SUPPORTING INFORMATION

Exposure to CuO nanoparticles changes the fatty acid composition of protozoa *Tetrahymena thermophila*

Monika Mortimer,[†,‡,] Kaja Kasemets,[†] Maša Vodovnik,[§] Romana Marinšek-Logar,[§] Anne Kahru[†]*

[†] Laboratory of Molecular Genetics, National Institute of Chemical Physics and Biophysics, Akadeemia tee 23, Tallinn, 12618, Estonia

[‡] Department of Chemistry, Tallinn University of Technology, Akadeemia tee 15, Tallinn, 12618, Estonia

[§] Chair for Microbiology and Biotechnology, Department of Animal Science, Biotechnical Faculty, University of Ljubljana, Groblje 3, 1230 Domzale, Slovenia

Summary

Number of pages: 10

Methods

Determining the effect of protozoa on the size of CuO nanoparticles

The hydrodynamic diameter of nanosized CuO (nCuO) was also measured after co-incubation with *T. thermophila* cells in Osterhout's medium to determine the possible effect of protozoa (direct contact, exudates) on the size distribution of nCuO in the test conditions. For that, protozoa (5×10^5 cells/mL) were incubated for 2 h with nCuO suspension at the concentration of 100 mg/L in Osterhout's medium in 24-well culture plates at 25 °C without shaking. Immediately after mixing *T. thermophila* cells with nCuO suspension and 2 h after the incubation, the cells were pelleted by centrifugation (700×g for 5 min) and the supernatant was used for the DLS analysis. *T. thermophila* cell culture and the suspension of nCuO in Osterhout's medium without the cells were subjected to the same procedure as the samples containing *T. thermophila* cells with nCuO.

Cultivation of protozoa

T. thermophila (strain BIII) was cultivated as described previously.[1] Briefly, the cultures were pre-grown in modified SSP medium[2] containing 2% proteose peptone (Fluka), 0.1% yeast extract (Lab M) and 0.2% glucose, supplemented with 250 µg/mL each of streptomycin sulphate (Sigma-Aldrich) and penicillin G (Gibco) on an orbital shaker at 100 rpm, 30ºC. During the exponential growth phase (at the cell density of 5×10^5 cells/mL) the cells were harvested by centrifugation at 300×g for 5 min, 4 °C, and washed twice with Osterhout's medium (0.01% NaCl, 0.0008% $MgCl_2$, 0.0004% $MgSO_4$, 0.0002% KCl, 0.0001% $CaCl_2$ in MilliQ water,[3,4]) pH 6.6, conductivity 170 µS/cm. Cell density was determined by counting the cells in haemocytometer (Neubauer Improved, bright line; Germany) after immobilising the cells in 5% formalin. For exposures to toxicants the density of the cells in Osterhout's medium was adjusted to 10^6 cells/mL (twice the final cell density used in the exposures).

Analysis of lipid peroxidation in the cells by thiobarbituric acid reactive substances (TBARS) assay

Lipid peroxidation upon exposure of protozoa to toxicants was measured by determining the generation of TBARS by means of quantifying the malondialdehyde (MDA) content. A modified method of Ohkawa et al (1979)[5] was used. To lyse the cells, 100 μL of 2% sodium dodecyl sulfate (SDS) was added to 100 μL of protozoan culture in Eppendorf tubes and incubated at room temperature for 5 min. Then 250 μL of 0.52% TBA in 7.5% acetic acid and 1% NaOH (pH 3.5) were added and the tubes were heated at 95 °C for 45 min. The reaction was stopped by placing the tubes on ice for 5 min. The tubes were centrifuged at 700×g for 15 min at room temperature and from each tube 200 μL of supernatant was transferred in two replicates on a 96-well microplate for fluorimetric reading (Fluoroskan Ascent FL, Thermo Labsystems, Helsinki, Finland) at 527 nm excitation and 590 nm emission. Quantification of TBARS was performed by comparison to a standard curve of MDA equivalents generated by acid hydrolysis of malondialdehyde bis(dimethyl acetal). To rule out possible interference of CuO NPs and the respective control chemicals with the TBARS assay, malondialdehyde bis(dimethyl acetal) was also analyzed in the presence of the tested chemicals at the EC50 concentrations used in the cell expose experiments. No interference of the chemicals with the test results was detected at the relevant MDA concentrations.

