Electronic supplementary information (ESI)

Metal-phenolic network-coated nanoparticles mitigate CuO nanotoxicity

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1. Additional methods

1.1. Asymmetric-flow field-flow fractionation (AF4)

The characterization of Au nanoparticles (NPs) and Fe-TA@Au NPs was performed using an AF4 instrument MF2000 connected to a diode array detector (DAD, Postnova Analytics, Germany). For separation, a similar flow program and channel set-up were used as reported previously,[1] with the exception of using a lower ionic strength carrier [0.1 mM 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES) buffer, pH 7] that minimized NP association with the AF4 membrane.[2] To measure the size distribution of NPs, a size-retention relationship was established in DAD fractograms using Au NPs of different core diameters having uniform size distribution (polyethylene glycol or PEG-coated Au NPs obtained from nanoComposix, California, US). Absorbance at 530 nm was used to measure the elution of Au NPs and Fe-TA@Au NPs. Surface plasmon resonance (SPR) spectra were obtained online from individual fractions of fractionated NPs as well as from unfractionated samples (obtained by directly injecting samples in the channel without applying focusing or cross-flow). A comparison of the spectra after normalization of the signals based on the intensities at maximum SPR signals was performed. The Au content of Au NPs and Au and Fe contents of Fe-TA@Au NPs were determined by linking the outlet of AF4 to an ICP-MS instrument (7700x, Agilent Technologies, Tokyo, Japan). The acquired ICP-MS fractograms (cps vs time) were converted into mass-flow (µg/L vs L) using the detector flow value and a post-channel calibration. Elemental standards were prepared by dilution in 1% HNO_3 (v/v) of a 1000 mg/L certified gold standard (TraceCERT, Merck) and 100 mg/L multi-element standard solution (ROTI®Star, Carl Roth). The analysis involved the use of a He collision cell for minimizing polyatomic interferences for ^{56}Fe quantification and a 0.7 mL/min

introduction flow. The Au and Fe (µg/L) concentrations were obtained by peak integration of the mass flow and correction for injection volume and sample dilution.

1.2. Total reflection X-ray fluorescence spectroscopy (TRXF)

Dissolved Cu in CuO NP suspensions and unbound Cu amd Fe in Fe-TA@Au NP suspensions mixed with $CuSO_4$ or CuO NP were quantified using TRXF Picofox S2 (Bruker AXS Microanalysis GmbH). For this measurement, the NP suspensions were either analyzed immediately after preparation or after being shaken at room temperature in the dark for 2 or 24 h. The suspensions were centrifuged at 13,000*g* for 10 min to pellet the CuO or Fe-TA@Au NPs, and the supernatants were collected for Cu and Fe analysis. Forty µL of acidified supernatant (containing 1% HNO_3) was mixed with 40 µL of gallium (Ga) internal standard and 5 µL of the mixture was pipetted onto a quartz carrier disc. After air-drying, the concentrations of Cu and Fe were quantified and the data were analyzed using the Spectra software (AXS Microanalysis GmbH). For quantification of Au and Fe content in Fe-TA@Au NPs, the NPs were pelleted by centrifugation at 13,000g for 10 min, the supernatant was removed and the pellet was dried in a miVac centrifugal evaporator (SP Genevac, UK) for 2 h at 36ºC. The dried pellet was dissolved in 50 µL aqua regia containing 10 mg/L internal standard Ga and analyzed by TRXF Picofox S2 as described before.

1.3. Copper quantification using bicinchoninic acid assay

The Cu-adsorbing capacity of Fe-TA@Au NPs was investigated using a colorimetric bicinchoninic acid (BCA) assay.[3] For this, $CuSO_4 \cdot 5H_2O$ (99%, ACS, ISO, Reag. Ph Eur) solutions were prepared in ultrapure water in a range of Cu^{2+} concentration between 0.25 and 5 mg/L. For the adsorption study, 150 µL of each Cu^{2+} solution were mixed with 150 µL of Fe-TA@Au NPs (100 mg/L) in 1.5-mL centrifuge tubes covered with foil and shaken on a mixer for 2 h at 22 ºC

and pH 6.0 (2 h contact time was sufficient to reach adsorption equilibrium and saturation capacity). Afterward, mixtures were centrifuged at 13,000*g* for 10 min and the supernatants were collected. A portion of 100 µL of each supernatant was pipetted into the wells of a polypropylene 96-well flat-bottom plate in three replicates. Twenty µL of freshly prepared L-ascorbic acid (99+%, Extra Pure, SLR, Fisher Chemical™) at 352 mg/L were added to each well, followed by the addition of 80 µL of BCA Protein Assay Reagent A (Pierce™) diluted 150 times with ultrapure water. Absorption at 354 nm of all samples was measured using a UV-Vis spectrophotometer, and the concentrations of Cu^{2+} in samples were calculated based on the calibration graph.

