



Ecotoxicological Assessment of Various Sizes and Shapes of Copper Nanoparticles on Soil Biological Indices

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Abstract

The increasing production and environmental release of copper (Cu) nanoparticles raise concerns about their ecotoxicological impact on terrestrial ecosystems. However, the effects of particle morphology and subtle size variations on soil biological properties remain unexplored. This study aimed to determine how shape (spherical vs. “torn” porous) and minor size differences (70 vs. 100 nm) influence Cu nanoparticle toxicity in Haplic Chernozem. A model experiment used 3 Cu forms: spherical nanopowders (70 and 100 nm), spherical nanopowders with a porous “torn” surface (70 and 100 nm), and macrodisperse particles (7,000 nm). Each form was applied at 5 concentrations: 1.5, 2, 3, 5, and 10 times the background level (30 mg kg⁻¹). Soil biological activity was assessed via integral indicators of biological state (IIBS). All Cu forms significantly suppressed key biological parameters. Nanoparticles exhibited higher toxicity than the macrodisperse form, and this was governed by concentration and particle shape. The highest dose (300 mg kg⁻¹) caused the most pronounced inhibition. “Torn” nanoparticles demonstrated greater ecotoxicity than spherical ones: spherical 70 nm particles reduced IIBS by 14 to 59%, whereas “torn” 70 nm particles reduced it by 27 to 63% relative to the control. Size-dependent effects were observed exclusively for the “torn” morphology: 70 nm particles decreased IIBS by 19 to 47%, while 100 nm particles caused a reduction of only 14 to 34%. These findings confirm that both size and shape are critical determinants of Cu nanoparticle toxicity in soil. This study provides a basis for refining environmental risk assessment and regulatory frameworks for metal nanoparticle contamination in agricultural soils.

Keywords: biodiagnostics; enzymatic activity; heavy metal pollution; microbiological indicators; phytotoxicity

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INTRODUCTION

Currently, as production and use of metal-containing nanomaterials, including in crop production and animal husbandry, increase, the risk of contamination of soils, biota, and food products with heavy metal (HM) nanoparticles is

rising (Wang et al., 2024; Zhang et al., 2026). However, the question of how HM ecotoxicity depends on the degree of particle dispersion remains unresolved (Ouyang et al., 2024; Wang et al., 2024). Nanoparticles are known to exhibit

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properties that differ from their bulk counterparts, including the potential to form novel compounds (Christian et al., 2008). Therefore, it is hypothesized that HM nanoparticles may interact with the environment differently than their macrosized forms (Li et al., 2024; Das et al., 2025).

Due to the increasing demand for the production and use of copper (Cu) nanoparticles, there is a risk of their unintentional release into the environment, particularly into the soil. Soil pollution with Cu is primarily related to the activities of mining and metallurgical enterprises, along with the operation of transport infrastructure facilities. Environmental problems associated with the operation of non-ferrous metallurgy enterprises are widespread all over the world (Ettler, 2016). Elevated total Cu concentrations in the topsoil have been reported in areas impacted by Cu smelters in Chile (Tapia-Gatica et al., 2020), Zambia (Mihaljevič et al., 2018), and Russia (Moshchenko et al., 2023).

At the same time, studies on the toxicity of nanomaterials are considerably far behind the pace of their application. Gaps in knowledge related to the toxicity risks of this new type of pollutant can lead to serious environmental consequences. In recent years, Cu-based nanoparticles have attracted significant scientific interest. Copper nanoparticles are widely used in agriculture as part of pesticides and fertilizers (Kamel et al., 2022; Nizamani et al., 2024), and in chemical, automotive, electronic, and other industries (AngelinThangakani et al., 2022; Chen et al., 2024; Dehcheshmeh et al., 2024; Van Impelen et al., 2024).

First of all, it is necessary to pay attention to the increase in Cu intake into the soil in the form of nanoparticles since their properties differ significantly from those of micro- and macroparticles. The results of studies comparing the toxicity of HMs and their nanoforms are contradictory. Most of the studies indicate a higher toxicity of HM nanoparticles (Seku et al., 2018; Kolesnikov et al., 2020; Tsepina et al., 2022), while other studies show that the nanoparticles have no adverse effects or even stimulate these effects (Rizwan et al., 2017; Asadishad et al., 2018).

For example, it has been established that Cu nanoparticles are capable of causing the death of earthworms (Gautam et al., 2018). Among microorganisms, *Azotobacter* are sensitive to such contamination (Concha-Guerrero et al., 2014). In addition to microorganisms, the adverse effect

of Cu nanoparticles on plants is manifested both in the inhibition of growth processes (Tiwari et al., 2019; Zhang et al., 2019) and in decreased seed germination and germination energy (Korotkova et al., 2018; Shang et al., 2019).

Copper nanoparticles can alter microbial diversity because they are essential for many enzymatic processes. High concentrations of Cu^{2+} can be toxic, killing sensitive species and allowing more resistant strains to thrive (Peixoto et al., 2024; Su et al., 2024). Copper nanoparticles can damage bacterial cell membranes, interfere with enzymatic activity, and generate reactive oxygen species (ROS) (excess production), leading to oxidative stress (Ghanbari Jahromi et al., 2025; Lai et al., 2025). In addition, the accumulation of Cu nanoparticles in plant tissues has been noted (Ruttkey-Nedecky et al., 2017; Velicogna et al., 2020).

In the work of Yang et al. (2024), it was shown that CuO nanoparticles deform wheat roots and penetrate into leaf cells, causing damage to cellular structures, reducing chlorophyll content, and increasing antioxidant enzyme activity. According to Abbasirad and Ghotbi-Ravandi (2025), CuO nanoparticles induce oxidative stress in barley (increased H_2O_2 , activation of catalase and ascorbate peroxidase), disrupt mineral nutrition, and cause an imbalance of key phytohormones (auxins, gibberellins, abscisic acid, jasmonic acid, and ethylene). Similar patterns indicating the suppression of root growth processes and the development of oxidative stress in the presence of CuO nanoparticles have been reported by other authors (Kovács et al., 2025; Ghosh et al., 2026).

Some studies indicate that Cu nanoparticles introduced at low concentrations stimulate plant growth processes (Badawy et al., 2021; Geremew et al., 2024). Several studies have confirmed the stimulatory effect of Cu nanoparticles at low concentrations. Kasana et al. (2017) noted enhanced germination and plant growth at low doses of Cu nanoparticles. Treatment of spring wheat seeds with Cu nanoparticles at concentrations of 0.01 to 100 g ha^{-1} increased germination energy by 6%, seedling length by 25.5%, and yield by 18%. According to Nagdalian et al. (2025), a concentration of 0.1 mg l^{-1} CuO nanoparticles provided maximum germination energy and length of pea roots and seedlings.