Analysis of reactive oxygen species (ROS) using 2',7'-dichlorofluorescein-diacetate (H_2DCFDA) assay

Generation of ROS in the cells was measured using ROS-sensitive fluorescent probe 2',7'-dichlorofluorescein-diacetate (H_2DCFDA). *Prior* to exposure to nCuO and the respective control chemicals the protozoa were "loaded" with 120 μM H_2DCFDA in Osterhout's medium for 45 min at 25 °C. To remove the excess loading solution, the cells were pelleted by

centrifugation at 300×g for 5 min, 4 °C. After centrifugation the protozoa tended to resuspend and thus small amount of extracellular dye remained in the cell suspensions. The extracellular dye remaining on the cells was diluted with Osterhout's medium during the re-suspension of the dye-loaded cells. For abiotic controls, the same volume of the cell-free dye-loading solution remaining on the pelleted cells (supernatant) was mixed with the same volume of Osterhout's medium used for resuspending the cells, so that the same concentration of extracellular dye was obtained as remained in the suspension of the dye-loaded cells. This mixture was used to prepare abiotic control suspensions/solutions for detecting possible toxicant induced fluorescence of DCFH. 50 µL of the dye-loaded cell suspension or cell-free loading solution in Osterhout's medium was then mixed with 50 µl of toxicant suspension/solution in 96-well black polypropylene microplates (Greiner Bio-One, Germany), each concentration in two replicates, and exposed for 2 and 24 h at 25 °C in the dark without shaking. After exposure the fluorescence of dichlorofluorescein (DCF) was quantified using the Fluoroskan Ascent FL microplate reader at excitation and emission wavelength of 485 and 527 nm, respectively. The experiments were repeated for three days.

After exposing the cells loaded with the fluorescent probe (dichlorofluorescein) to the toxicants for 2 and 24 h, the cells were also visualized with light and fluorescence microscope (Olympus CX41 equipped with a DP71 camera). Images were taken using software Cell B (Olympus).

T. thermophila is known to extensively secrete lysosomal enzymes, including esterases, into the surrounding environment.[6] H_2DCFDA used in the assay requires cleavage by esterases to become an active form of the dye, which is turned into a fluorescent form upon reaction with ROS.[7] During the toxicant exposure of the pre-loaded cells, the esterases secreted into the test environment by *Tetrahymena* also most likely contributed to the overall fluorescence (Figure S3). Also, as the intracellular DCF has been proven to diffuse out of the cells, especially after replacing the dye loading medium with the fresh medium,[8] after 24 h of

exposure no fluorescence was detected inside the cells. Nonetheless, we believe the extracellular fluorescence detected reflected the intracellular oxidative stress as the fluorescent probe was generated first inside the cells and then diffused out. Moreover, the fluorescence generated extracellularly in the abiotic controls was subtracted from the values measured in the cell culture samples.

Results and discussion

Aquatic organisms can influence the size of NPs in the aqueous suspensions also through secretion of enzymes and metabolites into the surrounding environment, which can reduce the size of NP aggregates.[6,9] In the current study, the incubation of nCuO suspension with *T. thermophila* in Osterhout's mineral medium for 2 h significantly reduced the hydrodynamic diameter of nCuO aggregates in the test medium (from 220 nm at 0 h to 28 nm at 2 h, Figure S4, green lines). The two peaks (at 28 nm and 105 nm Figure S4b, green line) detected in nCuO suspension at 2 h of incubation with protozoa indicated the possible agglomeration of protein-coated NPs. The DLS analysis also yielded peaks in the suspensions of protozoa cell cultures without any addition of CuO NPs, with peak maxima at 28 nm at 0 h and 16 nm at 2 h (Figure S4, red lines). These peaks characterized probably the enzymes secreted by *T. thermophila* cells to the medium or cell debris derived from the culture preparation procedures; which possibly contributed to the smaller size of CuO NPs after incubation with protozoa for 2 h.

