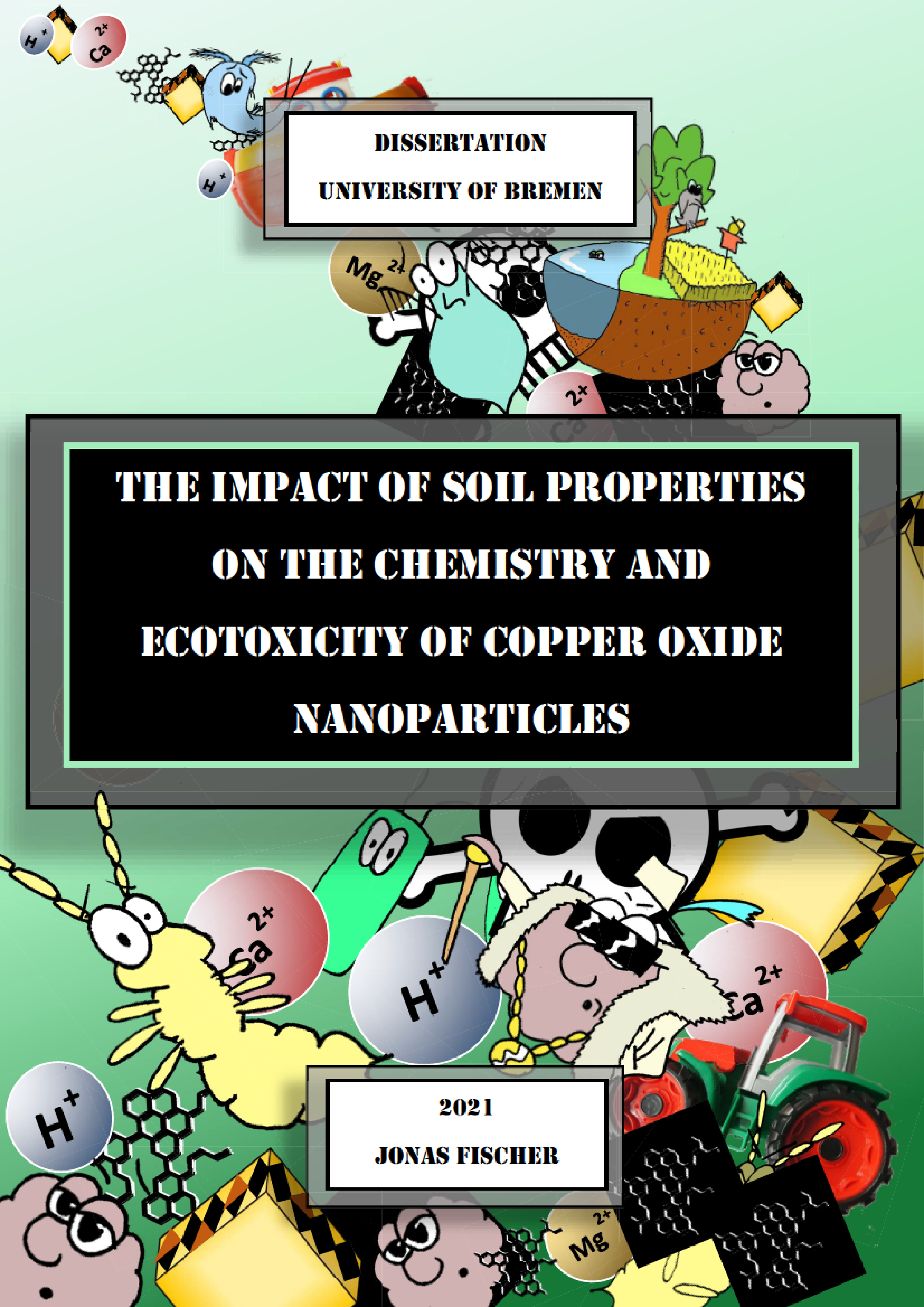

DISSERTATION

UNIVERSITY OF BREMEN

THE IMPACT OF SOIL PROPERTIES ON THE CHEMISTRY AND ECOTOXICITY OF COPPER OXIDE NANOPARTICLES

2021

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List of abbreviations

AAS: atomic adsorption spectroscopy

ASW: artificial seawater

CAT: catalase

CEC_{eff}: effective cation exchange capacity

CubNP: copper-based nanoparticles

CuO-NP: copper oxide nanoparticles

DLS: dynamic light scattering

ELS: electrophoretic light scattering

HA: humic acid

HDD: hydrodynamic diameter

IS: ionic strength

K: kaolin

M7: Elendt M7 medium

M: montmorillonite

MNP: metal-based nanoparticles

MQW: MilliQ water

MT: metallothionein

NNLS: non-negative least-square

NOM: natural organic matter

PDI: polydispersity index

ROS: reactive oxygen species

SDW: soil dry weight

SOM: soil organic matter

sp-ICP-MS: single particle inductively coupled mass spectroscopy

SPW: soil porewater

WHC_{max}: maximum water holding capacity

Summary

Copper is applied since decades in agriculture as fungicide and fertilizer, but can accumulate in top soils and cause negative effects to soil biota. Copper-based nanoparticles as ingredient could lead to more efficient applications and reduce the amount of applied copper, but at the same time raise novel risks towards the environment. The behaviour and the resulting risk of nanoparticles is strongly depending on their interaction with the environment.

In the present thesis, we studied the chemical behaviour and/or the resulting ecotoxicity of a model substance, uncoated copper oxide nanoparticles (CuO-NP, <50 nm), in different artificial test media (soil porewater, fresh- and seawater), field soils and artificial OECD soils. The impact of the media on size, surface characteristics and dissolution of CuO-NP under the influence of humic acid was studied. The size and dissolution of CuO-NP in the pore water of four field soils was also investigated, one sandy soil and three loamy soils of varying pH and organic content were used. The impact of CuO-NP as well as $CuCl_2$ on survival, reproduction and body weight of a model soil organism, the springtail *Folsomia candida,* was investigated in these field soils, but also in artificial OECD soils containing either kaolin or montmorillonite as representatives for the most common clay minerals in soils. For the OECD soils, also catalase activity, metallothionein level and moulting frequency of springtails were investigated. For all soils, mainly field-realistic Cu concentrations were applied.

In media of a high divalent cation fraction but low ionic strength, particle size and dissolution were remarkably higher, but the addition of humic acid roughly aligned these parameters for all media. The behaviour of CuO-NP in artificial media was clearly impacted by the interaction between humic acid, the ratio of dissolved ions of different valency and their competition for interaction sites on the NP surface.

The particle size in the pore water of field soils was independent of soil properties, but increased with increasing test concentrations. However, toxic effects occurred only in loamy soils and mainly at the lowest NP concentration of 5 mg Cu/kg, with reproduction and body

weight being reduced most by 61 or 28%, respectively, in a loamy-acidic soil. The effects were ascribed to the small size of CuO-NP at low concentrations providing more reactive sites and the higher content of clay minerals in loamy soils chemically interacting with CuO-NP, possibly supported by low soil pH and curbed by high SOM.

In artificial OECD soils containing montmorillonite and CuO-NP, survival, reproduction and body weight of springtails were reduced by up to 52, 33 and 48%, respectively, at soil concentrations of 1-3 mg Cu/kg, while at higher concentrations no negative effects occurred. Further, the catalase activity was depleted by up to 62% at 10 mg Cu/kg or lower. The metallothionein levels and the Cu content of exuviae shed by springtails was higher in these soils than for kaolin soils and/or $CuCl_2$ treated soils. We assume that CuO-NP-montmorillonite associations can provide and shuttle electrons for the formation of reactive oxygen species on the NP surface through Fe and Cu in a non-linear dose-response relationship. Furthermore, they may attach stronger to springtail gut cells and impact excretion mechanisms.

Overall, the thesis underlines the strong impact of environmental substances such as ion solutions, organic matter and clay minerals on the behaviour and toxicity of CuO-NP. In the soil environment, a negative impact on springtails can occur at field-realistic Cu concentrations in loamy soils, which are of high relevance for agriculture, containing Fe-rich reactive clays. Therefore, we suggest a focus on these soils and a denser testing at lower, field-realistic Cu concentrations for the risk assessment of agrochemicals containing Cu-based nanoparticles.

Zusammenfassung

Kupfer wird seit Dekaden in der Landwirtschaft als Fungizid und in Dünger verwendet, wobei es sich in Oberböden anlagern und Bodenorganismen beinträchtigen kann. Die Verwendung kupferbasierter Nanopartikel könnte die Effizienz von Kupferanwendungen erhöhen und dabei, jedoch mit bisher unbekannten Auswirkungen für Bodenökosysteme. Verhalten und resultierende Toxizität von Nanopartikeln hängen von deren Interaktion mit der Umwelt ab.

In der vorliegenden Dissertation wurde das chemische Verhalten und/oder die Ökotoxizität einer Modellsubstanz, unbeschichtete Kupferoxid-Nanopartikel (CuO-NP, <50 nm), in verschiedenen künstlichen Testmedien (Porenwasser aus Boden, Süß- und Salzwasser), Freilandböden und künstlichen OECD-Böden untersucht. Der Einfluss der Medien auf Größe, Oberflächeneigenschaften und Auflösung von CuO-NP unter dem Einfluss von Huminsäuren wurde erfasst. Ebenso wurde die Größe und Auflösung von CuO-NP im Porenwasser von vier Freilandböden untersucht, davon ein sandiger Boden und drei lehmige Böden mit unterschiedlichem pH und organischem Gehalt. Der Einfluss von CuO-NP und $CuCl_2$ auf Überleben, Reproduktion und Gewicht eines Modellbodenorganismus, dem Springschwanz *Folsomia candida*, wurde sowohl in diesen Böden als auch in OECD-Böden mit jeweils Kaolin oder Montmorillonit als Repräsentanten für die häufigsten Tonminerale in Böden untersucht. Für die OECD-Böden wurden zusätzlich auch Catalase-Aktivität, Metallothionein-Gehalt und Häutungsfrequenz der Springschwänze ermittelt. Für alle Böden wurden überwiegend umweltrelevante Kupferkonzentrationen appliziert.

In Medien mit hohem Anteil divalenter Kationen, aber geringer Ionenstärke, waren Partikelgröße und Auflösung deutlich erhöht, wurden jedoch durch Zugabe von Huminsäure in allen Medien angeglichen. Das Verhalten von CuO-NP in den Medien wurde stark von der Interaktion zwischen Huminsäuren, das Verhältnis gelöster Ionen unterschiedlicher Valenz sowie deren Konkurrenz um Bindungsstellen auf der NP-Oberfäche geprägt.

Die Partikelgröße im Porenwasser der Freilandböden wurde nicht durch die Bodeneigenschaften beeinflusst, nahm jedoch mit steigenden Testkonzentrationen zu. Toxische Effekte wurden nur in lehmigen Böden gefunden und dort v.a. in der niedrigsten NP-Konzentration von 5 mg Cu/kg, wobei Reproduktion und Gewicht am stärksten in einem lehmig-sauren Boden um 61 bzw. 28% reduziert wurden. Diese Effekte wurden durch die geringere Größe der CuO-NP bei niedrigen Konzentrationen und der daraus resultierenden erhöhten reaktiven Oberfläche erklärt sowie durch den höheren Gehalt an Tonmineralen in lehmigen Böden, die zur chemischen Interaktion mit CuO-NP zur Verfügung stehen und ggf. durch einen niedrigen Boden-pH unterstützt werden.

In künstlichen OECD-Böden mit Montmorillonit wurden durch Zugabe von CuO-NP Überleben, Reproduktion und Gewicht von Springschwänzen um jeweils bis zu 52, 33 und 48% reduziert. Dies geschah bei Bodenkonzentrationen von 1-3 mg Cu/kg, während bei höheren Konzentrationen keine negativen Effekte beobachtet wurden. Weiterhin wurde die Catalase-Aktivität um bis zu 62% bei 10 mg Cu/kg oder weniger gehemmt. Der Metallothionein-Spiegel und der Kupfergehalt der Springschwanz-Exuvien war in diesen Böden höher als in kaolinhaltigen und/oder mit $CuCl_2$ behandelten Böden. Es wird vermutet, dass CuO-NP-Montmorillonit-Konglomerate Elektronen für die Bildung von reaktiven Sauerstoffspezies and der NP-Oberfläche durch Kupfer und Eisen bereitstellen und transportieren können, und zwar in einer nicht-linearen Dosis-Wirkungsbeziehung. Zudem haften sie möglicherweise stärker an Mitteldarmzellen von Springschwänzen.

Zusammenfassend unterstreicht diese Dissertation den starken Einfluss von Umweltsubstanzen wie Ionenlösungen, organischer Substanz und Tonmineralen auf das Verhalten und die Toxizität von CuO-NP. In Böden können negative Effekte gegenüber Springschwänzen bei umweltrelevanten Kupferkonzentrationen auftreten, v.a. in lehmigen Böden, die besonders relevant für die Landwirtschaft sind, mit Fe-haltigen reaktiven Tonen. Daher schlagen wir einen Fokus auf diese Böden und ein verstärktes Testen in niedrigeren, umweltrelevanten Konzentrationen für die Risikoabschätzung von Agrochemikalien mit kupferbasierten Nanopartikeln vor.

- Chapter 1 -

General Introduction

Agricultural application of copper and copper-based nanomaterials

Copper-based compounds have been used for decades in agriculture to protect crops against fungal and bacterial diseases (Komárek et al., 2010; Lamichhane et al., 2018). In the EU, 158,000 tons of fungicides per year are sold, the biggest fraction of it being copper-based and mostly used in vineyards and horticulture (Panagos et al., 2018). Copper has the tendency to accumulate in surface soil layers, where it can have negative impact on soil biota such as microorganisms (Fernández-Calviño et al., 2010; Vázquez-Blanco et al., 2020; Wang et al., 2009), plants (Borkert et al., 1998) or macro- and mesofauna (Amorim et al., 2005; Caetano et al., 2016; Neves et al., 2019). Therefore, the use of copper in agriculture is caught between crop yield and direct economic benefit for farmers on the one hand and environmental protection and sustainable soil use on the other hand. For this reason, most industrial countries prescribe a strict legislation for the use of copper in agriculture. As an example, in the European Union the copper application is restricted to 4 kg/ha/year on average (The European Commission, 2018). However, the periodic application of copper still can lead to a remarkable accumulation of copper in agricultural top soils, due to the low vertical mobility of copper (Blotevogel et al., 2018), with the mentioned consequences for the environment. Therefore, the reduction of copper application rates in accordance with the needs of farmers is an ongoing challenge in agricultural research.

Currently, the use of nanomaterials seems promising for increasing the efficiency of agricultural copper applications (Adisa et al., 2019; Zhao et al., 2020). NM can be defined as materials containing particles, where at least one dimension is in the size range of 1 – 100 nm (Bleeker et al., 2013). Besides carbon-based structures, the use of metal and metal oxide nanoparticles (MNP) has rapidly developed within the last two decades (Kumar et al., 2021; Paladini et al., 2015). The ultrafine structuring of these substances leads to a very high surface-to-volume ratio surpassing their bulk counterparts in properties like reactivity or conductivity (Das et al., 2014; Gentile et al., 2016; Wang et al., 2019).

Therefore, copper-based nanoparticles (CubNP) are used in many applications such as optics, electronics or catalysts (Mohsen et al., 2017; Rubilar et al., 2013). With respect to the main applications of copper in agriculture, CubNP are currently developed and even applied in the field as ingredients in pesticides (Keller et al., 2017; Peixoto et al., 2021; Zhang et al., 2020) and fertilizers (Dimkpa & Bindraban, 2018; Wang et al., 2019). These materials show a number of benefits in comparison to conventional copper formulations, such as a continuous ion release (Cioffi et al., 2005), a higher toxicity towards target organisms (Kanhed et al., 2014) or a reduced wash-off from target crops (Kah et al., 2019). Furthermore, MNP offer the possibility for designing them to preferable properties by forming their size and shape (Gilbertson et al., 2016), by coating (Baumann et al., 2014) or doping them (Naatz et al., 2017); in principal, these designing tools can also be used to lower negative effects towards non-target organisms (Figueiredo et al., 2019). However, the chemical legislations in Europe are not yet prepared to account for nano-specific effects (Gottardo et al., 2021), so that impacts on the environment for these substances have not been investigated systematically.

Soil interactions of nanomaterials

The unique properties of nanomaterials lead to the previously mentioned benefits, but in turn, may also lead to unforeseen and unwanted negative effects. With respect to the soil environment, this can be understood as a high persistence, accumulation and negative impacts on soil biota. Most of the environmentally relevant reactions, i.e. ROS formation (Ali et al., 2019; Lops et al., 2019), catalysis (Ding et al., 2018; Skúlason et al., 2014), metal ion release (Hang et al., 2018; Liu and Hurt, 2010) and agglomeration (Conway et al., 2015; Ortelli et al., 2017) are surface related. MNP and their surfaces strongly react and interact with the soil environment, i.e. the soil matrix consisting out of sand, silt and fine clay particles, soil organic matter (SOM), the soil solution of a certain pH and ion composition (Jung et al., 2014; Tang and Cheng, 2018). Also, ions and SOM adsorb to the mineral surface of clays (Kaiser and Guggenberger, 2003), while at the same time, clay minerals disperse and SOM dissolves in the soil solution (Dexter et al., 2011); all these processes are impacted by the soil pH and, in turn, have a strong impact on the soil pH (see also Fig. 1). Therefore, the equilibrated milieu of each soil is unique, so that the behavior of MNP and their impact on organisms in the environment, but especially in soils, is hard to predict.

To reduce this complexity, it is widely established in soil ecotoxicology to use one single test soil in bioassays which is considered to represent the “worst-case-scenario”, mostly sandy soils like Lufa 2.2 or the artificial OECD soil (van Gestel, 2012). There are three good reasons for this approach: first, a widely harmonised use of the same test soils allows a good comparison of research results from different laboratories. Second, in many test setups, the interaction of test chemicals with specific soil compartments is of secondary interests, especially when complex ecological relations such as multispecies or multigenerational processes are investigated (e.g Bicho et al., 2017; Mendes et al., 2018). Third, both mentioned standard test soils are characterized by a low absorption capacity and, therefore, a potentially high availability of chemicals for organisms, so that they are justifiably considered as “worst-case-scenario”-soils.

However, there is some evidence that the latter argument cannot be asserted for the substance group of MNP. While conventional metals and metal oxides are steadily adsorbed and made unavailable by SOM and clay minerals, this pattern is not that obvious for MNP: on the one hand, analogously to metal ions, the presence of environmental adsorbants, i.e. organic matter (OM) or clay minerals, reduces their toxicity. This reduction occurs through the coating of reactive surfaces, the absorption of released toxic ions (Pradhan et al., 2016; Son et al., 2015; Thit et al., 2017), or through the destabilization and consequent agglomeration and sedimentation of MNP (Kansara et al., 2019; Zhou et al., 2012). On the other hand, OM and clay minerals can also increase the toxicity of MNP by increasing dissolution of toxic metal ions (Bagchi et al., 2013; Liu et al., 2020; Zhao et al., 2017), supporting ROS formation (Fathi et al., 2020) or modulating their uptake through and their toxicity to organisms (Das et al., 2014; Gupta et al., 2017; Wang et al., 2011; Zhu et al., 2020). In a study by Neves et al. (2019), a sand soil increased the toxicity of conventional copper pesticides compared to a sandy loam of higher clay content, while for CubNP, it was vice versa. Therefore, a focus on single test soils with low content of environmental adsorbants may overlook potential settings with a higher risk to soil biota which represent the real “worst-case-scenario”.

Copper soil concentrations

Another aspect where systematic overseeing of possible risks of MNP can occur is the choice of test concentrations. This choice is usually done with respect to expectable environmental concentrations as well as to assure observable effects in laboratory studies. While long-term copper applications can lead to enormous copper concentrations in top soils of >1000 mg/kg (Lamichhane et al., 2018), long-term aging in soils allows strong sorption towards the soil matrix, accordingly reducing toxicity (Lock and Janssen, 2003; McGrath et al., 2009; Pedersen and van Gestel, 2001). Therefore, simulating single or multiple exposure within one year in lab experiments may be more adequate than orientation on measured environmental concentrations, as done by Peixoto et al. (2021) for a CubNP containing nanofungicide. MNP behaviour in the environment is strongly concentration-driven: at higher NP concentrations, agglomeration rate increases, while dissolution decreases (Merrifield et al., 2017; Velicogna et al., 2021). Both processes lead to lower toxicity of MNP by reducing reactive surfaces (Schmid and Stoeger, 2016) and the release of toxic metal ions, which counteracts the toxicity merely caused by concentration increase. This may also explain why MNP toxicity in soils, where heteroaggregation and absorption of metal ions is much more likely than in aquatic environments, often does not occur at very high concentrations of 1 g/kg or even far above (Heckmann et al., 2011; Noordhoek et al., 2018; Pereira et al., 2011). All the more important it seems to also cover lower concentration ranges to (i) screen them for possible negative effects due to a non-linear dose-response relationship and (ii) give a clear statement on the behaviour of MNP at field-realistic concentrations.

The toxicity of copper towards the springtail *Folsomia candida*

As mentioned above, copper can have a high negative impact on soil biota. In our ecotoxicological studies we used the standard test organism *Folsomia candida* which is a widely distributed representative of the subphylum springtails (Collembola). Springtails are highly abundant in agricultural systems (Menta et al., 2020) and of great importance for carbon and nitrogen cycling in soils by breaking down organic matter (Filser 2002; Lakshmi et al., 2020); therefore, they play a central role in maintaining soil health and quality. *F. candida* are strongly associated to the pore water which is their main uptake route for toxicants (Fountain and Hopkin, 2005), rather than their body surface which is of a highly anti-adhesive surface structure (Helbig et al., 2011). Therefore, pore water analytics play a central role in assessing copper toxicity towards springtails (Lock and Janssen, 2003; Noordhoek et al., 2018; Pedersen et al., 1997). The sensitivity of *F. candida* towards copper salts has been investigated in many studies, and some showed that they were less sensitive than other soil invertebrates such as enchytraeids (Amorim et al., 2005; Mendes et al., 2018). One reason might be that some species of springtails are able to cope with copper contamination by internal regulation (Pedersen et al., 2000), so that contaminated sites can even provide beneficial conditions to springtails (Filser et al., 2000; Pfeffer et al., 2010).

Summarizing the data from Amorim et al. (2005), Criel et al. (2008). Mendes et al. (2018) and Neves et al. (2019), EC_{50} values of copper substances for the reproduction of *F. candida* are widely spread in a range from 45 to 3030 mg Cu/kg with a median of 532 mg Cu/kg. In these studies, toxicity strongly depended on soil properties and copper formulation. These data on the one hand state the potentially high resistance of *F. candida* towards copper contamination, and on the other hand suggest a potential environmental risk towards this species in certain soils as the lower EC_{50} values are close and comparable to copper amounts that are freshly applied in the field within one season (Peixoto et al., 2021). This underlines the necessity to include multiple soils for the risk assessment of copper substances towards *F. candida.*

Structure and aim of the thesis

The overall aim of the present thesis is to link the physico-chemical properties of soils to the toxicity of copper oxide nanoparticles (CuO-NP) towards the springtail *F. candida* at field-realistic test concentrations. In the following, the impact of soil properties on the colloidal and dissolution behaviour (Chapters 2 & 3) and the toxicity of CuO-NP towards the soil model organism *Folsomia candida* (Chapters 3 & 4) is studied. The soil properties of interest are the composition of the soil solution (Chapter 2), SOM (chapter 2 & 3), clay content (Chapter 3) and clay type present in soils (Chapter 4; see also Fig. 1).

In Chapter 2, surface interactions of CuO-NP with humic acid (HA) and differing artificial media are presented, with respect to colloidal stability, dissolution and copper speciation on the surface of CuO-NP. The media used here are representative for freshwater, seawater and soil pore water, differing in ionic strength and ion composition with respect to cation valency.

Chapter 3 aims to link the physico-chemical behaviour of CuO-NP in different field soils varying in clay content, pH and SOM content to ecotoxicological responses of a model soil organism, the springtail *Folsomia candida*. The parameters to be linked are, on the one side, the diffusion of copper into pore water, as well as its dissolution and agglomeration there, and on the other side, the impact on survival, reproduction and growth of *F. candida* as well as their bioaccumulation of copper under varying soil properties.

