Supporting Information

Risk assessment of metal/bio-based nanopesticides: Plant growth, soil environment, and non-target organisms

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Text S1 Determination of soil enzymes

Polyphenol oxidase was measured according to a previous study with minor modifications.[1] Briefly, 0.05 g of air-dried soil was passed through a 40-60 mesh sieve and placed in a 2 mL centrifuge tube. After incubation, add 0.1 mL of citric acid-phosphate buffer and 0.875 mL of ether, shake thoroughly, and extract at room temperature for 30 min. Transfer 0.2 mL of the extracted solution to a microplate and measure the absorbance at 430 nm. A control experiment was conducted by replacing the pyrogallol solution with distilled water, while following the same procedures as the sample. Finally, measure the absorbance value at 540 nm. A standard curve using potassium dichromate solution should be constructed to calculate the polyphenol oxidase activity in the sample.

Urease activity was measured according to a previous study with minor modifications.[1] Briefly, weigh 0.1 g of air-dried soil passing through a 40-60 mesh sieve and place it into a 2 mL centrifuge tube. Add 2 drops of toluene solution, mix thoroughly, and let it sit for 10 min. Then, add 0.2 mL of urea solution as the substrate and 0.4 mL of citrate buffer solution. Mix well and incubate at 37°C for 24 hours. After incubation, centrifuge at 10,000 rpm for 3 min and collect the supernatant as the sample solution. A control experiment should also be performed, where distilled water is used in place of the urea solution, with all other steps identical to those of the experimental procedure. Prepare nitrogen standard solutions of different concentrations. For each, pipette 0.025 mL of the respective nitrogen standard solution, along with the supernatant from the sample tube and the control tube, into a 96-well microplate. Add 0.1 mL of sodium phenolate solution and 0.075 mL of sodium hypochlorite solution. Mix thoroughly, incubate at 37°C for 20 min, and then measure the absorbance at 578nm using a spectrophotometer.

Sucrase activity was determined using the colorimetric method with 3,5-dinitrosalicylic acid.[2] Briefly, weigh 0.1 g of air-dried soil passing through a 40-60 mesh sieve and place it into a 2 mL centrifuge tube. Add 2 drops of toluene solution, mix thoroughly, and allow it to stand for 10 min. Then, add 0.2 mL of urea solution as

the substrate and 0.4 mL of citrate buffer solution. Mix well, and incubate at 37°C for 24 hours. After incubation, centrifuge at 10,000 rpm for 3 min and collect the supernatant as the sample solution. A control experiment should also be conducted, where distilled water is used in place of the urea solution, with all other experimental steps identical. Prepare nitrogen standard solutions of different concentrations. For each, pipette 0.025 mL of the respective nitrogen standard solution, along with the supernatant from the sample tube and the control tube, into a 96-well microplate. Add 0.1 mL of sodium phenolate solution and 0.075 mL of sodium hypochlorite solution. Incubate at 37°C for 20 min, then measure the absorbance at 578 nm using a spectrophotometer.

Acid phosphatase activity: was measured using the method with minor modifications.[3] Briefly, weigh 0.1 g of air-dried soil passsing through a 40-60 mesh sieve and place it into a 2 mL centrifuge tube. Add 0.15 mL of toluene, mix thoroughly, and allow it to stand for 10 min. Then, add 0.2 mL of disodium phosphate solution and 0.2 mL of acetate buffer solution. Mix well and incubate at 37°C for 24 hours. After incubation, centrifuge at 10,000 rpm for 3 min and collect the supernatant as the sample solution. A control tube should also be prepared, where distilled water is used instead of disodium phosphate solution, with all other steps identical to those of the experimental procedure.Prepare phenol standard solutions of varying concentrations. For each, pipette 0.03 mL of the respective phenol standard solution, along with the supernatant from the sample tube and the control tube, into a 96-well microplate. Add 0.1 mL of buffer solution and a solution of chlorinated dibromo-para-benzoquinone imine. Mix thoroughly, incubate at room temperature for 30 min, and then measure the absorbance at 570 nm using a spectrophotometer.

