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In *vivo* and *vitro* studies of Cu-based nanoparticle toxicity in invertebrate worms: A review

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Abstract
Nanotechnology has been progressively developed recently and used extensively in all disciplines. These nanoscale particles overpower the use of conventional technological metal particles. Applications of Cu nanoparticles in agriculture enhance production and soil fertility, albeit their usage in excess amounts causes toxicity for soil fauna. So, we studied and collated the toxicity research trends of copper nanoparticles in the worm's species (earthworms and enchytraeids) and their activities to assess the consequences of copper nanoparticles in varied sizes and oxidation states. Various Cu NPs have a high capacity for adsorbing biomolecules and interacting with biological receptors. Cu NPs can interact with the host organism's inherent immunity and impair the host's immune system when confronted with different dose concentrations. These artificially induced nanoparticles interpret the biological cell system and manipulate cell receptors *in situ*. Nations all across the world are currently attempting to establish a global policy on the regulation of nanomaterials as per their ecological safety. In some cases, they have been reported to be more hazardous than the comparable ions and micromaterials. As a result, nanoparticle safety research has far-reaching ramifications for national economies. This study will be extremely significant in regulating the environmental outcome of nanoparticles.

Keywords
Nanotechnology; Nanoparticles; Copper nanoparticles; Ecotoxicity; Worms

Introduction

Nanotechnology is the branch of material science concerned with particles or materials having one dimension in the range of nanometers. The nano size of the particle enhances the surface area and efficiency of the nanoparticles compared to their bulk counterparts. Different types of NPs (nanoparticles) or NMs (nanomaterials) have diverse industrial applications such as optics, automobiles, paint and coatings, medical, military, and agriculture. Nanoparticles have recently gained importance as competent agrochemical agents by improving crop production and soil fertility *via* enhancing nutrient uptake efficiency and reducing pests. When these nano-formulations are released in the environment to target a particular species to curb the pests, in turn, they not only kill pests but adversely affect flora and fauna of soil by entering into agro-ecosystems via spraying, washing, leaching, and attacking the non-targeted species in soil biota. Worms are the essential components of soil biota and are in direct contact with the upper layer of soil. Hence, it has a high potential for exposure to nanoparticles deposited into the soil through the trophic level and accumulated in worm tissues. Nanomaterials have the capability to

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pass the cellular barriers and interact directly because of their smaller size, structure, and surface charges. Terrestrial organisms are more subjected to nanoparticle interaction as approximately 8-28% of overall nanoparticle production is consumed in the soil (Keller et al., 2013). The Organisation for Economic Cooperation and Development (OECD) considers worms (Oligochaeta) as a terrestrial indicator for assessing physical and chemical changes in the terrestrial ecosystem. The wide-spreading practice of nano pesticides in the agricultural sector has made researchers contemplate toxicokinetics in worms. Copper nanoparticles or nanomaterials (Cu NPs or Cu NMs) have novel applicability in plastic/polymer, metallic coating, ink, as an additive oil, and sensors (Bouvy et al., 2007; Khangarot et al., 2022; Lin, 2007), batteries (Wang et al., 2009), as a coolant, adsorbent in environmental remediation and fungicides (Cioffi et al., 2004 & 2005; Park et al., 2007; Ren et al., 2009; Wang et al., 2008), soil remediation, fertilisers, plant growth regulators and food additives as well as packaging materials (Batsmanova et al., 2013; Llorens et al., 2012; Zhu et al., 2012). Usually, terrestrial and aquatic systems, along with plants, are vulnerable to copper toxicity, for instance, algae, fungi, crustaceans, worms, and fishes etc. (Aruoja et al., 2009; Braz-Mota et al., 2018; Katsumiti et al., 2018; Ruiz et al., 2015). In agriculture, Cu-based nano pesticides (e.g. Kocide) have been used extensively at a commercial scale to curb unwanted pests and pathogens (Anjum et al., 2015; Shahid and Khan, 2017). In this review paper, we have summarised the recent trends in aspects of the toxicity of copper nanoparticles (Cu NPs), copper oxide nanoparticles (CuO NPs), and copper nanowires (Cu NWs) to long tubular worms within the soil matrix and compiled the consequences of aforementioned nanoparticles in comparison with their salts and bulk counterparts to assess a thorough interpretation of Cu NPs ecotoxicological impression on soil invertebrates. Lately, much research has been published on Cu NPs and their impact on different species of soil invertebrates. Predominantly used annelidan species were *Eisenia fetida, Eisenia andrei, Lumbricus terrestris, Metaphire posthuma, Enchytraeus albidus, and Enchytraeus crypticus* (Fig. 1), which inhabit upper edaphic strata in an agro-ecosystem. Enchytraeus are the chief faunal species of soil (Rombke and Moser, 2002). In contrast, earthworms are sentinel species also named 'ecosystem engineers' due to their unique decomposing ability, and are both found in abundance in soil and have remarkable contributions in the refinement of soil pore structure by consuming organic matter or litter on upper soil layer (Amorim et al., 2005b). We have encapsulated the toxicological assay of copper nanoparticle exposure on various worm species at behavioural, physiological, cellular, molecular, and genetic levels.

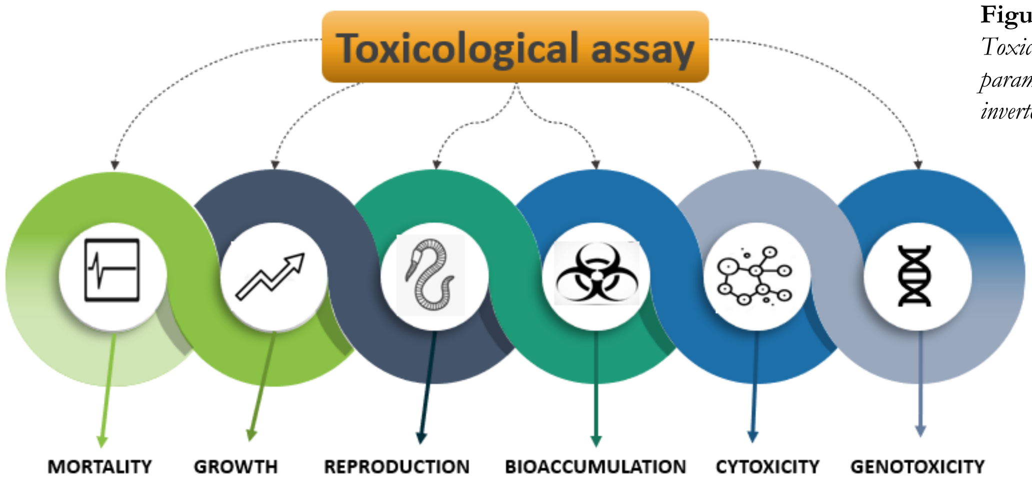

Figure 1
Toxicological end parameters studied on invertebrate worms

Apical toxicity endpoints (Growth, Mortality, Reproduction, and Histology)