The insights gained there are extended to Chapter 4, where a focus on single clay types and their contribution to the toxicity development of CuO-NP in soil is studied. To apply single clay

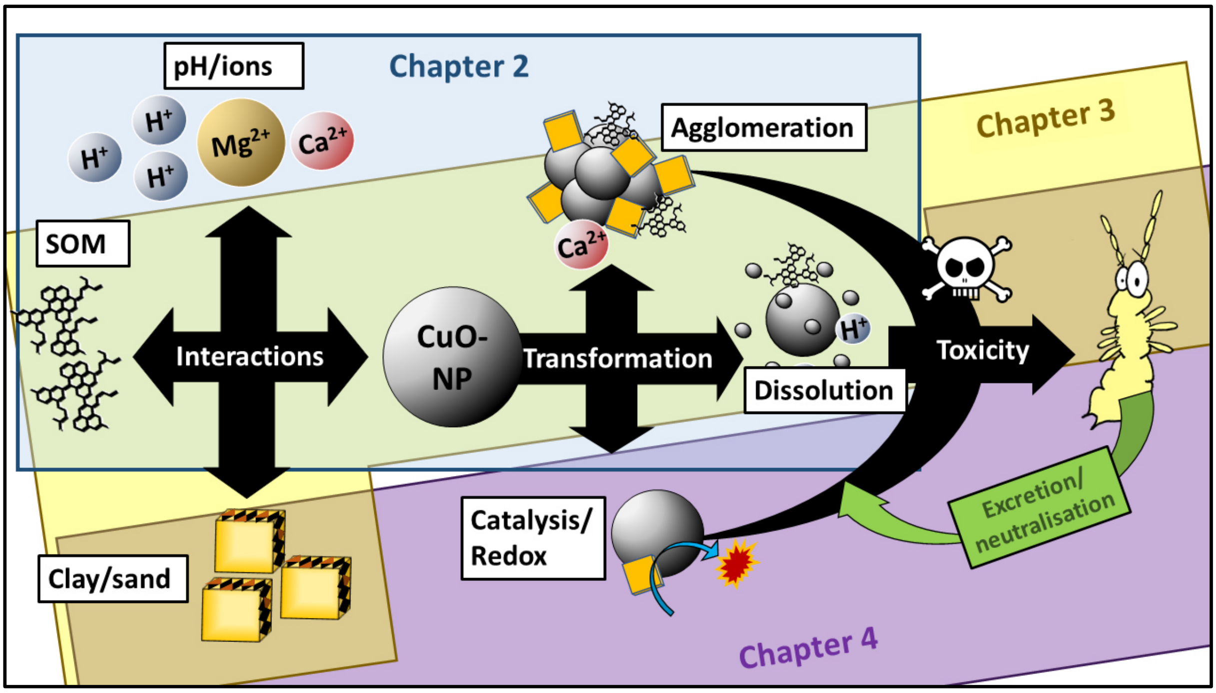

Fig. 1: Interactions of CuO-NP with substance groups in the soil environment, the resulting transformation and toxicity towards *Folsomia candida* (+ its defence mechanisms), and in which chapters of the thesis these coherences are studied

types, artificial OECD soils with varying proportion and content of the clay fraction were used. Besides the biological parameters previously tested in Chapter 3, also defense mechanisms on the molecular and behavioral level of *F. candida* against copper contamination were investigated; these mechanisms included the antioxidant defense represented by the activity of the enzyme catalase, the formation of HM binding proteins, metallothioneins, and an important excretion mechanism, the moulting frequency.

While in Chapter 2 a copper concentration allowing stable colloidal measurements was chosen, all soil bioassays in Chapters 3 and 4 were conducted with respect to environmental relevance, i.e. in the range of 1 to 158 mg Cu/kg (see also argumentation in Chapter 3). Consequently, the lowest test concentrations were in the range of a seasonal increase in soil copper concentrations in the top soil layer by copper application, as calculated in Chapter 3 and by Peixoto et al. (2021). To ensure comparability with the published literature, the higher concentrations slightly overlap with the concentrations of CuO-NP already tested on soil invertebrates.

- *Chapter* 2 -

The chemistry of CuO-NP in artificial media

The chapter is based on the publication in *Science of The Total Environment* (2021), *785*, 147241:

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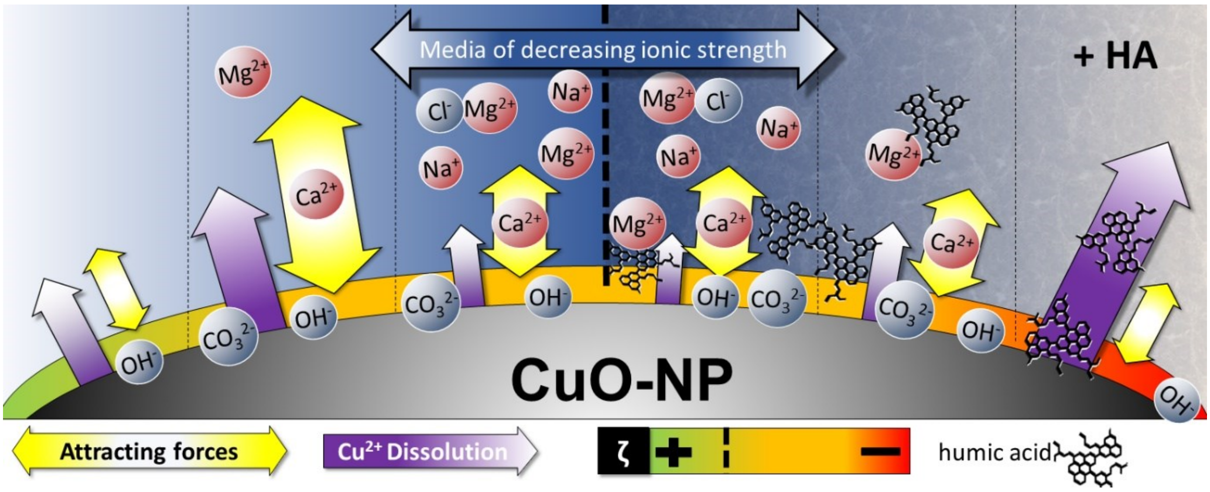
Ion compositions in artificial media control the impact of humic acid on colloidal behaviour, dissolution and speciation of CuO-NP

Jonas Fischer[1], Tonya Gräf[1], Yvonne Sakka, Christian Tessarek and Jan Köser



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[1] The authors have equally contributed to the manuscript

Abstract

The toxicity of copper oxide nanoparticles (CuO-NP) strongly depends on their interactions with the surrounding environment, impacting their dissolution and colloidal stability. This behaviour is studied quite extensively for simplified electrolytes, but information on the behaviour of CuO-NP in more complex artificial media are lacking.

In our study, we analysed the colloidal behaviour and considered the speciation of CuO-NP in pure water and three artificial media of different complexity which are used in ecotoxicology. Measurements were done over 7 days in the absence and presence of humic acid (HA) as a model organic molecule.

In pure water, the addition of HA lowered the zeta potential from +11 to -41 mV, while in all artificial media, it stayed constantly at about -20 mV. The hydrodynamic diameter of CuO-NP remained unaffected by HA in pure water and seawater, while in porewater and especially in freshwater, HA suppressed strong agglomeration. In pure water, HA strongly increased dissolution to the highest observed value (3% of total Cu), while HA reduced dissolution in all artificial media. Speciation calculations revealed that cations from the media competed with Cu from the NP surface for complexing sites of the HA.

This competition may have caused the reduced dissolution in the presence of ions. Furthermore, speciation calculations also suggest that ion composition drove agglomeration behaviour rather than ion concentration: agglomeration was high when divalent cations where the major interaction partner and dominant in relative terms. HA may have reduced the relative dominance and thus altered the agglomeration, aligning it in all media. Summarizing, ion composition and the presence of HA strongly drive the dissolution and agglomeration of CuO-NP in artificial media, consequently, analysing complexation can help to predict environmental behaviour and toxicity

CRediT author statement

Jonas Fischer and **Tonya Gräf** have equally contributed to the manuscript. **Jonas Fischer:** Conceptualization, Formal analysis, Investigation, Writing – Original Draft, Review & Editing, Visualization **Tonya Gräf:** Conceptualization, Investigation, Writing – Original Draft, Review & Editing, Project administration **Yvonne Sakka:** Conceptualization, Investigation, Writing – Original Draft, Review & Editing **Christian Tessarek:** Investigation, Visualization **Jan Köser:** Formal analysis, Writing – Original Draft, Review & Editing, Visualization

- *Chapter* 3 -

The chemistry and ecotoxicity of CuO-NP in field soils

The chapter is based on the publication in *Environmental Pollution* (2021), *270*, 116084:

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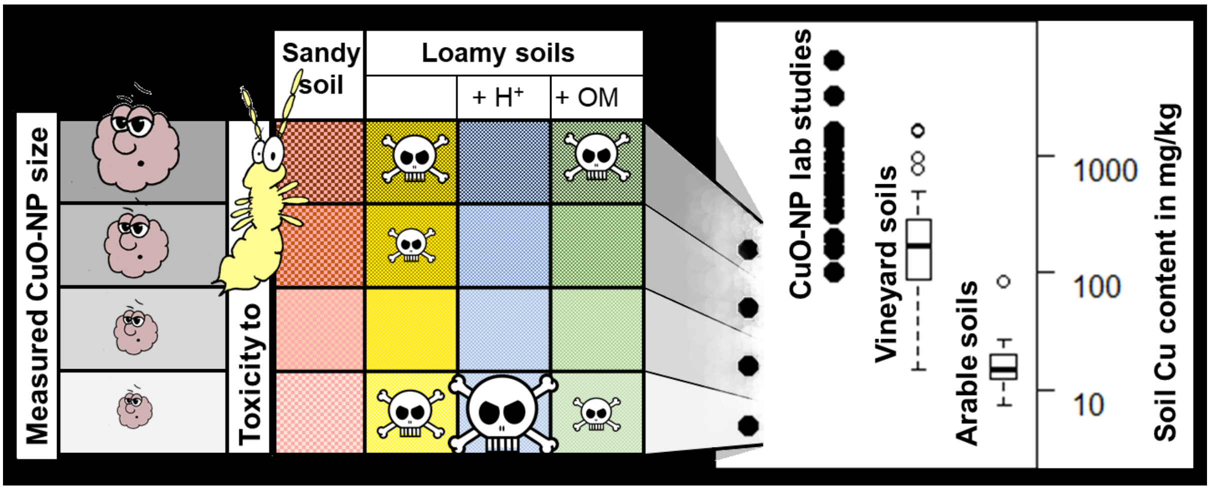
Soil properties can evoke toxicity of copper oxide nanoparticles towards springtails at low concentrations

Jonas Fischer, Anna Evlanova, Allan Philippe and Juliane Filser



ABSTRACT

Copper oxide nanoparticles (CuO-NP) are used as an efficient alternative to conventional Cu in agriculture and might end up in soils. They show a high toxicity towards cells and microorganisms, but only low toxicity towards soil invertebrates. However, most existing soil ecotoxicological studies were conducted in a sandy reference soil and at test concentrations ≥100 mg Cu/kg soil. Therefore, there is a knowledge gap concerning the effect of soil texture on the toxicity of CuO-NP at lower, more realistic test concentrations. In our study, a sandy reference soil and three loamy soils were spiked with CuO-NP at up to four concentrations, ranging from 5 to 158 mg Cu/kg. We investigated 28-day reproduction as well as weight and Cu content after 14-day bioaccumulation and subsequent 14-day elimination for the springtail *Folsomia candida*. For the first time we analyzed the size distribution of CuO-NP in aqueous test soil extracts by single particle-ICP-MS which revealed that the diameter of CuO-NP significantly increased with increasing concentration, but did not vary between test soils. Negative effects on reproduction were only observed in loamy soils, most pronounced in a loamy-acidic soil (-61%), and they were always strongest at the lowest test concentration. The observed effects were much stronger than reported by other studies performed with sandy soils and higher CuO-NP concentrations. In the same soil and concentration, a moderate impact on growth (-28%) was observed, while Cu elimination from springtails was inhibited. Rather than Cu body concentration, the diameter of the CuO-NP taken up, as well as NP-clay interactions might play a crucial role regarding their toxicity. Our study reports for the first time toxic effects of CuO-NP towards a soil invertebrate at a low, realistic concentration range. The results strongly suggest including lower test concentrations and a range of soil types in nanotoxicity testing.

CRediT author statement

Jonas Fischer: Conceptualization, methodology, investigation, Data curation, writing – original draft, visualization **Anna Evlanova:** Methodology, investigation, data curation **Allan Philippe:** Investigation, methodology, writing – review & editing **Juliane Filser:** Conceptualization, writing – review & editing, supervision

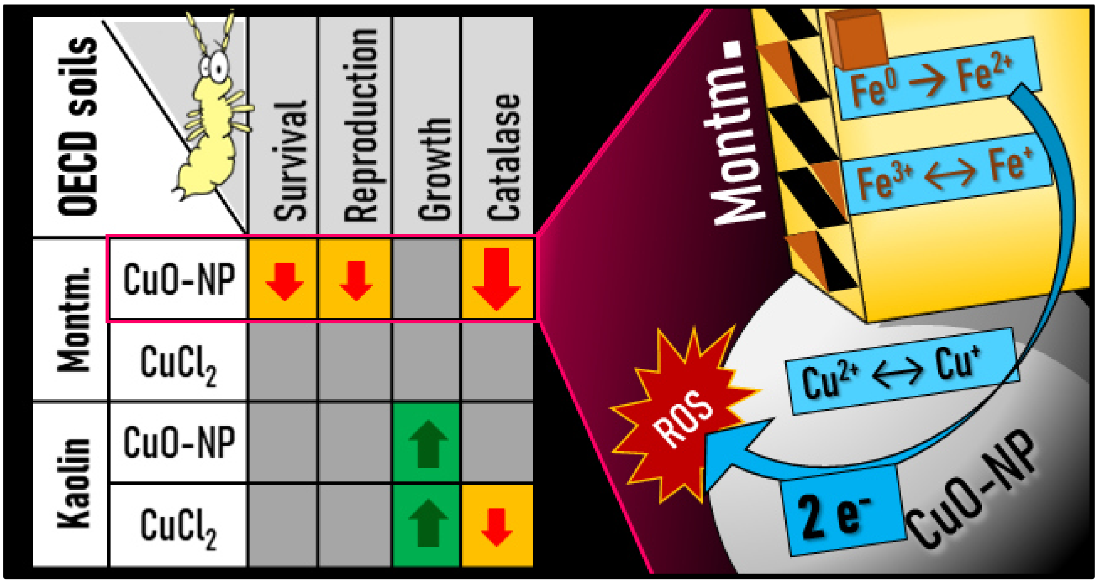
- *Chapter* 4 -

The ecotoxicity of CuO-NP in artificial test soils

The chapter is based on the manuscript submitted to *Environmental Science & Technology:*

Clay types modulate the toxicity of low concentrated copper oxide nanoparticles towards springtails in artificial test soils

Jonas Fischer, Ghanem D. A. Talal, Laura S. Schnee, Patricks V. Otomo and Juliane Filser



OECD soils		Survival	Reproduction	Growth	Catalase
Montm.	CuO-NP	↓	↓		↓
	$CuCl_2$				
Kaolin	CuO-NP			↑	
	$CuCl_2$			↑	↓

ABSTRACT

Copper oxide nanoparticles (CuO-NP) are currently applied as efficient alternative to conventional Cu in agriculture. We recently found negative effects of CuO-NP on the springtail *Folsomia candida* at field-realistic concentrations, but only in clay-rich loamy soils. In the current study, we produced artificial OECD soils containing 19% or 30% of kaolin or montmorillonite as clay type and spiked them with uncoated CuO-NP or $CuCl_2$. We performed 28-day reproduction tests and recorded survival, dry weight and Cu content of adults. In a second experiment, moulting frequency, metallothionein levels and catalase activity were investigated. Negative effects were mostly observed in 30% montmorillonite soil for CuO-NP (e.g. reproduction -33%, survival -52% and catalase activity -62% at 3, 1 and 10 mg Cu/kg, respectively), but in kaolin soils, Cu increased dry weight. Metallothionein levels and Cu content of exuviae were higher for CuO-NP than for $CuCl_2$, but lower than for the control. The reduced catalase activity may result from strong ROS formation overloading the antioxidant system, with negative consequences for reproduction and survival. The results underline that clay-CuO-NP interactions are striking for their toxic behaviour, especially at at low, field-realistic concentrations, which should be considered for risk assessment of CuO-NP.

Introduction

Copper-based nanoparticles are already in use as efficient alternative to conventional Cu formulations in agriculture, with two main manners of application: as Cu source in fertilizers (Adisa et al., 2019; Dimkpa and Bindraban, 2018) or as bactericides or fungicides (Kah et al., 2019; Keller et al., 2017; Peixoto et al., 2021; Su et al., 2020). While not being directly applied in the field, copper oxide nanoparticles (CuO-NP) are widely tested as model substance with respect to their toxicity to plants (Gao et al., 2018; McManus et al., 2018; Stewart et al., 2015), freshwater organisms (Heinlaan et al., 2008; Janani et al., 2020; Manusadžianas et al., 2012), marine organisms (Buffet et al., 2011; T. Gomes et al., 2011; Pang et al., 2013), soil organisms (Bicho et al., 2017; Mendes et al., 2018; Noordhoek et al., 2018) and microorganisms (Dimkpa et al., 2011; Khalid et al., 2021; Kim et al., 2013), also reviewed by Hou et al. (2017). In these studies, toxic effects tended to be lowest in soils, probably due to the binding capacity of the soil matrix for Cu (Jarvis, 1981; Wu et al., 1999) .

Recently we showed that CuO-NP can be toxic to soil invertebrates at field-realistic concentrations in clay-rich loamy soils; toxic effects occurred even at much lower Cu concentrations in soil pore water and body tissue compared to sandy soils, indicating a crucial interaction of CuO-NP with clay minerals (Fischer et al., 2021a). In the same study, we pointed out that most studies reporting no or only slight toxic effects of CuO-NP on soil invertebrates were conducted in sandy standard soils, where the lack of effects could be due to the lower availability of clay minerals for CuO-NP interactions.

The composition of a soil clay fraction is highly diverse, but the components can roughly be classified in two- and three-layer clay minerals. The latter have the ability to widen the distance between their mineral layers by incorporating water, resulting in a higher specific surface area and cation exchange capacity (CEC). Because of these properties, a high clay content in soils is expected to decrease the bioavailability and toxicity of heavy metals in soils, as it allows them to strongly adsorb heavy metals and metal-based nanoparticles (MNP) (Abbas et al., 2020; Golia et al., 2008; Lanno et al., 2019; Sungur et al., 2014). However, Cu and clays can also form highly reactive associations (Kalidhasan et al., 2017; Khanikar and Bhattacharyya, 2013), with strong toxicity to microbial cells (Bagchi et al., 2013; Das et al., 2014; Hundáková et al., 2013; Malachová et al., 2011; Pourabolghasem et al., 2016; Sohrabnezhad et al., 2014) and, as shown for some MNP, to protozoans (Gupta et al., 2017) and zebrafish (Gupta et al., 2016). In all of these studies, three-layer clay minerals, mainly montmorillonite, were used.

Springtails (Collembola) have multiple ways to react towards HM contamination in soils. In the case of Cu, its role as essential element has to be considered as this characteristic strongly drives HM kinetics in soil invertebrates (Ardestani et al., 2014). As an example, small amounts of Cu even can be beneficial towards springtails (Pfeffer et al., 2010). However, increasing soil Cu concentrations cause an upregulation of Cu excretion mechanisms in an attempt to maintain a bearable Cu body content (Fountain and Hopkin, 2001; Pedersen et al., 2000; van Straalen et al., 1987). One of these mechanisms is moulting which enables the removal of exuviae but also of the midgut epithelia where HM can be bound for detoxification (Fountain and Hopkin, 2001; van Straalen and Roelofs, 2005). This is often accompanied by and linked with the upregulation of HM-absorbing proteins, metallothioneins (MT) (Maria et al., 2014; Nota et al., 2011; Xiong et al., 2014). However, detoxification mechanisms are energy-consuming processes (Jager et al., 2013) which may reduce Cu body concentration, but also impact reproduction and growth (Bednarska et al., 2013). This may explain previous observations where toxic effects of CuO-NP occurred with low Cu body concentrations of springtails (Fischer et al., 2021a).

Besides excretion, antioxidative enzymes such as catalase (CAT) can be activated as defense mechanism against oxidative stress induced by Cu salts (Maria et al., 2014; Xiong et al., 2014) and nanosized Cu in the soil (Gautam et al., 2018; S. I. Gomes et al., 2011). CuO-NP are retained in the epithelial gut cells of invertebrates (Heinlaan et al., 2011; Velicogna et al., 2021) and are able to induce the formation of reactive oxygen species (ROS) directly in these cells (Baeg et al., 2018), which can be broken down by the catalase enzyme.

In the present study, we exposed the standard test organism *Folsomia candida* to CuO-NP and $CuCl_2$ in OECD soils either containing kaolin or montmorillonite as clay fraction. Both clays are either representative for two- or three-layer clay minerals and are widely used, e.g. kaolin in OECD soil and Montmorillonite K10 in different catalytic (Kalidhasan et al., 2017; Khanikar and Bhattacharyya, 2013) and ecotoxicological studies (Gupta et al., 2017, 2016; Kansara et al., 2019). We compared the impact of the soils on the toxicity of the Cu substances to the survival, reproduction, growth, moulting and biomarker responses of *Folsomia candida*. We hypothesized that montmorillonite soils would affect springtails more strongly by forming more reactive Cu-clay associations than kaolin soils.

Materials & methods

Test soils

Artificial OECD test soils differing in clay content and composition were produced according to OECD Guideline 232 (OECD, 2009) with some modificiations. C_{org}, pH and clay content were aligned to previously tested loamy field soils (Refesol 05-G; Fischer et al. (2021a). The soils contained two different clay fractions consisting either of kaolin (Erbslöh Lohrheim GmbH & Co. KG, Lohrheim, Germany) as suggested by OECD (2009) or chemically activated Montmorillonite K10 (CAS-No. 1318-93-0, Sigma-Aldrich, St. Louis, USA). Soils were mixed at two different levels of clay with 19% and 30% of the total solid fraction, ending up with four test soils in total (Table 4).

Table 1: Properties of test soils. C_{org} = organic carbon content, WHC = maximum water holding capacity. MMT = montmorillonite K10

Test soil	Clay fraction	Sand in mass %	Clay in mass %	C_{org} in mass %	pH ($CaCl_2$)	WHC In g/kg	Cu in mg/kg	Fe in g/kg
K19	Kaolin	81	19	1.59	5.15	304	3.4	1.45
K30	Kaolin	70	30	1.59	5.17	360	6.4	1.45
M19	MMT	81	19	1.59	5.10	410	0.31	5.57
M30	MMT	70	30	1.59	5.16	457	0.36	6.96

Peat (Einheitserdewerke Patzer GmbH & Co. KG, Sinntal, Germany) was air-dried and finely ground with a blender to achieve maximum-possible homogenous distribution of C_{org} in the soils. The pH of the test soils was measured (inoLab pH Level 1, WTW, Weilheim, Germany) in $CaCl_2$ and adjusted with $CaCO_3$ to a pH of 5.2±0.1 and the water holding capacity (WHC) of freshly mixed soils was investigated. The Cu content of the test soils was analysed by graphite furnace atomic adsorption spectroscopy (AAS, see 2.4) after acid extraction with 65%

HNO_3 for 2h at 80 °C (Fischer et al., 2021a; Pedersen and van Gestel, 2001) and the Fe content by energy dispersive X-ray fluorescence (ED-XRF) spectroscopy using a PANalytical Epsilon 3-XL benchtop ED-XRF spectrometer (Malvern Instruments, Malvern, UK; Wien et al. (2005)).

