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Considering safe and sustainable by design alternatives–Environmental hazards of an agriculture nano-enabled pesticide to non-target species

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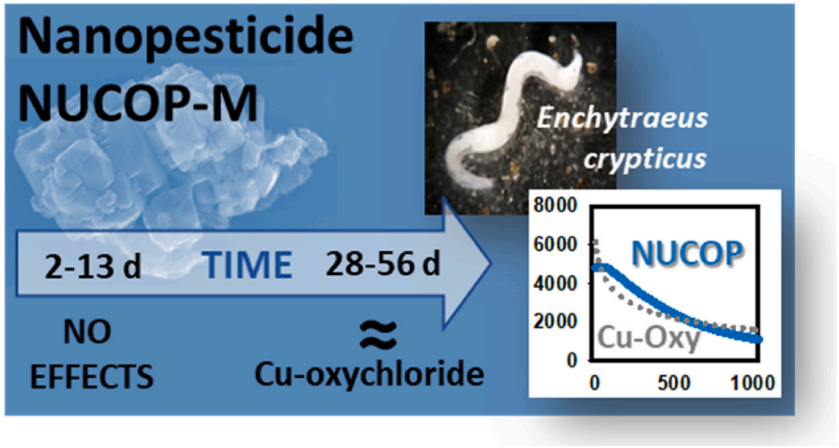
HIGHLIGHTS

- Environmental impacts of nano-pesticides (Npes) should be studied.
- Ecotoxicity of commercial Npe NUCOP-M was assessed in *E. crypticus*.
- High screening level (endpoints and exposure time) are relevant.
- NUCOP-M presented lower risks to enchytraeids than Cu-oxychloride.
- Differences might be related to a slower release of Cu^{2+} ions from NUCOP-M.

GRAPHICAL ABSTRACT



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ABSTRACT

Nanopesticides (Npes) offer improved efficacy compared to their conventional forms while reducing the usage/application rates, hence being more sustainable options. However, there is still a knowledge gap on the Npes environmental impacts. To support the safety of nano-enabled pesticides, the present study aimed at assessing the toxicity of the commercial Npe NUCOP-M and the active substance copper oxychloride, using the ecotoxicological soil model *Enchytraeus crypticus* and LUFA 2.2 soil. Bioassays were performed to assess various endpoints within short-to longer-term exposures: avoidance behaviour (2 d), hatching (13 d), survival, reproduction and organisms' size (based on the standard OECD test (28 d), the OECD extension (56 d), and the Full Life Cycle test – FLCt (46 d)). Based on the standard OECD test and its extension, NUCOP-M had a similar level of toxicity as copper oxychloride without indications of increase in toxicity over time (28 versus 56 d). The shorter-term exposures (2 and 13 d) showed higher toxicity for copper oxychloride. The exposure from cocoon stage (FLCt) seemed to provide an adaptative advantage (reduced toxicity) to NUCOP-M. The differences might be related to a

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slower release of Cu^{2+} ions from NUCOP-M, which seems to account for the toxicity at longer-term. Based on the recommended application doses (ca. 1.72 mg NUCOP-M kg^{-1}, i.e. 0.62 mg Cu kg^{-1} in the topsoil) there is no unacceptable risk of NUCOP-M on the enchytraeid population.

1. Introduction

While Plant Protection Products (PPPs) have a recognized importance in modern agriculture to protect crops against pests/diseases, its negative impacts to non-target species are also well-known (de Graaf et al., 2022; Mancini et al., 2019). Hence, and as patent in the EU Green Deal ambitions, a 50% reduction of the pesticide usage by 2030 is targeted. Nanopesticides (Npes) represent a potential for more sustainable alternatives compared to conventional pesticides, given the possibility to reduce application volumes due to the greater efficiency. Npes also aim improved stability and targeted delivery, all towards reducing environmental impact (An et al., 2022; Hayles et al., 2017; Wang et al., 2022). However, the current knowledge on the environmental fate and effects of Npes is still limited, and there is not enough data to support the efficacy gained from the use of nano-enabled products in a robust comparative assessment (Kah et al., 2018; Shekhar et al., 2021).

The soil compartment is the primary recipient and sink for pesticides and its residues in the environment (Hvězdová et al., 2018), with non-target soil living organisms being among the "first in line" to be exposed to these substances. Currently, there is limited information regarding the toxicity of Npes, and even less in comparison to conventional pesticides and/or active substances. The nano-enabled copper--based fungicide Kocide® 3000 is, probably, one of the currently most studied cases. Studies with aquatic organisms showed that Kocide® 3000 induced mortality (96hpf LC50 = 6.3 mg L^{-1}) to zebrafish embryos (Aksakal and Sisman, 2020) and to the water flea *Daphnia magna* (96h LC50 = 2.4 mg L^{-1}) (Aksakal and Arslan, 2020). For zebrafish embryos, Kocide® 3000 has shown to be more toxic than copper sulphate (Wang et al., 2021). Concerning soil invertebrates, Kocide® 3000 was more toxic to the collembolan *Folsomia candida* than the commercial non-nano formulations (Bordeaux mixture®, Cupravit®, and Nordox®), and similarly toxic as Champion® (Neves et al., 2019). On the other hand, Kocide® 3000 was the least toxic to the isopod *Porcellionides pruinosus* and the mealworm larvae *Tenebrio molitor*, in comparison to other copper hydroxide-based pesticide (Champion®) and the pure active substance, associated to a lower bioaccumulative potential (Morgado et al., 2022). Other examples are atrazine based products, where a synthesized nanoformulation containing atrazine was as toxic to the enchytraeid *Enchytraeus crypticus* as its active substance, and less toxic than the commercial non-nano formulation Gesaprim® (Gomes et al., 2019). Chlorpyrifos or tebuconazole nanoformulations presented increased risk in comparison to the pure active substances, because these bioaccumulated more in the earthworm *Eisenia fetida* and were bioavailable in soil for longer periods (increase in soil half-life and the amount of pesticides residues) (Fojtová et al., 2019). The above studies show that the fate, bioavailability and effects of Npes can differ from those of the active substances and conventional pesticides.