1.4. Adsorption isotherms

The experimental data were fitted using the Freundlich (1) and Langmuir (2) adsorption isotherm modeling equations.

$$Q_e = K_F\, C_e^{\frac{1}{n}} \qquad (1)$$

$$Q_e = \frac{Q_m\, K_L\, C_e}{1+K_L\, C_e} \qquad (2)$$

where Q_e is the equilibrium adsorbate load in the solid phase, i.e., the amount of adsorbed Cu^{2+} per gram of Fe-TA MPN [mg/g], Q_m is the maximum adsorption capacity [mg/g], C_e is the equilibrium concentration of the Cu^{2+} in the aqueous phase [mg/L], $\frac{1}{n}$ is a dimensionless parameter that describes adsorption capacity or adsorbent surface heterogeneity, K_F is the Freundlich isotherm constant associated with the adsorption strength [$mg^{1-n}L^n/g$], and K_L is the Langmuir adsorption constant [L/mg] reflecting the binding site affinity.

1.5. Cultivation of *Tetrahymena thermophila*

Protozoa *T. thermophila* were maintained axenically in sterilized 2 % proteose peptone containing mung beans at room temperature in the dark. For viability testing, protozoa were

cultivated in a modified SSP medium containing 2 % proteose peptone, 0.1 % yeast extract, 0.2 % glucose, and 0.003 % ethylenediaminetetraacetic acid iron(III) sodium salt (Fe-EDTA) supplemented with 250 mg/L each of streptomycin sulfate (Sigma–Aldrich) and penicillin G (Gibco) and 1.25 mg/L fungicide amphotericin B as described previously.[4] Cultures were grown overnight in sterile polystyrene Petri plates containing 10 mL of SSP medium, inoculated with 100 µL of protozoan stock culture, in a humidity chamber at 30 ºC, without shaking. At the late exponential growth phase (~5×10^5 – 10^6 cells/mL), the cells were collected by centrifugation (1000*g*, 5 ºC, 10 min) and resuspended in Dryl's starvation medium (2 mM sodium citrate, 1 mM NaH_2PO_4, 1 mM Na_2HPO_4, 1.5 mM $CaCl_2$, pH 7.4). The cell suspension was centrifuged again, the pellet was resuspended in Dryl's medium, pipetted into clean Petri plates (10 mL per plate), and incubated overnight at 30 ºC.

1.6. Growth inhibition assessment using microalgae *Raphidocelis subcapitata*

R. subcapitata was from the commercial kit Algal Toxkit F (MicroBioTests Inc., Nazareth, Belgium) and kept in liquid OECD growth medium[5] at 21 °C, in an incubator AlgaeTron AG230 (Photon Systems Instruments, Drasov, Czech Republic) under continuous illumination. For toxicity testing, the stock culture was diluted in fresh growth medium[6] approximately three days before the test. Cultures in the exponential growth phase were used for testing (approximately 5×10^5 to 1×10^6 cells/mL, as determined by a Neubauer hemocytometer). For the growth inhibition assays, the algal cell concentration was adjusted to approximately 1×10^4 cells/mL. Fe-TA@Au NPs were synthesized freshly before each experiment and suspended in the algal growth medium at the last step of the synthesis. The tests were conducted in 20-mL glass scintillation vials containing 5 mL of algal culture in Fe-TA@Au NP suspension at 50 mg/L or unamended growth medium for negative controls. Three vials with test samples and six vials with unamended control

samples were evenly distributed across the incubation platform (CERTOMAT MO II shaker, B. Braun Biotech International, Germany) and shaken at 100 rpm, 21 °C, under continuous illumination (3200 lm, SYLVANIA START eco Panel Flat 600x600, Feilo Sylvania Europe Limited, UK). At 0, 24, 36, 48, 60, and 72 h, 50 µL from each vial were transferred to black 96-well polypropylene plates (Greiner Bio-One) and frozen (−18°C). For biomass quantification, samples were thawed and chlorophyll was extracted by adding 200 µL of 96% ethanol and incubating the plates in the dark on a shaker (400 rpm) at ambient temperature for one hour. Fluorescence was quantified at Ex 440/Em 670 nm using a microplate fluorometer (Fluoroscan Ascent, Thermo Labsystems, Finland). Chlorophyll fluorescence data were used to construct growth curves and calculate specific growth rates.