Text S2 Soil element extraction

The total elements in soil samples were extracted by microwave digestion method, and the concentrations of phosphorus, sulfur, potassium, calcium, magnesium, copper, manganese, and iron elements in soil were determined by inductively coupled plasma

mass spectrometry (ICP-MS, Thermo Fisher, Germany).[4] Briefly, 15 mg of air-dried and finely ground soil (passed through a 100-mesh sieve) was weighed into a digestion tube, followed by the addition of 3 mL of nitric acid and 3 mL of Milli-Q water for pre-digestion, aimed at decomposing organic matter. The mixture was then digested using a microwave digestion instrument (Mars 6, 1600W, 190°C, USA). After digestion, the solution was filtered through a 0.22 μm membrane to remove undissolved particles, ensuring a clear extract. Finally, the solution was diluted to 50 mL with ultrapure water and stored at 4°C for analysis. Calibration curves of each element were obtained by standard addition method. The linear range and equation, detection limit and peak recovery rate are shown in Table S1.

Text S3. Soil DNA extraction and Illumina Miseq sequencing

Total genomic DNA was extracted from 0.3 g fresh soil using the OMEGA Soil DNA Kit (M5635-02, Omega Bio-Tek, Norcross, GA, USA), following the manufacturer's instructions. The extracted DNA was stored at −20 °C until further analysis. The quantity and quality of the DNA were assessed using a NanoDrop NC2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and agarose gel electrophoresis, respectively. For bacterial community analysis, the V3–V4 region of the 16S rRNA gene was amplified using the forward primer 338F (5'-ACTCCTACGGGAGGCAGCA-3') and the reverse primer 806R (5'-GGACTACHVGGGTWTCTAAT-3'), with sample-specific 7-bp barcodes integrated into the primers for multiplex sequencing.[5] Amplicons were purified using Vazyme VAHTS DNA Clean Beads (Vazyme, Nanjing, China) and quantified with the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, Carlsbad, CA, USA). The amplicons were pooled in equal amounts after individual quantification, and pair-end 2×250 bp sequencing was performed using the Illumina MiSeq platform with the MiSeq Reagent Kit v3 at Shanghai Personal Biotechnology Co., Ltd (Shanghai, China).

Text S4. Assessment of oxidative stress and antioxidant defense of tadpole tissue

The tadpole tissues were pre-treated as follows: Homogenate 0.1 g of tissue sample in a 2 mL centrifuge tube with 1 mL of distilled water and a steel bead using a high-

throughput homogenizer. Subsequently, centrifuge the mixture at 10,000 rpm for 3 min. The supernatant is used as the sample to be measured.

Hydrogen peroxide content: Mix 80 μL of the supernatant with 40 μL of hydrochloric acid solution and 80 μL of ammonium molybdate solution, then allow the mixture to stand at room temperature for 8 min. Measure the absorbance at 455 nm using a microplate reader. Additionally, prepare a standard curve for hydrogen peroxide.

Tadpole tissue malondialdehyde content: Transfer 400 μL of the extract and 600 μL of thiobarbituric acid solution into a 2 mL centrifuge tube, mix well, and then incubate in a boiling water bath for 10 min. After cooling, transfer 250 μL of the mixture into a 96-well plate and measure the absorbance at 532 nm, 600 nm, and 450 nm respectively.

Catalase activity in tadpole tissues: When preparing the reagents, take 25 μL of the supernatant and place it in a cuvette. For the detection, mix 15 μL of hydrogen peroxide solution with the sample solution in the cuvette, and allow the mixture to react for 10 min at 25°C. Subsequently, add 50 μL of sodium chloride solution and 110 μL of ammonium molybdate solution. After mixing well, immediately measure the absorbance at 410 nm.

Superoxide dismutase activity in tadpole tissues: Add 40 μL of phosphate buffer, 30 μL of methionine solution, 30 μL of nitro blue tetrazolium solution, 30 μL of Ethylene diamine tetraacetic acid solution, and 30 μL of riboflavin solution to both the sample tube and the light control tube. To the sample tube, also add 20 μL of the extract and 20 μL of distilled water, while the light control tube receives an additional 20 μL of distilled water instead of the extract, making the total volume 40 μL. Place all tubes under a light source to develop color for 20 min, and then measure the absorbance at a wavelength of 560 nm.

Table S1. Relative abundances of top 10 bacterial phyla in the rhizosphere soil of tomato seedlings.

