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Cu-nanoparticles ecotoxicity – Explored and explained?

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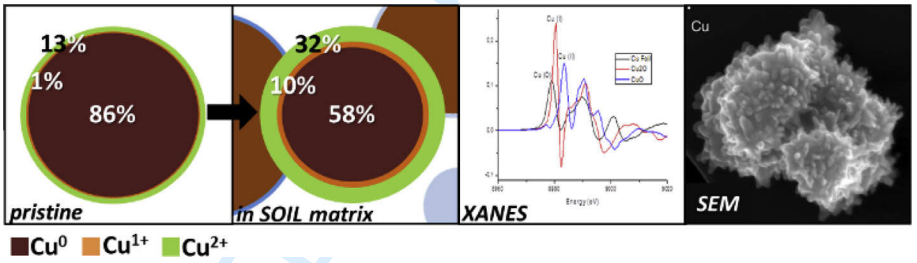
HIGHLIGHTS

- Exposure to various Cu materials caused different effect concentrations.
- Detailed in soil exposure characterization was performed.
- The characterization included XAS and Sequential Extraction.
- The analyses indicate NPs were partly, i.e. not fully oxidized in soils.
- Effects could be related to both toxicity of release of ions and of particles.

GRAPHICAL ABSTRACT



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ABSTRACT

The nano-form of copper (Cu-NPs) is already extensively used. In this paper the toxic effect of Cu in the worm *Enchytraeus crypticus* (Oligochaeta: Enchytraeidae) was assessed following exposure to (1) Cu-salt: freshly spiked soil with copper-nitrate, (2) Cu-NPs: freshly spiked soil with Cu nanoparticles (80 nm), and (3) Cu-field: historically Cu contaminated soil (80 years ago). Our main aims were to compare the three different exposure regimes and respective toxicity, and to determine how the oxidation state of the Cu and dissolution state of the particles differed. Characterization of *in situ*-exposure included identification of oxidation states with synchrotron generated X-ray absorption near-edge spectroscopy (XANES) analysis, activity of free Cu^{2+} in soil-solution (Ion Selective Electrode), and the relative distribution of the labile Cu-fractions (Sequential Extraction). Freshly spiked Cu-salt was the most toxic for reproductive output of the worms, followed by Cu-NPs and then Cu-field. XANES indicated only one oxidation state (II) in Cu-salt and Cu-field soil, whereas in Cu-NPs soil it was present in all oxidation states (0, I and II). The partial oxidation of the Cu-NPs (in soil) was evident and with limited dissolution.

1. Introduction

Cu nanoparticles (Cu-NPs) are already extensively used as wood-preservatives e.g. estimated to constitute 50% of the Cu containing wood-preservatives on the North American market (79.000 tonnes valued at $4.9 billion, see Evans et al. (2008)). The North America market represents 50% of the global market. Additional future sources may be Cu-NPs containing fertilizers and food additives/packaging (Batsmanova et al., 2013; Belli, 2012; Llorens et al., 2012). Hence, based on the above, direct exposure and hotspot scenarios in soils is very likely.

Copper toxicity is well documented to soil-dwelling organisms, e.g. enchytraeids (Amorim et al., 2008, 2005; Maraldo et al., 2006; Menezes-Oliveira et al., 2011), but very little is known about Cu-NPs. So far, only a handful studies reported effects of Cu-NPs

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via soil-exposure. Heckmann et al. (2011) observed that Cu-salt was more toxic (survival and reproduction) than Cu-NPs to the earthworm *Eisenia fetida*. Unrine et al. (2010), also using *E. fetida*, observed metallothionein induction following exposure to Cu-NPs (34 nm and 102 nm, TEM estimation) contaminated soil. Gomes et al. (2012a,b), using enchytraeids (*Enchytraeus albidus*), observed that both Cu-salt and Cu-NPs exposure caused oxidative stress and differential gene-expression, with different response-patterns for Cu-NPs and for Cu-salt. The same species indicated that Cu-NPs affected reproduction and avoidance to a higher extent than Cu-salt (Amorim and Scott-Fordsmand, 2012). Finally, Amorim et al. (2012), also using *E. albidus*, observed no differences in energy-reserves (lipids, proteins and carbohydrates) following exposure to Cu-salt or Cu-NPs.