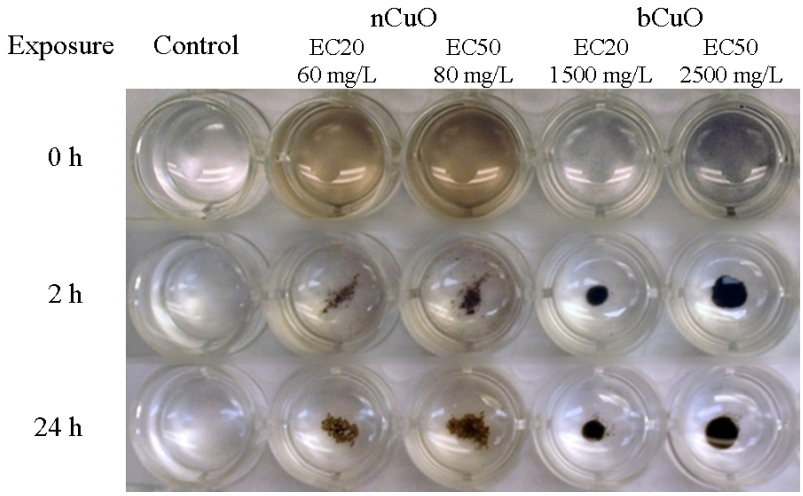

Figure S1. Agglomeration of nano- and bulk CuO in Osterhout's medium during the exposure of *Tetrahymen thermophila*. 500 µL of *T. thermophila* in Osterhout's medium was pipetted into the wells containing 500 µL of nano- or bulk CuO at the respective EC20 and EC50 concentrations. Control: *T. thermophila* in Osterhout's medium. The test plates were incubated on a microplate shaker (Heidolph Titramax 1000, 300 rpm) at 25°C.

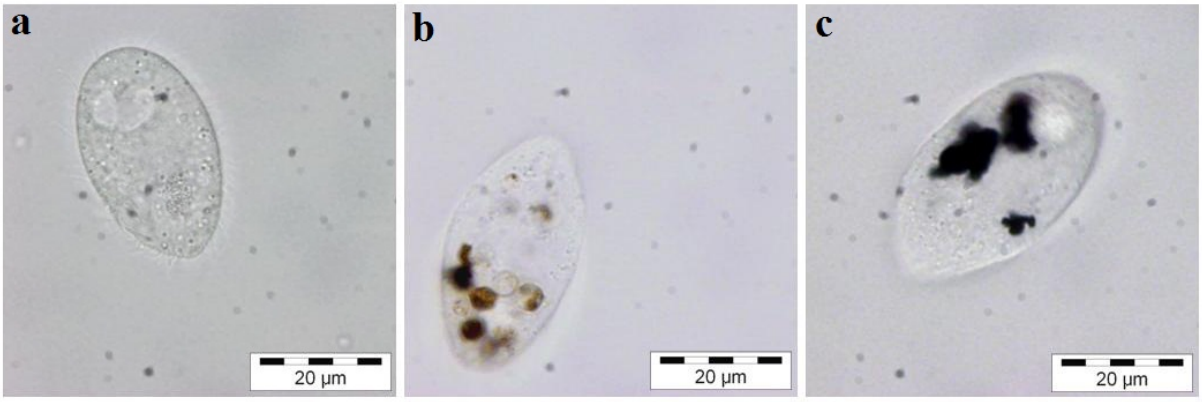

Figure S2. Bright field images of live *Tetrahymena thermophila* cells. Cells were exposed to 80 mg/L nCuO (b) and 2500 mg/L bCuO (c) (the respective EC50 concentrations) in Osterhout's mineral medium for 2 h. Panel a: control, i. e. *T. thermophila* cell after 2 h in Osterhout's medium.