Phylum	CK	nCuO1	Cu^{2+}	nCS100	CGA
Proteobacteria	41.52 ± 1.61 c	49.90 ± 1.26 a	46.41 ± 1.91 ab	45.37 ± 1.91 b	46.13 ± 3.35 ab
Actinobacteriota	16.55 ± 1.51 ab	17.64 ± 0.91 a	12.86 ± 1.32 d	15.35 ± 0.76 bc	13.75 ± 1.03 cd
Acidobacteriota	11.04 ± 1.26 a	8.34 ± 0.17 b	7.98 ± 0.77 b	10.37 ± 0.35 a	8.05 ± 0.61 b
Bacteroidota	8.52 ± 0.57 b	7.22 ± 0.60 c	10.39 ± 0.51 a	8.36 ± 0.13 bc	9.79 ± 1.11 a
Gemmatimonadota	8.87 ± 0.98 ab	6.48 ± 0.45 c	7.54 ± 0.68 bc	9.37 ± 0.36 a	7.70 ± 1.30 bc
Chloroflexi	5.45 ± 0.07 a	3.52 ± 0.07 c	4.14 ± 0.12 bc	4.37 ± 0.57 b	3.70 ± 0.52 bc
Myxococcota	2.91 ± 0.34 b	1.81 ± 0.15 c	4.13 ± 0.39 a	2.26 ± 0.30 c	3.94 ± 0.32 a
Verrucomicrobiota	1.19 ± 0.16 a	1.08 ± 0.31 a	1.23 ± 0.12 a	0.90 ± 0.36 a	1.10 ± 0.26 a
Firmicutes	0.66 ± 0.12 a	0.66 ± 0.09 a	0.72 ± 0.20 a	0.52 ± 0.11 a	0.52 ± 0.06 a
Bdellovibrionota	0.15 ± 0.04 b	0.11 ± 0.01 b	1.42 ± 0.11 a	0.11 ± 0.03 b	1.19 ± 0.27 a

Data were means ± SE (n = 3). Different lowercase letters indicated significant differences among treatments ($p < 0.05$)

Table S2. Relative abundances of top 20 bacterial genera in the rhizosphere soil of tomato seedlings.

Genus	CK	nCuO1	Cu^{2+}	nCS100	CGA
KD4-96	0.84 ± 0.03 a	0.54 ± 0.08 c	0.60 ± 0.02 bc	0.72 ± 0.13 ab	0.58 ± 0.06 bc
Iamia	2.20 ± 0.05 a	1.58 ± 0.20 b	1.25 ± 0.20 c	2.19 ± 0.09 a	1.50 ± 0.11 bc
Altererythrobacter	1.84 ± 0.07 a	1.23 ± 0.21 b	1.17 ± 0.13 b	1.63 ± 0.14 a	1.26 ± 0.10 b
Vicinamibacteraceae	3.65 ± 0.43 a	2.50 ± 0.09 c	2.03 ± 0.12 d	3.14 ± 0.12 b	1.93 ± 0.14 d
Ellin6067	1.70 ± 0.18 a	0.98 ± 0.19 c	0.63 ± 0.09 d	1.25 ± 0.10 b	0.75 ± 0.09 cd
Luteimonas	1.39 ± 0.38 a	1.25 ± 0.32 a	1.15 ± 0.32 a	1.26 ± 0.06 a	1.42 ± 0.11 a
Terrimonas	1.13 ± 0.29 a	0.70 ± 0.06 b	0.86 ± 0.03 ab	0.97 ± 0.08 ab	0.93 ± 0.07 ab
Ilumatobacter	0.95 ± 0.08 a	0.74 ± 0.16 b	0.91 ± 0.12 ab	1.00 ± 0.13 ab	1.10 ± 0.26 b
Limnobacter	0.87 ± 0.20 b	0.79 ± 0.03 b	0.94 ± 0.24 ab	0.79 ± 0.08 b	1.25 ± 0.23 a
Arenimonas	1.13 ± 0.03 a	1.07 ± 0.11 a	1.30 ± 0.11 a	1.13 ± 0.18 a	1.26 ± 0.17 a
Haliangium	0.99 ± 0.03 b	0.62 ± 0.08 c	1.33 ± 0.13 a	0.86 ± 0.16 b	1.22 ± 0.12 a
Rhodobacter	0.74 ± 0.06 b	0.84 ± 0.07 b	1.39 ± 0.07 a	0.80 ± 0.14 b	1.38 ± 0.15 a
Phaselicystis	0.50 ± 0.06 c	0.38 ± 0.06 c	1.11 ± 0.06 a	0.52 ± 0.07 c	0.94 ± 0.12 b
Streptomyces	0.43 ± 0.28 b	1.90 ± 0.78 a	1.58 ± 0.95 ab	1.76 ± 0.31 a	1.46 ± 0.95 ab
Flavihumibacter	1.45 ± 0.07 b	1.88 ± 0.12 a	1.66 ± 0.26 ab	1.71 ± 0.03 ab	1.68 ± 0.38 ab
Aeromicrobium	0.53 ± 0.02 c	0.90 ± 0.06 b	0.85 ± 0.14 b	0.83 ± 0.08 b	1.02 ± 0.09 a
Sphingomonas	1.71 ± 0.16 c	25.50 ± 0.44 a	1.94 ± 0.18 bc	2.54 ± 0.18 a	2.30 ± 0.08 ab
Aquicella	0.71 ± 0.08 b	0.91 ± 0.11 a	0.60 ± 0.04 b	1.04 ± 0.10 a	0.62 ± 0.03 b
Lysobacter	15.85 ± 1.97 b	23.61 ± 0.60 a	19.63 ± 2.79 b	19.34 ± 1.48 b	18.24 ± 2.90 b
Thiobacillus	1.26 ± 0.80 a	1.69 ± 0.50 a	0.95 ± 0.50 a	1.15 ± 0.33 a	1.15 ± 0.71 a

