile of the ecotoxicological response, as we have implemented (ECHA, 2008). Among the various factors influencing the choice between these two approaches, the quantity and quality of data play a crucial role. For example, the probabilistic approach requires data from at least five species, although a larger dataset is preferable to reduce uncertainties in data extrapolation (Posthuma et al., 2001; Del Signore et al., 2016; Environment Canada, 2007).

Regardless of the chosen approach, both methods link the PNEC to an AF. The selection of this factor should specifically consider (i) the quality of the data and the endpoints addressed, (ii) the diversity and representativeness of the evaluated taxonomic groups, and (iii) the availability of field assessment data or studies conducted in simulated ecosystems (ECHA, 2008; Environment Agency, 2022). However, guidance on selecting the most appropriate AF is limited to these general points. It is noteworthy that in the risk assessment of trace elements, employing an AF greater than 10 often yields values that fall below the geochemical background, as reported by Scott-Fordsmand et al. (1996). In such instances, the data become practically meaningless and cannot be utilized for legal purposes. This is one of several reasons why the Canadian Environmental Agency recommends that the AF should not exceed a value of 5 (Environment Canada, 2007). Chapman et al. (1998) emphasize that the application and selection of these AFs are matters of policy rather than purely scientific considerations, stemming from the need to establish a safety threshold, particularly for human health.

For these reasons, we suggest adopting PVs based on a more restrictive AF, as illustrated by the results (Table 4). The justification for this choice is that, while the ecotoxicological assessment has produced results capable of generating a realistic PV, it is essential to evaluate other taxonomic groups at different trophic levels that fulfill various ecosystem functions. It is important to note that a permissive PV may not accurately reflect the vulnerabilities of the ecosystem or support intervention actions according to the soil classification criteria established in Articles 13 and 20 of the CONAMA Resolution 420/2009 (Brasil, 2009). On the other hand, adopting an excessively restrictive PV, while protective, could adversely affect significant economic activities that do not cause environmental harm. Therefore, the solution lies not only in enhancing the robustness of the ecotoxicological assessment but also in fostering dialogue between regulatory policymakers and civil society.

Comparing the PVs for copper, specifically 172 mg kg⁻¹ in Latosol and 106 mg kg⁻¹ in Argisol, with the data from Oliveira et al., (2024) reveals a lower tolerance to contamination in the soils evaluated in this study. This is because the authors determined PVs for copper of 363.9 mg kg⁻¹ for a Nitisol and 151.2 mg kg⁻¹ for a Cambisol. It is important to highlight that the proposed PVs in the study were based on an AF of 1. In contrast, in our study we adopted an AF of 5; however, with a larger dataset covering additional trophic levels and ecosystem functions this assessment factor could potentially be reduced.

These differences, along with the limited number of assessments involving other soil types, pose challenges in determining a common PV for different soil types, as published for soils in the Brazilian State of São Paulo (Cetesb, 2021). Nevertheless, the United Kingdom's updated guidelines for Ecological Risk Assessment propose a common HC₅ for the same trace element, regardless of soil type. In this case, the prevailing concept of added risk suggests summing the contaminant's HC₅ with its respective geochemical background to obtain the PV (Crommentuijn et al., 2000), as we have done. However, the guideline precludes extrapolating the PV to an entire set of soils, as the method does not account for pedological influences on the contaminant's ecotoxicity

(Environment Agency, 2022).

Therefore, it is argued that it is not possible to determine a single PV that is common to the evaluated soils. To calculate a common PV, greater statistical robustness would be necessary, achievable only with a substantial amount of toxicity data on the contaminant. Furthermore, it is essential to emphasize that the inability to generate a common PV for both soils is also justified by differences in their clay content.

In regard to the comparison between the results of this study and the PVs outlined in CONAMA Resolution 420/2009, several observations are necessary. First, we decided not to suggest a PV based on EC₂₀ data due to the inability to calculate the no observed effect concentration for *F. candida* in the Latosol evaluation. Second, the aforementioned resolution does not clarify the procedure used for determining the PVs, specifically which approach was chosen. For this reason questions arise regarding the statistical methods used to validate the data. Although Article 9 of the aforementioned Resolution states that the PV was established based on phytotoxicity assays or ecological risk assessment, the data from the ecotoxicological evaluation is not published, nor the AF employed (Brasil, 2009). However, by comparison and considering the publication dates of the resolutions, it can be assumed that the national PVs were based solely on the analysis of soils from the Brazilian State of São Paulo (Cetesb, 2001).

For copper, the nationally adopted PV (60 mg kg⁻¹) is lower than the results presented here for both Latosol (172 mg kg⁻¹) and Argisol (106 mg kg⁻¹). Therefore, it can be concluded that the second hypothesis is not valid. This is because there was no evidence suggesting the need to update the PV for copper based on the findings from our ecotoxicological assessment. This same conclusion can be verified in the update of soil guidance values published by the Environmental Company of the State of São Paulo, as the regulation maintains the copper PV at 60 mg kg⁻¹ (Brasil, 2009; Cetesb, 2001). This consistency highlights the importance of ongoing assessments to ensure regulatory values align with the latest scientific findings while safeguarding environmental integrity.

