THESIS ON NATURAL AND EXACT SCIENCES B113

Evaluation of the Biological Effects of Engineered Nanoparticles on Unicellular Pro- and Eukaryotic Organisms

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Declaration:

Hereby I declare that this doctoral thesis, my original investigation and achievement, submitted for the doctoral degree at Tallinn University of Technology has not been submitted for any academic degree.

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LOODUS- JA TÄPPISTEADUSED B113

Sünteetiliste nanoosakeste bioloogiliste efektide hindamine üherakulistel pro- ja eukarüootsetel organismidel

MONIKA MORTIMER

TTÜ
KIRJASTUS

To my family and friends

Perele ja sõpradele

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications referred to by their Roman numerals in the text.

I **Mortimer, M**., Kasemets, K., Heinlaan, M., Kurvet, I., Kahru, A. 2008. High throughput kinetic *Vibrio fischeri* bioluminescence inhibition assay for study of toxic effects of nanoparticles . *Toxicology in Vitro* 22, 1412 – 1417.

II Blinova, I., Ivask, A., Heinlaan, M., **Mortimer, M**., Kahru, A. 2010. Ecotoxicity of nanoparticles of CuO and ZnO in natural water. *Environmental Pollution* 158, 41 – 47.

III **Mortimer, M**., Kasemets, K., Kahru, A. 2010. Toxicity of ZnO and CuO nanoparticles to ciliated protozoa *Tetrahymena thermophila*. *Toxicology* 269, 182 – 189.

IV **Mortimer, M**., Kasemets, K., Vodovnik, M., Marinšek-Logar, R., Kahru, A. Exposure to CuO nanoparticles changes the fatty acid composition of protozoa *Tetrahymena thermophila*. Manuscript, accepted for publication in *Environmental Science and Technology*.

AUTHOR'S CONTRIBUTION TO THE PUBLICATIONS

I The author participated in the study design, was responsible for the sample preparation and performing the Flash Assay in microplate format. She interpreted the data and prepared the manuscript.

II The author performed the ecotoxicity testing with protozoa *Tetrahymena thermophila*, analyzed the respective data and prepared the respective part for the manuscript.

III The author was responsible for the study design, performing the toxicity assays with *T. thermophila* and sample preparation for copper and zinc quantification. She was the major interpreter of the data and responsible for the preparation of the manuscript.

IV The author participated in the study design, performed the *T. thermophila* exposure assays, prepared the samples and performed the gas chromatography analysis, measured the metabolites of lipid peroxidation and reactive oxygen species. She was the major interpreter of the data and responsible for the preparation of the manuscript.

INTRODUCTION

Nanoscale particles (at least one dimension 1–100 nm) have existed on Earth for millions of years, created by geological (weathering, volcanic activity) as well as biological mechanisms. Thus, organisms have been exposed to nanosized materials since the formation of life. In addition, since the industrial revolution the significant source of nanoparticles (NP) of the anthropogenic origin (e.g., wear of car tires, diesel exhaust particles) has been released into the environment. During the last decades the production of engineered nanomaterials has emerged, evoking a special concern on the possible hazards on the human health and environment. The possible adverse effects of engineered NPs are of particulate concern because of their unique surface properties and chemistry that are not characteristic to natural NPs. Thus, for the sustainable and balanced development of nanotechnologies, hazard evaluation of engineered NPs is needed. The increasing applications of nanomaterials have resulted in a high risk of its accidental release to the environment, while the potential hazardous effects of engineered NPs to environmentally relevant test organisms are poorly known and understood.

Protozoa are ubiquitous in nature: they form an essential part in biological communities of the activated-sludge treatment processes. Also, compared to other aquatic organisms, ciliates show relatively high tolerance towards certain chemicals; such feature can be utilized to elucidate the mechanisms of organism adaptation to toxicants, including NPs. 2,400 industrial organic compounds have already been tested using *Tetrahymena* growth inhibition assay and the data has extensively been used for quantitative structure activity relationship (QSAR) studies. Moreover, the genome of *Tetrahymena thermophila* is sequenced and contains many genes similar to other eukaryotes, including humans. Despite of the favorable properties of protozoa there are only a few studies, where these organisms have been used for the evaluation of biological effects of engineered NPs.

The general objective of the present study was to evaluate the hazard of selected inorganic and organic engineered NPs to two environmentally relevant aquatic model organisms: (i) prokaryotic unicellular organism – bacteria *Vibrio fischeri* – that *a priori* are not internalizing NPs, and (ii) eukaryotic unicellular particle-ingesting organism – protozoa *Tetrahymena thermophila*. The focus was set on nanoZnO and CuO effects on particle-ingesting *T. thermophila* as a relevant organism to nanotoxicology and well characterized eukaryotic *in vitro* model.