Test substance and soil spiking

CuO-NP were purchased from Sigma-Aldrich (St. Louis, USA) with a particle diameter of <50 nm and a surface area of 29 m^2/g (supplier information). In a former study, the hydrodynamic diameter (NNLS) and zeta potential between 0 - 7 days in miliQ water (MQW) were found to be 500 - 1000 nm and -8 to -11 mV (Fischer et al., 2021b). For each test, a fresh stock dispersion of CuO-NP in MQW at a concentration of 300 - 600 mg Cu/L was prepared and ultrasonicated for 30 min at 35 kHz (Sonorex RE100H, BANDELIN electronic GmbH & Co KG; Berlin, Germany) directly before application. As a copper salt control, $CuCl_2*2H_2O$ (Merck KGaA, Darmstadt, Germany) was used and a stock solution of 1.000 mg/L was used for all assays. The respective stock solutions and dispersions were diluted in MQW to the required concentrations to achieve soil Cu concentrations of 1, 3, 10 and 32 mg/kg soil dry weight above the background and directly pipetted on and stirred into the soil until homogenous distribution. Soil moistened with MQW served as negative control. The water content of all soils was set to 50% of their WHC. Soils were stored at 17 °C in the dark for 2-3 days and water content was adjusted directly before use in the bioassays.

Bioassays

All *F. candida* adults originate from our laboratory cultures which are maintained on charcoal-plaster plates at 15 °C in the dark. Synchronisations were conducted at room temperature. In all bioassays, juveniles were 10 - 12 days old at the start of the test. All test vessels were stored at 20 °C under a 16:8h light:dark cycle during the test and provided with a small amount of dry yeast at test start.

The reproduction test was conducted according to a miniaturised protocol (Filser et al., 2014) which is based on OECD Guideline 232 (OECD, 2009). In brief, 5 juveniles of *F. candida* were exposed to 10 g of moist soil (50% WHC) in a 30 ml snap lid glass jar (VWR, Radnor, USA) with five replicates per concentration. Once a week, the jars were aerated and dry yeast was added as food source. The water content was controlled at the beginning and the end of the test by weighing the test vessels. After 4 weeks, the soil from test vessels was poured into plastic beakers, animals were floated by adding 100 ml of tap water and made visible by stirring and a small dash of black ink. The surface of the beakers was photographed (Panasonic Lumix DMC-FZ50, Kadoma Japan) and the number of juveniles was counted manually with the software ImageJ (Schneider et al., 2017). Adults were collected from the water surface with a small spatula and transferred onto clean culture plates of charcoal plaster for 1 hour. Afterwards, they were collected in Eppendorf cups, deep-frozen and freeze-dried (alpha 1-2 LDplus, Christ, Osterode am Harz, Germany) for further analysis. Tests were considered valid according to the OECD Guideline (OECD, 2009) when in the controls the average survival of adults was ≥ 80% and on average more than 100 juveniles per vessel were produced.

For the moulting and the biomarker experiments, all animals were exposed to 30 g of moist soil in 200 ml plastic beakers for 6 days. The animals again were extracted by floating. For the moulting experiments, 30 animals were transferred to clean culture plates and kept in a climate chamber for 1 week. After 2, 5 and 7 days, exuviae shed on the plates were counted and collected. Due to the high variation in moulting behaviour, these experiments were conducted twice with 5 replicates each.

For the catalase (CAT) and metallothionein (MT) measurements, 50 and 150 animals were transferred to culture plates after the 6 days of exposure, collected in Eppendorf cups, deep-frozen in liquid nitrogen and stored at -80 °C until further analysis, respectively. The MT assay was conducted according to Maria et al. (2014) and Viarengo et al. (1997) with slight adaptions. Briefly, animals were manually homogenised with a micro-pestle and ultrasonicated at 35 kHz for 1 min in 600 µl of 0.1 mM potassium phosphate buffer (pH=7.4) containing 1 mM EDTA (Sigma-Aldrich, St. Louis, USA) and 1 mM DTT. To guarantee complete dissolution of EDTA, the buffer had been ultrasonicated for 5 min at 30 °C. Then, 500 µl of the homogenate was mixed with 500 µl of an EtOH/Chloroform/MQW solution (87/8/5%) and centrifuged at 6,000 g (10 min, 4 °C). All used ethanol had been cooled to -20 °C. 750 µl of the supernatant were mixed with 1.2 ml of EtOH and 50 µl of an RNA dispersion (20 g/L). After centrifuging at 6,000 g (1 min, 4 °C), the supernatant was discarded and the precipitate was resuspended in 300 µl of an EtOH/Chloroform/MQW solution (87/1/12% v/v) and again centrifuged at 6,000 g (1 min, 4 °C). Again, the supernatant was discarded and the precipitate was resuspended in 150 µl of 0.25 M NaCl, 150 µl of Tris-EDTA buffer (5 and 4 mM, respectively, pH=7), and 300 µl of Ellman's reagent (0.4 mM DTNB (Sigma-Aldrich, St. Louis, USA) and 2 M NaCl in 0.2 M KH_2PO_4, pH=8). As a standard, reduced glutathione (Sigma-Aldrich, St. Louis, USA) was diluted to different concentrations in the Tris-EDTA buffer and mixed in a 1:1 ratio with Ellman's reagent. From each sample and standard concentration, 190 µl were transferred to a 96–well plate (Sarstedt, Newton, USA) in triplicate and the absorbance was measured after 5 min at 405 nm (Wallac Victor2 1420 Multilabel Counter, Perkin Elmer, Norwalk USA).

The CAT assay was conducted according to Cohen et al. (1970). In brief, 50 juveniles were manually homogenized in 0.01 M sodium phosphate buffer (pH=7.4). After centrifugation at 10,000 g (4 °C, 10 min), 10 µl of supernatant were transferred in triplicate to a 96 well plate. 100 µl of 6 mM H_2O_2 were added and after 3 min, the degradation of H_2O_2 was interrupted by the addition of 25 µl H_2SO_4 (6 N). Then, 125 µl of 0.02 mM $KMnO_4$ was added and the absorbance was immediately measured at 492 nm. The CAT activity was calculated based on the absorbance of the samples in relation to the absorbance of a standard containing no H_2O_2. MT level and CAT activity per mg protein of the used homogenate or supernatant were calculated, respectively. Protein content was assessed according to Bradford (1976) with a microassay (Bio-Rad, Munich, Germany) using BSA as standard and Bradford reagent as dye. 5 µl of sample were transferred in triplicate to a 96 -well plate, 250 µl of Bradford reagent was added and the absorbance was read at 595 nm after 5 min.

Cu analytics

All springtail and exuviae samples were digested in 125 µl of 65% HNO_3 and 125 µl of 30% H_2O_2 for 1 h at 65 °C, then for 1 h at 85 °C and, finally, overnight at 95 °C with lids open until complete dryness. The samples were dissolved in 1% HNO_3 and the Cu concentration was measured with AAS using a Uni- cam989QZ AA Spectrometer (Unicam, Cambridge,UK) with a GF-90 plus furnace and an FS-90 plus autosampler.

Statistics

Statistical analyses were conducted with RStudio (R Core Team, 2018). All data were transformed to normal distribution and checked for variance homogeneity, if required for the respective analysis. For the reproduction assay and the biomarkers, all endpoints were compared to the control or another substance group by a glm. Due to the two test runs, the data from the moulting experiment were fitted by a nested ANOVA using a linear mixed effect model from the package nlme (Pinheiro et al., 2019) with the test number as random factor; in this case, differences to the control were analysed by a Dunnett post-hoc test.

Results and discussion

Survival, reproduction and dry weight

In the 28-day reproduction test, negative effects on springtails were mainly found in the montmorillonite (M) soils, and only for NP treatments (Fig. 10). The survival of springtails in kaolin (K) soils was never impaired by more than 30% of the reproduction rate in the control, except for K19-NP-32 (soil-substance-concentration) where a decrease by -47% occurred (Fig. 10A); however, survival in this treatment strongly deviated between the two conducted tests (-85% vs. -9%). In M soils, survival was significantly reduced in M30-NP-1 by -52% ($p=0.048$, Fig. 10B). The reproduction in all tests was lowest in M soil with CuO-NP, yet significantly impaired only in M30-NP-3 by -33% ($p=0.033$, Fig. 10D). The dry weight of springtails in K soils was constantly elevated for both salt (S) and NP-treatments, being 5 to 90% above the control, even if never significant due to high variability of the data (Fig. 10E). In contrast, dry weight in M soils was close to the control or significantly impaired by -48% in M19-NP-1 ($p=0.012$, Fig. 10F). Also, in M30-NP-3 dry weight was remarkably lowered by -42%, but not significantly ($p=0.068$)

The Cu content of adults was similar within the soils of the same clay type, but was generally much higher in K soils (Fig. 11A), which can be attributed to the higher background Cu content of the kaolin (Table 4). There was no remarkable difference between NP and S treatments in all soils. In K30 soil, the control animals contained 33.5 ± 7.9 µg Cu/g dry weight (DW), while in NP-treatments, Cu content increased from 81 ± 31 to 107 ± 22 µg Cu/g DW and in S-treatments from 52 ± 9 to 118 ± 18 µg Cu/g DW. The values in K19 were similar, but at the highest test concentrations, a sudden drop occurred down to the Cu level of the control. For M30 soil, all treatments were below the LOQ, except the treatments M30-NP-32 and M30-S-32, where Cu body content was remarkably elevated with 74 ± 43 and 62 ± 27 µg Cu/g DW. For M19 soil, the pattern was similar, but in addition, also M19-NP-10 and M19-S-10 samples showed higher Cu body content (Fig. 11B).

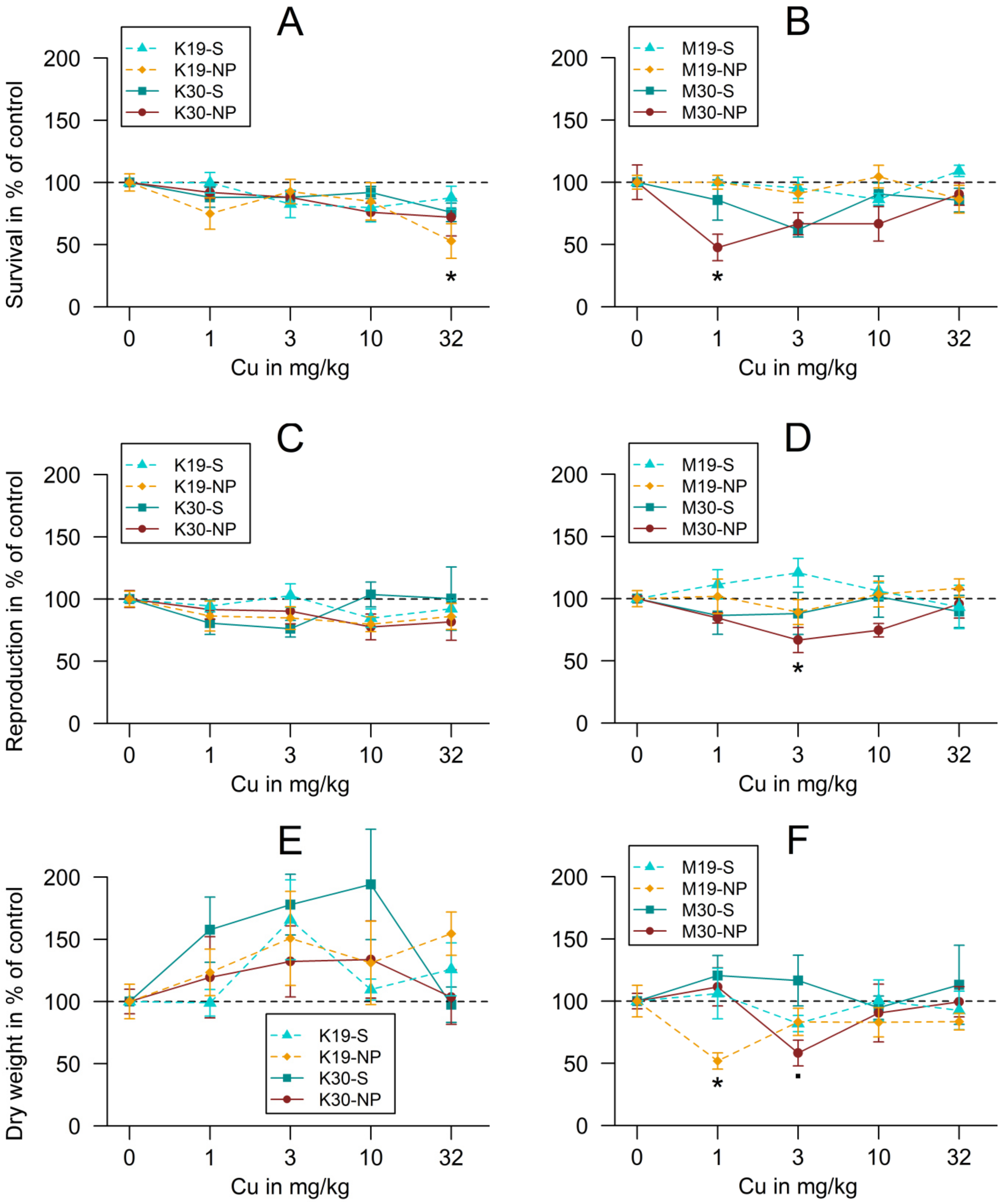


Fig. 2: Mean effects in % of control ± SE (n=5) on the survival (A, B), reproduction (C, D) and dry weight of springtails exposed towards CuO-NP (NP) or $CuCl_2$ (S), either exposed in kaolin soils K19and K30 (A, C, E) or montmorillonite soils M19 and M30 (B, D, F). Significant differences to the control analysed by glm with $0.01<p<0.05$ are marked by an asterisk, trends with $0.05<p<0.1$ by a dot.

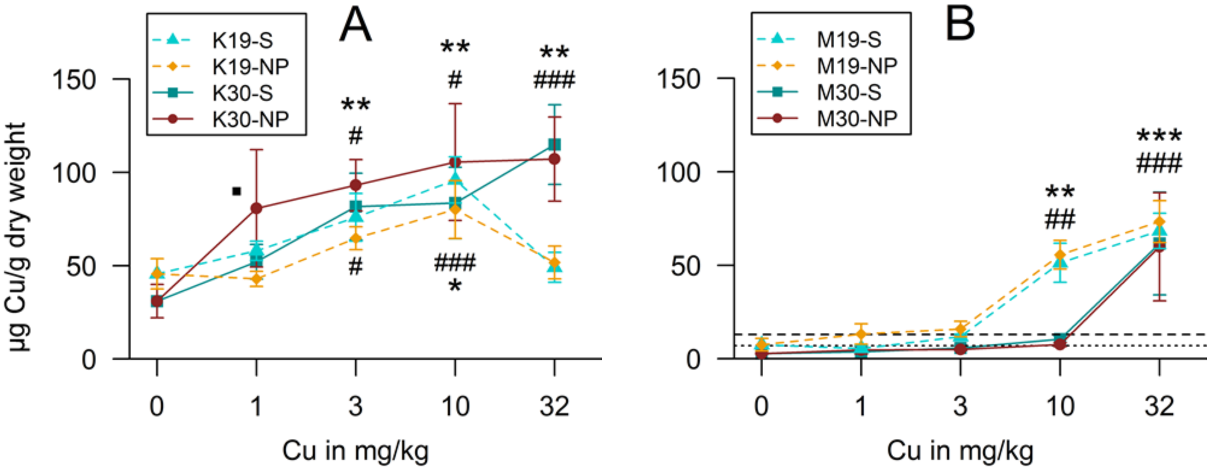


Fig. 3: Cu body concentration ± SE (n=5) of springtails in in kaolin soils K19 and K30 (A) and montmorillonite soils M19 and M30 (B). Significant differences to the control analysed by glm are marked by asterisks for NP and by hashs for S, with */# = $0.01<p<0.05$, **/## = $0.001<p<0.01$ and ***/### = $p<0.001$. Trends with $0.05<p<0.1$ are marked by a dot. The dotted line indicates the LOQ divided by the average dry weight in M19 soil, the dashed line in M30 soil. As most values from 0-10 mg Cu/kg in M30 soil were <LOQ, no statistical analysis was conducted for that soil.

The results in M30 soil corroborate the observation of our previous study that, in contrast to $CuCl_2$, CuO-NP can impact the reproduction of *F. candida* in clay rich soils in a non-linear dose-response relationship (Fischer et al., 2021a). While in that study reproduction was impaired the most at 5 mg Cu/kg, in the present study the highest effect occurred at 3 mg Cu/kg, with no effects at higher concentrations. The reason for this non-linear dose-response relationship may be the stronger agglomeration behaviour of CuO-NP at higher test concentrations, reducing their reactive surfaces, which may also explain why no toxic effects on soil invertebrates were observed at much higher concentrations tested in other studies (Fischer et al., 2021a). Also for other MNP, no toxicity at very high soil concentrations was observed (Heckmann et al., 2011; Noordhoek et al., 2018; Pereira et al., 2011). The concentrations tested here are in the range of the soil Cu increase caused by one season of Cu fungicide application and, therefore, can be considered to be field-realistic (Peixoto et al., 2021). For the other endpoints, the highest effects occurred even at the lowest concentration

(1 mg Cu/kg) in M30 soil for reproduction and in M19 soil for dry weight. However, in both cases the effect on reproduction was not significant. In M30 soil, possibly only the fittest adults survived the chemical stress caused by CuO-NP and, consequently, were able to use all available food and space resources for an above-average growth and reproduction per individual (Fig. S17). For *F. candida* it is known that lower initial population densities allow a higher egg production per individual (Filser et al., 2014), even under chemical stress (Noel et al., 2006). In general, for *F. candida* a trade-off between growth, reproduction and survival in response to chemical stress is possible (Crommentuijn et al., 1997).

The absence of negative effects on survival and reproduction plus the positive effect on dry weight in K30 soil underline how the clay type interacting with Cu influences its toxic behaviour. The Cu uptake via food results in a positive hormetic effect on reproduction of *F. candida* at low Cu concentrations (Pfeffer et al., 2010). This may also be the case for kaolin-Cu associations which seem to support growth. The data of the Cu body content give further support to the previous observation that the accumulated Cu quantity cannot explain toxic effects (Fischer et al., 2021a), as they were never found in treatments with high Cu body contents of springtails (Figs. 10 & 11). Detoxification mechanisms can have direct or indirect effects on organisms' energy-budget (Jager et al., 2013) and, consequently, on their reproduction and growth (Bednarska et al., 2013). Therefore, we hypothesised that especially excretion mechanisms of springtails, which reduce their Cu body concentration, were involved in the detoxification of Cu.

Conversely, Cu excretion may be one reason for the strong difference in Cu body content between the test soils of different clay fractions, as discussed below. The main reasons for this difference, however, are rather the higher Cu background (Table 4), resulting in a higher Cu pore water (Fig. S18) and body content in the K soil control treatments (Fig. 11), and the lower CEC of kaolin for Cu compared to montmorillonite (Farrah and Pickering, 1976), leading to higher Cu pore water (Fig. S18) and body contents in lower concentrated Cu treatments of K soils (Fig. 11).

CAT activity and MT levels

Overall, the CAT activity of springtails was reduced by Cu in both soils, but in M30 soil, the effect was strongest for NP treatments and the overall difference between the substances NP and S was significant (p=0.016, Fig. 12B). The most pronounced reduction by -62% (p=0.012) was observed in M30-NP-10, followed by -39% (p=0.093) in M30-NP-3. In K30 soil, the CAT activity was significantly reduced in K30-NP-1 (-40%, p=0.018), and K30-S-10 (-36%, p=0.033), but the overall difference between NP and S treatments was not significant (p=0.91, Fig. 12A).

For invertebrates, CAT activity has been reported to increase (Buffet et al., 2011; Gomes et al., 2012; Maria et al., 2014; Ribeiro et al., 2015) or decrease after exposure towards HM and MNP (S. I. Gomes et al., 2011; Li et al., 2020; Xiong et al., 2014). In some studies on organic and inorganic contaminants, CAT activity has been reported to increase at low concentrations or for short durations, and to diminish at higher concentrations or longer exposure (Hu et al., 2010; Lin et al., 2010; Ma et al., 2017; Wang et al., 2014). In these studies, this effect was explained by an overload of the antioxidant defence system by an increased presence of ROS, which in turn caused damage to the antioxidant system thus preventing further neutralisation of the ROS. Ma et al. (2017) showed that the higher the CAT activity in snails was increased by Cu in the short term, the more it was depleted in the long-term. Sustained decreases in CAT activity has been observed in several species following exposure to ionic Cu (Garcia Sampaio et al., 2008; Gautam et al., 2018; Vutukuru et al., 2006), CuO-NP (Canli et al., 2017; Janani et al., 2020) and Cu-NP (Gautam et al., 2018).

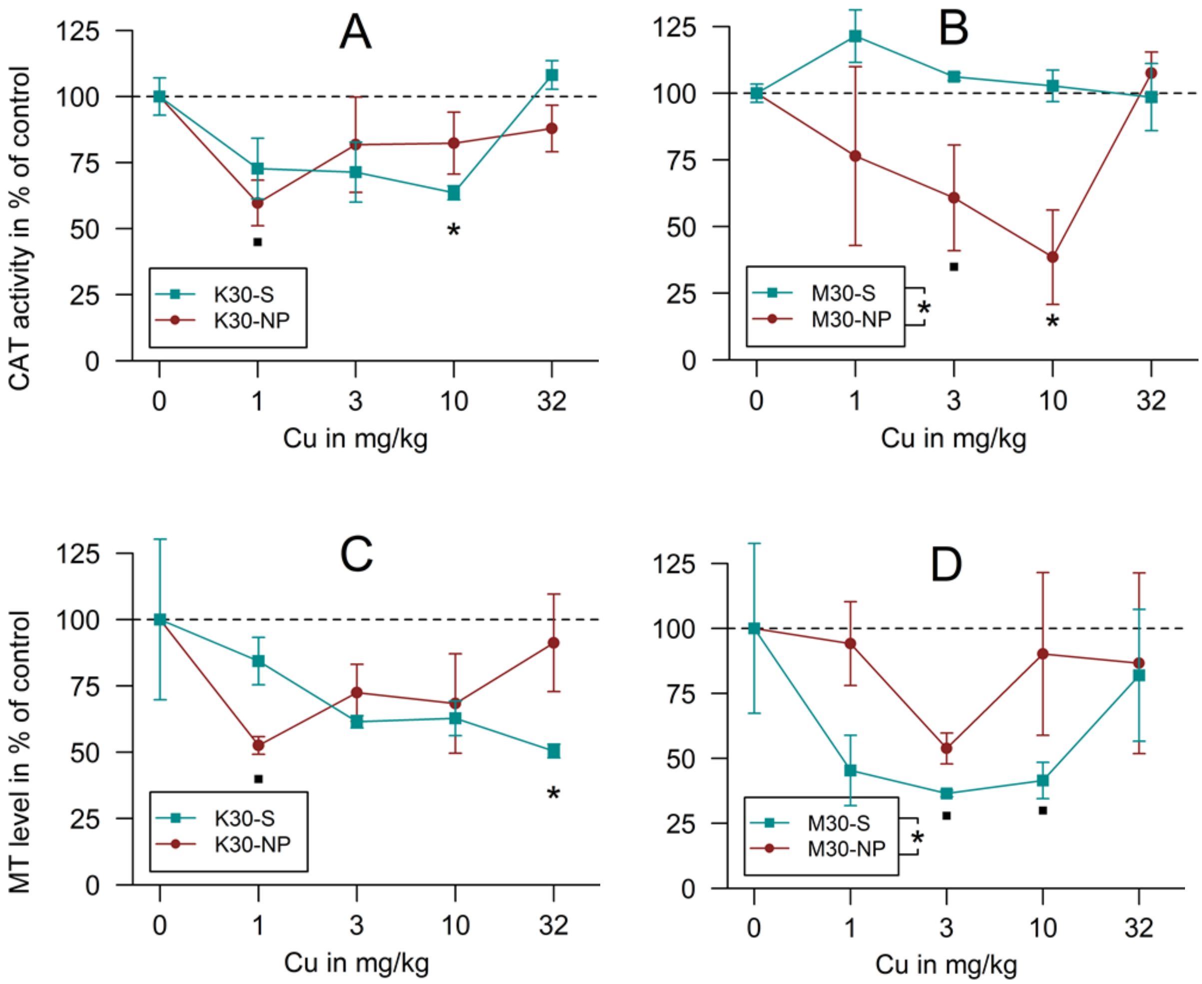


Fig. 4: Mean effects in % of control ± SE (n=3) on the catalase activity (A, B) and metallothionein levels (C, D) of springtails exposed towards CuO-NP (NP) or $CuCl_2$ (S), either exposed in kaolin soil K30 (A, C) or montmorillonite soil M30 (B, D). Significant differences between substances NP and S and of treatments to the control analysed by glm with $0.01<p<0.05$ are marked by an asterisk, trends with $0.05<p<0.1$ by a dot.