This review paper summarises the trends of ecotoxicity caused by Cu and CuO NPs on the growth, mortality, survivability, reproduction, and histology of invertebrates. OECD guidelines (2004) were followed for reproduction, acute, and subchronic lethality of terrestrial oligochaetes (Lumbricidae and

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Enchytraeidae). The standard test followed for *Eisenia sp.* is test no. 207 for acute toxicity for the duration of 48 hrs and extended up to 72 hrs and test no. 222 for reproduction for the period of 28 days. *Enchytraeus* reproduction test (ERT) test no. 220 for the period of 28 days is followed in the case of enchytraeid species. These invertebrates' growth, mortality, survivability, and reproduction are affected by test conditions such as test soil, test species, dose concentration, nanoparticle size, reactivity and dissolution, and exposure duration. We have summarised the available data in Table 1 and Figure 2. Unrine and his colleagues (2010) spiked the soil with various-sized Cu NPs (<100 nm and 20-40 nm) upto 65 mg/kg and $CuSO_4$ at concentrations up to 20 mg/kg concentrations. They found 90% survivability of earthworm sp. *Eisenia fetida* in all experimental set up of Cu NPs. Only in 20 mg kg^{-1} of Cu NPs (<100 nm), 87% survival was recorded. They concluded no significant toxicity was observed due to $CuSO_4$ or Cu NP exposure at concentrations ranging up to 20 mg Cu kg^{-1} soil as $CuSO_4$ and up to 65 mg Cu kg^{-1} soil as Cu NP based on subchronic endpoints (Unrine *et al.* 2010). Another study conducted in the same year by different researchers revealed the toxic effects of inorganic (Ag, Al_2O_3, Cu, Ni, SiO_2, TiO_2 and ZrO_2 NPs and their salt analogues ($AgNO_3$, $CuCl_2$ and $NiCl_2$) on the earthworm sp. *Eisenia fetida* was dosed with 1000 mg/kg of dry soil for 28 days. The author and Co-workers found that Ag, Cu, and TiO_2 nanoparticles all caused more toxic effects on *Eisenia fetida*; $AgNO_3$, $CuCl_2$, and $NiCl_2$ metal salts interrupted the surviving ability of earthworms. Furthermore, reproductive stress was found in earthworms exposed to Ag, Cu, and TiO_2 NPs and $AgNO_3$, $CuCl_2$, and $NiCl_2$ metal salts; Ag NPs and $AgNO_3$ (100%) completely hamper the reproduction whereas, Cu NP and $CuCl_2$ halt the reproduction in worms, but $CuCl_2$ was more toxic than Cu NPs (Heckman et al., 2011). The study on the toxicokinetic depicted the effects of used conventional engine oil (uCEO), used nano engine oil (uNEO), virgin conventional engine oil (vCEO), virgin copper nano engine oil (vNEO) sized 6–20 nm by conducting filter paper test and artificial soil test as per OECD guidelines in the range 3×10^{-3}, 24×10^{-3} ml/cm^2 and 0.1 mg/kg, 100 g/kg, respectively on earthworm sp. *Eisenia fetida*.

The mortality results for 48 hrs LC_{50} found for all four engine oils were at concentrations 6×10^{-3}, 23×10^{-3}, 24×10^{-3}, and 16×10^{-3} ml/cm^2 in the following order: vCEO > uNEO > vNEO > uCEO, and LC_{50} for artificial soil test were at a concentration more than 100 g/kg. Fragmentation, bleeding, and separation were clearly observed in all the earthworms treated with engine oils except used conventional engine oil (uCEO). Histopathological results depicted epidermal separation, necrosis, and nerve leukomalacia for all four engine oils. Among all engine oils, virgin copper nano engine oil (vCEO) was found highly toxic (Khodabandeh et al., 2011). A similar study in the later years was conducted on the effects of copper nanoparticle-enriched new engine oil and conventional engine oil in earthworm sp. *Eisenia fetida* by contact filter paper and artificial soil test. The test substance i.e., conventional and new engine oil (enriched with nano copper or without) applied to worms during filter paper test was in the following ratio with chloroform as solvent: 2-0, 1.75-0.25, 1.5-0.5, 1.25-0.75, 1-1, 0.75-1.25, 0.5-1.5, 0.25-1.75, 0-2 whereas, 1, 10, 100, 1000, 10000, and 100000 ppm solutions were mixed with artificial soil for 7 and 14 days experiment period.

The results illustrated from the experimental setup inferred that new engine oil and conventional engine oil enriched with nanoparticles cause higher toxicity, and new engine oil enriched with nanoparticles at a concentration of ≥1.25ml was found to be more lethal (Armand et al., 2019). The toxicological assay of CuO and ZnO NPs on mortality and reproduction of *Eisenia fetida* tested at a dose concentration of 0, 0.4, 0.8, and 1.2 g/kg in two different mediums, i.e., cow manure and spent mushroom compost (SMC) for 7 and 14 days. They found a reduction in cocoon production, weight and reproduction and no mortality at the end of the test period. However, in the SMC medium, the worm tissue was damaged (Alahdadi et al., 2015). Similar results as in the previous studies were concluded in this study: Researchers investigated the toxicity assay of CuO and ZnO engineered nanoparticles (ENPs), CuO and ZnO micro-particles, and $CuCl_2$ and $ZnCl_2$ salts on *Eisenia fetida* in aged sandy loam and silt loam with 10 mg/kg spiked concentration.