In the present study we aimed to investigate the toxicity of the commercial Cu-based fungicide NUCOP-M 35% HIBIO (further referred to as NUCOP-M), and additionally the active substance copper oxychloride. NUCOP-M is described has having reduced particles size (https://www.agrototal.pt/detalhe-produto.php?cd_produto=18&cd_categoria=205&cd_sub=207), thus being a potential nanoformulation. The toxicity test followed the OECD (Organisation for Economic Co-operation and Development) guidelines for the species *Enchytraeus crypticus* which meets the standards for regulatory use. Furthermore, other test/endpoints were included, that are relevant and increase the level of detail and interpretation (avoidance test to assess avoidance behaviour, the Full Life Cycle test – FLCt to assess hatching, in addition to survival and reproduction), and the OECD standard test extension), covering short- and longer-term effects, and are available for the selected test species (Bicho et al., 2015a, 2015b; Ribeiro et al., 2018). Hence, the soil model invertebrate *Enchytraeus crypticus* (Oligochaeta) (OECD, 2016) was used as test species. Effects were assessed using the standard LUFA 2.2 soil, covering various endpoints and life-stages as based on: avoidance test [2 d: avoidance behaviour (Bicho et al., 2015a)], OECD standard reproduction test (OECD, 2016) [28 d: survival, reproduction and adults' size], its extension (Ribeiro et al., 2018) [56 d: total organisms], and the Full Life Cycle test (Bicho et al., 2015b) [13 d: hatching and juveniles' size; 46 d: reproduction and adults' size].

2. Materials and methods

2.1. Test organisms

The test species used was *Enchytraeus crypticus* (Oligochaeta: Enchytraeidae). The cultures were kept in agar, consisting of Bacti-Agar medium (Oxoid, Agar No. 1) and a sterilized mixture of four different salt solutions at the final concentrations of 2 mM $CaCl_2{\cdot}2H_2O$, 1 mM $MgSO_4$, 0.08 mM KCl, and 0.75 mM $NaHCO_3$, fed *ad libitum* with dried, ground autoclaved oats twice a week, and maintained in laboratory under controlled conditions at temperature of 20 ± 1 °C and a photoperiod of 16:8 h (light:dark).

2.2. Test soil

The standard LUFA 2.2 natural soil (Speyer, Germany) was used. The main characteristics were as follows: pH (0.01 M $CaCl_2$) = 5.6, organic matter = 1.77%, CEC (cation exchange capacity) = 8.5 meq/100 g, WHC (water holding capacity) = 43.3%, grain size distribution of 10.6% clay, 15.0% silt, and 74.4% sand. The soil was dried for 48 h at 60 °C before use.

2.3. Materials and characterization

NUCOP-M 35% HIBIO (Agrototal®) containing 35% Cu w/w in the form of copper oxychloride (other components include calcium carbonate, polyglycolic ether of fat alcohol, among other non-specified elements) and the commercially available copper oxychloride ($H_6Cl_2Cu_4O_6$, Aldrich® CPR) were used as purchased.

NUCOP-M was characterized by dynamic light scattering (DLS) (Malvern Zetasizer Nano, Malvern Instruments Ltd, UK) to determine the hydrodynamic diameter (zeta average), polydispersity index (PDI), and zeta potential (ζ). All measurements were performed in auto-mode at 25 °C, with 3 consecutive measurements for each sample, using the same samples as to spike the soil. The morphology of NUCOP-M particles was analyzed by scanning and transmission electron microscopy (SEM/TEM), using a JEOL 2200FS HR-TEM instrument (JEOL, Tokyo, Japan) operating at 200 kV. The sample was prepared by dropping (twice) 20 μl of a NUCOP-M aqueous suspension (50 mg Cu L^{-1}) on a carbon-coated Cu grid and drying, at room temperature, before imaging.