These contrasting findings indicate that research on the toxicity of HMs, including Cu, in relation to particle size and shape is still in its early stages. However, the following questions

remain unresolved. First, it is unknown whether particle shape influences the ecotoxicity of Cu nanoparticles in soil, as most studies have compared nanoparticles only with bulk materials without comparing shape. Second, it is unclear whether relatively small differences in particle size (e.g., 70 vs. 100 nm) lead to significantly different results when other variables (e.g., shape, concentration) are held constant. Third, no study has compared the effects of Cu nanoparticles of different sizes and shapes on a wide range of biological parameters—including enzymatic activity, phytotoxicity, and microbiological parameters—within a single study. Given that Cu nanoparticles may in some cases exhibit higher toxicity than their larger counterparts, the development of more precise environmental standards for HM concentrations in soils and biota may be warranted.

To address these gaps, this study conducted a comparative assessment of the ecotoxicity of Cu in nanoparticles (70 and 100 nm, spherical and “torn” forms) and macrodispersed form (7,000 nm) on the biological properties of Haplic Chernozem, using a wide range of sensitive and informative biological indicators. The research hypothesizes that the toxicity of Cu nanoparticles to soil biological properties depends on both particle size and shape, with smaller and irregularly shaped (“torn”) particles exhibiting greater ecotoxicity due to their larger specific surface area and reactivity. The aim of this study was to compare the effects of Cu particles of different sizes and shapes on the biological properties of Haplic Chernozem.

MATERIALS AND METHOD

Soil and plant samples preparation

The ordinary Chernozem of the Southern European facies from the Botanical Garden of the Southern Federal University (Russia, Rostov-on-Don), classified as Haplic Chernozem (Loamic) according to WRB (2015), was selected for the model experiment. The selection site is a specially protected natural area, which makes it possible to exclude additional Cu inputs and the use of this soil as a standard for conducting research. Haplic Chernozem has a heavy loamy granulometric composition. Compared with other soil types, Chernozems exhibit advantageous soil-forming conditions. The average organic matter content was 3.7%, and pH was 7.8 (nearly neutral). The cation exchange capacity (CEC) of the studied horizon was 33.3 cmol(+) kg⁻¹. Calcium was the

dominant exchangeable cation (up to 80–90% of the CEC). Magnesium accounted for a smaller share (about 15%). The structure was lumpy-granular, the soil bulk density was 1.18 g cm⁻³, particle density was 2.68 g cm⁻³, and the total porosity was 56%. Silt particle content (< 0.001 mm) was 35.6%, and clay particle content (< 0.01 mm) was 64.8% (according to the Russian soil classification of N.A. Kachinsky).

Chernozems are the most fertile soils, and the quality and quantity of the crop depend on their condition. In addition, studies confirm a greater resistance to contamination in Chernozems compared with other soils (Timoshenko et al., 2023; Kolesnikov et al., 2024). It means that if the negative effects of Cu nanoparticles on Haplic Chernozem are detected, it can be argued that the adverse effects of this substance on other soil types would be even greater. Soil samples were taken from the topsoil (0 to 10 cm). After air-drying, all soil samples were sifted into fractions of 2 to 5 mm particle size to lay a model experiment.

Plan of the experiment

A model experiment was conducted to investigate the effects of Cu contamination on the biological activity of Haplic Chernozem. Three forms of Cu were applied: (1) spherical Cu nanopowders with particle sizes of 70 and 100 nm, (2) Cu nanopowders of approximately spherical shape with a porous surface (“torn” shape) and particle sizes of 70 and 100 nm, and (3) macrodispersed Cu particles with a mean size of 7,000 nm. The manufacturer provided these specifications. All Cu forms used in the work were manufactured and supplied by “Advanced Powder Technologies” LLC, Tomsk, Russia. It was assumed that, due to the porous surface, nanopowders of the “torn” shape would have a larger surface contact area and a higher reactivity compared to similar spherical particles, and therefore greater ecotoxicity.

The applied concentrations were calculated based on the background Cu content in the soil. Quantitative determination of the Cu content in the studied soil was carried out at the Laboratory of the A.P. Karpinsky Russian Geological Research Institute (VSEGEI). The analysis was conducted using inductively coupled plasma mass spectrometry (ICP-MS, ELAN-DRC-e, PerkinElmer). This mass spectrometer can detect chemical elements (metals, metalloids, and nonmetals) in the range of 10⁻⁵ to 0.1%. The background Cu content in the studied soil was 30

mg kg⁻¹, and technogenic emissions significantly exceeded this value. The effects of the following Cu concentrations in the soil were studied: 1.5, 2, 3, 5, and 10 times the background Cu content, which corresponds to 45, 60, 90, 150, and 300 mg kg⁻¹, respectively. Uncontaminated soil was used as a control.

The scheme and conditions of the experiment are represented in Figure 1. Cu was initially incorporated into a small amount of soil (10 g), meticulously pulverized, and subsequently mixed with the bulk soil samples (300 g) to achieve a uniform mixture. The soil was kept at room temperature (22 to 24 °C) and 30% humidity while being incubated in vegetative vessels. The soil was incubated for 30 days. This incubation period is the most informative (Kolesnikov et al., 2020).

The studied indicators

To assess the condition of the soil post-Cu contamination, the following critical and informative indicators were examined: enzymatic activity (catalase, dehydrogenase, invertase, urease, and phosphatase), phytotoxicity indicators

(germination rates, root length, and shoot length), and microbiological parameters (abundance of *Azotobacter* and total bacterial count).

Catalase activity was assessed using the gasometric approach by measuring the dissolution rate of 3% H₂O₂ following its interaction with soil (temperature: 20 to 22 °C), whereas dehydrogenase activity was quantified spectrophotometrically on a PE 5800VI spectrophotometer at a wavelength of 540 nm (Kazeev et al., 2016). The activities of dehydrogenases and ureases were assessed using the modified Galstyan method, invertase activity was measured by the colorimetric approach employing the Fehling's reagent, and phosphatase activity was evaluated using the modified Tabatabai and Bremner method (Kazeev et al., 2016).