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Results represented no significant changes in mortality, survivability, reproduction, and behaviour except an increase in the weight of juveniles (Josko et al., 2021). Earthworm sp. *Eisenia andrei* was dosed with CuO NPs in the range of 98-149 mg/kg dry soil with or without biosolids amendment affected the reproductive activity (Velicogna et al., 2021). In this study, the possible effects of metal colloids of Cu, Ni, and Co NPs doped with Chitosan; bioassay was conducted on *Eisenia fetida* for 48 hrs that demonstrated 50% mortality at low concentration (Trivino et al., 2017).

The potential effects of CuO NPs sized less than 100 nm were examined by authors on *Eisenia fetida* at a concentration of 100-300 mg/ml (5 doses at an interval of 50) by direct contact method. Their investigation exhibited that average mortality was not dose-related, whereas weight loss was observed at the end of the exposure period. Furthermore, their findings supported the dissolution of CuO NPs in water as a crucial factor in earthworm mortality assessment (Pavani et al., 2018). Researchers conducted an artificial soil toxicity assay of Cu and CuO NPs on earthworm sp. *Eisenia fetida* for 14 days duration at a dose concentration of 0, 50, 100, and 500 mg/kg of dry soil. In this paper, the author and his co-worker assessed the mortality and biomass in earthworms. The mortality rate was found higher for Cu NPs as compared to CuO NPs with 70% and 20%, respectively; reduction in worm mass on the 7th and 14th day of the experiment amounted to 45% and 28% only at the highest concentration of Cu NPs and CuO NPs in the soil; although CuO NPs also triggered the weight loss at 50 mg/kg dose concentration by 21.3% against the background. Overall, Cu NPs stimulated more toxicity as compared to CuO NPs (Lebdev et al., 2020). Some studies on *Enchytraeus sp.* also displayed the toxicity of Cu and CuO NPs on growth, reproduction, and survivability. The authors studied the toxicity effects of Cu NPs and $CuCl_2$ salt on the survival, reproduction, and avoidance behaviour of *Enchytraeus albidus*. They demonstrated that reproductive activity was reduced and avoidance behaviour of worms' was enhanced after exposure. Reproductive and avoidance behaviour for Cu NPs ($EC_{50\text{-reprod}}$=95 mg/kg and $EC_{50\text{-avoid}}$ =241 mg/kg) was more effective than $CuCl_2$ ($EC_{50\text{-reprod}}$=251 mg/kg and $EC_{50\text{-avoid}}$=475 mg/kg). Hence, Cu NPs were found toxic compared to $CuCl_2$ salt (Amorim *et al.* 2012a). The author and his coworkers investigated the toxicity of different Cu forms such as Cu salt [$Cu(NO_3)_2$], Cu NPs (80nm) and Cu-field (80 years ago) in soil-dwelling worm sp. *Enchytraeus crypticus*. The results showed that Cu salt [$Cu(NO_3)_2$] was found highly toxic to the reproductive functionality of worms and least affected by Cu-field when compared. XANES (X-ray absorption near-edge spectroscopy) study disclosed that Cu was found in oxidation state II in Cu-field and $Cu(NO_3)_2$, whereas Cu released from Cu NPs was in 0, I, and II oxidation states (Gomes et al., 2015b). Researchers investigated the toxicity effects of CuO NMs released into the environment from sewage sludge and $CuCl_2$ on *Enchytraeus crypticus* through the reproductive test (ERT) and full life cycle test (FLCt) as suggested in OECD guidelines. Their study displayed that CuO NMs affected the growth and development of juvenile worms, whereas $CuCl_2$ interfered with embryo development, hatching, and survivability of adults. When both ERT and FLCt were compared, FLCt was preferred as it clearly demarcated various effects between all life stages and enhanced reproductive actions for CuO NMs such as effective concentration (EC_{10}) at 8 mg/kg and EC_{10} at 421 mg/kg, respectively. In this paper, a full life cycle test was suggested to assess NM toxicity over the Enchytraeus reproductive test (Bicho et al., 2017a). A similar group of researchers studied the effects of CuO NMs and $CuCl_2$ on *Enchytraeus crypticus* for a year (F1 to F7 generations, wherein 4 generations in dosed soil and 2 in control soil) to investigate the multigeneration by ERT (EC_{10} and EC_{50}) on worms' survival and reproduction. In this study, they demonstrated that CuO NMs induced toxicity in worms to EC_{10} exposure, whereas no significant changes were found for EC_{50}, and continued the similar effects in control soil in F7 generation. $CuCl_2$ exposure reduced the EC_{10} and EC_{50} toxicity; however primary effects were resuscitated in control soil in F7 generation (Bicho et al., 2017b). The author and his co-workers studied the reproduction (ET_{50}= effect time) and survival (LT_{50}= lethal time) of *Enchytraeus crypticus* through a lifespan test for the period of 202 days on exposure to CuO NPs and $CuCl_2$ against the background (control). They demonstrated that survival [LT_{50}: 218 days (control) > 175 days ($CuCl_2$) > 145 days (CuO NPs)] and reproduction [ET_{50}: 158 days (control) > 138 days ($CuCl_2$) > 92 days (CuO NPs)] were affected prominently in case of CuO NPs than $CuCl_2$ against