2.4. Soil spiking

The nominal tested concentrations were 0-1-10-100-500-1000 mg $Cu kg^{-1}$ soil dry weight (DW) for both materials. The concentration range covers the recommended application dose (ca. 0.62 mg Cu kg^{-1} soil, considering 3 annual applications of NUCOP-M at 8.6 g L^{-1}, for prune and cherry threes, at ca. 1L ha^{-1}) up to higher doses, aiming the visualization of the full dose-response curve. Spiking procedures

followed the guidelines for nanomaterials (OECD, 2012) with the spiking of the soil being done per replicate, individually, to ensure total raw/nominal amounts of the test materials. NUCOP-M is commercialized as water dispersible granules, thus an aqueous stock suspension was prepared, serially diluted, and added onto the pre-moistened soil to reach 50% of the maximum WHC (maxWHC), being homogeneously mixed. Copper oxychloride is not soluble in water or organic solvents, thus it was added to soil as dry powder to obtain the desired final concentration range. The soil was homogeneously mixed, deionized water was added until 50% of soil's maxWHC, and the soil was mixed again. The soil equilibrated for 1 d prior test start, for both materials.

2.5. Test procedures

2.5.1. Avoidance tests

Avoidance tests were performed following the earthworm avoidance test guideline (ISO 17512-1, 2008), with some adaptations as described (Bicho et al., 2015a). Briefly, circular plastic containers (6.5 cm diameter × 2.5 cm height) and one removable plastic divider were used. In each container (replicate), the divider was placed in the middle, 20 g (wet weight) of soil introduced in each side – the control in one side and spiked on the other. The divider was then gently removed, ten adult organisms (with well-developed clitellum) were placed on the contact line of the soils, and each container was covered with a lid (containing small holes). The test ran for 48 h, at 20 ± 1 °C and a photoperiod of 16:8 h (light:dark). Five replicates per treatment were used, including controls (control soil in both sides of the containers). After 48 h, the divider was again placed between the two soils, and each side of the box was independently searched for enchytraeids.

2.5.2. Enchytraeid Reproduction Tests: OECD standard and extension

The Enchytraeid Reproduction Test (ERT) was performed following the procedures as described in the standard guideline (28 d) (OECD, 2016), plus the OECD extension (56 d) as described in (Ribeiro et al., 2018). In short, the test is extended for 56 d in total and had 5 monitoring sampling times at days 7, 14, 21, 28 and 56. Endpoints included survival (all sampling times), reproduction at days 28 and 56, i.e., number of juveniles and population, respectively, and size at day 28. Four replicates per treatment were carried out, except at days 7, 14 and 21 (1 replicate). At test start, ten juveniles (18-d old after cocoon laying, for details on cultures synchronization see (Bicho et al., 2015b)) were selected and introduced in each test vessel (7, 14, 21, and 28 d exposure: 4 cm diameter vessel, 20 g of soil, and 56 d exposure: 5.5 cm diameter vessel, 40 g of soil). Food supply (22 ± 2 mg autoclaved grinded oats) was added to each test vessel. The test ran at 20 ± 1 °C and 16:8 h (light: dark) photoperiod. Food was replenished every week (11 ± 1 mg up to day 28; 33 ± 3 mg from day 28–56) as well as water, based on weight loss. On sampling days 7, 14, 21, and 28, adults were carefully removed from the soil and counted (survival). The juveniles were counted at day 28 and 56 using a stereo microscope (Zeiss Stemi, 2000C), to assess reproduction. After being fixed with ethanol and Bengal rose (1% in ethanol) for 24 h, soil samples were sieved through meshes with decreasing pore size to separate the enchytraeids from most of the soil and facilitate counting. For the replicates that continued until day 56, adults were carefully removed from the soil at day 28. The adult organisms collected at day 28 were photographed after staining with Bengal rose, and size (length, mm) was assessed using the software ImageJ 209 (v.1.52a).

2.5.3. Full life cycle tests

A reduced version of the full life cycle test (FLCt), as described in (Bicho et al., 2015b) was performed. Endpoints included hatching success and juveniles' length (13 d), and reproduction and adults' length (46 d). Four replicates per treatment were done. At test start, ten cocoons (1–2 d old) were selected and introduced in each test vessel (4 cm diameter) containing 20g of moist soil. The test ran at 20 ± 1 °C and 16:8 h (light:dark) photoperiod. Food supply (11 ± 1 mg) was added first at day 13 and replenished weekly (33 ± 3 mg, from day 32), as well as water, based on weight loss. At each sampling time point, the respective replicates were processed, and organisms were counted, using a stereomicroscope, following the method described above. A sub-sample of the organisms in each replicate (n = 10) was photographed for size measurement (length, mm), as described above.

2.6. Data analysis

Avoidance response (A) was calculated as the percentage of worms that avoided the treated soil in the test container from the total number of worms in that container, calculated as follows:

$$A = ((C\text{-}T) / N)*100$$

where C is the number of organisms observed in the control soil; T is the number of organisms observed in test soil; N is the total number of organisms per replicate. A positive A indicates avoidance behaviour, and a negative A indicates a non-response (or attraction) to the tested substance.

One-way analysis of variance (ANOVA) followed by Dunnett's comparison post-hoc test ($p \leq 0.05$) was used to assess differences between treatments and respective controls (Sigma Plot, 14.0). Effect Concentrations (ECx) estimates were performed for the various endpoints, modelling data to logistic or threshold sigmoid two parameters regression models, as fully detailed in Table S1, using the Toxicity Relationship Analysis Program (TRAP v1.22). For the application of the regression models, avoidance data were inverted.