Germination rate, root length, and shoot length of barley, as an important agricultural crop, were taken as indicators of phytotoxicity. Spring barley seeds of the "Ratnik" variety were used. The method is based on the high sensitivity of plant seeds to toxic substances, established by

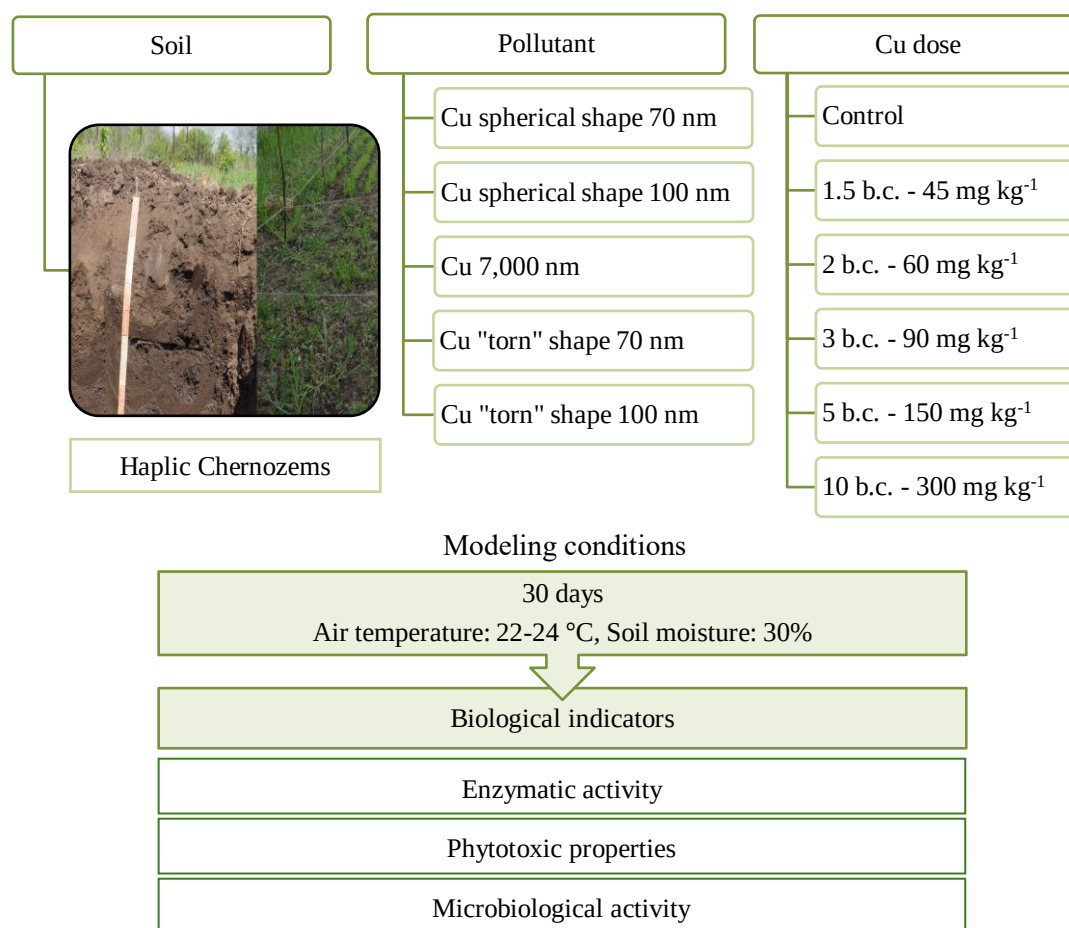


Figure 1. The scheme of the model experiment

measuring changes in root and shoot length and germination relative to the control, expressed as a percentage. The ratio of each indicator in contaminated soil to that in the control was calculated and expressed as a percentage.

Seeds were sown in petri dishes after collecting the soil from the incubation vessels. To assess phytotoxicity, 40 g soil samples were taken from each vegetation vessel 30 days after the onset of contamination, in a 3-fold replicate. The soil samples were placed in petri dishes, moistened to 80% of the full moisture capacity, and mixed until a uniform consistency was achieved. Twenty-five barley seeds were planted in the soil prepared in this way. The samples studied were kept under conditions of constant optimal temperature, humidity, and lighting in a KBW240 climate chamber. The barley growth indicators were assessed after 7 days.

The total bacterial count was assessed using luminescence microscopy, whereas the abundance of *Azotobacter* genus bacteria was evaluated using the soil grain-sowing method on Ashby medium (Kazeev et al., 2016).

Based on the most sensitive and informative biological indicators, an integral indicator of the biological state (IIBS) of the soil was determined (Kazeev et al., 2016). To calculate the IIBS in the sample, the maximum value of each indicator was set to 100%, and the indicator value in the remaining samples was expressed as a percentage relative to that maximum (Equation 1).

$$B_1 = B_x / B_{\max} \times 100\% \quad (1)$$

Where, B_1 is the relative score of the indicator, B_x is the actual value of the indicator, and $B_{\max}$ is the maximum value of the indicator. After that, the average estimated score of the studied indicators for the sample (option) was calculated. The absolute values cannot be summed up since they have different units of measurement (mg, mm, %). The IIBS of the soil was calculated using a similar formula (Equation 2).

$$\text{IIBS} = B_{\text{aver.}} / B_{(\text{aver. max})} \times 100\% \quad (2)$$

Where, $B_{\text{aver.}}$ is the average score of all indicators, $B_{\text{aver. max}}$ is the maximum score of all indicators.

Based on the IIBS calculations, regression equations were developed, and critical Cu input doses that do not lead to deterioration of soil conditions were calculated. Calculations were carried out on the basis of a decrease in the IIBS of Chernozem when contaminated with the most toxic form of Cu.

Statistical analysis

The data were processed and analyzed using the Statistica 13.1 software. Tests for normal distribution and homogeneity were performed using the Box-plot method and the Shapiro–Wilk test. Results were expressed as mean $\pm$ standard deviation. The statistical differences between treatments for soil enzymatic activity, an indicator of phytotoxicity, and microbiological characteristics caused by Cu particles of various sizes and shapes were analyzed using analysis of variance (ANOVA), followed by Duncan's multiple range test (DMRT). The relationship between soil biological activity and Cu concentration was evaluated using Pearson correlation analysis. Additionally, a variation analysis was conducted to evaluate the variability of the measured values.

RESULTS AND DISCUSSION

Enzymatic activity

Figure 2 shows the changes in the enzymatic activity of Haplic Chernozem after contamination with Cu nanoparticles of various sizes and shapes. It can be noted that the presence of Cu in all forms in the soil led to a decrease in the activity of the studied enzymes.

Two-way ANOVA showed that both the concentration of the pollutant ($p < 0.01$) and the form of Cu ($p < 0.05$) significantly affected the enzyme activity. The degree of reduction depended on whether Cu was used in nanoparticles or a macrodispersed form. According to the degree of enzyme inhibition, the pollutants formed the following series: $\text{Cutr } 70 \text{ nm} \geq \text{Cutr } 100 \text{ nm} > \text{Cusf } 70 \text{ nm} \approx \text{Cusf } 100 \text{ nm} > \text{Cu } 7,000 \text{ nm}$. The activities of catalase and dehydrogenase were most sensitive to Cu contamination, whereas invertase and phosphatase were comparatively less sensitive. Notably, “torn”-shaped nanoparticles demonstrated significantly greater inhibition compared to spherical particles of the same size, and this shape-dependent effect was more pronounced at higher concentrations. Importantly, significant size-dependent differences (70 vs. 100 nm) were observed exclusively for the “torn”-shaped particles. These results indicate that the toxic effect of Cu depends on both particle size and shape, with the “torn” shape enhancing the negative impact.