Our aim here is to study the Cu toxicity to *Enchytraeus crypticus* between three exposure regimes, i.e. Cu-NPs spiked soil, Cu-salt spiked soil, and soil Cu-contaminated in the field 80 years ago (Cu-field) (Scott-Fordsmand et al., 2000a). Toxicity is measured as survival and reproductive-output. To help explain the difference in toxicity exposure is characterized as total Cu concentration, the labile concentration, the free Cu^{2+} concentration in the soil-solution, and in supplement to this the Cu oxidation state is measured in the soil.

E. crypticus (Enchytraeidae, Oligochaeta) was used; this is a standardized species (ISO, 2005; OECD, 2004), hence comparable to other studies. Enchytraeids inhabit the litter layer and the upper mineral soil where they feed on fungal hyphae, microorganisms and dead organic matter (OM) (Jansch et al., 2005; Løkke and Van Gestel, 1998). Further they actively contribute to the acceleration of OM decomposition and nutrient recycling processes. As a soft-bodied soil living invertebrate, uptake is made not only via ingestion (e.g. food and soil particles) but also via the body surface or skin (used for respiration and water uptake) (Peijnenburg et al., 2012).

2. Material and methods

2.1. Experimental plan

To study the Cu toxicity, three Cu forms were used, i.e. Cu-NPs spiked soil, Cu-salt spiked soil, and Cu-contaminated soil from the field 80 years ago (Cu-field). The Cu-exposure concentrations aimed to cover the full concentration–response (reproductive output) curve for the organisms. Since difference in toxicity between the three Cu forms was observed in range-finding experiments, the exposure-concentrations were adjusted accordingly. Biological and physicochemical measurements were performed (see sections ahead).

2.2. Test soil

Test soil was collected at Hygum, Jutland, Denmark. The general physicochemical characteristics of the soil from the Hygum-site are as follows: 20–32% coarse sand (>200 μm), 20–25% fine sand (63–200 μm), 11–20% coarse silt (20–63 μm), 12–20% silt (20–20 μm), 12–16% clay (<2 μm), 3.6–5.5% organic matter, Cation Exchange Capacity 6.8–10 (cmolc/kg dw), N 0.25–0.31% and P 0.10–0.12%. The clay mineralogy analyzed by X-ray diffraction were dominated by illite, kaolinite, chlorite and vermiculite.

Previous (more than 80 years ago), this site was a timber preservation site using copper sulphate to preserve and consequently contaminating the soil. There is a known contaminated gradient of Cu, ranging from the natural background levels of thousands of mg Cu/kg dry soil (Scott-Fordsmand et al., 2000a).

For the experiments, soil was sampled in the field to a depth of 20 cm. To exclude existing soil animals, major stones and plant material, the soil was dried at 80 °C for 24 h in an oven (Memmert, Type UL40, Braunschweig, Germany) and then sieved to <2 mm. Besides the control soil (with background Cu levels), the field test soil included Cu concentrations of 525, 1155, 2880 and 3900 mg Cu/kg, as picked from different locations along the field gradient and measured by flame Atomic Absorption Spectrometry (AAS). The physicochemical parameters (described above) vary little with increasing Cu concentrations; the only monotonic trend observed was for the organic matter, 3.6–5.5% positively correlating with the Cu content.

In the experiment, the control soil was spiked with Cu-salt or Cu-NPs, and the field soil with original concentrations. For the Cu-salt spiked soil the pH was regulated (with $CaCl_2$) to 6.5. During the experiment the soil pH was measured at the beginning (day 0) and at the end of the experiment (3 weeks).

2.3. Test chemicals and characterization

Compounds used included Cu-salt: copper (II) nitrate trihydrate ($Cu(NO_3)_2{\cdot}3H_2O$) (⩾99% pure, Merck, Darmstadt, Germany) and Cu-NPs: elemental Cu (nominal particle size 80 nm) without coating (American Elements). The particles were characterized (see Heckmann et al., 2011) by transmission electron microscopy – TEM (Phillips/FEI, Eindhoven, The Netherlands), scanning electron microscopy – SEM (FEI NOVA 600, FEI Company, Hillsboro USA), powder X-ray diffraction – PXRD (STOE STAPI PSTOE & Cie GmbH, Darmstadt, Germany) and dynamic light scattering – DLS (Malvern Zetasizer Nano, Malvern Instruments Ltd, Worcestershire, UK).