ABBREVIATIONS

3,5-DCP	3,5-dichlorophenol
AFW	artificial fresh water
ATP	adenosine 5'-triphosphate
BET	Brunauer-Emmet-Teller
CB	carbon black
CNT	carbon nanotubes
DCF	fluorescent 2',7'-dichlorofluorescein
DCFH	non-fluorescent 2',7'-dichlorofluorescein
DLS	dynamic light scattering
DNA	deoxyribonucleic acid
EC_{20}	the effective concentration of the toxicant that induces a designated effect in 20% of the test organisms after a specified exposure time
EC_{50}	the median effective concentration of the toxicant that induces a designated effect in 50% of the test organisms after a specified exposure time
EDTA	ethylenediaminetetraacetic acid
eNPs	engineered nanoparticles
FAMEs	fatty acid methyl esters
$FMNH_2$	reduced flavin mononucleotide
GC	gas chromatography
GSH	glutathione
H_2DCFDA	2',7'-dichlorofluorescein-diacetate
ISO	International Organization for Standardization
LDH	lactate dehydrogenase
MDA	malondialdehyde
NADH	reduced nicotinamide adenine dinucleotide
NICPB	National Institute of Chemical Physics and Biophysics
NOM	natural organic matter
NP	nanoparticle
OD	optical density
OECD	Organization for Economic Co-operation and Development
PAMAM	polyamidoamine
PEI	polyethylenimine
PI	propidium iodide
QSAR	quantitative structure–activity relationship
RLU	relative light unit
RNA	ribonucleic acid
ROS	reactive oxygen species
SD	standard deviation
SDS	sodium dodecyl sulfate

SEM	scanning electron microscopy
SFA	saturated fatty acids
SOD	superoxide dismutase
SSA	specific surface area
SWNT	single wall carbon nanotubes
TBA	thiobarbituric acid
TBARS	thiobarbituric acid reactive substances
UFA	unsaturated fatty acids
UV	ultraviolet

1. LITERATURE REVIEW

1.1. The increasing production of nanomaterials and the need for their hazard evaluation

1.1.1. Applications of nanomaterials

By year 2015, the nanotechnology economy is estimated to be valued at 2.2 trillion Euros (Lux Research, 2008). By the end of 2009 there were already more than 1000 nano-enabled consumer products listed in the Nanotechnology Consumer Product Inventory. While a few years ago carbon was the most commonly used nanomaterial in consumer products, currently nanosized silver is utilized most extensively (259 products registered by Aug 25, 2009), mainly in antimicrobial applications. Carbon, which includes fullerenes, was used in 82 products, titanium (including titanium dioxide) in 50, silica in 35, zinc (including zinc oxide) in 30, and gold in 27 consumer products (Project on Emerging Nanotechnologies, 2009; www.nanotechproject.org).

1.1.1.1. Carbon nanotubes

The most popular uses of carbon nanotubes and their derivatives include additives in plastics, catalysts, battery and fuel cell electrodes, supercapacitors, water purification systems, orthopedic implants, conductive coatings, adhesives and composites, sensors, and components in the electronics, aircraft, aerospace, and automotive industries (Klaine et al., 2008). In addition, carbon nanotubes are actively studied for their potential biomedical applications. Their favorable electronic properties and their size enable their usage as bioelectronic devices, such as biosensors, and as drug or DNA delivery devices in biomedicine (Yang et al., 2007).

1.1.1.2. Silver nanoparticles

As mentioned above, silver is currently the most extensively used material in nanotechnology. Silver has been known for its biocidal properties for centuries, thus the most widely employed field of silver NPs has been in antibacterial applications (Weir et al., 2008). Silver NPs have been incorporated into domestic appliances, e.g. fridges, vacuum cleaners, air conditioners; added to paints, textiles, plastics, varnish etc. (Bystrzejewska-Piotrowska et al., 2009). In addition to antibacterial, antiviral and antifungal properties, nanosilver has also been shown to facilitate wound healing (Nair and Laurencin, 2007).

1.1.1.3. ZnO and TiO_2 nanoparticles

Similarly to silver NPs, also zinc oxide and titanium dioxide NPs have been shown to exert antibacterial properties (Adams et al., 2006). NanoTiO_2 is the most widely used photocatalyst antimicrobial metal oxide which photocatalytic properties are used for solar-driven self-cleaning coatings (Cai et al., 2006) as well as for biocidal/antiproliferative applications (Blake et al., 1999). In addition, TiO_2 NPs have found extensive use as UV protecting agents, gas sensors, and in high-efficiency solar cells (Chen and Mao, 2006). Both, TiO_2 and ZnO NPs are included in personal care products—toothpaste, beauty products and sunscreens (Serpone et al., 2007), as well as in textiles (Dastjerdi and Montazer, 2010).

1.1.1.4. CuO nanoparticles

Also copper NPs have been shown to inhibit the growth of microorganisms and exert antiviral properties (Borkow and Gabbay, 2004; Gabbay et al., 2006). Technologies have been developed to incorporate copper NPs into textile fibers, latex and other polymers (Cioffi et al., 2005; Gabbay et al., 2006). Using these technologies copper oxide has been used to impregnate face masks (Borkow et al., 2010A), wound dressings (Borkow et al., 2010B), socks (Borkow et al., 2009) to give them biocidal properties. CuO NPs have potential wide industrial use in applications such as gas sensors (Li et al., 2007), catalytic processes (Carnes and Klabunde, 2003), high temperature superconductors, solar cells, lithium batteries (Gou et al., 2007). Commercially available NanoArc® Copper Oxide is advertised by Nanophase as a nanomaterial based additive which can be included into wood preservative formula, textile fibers, marine antifouling paints, permanent coatings and thermoplastics (Nanophase website). Copper and other metallic NPs, e.g. silver, gold and certain alkali metals, have been used to improve the performance of several analytical tools such as surface enhanced Raman scattering (SERS) and metal enhanced fluorescence (MEF) (Sau et al., 2010).