Data were means ± SE (n = 3). Different lowercase letters indicated significant differences among treatments ($p < 0.05$)

Table S2. Typical sensitivity, detection limits and R^2 for element detection by ICP-MS.

Element	Linearity range (ug/L)/R	Linearity equation	Detection limit (ug/L)	Bush branches and leaves (GBW07602): Standard value (μg/g)	Bush branches and leaves (GBW07602): ICP-MS measured value (μg/g, n = 5)
Phosphorus	0-10/0.9998	y = 738.39x − 764.17	203	830 ± 40	838 ± 35
Sulphur	0-10/0.9999	y = 1219.99x − 55104.26	552	3200 ± 300	3255 ± 14
Potassium	0-10/0.9999	y = 101.98x − 3395.61	499	8500 ± 500	8720 ± 187
Calcium	0-10/0.9950	y = 8.04x − 357.13	1768	22200 ± 1300	23398 ± 1385
Magnesium	0-10/0.9990	y = 155.53x − 210.58	129	2870 ± 180	2863 ± 29
Copper	0-0.1/0.9998	y = 23906.23x − 91149.17	85	5.2 ± 0.5	5.3 ± 0.05
Manganese	0-0.1/0.9950	y=2187.52x − 143.70	10	58 ± 6	63 ± 0.35
Iron	0-10/0.9996	y=118.72x − 73.85	140	1020 ± 67	1085 ± 1.86

Figure S1

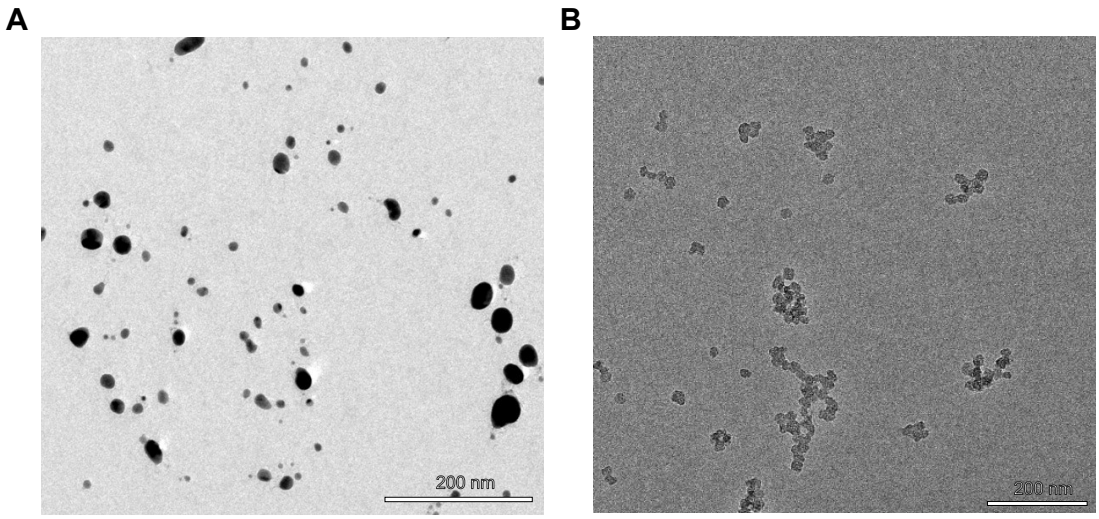

Fig. S1. TEM images of nCuO (A) and nCS (B).