Oxidations states, of pure particles and mixed in soil, were analyzed by X-ray absorption near edge structure (XANES) measurements, performed at Beamlines C and X1 at DORIS (DESY, Hamburg, Germany) using a Si (1 1 1) crystal pair monochromator for the Cu energy range with a resolution $\Delta E/E$ of $<10^{-4}$ (<1 eV). Powder Standards were mixed with cellulose and pressed into pellets of 13 mm in diameter with ~2 absorption lengths of material ensuring sufficient uniformity and thickness for transmission measurements. Soil samples (three replicates) were sealed in Kapton tape and measured in fluorescence mode using a Canberra 7 element solid state (Ge) detector to monitor the Cu Kα fluorescence line. Spectra were normalized to the incoming flux and a uniform edge step. Background subtraction, normalization and linear combination analysis of the spectra was performed using Athena (Ravel and Newville, 2005). To enhance resolution the soil samples contained 3000 mg Cu/kg soil (spiked), similar but lower resolution spectra (not shown) were obtained at lower soil Cu concentrations.

To verify exposure, the total soil Cu-concentrations were analyzed by AAS (Perkin Elmer 4100, Ueberlingen, Germany). The AAS analyses were checked using a standard reference sample (standard oyster tissue (NBS 1566a Oyster Tissue, U.S. Department of Commerce, National Bureau of standards, Gaithersburg, MD 20899)). The soil (1 g dry weight) was digested using 7 N HNO_3 and heating up to 110 °C until all brown fumes were gone (Scott-Fordsmand et al., 2000b).

To estimate the labile Cu fraction two concurrent experiments were performed. For these experiments the labile Cu-fractions were estimated by measures of free active Cu^{2+} in soil-solution and estimating the distribution of Cu between the various soil fractions using sequential extraction of Cu from soil. The free Cu-activity (Cu^{2+}) was measured on soil-solutions using an ion-selective electrode (ISE25CU, Radiometer) see (Gomes et al., 2012a). The soil-solution extract was the supernatant of a (1:5) soil:water solution which was mixed for 5 min at 250 rpm (lab shaker) and then settled for 2 h. This soil-solution contained other

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elements that possibly could interfere with the ISE measures, hence to support the soil–water observations, the possible dissolution of the Cu-NPs was measured in a simpler solution, a modified-water solution. The modified-water solution was demineralized water with the same ionic strength and pH as soil-solution extracts, by adding KNO_3 to the demineralized water. The distribution between soil fraction was estimated by sequential extraction following the Rauret et al. (1999) modifications of the BCR-method. In short, 1st extraction: 0.11 M acetic acid [targeting carbonates], 2nd extraction: 0.5 M hydroxylammonium chloride [to dissolve Mn-and Fe-oxides], and the 3rd extraction: 8.8 M hydrogen-peroxide plus heating [to digest organic matter] followed by 1.0 M ammonium acetate [to complex re-adsorbed Cu]. The extraction analyses were performed on 1.3 g dry soil at the exposures scenario similar to that of the XANES analyses. The extracted fractions were analyzed for Cu on AAS (as described above).

2.4. Test organism

The test organism used for this was the specie *E. crypticus*, Westheide and Graefe 1992. Individuals were cultured in Petri dishes containing agar medium, consisting of a sterilized mixture of four different salt solutions ($CaCl_2{\cdot}2H_2O$; $MgSO_4$; KCl; $NaHCO_3$) and a Bacti-Agar medium (Oxoid, Agar No. 1), kept at 20 °C and fed on dried, ground oats.

2.5. Spiking procedure

Spiking of uncontaminated field collected soil was done according to OECD recommendations for the testing of non-soluble substances (OECD, 2004). In short, the equivalent of 2.5 g of dry soil per test vessel (10 g per treatment) was mixed with the required quantity of the test substances (Cu-salt or Cu-NPs), as dry powders, to obtain the desired final concentrations. This portion of spiked soil was added to the remaining pre-moistened soil (20% of water) and mixed manually for 10 min. The procedure was repeated for each test concentration and the soil was then divided into 4 replicates per treatment. The spiked concentrations used were 100, 200, 400, 600, 800, 1000 and 1500 mg Cu/kg for Cu-salt and 100, 400, 800, 1000 and 1500 mg Cu/kg for Cu-NPs.