1.1.1.5. Polycationic polymers

A group of NPs extensively studied for possible biomedical applications is water soluble polycationic polymers. For instance, nanoscale polymers polyethylenimine (PEI) and polyamidoamine (PAMAM) dendrimers are attractive for different biomedical applications such as drug delivery, gene transfection and bioimaging (Godbey et al., 1999; Svenson and Tomalia, 2005). Both, PEI and PAMAM dendrimers are organic macromolecules forming highly branched networks (Fig. 1.1). The molecules have aliphatic properties and are polycationic due to primary, secondary and tertiary amino groups in the structure. The most widely employed method for the preparation of branched PEI is based on polymerization of ethylenimine (von Harpe et al., 2000). Also the synthesis of PAMAM dendrimers starts from the ethylenediamine core, but the branched units are built from methyl acrylate and ethylenediamine (Peterson

et al., 2001; 2003). The branched units are organized in layers called "generations". The third, fourth, fifth and sixth generations of PAMAM-NH_2 dendrimers (PAMAM G3, G4, G5 and G6) have molecular weights of 6.9, 14.2, 28.8 and 60 kDa, respectively, with corresponding molecular diameters 3.1, 4, 5.3 and 6.7 nm (Shakhbazau et al., 2010). Due to their unique structure high numbers of drug molecules can be attached to terminal groups of PAMAM dendrimers through covalent bonds, or, alternatively, encapsulated inside the cavities of the dendrimer (Klajnert and Bryszewska, 2001). In the field of analytical testing, PAMAM dendrimers are studied as carriers of contrast agents for magnetic resonance imaging (Yan et al., 2010) and used for enhancement of the analytical sensitivity of enzyme-linked immunosorbent assay (Speroni et al., 2010). In molecular biology, PEI is utilized to flocculate cellular contaminants to facilitate purification of soluble proteins (Cordes et al., 1990), as an immobilizing agent for biocatalysts (Bahulekar et al., 1991) and as a soluble carrier of enzymes (Cong et al., 1995).

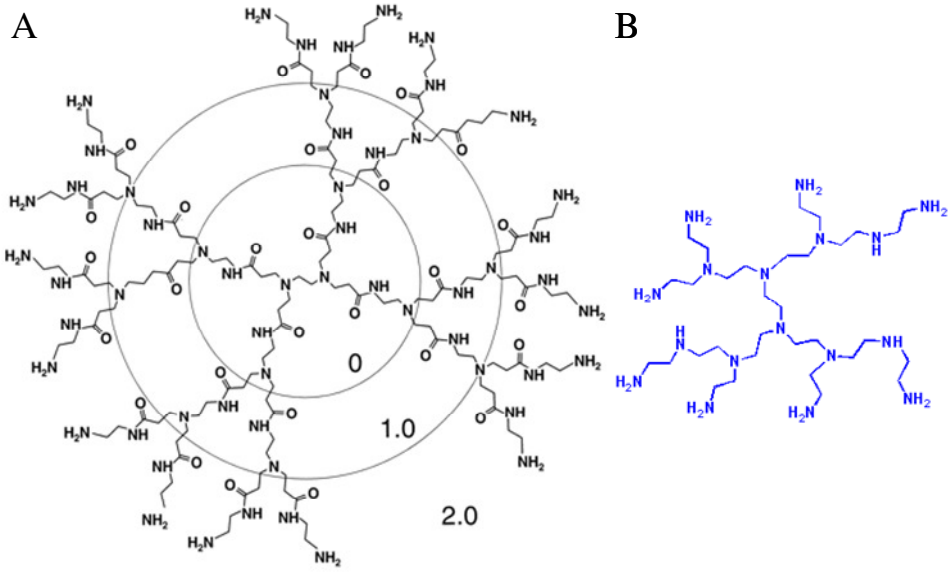

Figure 1.1. Schematic diagram of PAMAM dendrimer (A), generation 2.0, where the successive full generations are indicated by concentric circles (Supattapone et al., 1999), and the scheme of polyethylenimine (B).

In addition to biomedical applications nanoscale polycationic polymers can be used to improve industrial processes. PEI for example has widely been used as a flocculation agent in paper industry and waste water treatment (Geckeler and Volchek, 1996), is a common ingredient in a variety of formulations ranging from washing agents to packaging materials, and is also involved in microbicidal compositions (Helander et al., 1997). PAMAM dendrimers have been proposed as nano-biosensors for ethanol detection (Perinotto et al., 2008), and have also

been developed for removal of heavy metals from soil by acting as water-soluble chelators (Xu et al., 2005). Fluorescently labeled PAMAM dendrimers anchored on a gold nanowire have been suggested for sensitive copper detection (Bhattacharya et al., 2011). Using molecular dynamics simulations, Lard et al. (2010) recently showed that due to the tunable binding of phenantrene with PAMAM dendrimers, they may also be used for water purification from polycyclic aromatic hydrocarbons (PAHs) and environmental remediation.