Figure S2

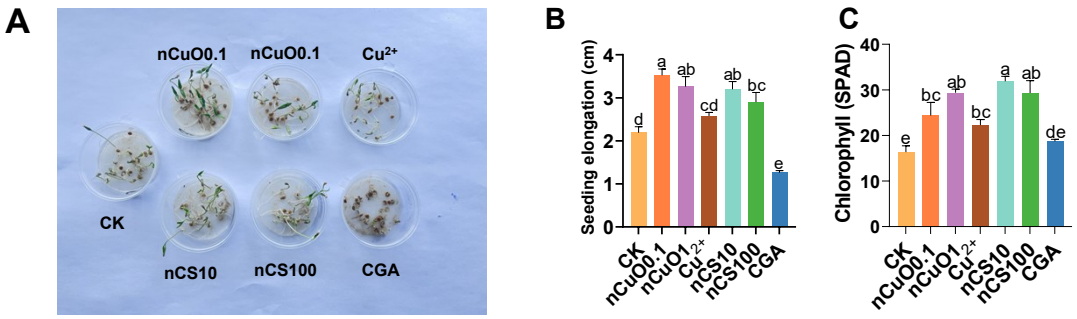

Fig. S2. Effects of nCuO and nCS on tomato seed germination: Photograph of seed germination (A) and seedling elongation (B). Effects of nCuO and nCS on tomato leaves chlorophyll content (C). Different letters on bars represent significant difference among different treatments ($p < 0.05$, n = 3).