High concentrations of Cu in the soil can have a negative impact on the microbiological and biochemical properties of the soil (Gautam et al.,

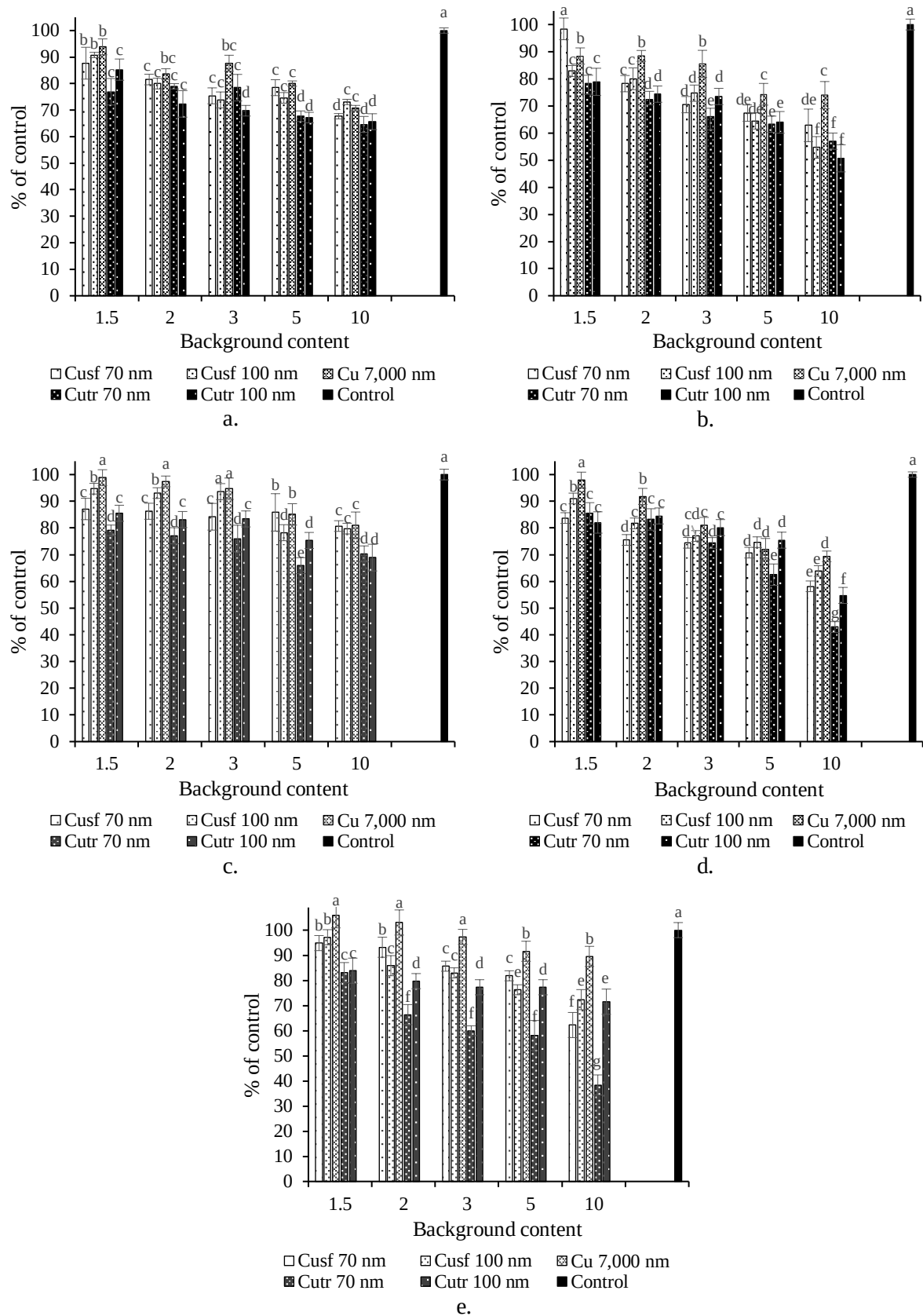


Figure 2. Effect of Cu contamination with nanoparticles of various sizes and shapes on the enzymatic activity of Haplic Chernozem (Loamic): a) catalase; b) dehydrogenase; c) invertase; d) urease; e) phosphatase

Note: Different letters mean that there are significant differences between treatments at $p < 0.05$ according to DMRT

2018; Kuziemska et al., 2020) and plant growth (Tiwari et al., 2019; Zhang et al., 2019). These violations were observed in this study and are consistent with previously obtained data. In the experiment of Kuziemska et al. (2020), it was found that the introduction of Cu into the soil, regardless of its dose, led to decreases in urease, dehydrogenase, and phosphatase activities, while the inactivating effect increased with an increase in the amount of the substance in the soil. A close correlation of the degree of enzyme reduction with the contaminant concentration was also observed in the present study. A study by Di et al. (2023) showed that applying CuO nanoparticles at concentrations of 20 and 80 mg kg⁻¹ to soil reduced the activities of urease and alkaline phosphatase by 25.7 to 29.1% and 31.0 to 39.4%, respectively. According to the results of a study by Xu et al. (2015), the application of CuO nanoparticles at concentrations of 100 to 1,000 mg kg⁻¹ resulted in a decrease in the activity of soil enzymes in flooded rice soil. Higher toxicity of CuO nanoparticles compared to TiO₂ nanoparticles was noted.

In the present study, urease, catalase, and dehydrogenase were more sensitive to Cu contamination than invertase and phosphatase. These results are consistent with the data of the study by Wyszowska et al. (2017), which observed a similar pattern. Sun et al. (2007) also found that urease activity was the most sensitive among the enzymes studied. The different sensitivities of the enzymes are related to their cellular localization and molecular structure. The sensitivity of soil enzymes depends on several factors, such as particle size, pollutant concentration, and enzyme type (Isildak et al., 2026). Furthermore, the effects of Cu nanoparticles on soil enzymes vary depending on soil type and land use (Timoshenko et al., 2021).

The mechanisms underlying enzyme inhibition by Cu nanoparticles are multifaceted. Adhikari et al. (2024) demonstrated that the size, concentration, surface coating, and exposure duration of CuO nanoparticles collectively impact their interactions with soil enzymes. Their study showed that 10 nm CuO nanoparticles had the highest extractable Cu across all incubation times. After 24 hours of short-term exposure, CuO nanoparticles significantly inhibited both acid phosphatase and dehydrogenase activities across all tested concentrations, with smaller nanoparticles showing a greater effect. A strong negative correlation was observed between CuO

dissolution and enzyme activities, indicating that Cu ions are the main toxicity source.