1.1.2. Fate of engineered nanomaterials in the environment

Considering the increasing application of NPs in biomedical field as well as industrial processes, the possible routes of exposure can be either deliberate – e.g. medical diagnostics, medicines (Shi et al., 2010), cosmetics (Serpone et al., 2007), food additives (Chaudhry et al., 2008), or accidental – e.g. occupational exposure, consumer misuse, waste streams (Thomas et al., 2009). Although currently the production volumes of engineered NPs are marginal compared to unintentionally released nanosized particulate matter, e.g. through incineration processes, it is expected that the level of exposure to intentionally produced NPs will increase rapidly (Farre et al., 2009). During the typical life-cycle of an engineered nanomaterial the main sources of NP exposure to the environment are the production facilities, wastewater treatment plants and accidents during the transport (Farre et al., 2009). The need to conduct life-cycle based assessments of nanomaterials as early in the new product development process as possible is crucial for a better understanding of the potential environmental and human health consequences of nanomaterials over the entire life cycle of a nano-enabled product (Dhingra et al., 2010, Fig. 1.2). After being released into the environment, the most important pathways of NPs in natural waters are expected to be adsorption to the aquatic organisms or organic matter, uptake by filter feeders and phagocytotic organisms, and subsequent transfer through the food chain or biodegradation (Fig. 1.3). The stability and mobility as well as aggregation and deposition of NPs in the aquatic environment is governed by the surface properties of the NPs, which depend primarily on the parameters such as temperature, ionic strength, pH, particle concentration and size (Filella and Buffle, 1993). The complexity of the interactions of NPs is further increased by the presence of natural organic matter (NOM), which may be sorbed to the surface of NPs by hydrogen bonding, electrostatic and hydrophobic interactions, and consequently, through stabilization, prolong the residence time of NPs in aquatic systems (Navarro et al., 2008). Upon the release of the nanomaterial into the environment the possible changes in NP surface chemistry and biotransformation could significantly alter the physicochemical properties as well as the toxicity potential of NPs (Thomas et al., 2009).

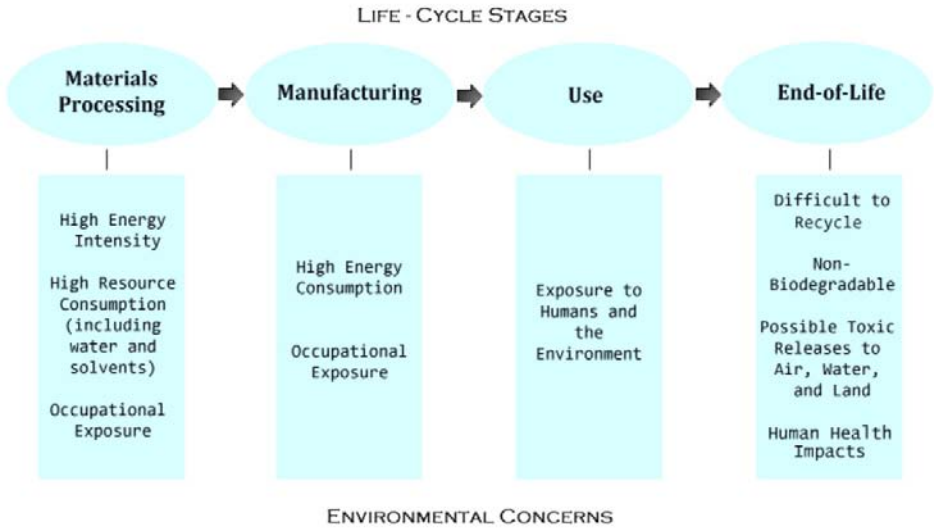

Fig. 1.2. Typical life cycle of nanomaterials (from Dhingra et al., 2010).

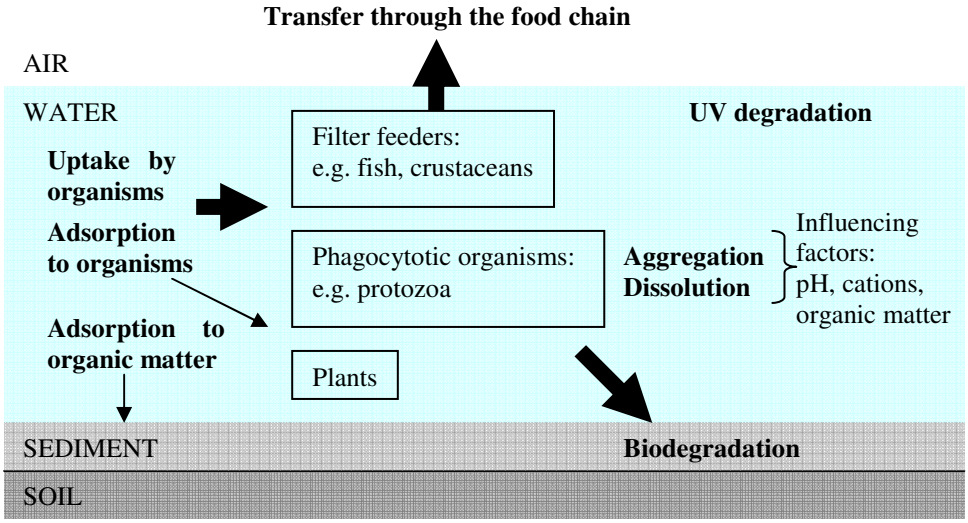

Figure 1.3. Main pathways of engineered nanoparticles in natural waters. Based on the review papers of Oberdörster et al., 2005 and Farre et al., 2009.