Figure S3

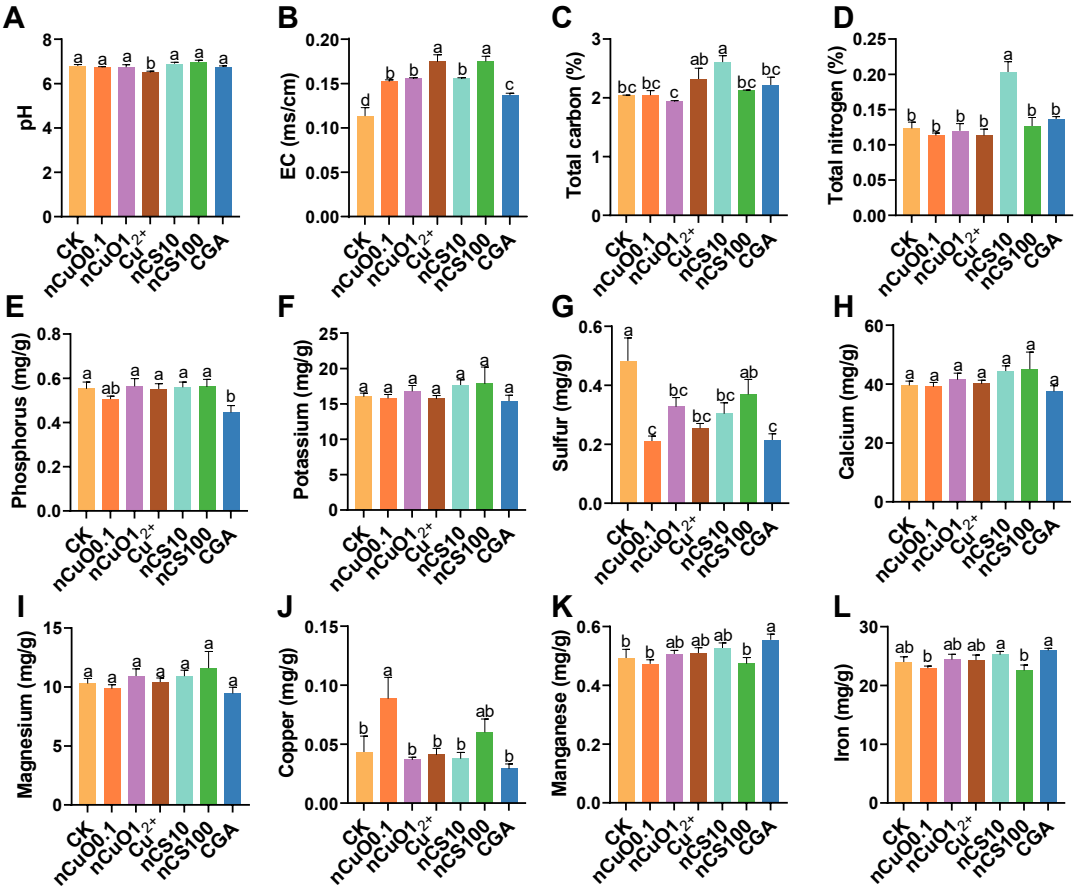

Fig. S3. Effects of nCuO and nCS on physical and chemical properties of soil: pH (A), electrical conductivity (EC) (B). Effects of nCuO and nCS on the contents of elements in soil: total carbon (C), total nitrogen (D), phosphorus (A); sulphur (B); potassium (C); calcium (D); magnesium (E); copper (F); manganese (G); iron (H). Different letters on bars represent significant difference among different treatments ($p < 0.05$, n = 3).

Figure S4

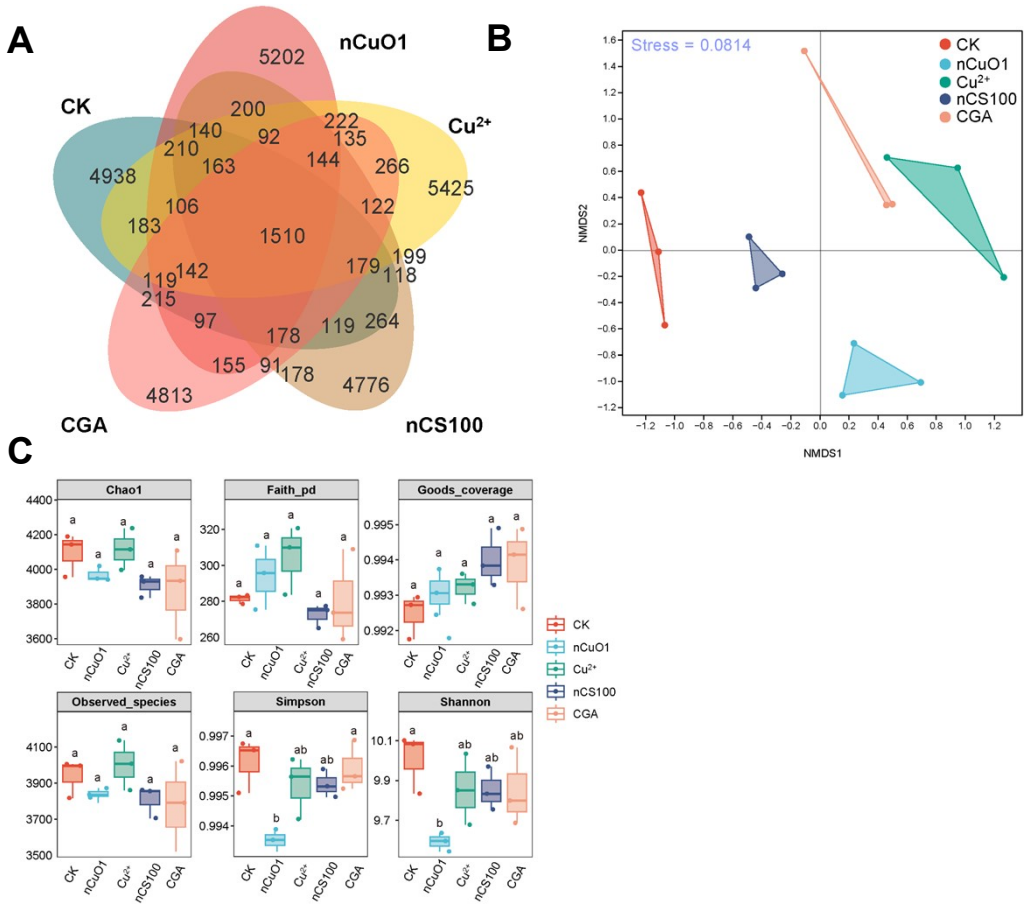

Fig. S4. Effects of nCuO1 and nCS100 on rhizosphere soil microorganisms. Venn diagram revealing the distinct and overlapping microbial populations across the different treatments (A). β-diversity analysis, employing nonmetric multidimensional scaling (NMDS) to visualize disparities in microbial community structure (quantified by Bray-Curtis distances) (B). Assessment of α-diversity, encompassing Chao1, Faith_pd, Goods_coverage, Observed_species, Simpson and Shannon estimators (C). The superscript letters indicating significant differences among treatments (LSD test, $p < 0.05$, n = 3).