3. Results

3.1. Materials characterization

NUCOP-M aqueous suspensions are polydisperse, presenting high degree of agglomeration (zeta average ranging from 876 to 1754 nm, Table S2). The presence of smaller peaks (ca. 300 nm, as depicted in Fig. S1) was detected, corroborating the polydispersity of the sample. The surface charge of NUCOP-M suspensions was below −24.8 mV, up to 100 g Cu L^{-1} (Table S2), indicating stable suspensions. At 500 and 1000 mg Cu L^{-1}, the zeta potential is less negative (close to 0 mV), hence the suspensions are no longer stable and sedimentation might occur. SEM/TEM images of NUCOP-M suspensions (Fig. 1) revealed the presence of faceted particles of irregular shape, densely agglomerated – it was not possible to detect single particles.

3.2. Avoidance tests

The validity criteria for the standard guideline for earthworms (ISO 17512-1, 2008) were fulfilled, i.e., mortality <20% and random distribution of organisms in controls. Soils' pH, measured at the start of the test, varied less than 0.5 between test concentrations, for both materials [pH = 5.55 and 5.64 for 0 and 1000 mg Cu kg^{-1} of NUCOP-M, and pH = 5.59 and 5.93 for 0 and 1000 mg Cu kg^{-1} of copper oxychloride].

There was no significant avoidance of NUCOP-M (Fig. 2), but a dose-dependent avoidance response for copper oxychloride was observed, with nearly total avoidance (100%) at 500 mg Cu kg^{-1} soil.

ECx estimates show an EC10 of 79 and EC50 of 208 mg Cu kg^{-1} soil DW for copper oxychloride (Table 1; for further details see Table S1).

3.3. Enchytraeid Reproduction Tests: standard OECD and extension

Validity criteria were fulfilled as within the standard OECD test (OECD, 2016), i.e., mortality ≤20%, number of juveniles ≥50, and coefficient of variation <20% in the controls. Soils' pH increased (although still within the ±0.5) with increasing concentrations, for both

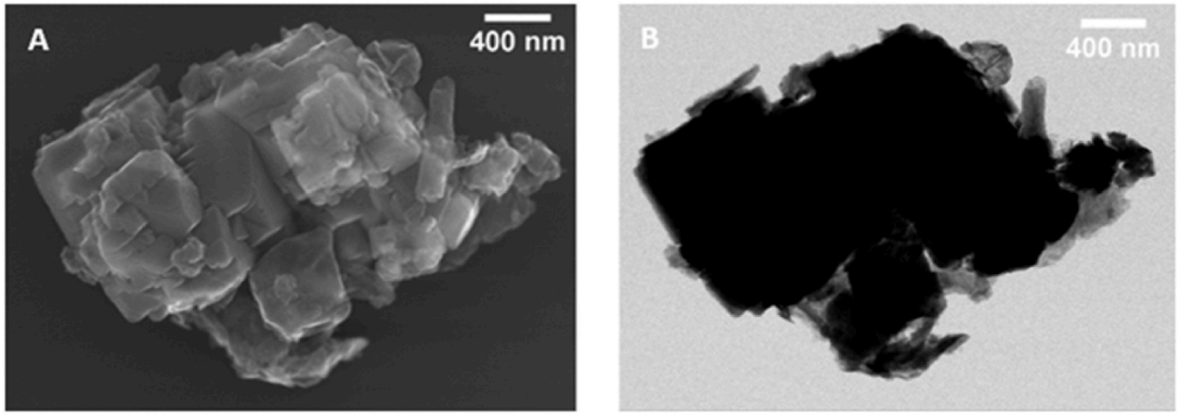

Fig. 1. Scanning Electron Microscopy (A) and Transmission Electron Microscopy (B) images of NUCOP-M particulates, deposited from an aqueous suspension.

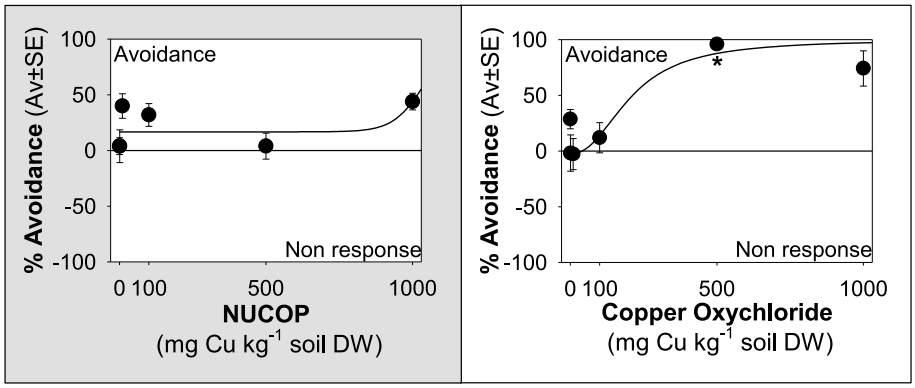

Fig. 2. Avoidance response of *Enchytraeus crypticus*, exposed for 48 h, to NUCOP-M and copper oxychloride, in LUFA 2.2 soil. Data is expressed as average ± standard error (Av ± SE). *p < 0.05 (Dunnett's method). Lines represent the models fit to data.