The inhibition mechanism is thought to involve interaction of metal ions with the sulfhydryl groups of enzymes. Zaynitdinova et al. (2022) established that Cu nanoparticles completely inhibited the activity of both the cellulase complex and amylase in soil-borne microorganisms. Thus, the entry of Cu into the soil will disrupt its biological processes and change the biochemical parameters of the soil condition.

In the study by Wyszowska et al. (2017), dehydrogenase and urease activities showed higher sensitivity to Cu sulfate and chloride contamination than phosphatase activity. There was also a high degree of correlation between the decrease in indicators and the Cu concentration in the soil. A similar pattern was observed in the study of Sun et al. (2007): urease activity was the most sensitive indicator. However, unlike the previous study, catalase activity, which belongs to the same class of enzymes as dehydrogenase activity, was the most resistant to the effects of Cu. The current investigation revealed that invertase and phosphatase showed comparatively reduced sensitivity to Cu contamination, while urease, catalase, and dehydrogenase exhibited more inhibition, signifying their increased susceptibility to Cu stress (Wyszowska et al., 2017).

Phytotoxic indicators

Phytotoxicity indicators are among the most important in biodiagnostics, as plants are primary producers in terrestrial ecosystems and serve as an integrated indicator of soil quality. This study found that all forms of Cu tested significantly inhibited barley growth. Patterns were identified that allow assessment of toxic effects depending on the form, size, and concentration of the pollutant. Figure 3 shows the changes in the phytotoxicity of Haplic Chernozem after contamination with Cu nanoparticles of different sizes and shapes.

The *p*-values from the two-way ANOVA showed that changes in phytotoxicity indicators were significantly influenced by pollutant concentration (*p* < 0.01) and the form of Cu input to the soil (*p* < 0.05). The degree of reduction depended on whether Cu was used in nanoparticles or a macrodispersed form. The pollutants formed the following order of inhibition of plant growth and development:

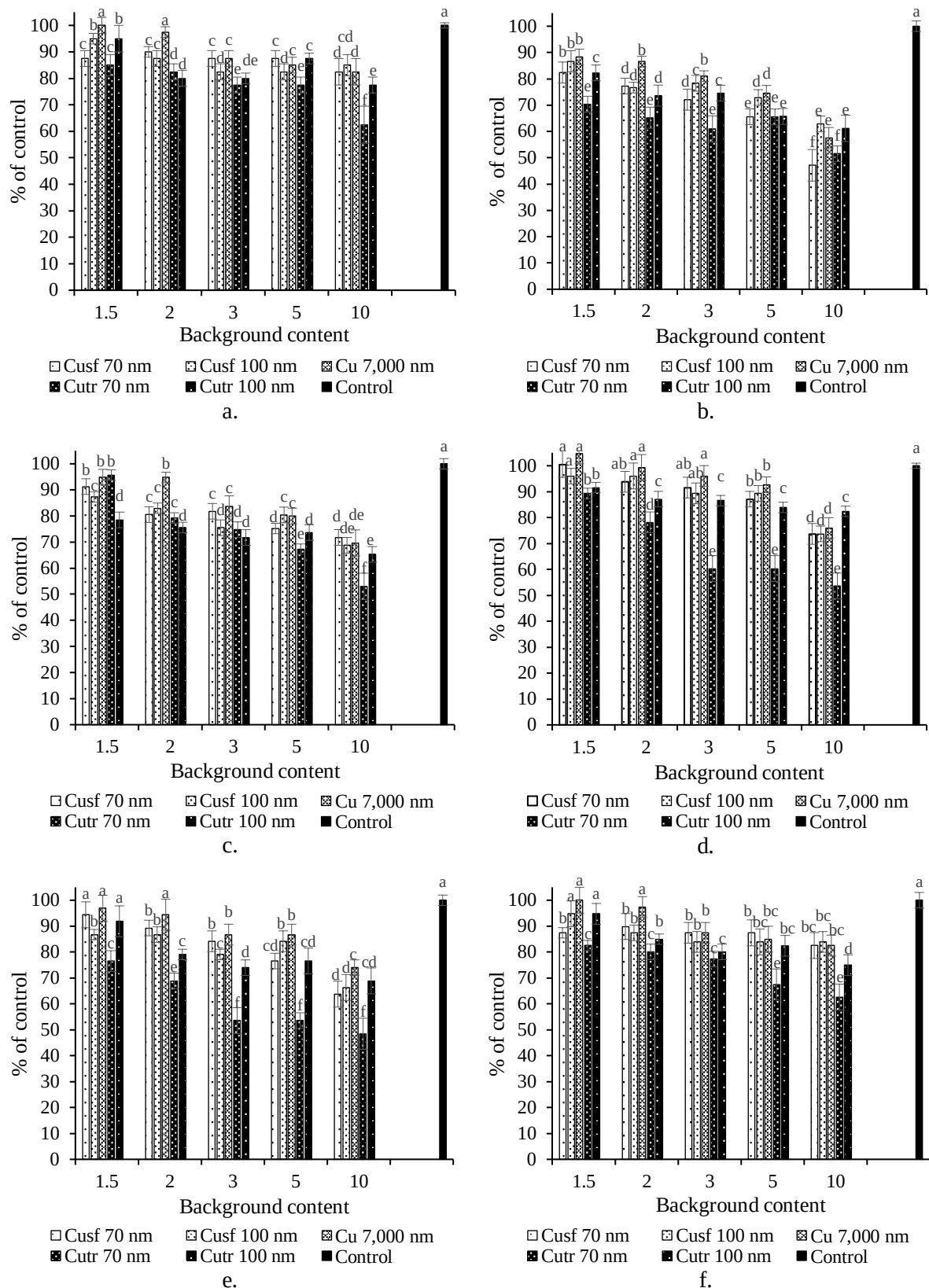


Figure 3. Effect of Cu contamination of various sizes and shapes on the phytotoxicity indicators of Haplic Chernozem (Loamic): a) germination; b) root length; c) shoot length; d) germination rate; e) germination energy; f) germination evenness

Note: Different letters mean that there are significant differences between treatments at $p < 0.05$ according to DMRT

Cutr 70 nm $\geq$ Cutr 100 nm > Cusf 70 nm $\approx$ Cusf 100 nm > Cu 7,000 nm. Among the phytotoxicity parameters studied, root length was the most sensitive to Cu contamination. Cutr had a significantly greater negative effect than Cusf of the same size. Significant differences in the effects of nanoparticles of different sizes were typical only for torn-shaped nanoparticles. Differences in the influence of spherical nanoparticles of sizes 70 and 100 nm were recorded only for the length of the roots.

This study confirmed and supplemented the available results on the negative effects of Cu compounds on plant growth. Excess Cu is known to have a number of toxic effects on various biophysicochemical processes (Jaime-Pérez et al., 2019; Marastoni et al., 2019; Ryszka et al., 2019). Excessive Cu can interfere with plant growth and development and with the absorption of essential nutrients (Tiwari et al., 2019; Zhang et al., 2019).