Figure S5

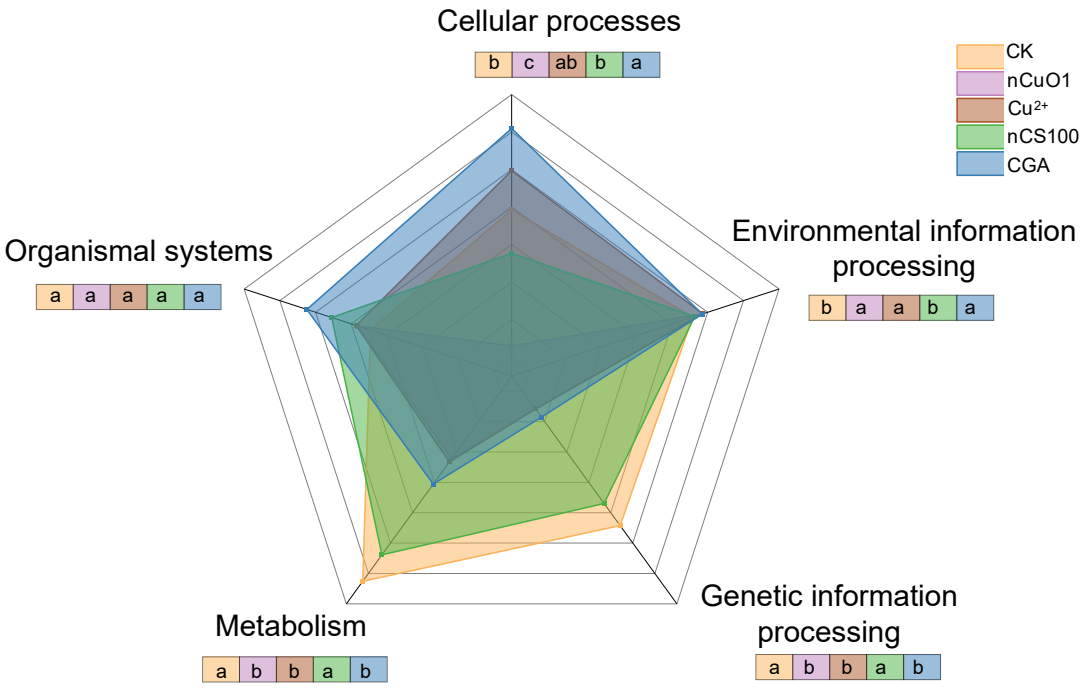

Fig. S5. Effects of nCuO1 and nCS100 on functional primary taxon of rhizosphere soil microorganisms. Different letters represent significant difference among different treatments ($p < 0.05$, n = 3)