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the background. Furthermore, they depicted that the torjan horse mechanism was responsible for higher Cu effects of CuO NPs (Goncalves et al., 2017). Similar two studies on *Enchytraeus crypticus* were published: first revealed the effects of CuO NMs and $CuCl_2$ for EC_{10} and EC_{50} and concluded that CuO NMs were responsible for the reduction in some metabolites in contrast to $CuCl_2$, and later one displayed the effects of Cu^{2+} released in the soil from CuO NPs and $Cu(NO_3)_2$ remarkably inhibited the growth and reproduction (Maria et al., 2018a and Ma Jun et al., 2020). Researchers and his colleagues set up an experiment to demonstrate the toxicokinetics of two Cu NMs (spherical and wire-shaped) and Cu salts ($CuNO_3$ and Cu salt field contaminated) on *Enchytraeus crypticus* for the period of 3 and 7 days. In this paper, they examined the reproductive activity at EC_{20} and EC_{50} and displayed that Cu Nwires obstructed the genitalia development and reproduction by disturbing the male gamete generation; $CuNO_3$ impeded locomotion due to worm's avoidance behaviour; Cu salt-aged also influenced the reproductive system (differently than Cu Nwires) during the exposure period (Gomes et al., 2018). The researchers evaluated the effects of Cu, CuO and ZnO NPs/bulk counterparts in both urban and artificial soils on earthworm sp. *Eisenia fetida.* Soils spiked in the ranged from 100-500 mg/kg Cu, 100-4000 mg/kg CuO, and 100-4000 mg/kg ZnO for 14 days to investigate the toxicity caused by different NPs/bulk counterparts in urban [1.5% soil organic matter (SOM)] and artificial soils (>35% SOM). Dose-related weight loss was noticeable in both soils (Mwaanga et al., 2017). The author and his co-workers exposed the *Eisenia fetida* with $CuSO_4$ and CuO ENMs with different coatings, i.e., CuO-core, CuO-polyethylene glycol, CuO-carboxylate, and CuO-ammonium by using two concentrations 200 and 1000 mg/kg spiked for 14 days against background concentration in fresh soil and aged soil. They observed that in fresh soil setup, there was no significant change found in mortality, survivability, and biomass for $CuSO_4$ and Cu ENMs at 200 mg/kg against control with the highest survival rate among all the ENMs with 20 ± 50% for CuO-carboxylate. However, significant mortality with a reduction in biomass was notable at 1000 mg/kg concentration of CuO-carboxylate and CuO-ammonium. Whereas, in aged soil, no effects were observed in $CuSO_4$ and CuO ENMs at 200 mg/kg, but a reduction in the survivability of worms was found at 1000 mg/kg for CuO-core and CuO-ammonium (Tatsi et al., 2018). Similar, comparative toxicity study of Cu NPs and $CuSO_4$ on earthworm sp. *Metaphire Posthuma* was performed for the duration of 7 and 14 days at 100, 500, and 1000 mg/kg concentrations in dry soil leading to the shrinkage of the worm's population in the wild (Gautam et al., 2018).

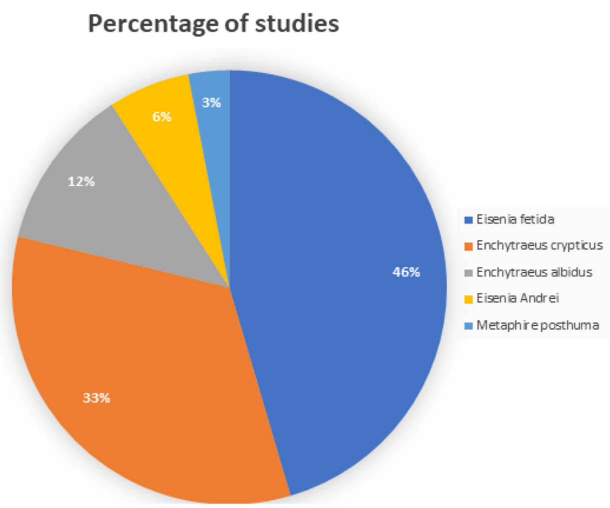

Figure 2. *Studies of Cu-based NPs on worm species*

Bioaccumulation

Copper is a micronutrient found in the body of various species. So, it is not surprising to detect Cu in the body of soil-dwelling tubular organisms. Here, we have summarised the findings of various researchers to track down whether worms accumulate Cu in their bodies when exposed to Cu and CuO NPs, Zn and ZnO NPs, salts, and bulk analogues. Alahdadi and co-workers investigated the Cu accumulation in *Eisenia fetida* when exposed to CuO NPs at the concentration of 0, 0.4, 0.8, and 1.2 g/kg in two substrates, i.e., cow manure and spent mushroom compost (SMC) for the period of 7 to 14 days, and found out that absorption and accumulation of CuO NPs in earthworm tissues were found less as compared to ZnO NPs in both the substrates, however, overall accumulation was found high in cow manure substrate (Alahdadi et al., 2015). A similar investigation was done by Mwaanga and his co-authors, and they assessed Cu accumulation in earthworm sp. *Eisenia fetida* after the exposure of Cu and CuO NPs/bulk counterparts in both urban and artificial soils. The dose concentra-

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tion administered to earthworms ranged from 100-500 mg/kg Cu NPs and 100-4000 mg/kg CuO NPs for 14 days to investigate accumulation in the tissues of earthworms caused by different NPs/bulk counterparts in urban [1.5% soil organic matter (SOM)] and artificial soils (>35% SOM). Earthworms accumulated NPs/bulk material in their tissues in both the soils, but more in the case of Cu and CuO NPs with no indicated difference (Mwaanga et al., 2017). Another group of researchers found that the accumulation of Cu ions in tissues of *Enchytraeus crypticus* from $Cu(NO_3)_2$ was more than CuO NPs (Ma Jun et al., 2020). Galaktionova and his co-researchers examined the Cu and Zn NP spiked soil for the degree of absorption, bioaccumulation, and rate of accumulation in earthworm sp. *Eisenia fetida* was administered with different dose concentrations such as 0, 50, 100, 200, and 400 mg/kg, and depicted that soil contaminated with Cu NPs had more accumulation potential than Zn NPs. Earthworms accumulated maximum Cu concentration at 100 mg/kg dose with 16.03 mg/kg followed by its reduction with increasing dose concentration such as at 200 mg/kg and 400 mg/kg with 2.83 mg/kg and 2.57 mg/kg, correspondingly. However, the accumulation rate and degree of absorption were 83.0% and 83.8%, respectively (Galaktionova et al., 2019). Another study by Josko and co-workers evinced the bioavailability and bioaccumulation of ENPs (CuO and ZnO), microparticles (CuO and ZnO), and metal salts ($CuCl_2$ and $ZnCl_2$) in two different soils: aged sandy loam and silt loam at 10 mg/kg concentration in earthworm sp. *Eisenia fetida*. Bioavailability of Cu and Zn in soil was extracted by H_2O, $MgCl_2$ with CH_3COONa or EDTA, among which EDTA was found highly effective (2.69-3.52 mg/kg Cu and 10.06-11.65 mg/kg Zn), while H_2O was least effective with 1.98-2.12 mg/kg Zn and 0.54-0.82 Cu mg/kg concentration; Bioavailability of Cu and Zn metals were found high in silt loam compared to sandy loam because of its high pH. Further, they also concluded that juveniles accumulated Cu and Zn metals (20.68-33.01 mg/kg for Cu and 75.82-90.53 mg/kg for Zn) in a higher proportion than adults (10.04-17.00 mg/kg for Cu and 69.07-83.75 mg/kg for Zn) regardless of the forms (nano, micro, and salts) (Josko et al., 2021). Tatsi and co-researchers exposed *Eisenia fetida* to CuO nanomaterials having four different chemical coatings, i.e., CuO-core, CuO-polyethylene glycol, CuO-carboxylate, and CuO-ammonium at a dose of 200 and 1000 mg/kg of soil for the period of 14 days, were compared to $CuSO_4$ in fresh and aged soil. In the case of aged soil, Cu accumulation was found less than in fresh soil. Cu accumulation in earthworm for lower (200 mg/kg Cu) dose concentration in fresh were found 0.29, 0.35, 0.74, 0.76, and 0.76 for $CuSO_4$, CuO-core, CuO-polyethylene glycol, CuO-carboxylate, and CuO-ammonium and in aged soil were 0.31, 0.24, 0.55, 0.54, and 0.46, respectively (Tatsi et al., 2018). Similar research on fate, toxicity, and bioaccumulation of CuO NPs and $CuSO_4$ on earthworm sp. *Eisenia andrei* in soil amended with or without biosolids was performed by Velicogna and his co-workers with the help of inductively coupled plasma mass spectrometry (ICP-MS) characterisation technique. The results indicated that the kinetic bioaccumulation factor (BAFk) was found at 2.31 in soil amended with biosolids and 1.12 without biosolids for $CuSO_4$; however, for CuO NPs, it was 0.80 and 0.81, respectively. Biota to soil accumulation factor (BSAF) was found to be -0.21-0.35 for CuO NPs, and bioaccumulation was found to be higher in the case of $CuSO_4$ than CuO NPs. Furthermore, bioaccumulation of CuO NPs in worm tissues was depicted by enhanced darkfield hyperspectral imaging (Velicogna et al., 2021).