In the present study, the marked ROS formation in M30-NP treatments can be explained by the high reactivity of Cu-montmorillonite associations: they are able to break down organic compounds, and they are more reactive than Cu-kaolin (Khanikar and Bhattacharyya, 2013) or Cu-sand associations (Kalidhasan et al., 2017). For example, Cu on montmorillonite showed a high degradation rate of atrazine through the activation of molecular oxygen and the subsequent hydroxyl radical formation (Hong et al., 2017). The Montmorillonite K10 clay used in the present study is rich in structural and exchangeable iron (Table 4; Wirth, 2005) which can act as electron shuttle to reduce HM ions (Liu et al., 2014). We suggest that metallic Fe associated with Montmorillonite K10, which was found in XRD spectra (Santangelo et al., 2011, Fig. S19) acts as a Lewis base upon dissolution, thus fuelling the electron shuttle via octahedral Fe(III) (Wirth, 2005) to the CuO-NP. Finally, the reduced Cu(I) can provide electrons for ROS formation on the NP surface. The addition of Montmorillonite K10 also increased the ROS formation in protozoans by ZnO-NP in the long-term (Gupta et al., 2017). In zebrafish, ROS formation caused by CuO-NP was reduced by Montmorillonite K10, but also sedimentation of CuO-NP was increased (Kansara et al., 2019).

Moreover, Cu^{2+} ions have the ability to reduce the CAT activity by interacting with the CAT enzyme and changing its secondary structure (Hao et al., 2015). However, this does not explain why the most pronounced reduction in CAT activity occurred in the soil with expectably lower Cu pore water concentration (Fig. S18). Therefore, an inhibition of CAT activity through ROS formation seems more likely.

The MT expression was constantly lowered by Cu in both soils, and again, the difference between the substances NP and S was only significant in M30 soil (p=0.035, Fig. 12D), but not in K30 soil (p=0.97, Fig. 12C). Except for S treatments in K30 soils, MT levels occurred as U-shaped curves in relation to Cu concentrations. Due to the large variability of the controls, only in K30-S-32 a significant difference was found (-50%, p= 0.038). This was never the case in M30 soil, even for M30-S-3 with the most MT reduction of -64% (p=0.063).

MT levels in Cu treatments were constantly lower than in controls, but never significantly due to the high variability of controls. A higher MT level in the control compared to Cu treated soils was also observed elsewhere (Xiong et al., 2014), but cannot be explained that easily. Therefore, a comparison between the substances NP and S may be more meaningful. In M30 soil, the significant difference between NP and S and the constantly higher MT levels after exposure to NP suggest higher elimination processes in springtails for CuO-NP than for Cu^{2+}. Furthermore, absolute MT levels are about twice as high in M30 as in K30 soil (Table S5). Overall, the highest absolute MT levels within Cu treatments coincide with the highest observed toxicity and CAT depletion, namely in M30-NP treatments. This could mean that, in relation to S treatments, MT upregulation is a response towards the toxic properties of CuO-NP-montmorillonite associations. Another reason for the increased MT levels might be that montmorillonite in general may attach stronger to the cell membranes of the springtail midgut, allowing the CuO-NP being adsorbed on it to interact more directly with the gut cells. This assumption is supported by the analytical data presented and discussed below, assuming that MT levels and moulting behaviour are linked.

We conclude that Fe-rich montmorillonite, in contrast to kaolin, impacts the toxic properties of CuO-NP and $CuCl_2$ differently, causing different reactions of springtails at a physiological level which shows in the significantly different MT levels and CAT activities in M30 soil. The reason may be that kaolin adsorbs both Cu substances in the same way on its surface. In contrast, montmorillonite can absorb Cu^{2+} ions even stronger due to its higher surface area and CEC, which mainly comes from their interlayer spaces (Dau and Lagaly, 1998). CuO-NP may also enter these spaces if their diameter is below the layer distance (~1 nm) (Pourabolghasem et al., 2016; Sohrabnezhad et al., 2014), which is not the case for the CuO-NP used in this study (~50 nm). Instead, they may stay adsorbed on the clay mineral surface forming ROS as stated above.

Number and Cu content of exuviae

The number of exuviae was more or less similar between treatments in all soils, but in K30 soil, it was significantly increased by 57% ($p < 0.001$) compared to M30 soil (Fig. S20). The highest increase in the number of exuviae compared to the control was found in M30-NP-3, the treatment with the most observed negative effects. In this treatment, moulting was increased by 52% within 3-5 days after exposure, although not significantly (Fig. S20C).

The Cu content of the exuviae was in most cases below LOQ, which did not allow a proper quantification. For values above LOQ, a maximum is indicated for M30-NP-3 (Fig. 13). Therefore, the probability of samples in the respective soils and substances (control, S or NP) to exceed LOD or LOQ was analysed. The odd ratios of the likelihood to exceed one of these ordinal limits for the respective substance or soil groups are given in Table S6. On average, the likelihood of exceedance was 35% lower for NP samples and 66% lower for S samples compared to the control. With respect to soil types, this likelihood was about 5 times higher in M30 soil than in K30 soil.

We had hypothesized that energy-consuming detoxification processes, especially moulting, would significantly contribute to the toxic effects of CuO-NP towards springtails (Fischer et al., 2021a). In the present study, a slight increase in moulting activity in M30-NP-3 was observed at days 3-5 of exposure, however, this difference is to small and inconsistent to draw a conclusion on the impact of moulting activity on the observed toxicity in the reproduction assays. Roughly estimated from the number and Cu content of exuviae, moulting may have contributed about 10 - 20% to the difference between M30 and K30 soil in the total Cu body content of springtails after 28-day exposure, so that the main contribution should come from the differing Cu background and CEC of the clays, as mentioned above.

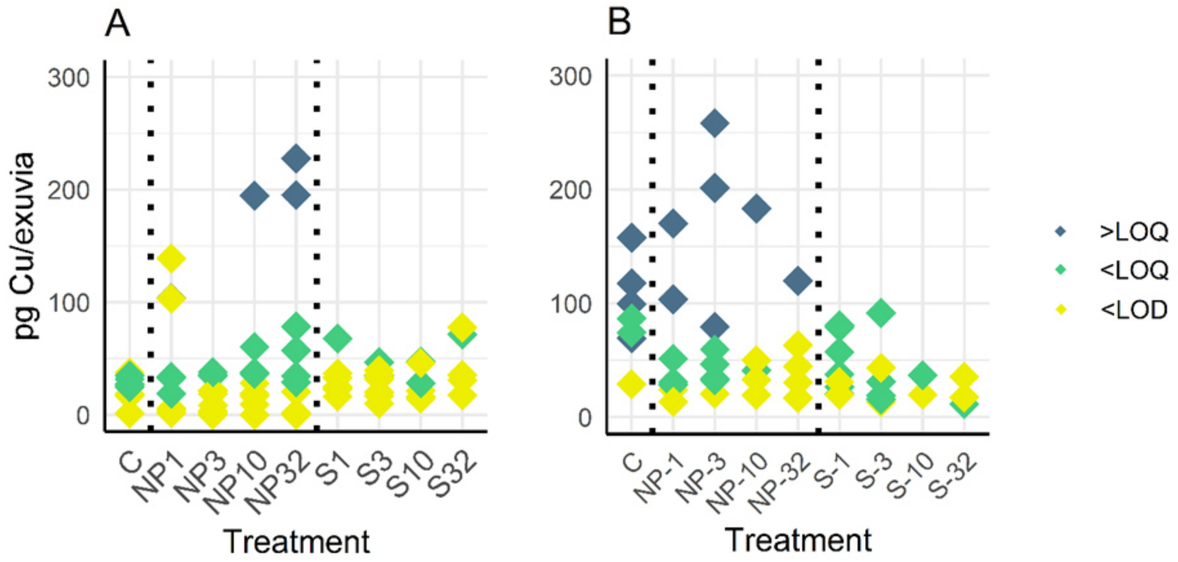


Fig. 5: Cu content ± SE (n=10) of exuviae from springtails in pg Cu/skin exposed towards CuO-NP (NP) or $CuCl_2$ (S), in kaolin soil K30 (A) or montmorillonite soil M30 (B). Cu contents are based on measured values >LOQ (blue), <LOQ and >LOD (green) and <LOD (red). Only samples containing >9 exuviae were considered; explanations for this decision are given in Figs. S21 and S22.

The Cu content of exuviae and the MT level showed the same order of increase for the soil type (M30 > K30) and for the substances (control > NP > S). Again, the reason why the likelihood for the Cu content of exuviae to be above LOQ is highest in the control remains unclear. As mentioned before, one reason for this coincidence might be the stronger attachment of Cu-montmorillonite associations towards the gut cell membranes of springtails. In some studies, the high toxicity of this type of associations was linked to the strong attachment of montmorillonite towards microbial cell membranes (Bagchi et al., 2013; Das et al., 2014; Pourabolghasem et al., 2016). Smectite clays like montmorillonite were observed to attach stronger to bacterial surfaces than kaolinite, while the ability to absorb Cu compared to these surfaces is also higher for smectites than for kaolinites (Walker et al., 1989). This would explain why $CuCl_2$ does not show any toxicity in M soils. In contrast, CuO-NP could be closely bound to cell surfaces via montmorillonite and produce ROS in spatial proximity to these cells. This toxic behaviour of the associations could cause detoxifying processes in springtails by upregulating MT production. Consequently, the increased binding and deposition in the midgut of Cu by MT would reflect in the higher Cu content of exuviae.

The present study could show that reactive three-layer clay minerals like montmorillonite can increase the toxicity of CuO-NP towards springtails. Altered detoxification mechanisms may have contributed to the observed toxicity. However, evidence is more robust for an increased ROS formation as the main toxicity driver. We propose the presence of an autochthonous electron donor and subsequent Fe(III) mediated electron shuttling as the mechanism for Cu(II) mediated ROS formation. According to our hump-shaped data this process is highly concentration-dependent, with a maximum at field-realistic soil concentrations. Therefore, future risk assessment for CuO-NP in soils should consider the role of redox-reactive (clay) minerals - importantly, with a focus on low test concentrations.

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CRediT author statement

Jonas Fischer: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Visualization. **Ghanem A. D. Talal:** Methodology, Investigation. **Laura S. Schnee:** Conceptualization, Writing – original draft, Visualization. **Patricks V. Otomo:** Methodology, Writing – review & editing. **Juliane Filser:** Writing – review & editing, Supervision.

- Chapter 5 -

General discussion, conclusions and outlook

Size and dissolution characterization of CuO-NP

The current thesis has revealed up to now unknown physico-chemical reactions and toxic effects of CuO-NP towards soil invertebrates. The model substance used here, spherical uncoated CuO-NP from Sigma-Aldrich (Munich, Germany), is widely used in many chemical and ecotoxicological studies; in Chapter 2, a comprehensive characterization of these CuO-NP with respect to size measurements (TEM, sp-ICP-MS, DLS), surface chemistry (ELS, Raman spectroscopy) and dissolution behaviour (AAS, numerical speciation) was done. These results and other parameters (surface area, crystallinity) compared to and within the literature showed that constant properties for this model substance can be expected. Strongest deviations occur for zeta potential data, probably due to sample handling.

It was shown that interactions between the soil solution and HA highly impact the agglomeration and dissolution of CuO-NP in aqueous media (Figs. 4 and 5). The ion composition of the medium seems to be striking for these transformation processes here, rather than ionic strength and divalent cation concentration as assumed in many studies before. This can be explained by the competition between HA and ions for binding sites on the surface of CuO-NP as well as competition between ions of different charge for bridging interactions

A reduced dissolution and agglomeration was also observed in the artificial soil medium, however, the impact on the toxicity was not investigated. In Chapter 3, DLS measurements of CuO-NP added to extracted pore water (Fig. S11) and sp-ICP-MS measurements of pore water extracts from spiked field soils (Fig. 7) indicated that the CuO-NP particle diameter was strongly influenced by the applied test concentration, but did not vary between test soils, even if they were highly variable in their pH, SOM and clay content. The different properties of the soils should lead to differently composited pore water solutions. Therefore, the results suggest that the impact of the soil solution in field soils on particle agglomeration might be limited, whereas it is strongly impacted by NP concentration; this effect is also stated by other sp-ICP-MS measurements (Velicogna et al., 2021).

Cu quantities vs. toxic effects

The size and dissolution characteristics of CuO-NP in different artificial media also aimed to draw first conclusions of the environmental impact on their toxicity. On the one hand, a link between the toxicity of soil organisms exposed via aquatic media or soils can strongly differ in their outcomes (Zhang et al., 2018). Therefore, it may be difficult to conclude from the chemical behaviour in a soil medium on the toxic properties of CuO-NP in a soil matrix. On the other hand, observations from both systems can be combined for a more comprehensive understanding. As an example, the dissolution-reducing effect of HA observed in the soil medium (Fig. 5) suites to the behaviour of CuO-NP in the loamy-organic field soil LO tested in Chapter 3, where the organic content was highest of all loamy soils, but dissolution (sp-ICP-MS, Fig. S13), was lowest. Here, the complexation of Cu^{2+} by organic matter may have rather led to a retainment in the soil matrix than to a release into the pore water.

A basic understanding of chemical transformation processes of CuO-NP under environmental conditions is necessary as they determine the toxic properties of CuO (see also Chapter 1, Fig. 1). This gets even more obvious considering the limitation of mere Cu quantification for interpreting our toxicity data. In all bioassays of this thesis, the concentration of Cu in soil, pore water and springtails did not correlate with toxicity. As an example, Cu pore water and springtail body concentration were highest in the sandy field soil used in Chapter 3, where no negative effects occurred at all, and about three times lower in loamy soils, where the strongest effects occurred (Figs. 8, 9 & S15). The same observation was stated in Chapter 4 for the artificial soils, where kaolin soils did not cause toxic effects but a high Cu bioaccumulation, and vice versa for montmorillonite soils (Figs. 10, 11 & S18).

The dissolution of CuO-NP is a transformation process which is comparably easy to quantify e.g. as the dissolved Cu pore water or medium concentration (see Chapters 2 and 3). However, it also seems not to be the striking factor for toxicity development in our studies. First, the dissolved Cu fractions in the pore water measured by sp-ICP-MS did not correlate with the observed toxicity in Chapter 3 (Fig. S13). Second, in Cu salt treatments, which can be seen equivalent to the complete dissolution of CuO-NP in the soil, were never more toxic than their NP counterparts (Chapters 3 and 4).

Furthermore, it should be noted that the Cu concentrations of pore water and springtails were similar between CuO-NP and Cu salts in Chapter 4 (Figs. 11 & S18). In Chapter 3, some differences occurred for Cu body concentration, but not in a coherent way (Fig. 8). This suggests that the dissociation into the pore water and the uptake by organisms is independent of the Cu form, while for toxicity, the opposite can be the case.

Summarizing, in the case of CuO-NP, toxic effects and the Cu quantity present in soil, pore water or body are uncoupled, which may also be one reason for the observed U-shaped dose-responses.

U-shaped dose-responses or low-concentration effects

In ecotoxicology and risk assessment, there is an ongoing debate on so-called hormetic effects, also with respect to NP (Iavicoli et al., 2018) and heavy metals (Muszyńska and Labudda, 2019). The concept describes the occurrence of biphasic dose-responses, usually with positive effects of substances at low, but negative effects at high concentrations. This effect by Cu on the dry weight of springtails was observed in artificial kaolin soils (Fig. 10E) and also occurred in other studies for Cu and springtails (Pfeffer et al., 2010). However, the occurrence of negative effects at low concentrations is not yet described in this context. Therefore, we explicitly do not apply this concept to our data. We rather interpret our findings as specific for our experimental setup (CuO-NP + clay + springtails) and summarize them under low-concentration effects.

The toxic effects in Chapters 3 and 4 mostly occurred in a very narrow concentration range of 1-10 mg Cu/kg, in the case of reproduction even of 3-5 mg Cu/kg, always with at least one higher concentration showing lower or no toxicity (Figs. 8, 9, 10 and 12). It should be noted that most rangefinding experiments are conducted with a distance of factor 10 between the test concentrations. This underlines that low-concentration effects as observed in these studies can be overseen quite easily in common rangefindings.

Low-concentration effects occurred in the Chapters 3 and 4 for the endpoints survival, reproduction, body weight, CAT activity and, to some extent, the Cu content of exuviae. They can be explained by the reduced agglomeration, providing more reactive surfaces per mass on the one hand, and allowing more easy retention of particles in biological structures on the other hand, e.g. in microfibrils of the springtail midgut. We assume that with a concentration increase of CuO-NP in the soil the total amount of surfaces increases, while by agglomeration processes the fraction of unbound and free surfaces decreases. Both processes running in parallel lead to a maximum of available reactive surfaces and a consequent maximum toxicity in the range of 1 to 10 mg Cu/kg (Figure 14). As an example, it can be expected that the ROS formation by CuO-NP-montmorillonite associations was related to their available reactive surfaces, which probably reached their maximum at 10 mg Cu/kg where CAT activity was most depleted (Fig. 12). Also indirect effects besides increased reactivity could play a role: regarding the increased Cu content of exuviae in montmorillonite-CuO-NP soil (Fig. 13), the increased surfaces could also have offered more space for the attachment of montmorillonite particles, which in turn could have increased the attachment of CuO-NP towards cell surfaces (Walker et al., 1989). However, with respect to moulting, more reliable data have to be produced to allow clear conclusion of its role for the detoxification of CuO-NP and its contribution to the observed low-concentration effects.

Still, the particle size alone cannot explain the observed toxic effects, as the size profile of CuO-NP was similar in sandy and loamy soils (Fig. 7), but toxic effects did only occur in loamy soils (Figs. 8 and 9). This indicates that the toxicity observed in loamy soils might be due to interactions of CuO-NP with their clay fraction, i.e. that CuO-NP-clay associations may be more toxic than CuO-NP alone.

Soil properties impacting CuO-NP

Field soils are of highly diverse composition regarding their organic and mineral fractions. The different properties of clay minerals let suggest that each clay mineral can interact differently on CuO-NP, inducing different physicochemical reactions (Ammann et al., 2005; Khanikar and Bhattacharyya, 2013; Shi et al., 2013). In Chapter 4, we could show that montmorillonite and kaolin (mainly consisting out of the clay mineral kaolinite) as representatives of two- and three-layer clay minerals can have very contrasting effects on the toxicity of CuO-NP.

For CuO-NP-montmorillonite associations, three main differences to their counterparts with $CuCl_2$ and/or kaolin affecting *F. candida* were found: (i) they negatively impacted survival and reproduction (Fig. 10), (ii) they most depleted CAT activity (Fig. 12) and (iii) from all Cu treatments, they showed the highest MT level and the highest Cu concentrations in exuviae (Figs. 12 & 13). As mentioned above, for all these observations, except MT level, a low-concentration effect was observed, which was due to surface-dependent interactions of NP-clay associations increasing their reactivity and toxicity. This clearly shows that the soil properties, possibly supporting redox reactions and subsequent ROS formation, and NP size and surface-related processes must be thought together to explain the observed low-concentration effects in Chapters 3 and 4 (Fig. 14).

The results from Chapter 4 allow a reinterpretation of those from Chapter 3. In field soils which are of highly diverse composition, all present clay mineral fractions can play different roles parallelly, e.g. kaolin-associated Cu can increase Cu springtail body concentration and support growth, while at the same time, montmorillonite-associated Cu can produce ROS and impact reproduction. This would explain why the data on reproduction, growth and Cu body concentration in Chapter 3 did not follow a clear pattern (Fig. 8). In Chapter 3, we also discussed the role of moulting for the unexpected results in cu body content e.g. increasing Cu body concentration in elimination phase. In Chapter 4, we could see that clay minerals highly impact the moulting frequency and the amount of Cu adsorbed to exuviae. Therefore, each soil with its unique clay mineral composition could lead to different moulting and Cu detoxification patterns.

In Chapter 4, pH was not investigated as variable in soil properties. We had assumed that the pH difference between LA soil and L soil in Chapter 3 could drive toxicity by increasing dissolution. However, this was not the case as discussed above. We concluded that Ca^{2+} input through liming was more relevant for the differences in toxicity than the change in soil pH. In spite of the results from Chapter 4, this conclusion should be revised. Hong et al. (2019) found out that hydroxyl radical formation by Fe^{3+}-montmorillonite systems highly increased with decreasing pH values. Therefore, the highest observed toxicity found in the acidic LA soil (Figs. 8 & 9) could be rather explained by the low soil pH supporting ROS formation than dissolving Cu^{2+} ions.

Also the reduction of Cu(II) to Cu(I), which we assume to be the last step in the electron shuttling to produce ROS at the CuO-NP surface is pH sensitive: at low pH values, the formation of $Cu(OH)_2$ is supported, resulting in Cu_2O by water dissociation. In Chapter 2, we concluded that CuO-NP are covered by a thin layer of $Cu(OH)_2$ even at pH 8 due to interactions with the surrounding water. Therefore, a support of Cu(II) reduction by the soil pH could also be possible for our test soils ranging from 4.8 – 5.8, with the support being highest in the acidic LA soil.

As discussed above, the reduced dissolution in the soil medium by the addition of HA (Fig. 5, Table 2) is in line with the reduced dissolution observed in the loamy-organic LO soil (Figs. S13 and S15). We also had discussed that dissolution may not be a key factor driving the toxicity of CuO-NP. However, organic matter adsorbs to reactive surfaces of clay minerals, reducing specific surface areas of soils (Kaiser and Guggenberger, 2003), and also towards CuO-NP surfaces as observed in Chapter 2 (Figs. 3 and S3). This may also rather explain the reduced toxicity in LO soil compared to the other loamy soils of lower organic content.

Summarizing, the concept developed in Chapter 4, a central role of ROS formation depending on the availability of reactive NP-clay reactive sites, can help to explain the observed toxicities in Chapter 3, with an increase in toxicity by lower soil pH and a decrease in toxicity with higher

SOM content. Some chemical prerequisites for this concept are investigated in Chapter 2. In general, the concept encourages to shift the interpretation of NP related toxicity data from a dissolution-driven to a surface reactivity-driven discourse.

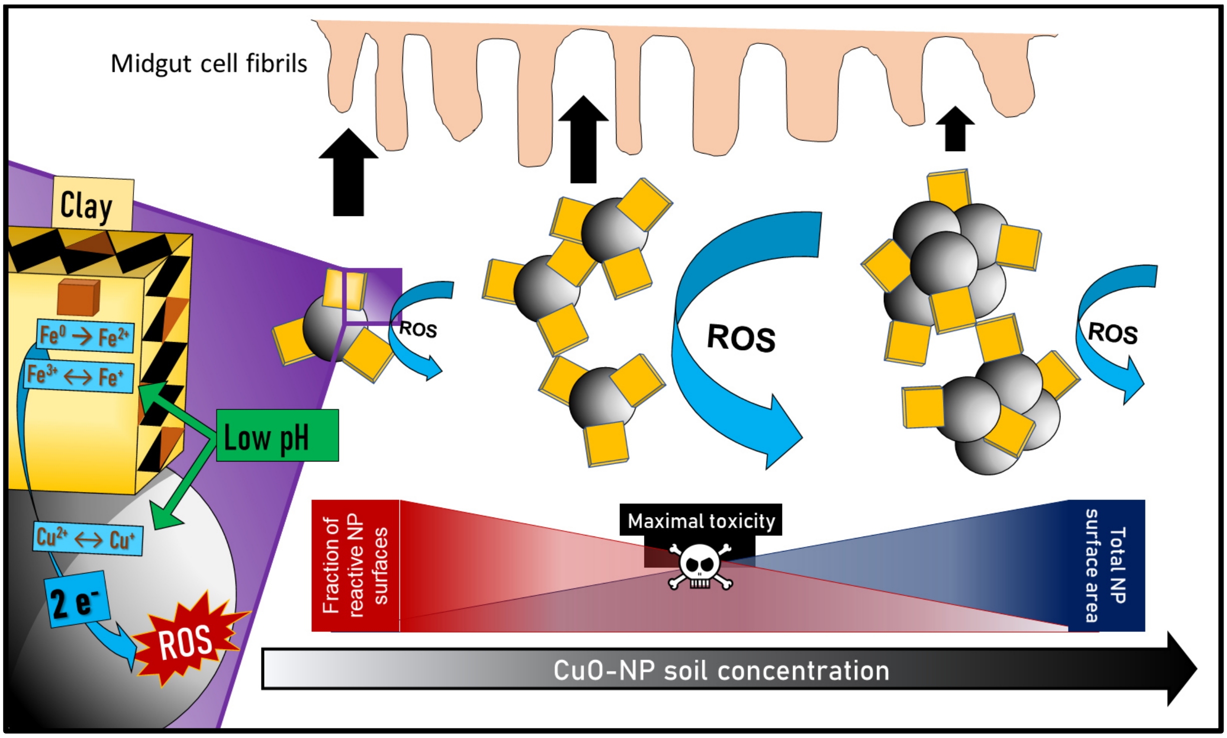

Fig. 6: Scheme for hump-shaped toxicity curve of CuO-NP in loamy soils, due to increased agglomeration and consequent reduction of reactive NP surfaces. Purple: detailed mechanism of ROS formation through structural or interlayer Fe in clay minerals as electron shuttle for Cu impacted by soil pH.