2.6. Test procedure

Test procedures followed the Enchytraeid Reproduction Test (ERT) (OECD, 2004). In short ten adult organisms with well-developed clitellum and similar size were selected and introduced in each test vessel containing 25 g of moist soil and fed with 25 mg of finely ground and autoclaved rolled oats. The vessels were covered with a lid containing small holes and the test ran for 3 weeks, at 20 °C and 16:8 h photoperiod. Four replicates per treatment were used. Weekly, 12.5 mg of food was supplied and soil moisture was adjusted by replenishing weight loss with the proper amount of demineralized water. At the test end enchytraeids were extracted by spreading the soil of each test vessel into plastic containers (200 ml volume and diameter of 7 cm), which were filled with demineralized water, gently shaken and left for 24 h at 5 °C for sedimentation to occur. After that, adult and juvenile enchytraeids appeared on the soil surface and were collected and counted under dissection microscope within 24 h to assess their survival and reproduction.

2.7. Statistics

Results were analyzed trough a one-way ANOVA (Dunnett's Method) (SigmaPlot 11.0) to assess differences between control and treatments and to determine the No Observed Effect Concentration (NOEC) and the Lowest Observed Effect Concentration (LOEC) as the highest tested concentration with no statistically significant effect and the lowest tested concentration with a statistically significant effect, respectively. The reproduction effect concentrations (EC_x) and the lethal concentration to 50% of the organisms (LC_{50}), were calculated using the Toxicity Relationship Analysis Program (TRAP). For the LC_{50} probit was used and for the EC_x determination a 2-parameters non-linear sigmoid threshold model was used.

3. Results

3.1. Particles characterization

The tested nanoparticles (American elements) had a mean nominal diameter of 80 nm and purity of 99.5%. Measured by PXRD the crystal particle size was $\geqslant$66 nm with a *z*-average of 419 ± 1 nm (measured by DLS) indicating agglomeration of particles, and a Zeta potential of 15.3 ± 0.3 mV (measured in demineralized water). Correspondence between the Cu and CuO crystallite sizes and the TEM and SEM images, suggest that the particle morphology was comprised by a Cu crystalline core with a Cu_xO_y (shell) surface oxidation (Heckmann et al., 2011), see Supplementary data (Table S1, Fig. S1A/B, and S2).

3.2. Exposure characterization

The soil "background" Cu concentration was 30 mg Cu/kg. For all test-exposures the total concentration was within 5–7% of the nominal (i.e. "background" plus added Cu). The pH of the soil remained constant throughout the experiments.

3.2.1. XANES characterization (Cu oxidation states)

The XANES (using linear combination fit (LCF) in the program Athena (Ravel and Newville, 2005)) indicated that oxidation state distribution of Cu-NPs before addition to soil was approximately 86% Cu(0), 1% Cu(I) and 13% Cu(II) (Table S1). For the Cu-NPs spiked soil, oxidation state distribution was approximately 58–59% Cu(0), 10% Cu(I) and 32% Cu(II), based upon analysis of a soil containing 3000 mg Cu-NPs/kg soil (Fig. 1 and Table 1, see also Supplementary data). For the Cu-salt and Cu-field contaminated soils, XANES showed that only the oxidation state Cu(II) could be observed. For Cu-NPs spiked soil the "background" soil Cu concentration (approx. 30 mg Cu/kg) explained 1% of the oxidation state. The XANES spectra indicate that Cu(II) mainly exists as Cu—O bonds (although not possible to exclude N bounds). For both the Cu-salt and the Cu-field the indications are also of Cu—O bonds (or N).

3.2.2. Ion selective electrode (Cu^{2+} activity)

The active Cu^{2+} fraction (as measured by Cu-ISE) in modified-water and the soil-solution extracts was below the detection limit and quantification ($3 * 10^{-9}$ M Cu^{2+}). The detection limit corresponded to that less than $5 * 10^{-7}$% of the total-content that could be detected.