Cytotoxicity

Many researchers found that engineered nanomaterials are transcutaneous in nature and cause cellular dystrophy and damage to the gut tissues in worms. Here are some research papers delineating the intracellular and intercellular transfer of NPs and their respective ionic forms. Gautam and his co-workers performed the comparative immunotoxicity of Cu NPs and $CuSO_4$ towards earthworm sp. *Metaphire posthuma* under three doses of 100, 500, and 1000 mg/kg in the soil for the duration of 7 and 14 days. They highlighted the immunological status of earthworms, phagocytic activity, and total count. Cell viability measured at the highest concentration for Cu NPs and $CuSO_4$ was found at 94.19±1.10% and 86.82±0.93%, respectively. Under all three concentrations, a complete reduction in the total coelomocyte count was recorded for 7 days period, and concentration-dependent effects in the case of 14 days exposure, i.e., 15.45±2.2 and $12.5\pm2\times10^4$

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cells/ml for CuO NPs and $CuSO_4$, respectively. Dose-dependent obstruction in phagocytic activity of worm against yeast particles *in vitro* for both Cu NPs and $CuSO_4$ were recorded as 21.5±2 and 14.67 ±2.58, respectively at 1000 mg/kg dose concentration for 14 days (Gautam et al., 2018). Tatsi and his co-workers exposed *Eisenia fetida* to CuO nanomaterial having four different chemical coatings, i.e., CuO- core, CuO- ammonium, CuO-carboxylate, and CuO- polyethene glycol at a concentration of 200 and 1000 mg/kg of soil for the period of 14 days and compared to $CuSO_4$ in fresh and aged soil. In the fresh soil experimental setup, sodium pump activity decreased in all four coated CuO NPs against control. Very little hypoplasia of mucous cells in the epidermal lining of earthworms was observed in some ENMs. Additionally, significant ionoregulatory disturbances were noticed in both cases (Tatsi et al., 2018). Ribeiro and his co-workers studied the effects of CuO NMs with a different surface coating, such as ascorbate, citrate, polyethyleneimine, and polyvinylpyrrolidone, compared to $CuCl_2$ on *Eisenia fetida* for the duration of 24 hrs. The core purpose of this research was to identify *in vitro* effects of the NMs' characteristics as to dispersibility and agglomeration on worms at the cellular level using flow cytometry and depicted that $CuCl_2$ was highly toxic among all followed by CuO-citrate NMs. In addition to this, a correlation was found between effective concentration (EC_{50}), hydrodynamic size, and characteristics of NMs (Ribeiro et al., 2019). A similar study of CuO NPs and their salt analogues on *Eisenia fetida* was conducted by Swart and his co-authors in soil for 28 days. Earthworms exposed to CuO NPs to assess their gut microbiome, immunity, and susceptibility against soil bacteria *Bacillus subtilis* showed that earthworms' gut microbiome was affected after the treatment and revealed zero susceptibility to bacteria (Swart et al., 2020a). Scientists like Pacheco and his co-workers examined the effects of CuO NPs on the cellular and subcellular levels of earthworm sp. *Eisenia andrei* at the concentration of 1, 10, and 100 μg/mL of Cu. CuO NPs at the concentration of 100 μg/mL of Cu reduced the amoebocyte subpopulation by 40%, and phagocytic activity of hyaline amoebocytes was decreased by 37%, and 25% after 6 and 24 hrs of exposure, respectively. Furthermore, they concluded that the Cu_2^+ ions released from the dissolution of CuO NPs played a major role in affecting the earthworms' coelomocytes (Pacheco et al., 2021). Cu NPs influenced the aging and cuticle formation pattern in *Enchytraeus crypticus* when exposed for 3 to 7 days (Gomes et al., 2018).