1.7. Confocal laser scanning microscopy

Protozoan samples were prepared for microscopy by fixing the cells in 5% formaldehyde and then concentrating by centrifugation for 10 min at 1000*g*. The supernatant was removed and the pellet was resuspended in 20 µL HEPES buffer. Microscopy samples were prepared by pipetting 5 µL of fixed protozoa onto a glass microscopy slide and mixed by pipetting with 5 µL Mowiol 4-88 (Sigma-Aldrich) solution. Glass coverslips were carefully placed on the liquid samples and then let harden at room temperature in the dark for 24 h. The slides were stored at 4 ºC until imaging. Imaging was done with a Zeiss LSM800 confocal laser scanning microscope (Darmstadt, Germany) and the images were processed using ZEN software (Carl Zeiss Microscopy, Oberkochen, Germany) and ImageJ (National Institutes of Health, NIH, Bethesda, MD).

2. Additional figures and tables

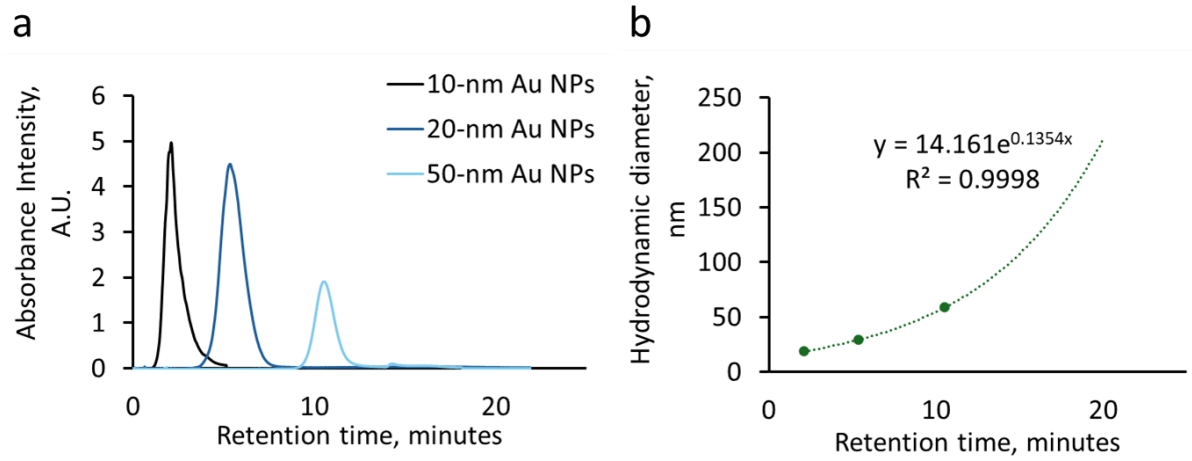

Figure S1. External calibration of the hydrodynamic size obtained by AF4-DAD. The retention times of standard Au NPs at the maximum peak intensities (a) were used to construct the calibration curve (b).

Table S1. Quantitative measurements of gold and iron contents in the Au NP and Fe-Ta@Au NP suspensions before and after AF4 fractionation as determined by ICP-MS.

	Au NPs[a]	**Fe-TA@Au NPs**		
	Au (mg/L)	Au (mg/L)	Fe (mg/L)	Fe/Au ratio[b]
Suspension	55.5 ± 3.4	55.6 ± 2.7	0.34 ± 0.004	0.0061
AF4	50.9 ± 1.6	41.2 ± 3.8	0.15 ± 0.01	0.0036
Recovery in AF4[c]	92%	74%	44%	n.a.

[a]Au NPs also contained traces of Fe at 0.02 mg/L, likely as impurities of $HAuCl_4$ or TA; [b]ratio of Fe and Au content in Fe-TA@Au NPs; [c]recovery percentages were calculated by dividing the values obtained after AF4 fractionation by the metal contents in the NP suspensions; the recovery % reflects the loss of NPs or their components during the AF4 fractionation.