Conclusions and outlook

The present thesis could show that CuO-NP highly interact with their environment and that these interactions can impact their toxicity towards springtails. The fraction of divalent cations in combination with humic acid (HA) determine the degree of agglomeration and dissolution in artificial media. The interaction of CuO-NP with reactive clay minerals like Fe-rich montmorillonite can deplete the oxidative defense system, probably by increased ROS formation on the NP surface, possibly also through stronger attachment to midgut cells. In consequence, survival, reproduction and body weight of springtails are affected in a non-linear dose-response over a soil concentration range of 1 to 158 mg Cu/kg, with a focal point between 1 and 10 mg Cu/kg. The reactivity of NP-clay associations might be supported by low soil pH and curbed by high SOM content and, thereby, this factor may impact the toxicity stronger than Cu dissolution or bioaccumulation; a similar approach to explain the toxicity of CubNP is suggested by (Denluck et al., 2018).

The concentration range for springtails being most sensitive towards CuO-NP can quickly be reached within one or few years of Cu application. This underlines that the observed effects become environmentally highly relevant when also occurring in this way in the field. Therefore, a next step in research could be more complex multispecies (Mendes et al., 2018) or aging studies (van Gestel et al., 2012). As nano $Cu(OH)_2$ is already applied as fungicide in the field (Keller et al., 2017; Neves et al., 2019; Zhang et al., 2020), one step further could be an accompanying field monitoring as suggested by Schäfer et al. (2019).

It was also shown how important the choice of the test concentration range is. Future studies on MNP in soils should account for field-realistic behaviour in their choice of concentrations, which could more than ever reveal toxic properties of these substances. For nano-$Cu(OH)_2$, some studies exist where field-realistic test concentrations were included (Neves et al., 2019; Peixoto et al., 2021). The same accounts for the choice of test soils. We could clearly show that in the case of CuO-NP, a sandy test soil may be not sufficient to depict the worst-case scenario. In general, the use of different test soils regularly shows contrasting outcomes for the toxic effects in these soils (Neves et al., 2019; You et al., 2018). Further, agrochemicals in general should be tested on soils where they are preferably applied, such as loamy soils which are widely spread and farmed due to their favourable properties for agriculture.

Some possible mechanisms behind the toxicity of CuO-NP towards springtails were proposed. Future research should focus on these mechanisms to design MNP in a way that the risk towards organisms is lowered (e.g. Figueiredo et al., 2019). The design options such as size and shape (Gilbertson et al., 2016), coating (Baumann et al., 2014) or doping (Naatz et al., 2017) could focus on the reduction of ROS formation or the adhesion to eukaryotic cells.

To enable the design of environmentally-friendly CubNP, a deeper investigation of interactions with environmental substrates such as clay minerals and organic matter is required. As discussed before, the generation of ROS is based on electrochemical reactions which may be one reason for CAT enzyme depletion. In an electrochemical pilot study using accelerated voltammetric stripping, we could show that montmorillonite K10 is able to increase the current produced by CuO-NP in a phosphate-buffer more strongly than kaolin and when $Cu(NO_3)_2$ was used instead (Fig. S24); the formation of ROS and charge carriers can co-occur (He et al., 2018). In future experiments with this test setup, the impact of clay mineral and organic matter combinations on the electrochemical behaviour of CubNP could be investigated and the presence of ROS could be proven spectrophotometrically (Denluck et al., 2018) or by electron spin resonance (ESR; He et al., 2018; Zhao et al., 2019).

As shown in the present thesis, the interaction partner of CuO-NP is striking for their toxic properties. Therefore, it is important to know which interaction partner is preferred when CubNP enter the soil environment. This question is currently investigated in a pilot study using X-ray absorption near-edge structure spectroscopy (XANES) on the L-edge of Cu. The spectra gained from field soils spiked in the lab with CuO-NP, $Cu(NO_3)_2$ or field-contaminated by viticulture will be compared by linear combination with reference spectra of Cu substances adsorbed to substances being representative for different soil fractions, namely kaolinite, montmorillonite, HA and goethite (Figs. S25 & S26).

On the one side, the present thesis has revealed the potential risk of CuO-NP towards soil organisms under varying environmental conditions and, on the other side, suggested potential approaches to further investigate, but also reduce the risk of CubNP towards soil organisms, e.g. by targeted NP design.

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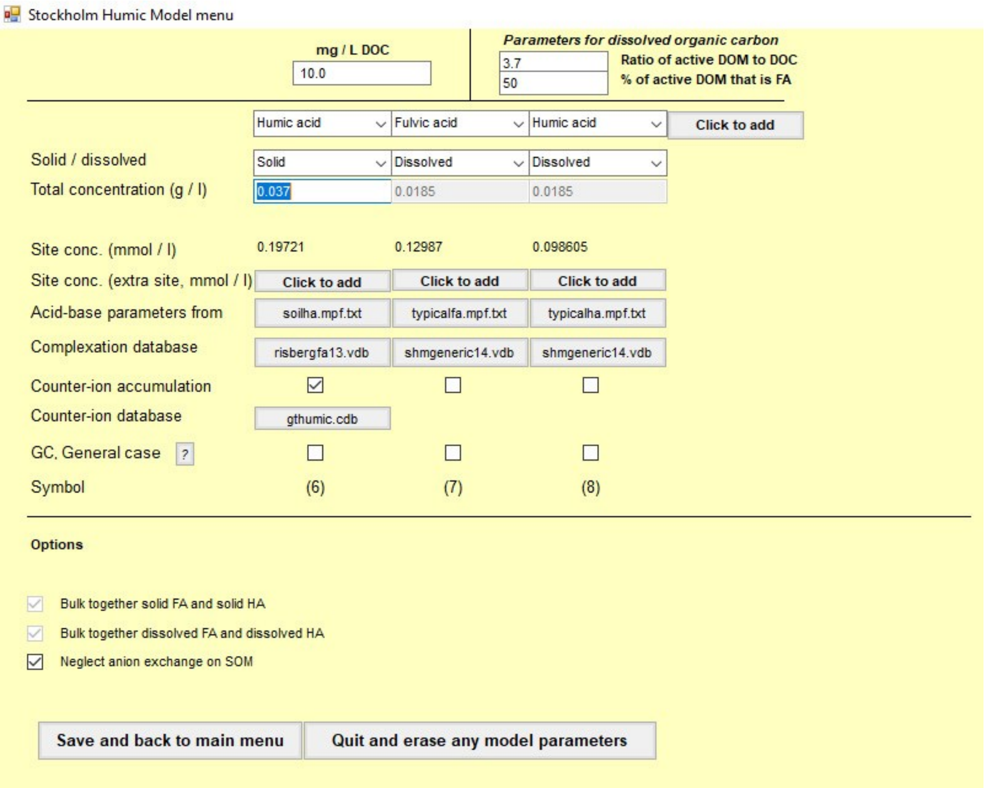
Supporting information Chapter 2

Table S1: Complete list of components of the different media as input parameters for the speciation calculations. Ion concentrations of the media were calculated according to the literature (OECD, 2004); (Voua Otomo and Reinecke, 2010); (ASTM, 2008).

Components	**Elendt M7 [mmol/L]**	**Artificial seawater [mmol/L]**	**Soil porewater [mmol/L]**
Ionic strenght	8.00	643.1	10.21
Ca^{2+}	2.00	10.43	1.36
Mg^{2+}	0.50	54.60	1.50
K^{+}	0.08	10.15	0.20
Na^{+}	0.87	479.43	1.19
Cl^{-}	4.08	559.07	2.72
CO_3^{-}	0.77	2.39	1.39
SO_4^{-2}	0.50	54.60	1.50
Components	**Elendt M7 [µmol/L]**	**Artificial seawater [µmol/L]**	
Sr^{2+}	0.14	158.40	
$B(OH)_3$	11.60	438.50	
Br^{-}	0.04	844.30	
NO_3^{-}	3.22	1.61	
Co (II) $^{2+}$	0.04		
Fe^{3+}	3.60		
Li^{+}	1.80		
NH_4^{+}	0.01		
Rb^{+}	0.15		
Zn^{2+}	0.10		
I^{-}	0.02		
MoO_4^{-2}	0.07		
PO_4^{3-}	2.10		
SeO_3^{-2}	0.01		
SiO_4^{-4}	35.20		
VO_3^{-}	0.01		
EDTA	6.72		
F^{-}		71.45	
NO_3^{-}		0.27	

Table S2: pH mean values ± SE (n=4) for the four test media MilliQ water (MQW), soil porewater (SPW), fresh water (M7) and artificial seawater (ASW) with or without HA at four time points.

	Mean ± SE			
HA-	**MQW**	**M7**	**ASW**	**SPW**
Day 0	8.07 ± 0.28	8.16 ± 0.15	8.35 ± 0.12	8.07 ± 0.02
1	8.13 ± 0.21	7.99 ± 0.06	8.28 ± 0.06	8.10 ± 0.13
3	8.10 ± 0.00	7.90 ± 0.04	8.18 ± 0.11	8.09 ± 0.08
7	8.40 ± 0.13	7.86 ± 0.10	8.30 ± 0.03	8.16 ± 0.08
HA+				
Day 0	8.21 ± 0.21	8.06 ± 0.08	8.32 ± 0.14	8.10 ± 0.02
1	8.27 ± 0.13	7.97 ± 0.02	8.29 ± 0.07	8.12 ± 0.08
3	7.85 ± 0.14	7.91 ± 0.10	8.16 ± 0.06	8.05 ± 0.14
7	7.95 ± 0.15	7.86 ± 0.08	8.28 ± 0.00	8.21 ± 0.06


Stockholm Humic Model menu

mg / L DOC: 10.0

Parameters for dissolved organic carbon
3.7 Ratio of active DOM to DOC
50 % of active DOM that is FA

	Humic acid	Fulvic acid	Humic acid	Click to add
Solid / dissolved	Solid	Dissolved	Dissolved	
Total concentration (g / l)	0.037	0.0185	0.0185	
Site conc. (mmol / l)	0.19721	0.12987	0.098605	
Site conc. (extra site, mmol / l)	Click to add	Click to add	Click to add	
Acid-base parameters from	soilha.mpf.txt	typicalfa.mpf.txt	typicalha.mpf.txt	
Complexation database	risbergfa13.vdb	shmgeneric14.vdb	shmgeneric14.vdb	
Counter-ion accumulation	☑	☐	☐	
Counter-ion database	gthumic.cdb			
GC, General case ?	☐	☐	☐	
Symbol	(6)	(7)	(8)	

Options

☑ Bulk together solid FA and solid HA
☑ Bulk together dissolved FA and dissolved HA
☑ Neglect anion exchange on SOM

Fig. S1: The applied settings of the Stockholm Humic Model for conducted speciation calculations with the software Visual MINTEQ (Gustafsson, 2013).

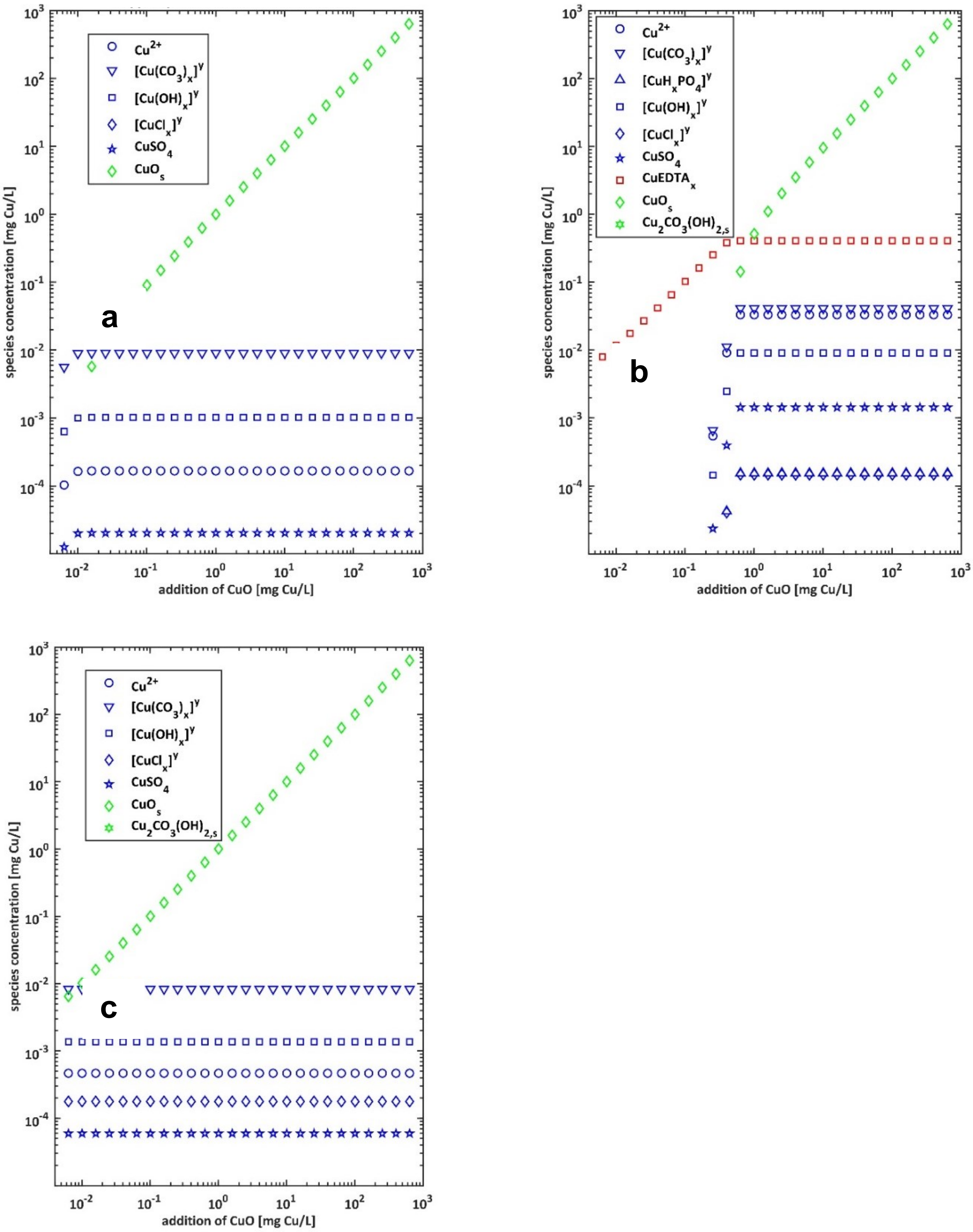


Fig. S2: Copper speciation of CuO-NP in different test media: **(a)** soil porewater (SPW), **(b)** fresh water (M7) and **(c)** artificial seawater (ASW), using the software PHREEQCi (Parkhurst, D.; Appelo, 2010) and the database files *llnl.dat* (for SPW and ASW) and *minteq.v4.dat* (for M7).

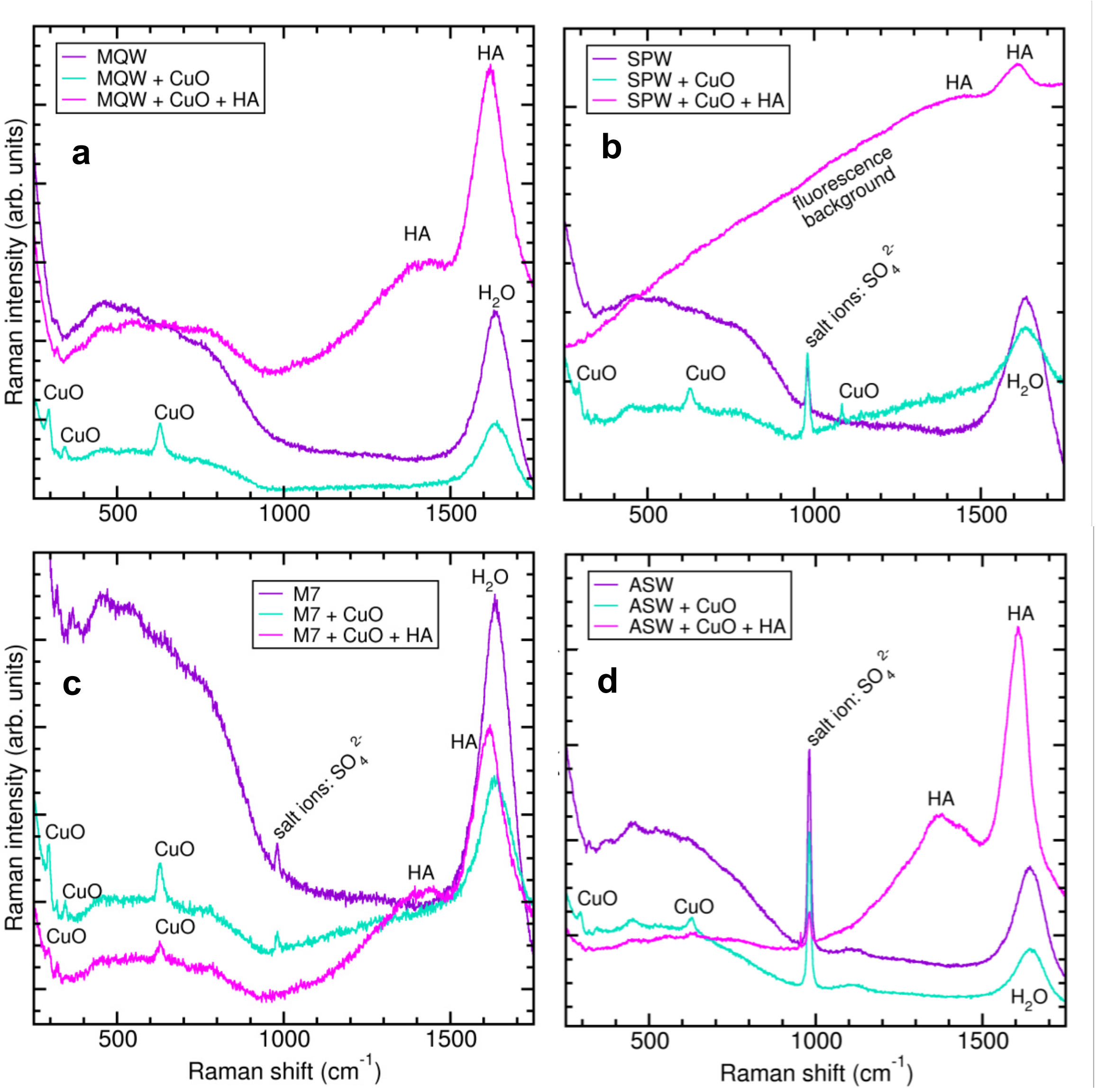


Fig. S3: Raman spectra of CuO-NP in different media: **(a)** MilliQ water (MQW), **(b)** soil porewater (SPW), **(c)** fresh water (M7) and **(d)** artificial seawater (ASW) with or without HA.

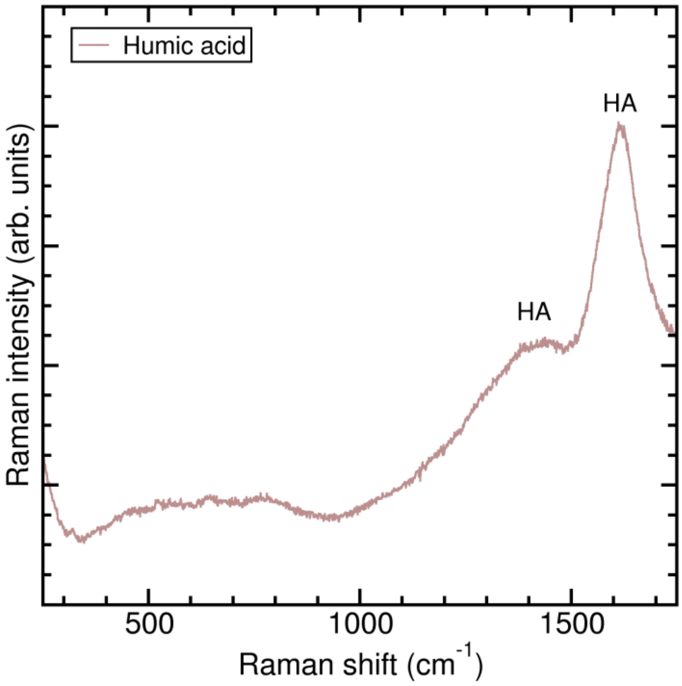


Fig. S4: Raman spectra of humic acid.

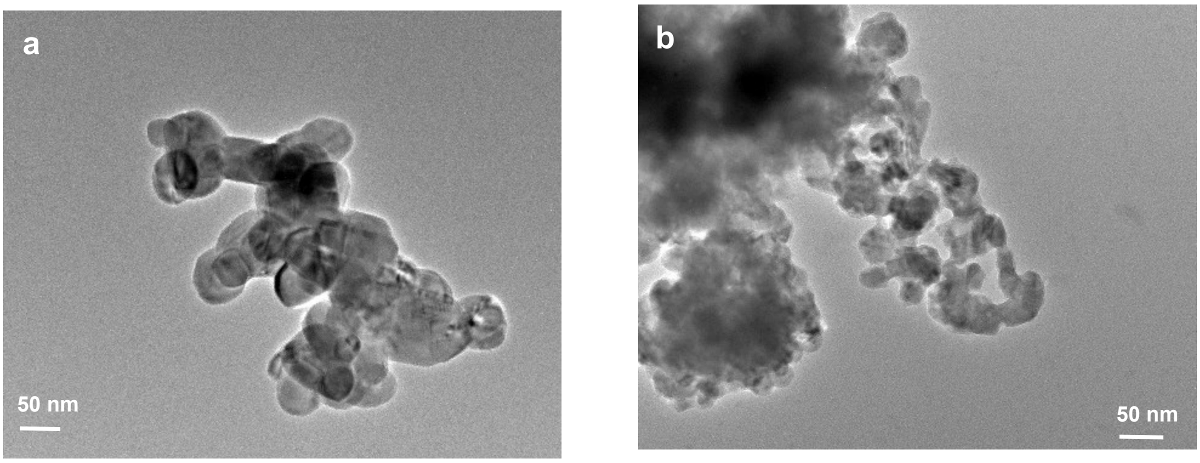


Fig. S5: Characterization of CuO-NP: Transmission electron microscopic image of 100 mg Cu/L CuO-NP suspension in **(a)** EtOH and **(b)** MilliQ water + humic acid.