Table 1
Summary of the effect concentrations (EC10 and EC50) for *Enchytraeus crypticus* when exposed to NUCOP-M and copper oxychloride, in LUFA 2.2 soil. Effect concentrations (ECx) are expressed as mg Cu kg^{-1} soil dry weight, and the 95% confidence intervals (CI) are presented in brackets. n.e.: no effect; n.d.: not determined; ERT: Enchytraeid Reproduction Test; FLCt: Full Life Cycle test. (For further details on models and ECx see Table S1).

Endpoint	Test	Time (days)	NUCOP-M		Copper oxychloride	
			EC10	EC50	EC10	EC50
			(95% CI)	(95% CI)	(95% CI)	(95% CI)
Avoidance behaviour	Avoid	2	922 (–65812-67657)	1038 (–31640-33716)	79 (28–224)	208 (103–421)
Survival	ERT	28	370 (126–613)	893 (775–1011)	678 (313–1043)	1123 (891–1355)
Reprod.	ERT	28	101 (7–195)	306 (228–384)	138 (49–340)	457 (315–663)
Size (adults)	ERT	28	n.e.	n.e.	n.e.	n.e.
Total organisms	ERT	56	145 (62–340)	497 (374–659)	24 (5–113)	286 (164–500)
Hatching	FLCt	13	n.e.	n.e.	396 (–50982-51775)	482 (–8512–9476)
Size	FLCt	13	931 (242–3584)	n.e.	397 (231–563)	839 (365–1313)
Reprod.	FLCt	46	25 (4–176)	n.e.	148 (1–294)	357 (272–442)
Size (adults)	FLCt	46	607 (117–3155)	n.e.	n.e.	n.e.

materials, with the highest variation for the 56 days: variation of 0.62 for NUCOP-M and 0.74 for copper oxychloride [at day 56, pH = 5.52 and 6.14 for 0 and 1000 mg Cu kg^{-1} of NUCOP-M, and pH = 5.52 and 6.26 for 0 and 1000 mg Cu kg^{-1} of copper oxychloride].

Both NUCOP-M and copper oxychloride caused a dose-dependent decrease in terms of survival and reproduction, based on the 28 d OECD standard test (Fig. 3A). Survival was not significantly different between the two exposures, neither at the EC10 nor at the EC50 level (e.g., LC50 = 893 versus 1123 mg Cu kg^{-1} soil, for NUCOP-M and copper oxychloride, respectively), although the means were not the same. For reproduction there were no significant difference in effect, however at the EC50 level the overlap on the confidence intervals were smaller and no overlap at EC80 (e.g., reproduction EC50 = 306 (228–384) and 457 (315–663) mg Cu kg^{-1} soil, for NUCOP-M and copper oxychloride, respectively) (Table 1 and Table S1). This is also seen in the slopes of the different treatments, where the slopes for the survival was the same (i.e. S = 0.001 in both cases) but different for reproduction (i.e. S–NUCOP-M = 0.0001 and S-copper oxychloride = 1.07). The size of the adults (28 d) was not affected (Fig. S2).

Toxicity did not increase with the extension of exposure (56 d, Fig. 3B, C), i.e., from 28 to 56 d. In fact, for NUCOP-M there is a slight decrease in toxicity (28 d_reproduction EC50 = 306 mg Cu kg^{-1} soil, 56 d_EC50 = 497 mg Cu kg^{-1} soil), while for copper oxychloride the EC50 are similar (with overlapping confidence intervals) (Table 1).