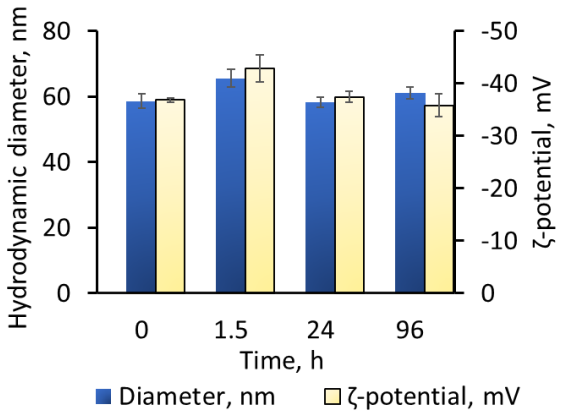

Figure S2. Hydrodynamic diameters (Z-average values) and ζ-potential values of Fe-TA@Au NPs suspended in ultrapure water for 96 h. Data bars are average values of three replicate measurements and error bars indicate standard deviations.

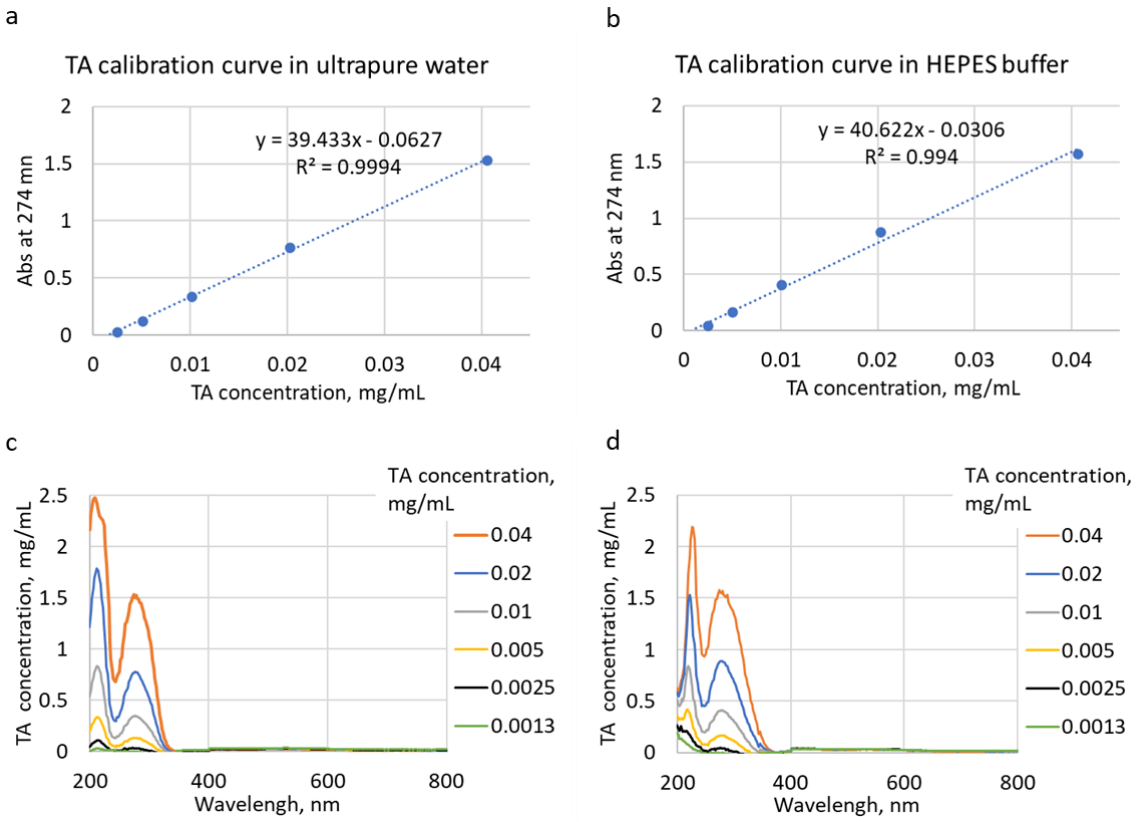

Figure S3. Determination of tannic acid (TA) concentration using UV-Vis. Relationship between TA concentration in ultrapure water (a, c) and 10 mM HEPES buffer, pH 7.5 (b, d) and UV-Vis absorbance. Absorbance at 274 nm was plotted against TA concentration to achieve calibration curves.