3.2.3. Sequential extraction characterization (Cu binding)

The sequential extraction (Fig. 2) showed nearly similar extraction patterns for the Cu-NPs and the Cu-salt samples, with approximate values of 69% (Cu-NPs) and 77% (Cu-salt) in the first extraction, 28% (Cu-NPs) and 22% (Cu-salt) in the second extraction, 3% (both) in the third extraction, and less than 1% (both) in the residues. For the field-contaminated soil the equivalent % was: 1st extraction 34%, 2nd extraction 40%, 3rd extraction 13%

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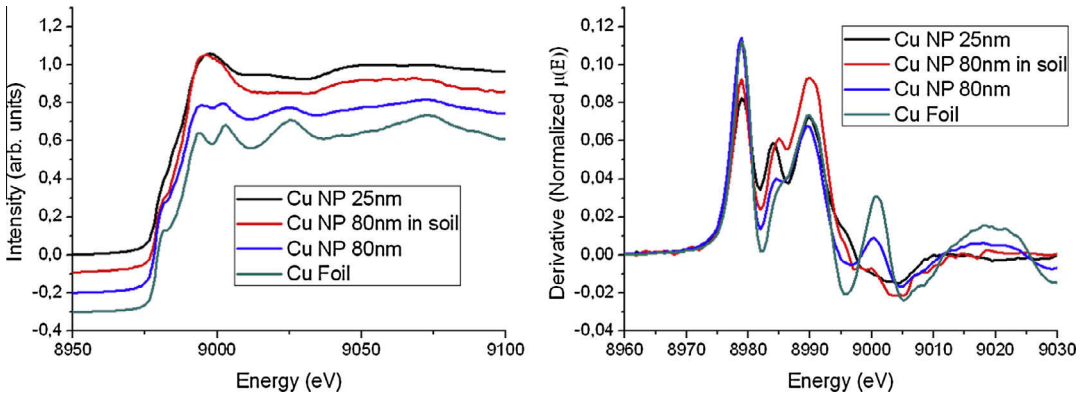

Fig. 1. Left, normalized XANES spectra at the Cu *K*-edge. Right, 1st derivative of the normalized XANES spectra.

Table 1
Effect concentrations (EC_x), no observed effect concentrations (NOEC) and lowest observed effect concentrations (LOEC) for survival and reproduction of *Enchytraeus crypticus* exposed to $Cu(NO_3)_2$ (Cu-salt), copper nanoparticles (Cu-NPs) and Cu field historically contaminated soil (Cu-field). NE: No Effect.

	Survival		Reproduction				
	LC_{50}	NOEC/LOEC	EC_{10}	EC_{20}	EC_{50}	NOEC/LOEC	Steepness (SE) Y_0
Cu-salt	595 (555–653)	400/600	254 (150–358)	290 (211–369)	361 (326–398)	200/400	$5.1 * 10^{-3}$ ($1.8 * 10^{-3}$) 239
Cu-NPs	NE	>1500	590 (−29–1208)	982 (575–1389)	1760 (1038–2482)	>1500	$4.7 * 10^{-4}$ ($2.1 * 10^{-4}$) 227
Cu-field	3508 (3292–3726)	2880/3990	NE	564 (−352–1482)	2068 (1391–2747)	<525	$2.4 * 10^{-4}$ ($0.6 * 10^{-4}$) 224

LC_{50} = 50% Lethal Concentration; EC_{10} = 10% Effect Concentration; EC_{20} = 20% Effect Concentration; EC_{50} = 50% Effect Concentration; NOEC = No Observed Effect Concentration; LOEC = Lowest Observed Effect Concentration; 95% CL = 95% Confidence Limits; NE: No Effect;

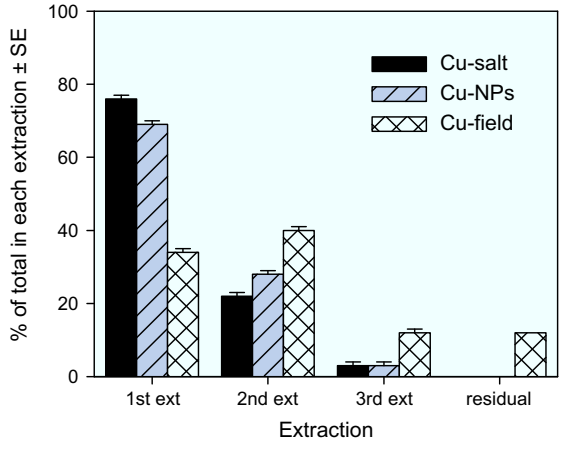

Fig. 2. Relative distribution between fractions from sequential extraction of copper from Hygum-soil contaminated with 2000 mg/kg of $Cu(NO_3)_2$ (Cu-salt), copper nanoparticles (Cu-NPs) and $CuSO_4$ historical contamination (Cu-field).