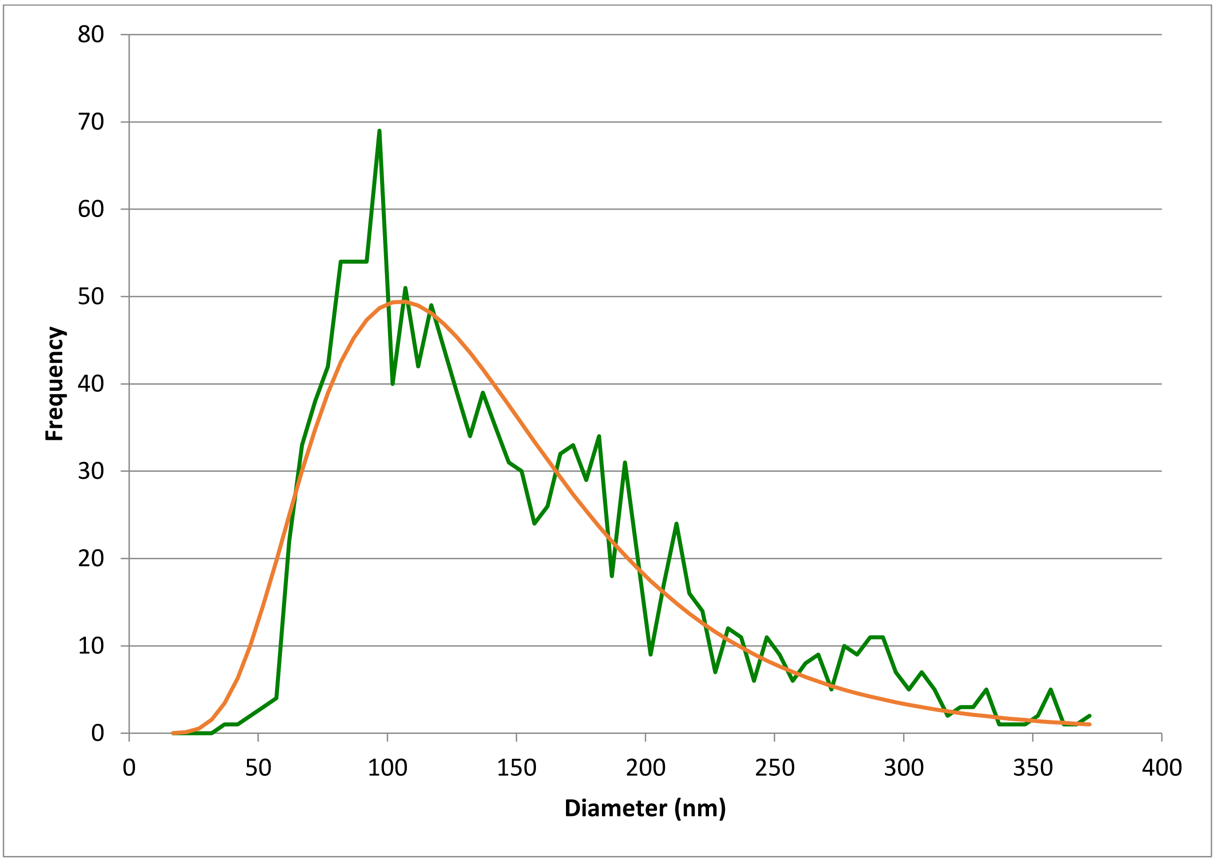


Fig. S6: Particle size distribution of CuO-NP dispersed in MQW, analysed by single particle ICP-MS. CuO-NP were ultrasonicated at 100 mg Cu/L for 30 min and afterwards diluted to 10 µg/L to achieve a suitable signal. Bin size=2 nm; green=particle count, orange=lognormal fit. For detailed methodology, see (Fischer et al., 2021a)

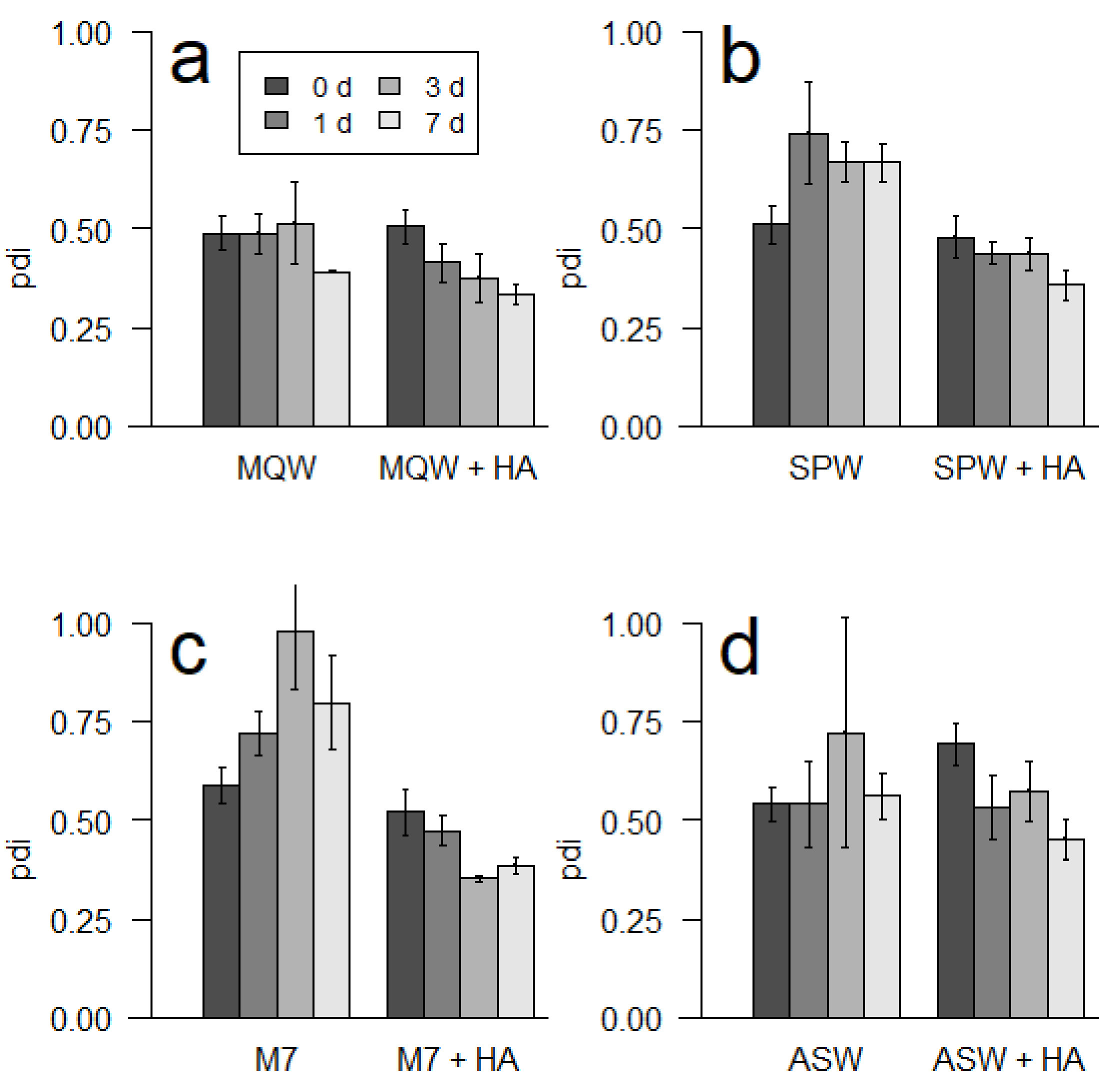


Fig. S7: Mean polydispersity index ± SE (n=4) in nm for **(a)** MilliQ water (MQW), **(b)** soil porewater (SPW), **(c)** fresh water (M7) and **(d)** artificial seawater (ASW) with or without HA at four time points.

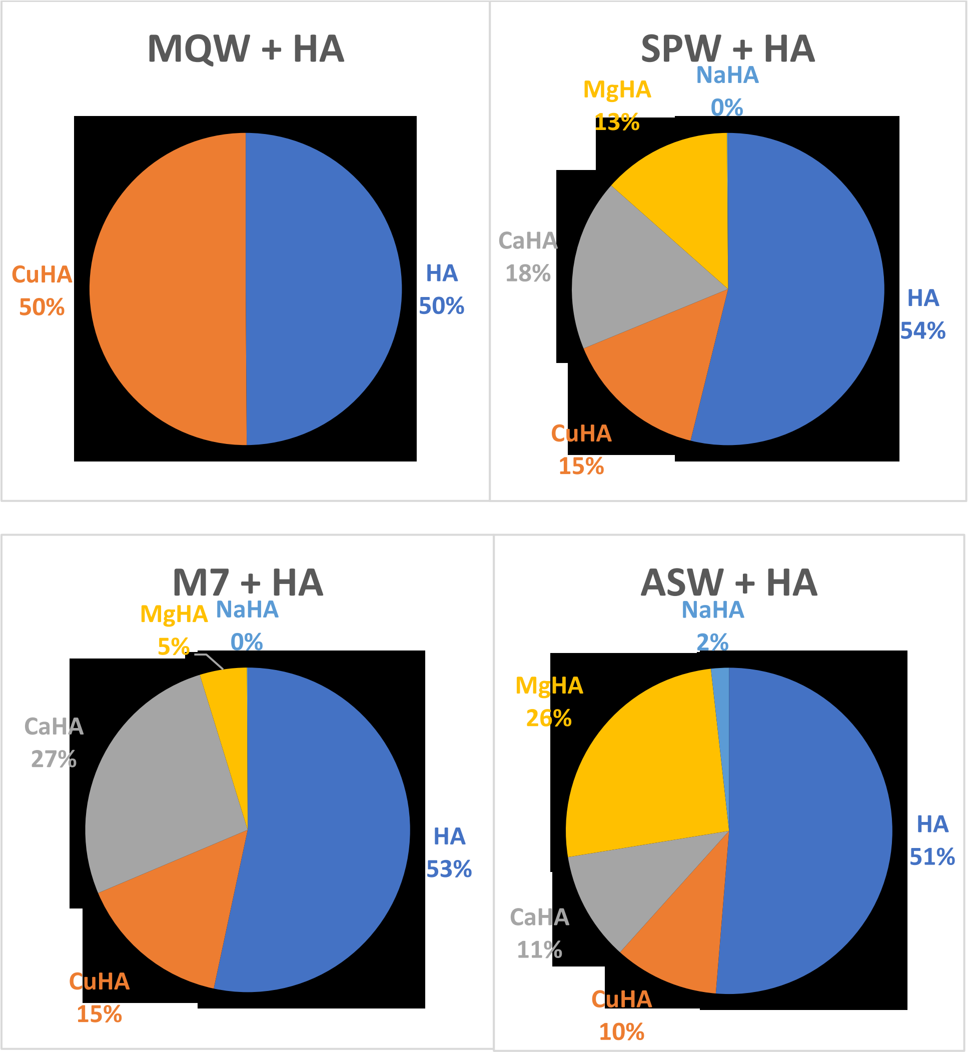


Fig. S8: Pie charts of the fractions of HA complexes with the cations of Cu. Ca, Mg and Na or uncomplexed HA in the four different test media (MilliQ water (MQW), soil pore water (SPW), fresh water (M7) and artificial seawater (ASW)) as calculated by Visual MINTEQ (Gustafsson, 2013).

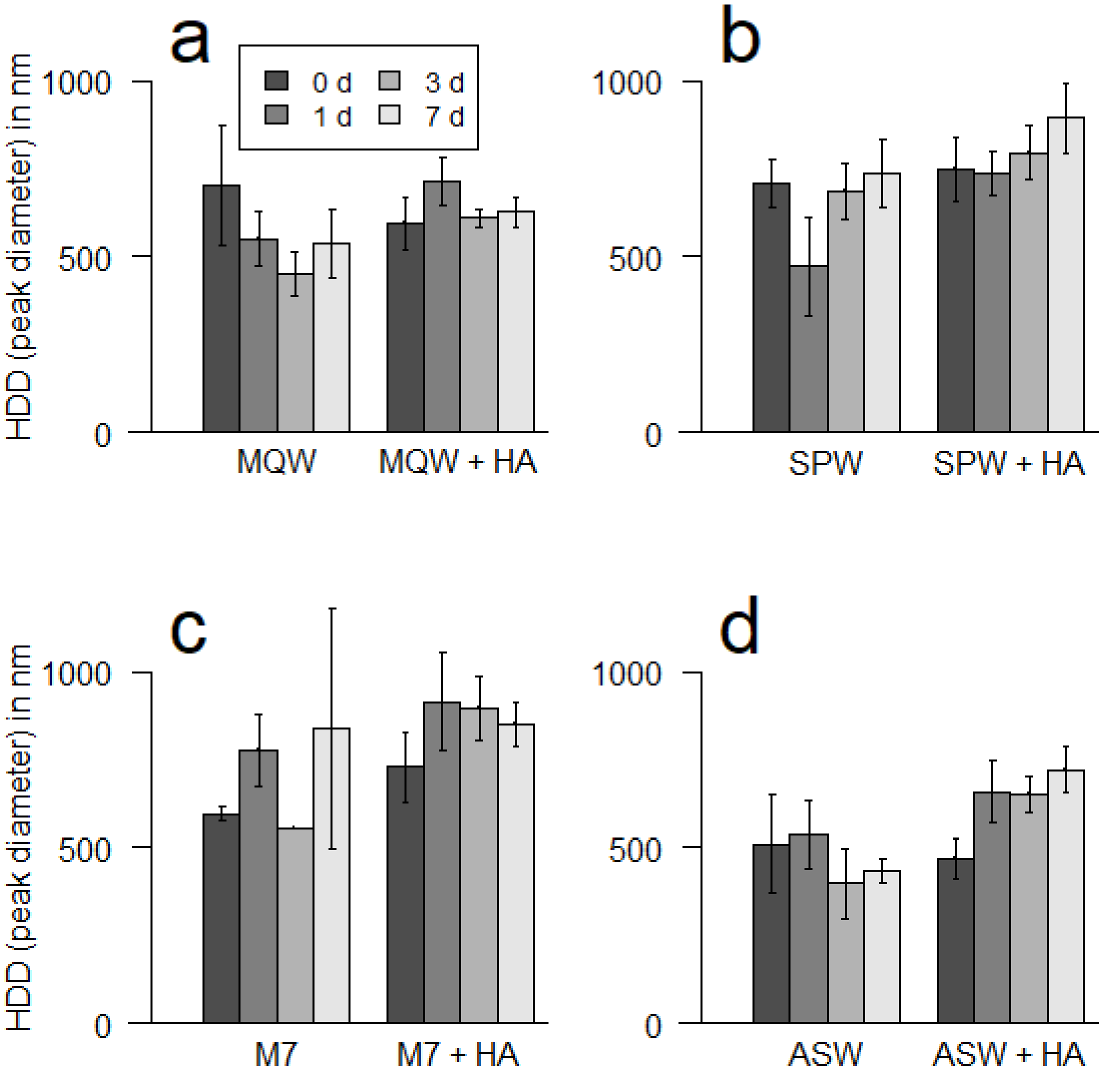


Fig. S9: Mean peak diameter analysed by NNLS ± SE (n=4) in nm for **(a)** MilliQ water (MQW), **(b)** soil porewater (SPW), **(c)** fresh water (M7) and **(d)** artificial seawater (ASW) with or without HA at four time points.

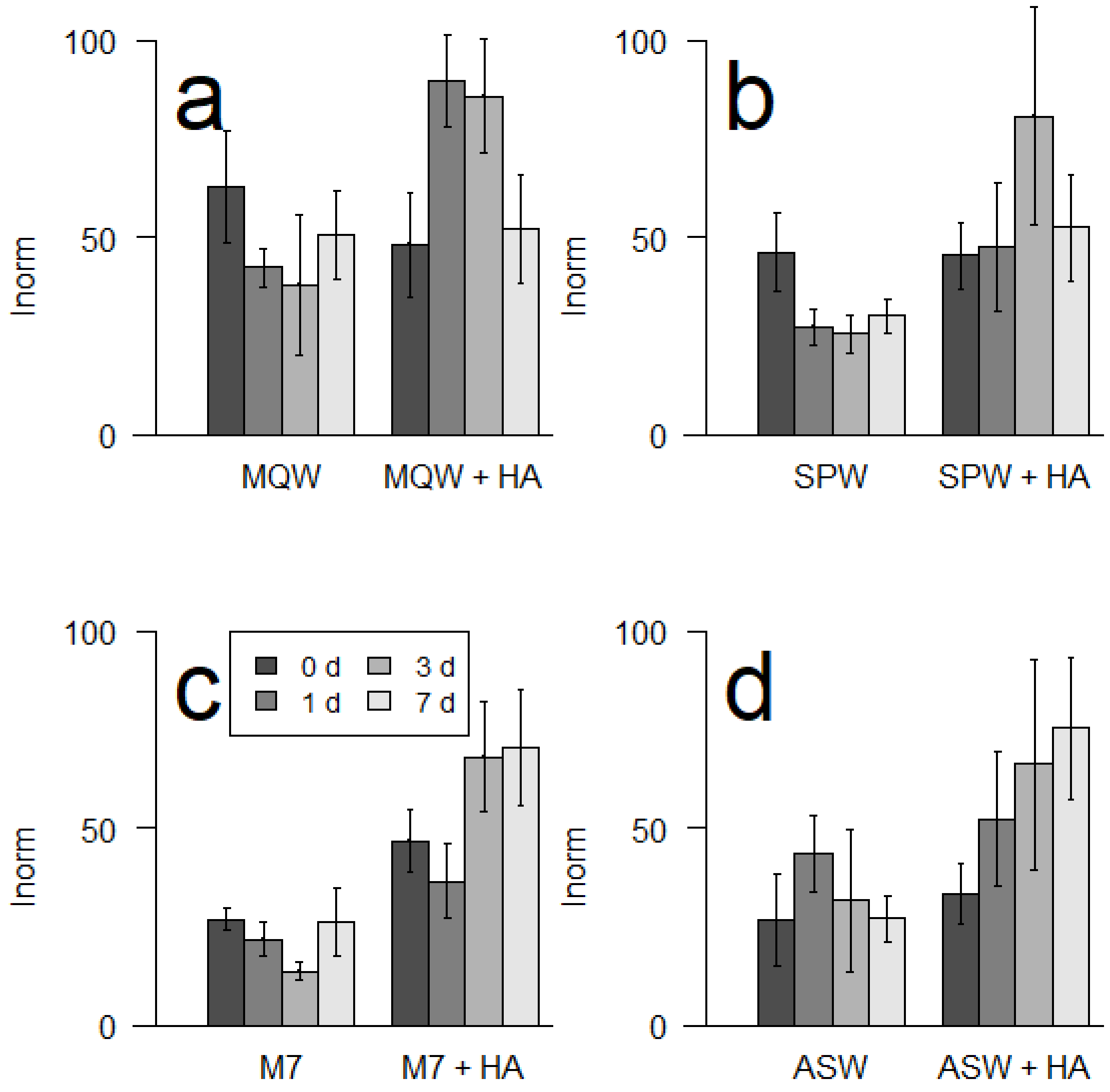


Fig. S10: Mean normalised intensity (Inorm) ± SE (n=4) for **(a)** MilliQ water (MQW), **(b)** soil porewater (SPW), **(c)** freshwater (M7) and **(d)** artificial seawater (ASW) with or without HA at four time points.

Supporting information Chapter 3

Table S3: p-values of statistical analysis of growth and bioaccumulation assay. Data of all four time points were analysed with a linear mixed effect model where the communities of 30 animals in each replicate at the beginning of the test, from which animals were removed repeatedly, were set as random factor to account for time repeated measure.

Soils	**Treatments**			**Transformation**
	s-5	**n-5**	**n-158**	
		Growth		
S	0.999	0.867	0.999	-
L	0.945	0.363	0.163	^-1
LA	0.299	0.002 (**)	0.383	^0.4
LO	0.998	0.999	0.020 (*)	^0.4
		Cu body content		
S	<0.001 (***)	<0.001 (***)	<0.001 (***)	^0.1
L	0.283	0.970	0.874	^0.1
LA	0.051 (.)	0.008 (**)	0.003 (**)	^0.4
LO	0.003 (**)	0.016 (*)	<0.001 (***)	^-0.5

Table S4: We calculated the theoretical increase in soil Cu content in mg/kg after periodic Cu-based fungicide application. In our assumptions, the application rates of 4 and 1.6 kg Cu/ha/yr relate to the maximum allowed application rate in the EU and the average application in German organic horticulture in the years 2010-2015. For the calculations, spray drift towards soil, i.e. the fraction which will directly end up there, was estimated to be 30%, whereas field data on that topic strongly vary(Berenstein et al., 2017; Cross et al., 2003; Herrington et al., 1981). Further, a homogenous distribution of all applied Cu over a 10 cm deep soil layer with a bulk density of 1kg SDW/L was assumed. Wash-off from leaves for Cu-based compounds was reported with 2%(Kah et al., 2019) and, therefore, is neglected here.

Application rate ha-1 yr-1	Duration of application		
	1 year	5 years	20 years
	Soil Cu increase in mg/kg		
4.0 kg	1.20	5.99	23.94
1.6 kg	0.48	2.38	9.50

Method S1: Methodology of Cu field soil concentration analysis

We evaluated selected studies from different regions of the world to get a rough idea of common soil Cu concentrations for different land use types. The investigated regions or countries were Nordrhein-Westfalen (Landesumweltamt Nordrhein-Westfalen, 2003) and Schleswig-Holstein in Germany (Kuhnt et al., 2011), Austria (Freudenschuß et al., 2007), Hungary (Rékási and Filep, 2012), Greece (Vavoulidou et al., 2005), Europe (Ballabio et al., 2018), Chile (De Gregori et al., 2003), Florida in USA (Fan et al., 2011) and Victoria in Australia (Pietrzak and McPhail, 2004). From these studies all mentioned soil Cu median concentrations were extracted for the land use types arable land, forest, grassland and vineyards/orchards, regardless of sample and test field size. Further, the results of two meta studies summarizing soil Cu concentrations in worldwide vineyards and orchards were included (Komárek et al., 2010; Lamichhane et al., 2018).

Method S2: Materials and methods of pore water and total soil Cu analysis

For pore water analysis, soils prepared for growth and bioaccumulation assays were aliquoted to 20 g dry soil equivalent (n=5) and aged for three weeks at 16 °C in the dark. After that, soils were brought to 100% water holding capacity by adding miliQ water and shaken shortly. After 24 h of equilibration, soil pore water was extracted by a method based on Topuz and van Gestel(Topuz and Gestel, 2017) with slight deviations. Soil samples were transferred into 20 ml plastic syringes with a bottom of glass wool which were put into 50 ml centrifuge tubes. The tubes were centrifuged for 30 min at 4.000 g so that the pore water was extracted through the syringe whole and collected in the centrifuge tube. 100 µl of the collected pore water were sampled for Cu analysis. For total Cu soil content, a method based on Pedersen and van Gestel (2001) was applied with slight deviations. 1 g of the three weeks aged dry soil (n=3) was mixed with 5 ml of 52% HNO_3 (Merck, Darmstadt, Germany) and digested at 65°C for 2 hours with shaking steps every 30 min. 100 µl of sample was taken and diluted in 1% HNO_3, if necessary. Cu concentrations in all samples were analysed by graphite furnace atomic absorption spectroscopy using a Unicam989QZ AA Spectrometer (Unicam, Cambridge,UK) with a GF-90 plus furnace and an FS-90 plus autosampler.