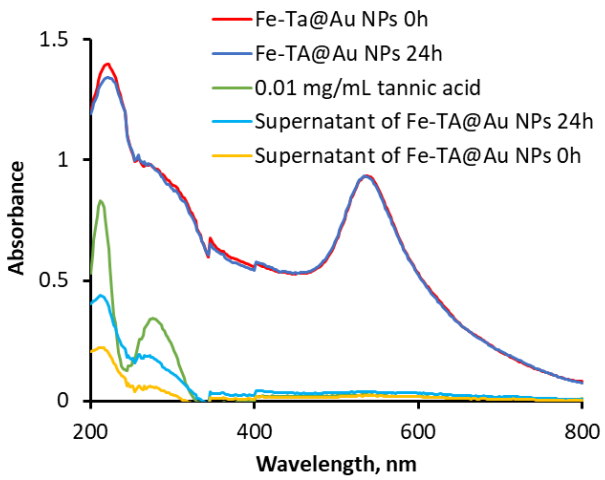

Figure S4. UV-Vis spectra of Fe-TA@Au NPs in ultrapure water and respective supernatants collected from the pelleted Fe-TA@Au NPs (13,000*g*, 10 min) immediately after synthesis and after 24 h of shaking at room temperature. The absorbance spectrum of 0.01 mg/mL tannic acid (TA) demonstrates TA-characteristic peaks in the spectra and illustrates the peak height at this TA concentration.

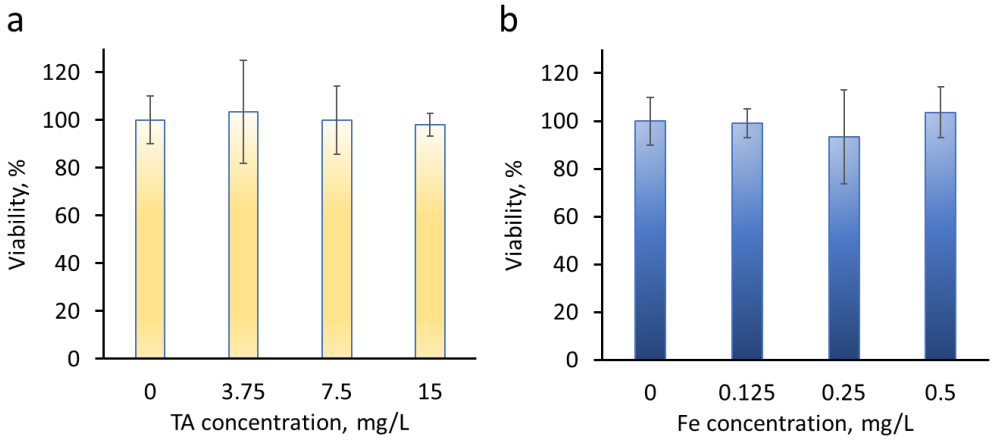

Figure S5. Relative viability of *T. thermophila* after exposure to Fe-TA@Au NP MPN components. *T. thermophila* was exposed to tannic acid, TA, (a) or $FeCl_3 \cdot 6H_2O$ (b) for 24 h in ultrapure water at the respective chemical concentration relevant to the content in MPN. Data bars are average values of three replicates and error bars indicate standard deviations.