Enzymatic, Genetics and Transgenerational Toxicity

Extensive molecular-level effects of NPs on oligochaetes (earthworms and pot worms'), for instance, gene expression, oxidative stress, enzyme activity, and metabolomic responses, were also actively studied by many researchers. When *Enchtraeus crypticus* exposed to Cu (Cu-NPs, Cu-nanowires Cu-field, and $CuNO_3$) and Ag (Ag NM300K, Ag-NPs Non-coated, Ag-NPs PVP-coated and $AgNO_3$). They interfere with the worms' energetic status, such as lipid, carbohydrate, protein, and energy consumption (EC), which were investigated by cellular energy allocation (CEA). Results depicted that in the case of Cu, energy consumption, protein budget, and metabolic rate increased. This study also concluded that the size and shape of Cu NPs caused distinct effects on worms (Gomes et al., 2015a). In a similar study, no prominent effects were reported on *Enchytraeus albidus* after the exposure of Cu NPs and its salt against control for the duration of six weeks. The apparent reduction in energy reserves of worms *Enchytraeus albidus* was assessed after three weeks, whereas no obvious demarcation in worms' basal energy allocation was observed after six weeks. Furthermore, a reduction in carbohydrates was noticed (Amorim et al., 2012b). The comparative immunotoxicity study of Cu NPs and $CuSO_4$ towards earthworm sp. *Metaphire posthuma* under concentration 100, 500 and 1000 mg/kg dry soil medium for the duration of 7 and 14 days highlighted the immunological status of earthworms' enzyme activities [enzymes: superoxide dismutase (SOD), catalase (CAT), phenoloxidase, alkaline phosphatase, and acid phosphatase], and total protein. The 1000 mg/kg concentration of CuO NPs and $CuSO_4$ in earthworms for 7 days, significantly decreased the phenoloxidase activity of coelomocytes, and the highest reduction was recorded at 0.017±0.0014 and 0.01±0.0013 unit/mg protein/minute, respectively. The noteworthy reduction was contemplated in the activity of SOD and CAT when coelomocytes of earthworm treated with 1000 mg of CuO NPs and $CuSO_4$ for 14 days and for SOD ob-

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Table 1. *Studies of various parameters investigated and Cu- based NPs on selected oligochaetes*

S. No	Nanoparticle and Size	Faunal Species	Studied Parameter Cu-based	Reference
1.	Cu NPs (<100 nm and 20-40 nm)	*Eisenia fetida*	Growth, Mortality, Reproduction, and Accumulation	Unrine et al. 2010
2.	Cu NPs (80 nm)	*Eisenia fetida*	Limit test toxicity (range finding test)	Heckman et al. 2011
3.	Virgin copper nano engine oil (vNEO) and used nano engine oil (uNEO) (6-20 nm)	*Eisenia fetida*	Survival, Reproduction, Accumulation, and Enzymatic activity	Khodabandeh et al. 2011
4.	Cu NPs (80 nm)	*Enchytraeus albidus*	Survival, Reproduction, and Avoidance	Amorim et al. 2012a
5.	Cu NP (80 nm)	*Enchytraeus albidus*	Energy Basal Levels (protein, carbohydrates and lipids)	Amorim et al. 2012b
6.	Cu NPs (80 nm)	*Enchytraeus albidus*	Survival, Reproduction, Avoidance, and Gene expression	Gomes et al. 2012a
7.	Cu NPs (80 nm)	*Enchytraeus albidus*	Reproduction, Stress biomarker, and Enzymatic activity	Gomes et al. 2012b
8.	Cu Nanowires and Cu NPs (20-30 nm)	*Enchytraeus crypticus*	Cytotoxicity and Biomolecular response	Gomes et al. 2015a
9.	Cu NPs (80 nm)	*Enchytraeus crypticus*	Reproduction and Bioavailability	Gomes et al. 2015b
10.	CuO NPs (<60 nm)	*Eisenia fetida*	Growth, Mortality, Reproduction, and Accumulation	Alahdadi et al. 2015
11.	CuO NMs (9.3 nm)	*Enchytraeus crypticus*	Reproduction and Full life cycle test	Bicho et al. 2017a
12.	CuO NMs (9.3 nm)	*Enchytraeus crypticus*	Multigenerational test	Bicho et al. 2017b
13.	CuO NPs (9.3 nm)	*Enchytraeus crypticus*	Lifespan test	Goncalves et al. 2017
14.	Cu NPs (10 nm) and CuO NPs (20 nm)	*Eisenia fetida*	Survival, Bioaccumulation, and Oxidative stress biomarkers	Mwaanga et al. 2017
15.	Cu NPs (8-13 nm)	*Eisenia fetida*	Range finding and Bioassay	Trivino et al. 2017
16.	CuO NPs (40.38±3.6 nm)	*Metaphire Posthuma*	Cytotoxicity and Enzymatic activity	Gautam et al. 2018
17.	Cu NMs and Cu Nanowires (20 and 30 nm)	*Enchytraeus crypticus*	Survival, Reproduction, and Genomics	Gomes et al. 2018
18.	CuO NMs	*Enchytraeus crypticus*	Stress metabolome and Reproduction	Maria et al. 2018a
19.	CuO NMs	*Enchytraeus crypticus*	Stress response and Proteomic mechanism	Maria et al. 2018b
20.	CuO NPs (<100 nm)	*Eisenia fetida*	Growth, Mortality, and Reproduction	Pavani et al. 2018
21.	CuO ENMs (With different coatings i.e., polyethylene glycol, carboxylate and ammonium) (10-20 nm)	*Eisenia fetida*	Growth, Survival, Behaviour, Bioaccumulation, and Oxidative stress markers	Tatsi et al. 2018
22,	Cu NPs (Engine oil with Cu NP)	*Eisenia fetida*	Acute toxicity and Reproduction	Armand et al. 2019
23.	Cu NPs (50-100 nm)	*Eisenia fetida*	Growth, Mortality, and Reproduction	Galaktionova. et al. 2019
24.	CuO NMs (12 ± 8 nm)	*Eisenia fetida*	Cytotoxicity	Ribeiro et al. 2019
25.	CuO NPs (9.3 nm)	*Enchytraeus crypticus*	Reproduction and Epigenetic Changes	Bicho et al. 2020
26.	Cu NPs (80±9 nm) and CuO NPs (70±5 nm)	*Eisenia fetida*	Survival, Reproduction, Bioaccumulation and Antioxidant enzymes	Lebdev et al. 2020
27.	CuO NPs (<50 nm)	*Enchytraeus crypticus*	Reproduction, Growth, and Bioaccumulation	Ma Jun et al. 2020
28.	CuO NPs (20-50 nm)	*Eisenia fetida*	Immune responses and Genotoxicity	Swart et al. 2020a
29.	CuO NPs (20-50 nm)	*Eisenia fetida*	Survival, Reproduction, and Microbiome resilient	Swart et al. 2020b
30.	Cu NMs	*Enchytraeus crypticus*	Growth, Survival, Reproduction, Histology Immunohistochemistry, Epigenetic, and Stress responses	Bicho et al. 2021
31.	CuO NPs (100±25 nm)	*Eisenia fetida*	Reproduction and Bioaccumulation	Josko et al. 2021
32.	CuO NPs	*Eisenia andrei*	Growth, Mortality, Reproduction, Stress responses (protein, carbohydrates and lipids), and Genotoxicity	Pacheco et al. 2021
33.	CuO NPs (<50 nm)	*Eisenia andrei*	Reproduction and Bioaccumulation	Velicogna et al. 2021