1.1.3. Nanotoxicology and nanoecotoxicology – new disciplines on potential harmful effects of engineered nanoparticles

The goal of studying the potential of engineered nanomaterials to cause adverse effects gave rise to nanotoxicology (Oberdörster et al., 2005). The research of the potential toxicity of very small particles dates back to inhalation studies of ultrafine particles in 1990s (Oberdörster et al., 1994). With increasing exposures to NPs nanotoxicology has become an important discipline in risk assessment of NPs (Gil et al., 2010). When evaluating the toxic effects of NPs to ecosystems, the term nanoecotoxicology has been used (Baun et al., 2008; Behra and Krug, 2008; Hassellöv et al., 2008). While nanotoxicological research started in the beginning of 1990s, it took another 10 years for the first papers on nanoecotoxicology to be published, the first ones published by Colvin (2003) and Oberdörster (2004) (Kahru and Dubourguier, 2010).

According to the International Organization for Standardization (ISO), nanoscale objects are of a size range from approximately 1 nm to 100 nm (ISO/TS 27687:2008 (E)). Analogously, according to the definition by US National Nanotechnology Initiative (NNI, www.nano.gov) nanotechnology is science, engineering, and technology conducted at the nanoscale (1–100 nm). As carbon nanotubes, for example, can be remarkably longer than 100 nm, currently most scientists refer to NPs as particles with at least one dimension < 100 nm. However, considering the tendency of NPs to aggregate in aqueous systems, it has been proposed that in ecotoxicology the definition of NPs should be extended to particles with a size up to 500 nm, including primary particles larger than 100 nm and larger aggregates of NPs, which could be as large as few hundred nanometers (Handy et al., 2008B).

The same properties which give NPs their unique favorable properties for novel applications in wide range of areas (see above) could potentially exhibit harmful effects to biological systems (Fig 1.4).

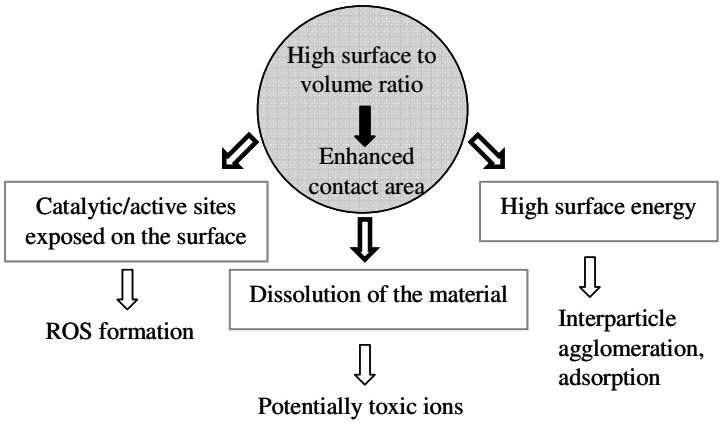

Figure 1.4. Possible toxic mechanisms of nanoparticles triggered by the physico-chemical properties. Based on the review paper of Gil et al., 2010.

1.2. Mechanisms of toxic actions of engineered nanoparticles

In the following sections some of the best-developed paradigms of the possible mechanisms of toxic actions of eNPs, including generation of oxidative stress, effects of dissolved ions from metalbased NPs and interactions of NPs with biomolecules, are discussed.

1.2.1. Engineered nanoparticles, reactive oxygen species and oxidative stress

Oxygen-derived radicals or reactive oxygen species (ROS) are generated constantly in aerobic living systems. They are formed in mitochondria in the electron transport chain, during autooxidation and photochemical reactions, and as intermediates in enzymatic reactions, involving enzymes such as cytochromes P450, various oxidases, peroxidases, lipoxygenases and dehydrogenases. Sequential reduction of molecular oxygen generates a number of reactive species, which among others include hydrogen peroxide and organic hydroperoxides (half lives in the range of minutes), superoxide anion (half life of a microsecond) and hydroxyl radical with a half life of about a nanosecond (Kehrer, 2000). In addition to normal physiological stimuli, production of ROS is also triggered by stress. ROS are involved in inter- and intracellular signaling, and, as described above, are necessary intermediates in a variety of enzyme reactions (Fedoroff, 2006). In certain conditions ROS are overproduced in the cells, for example in hypoxic or hyperoxic conditions, as a response to ionizing radiation or xenobiotics, and in phagocytic cells in response to invading pathogens. Under normal conditions, damage by ROS is kept under control by an antioxidant cascade that includes enzymatic and non-enzymatic components. Most important antioxidant enzymes in the cells are superoxide dismutases (SOD), catalyzing the conversion of superoxides into hydrogen peroxide and oxygen, catalase, which degrades hydrogen peroxide to water and oxygen, and glutathione peroxidase, reducing lipid hydroperoxides and hydrogen peroxide; the main non-enzymatic antioxidants include α-tocopherol, ascorbic acid and glutathione (Blokhina et al., 2003). As a result of excessive production of oxygen-based radicals they become prominent toxicological intermediates, and are commonly involved in oxidative stress, defined as an imbalance between the production of ROS and the ability of biological system to detoxify the reactive intermediates or to repair the resulting damage (Sayre et al., 2005).