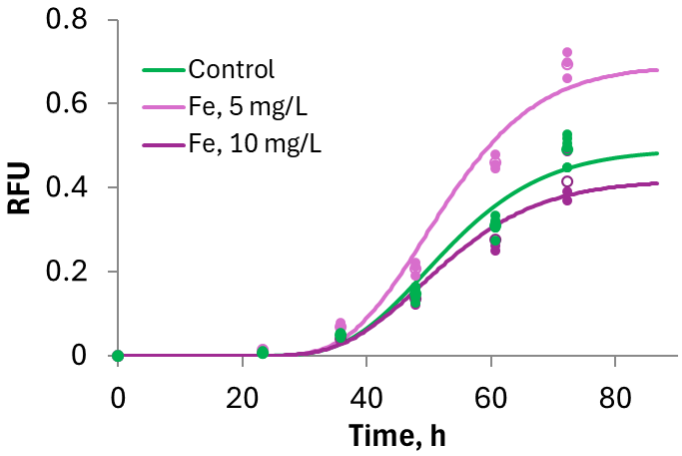

Figure S6. Growth of *R. subcapitata* control and Fe-exposed cultures. Fe was added as $FeCl_3$ at nominal total Fe concentrations of 5 mg/L or 10 mg/L. Biomass was quantified by extracted chlorophyll fluorescence measurements (RFU – relative fluorescence units). The experimental data from replicates were fitted into a logarithmic model (log-normal) using MS Excel macro REGTOX. The specific growth rates were calculated based on the natural logarithm-transformed RFU data in time points 24 – 60 h and were as follows: 0.1 ± 0.007 h^{-1} for the control culture, 0.09 ± 0.004 h^{-1} for the 5 mg/L Fe-exposed culture, and 0.09 ± 0.004 h^{-1} for the 10 mg/L Fe-exposed culture. There was no statistically significant difference between these values (t-test, $p > 0.05$).