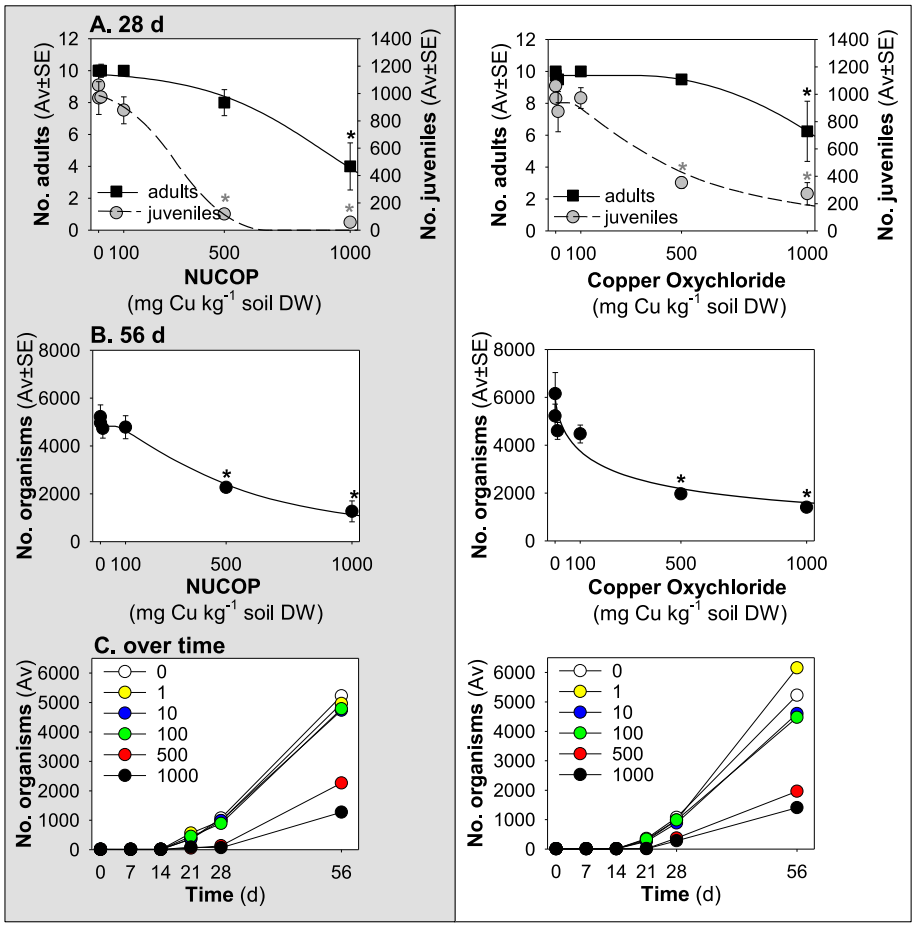

Fig. 3. Results in terms of survival and reproduction from the Enchytraeid Reproduction Test (ERT) when exposing *Enchytraeus crypticus* to NUCOP-M and copper oxychloride, in LUFA 2.2 soil for **(A)** 28 d (OECD Standard), **(B)** 56 d (OECD standard extension), and **(C)** overview of the time series sampling at days: 7, 14, 21, 28 and 56. All values are expressed as average value ± standard error (Av ± SE). *p < 0.05 (Dunnett's method).

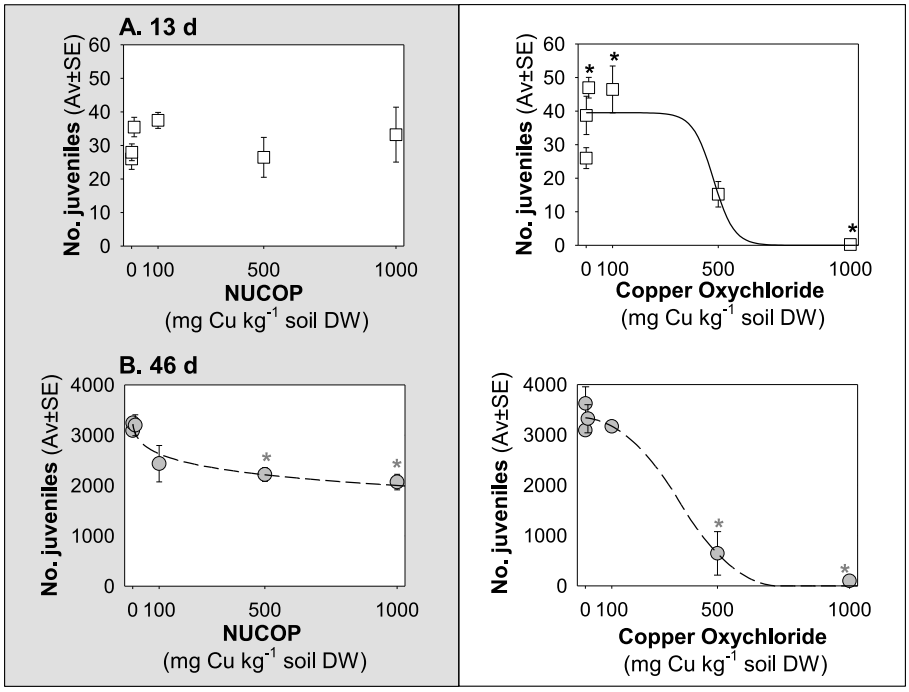

Fig. 4. Results in terms of hatching (13 d) and reproduction (46 d), from the Full life cycle test, when exposing *Enchytraeus crypticus* NUCOP-M and copper oxychloride, in LUFA 2.2 soil for **(A)** 13 d and **(B)** 46 d. All values are expressed as average value ± standard error (Av ± SE). *p < 0.05 (Dunnett's method).

3.4. Full life cycle test (FLCt)

Soils' pH varied less than 0.5 between test treatments (concentrations and time), for both test materials [the highest variation was observed for day 46, with pH = 5.85 and 6.15 for 0 and 1000 mg Cu kg^{-1} for both NUCOP-M and copper oxychloride]. NUCOP-M caused no significant effects on hatching (13 d), while copper oxychloride inhibited hatching in a dose-response pattern (hatching_EC50 = 482 mg Cu kg^{-1} soil) (Fig. 4A). Both materials caused an increase in hatching rates at lower concentrations (up to 100 mg Cu kg^{-1} soil), significantly for copper oxychloride at 10 and 100 mg Cu kg^{-1} soil. The size of the hatched juveniles was minutely inhibited, for NUCOP-M, at 1000 mg Cu kg^{-1} soil, although it was the least sensitive endpoint (13 d_size_EC50 ≫1000 mg Cu kg^{-1} soil) (Fig. S3A). For copper oxychloride juveniles' size was affected with 13 d_size_EC50 = 839 mg Cu kg^{-1} soil; measurements at 1000 mg Cu kg^{-1} soil were not possible as there was no hatching (Fig. S3A).