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tained as 0.078±0.0029 and 0.055±0.0057 unit/mg protein/minute, and for CAT 0.0087±0.00019 and 0.0085±0.00014 K/mg protein, respectively. A dose-dependent inhibition was illustrated the acid phosphatase and alkaline phosphatase activity when tested with CuO NPs and $CuSO_4$ at 1000 mg/kg soil for 14 days; a maximum reduction recorded for acid phosphatase was 0.028±0.0028 and 0.022±0.0026 µm PNP/mg protein/minute and minimum for alkaline phosphatase was 0.019±0.0022 and 0.001±0.0027 µm PNP/mg protein/minute, respectively. A remarkable reduction in total protein content was documented as 0.113±0.0016 and 0.095±0.0015 mg protein/mL when spiked with CuO NPs and $CuSO_4$ at 1000 mg/kg soil for 14 days, individually (Gautam et al., 2018). Researchers and his team evaluated the ecotoxic effects of Cu and CuO NPs/bulk counterparts in both urban (1.5% SOM) and artificial soils (>35% SOM) on earthworm sp. *Eisenia fetida* at the concentration of 100-500 mg/kg Cu and 100-4000 mg/kg CuO for 14 days to investigate the oxidative stress such as superoxide dismutase (SOD), glutathione (GSH), and hydrogen peroxide (H_2O_2). In urban soils, SOD, GSH, and H_2O_2 increased initially with increasing concentrations and then decreased at relatively higher concentrations for both NPs, somewhat indistinguishable results were observed with less intensity for bulk counterparts. In urban soils (1.5% SOM) SOD activity initially increased with NPs (Cu and CuO) dose concentration up to a certain limit (200 mg/kg for Cu and 1000 mg/kg for CuO), then dropped down. However, no remarkable changes were observed for both NPs and their counterparts in artificial soils (>35% SOM). A similar trend was also observed for GSH and H_2O_2 activities in both urban and artificial soils (Mwaanga et al., 2017). Further earthworm sp. *Eisenia andrei* was investigated to evaluate the effects of CuO NPs at the concentrations of 1, 10, and 100 µg/mL of Cu. CuO NPs at 100 µg/mL of Cu elevated the level of lipid peroxidation and malondialdehyde by 10 folds after 6 hrs of exposure. However, the author and team illustrated that malondialdehyde might trigger DNA breaks, cell cycle arrest and apoptosis (Pacheco et al., 2021). The researchers depicted the toxic effects of Cu and Zn NPs on enzymatic activities of earthworms administered with different dose concentrations such as 0, 50, 100, 200, and 400 mg/kg in soil. Cu NPs increased the enzymatic activity at lower dose concentrations (50 and 100 mg/kg and decreased at higher concentrations (200 and 400 mg/kg). Cu NPs increased the urease activity at 50 and 100 mg/kg dose concentrations from 25% to 30.1%, respectively and a further increase in NP concentration reduced the urease activity. Catalase activity was reduced from 9.2 mL O_2 per 1g/1min in 50 mg/kg soil to 8 mL O_2 per 1g/1min in 400 mg/kg soil by X% and Y%, respectively. The peroxidase and invertase activities increased initially at 50 and 100 mg/kg Cu NP concentrations by 9.4%, 12.5%, 34.5%, and 27.6%, respectively. However, the polyphenol oxidase activity was decreased on the introduction of Cu NPs and decreased by 10.5% at 400 mg/kg dose concentration (Galaktionova et al., 2019). The authors exposed the *Eisenia fetida* to CuO NMs having four different chemical coatings, i.e., CuO-core, CuO-polyethylene glycol, CuO-carboxylate and, CuO-ammonium at a concentration of 200 and 1000 mg/kg of dry soil for the period of 14 days, and compared to $CuSO_4$ in fresh soil and aged soil (same soil after a year). In the fresh soil experimental setup, no change was observed in glutathione and superoxide dismutase activities in all treatments. In aged soil, no attributed effects were observed in glutathione activity (Tatsi et al., 2018). *Eisenia fetida* was exposed to CuO NPs in soil for 28 days and then subjected to *Bacillus subtilis* to assess its susceptibility against bacteria. The earthworms' immune responses were recorded by recording mRNA levels which revealed no effects of the treatment. A similar study by the same group of researchers demonstrated the toxicity of CuO and Ag NPs on *Eisenia fetida*'s gut microbiome bacteria *Candidatus lumbricincola* using a metabarcoding process. They illustrated that the relative abundance of microbiome bacteria was affected negatively in the case of CuO NPs (Swart et al., 2020a & 2020b). An artificial soil toxicity assay of Cu and CuO NPs on earthworm *Eisenia fetida* was conducted for 14 days at the dose concentrations of 0, 50, 100, and 500 mg/kg of dry soil. In this study, the author and his co-worker assessed the enzymatic activities (malondialdehyde (MDA), CAT, and SOD) of the earthworm on exposure to Cu and CuO NPs. They showed different trends: MDA activity increased for Cu NPs by 33.3% and 67% at 100 and 500 mg/kg dose concentrations, respectively while decreased at 50 mg/kg dose by 12.3%, and for CuO NPs it was found lower than control, CAT activity for Cu NPs was increased remarkably at 50 and 500 mg/kg dose concentrations by 83% and 69.9%, while in case of CuO NPs, it was increased by 18.7% at highest dose concentration and decreased at rest of