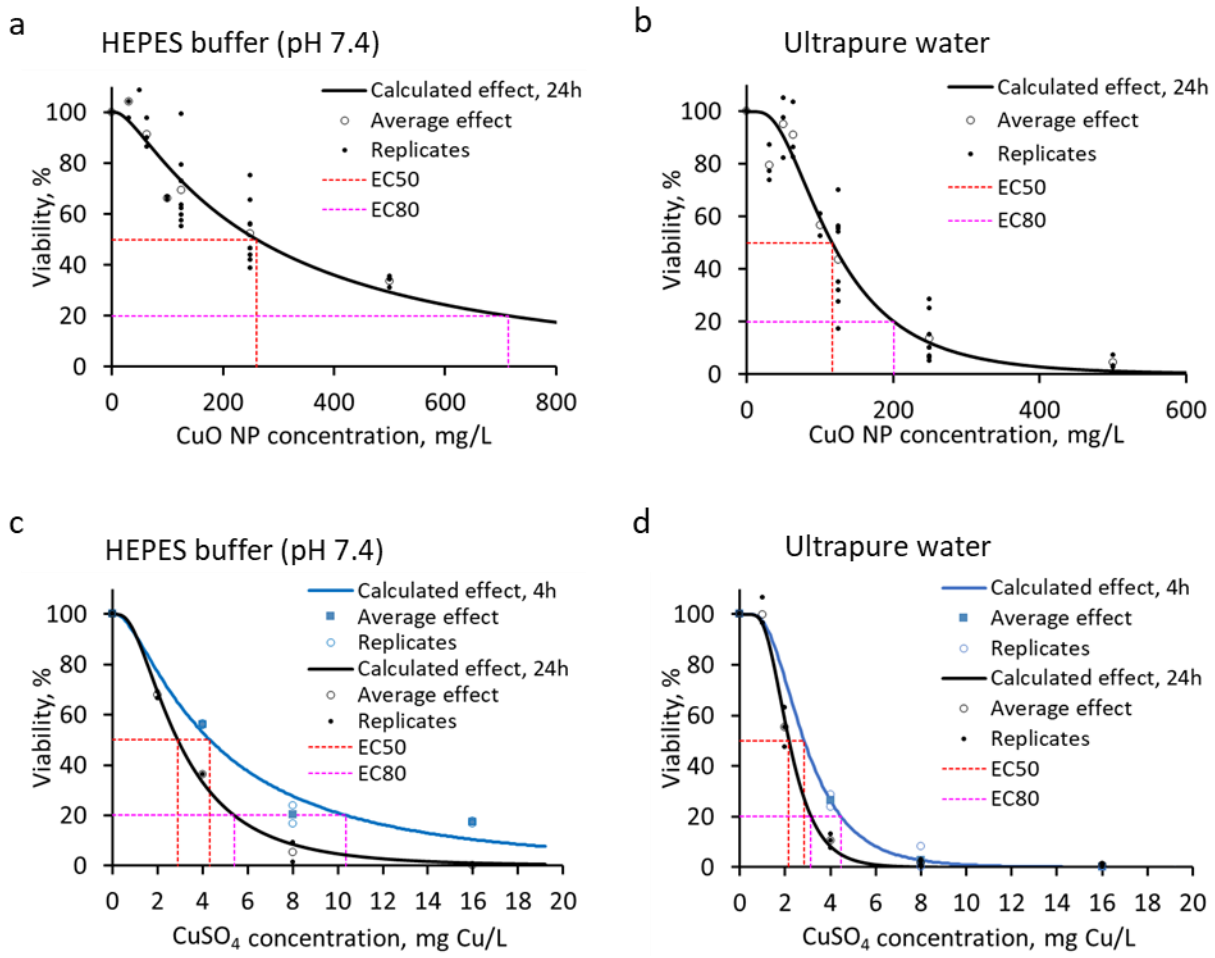

Figure S7. Dose-response curves for CuO NPs (a, b) and $CuSO_4$ (c, d) after exposure of *T. thermophila* for 24 h (in the case of CuO NPs) or 4 and 24 h (in the case of $CuSO_4$) in 10 mM HEPES buffer, pH 7.4 (a, c) or ultrapure water, pH 6 (b, d). The graphs were generated using an Excel Macro REGTOX (https://www.normalesup.org/~vindimian/en_index.html). The numerical values of EC_{50} and EC_{80} are in **Table S2**.

Table S2. Average EC_{50} and EC_{80} values of CuO NPs to *T. thermophila* after 24-h exposure and of $CuSO_4$ after 4- and 24-h exposure in ultrapure water or 10 mM HEPES buffer, pH 7.4. Average values and 95% confidence intervals were calculated using an MS Excel Macro REGTOX.

	10 mM HEPES, pH 7.4		**Ultrapure water**	
	4-h exposure	24-h exposure	4-h exposure	24-h exposure
CuO NPs				
EC_{50}, mg/L	ND[a]	260 (229 – 346)	ND	116 (102 – 129)
EC_{80}, mg/L	ND	713 (509 – 1190)	ND	201 (164 – 240)
$CuSO_4$				
EC_{50}, mg Cu/L	4.3 (3.1 – 5.1)	2.9 (2.7-3.0)	2.8 (2.3-3.4)	2.2 (2.1-2.3)
EC_{80}, mg Cu/L	10.4 (8.4 – 13.8)	5.4 (4.8 – 5.7)	4.5 (4.1-4.8)	3.2 (2.9-3.5)

[a]ND – not determined (i.e., not tested)

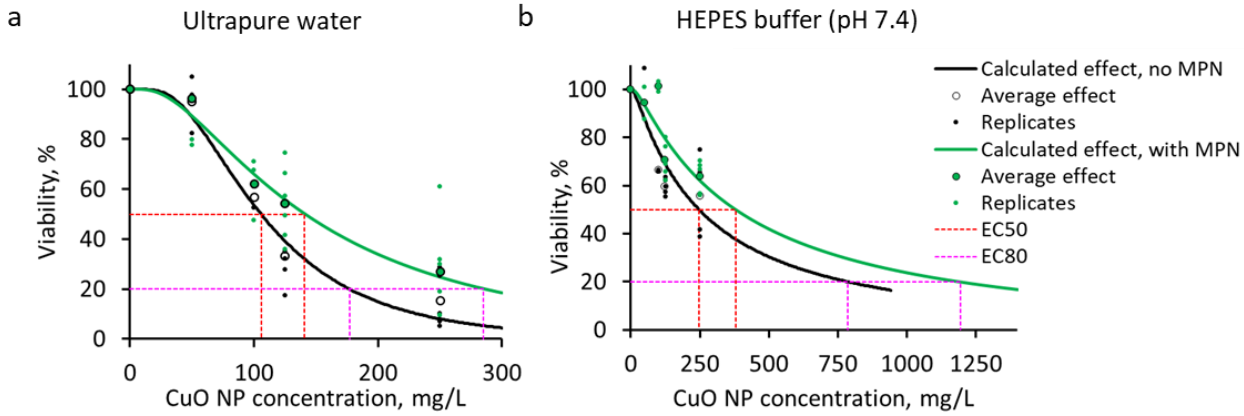

Figure S8. Dose-response curves upon 24-h exposure of *T. thermophila* to CuO NPs with and without Fe-TA@Au NPs (MPN or metal-phenolic networks). (a) Toxicity test was done in ultrapure water, pH ~6.0, and (b) in 10 mM HEPES buffer, pH 7.4. Black lines and data points indicate results obtained without Fe-TA@Au NPs, and green lines and data points show results from tests with Fe-TA@Au NPs. The dose responses were modeled using an Excel Macro REGTOX.