Oxidative stress induced by NPs is one of the best-developed paradigms for the potential toxicity of eNPs (Li et al., 2003; Nel et al., 2006). Three main scenarios have been proposed for the NPs to induce the excess of intra- and extracellular ROS in eukaryotic organisms (Petersen and Nelson, 2010): 1) NPs might cause oxidative stress due to the redox-active characteristics of their composition or surface properties; 2) biopersistent NPs might remain in the system and cause site-specific or systemic inflammation, inducing excess ROS

production; 3) upon entering the cell NPs might physically damage subcellular compartments such as mitochondria leading to disruption of the electron transport chain, which could cause the generation of excess ROS. It has been demonstrated both in *in vitro* and *in vivo* studies that various types of NPs induce the formation of ROS. Also, it has been concluded that particle surface properties determined by chemical composition play a critical role in ROS generation (Yang et al., 2009). For example, in a comparative *in vitro* study of carbon black (CB), single wall carbon nanotubes (CNTs), silicon dioxide (SiO_2) and zinc oxide (ZnO) Yang et al. (2009) found that although all the tested NPs induced statistically significant cytotoxicity through oxidative stress mechanism, ZnO NPs were the most cytotoxic and oxidatively damaging, whereas CNTs caused the highest DNA impairment in primary mouse embryo fibroblast cells. Oxidative stress mediated cytotoxicity *in vitro* has been detected in case of both metal-based and organic NPs, e. g. iron oxide NPs (Naqvi et al., 2010), ambient ultrafine particles and cationic polystyrene nanospheres (Xia et al., 2006) induced ROS in murine macrophage cells, Ag NPs in rat liver derived cell line (BRL 3A) (Hussain et al., 2005), nano-C60 in human dermal fibroblasts, human liver carcinoma cells and neuronal human astrocytes (Sayes et al., 2005). The ability of copper oxide NPs to generate ROS has been mostly studied using human airway epithelial cell lines (A549 cells). In addition to causing oxidative lesions in A549 cells, CuO NPs were the only particles among different types of metal oxide NPs tested, which induced an almost significant increase ($p = 0.058$) in intracellular ROS at dose of 80 mg/l (Karlsson et al., 2008). NanoCuO induced oxidative stress has also been detected in human laryngeal epithelial cells *in vitro* (Fahmy and Cormier, 2009). It has been shown that CuO NPs have a genotoxic potential in A549 cells which may be mediated through oxidative stress (Ahamed et al., 2010).

The photocatalytic reactivity of TiO_2 and ZnO NPs has been a special concern because of their incorporation in the sunscreens and production of ROS that can potentially cause oxidative stress to tissues or damage DNA (Serpone et al., 2007). There has also been raised an issue of ROS generation by NPs in the sunscreens interacting with other materials or organisms in the environment. Indeed, in illuminated conditions TiO_2 and ZnO NPs have been shown to exert enhanced damaging effects on bacteria (Adams et al., 2006) and algae (Kim and Lee, 2005). However, TiO_2 inhibited bacterial growth also in the dark conditions (Adams et al., 2006). Interestingly, although TiO_2 NPs were not cytotoxic in the absence of UV irradiation, they were genotoxic to fish cells *in vitro*, mainly due to the hydroxyl radicals generated by TiO_2 NPs (Reeves et al., 2008).

In addition to TiO_2 and ZnO, also other types of NPs may induce deleterious effects in aquatic life on environmental release. Oxidative stress has been associated with the toxicity of nanosilver in the liver of adult zebrafish (Choi et al., 2010A) and nanogold in mussel *Mytilus edulis* (Tedesco et al., 2010). ROS-generating potential of nanoCuO and nanosilver aqueous supensions has been demonstrated in recombinant *E. coli* strains, using high-throughput luminescent

bacterial tests (Ivask et al., 2010). The role of oxidative stress in toxic effect of CuO NPs has also been suggested in case of *Daphnia magna* (Heinlaan et al., 2011) and *Saccharomyces cerevisiae* (Kasemets et al., 2009). While it has been shown in several studies that fullerene exerts ROS-independent toxicity in bacteria (Lyon and Alvarez, 2008; Ivask et al., 2010), it has been associated with extensive ROS generation in embryonic zebrafish (Usenko et al., 2008). Using atomistic simulations on artificial membrane bilayers Qiao et al. (2007) showed that pristine C_{60} molecules, but not OH-functionalized fullerenes, can diffuse easily into the bilayer through transient micropores in the membrane. Once C_{60} molecules reach the bilayer interior, they can facilitate the formation of micropores, which may contribute to the membrane-related adverse effects.

While in case of carbon based NPs, like fullerenes the mechanism of oxidative damage seems to require direct contact between the NP and the cell (Lyon and Alvarez, 2008), metal-containing NPs may exert their ROS generating potential also through the metal ions released from the surface of the NPs (Griffitt et al., 2008; Heinlaan et al., 2008; Kahru and Dubourguier, 2010).