and 12% in the residual. Technically speaking, the 1st extraction targets exchangeable and acid soluble Cu, the 2nd extraction the reducible Cu, and the 3rd extraction targets the oxidisable Cu in the soil sample.

The chemical exposure analyses (3.2.1–3.2.3) showed a partial dissolution of the nano-form (XANES), and that for the $CuNO_3$ and the Cu-NPs exposure Cu was bound primarily in the exchangeable and acid soluble part (i.e. assumed to be "more easily" available), whereas a higher fraction of the Cu in the Cu-field exposure was bound in the reducible and oxidisable fraction of the soil (i.e. "more hardly" available). Since, the Cu^{2+} activity (ISE measures) was very low in all soils, is seem that virtually all Cu was bound to soil surfaces. The picture (see Fig. 3) seem to be that for $CuNO_3$ and Cu-NPs a large fraction of the Cu is bound "loosely" to soil surfaces, with Cu-NPs remaining on the particulate form, whereas for the Cu-field soil the Cu is incorporated into less extractable sites (see Fig. 4).

3.3. Effect characterization

All the tests fulfilled the biological validity-criteria as described in the OECD guideline, i.e. controls mortality <20%, number of juveniles >25 and coefficient of variation <50%. The soil pH was not significantly different between test conditions and did not significantly change during the test duration.

Survival was affected by Cu-salt and Cu-field treatments, but not by Cu-NPs (Table 1, Fig. 3); reproduction was affected by all Cu materials. All effect scenarios showed a continuous concentration–response relationship. The freshly spiked Cu-salt caused an LC_{50} of 595 mg Cu/kg (adult-survival) and an EC_{50} at 361 mg Cu/kg (reproductive output). The Cu-NPs caused an EC_{50} at 1760 mg Cu/kg (reproductive output), which was similar for the historically Cu-contaminated field soil (Cu-field) i.e. the EC_{50} was 2068 mg Cu/kg. For Cu-field adult survival was affected (LC_{50}) at 3508 mg Cu/kg (Table 1).

4. Discussion

This study showed that in a 21-day test the Cu toxicity to the soil organism *E. crypticus* depended on the Cu-form, with up to a sixfold difference in toxicity level among the three exposure regimes: $CuNO_3$ (most toxic), followed by Cu-nanoparticles (Cu-NPs) and Cu-field soil (Cu-field) exposure. The chemical exposure analyses show that for $CuNO_3$ and Cu-NPs a large fraction of the Cu is bound "loosely" to soil surfaces, with Cu-NPs remaining on the particulate form, whereas for the Cu-field soil the Cu is incorporated into less extractable sites. Hence, this sixfold toxicity difference observed between $CuNO_3$ exposure and the Cu-NPs and Cu-field, seemed to be partially due to a difference in available Cu