At day 46, both tested materials caused a dose-dependent decrease in terms of reproduction (Fig. 4B), with higher toxicity for copper oxychloride (46 d_EC50 = 3101 and 357 mg Cu kg^{-1} soil for NUCOP-M and copper oxychloride, respectively). The size of the adults (46 d) was not significantly affected (Fig. S3B).

4. Discussion

The ecotoxicity of NUCOP-M was investigated here, and additionally compared to copper oxychloride, revealing that the hazards of NUCOP-M to *E. crypticus* were overall similar to lower.

Our characterization revealed the presence of aggregated particles, some in the nanometer size range (ca. 300 nm). Hence, NUCOP-M presents nanofeatures, although this was not included in the product information. This is similar to other commercial formulations like the fungicide Kocide®3000 (Li et al., 2019), the insecticide KarateZeon® (Meredith et al., 2016), or the fertilizer WELGRO® Cu + Zn (Gomes et al., 2023). This brings awareness on the lack of clearly acknowledging nanofeatures in commercial products and probably the lack of up-to-date governance on the matter for novel and advanced materials.

Despite the dose-dependent toxicity reported here (e.g., Table 1), both NUCOP-M and copper oxychloride trigger a positive response (or biological stimulation) at one or more of the lower tested concentrations in the assessed endpoints (e.g. hatching at 13 d). This is likely hormesis, a phenomenon often reported in the literature, a response observed to several classes of chemicals, including copper (Rix et al., 2022).

NUCOP-M was not avoided by enchytraeids, while copper oxychloride caused a dose-dependent avoidance. Previous we have shown that *Enchytraeus albidus* were more sensitive to copper nanoparticles (Cu-NPs) than to a copper-salt ($CuCl_2$) (EC50 = 241 and 475 mg Cu kg^{-1} soil, for Cu-NPs and $CuCl_2$, respectively) (Amorim and Scott-Fordsmand, 2012). The differences could be related to the species and the nature of the copper form (oxychloride in the current study). For the "nano"-forms the presence of the inert ingredients in NUCOP-M formulation may prevent either the sensing of the NPs, or the release of Cu ions from copper oxychloride particles. Previous studies have shown that the avoidance of formulations could not be predicted based on the active substances alone, partly because of the influence of the inert ingredients present in those formulations (Guimarães et al., 2018; Marques et al., 2009; Nagy et al., 2020). NUCOP-M adverts for increased rate of release of Cu ions, allowing to apply lower amounts of copper per hectare (https://www.agrototal.pt/detalhe-produto.php?cd_produto=18&cd_categoria=205&cd_sub=207), but with release of larger quantity of the active in the form of Cu^{2+}. The avoidance of the contaminated soil represents an immediate important ecological advantage for enchytraeids, but the soil function can be dramatically affected by such avoidance. Such concerns have been raised by Van Zwieten et al. (2004) who found that earthworms occurred at lower densities in avocado farm soils with a history of copper-based fungicides use.