The mechanism of Cu toxicity in plants is based on its redox properties. The conversion of this element from Cu^+ to Cu^{2+} can lead to increased formation of highly toxic ROS and other hydroxyl radicals. These free radicals can oxidize and damage a number of key biomolecules, such as DNA, proteins, RNA, and lipids (Shahid et al., 2017; Shabbir et al., 2020). Protein oxidation in plants typically occurs under the influence of ROS or other oxidative damage products (Farooq et al., 2019; Kapoor et al., 2019; Savelli et al., 2019). In most cases, protein oxidation is irreversible, except for some sulfur-containing amino acids (Kapoor et al., 2019). Thus, oxidative damage to plant biomolecules is often used as a biomarker of oxidative stress and ROS formation (Pisoschi and Pop, 2015; Kapoor et al., 2019).

Several studies have indicated that nanoforms exhibit lower toxicity relative to bulk forms for Ag (Tsepina et al., 2022) and Pt (Timoshenko et al., 2023), suggesting that nanoparticle toxicity is contingent on the metal type, particle characteristics, and environmental factors. The effect of TiO_2 particle size on radish growth has previously been established (Bryan et al., 2025). In contrast, a high degree of toxicity to plants has been established for Cu nanoparticles (Kolesnikov et al., 2021), which was higher than that of Cu in the macrodispersed form (Fedorenko et al., 2020; Timoshenko et al., 2021). According to the results of this study, Cu nanoparticles of both spherical and “torn” shapes were more toxic to spring barley than the macrodispersed Cu form. Meanwhile, the “torn” shape was more toxic

than the spherical one. Among the indicators of phytotoxicity, root length was the most sensitive indicator to Cu contamination, while germination rate, speed, and germination seedling vigor were the least sensitive. As in the study by Wyszowska et al. (2017), the sensitivity of spring barley to Cu compounds was lower than that of the studied enzymes.

Microbiological indicators

An analysis of Cu effects on soil microorganisms revealed low sensitivity for *Azotobacter* and high sensitivity for total bacterial counts (Figure 4). A two-way ANOVA confirmed that Cu toxicity to total bacterial counts depended on both the pollutant concentration in the soil ($p < 0.01$) and the form of its introduction ($p < 0.05$). Particle size was a critical factor only for “torn” nanoparticles ($p = 0.03$), while no significant correlation was found for spherical particles ($p = 0.38$).

The most pronounced reduction in total bacterial counts (up to 37 to 55% of the control at the maximum concentration) was observed with “torn” nanoparticles. Nanoparticles measuring 70 nm exhibited higher toxicity than those measuring 100 nm across the entire concentration range. Unlike spherical particles, “torn” nanoparticles showed a clear size dependence: the smaller the size, the greater the inhibitory effect. This indicates that the larger specific surface area and imperfections in the crystal lattice of the “torn” particles ensure a more intense release of toxic Cu^{2+} ions and, consequently, a more pronounced bactericidal effect.

In contrast to the total bacterial count, *Azotobacter* were not sensitive to Cu contamination at concentrations of 1.5, 2, 3, and 5 b.c., with the exception of Cutr 100 nm at a concentration of 3 b.c., where the value decreased by $12 \pm 0.4\%$.

It should be noted that for 100 nm particles, the “torn” shape was more toxic to the total count of bacteria than the spherical shape. In contrast, for *Azotobacter* at the same concentration, spherical particles exerted more pronounced inhibition than the “torn” shape. This multidirectional effect can be explained by differences in cellular organization and metabolic needs of different bacterial groups. *Azotobacter*, as Gram-negative bacteria with a more complex cell wall, can exhibit specific adaptive responses to different types of nanoparticles.

Thus, it has been established that total bacterial abundance is an integral and highly sensitive

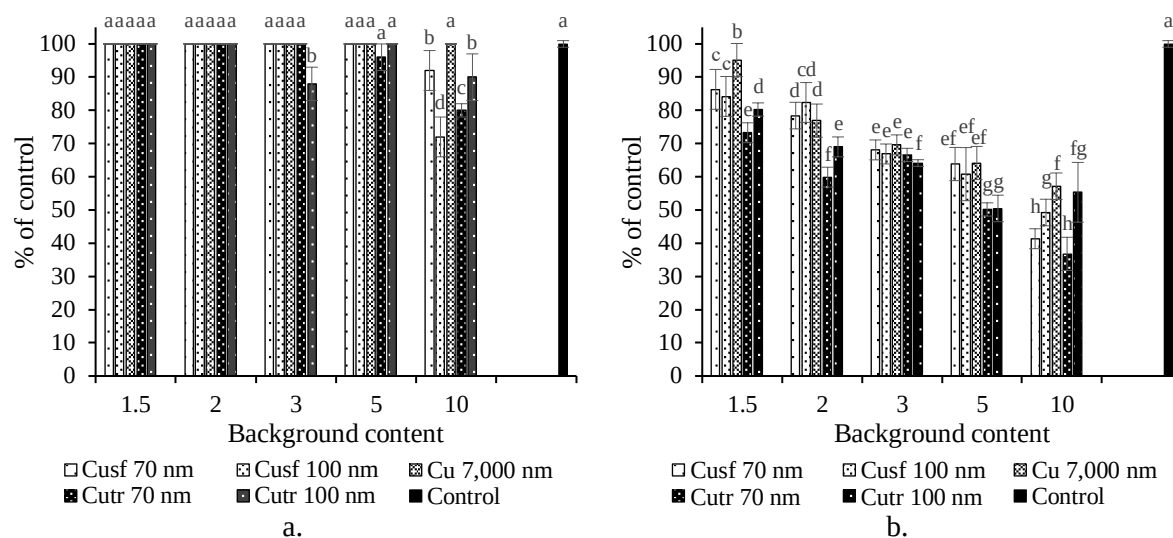


Figure 4. Effect of Cu contamination of various sizes and shapes on the microbiological indicators of Haplic Chernozem (Loamic): (a) the abundance of *Azotobacter*; b) the total count of bacteria

Note: Different letters mean that there are significant differences between treatments at $p < 0.05$ according to DMRT

indicator of soil contamination with Cu nanoparticles, while *Azotobacter* abundance can serve only as a marker at high contamination levels (≥ 10 b.c.) or for specific particle shapes. The identified sensitivity of total bacterial abundance indicates disruption of key soil processes: organic matter degradation, mineralization, and humification. At the same time, the stability of nitrogen-fixing bacteria may indicate the preservation of the soil's potential for biological nitrogen fixation even under anthropogenic stress.

Previously, the negative impact of Cu on the soil bacterial community was established in several studies, with the degree of sensitivity depending on the type of bacteria (Asatiani et al., 2021). Research by Kolesnikov et al. (2019) showed that contamination of Chernozem with Cu nanoparticles leads to a decrease in the total bacterial count and the abundance of *Azotobacter*.