Figure S6

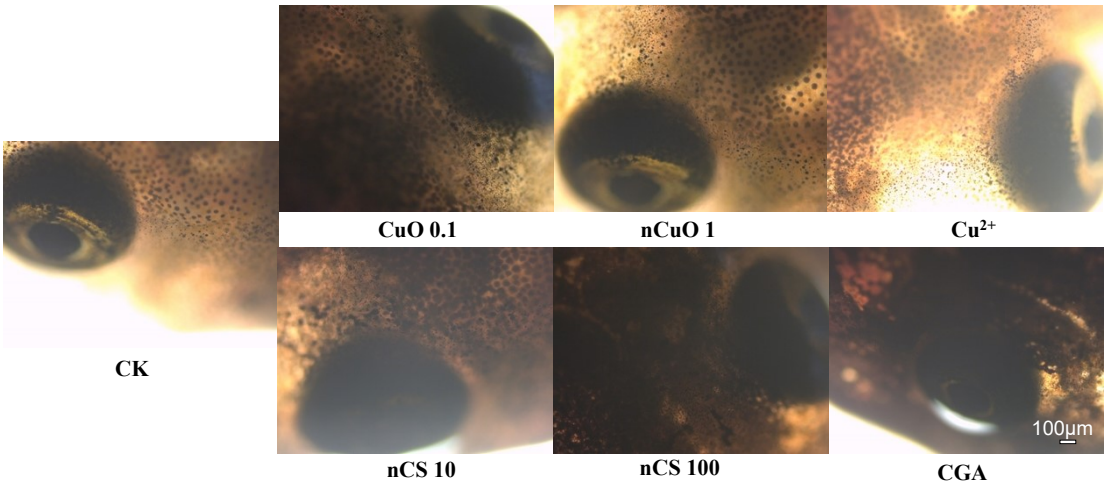

Fig. S6. Effects of nCuO and nCS on the distribution of melanin pigmentation around the eyes of tadpoles.

Figure S7

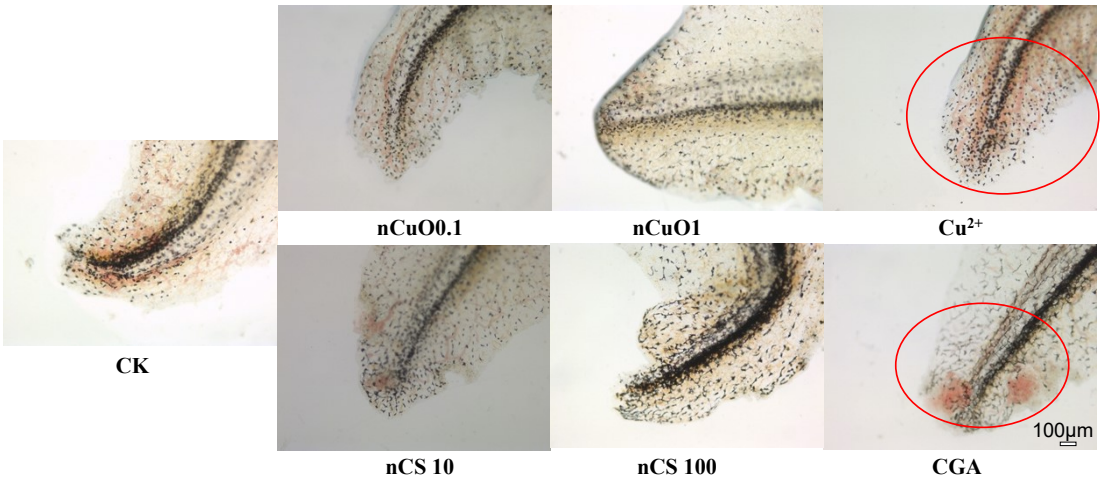

Fig. S7. Effects of nCuO and nCS on mechanical damage to tadpole tails. The red circle in the figure indicates obvious caudal damage.

References

1. H. Li, Y. Guo, A. Liang, X. Xu, Y. Hao, H. Shang, C. Li, Z. Cai, L. Han, J. Zhao, W. Jia, J. C. White, C. Ma and B. Xing, Commonly used engineered nanomaterials improve soil health via suppressing soil-borne fusarium and positively altering soil microbiome, *ACS EST Engg.*, 2024, **4**, 915-927.
2. L. Wang, Z. Dou, C. Ma, X. Jia, H. Wang, W. Bao, L. Wang, J. Qu and Y. Zhang, Remediation of di(2-ethylhexyl) phthalate (DEHP) contaminated black soil by freeze-thaw aging biochar, *J. Environ. Sci*, 2024, **135**, 681-692.
3. Y. Ding, M. Tao, L. Xu, C. Wang, J. Wang, C. Zhao, Z. Xiao and Z. Wang, Impacts of nano-acetamiprid pesticide on faba bean root metabolic response and soil health, *Sci. Total Environ.*, 2024, **927**, 171976.
4. J. Zuluaga, N. Rodríguez, I. Rivas-Ramirez, V. de la Fuente, L. Rufo and R. Amils, An Improved Semiquantitative Method for Elemental Analysis of Plants Using Inductive Coupled Plasma Mass Spectrometry, *Biol Trace Elem Res*, 2011, **144**, 1302-1317.
5. Z. Xiao, N. Fan, X. Wang, H. Ji, L. Yue, F. He and Z. Wang, Earthworms Drive the Effect of La2O3 Nanoparticles on Radish Taproot Metabolite Profiles and Rhizosphere Microbial Communities, *Environ. Sci. Technol.*, 2022, **56**, 17385-17395.