1.2.2. Metal-containing engineered nanoparticles and their solubility

In case of metal-containing NPs one of the factors, which could influence their harmful effects in biological systems is the release of metal ions. Metal NPs are expected to dissolve at a greater extent due to the largely increased surface area compared to the corresponding micro- or macrosized particles. Ions of the redox-active metals may contribute to yield free radicals via the Fenton-type reaction and to elicit intracellular oxidative stress (Valko et al., 2005). The production of hydroxyl radicals can be explained by the following reactions:

$Fe(III) + O^{2\bullet-} \leftrightarrow Fe(II) + O_2$

$Fe(II) + H_2O_2 \rightarrow Fe(III) + {}^{\bullet}OH + OH^-$ (Fenton reaction)

The overall reaction of the combined steps is called Haber- Weiss reaction:

$O^{2\bullet-} + H_2O_2 \leftrightarrow O_2 + {}^{\bullet}OH + OH^-$

Also copper can participate in Fenton-type reactions. The cupric ion (Cu (II)) can be reduced to cuprous ion (Cu (I)) in the presence of biological reductants such as ascorbic acid or glutathione (GSH), and consequently reactive hydroxyl radicals will be generated from hydrogen peroxide via Fenton reaction:

$Cu(I) + H_2O_2 \rightarrow Cu(II) + {}^{\bullet}OH + OH^-$

The dissolution of metal-containing NPs depends on the metal solubility in a given environment as well as on NP functionalization and storage temperature of the suspension. Kittler et al. (2010) showed that dissolution of silver NPs was time dependent and at the NP concentration of 100 mg/l the amount of released ions reached the limiting value of 90% of NP weight in several days. The release of silver ions increased the toxicity of silver NPs towards human mesenchymal

stem cells *in vitro* (Kittler et al., 2010). It has been also suggested that antibacterial and antifungal activities of Ag and Cu NPs depend on the release of Ag- and Cu-ions (Auffan et al., 2009, Ivask et al., 2010). Also the toxicity of ZnO NPs to algae, bacteria, yeast and crustaceans has been attributed to the dissolved fraction of NPs (Franklin et al., 2007; Heinlaan et al., 2008; Aruoja et al., 2009; Kasemets et al., 2010). However, considering that there are great differences in metal homeostasis and regulation in pro- and eukaryotic cells, the type of the test organism should also be given considerable attention. For example, while in case of mammalian cells the iron-doping of ZnO NPs reduced the cytotoxicity of ZnO by slowing down the release of zinc ions (George et al., 2010) no change in NP toxicity was detected in the bacterial assay (Li et al., 2011). In the same study also the importance of water chemistry was pointed out in the NP dissolution. The rate of solubility of metal-containing NPs is of crucial importance as it is not fully understood yet, to what extent the dissolved metal ions contribute to the toxicity of metal NPs. It was shown recently by gene expression profiling that the dissolved fraction of ZnO NPs does not account for the toxicity of ZnO NPs to *Daphnia magna* (Poynton et al., 2011). In response to ZnO NP exposure the expression of several cytoskeletal transport proteins was upregulated, but unexpectedly, antioxidant response genes were not induced. The latter was also observed in a study on zebrafish embryos, where it was inferred that the cell does not recognize ZnO NPs as Zn^{2+} and consequently does not initiate an antioxidant response (Zhu et al., 2009). Moreover, it has been shown that partially soluble NPs, such as cobalt oxide and manganese oxide, can exert their toxic effects through Trojan-horse type mechanism, by entering the human lung epithelial cells *in vitro* and releasing metal ions, which would not normally be internalized by the cell as their transport is strictly controlled (Limbach et al., 2007). Even inherently insoluble metallic NPs such as TiO_2 could contribute to the increased toxicity of metal ions to the organisms: TiO_2 NPs remarkably enhanced the toxicity of copper ions to *D. magna* (Fan et al., 2011) as copper ions adsorbed on TiO_2 NPs, and leading to the ingestion of excess amounts of copper increased the toxic effect. Moreover, TiO_2 was proposed to compete with copper ions for the binding to sulfhydryl groups, inhibiting the detoxification of copper (Fan et al., 2011).

1.2.3. Effects of engineered nanoparticles on the cell membranes

1.2.3.1. Internalization of nanoparticles by the cells

The applications of nanomaterials such as gene and drug delivery systems, biosensors, and bioimaging agents require the internalization of NPs by the cells. To facilitate the internalization process, NPs with specific properties have been designed, for example by modifying the surface of the metal NPs with various surfactants, polymers and chelating groups to ensure suitable dispersion, or by employing surface functionalization or attaching biomolecules such as antibodies, peptides or enzymes on the surface of NPs for targeted delivery and