Copper toxicity to bacteria is realized through a complex set of interconnected mechanisms: ROS generation, metal substitution at enzyme active sites, membrane damage, and the induction of specific cellular responses (Chen et al., 2025; Pellosi et al., 2025). Copper nanoparticles enhance these effects due to their high reactivity, intense ion release, and direct physical damage to cellular structures (Pellosi et al., 2025).

A study by Qian et al. (2025) found that the nanospecific effect plays a decisive role in shaping the response of microbial communities to CuO. A 63-day experiment demonstrated

that CuO nanoparticles significantly ($p < 0.05$) reduced bacterial diversity and altered the overall structure of the bacterial community. Moreover, the dissolution rate of CuO nanoparticles was significantly lower than that of $\text{Cu}(\text{NO}_3)_2$ in soil, confirming the dominant role of the nanospecific effect.

Thus, the results of this study are consistent with the current understanding that the differential sensitivity of microbial groups to Cu is determined by a complex set of factors, including microbial type, particle size and shape, and biofilm formation ability. The increased resistance of *Azotobacter* compared to the general bacterial population may be due to both biofilm formation and exopolysaccharides production, which create a protective barrier, as well as to the activation of specific detoxification systems, consistent with the data of Reyes et al. (2015).

Integral indicator of soil condition

To assess the biological state of Haplic Chernozem after Cu contamination, the IIBS was calculated (Figure 5). Since *Azotobacter* abundance showed relatively low sensitivity at lower concentrations, it was excluded from the IIBS calculation. All other indicators were used for the calculation. The degree of change in the IIBS is shown in Figure 5.

As shown in Figure 5, there was no difference between the IIBS values for Cusf 70 nm and Cusf 100 nm, and Cutr 70 nm reduced the IIBS to a greater extent than Cutr 100 nm. In addition,

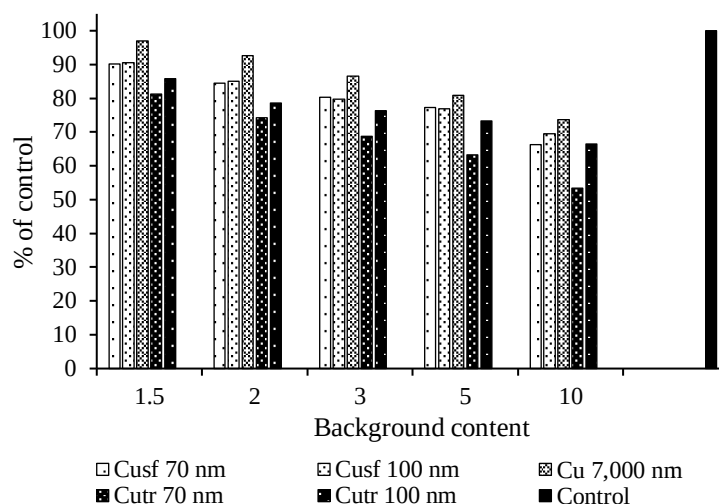


Figure 5. The effect of Cu contamination of various sizes and shapes on IBS Haplic Chernozem (Loamic)

the difference in IBS was obvious when soil was contaminated with different forms of Cu. The “torn” shape of Cu had a greater negative impact than the spherical one. For example, for nanoparticles with sizes of 70 nm at concentrations of 1.5, 2, 3, 5, and 10 b.c. reduced the value of IBS for a spherical shape by 14, 22, 32, 36, and 59% from the control, and for a “torn” shape by 27, 40, 37, 50, and 63% from the control, respectively. This is most likely due to the porous surface of “torn” nanopowders, which provides a larger contact area and higher reactivity compared to similar spherical particles, thereby leading to greater ecotoxicity. Both forms of Cu nanoparticles were characterized by a greater reduction in IBS than the 7,000 nm Cu, which indicates a greater toxicity of the nanoparticles compared to the macrodispersed form.

Sensitivity and informativeness of indicators

Based on the correlation analysis, correlation matrices of indicators for the studied substances were constructed (Figure 6). In most cases, a close inverse correlation was established between the concentration of the pollutant and the values of the studied indicators, which indicates the high informativeness of the indicators used in the work. A significant positive correlation was observed between enzyme activity and total bacterial count. The high positive correlation ($r \approx 0.7$ to 0.9 , as shown by the matrices in Figure 6) between soil enzymatic activity and total bacterial counts suggests that these 2 parameters are closely interrelated and respond jointly to Cu nanoparticle contamination. The abundance of *Azotobacter* correlated weakly with other indicators. *Azotobacter* are nitrogen-fixing bacteria that

possess heavy-metal tolerance mechanisms, including active release of Cu^{2+} ions from the cell, metal binding by metallothioneins and other metal-binding proteins, and enzymatic detoxification. Therefore, *Azotobacter* exhibits a unique response to Cu contamination that is distinct from that of other bacteria.

The observed correlation ($r \approx 0.5$ to 0.8) between phytotoxicity indices and enzymatic activity indicates that the toxic effects of Cu on plants and soil enzymes are mediated by common mechanisms and have a single concentration-dependent nature.

The sensitivity of the indicators varied based on the size and shape of the Cu particle. For Cusf 70 nm, the most informative indicators were the total count of bacteria, germination rate, and germination energy of radish, and the least informative were invertase activity and germination seedling vigor. For Cusf 100 nm, the greatest correlations with contaminant concentration were observed for radish germination rate, the abundance of *Azotobacter*, and the total count of bacteria. The lowest correlations with the contaminant concentration were noted for radish germination rate, shoot length, and germination seedling vigor. For Cu 7,000 nm, phytotoxicity indicators were the most informative: the length of radish roots, germination rate, and germination energy, while the length of radish shoots turned out to be the least informative indicator in this case.