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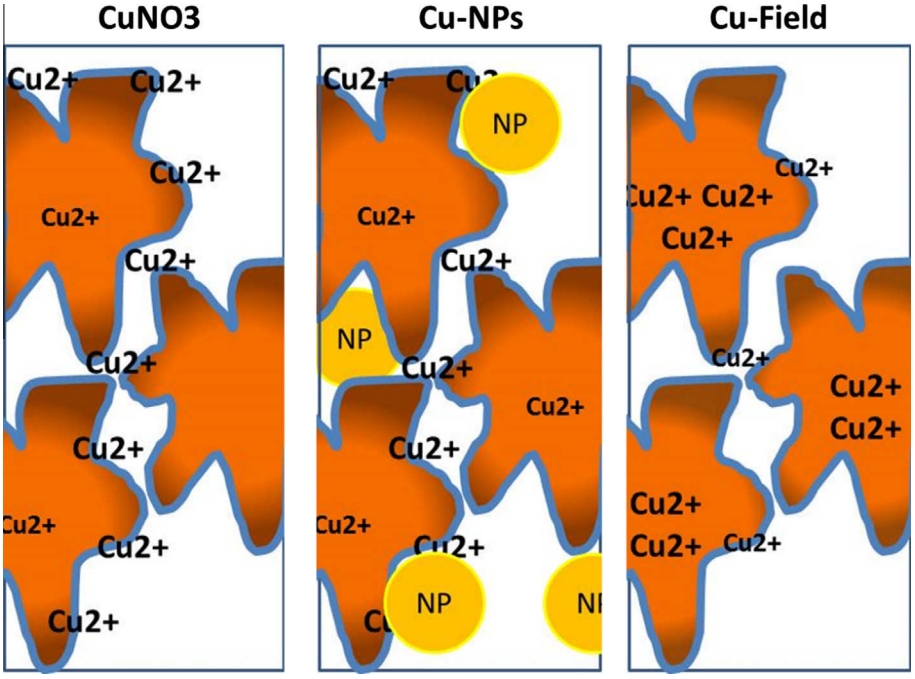

Fig. 3. Schematic overview of Cu distribution in the soil core for the three different Cu exposures ($CuNO_3$, Cu-NPs, Cu-field), based on X-ray absorption near edge structure, Sequential Extraction and Ion Selective Electrode results. Brown shapes represent soil particles, blue surrounding is the water biofilm. The "Cu^{2+}" on the surface of the same shapes illustrates the easily extractable Cu; "Cu^{2+}" in the centre of the soil particles illustrates the Cu that required stronger methods (reduced or oxidisable condition) for extraction. The relative presence of Cu^{2+} is also illustrated by the different amount/size of the added Cu^{2+}. For the Cu-NPs exposure Cu-NPs was remaining in the soil, illustrated by spherical shapes. No Cu was observed as free ions in solution. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

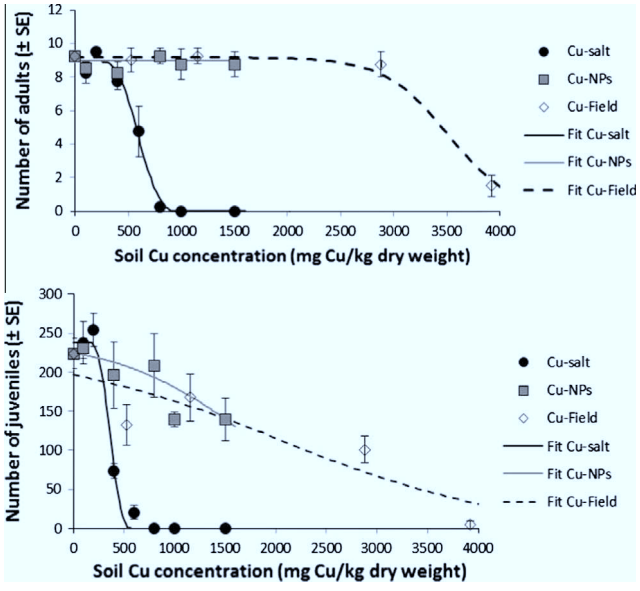

Fig. 4. Effects of copper nitrate (Cu-salt), copper nanoparticles (Cu-NPs) and historically Cu-salt contaminated field soil (Cu-field) on survival (up) and reproduction (down) on *Enchytraeus crypticus*. Results are expressed as average (AV) ± standard error (SE); $n = 4$. The fitted lines are the models (threshold sigmoid) used to estimate EC_x values, see Table 1.

combined with an only partial oxidation of the nanoparticles. The study also show that spiking soils with $CuNO_3$ does not provide an exposure scenario (based on chemical measures) similar to that of field contaminated soil, in the latter soil the long-term development of the soil incorporates the Cu to less extractable fractions.