5. Conclusions

Copper contamination in soil reduced the reproductive capacity of enchytraeids and springtails, with the extent of this effect varying according to soil type. The fragmenting species *E. bigeminus* was the most sensitive to the deleterious effects of copper. Among the springtails, *S. curviseta* showed the highest EC₂₀, while the highest EC₅₀ values were verified for *P. minuta*. These observations highlight the necessity of including a wide range of organisms in ecotoxicological assessments, as we observed that the effects vary even within the same biological group.

It is also important to note that all enchytraeid species were more sensitive to contamination when exposed to copper in Argisol. This sensitivity may be attributed to the lower organic matter content in this soil type. However, no differences in the sensitivity of springtails between the soils were observed. In this context, it is noteworthy that Latosol has a significantly higher copper geochemical background than Argisol; however, this characteristic does not appear to have directly influenced the measured effects.

The ecotoxicological assessment data involving six different species of soil fauna allowed for the construction of SSDs and the subsequent determination of HC₅ for both Latosol and Argisol. However, due to statistical limitations, primarily associated with uncertainties in the extrapolation of the results, the application of a restrictive AF (=5) was necessary for the PVs calculation. Consequently, when comparing the results with those outlined in CONAMA Resolution 420/2009, it was observed that the Brazilian adopted PV is sufficiently protective, as it is lower than the PV we calculated.

Finally, it is important to emphasize that a common PV for soils was not proposed due to the contrasting clay content of the soils evaluated. Therefore, it was deemed imprudent to combine the ecotoxicological assessment results for the soils into a single SSD. Moreover, assessments involving other taxonomic groups are necessary to reduce the statistical

uncertainties arising from the extrapolation of the data to the entire soil ecological community.

CRediT authorship contribution statement

Thiago Ramos Freitas: Writing – original draft, Visualization, Validation, Investigation, Formal analysis. **Rafaela Alves dos Santos Peron:** Investigation, Formal analysis. **Luís Carlos Iuñes de Oliveira Filho:** Writing – review & editing, Validation, Supervision. **Mari Lucia Campos:** Writing – review & editing, Supervision, Funding acquisition. **Osmar Klauber-Filho:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Dilmar Baretta:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

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.

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Appendix A Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.chemosphere.2025.144079>.

Data availability

Data will be made available on request.

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9. TRANSFORMAÇÕES MICROBIANAS DO CARBONO NO SOLO

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9.1 Introdução

O carbono é a estrutura básica de qualquer molécula orgânica, tanto fazendo parte da estrutura da célula tanto como componente das membranas e paredes celulares, ou em compostos relacionados ao metabolismo (enzimas). A matriz energética ou conjunto de energia disponível evolui junto com a humanidade. Nesse contexto fica exposta a importância do carbono, que é componente das principais bases energéticas, assim como os combustíveis fósseis formados por hidrocarbonetos (CH_4 , C_4H_{10} , C_8H_{18}), madeira e carvão.

A relevância do carbono no solo está intrinsecamente ligada às características que esse elemento confere, envolvendo aspectos da estrutura física e sua associação com minerais do solo. Além disso, certos compostos de carbono, produzidos por fungos, atuam como uma espécie de cola, promovendo a agregação das partículas do solo e, conseqüentemente, aprimorando sua porosidade. As transformações relacionadas ao carbono no solo não apenas otimizam a infiltração e o armazenamento de água, mas também aumentam a disponibilidade de nutrientes, contribuindo para uma maior qualidade biológica. Em termos práticos, essa dinâmica será abordada no contexto da matéria orgânica do solo (MOS).

10. ECOTOXICOLOGIA TERRESTRE

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10.1 Introdução

O solo, como discutido nos capítulos anteriores, desempenha diversos serviços ecossistêmicos e serve de hábitat e fonte de alimento para vários organismos. No entanto, está suscetível à contaminação por diversos tipos de poluentes, o que pode acarretar impactos negativos no ecossistema. A contaminação do solo por agrotóxicos, fármacos, dejetos, resíduos e outros produtos resultantes da atividade humana é uma realidade preocupante. Essa contaminação pode levar à perda de serviços ecossistêmicos e afetar toda a cadeia alimentar. A avaliação de áreas contaminadas geralmente se baseia em parâmetros químicos, mas essas análises, muitas vezes, não são suficientes para prever os efeitos nos organismos do solo, mesmo em concentrações muito baixas. Por esse motivo, para uma avaliação mais precisa, é recomendável que as análises químicas sejam complementadas por testes ecotoxicológicos.

A **ecotoxicologia** é a ciência que busca associar conceitos em ecologia e toxicologia, de modo que tem por intuito estudar os efeitos tóxicos de contaminantes dentro do contexto dos ecossistemas. O ramo da ecotoxicologia terrestre tem avaliado o efeito potencial de contaminantes no solo, objetivando determinar quais são os limites críticos das substâncias, isto é, a concentração capaz de resultar em efeitos deletérios aos organismos