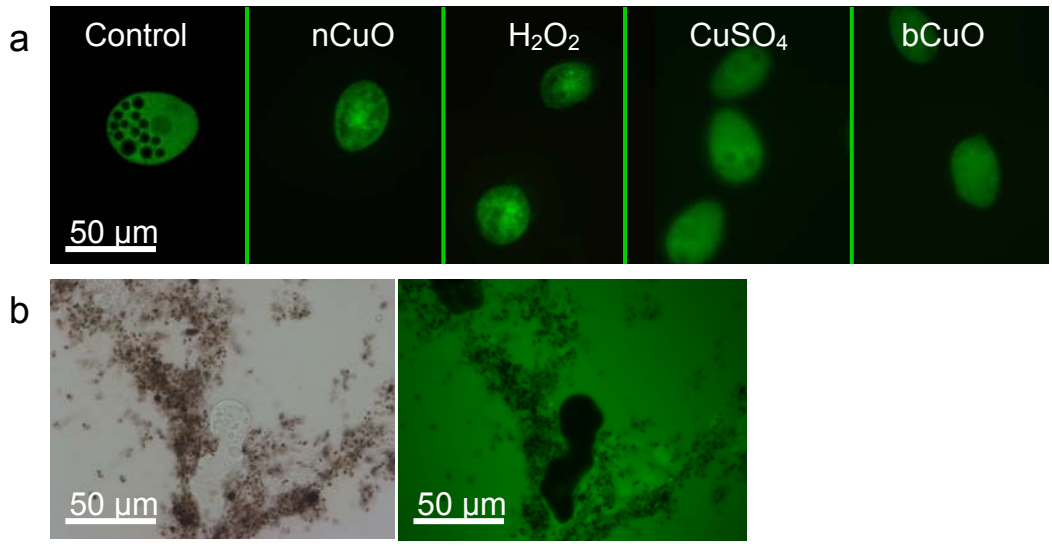

Figure S3. Dichlorofluorescein (DCF) assay. *Tetrahymena thermophila* cells were loaded with H_2DCF-DA, washed and exposed to nCuO and the respective control chemicals for 2 h and for 24 h. Panel a: fluorescence images of *T. thermophila* cells after 2-h exposure to the EC50 concentrations of the chemicals. Panel b: images of *T. thermophila* cells (left - bright field, right – fluorescence) after 24-h exposure to the EC50 concentration of nCuO (80 mg/L). After 24 h no intracellular fluorescence was detected in live and moving cells, instead the extracellular space was found to be fluorescent, likely due to DCF diffusing out of the cells during 24-h exposure.