When in longer exposure periods or in aged soils, often the realistic field contamination scenario, the Cu release from NUCOP-M can be expected higher. Results from the OECD standard test (28 d ERT) and its extension (56 d) showed NUCOP-M toxicity was similar or higher than the active substance copper oxychloride (lower LC/EC values for NUCOP-M, with overlapping confidence intervals with copper oxychloride), although without indications of increased toxicity from 28 to 56 d exposure. Although, ECx values between 10 and 50% were not different it was noticed that the dose-response curves differed in steepness – with the lowest steepness for copper oxychloride, perhaps indicating that at higher NUCOP-M concentration the particles agglomerated (as seen from the aqueous stability measures). For both materials, reproduction was a more sensitive endpoint than survival, as often reported for several classes of chemicals, including for copper oxychloride. For example, for *E. fetida* exposed in OECD soil to a copper oxychloride-based fungicide (commercial name 'Virikop') no mortality was observed up to 640 mg Cu kg^{-1} soil, while reproduction was inhibited (EC50 = 309 mg Cu kg^{-1} soil) (Owojori et al., 2009). Despite the differences in test species and soil, these results corroborate ours: significant mortality at 1000 mg Cu kg^{-1} soil (no effects at 500 mg Cu kg^{-1} soil) and reproduction EC50 = 306 and 457 mg Cu kg^{-1} soil, for NUCOP-M and copper oxychloride, respectively. Also, in agreement with our results (LC50 = 893 and 1123 mg Cu kg^{-1} soil, for NUCOP-M and copper oxychloride, respectively), Maboeta et al. (2004) determined a 28 d LC50 = 883 mg Cu kg^{-1} soil for *E. fetida* exposed to a copper oxychloride-based pesticide (not identified) in OECD soil. To note that these studies were performed with commercial pesticides and not compared to the individual active substance. Overall indications are that the active substance causes the toxicity in NUCOP-M, i.e., as advertised, the Cu^{2+} are released from NUCOP-M. Accordingly, previous studies on *E. crypticus* showed that copper nanoparticles (Cu-NPs) (Gomes et al., 2015) and copper-oxide nanoparticles (CuO-NPs) (Bicho et al., 2017a) were less toxic than Cu-salts ($CuNO_3$ and $CuCl_2$), with low release of Cu ions from the NPs. The reproduction EC50 reported for the Cu-salts ($CuNO_3$_EC50 = 361 mg Cu kg^{-1} soil (Gomes et al., 2015); $CuCl_2$_EC50 = 303 mg Cu kg^{-1} soil (Bicho et al., 2017a)) are in good agreement with current results. Although based on different copper forms (copper hydroxide versus copper oxychloride), the toxicity of the nano-fungicide Kocide®3000 to *F. candida* (EC50 = 156 and 265 mg Cu kg^{-1} soil, for 0 h and 48 h of soil equilibration times, respectively (Neves et al., 2019)) was not very different from the toxicity of NUCOP-M to *E. crypticus*, based on the standard OECD test (EC50 = 306 mg Cu kg^{-1} soil). The lack of effects on hatching (13 d) as observed for NUCOP-M, as opposed to copper oxychloride, indicate that the cocoons/embryos were probably not exposed to Cu ions. It is known that $CuCl_2$ affects embryo development and hatching in *E. crypticus*, even at lower levels ($CuCl_2$_hatching_EC50 = 210 mg Cu kg^{-1} soil (Bicho et al., 2017a)) than the reported here for copper oxychloride (hatching_EC50 = 482 mg Cu kg^{-1} soil). These results support a slow (>13 d) release of Cu ions from NUCOP-M, as also suggested by the avoidance results. It seems that the juveniles hatched in the presence of Cu^{2+} (based on the standard ERT, Cu^{2+} is released from NUCOP-M within 28 d) which managed to survive and reproduce, were among the least sensitive to Cu^{2+} toxicity, reproduction at day 46 was higher than at day 28 (standard test) and day 56 (extension). Considering the long(er)-term exposure aspect, a multigenerational study with *E. crypticus* exposed to CuO-NPs and $CuCl_2$ showed that the impact on reproduction of $CuCl_2$ decreased over generations, while for CuO-NPs the toxicity increased and then stabilized (Bicho et al., 2017b). The decrease in toxicity for $CuCl_2$ was explained by the authors as possibly related to increased tolerance or resistance after the first generation, as due to the development of defences or activation of mechanisms to keep new homeostasis of Cu (e.g. metallothionein or Cu binding proteins activation), which is an essential element (Bicho et al., 2017b). In agreement with our findings, a study on the effects of the copper oxychloride based fungicide Cuprafor micro® on the earthworm *Aporrectodea caliginosa* showed that the cocoons exposed from

cocoon stage (similar to current FLCt) were less sensitive than the cocoons produced by exposed adults (Bart et al., 2019). The fungicide was not characterized for particles size distribution, but it is advertised (as the formulation name also suggests) to have (at least) micron sized particles (<300 μm). It is likely that this micron size also causes a delayed release in Cu^{2+} ions from Cuprafor micro®, but here the active substance alone was not tested in *A. caliginosa* for comparison.

Size was not a very sensitive endpoint for NUCOP-M or copper oxychloride, as it was only significantly affected, for the hatched juveniles (13 d), exposed to 1000 mg Cu kg^{-1} soil of NUCOP-M. Nevertheless, this might be a (nano) size related effect, as for similar test design (FLCt) $CuCl_2$ did not affect the size of the hatched juveniles, while CuO-NPs did (Bicho et al., 2017a).

As long as the recommended application doses for NUCOP-M (the highest being 3 applications at 8.6 g L^{-1}, for prune and cherry threes, at ca. 1 L ha^{-1}, which corresponds to ca. 1.72 mg NUCOP-M kg^{-1} (and 0.62 mg Cu kg^{-1}) in the top soil) are met, it poses no unacceptable risk to enchytraeid populations.

5. Conclusions

To the best of our knowledge, this is the first time that the ecotoxicity of NUCOP-M, is reported. NUCOP-M has nanoscale components confirmed in its composition. Based on the standard OECD test (28 d), the toxicity of NUCOP-M was overall similar to copper oxychloride, with no indication of increased toxicity with the prolonged exposure (56 d, OECD test extension). However, for short exposure periods (2 d avoidance test, and 13 d hatching - FLCt) copper oxychloride had higher effect than NUCOP-M. Based on the recommended application doses (3 applications at 8.6 g L^{-1}, for prune and cherry threes, at ca. 1 L ha^{-1}), which corresponds to ca. 1.72 mg NUCOP-M kg^{-1} (and 0.62 mg Cu kg^{-1}) in the top soil, NUCOP-M may have no unacceptable risk on enchytraeid population. This may of course vary depending on soil conditions, such as soil type, flooding etc. Repeated application, e.g., yearly, may of course lead to higher soil values.

CRediT authorship contribution statement

Sekerani B. Chidiamassamba: Writing – review & editing, Methodology, Investigation. **Susana I.L. Gomes:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Mónica J.B. Amorim:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Janeck J. Scott-Fordsmand:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Formal analysis, Data curation, 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.2024.143582.

Data availability

Data will be made available on request.

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