For “torn”-shaped Cu nanoparticles, regardless of size, urease activity and shoot length were the least informative indicators. Some indicators correlated differently with the concentrations of

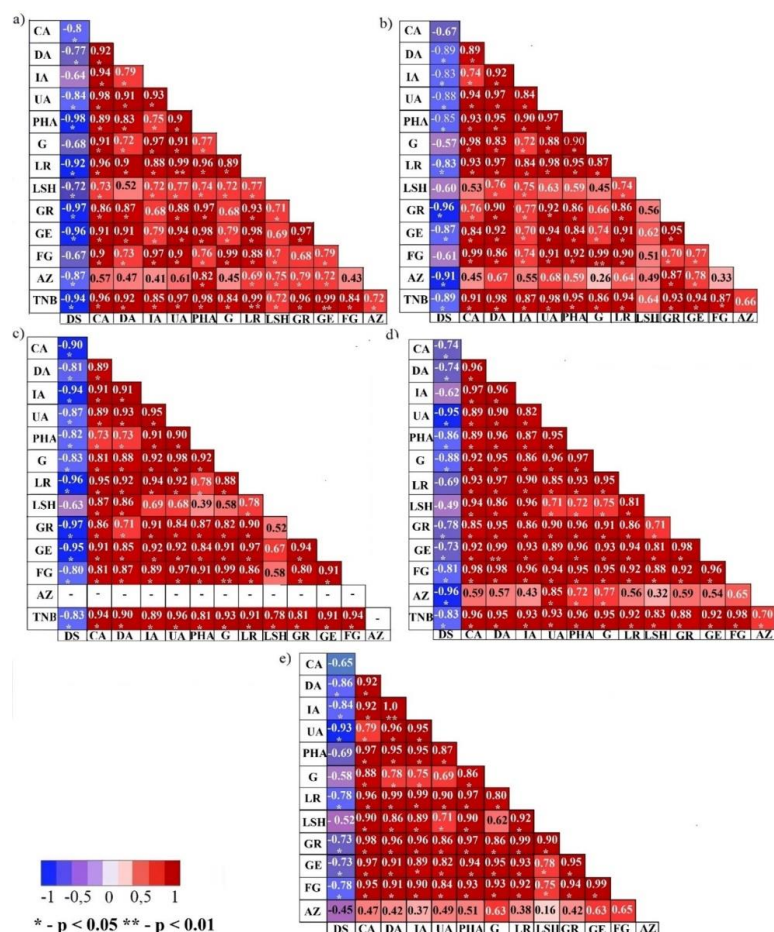


Figure 6. Correlation matrices of indicators: a) Cusf 70 nm, b) Cusf 100 nm, c) Cu 7,000 nm, d) Cutr 70 nm, e) Cutr 100 nm

Note: DS = Dose, CA = Catalase activity, DA = Dehydrogenase activity, IA = Invertase activity, UA = Urease activity, PHA = Phosphatase activity, G = Germination, LR = Length of roots, LSH = Length of shoots, GR = Germination rate, GE = Germination energy, FG = Evenness of germination, AZ = *Azotobacter*, TNB = Total number of bacteria

Cutr 70 nm and Cutr 100 nm. For example, the abundance of *Azotobacter* was strongly correlated with the concentration of Cutr 70 nm in the soil but weakly correlated with the concentration of Cutr 100 nm in the soil. In contrast, invertase activity was one of the most informative indicators for Cutr 100 nm and uninformative when contaminated with Cutr 70 nm.

Thus, when studying the toxicity of Cu, both particle size and shape must be considered to select the most informative indicators. The study found that Cu nanoparticles had a greater toxic effect on soil biological parameters than the macrodispersed form. This result is corroborated with the previous study by Tsepina et al. (2022). Nanoparticles have greater toxicity than macrodispersed forms because of their larger specific surface area, higher surface energy, and greater chemical reactivity, which allow for enhanced interactions with microbial cells or plant

tissues. It has been suggested that nanoparticles smaller than 100 nm may interact more readily with biological membranes, hence enhancing their potential biological effects (Manikandana et al., 2012).

In the present study, the toxicity of nanoparticles with sizes of 100 nm and 70 nm was studied, but even for such an insignificant size difference, it was found that Cu nanoparticles in the “torn” shape were more toxic at a size of 70 nm than at 100 nm. There were no differences for the spherical shape. This indicates that the toxicity of nanoparticles is determined not only by their size, but also by their shape. The study established a high degree of toxicity for “torn” nanoparticles in comparison with the spherical shape. Perhaps this is due to the fact that the nanoparticles with a “torn” shape have a loose porous surface, which provides a larger contact area and higher reactivity. It was previously found

that dendritic and spindle-shaped nanoparticles had higher cytotoxicity than spherical particles (Sajjad et al., 2023).

Critical doses of Cu intake into the soil

An important result of this study is the establishment of critical doses of Cu intake into the soil that do not cause disruption of the most important ecosystem functions. Based on the results of the IIBS, regression equations were constructed for all the studied sizes and shapes of Cu (Equations 3 to 7).

$$\text{For Cusf 70 nm: } y = 170.82 \cdot x^{-0.165} \quad (3)$$

$$\text{For Cusf 100 nm: } y = 161.17 \cdot x^{-0.15} \quad (4)$$

$$\text{For Cu 7,000 nm: } y = 161.89 \cdot x^{-0.138} \quad (5)$$

$$\text{For Cutr 70 nm: } y = 217.85 \cdot x^{-0.25} \quad (6)$$

$$\text{For Cutr 100 nm: } y = 160.13 \cdot x^{-0.159} \quad (7)$$

Previously, it was established that when the IIBS decreases by less than 5%, soil functions normally. A decrease of 5 to 10% disrupts information functions; 10 to 25% disrupts chemical, physico-chemical, biochemical, and integrative functions; and a decrease of > 25% disrupts physical functions. The critical Cu doses in soil were defined as the concentrations at which the IIBS value reached 90% of the control. Thus, the critical dose was 48.6 mg kg⁻¹ for both Cusf 70 nm and Cusf 100 nm, 70.4 mg kg⁻¹ for Cu 7,000 nm, 34.3 mg kg⁻¹ for Cutr 70 nm, and 37.7 mg kg⁻¹ for Cutr 100 nm. It is obvious that Cutr 70 nm exhibited the highest toxicity, with a critical concentration significantly lower than those of other Cu forms and sizes studied in this work.

Limitations

This study has several limitations that should be acknowledged. First, the experiments were conducted under controlled laboratory conditions using a single soil type (Haplic Chernozem). As noted earlier, the high buffering capacity of Chernozem may result in less pronounced toxicity effects compared to more sensitive soil types. Therefore, the observed effects may be conservative estimates of Cu nanoparticle toxicity in other soils.

CONCLUSIONS

This study demonstrated that the ecotoxicity of Cu nanoparticles in soil is critically determined by particle morphology and size. The “torn” porous morphology exhibited greater toxicity than spherical particles due to enhanced surface

reactivity and a higher density of reactive sites. Size-dependent differences were observed exclusively for the “torn” shape, with 70 nm particles more toxic than 100 nm, indicating that surface structure modulates the biological impact of particle size. The most sensitive indicators—catalase, dehydrogenase, urease, and root length—are recommended for soil biomonitoring. Current permissible Cu concentrations (100 to 150 mg kg⁻¹) may be inadequate, as critical thresholds for Cutr 70 nm reached 34.3 mg kg⁻¹, necessitating revision of regulatory standards to account for nanoparticulate Cu forms. Future research should focus on long-term chronic effects, studies across diverse soil types and plant species, and field validation of laboratory findings to develop robust risk assessment frameworks for nanotechnology in agriculture.

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DECLARATION OF GENERATIVE AI USE

The AI tools were used for any purpose of this manuscript.

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