The XANES observations indicated partial oxidation of the Cu-NPs. Since the Cu-NPs soil spectra resemble that of a non-soil 25 nm particle [more similar than the non-soil 80 nm spectra] the observations suggests that the NPs were present as smaller and more oxidized particles in the soil matrix. This is in agreement with Unrine et al. (2010), who observed partial oxidation of Cu-NPs (32 and 102 nm) in OECD soil. Within the Cu-NPs spiked soil, the XANES spectra shows approximately 42% Cu oxidation, i.e. 10% Cu(I) and 32% Cu(II). Considering that there was a sixfold difference in toxicity between Cu-salt and Cu-NPs exposure a 17% release would be enough for the toxicity in the Cu-NPs to be explained by released Cu^{2+}. Possible released ions must have been immediately bound in the soil matrix, since there was no measurable Cu-activity in the soil-solution (detection limit 10^{-9}, ion selective Cu-activity). However, since there was also no measurable Cu-activity when adding Cu-NPs to the modified-water (same ionic strength and pH as the soil-solution), the release of Cu from Cu-NPs seems limited despite oxidation of the particle surface. The sequential extraction showed that Cu from both Cu-NPs and Cu-salt are in relative loosely bound fractions (first extraction), which correspond to the higher toxicity in these soils compared to Cu-field soil. This first extraction probably covers Cu specifically sorbed on surfaces of clays, OM, Fe/Mn oxyhydrooxides and to some degree silicates (Gleyzes et al., 2002), whereas the next extraction covers Cu bound in Fe- and Mn-oxides. The Cu-NPs are a little less extractable in 1st extraction (5% difference) than Cu-salt indicating a little stronger binding of Cu-NPs in soil i.e. equivalent to a higher K_d. For the field contaminated soil, a larger fraction remained in the residual fraction (believed to be less bio-available) which corresponded to a lower toxicity in this soil. A concern with the sequential extraction procedure is that it could have in itself influenced Cu-NPs e.g. dissolving them causing the measurement to reflect free ions and not the particles behavior. The 1st extraction, a weak acid, probably did not increase dissolution since it is also used as a capping agent in NPs, e.g. Ag-NPs (see e.g. Deng et al., 2013), the reducing power of hydroxylammonium

chloride (2nd extraction) would also not dissolve the particles, but rather reduce Cu (I and II) to Cu(0) during the extraction process. The final extraction (3rd extraction) using hydrogen peroxide likely oxidizes the nanoparticles to Cu I or II. Hence, the first two extractions probably reflect the particle behavior, whereas the third reflected the free-ions. Given this, the exposure measures indicated that the enchytraeids in the Cu-NPs contaminated soil were partly exposed to Cu in the form of nanoparticles and less [in terms of total-concentration (weight)] to the released dissolved Cu^{2+} ions.

Hence, the chemical analyses supported that the Cu-salt and the Cu-field toxicity was related to the availability of Cu^{2+}, and that even the organisms ingest soils they do not extracted the harder bounded Cu fraction. For the Cu-NPs exposure the toxicity was probably due to both nanoparticles and Cu^{2+} toxicity, although released ions (ca. 20% released) may also explain toxicity. At the same time, and assuming that particles are causing the toxicity, then a slower uptake-rate could explain the observed toxicity differences. This would also fit with all the exposure measurements, i.e. primary oxidation state of 0 in the soil (with oxidation state I and II still associated with the particle), low Cu^{2+} activity in the soil-solution and a similar distribution in the sequential extraction (the toxicity could be expressed based on the first extraction which would overlap the differences). This is well in line with the organisms' uptake mechanisms, i.e. both adsorption of via the body surface and ingestion of soil particles occurs. Adsorption would occur from the water soil biofilm and mostly for the bioavailable ionic fraction, whereas soil ingestion would include both readily and less bioavailable fraction. Once inside the organisms' gut the soil bounded Cu-NPs can be released at a slower rate. Given this, Cu-NPs would simply be less toxic (than free ions) during a 3 week exposure, with the Cu-NPs toxicity becoming expressed over longer exposure time.

5. Conclusions

The three Cu-exposure scenarios used showed induced toxicity at different levels. The freshly spiked Cu-salt was the most toxic for reproductive output of the worms, followed by Cu-NPs and then Cu-field. XANES indicated only one oxidation state (II) in Cu-salt and Cu-field soil, whereas in Cu-NPs soil it was present in all oxidation states (0, I and II). The Cu-NPs became partially oxidized in soil, but only a limited dissolution occurred.

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Appendix A. Supplementary material

Supplementary data is available with calculations, derivations and additional graphs for XANES measurements (see relevance in main text). Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chemosphere.2015.06.045.

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