Table S3. Average EC_{50} and EC_{80} values of CuO NPs with and without Fe-TA@Au NPs for THP-1-derived macrophages after 24-h exposure in RPMI 1649 medium. Average values and 95% confidence intervals were calculated using an MS Excel Macro REGTOX.

	CuO NPs	**CuO NPs with Fe-TA@Au NPs**
EC_{50}, mg/L	4.7 (4.3 – 5.2)	15.4 (13.6 – 17.4)
EC_{80}, mg/L	16.9 (15.2 – 19.2)	61.9 (48.7 – 79.9)

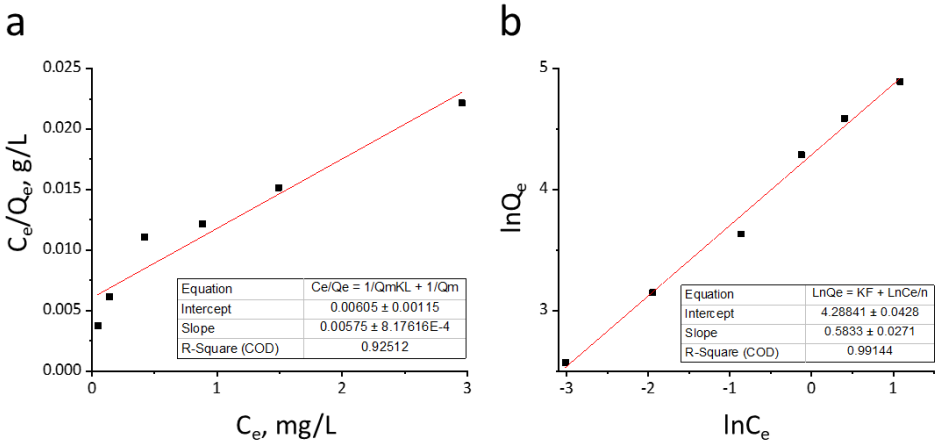

Figure S9. Linearized Langmuir (a) and Freundlich (b) isotherms characterizing Cu^{2+} adsorption by Fe-TA@Au NPs.

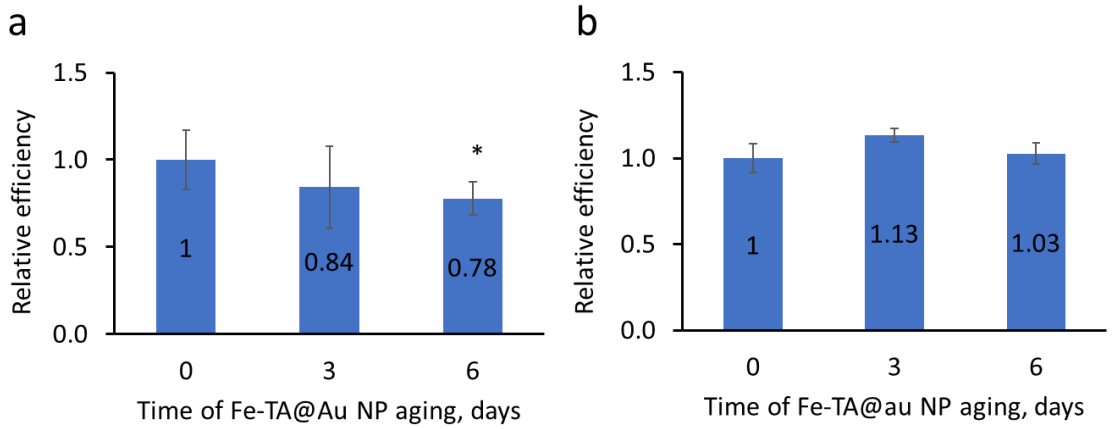

Figure S10. Relative efficiency of Fe-TA@Au NPs in reducing $CuSO_4$ toxicity at 2 mg Cu/L after aging of Fe-TA@Au NPs for 3 and 6 days (at 4°C in the dark). *T. thermophila* was exposed to $CuSO_4$ and fresh or aged Fe-TA@Au NPs in ultrapure water for 4 h (a) or 24 h (b). Data bars are the average of three replicates and the error bars represent error %. The asterisk indicates a significant difference from the efficiency of fresh Fe-TA@Au NPs (Student's t-test, $p < 0.05$).

References

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