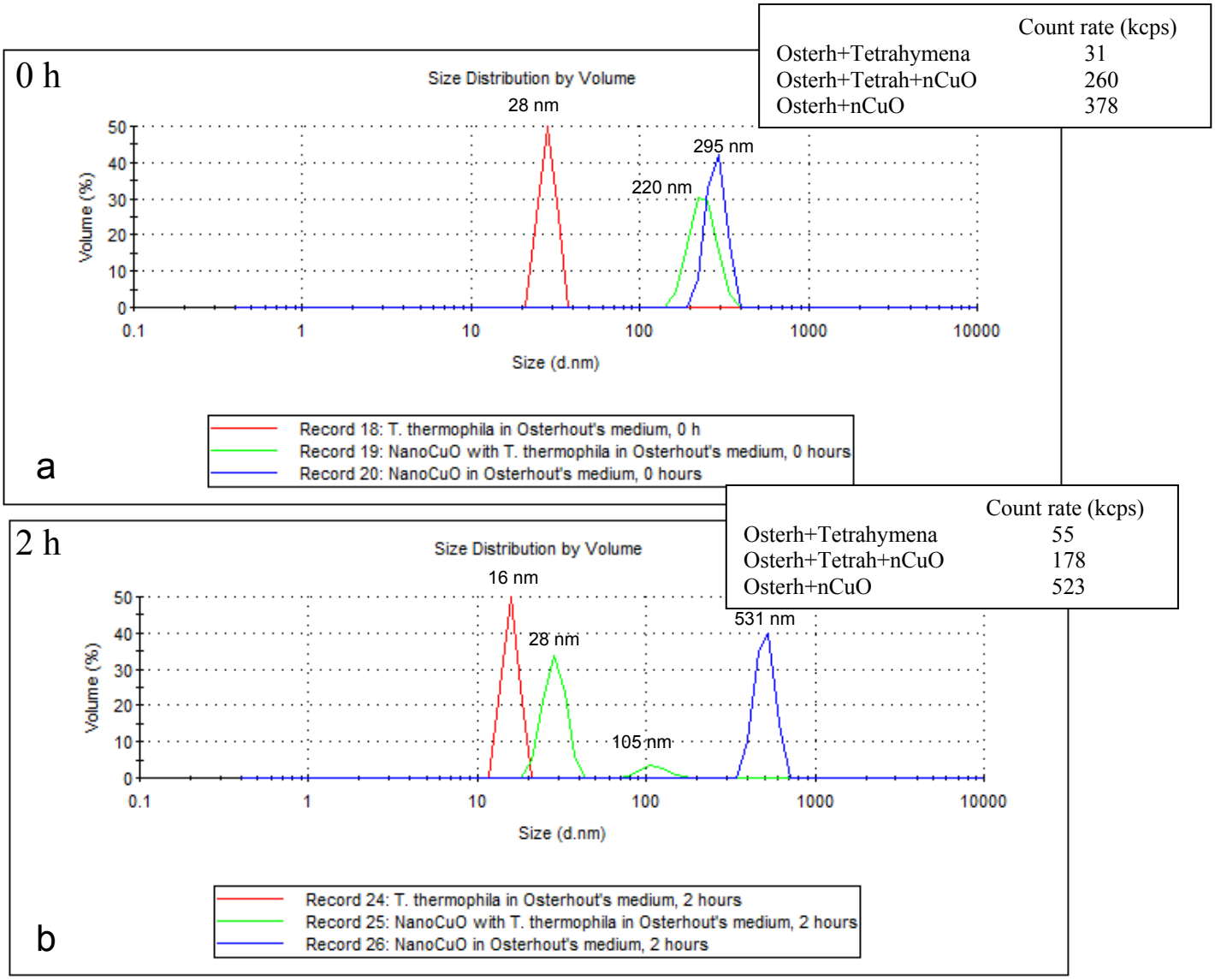

Figure S4. The effect of direct contact of *Tetrahymena thermophila* cells on the hydrodynamic diameter of nCuO in Osterhout's mineral medium, measured by DLS. Panel a: at the beginning of incubation (0 h); panel b: after 2-h incubation at 25 °C. Before the DLS analysis the incubation mixture was centrifuged (700×g for 5 min) to remove the cells and the supernatant was used for the DLS assay. Although the peaks of *T. thermophila* supernatant in Osterhout's medium without nCuO appear relatively large, the values of count rate (number of photons detected per second displayed in kilocounts per second, kcps) indicate that the signal collected from samples mainly reflected the size of CuO particles and not the exudates released by protozoa.

References

(1) Mortimer, M.; Kasemets, K.; Kahru, A. Toxicity of ZnO and CuO nanoparticles to ciliated protozoa *Tetrahymena thermophila*. *Toxicology* **2010**, *269*, 182-189.

(2) Gorovsky, M. A.; Yao, M. C.; Keevert, J. B.; Pleger, G. L. Isolation of micro- and macronuclei of *Tetrahymena pyriformis*. *Methods Cell Biol.* **1975**, *9*, 311–327.

(3) Osterhout,W. J. V. On the importance of physiologically balanced solutions for plants. I. Marine plants. *Bot. Gaz.* **1906**, *43*, 127–134.

(4) Society of Protozoologists. A catalogue of laboratory strains of free-living and parasitic protozoa. *J. Protozool.* **1958**, *5*, 1–8.

(5) Ohkawa, H.; Ohishi, N.; Yagi, K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction, *Anal. Biochem.* **1979**, *95*, 351–358.

(6) Banno, Y.; Sasaki, N.; Nozawa, Y. Secretion heterogeneity of lysosomal enzymes in *Tetrahymena pyriformis*. *Exp. Cell Res.* **1987**, *170*, 259-268

(7) Gomes, A.; Fernandes, E.; Lima, J. L. Fluorescence probes used for detection of reactive oxygen species. *J. Biochem. Biophys. Methods* **2005**, *65*, 45-80.

(8) Hafer, K.; Schiestl, R. H. Biological aspects of dichlorofluorescein measurement of cellular reactive oxygen species. *Radiat. Res.* **2008,** *170*, 408.

(9) Horst, A. M.; Neal, A. C.; Mielke, R. E.; Sislian, P. R.; Suh, W. H.; Mädler, L.; Stucky, G. D.; Holden, P. A. Dispersion of TiO_2 nanoparticle agglomerates by *Pseudomonas aeruginosa*. *Appl. Environ. Microbiol.* **2010**, *76*, 7292-7298.