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the concentrations relative to control, and SOD activity for Cu NPs decreased at 100 and 500 mg/kg dose concentrations, though found equal to control in case of 50 mg/kg dose concentration, although CuO NPs enhanced the SOD activity with highest 98.1% at 500 mg/kg dose concentration against the background concentration. Overall, Cu NPs stimulated more toxicity than to CuO NPs (Lebdev et al., 2020). Researchers studied the epigenetic and transgenerational (after removal of dose) effects of CuO NMs and Cu salts ($CuCl_2$) on *Enchytraeus crypticus* for 7 generations (F1-F5 dosed with NMs) and (F6-F7) in the soil as per OECD guidelines for 244 days consecutively. They analysed epigenetic alterations in worms' bodies concerning gene expression qPCR for DNA methylation, histone modifications, non-coding RNA, stress response DNA methylation, methylation-sensitive – high resolution melting (MS-HRM), and gene-specific methylation through bisulphite sequencing. These effects were also observed in post exposure generations (Bicho et al., 2020). The authors demonstrated the ecotoxicogenomics effects of Cu NPs and Cu salts ($CuCl_2$) on Enchytraeus albidus by studying the cDNA microarray. They spiked the soil and dosed at 400 to 1000 mg/kg for 48 hrs. Results found that differently expressed genes (DEG) reduced with consecutively increased dose concentrations of $CuCl_2$ spiked soil, while no changes were observed for Cu NPs. Different DEG pattern was noticed with increasing dose, and these transcripttomic differences interfered with energy metabolism (monosaccharide transporting ATPase, NADH-dehydrogenase, NADH dehydrogenase subunit 1, Fe–S protein 4, and cytochrome c), transcription and translation (ribosomal protein L26), and stress-related enzymes (SOD). In addition, they found out that Cu_2^+ ions were not released in soil from Cu NPs, thus confirming Cu NP toxicity caused by nanoparticles rather than released ions. A negative concentration correlation was noticed on Cu salt exposure for DEG, while the same number of DEG was released on exposure to various concentrations of Cu NPs (Gomes et al., 2012a). Another complete life cycle, multigenerational (46 and 224 days) and immunohistochemistry at 12 points assayed on *Enchytraeus crypticus* by spiking the soil with Cu NMs and $CuCl_2$ salt. Both CuO NMs and $CuCl_2$ exerted indistinguishable stress responses at molecular initiating events (MIE) or key events at a higher level of biological organisation. Furthermore, both microRNA related protein, and post-transcriptional and transcriptional mechanisms at cellular, tissue, and organ levels (through histone modifications) showed similar results. Exposure to CuO NMs and $CuCl_2$ affected notch signalling pathways and activated DNA methylation at molecular initiating events, whereas $CuCl_2$ induced oxidative stress (Bicho et al., 2021). Researchers studied the effects of CuO NMs and $CuCl_2$ on *Enchytraeus crypticus* at the protein level by evaluating differently expressed proteins after exposure. CuO NMs were more responsible for releasing differently expressed proteins than $CuCl_2$. CuO NMs' toxicity occurred due to different cell uptake courses, the Torjan-horse effect, time exposure, and different life stages (Maria et al., 2018b). The author and his co-workers studied the effects of two Cu NMs (spherical and wire-shaped) and Cu salts ($CuNO_3$ and Cu salt field contaminated) by analysing gene expression with higher effects on *Enchytraeus crypticus* for the period of 3 and 7 days. All the adverse outcome pathways (AOP) were assayed for the first time in this paper. They depicted that transcriptomic responses appeared after 3 days, and assumed that exposure time was also a critical factor due to varied Cu uptake rates. $CuNO_3$ interfered with the worms' neurotransmission and locomotion activity. All the Cu forms except $CuNO_3$ showed epigenetic effects with ROS and DNA damage as well as alterations in protein (methylation and ubiquitination) (Gomes et al., 2018). Another study reported on *Enchytraeus albidus* on exposure of Cu NPs and ionic Cu to assess the stress biomarkers. They observed different lipid peroxidation (LPO) levels in worms due to both forms (clearer in ionic form), while other antioxidant stress biomarkers such as glutathione peroxidase (GPx) and glutathione content (GSH and GSSG) were more pronounced in both the cases (Gomes et al., 2012b).

Conclusions

The study of copper nanoparticle toxicity on worms is yet not elucidated well. In this paper, we collated the toxicity trends of various nanoparticles of copper and their derivatives on soil invertebrate species (earthworms and enchytraeids). This study illuminates the hazards of nanoparticles reached within the soil targeting the soil biota and interfering with their physiological and biochemical interceptors. Here, we documented the organismal (growth, biomass, behaviour, reproduction, survival, mortality, histology,

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and full life cycle test), cellular (cytotoxicity), genetic (enzymatic activities, gene expression, and post-generational effects), and epigenetic effects on selected worms. The results indicated a reduction in worms' population, biomass, survival reproduction, cocoon production, genitalia development, and tissue damage; however, no significant changes were observed in mortality in most of the cases when incorporated with different dose concentrations. In the majority of the studies, the nanoparticles were found more toxic than comparable bulk/ salt analogs. Researchers obtained similar results to different dose exposure for bioaccumulation and cellular toxicity. Genetic studies revealed mixed elevation trends and reduction of enzymatic activities with increased dose concentration. In this study, we observed that soil medium, soil substrate, soil organic matter, soil age, soil pH, NP size, NP shape, NP surface properties, NP surface coatings, NP oxidation states, and NP dissolution alters the functioning of soil invertebrates. Further, statistical and experimental data is needed to gain insights into the toxicokinetics of ENMs (engineered nanomaterials). We need to impose stringent guidelines to regulate the fate of NPs, paving their way to the agro-ecosystems.

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Contribution

Bhavya Singh: conceptualisation, formal analysis, resources, investigation, funding acquisition, writing the original draft. Devendra Singh Rathore: investigation, supervision, editing, and funding acquisition. Kapil Kumar: formal analysis and resources. Tanushree Kain: reviewing and draft editing.

Data availability

This manuscript has no associated data; however, some data will be made available on reasonable request

Declarations

Competing interests: The authors declare no competing interests.

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