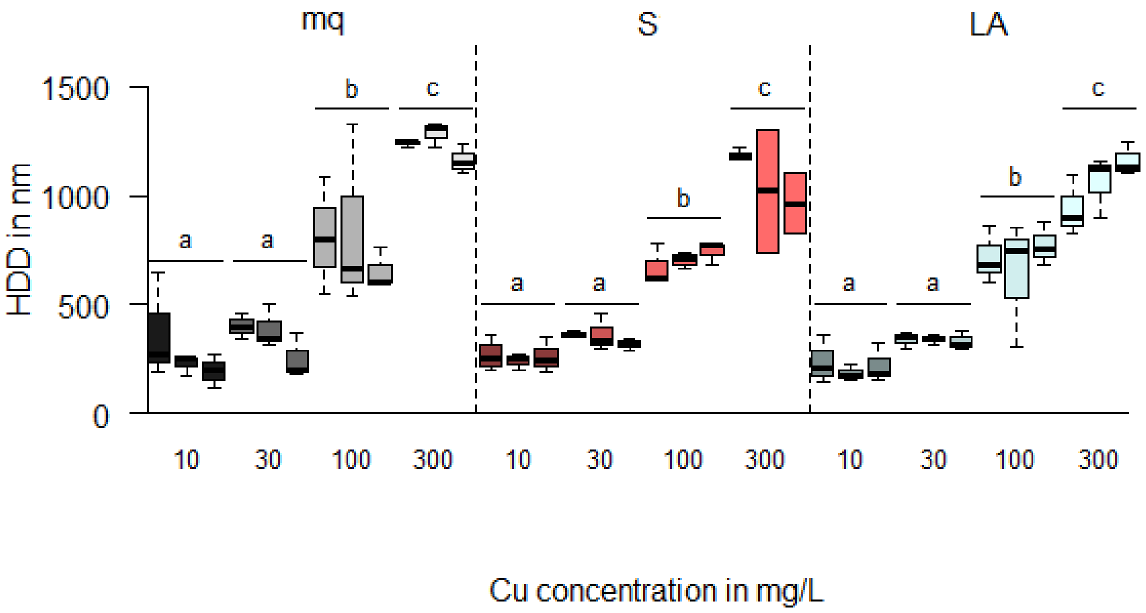


Fig. S11: Hydrodynamic diameter of CuO-NP dispersed in miliQ water (mq) and eluates of the test soils S and LA at concentrations between 10 and 300 mg Cu/L. The peak diameters analysed by NNLS are displayed. Three boxplots of the same colour show results of three time points 0, 24 and 48 hours after spiking from left to right. Letters a-c indicate significant difference between concentrations within one medium ($p<0.05$, Tukey test after linear mixed effect model)

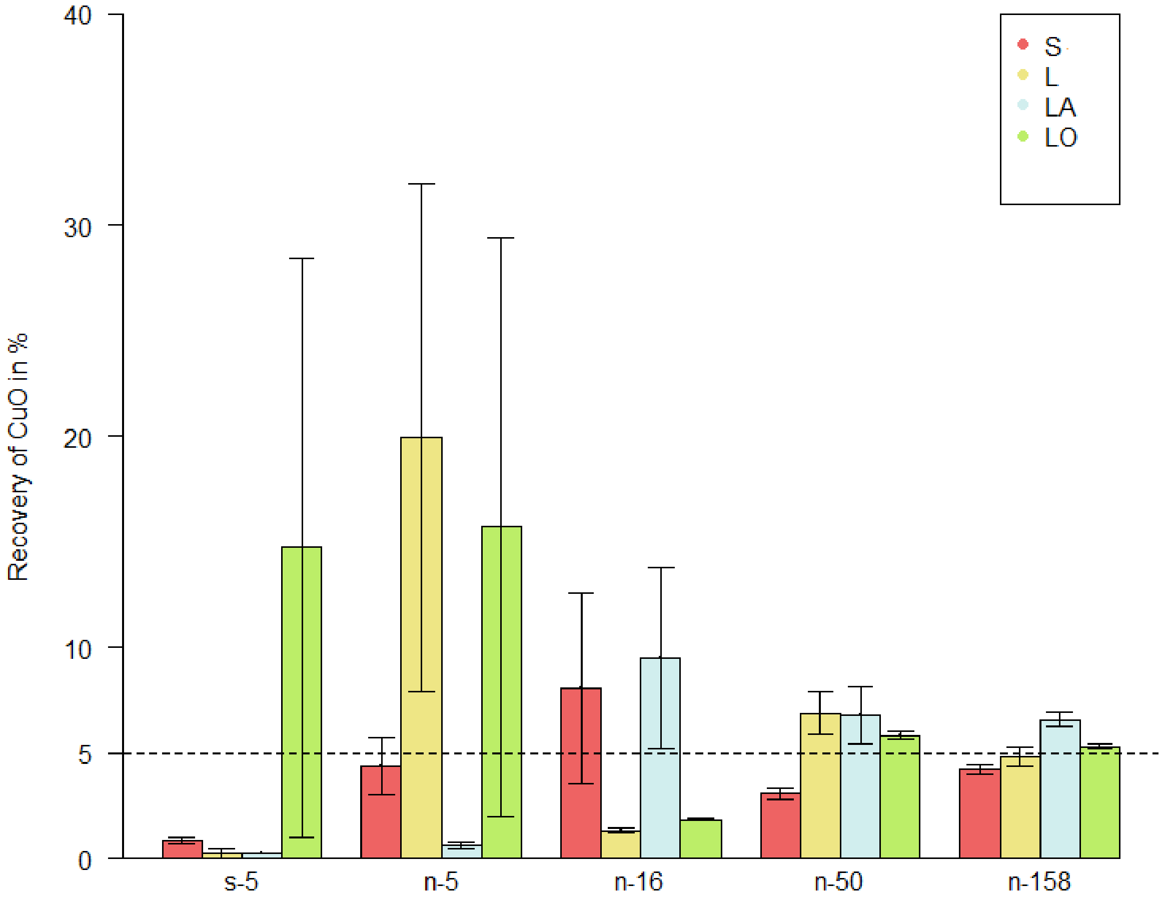


Fig. S12: Total recovery of CuO from test soil eluates by sp-ICP-MS in %. The mass of detected particles was derived from the measured diameter by calculating its volume, assuming spherical particles, and multiplying it with the density of CuO (6.31 kg/L). The accuracy of this calculation method was stated with a pure CuO-NP dispersion, ending up with a recovery rate of app. 100%. However, due to this calculation method based on CuO particle size and density, the recovery had to be based on CuO rather than Cu, and, in terms of comparability, also had to be applied to s-5 treatment. Cu^{2+} concentration was measured by sp-ICP-MS.

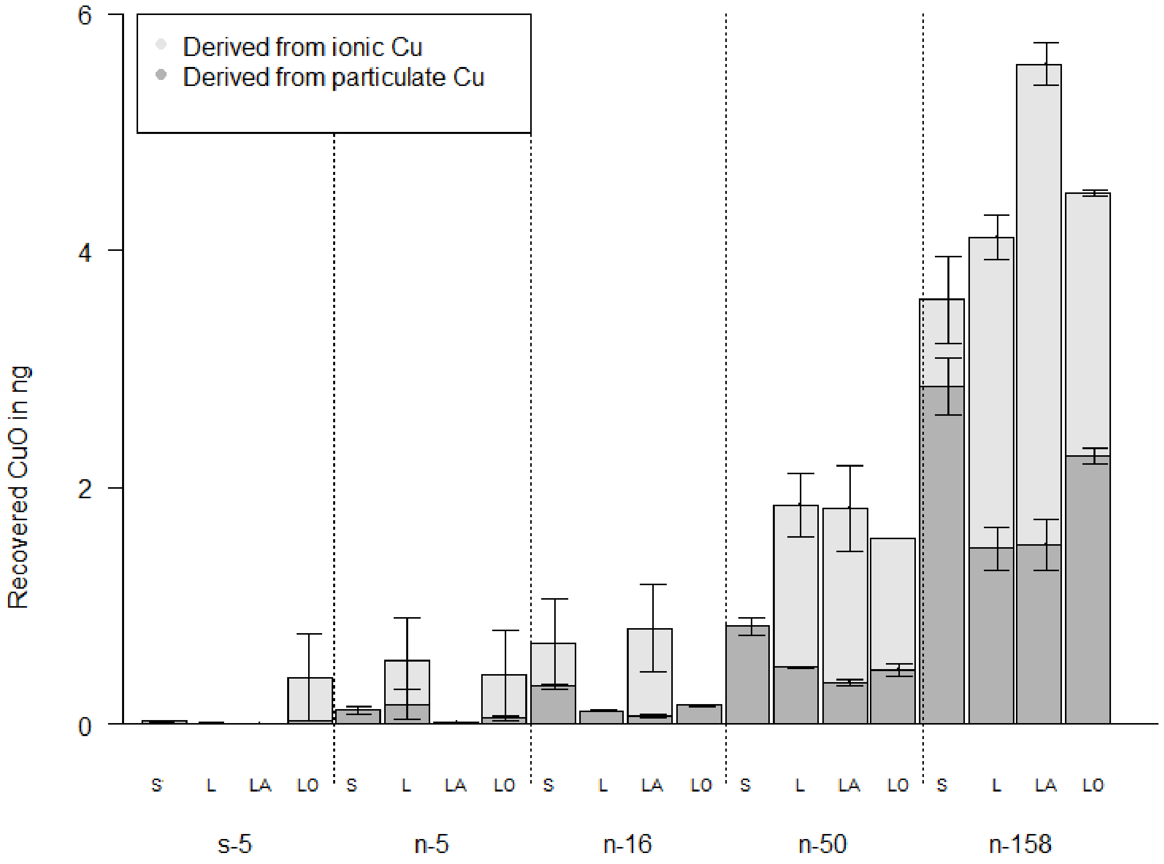


Fig. S13: Total amount of recovered CuO from test soil eluates by sp-ICP-MS in ng. Particle mass was derived from diameter of detected particles by calculating volume and mass, assuming pure, spherical CuO-NP. The accuracy of this calculation method was stated with a pure CuO-NP dispersion, ending up with a recovery rate of app. 100%. Cu^{2+} concentration was measured by sp-ICP-MS and recalculated into the CuO mass it originates from. Due to the calculation method based on CuO particle size and density, the recovery rate had to be based on CuO rather than Cu, and, in terms of comparability, also had to be applied to s-5 treatment.

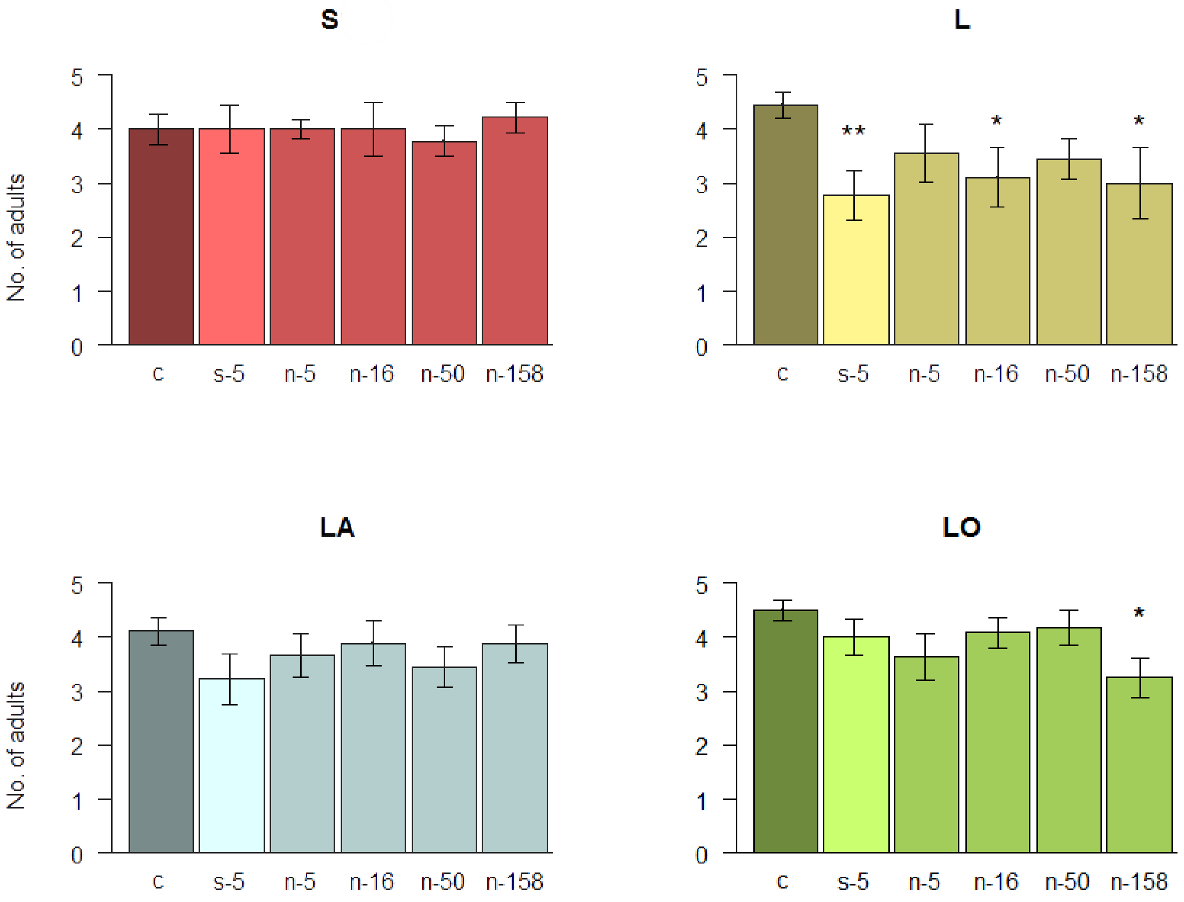


Fig. S14: Survival of *F. candida* adults after 28 days in four different soils: sandy soil S1, loamy soil L, loamy-acidic soil LA and loamy-organic soil LO. Five adults are introduced at the beginning of the test. The mean numbers of recovered adults at test end ± standard error of 3-4 tests (each n=3) are shown for each treatment, ending up with a total number of replicates of n=9 (S1, L and LA) and n=12 (LO). Small letters indicate the applied substance (c=control, s=$CuCl_2$, n=CuO-NP) and numbers the respective test concentrations in mg Cu/kg SDW. Asterisks indicate a significant difference towards the control (*: $p<0.05$, **: $p<0.01$, $p<0.001$), whereas the dot indicates a trend ($0.05<p<0.1$, Dunnett-test after nested ANOVA).

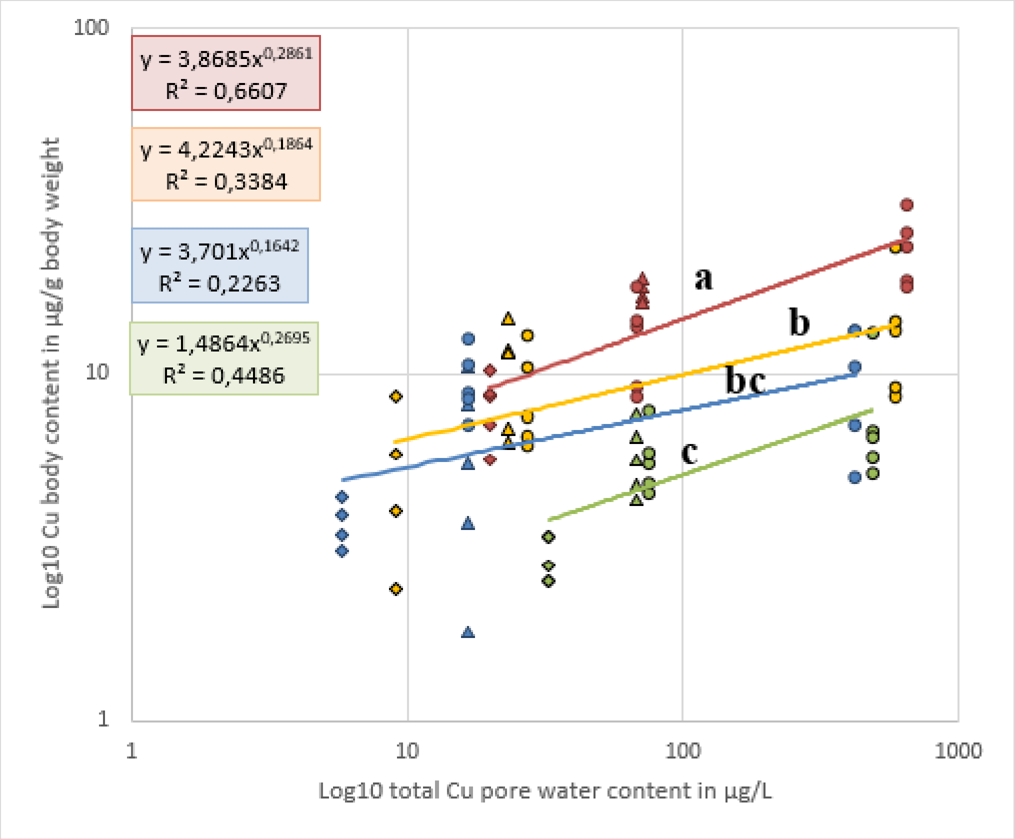


Fig. S15: Bioaccumulation of Cu in *F. candida* after 14 days in relation to mean pore water Cu content (n=5) at the same time point. Cu body contents of *F. candida* in three test soils are shown: sandy S soil (red), loamy L soil (yellow), loamy-acidic LA soil (blue) and loamy-organic LO soil (green). ◊=control, Δ=$CuCl_2$, ○=CuO-NP. Formula of trend lines are given in the respective coloured boxes. Data were compared with respect to test soil and total Cu pore water content by a glm and a Tukey post-hoc test. Soils which do not share a common small letter are significantly different.

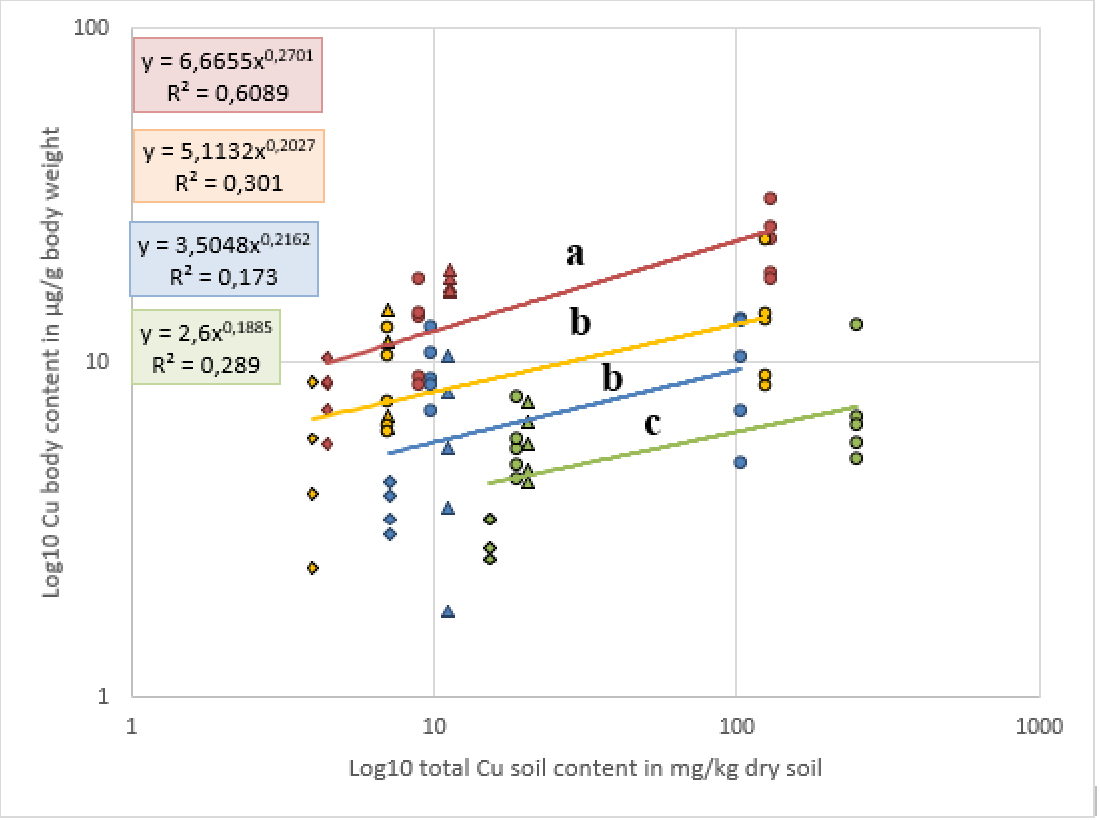


Fig. S16: Bioaccumulation of Cu in *F. candida* after 14 days in relation to mean total soil Cu content (n=3) at the same time point. Cu body contents of *F. candida* in three test soils are shown: sandy S2 soil (red), loamy-acidic LA soil (blue) and loamy-organic LO soil (green). ◊=control, Δ =$CuCl_2$, ○=CuO-NP. Formula of trend lines are given in the respective coloured boxes. Data were compared with respect to test soil and total Cu soil content by a glm and a Tukey post-hoc test. Soils which do not share a common small letter are significantly different.

Supporting information Chapter 4

Table S5: Absolute mean values ± SE of the control treatments in the reproduction assays (survival, reproduction, dry weight) and the biomarker assays (catalase activity, Metallothionein levels) for all four test soils

	Test soils			
Endpoints	**K19**	**K30**	**M19**	**M30**
Survival in number of adults (n=5)	4.2 ± 0.3	5 ± 0.0	4.4 ± 0.2	4.2 ± 0.6
Reproduction in number of juveniles (n=5)	357 ± 36	322 ± 22	412 ± 27	280 ± 6
Dry weight in µg/individual (n=5)	67 ± 11	42 ± 4	93 ± 12	46 ± 3
Catalase activity in µmol H2O2min/mg protein (n=3)	-	342 ± 25	-	399 ± 12
Metallothionein level in nmol MT/mg protein (n=3)	-	53 ± 16	-	96 ± 31

Table S6: Odd ratios of the likelihood to exceed ordinal limits LOD or LOQ for the factors “substance”, “test soil” and “number of exuviae”

Factors	Odd ratios	CI 2.5 %	CI 97.5 %
Substance NP	0.6017693	0.20462126	1.7750443
Substance S	0.2728979	0.08508081	0.8564579
Soil M30	4.9265389	2.30378768	11.0360181
No. exuviae	1.0631072	1.00631211	1.1242383

The Cu contents of exuviae were categorised in three groups separated by the limit of detection (LOD) and and the limit of quantification (LOQ) , i.e. <LOD, <LOQ and >LOQ, and analysed by ordinal logistic regression with the MASS package (Ripley et al., 2002). Out of the model coefficients for the factors soil, substance and number of collected exuviae the odds for exceeding one of the limits, holding constant all other factors, were calculated. Further details on this type of evaluation can be found here:

https://stats.idre.ucla.edu/r/dae/ordinal-logistic-regression

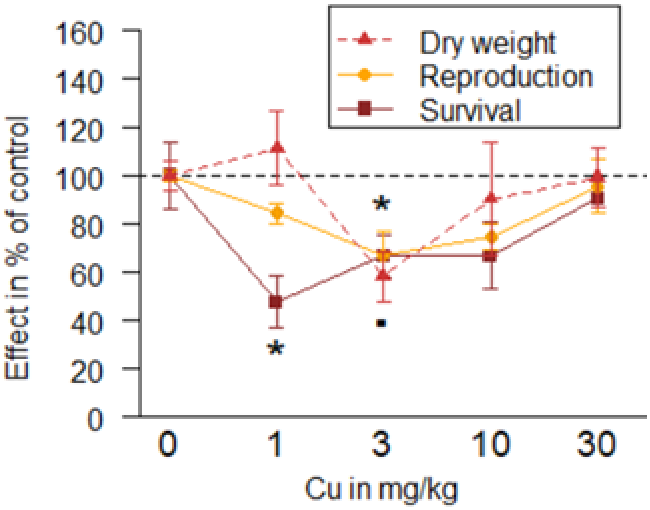


Fig. S17: Mean effects in % of control for the survival, reproduction and dry weight of *F. candida* exposed to CuO-NP in M30 soil. Significant differences to the control analysed by glm with $0.01<p<0.05$ are marked by an asterisk, trends with $0.05<p<0.1$ by a dot.

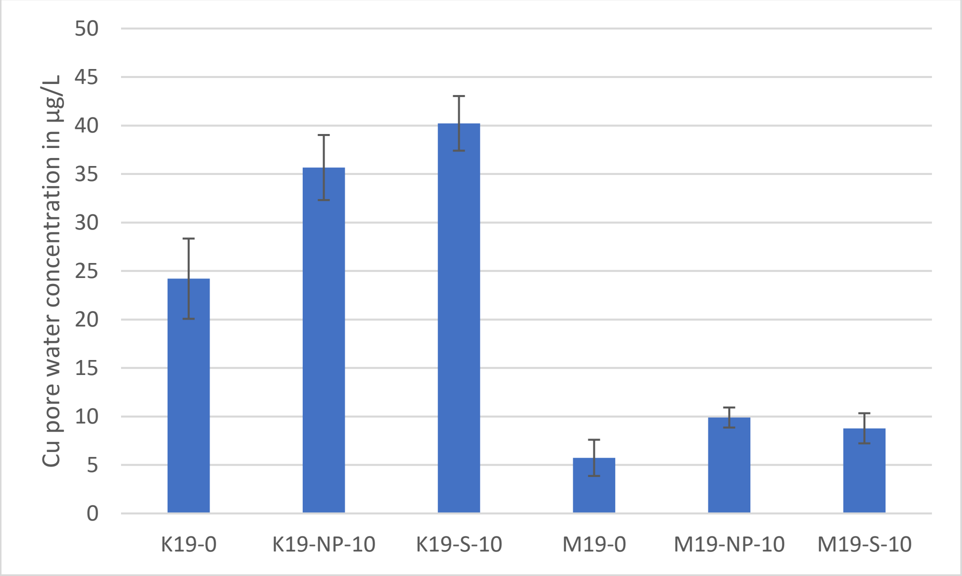


Fig. S 18: Mean Cu pore water concentration ± SE of the test soils K19 and M19. Shown treatments are the control, CuO-NP- and $CuCl_2$ spiked soils with 10 mg Cu/kg, respectively (NP-10/S-10). n=3 for controls and n=9 for all Cu treatments.

[redacted]

Fig. S19: XRD scan of montmorillonite K10. The arrow indicates the metallic Fe peak in accordance with Santangelo et al. (2011).

Method S3: XRD measurements

XRD measurements were done with a Bruker D8 Discover diffractometer (Bruker, Billerica, USA) equipped with a Cu-tube (k_α 1.541, 40 kV, 40 mA), a fixed divergence slit of ¼° , a 90 samples changer, a monochromatisation via energy discrimation on the highest resolution, Linxeye detector system. The measurements have been done as a continuous scan from 5 – 70° 2θ, with a calculated step size of 0.016° 2θ. Mineral identification has been done by means of the Philips software X'Pert HighScore™ Vs. means of the Philips software X'Pert HighScore™ Vs. 1.2 (Degen et al., 2014) and identification of sheet silicates have been done with the freely available Apple MacIntosh X-ray diffraction interpretation software MacDiff 4.25 (http://www.geologie.uni-frankfurt.de/Staff/Homepages/Petschick/Rainer.html#MacDiff) freeware (Petschick et al., 1996). This was followed by full quantification of mineral assemblage of the bulk fraction via the QUAX full pattern method (Vogt et al., 2002)

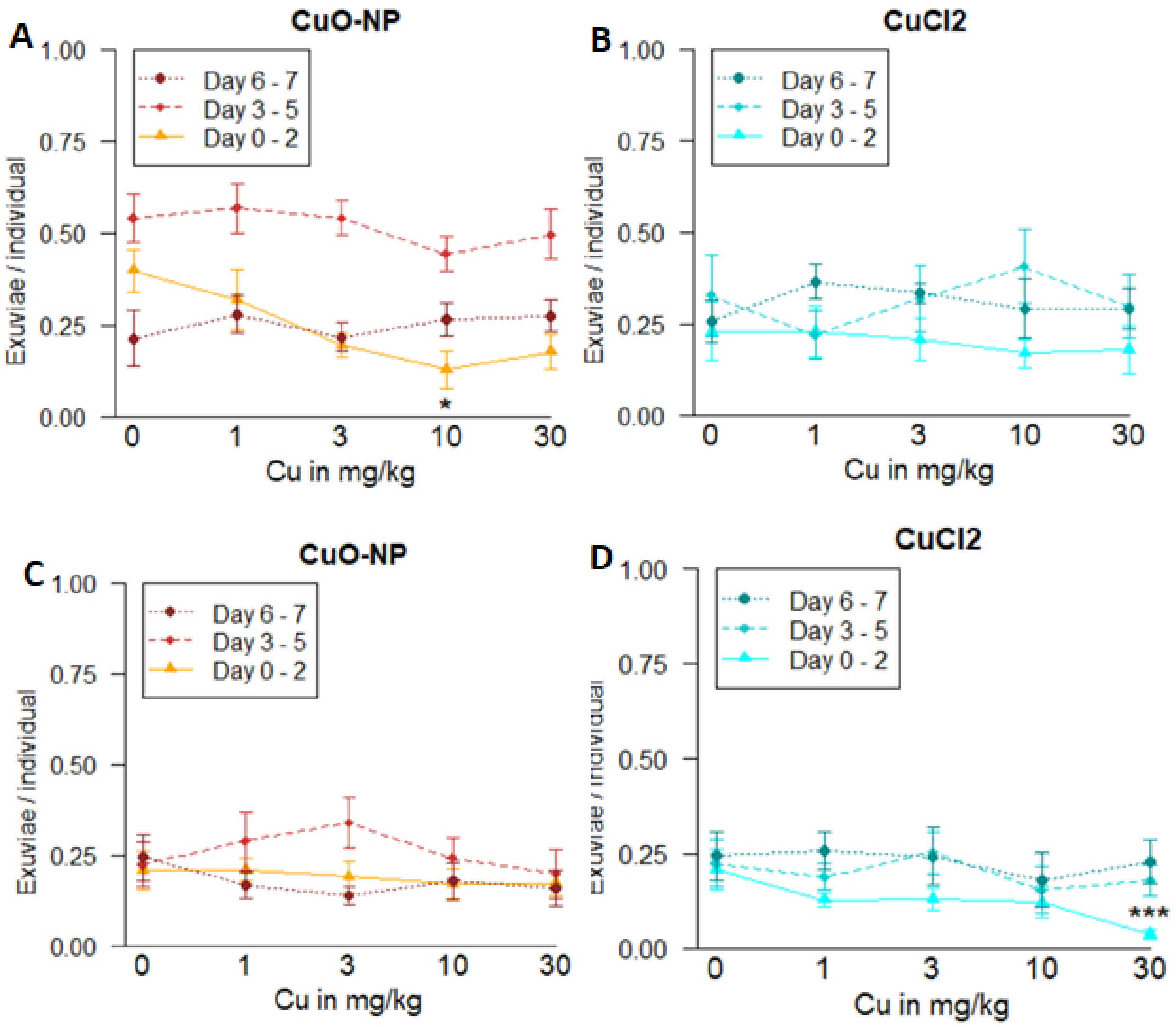


Fig. S 20: Mean number of shed exuviae per individual of *F. candida* ± SE (n=10). Springtails were exposed either in K30 (A, B) or in M30 soil towards CuO-NP (A, C) or $CuCl_2$ (B, D). Exuviae were collected in three periods within one week, ranging from days 0-2, 3-5 and 6-7 after exposure start. Significant differences to the control analysed by glm are marked by asterisks: *= 0.01<p<0.05, ***=p<0.001.

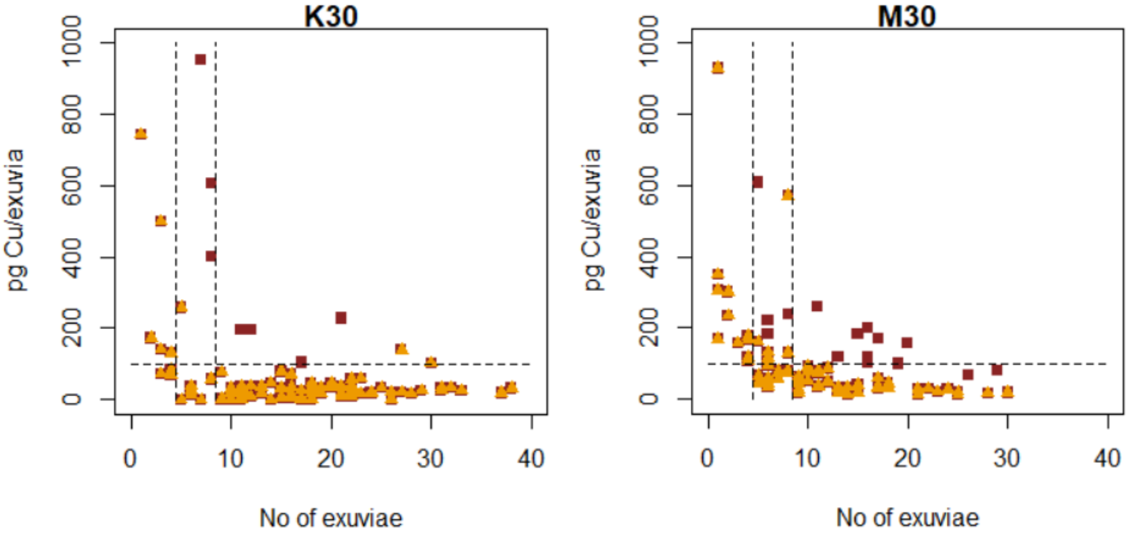


Fig. S 21: Relation between the number of collected exuviae and the Cu content of a sample in pg Cu of *F. candida* exposed in either K30 or M30 test soil (all treatments included). Vertical lines indicate the limits which were chosen in Fig S22 with <5 and, in Fig. 13 of the main text, <9 exuviae, respectively. The horizontal line indicates a Cu content of 100 pg/exuvia for optical orientation. Dark red points are data points with Cu measurements being above LOQ, while for orange orange points they are <LOQ.

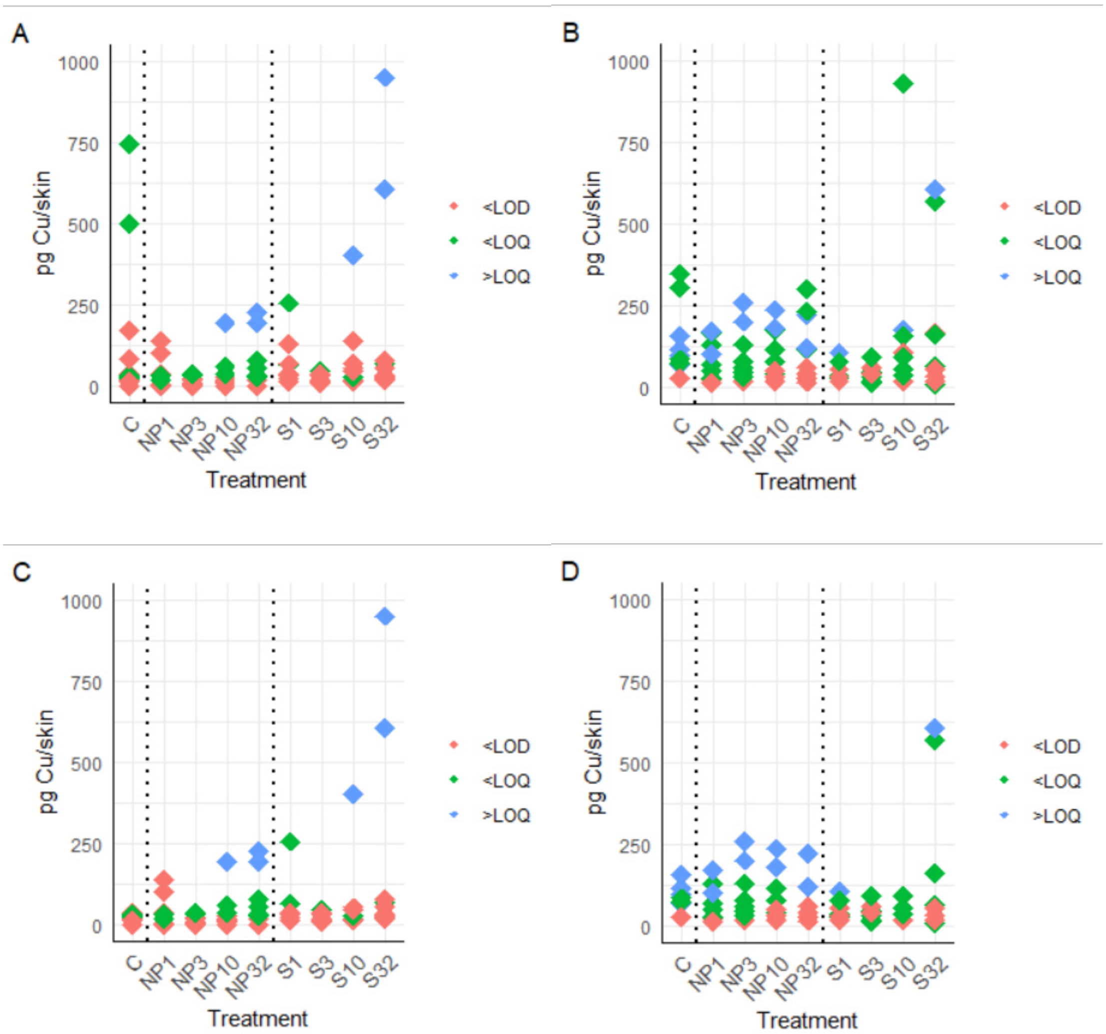


Fig. S 22: Cu content ± SE (n=10) of exuviae from springtails in pg Cu/skin exposed towards CuO-NP (NP) or $CuCl_2$ (S), in kaolin soil K30 (A, C) or montmorillonite soil M30 (B, D) with consideration of all samples (A, B) and only samples containing >4 exuviae (C, D). Cu contents are based on measured values >LOQ (blue), <LOQ and >LOD (green) and <LOD (red). For a comparison to data containing >8 exuviae, see Fig. 13 in the main text.

The Figs. S21 and S22 show that the limitation of data points to a minimum number of exuviae reduces the number of outliers. The high Cu content/exuviae probably origins rather from contaminations and/or are arithmetically based as measured values are divided by the number of exuviae which is smaller in these samples.

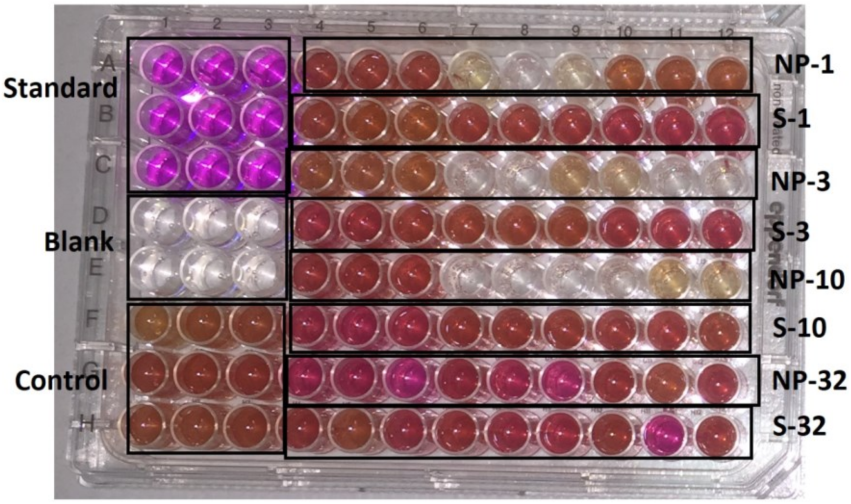


Fig. S 23: Test plate of CAT activity assay for M30 soil, including the standard without any H_2O_2, the blank without $KMnO_4$ and all treatments with three replicates measured in triplicate each

Supporting information Chapter 5

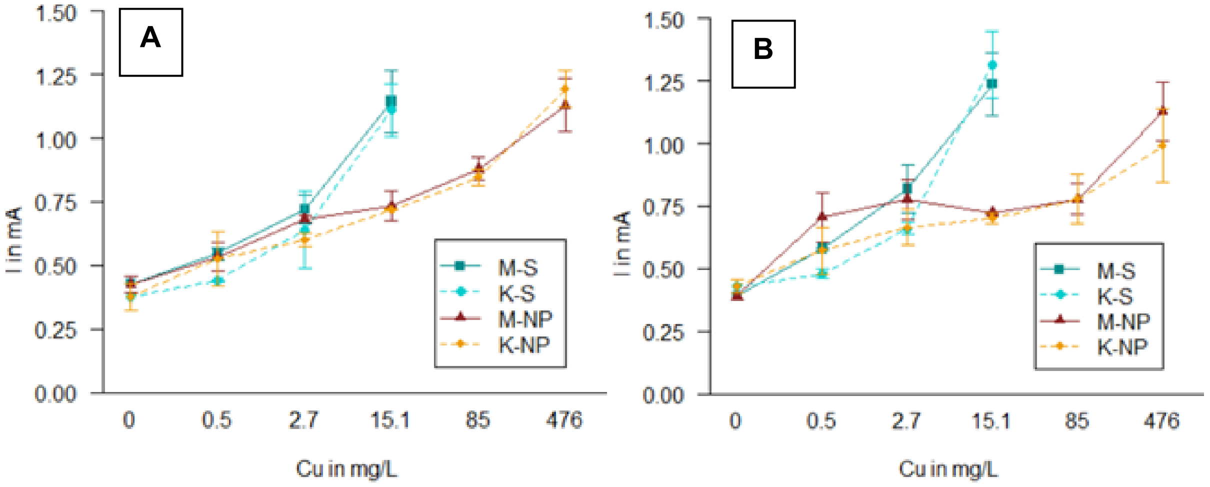


Fig. S24: The current in mA produced by Cu-clay mixtures at different Cu concentrations. Clay types were applied at different concentrations so that the provided surface areas of clays was comparable. A) c(kaolin) = 100 mg/L and c(montmorillonite)= 8,6 mg/L B) c(kaolin) = 300 mg/L and c(montmorillonite) = 26 mg/L. Jorg Thöming from University of Bremen was involved in development of methodology.

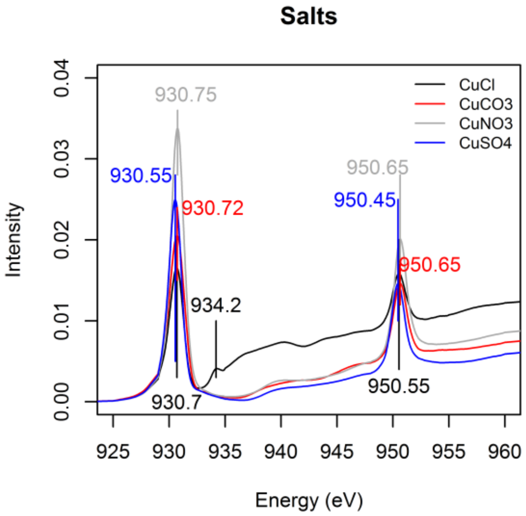


Fig. S25: XANES spectra of four Cu salts. The graphs were created by Simon Blotevogel, Institut National des Sciences Appliquées de Toulouse, France.

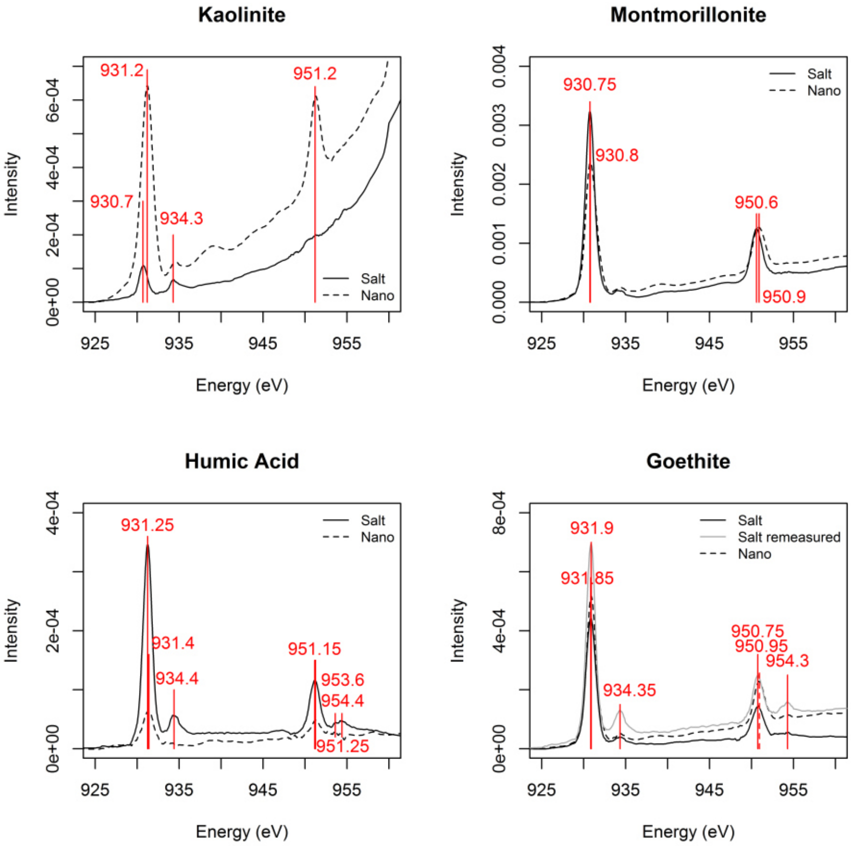


Fig. S26: XANES spectra of CuO-NP (nano) and Cu(NO$_3$)$_2$ adsorbed to four environmental substances. The graphs were created by Simon Blotevogel, Institut National des Sciences Appliquées de Toulouse, France

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Publikationsliste

Fischer, J., Gräf, T., Sakka, Y., Tessarek, C., & Köser, J. (2021). Ion compositions in artificial media control the impact of humic acid on colloidal behaviour, dissolution and speciation of CuO-NP. *Science of The Total Environment*, *785*, 147241.

Fischer, J., Evlanova, A., Philippe, A., & Filser, J. (2020). Soil properties can evoke toxicity of copper oxide nanoparticles towards springtails at low concentrations. *Environmental Pollution*, *270*, 116084.

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Konferenzbeiträge

Fischer, J., Talal, G. D. A. & Filser, J. Clay type matters: toxicity and bioaccumulation of copper oxide nanoparticles to springtails in artificial soils. SETAC Europe 31st Annual Meeting SETAC SciCon from 03 – 06 May 2021, oral presentation.

Fischer, J., Evlanova, A., Philippe, A. & Filser, J. Low concentrations of copper oxide nanoparticles are toxic in three loamy soils. SETAC Europe 30th Annual Meeting SETAC SciCon from 03 – 07 May 2020, poster presentation.

Fischer, J., Evlanova, A., & Filser, J. High clay content in soil increases toxicity of low concentrated CuO-NP towards springtails. 9th International Conference on Nanotoxology (NanoTox 2018), 18 - 21September 2018 in Neuss/ Germany, poster presentation.

Schreiber, B., **Fischer, J.,** Petrenz, M., Monka, J., Schiwy, S., Hollert, H., Schulz, R.: Weatherfish embryos (*Misgurnus fossilis*) as potential alternative for sediment toxicity testing. Fish and Amphibian Embryos as Alternative Models in Toxicology and Teratology (Paris, France), 2016.

Schreiber, B., Korte, E., Monka, J., Petrenz, M., **Fischer, J.,** Hundt, M., Schmidt, T., Stoll, S., Gergs, R., Schulz, R.: Untersuchungen zur Wiederansiedlung des Schlammpeitzgers (*Misgurnus fossilis*). DGL-Tagung (Vienna, Austria), 2016.

Schreiber, B., Petrenz, M., **Fischer, J.,** Gergs, R., Hollert, H., Schulz, R.: Das Gefährdungspotential von sedimentassoziierten Schadstoffen für eine bedrohte benthische Fischart - Bedroht der Schlamm den Schlammpeitzger (*Misgurnus fossilis*)?. DGL-Tagung 2015 (Essen, Germany), 2015.

Schaumann, G., Peikert, B., Tamini, N., Steinmetz, Z., **Fischer, J.,** Bibus, D., Marei, A., Dag, A.: Effect of climatic conditions on the development of soil water repellency in soils treated with the wastewater of the olive oil production. EGU General Assembly (Vienna, Austria) 2014.

Antrag auf Zulassung zur Promotion

Hiermit beantrage ich, Jonas Fischer, geb. am 2.4.1988 in Bad Mergentheim, die Zulassung zur Promotion zur Erlangung des Grads Doktor der Naturwissenschaften (Dr. rer. nat.) durch den Fachbereich 2 der Universität Bremen.

Zudem erkläre ich, dass ich mich bisher noch keinem weiteren Promotionsvorhaben unterzogen habe und dass ich eine Überprüfung meiner Dissertation mit qualifizierter Software im Rahmen der Untersuchung von Plagiatsvorwürfen gestatte.

Diese Arbeit wird in Form einer kumulativen Dissertation eingereicht. Für die Kapitel 2, 3 und 4, welche als wissenschaftliche Publikationen bei einer Fachzeitschrift veröffentlicht bzw. eingereicht wurden, ist mein Eigenanteil an den jeweiligen Publikationen in Form eines CRediT authorship statements am Kapitelende kenntlich gemacht.

Ort, Datum Unterschrift

Versicherung an Eides Statt

Ich, Jonas Fischer, geboren am 2.4.1988 in Bad Mergentheim (Matr. Nr.: 3082395), versichere an Eides Statt durch meine Unterschrift, dass ich die vorstehende Arbeit selbständig und ohne fremde Hilfe angefertigt und alle Stellen, die ich wörtlich dem Sinne nach aus Veröffentlichungen entnommen habe, als solche kenntlich gemacht habe, mich auch keiner anderen als der angegebenen Literatur oder sonstiger Hilfsmittel bedient habe.

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Declaration on the contribution of the candidate to a multi-author article/manuscript which is included as a chapter in the submitted doctoral thesis

Chapter: 2

Contribution of the candidate in % of the total work load (up to 100% for each of the following categories):

Experimental concept and design: ca. 25 %
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Preparation of Figures and Tables: ca. 100 %
Drafting of the manuscript: ca. 80 %

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