bioconjugation (Sharma et al., 2006). Different types of NPs can exploit different mechanisms to enter the cell: these may include phagocytosis, macropinocytosis, clathrin- or caveolin-mediated endocytosis, or direct penetration (Dawson et al., 2009; de Planque et al., 2011). Also, the internalization mechanism of the same NP varies between different cells: *in vitro* studies have shown that polypyrrole NPs entered human lung fibroblasts by endocytosis, but in mouse alveolar macrophages by both, phagocytosis and endocytosis (Kim et al., 2011). Positively charged particles have been found to exhibit the highest uptake by cells *in vitro*, explained by the influence of negatively charged cell membrane and thus faster concentration of NPs on the cell surface due to electrostatic interactions (Hauck et al., 2008). The regulation of surface charge and functional groups to optimize cell uptake may be useful in targeting tissues during cancer therapy. For instance, the cellular uptake of polyelectrolyte coated gold nanorods by HeLa cervical cancer cells was regulated from very high to very low by manipulating the surface charge and surface functional groups, while no significant cell toxicity was detected (Hauck et al., 2008). However, it has also been demonstrated that cationic particles or particles with high surface reactivity are more likely to be toxic than anionic particles (Goodman et al., 2004). NPs may have a direct damaging effect on the membrane composition, or the NPs could disrupt the intracellular balance after internalization through the cell membrane (Nel et al., 2009). *In vitro* studies have shown that for example, PEI-coated cationic NPs can bind with high affinity to lipid groups on the surface membrane of mammalian cells and can be endocytosed, ending up in an acidifying lysosomes. These NPs can cause the rupture of lysosomes by sequestering protons that are supplied by the lysosomal proton pump and thereby keeping the proton pump functioning, leading to the retention of Cl^- anions and water molecules which enter the lysosome concurrently with the protons. As a result the lysosome bursts and NPs are released in the cytoplasm (Nel et al., 2009). In addition to damaging effects on lysosomal function, cationic molecules have also been shown to target cell membranes through strong binding to phospholipid components, which can lead to membrane disruption. Silica nanospheres with $-NH_3^+$ surface functionalization have been shown to adsorb to pure-lipid membranes and compromise their structural integrity at very low number of membrane-associated NPs, indicating that NPs can cause a direct damage to lipid bilayers and could enter the cells also by passive transportation (de Planque et al., 2011). Nonetheless, despite of extensive uptake of cationic particles their effects on the cell viability could vary depending on the cell type. Polystyrene nanospheres were shown to be highly toxic in macrophage (RAW 264.7) and epithelial (BEAS-2B) cells, while human microvascular endothelial (HMEC), hepatoma (HEPA-1), and pheochromocytoma (PC-12) cells were relatively resistant to particle injury upon their uptake into the cells (Xia et al., 2008). After extensive endocytosis of NPs, one of the possible ways for the cells to avoid NP toxicity could be the excretion of NPs by exocytosis. Extracellular calcium enhanced exocytosis of pre-

endocytosed gold NPs has been demonstrated in HT-29 human colonic adenocarcinoma cell lines (Chen et al., 2010).

1.2.3.2. Effect of coating of nanoparticles with natural organic matter or biomolecules on their cellular internalization

Also variations in NP size and hydrophobicity influence NP interactions with cells and cell damage. In a comparative study, fullerene C_{70} and fullerol $C_{60}(OH)_{20}$ were shown to be differentially taken up by plant and mammalian cells (Chen et al., 2010). Fullerene C_{70} suspended in natural organic matter (NOM) was found to bind to or become embedded in membranes of the HT-29 human colonic adenocarcinoma cells *in vitro* causing cell lysis, while a water-soluble fullerene derivative $C_{60}(OH)_{20}$ did not damage the mammalian cells. On the other hand, the hydrophobic C_{70}-NOM NPs exerted little damage on the plant cell as the physiology of the plant cell wall favors uptake of smaller and more hydrophilic NPs (Chen et al., 2010). The high content of NOM in soil and water facilitates its binding to NPs modulating their surface properties, and thus interaction pattern with living organisms (Ke and Lamm, 2011). Similar NP surface modifying takes place when NPs enter a biological fluid, for example blood or plasma, where the NPs tend to lower their free surface energy by becoming coated with proteins (Monopoli et al., 2011). The interactions between NPs and proteins are governed by the composition and size of the NP, leading to different types of corona, which are composed of relatively few proteins compared with the numbers found in the biological environment, indicating that NPs bind proteins with high selectivity. Furthermore, it has been proposed that the corona is persistent enough to be the most important interface between cells and NPs (Walczyk et al., 2010). Several different types of peptide units could be presented on the surface of the corona, some of which could target a specific receptor, facilitating the uptake of NP into the cell by receptor-mediated endocytosis (Nel et al., 2009). NPs may become coated also with lipids and other biomolecules (Monopoli et al., 2011).

1.2.3.3. Membrane integrity as an endpoint for nanoparticle cytotoxicity *in vitro*

Many of cytotoxicity assays used in NP studies, analogously to *in vitro* toxicity testing of the 'conventional' chemicals, evaluate cell viability *via* methods that measure plasma membrane integrity (reviewed in Lewinski et al., 2008). For example, the dyes neutral red and Trypan blue have been used in the cytotoxicity studies of CuO NPs in human airway epithelial cells *in vitro* (Midander et al., 2009), TiO_2 in goldfish skin cells (Reeves et al., 2008) and Au NPs in *Mytilus edulis* (Tedesco et al., 2010). Also several fluorescent dyes used in the viability assays are based on the membrane integrity endpoint. For instance, membrane impermeable molecules ethidium homodimer and propidium iodide, which can only enter the cells with damaged membranes have been widely used in

assessing the cytotoxicity of both metal-based and organic engineered NPs (Choi et al., 2010B; Naqvi et al., 2010). Also, lactate dehydrogenase (LDH) release from the cells, which is another biochemical assay for plasma membrane damage, has been observed for a wide range of NP, for instance copper NPs in rat neurons *in vitro* (Prabhu et al., 2010), iron oxide NPs in murine macrophages (Naqvi et al., 2010) and, PEI and PAMAM dendrimers in Jurkat T-cells (Choi et al., 2010B).

1.2.3.4. The effect of nanoparticle-induced oxidative stress on membrane lipids

It has been demonstrated both in *in vitro* and *in vivo* studies that various types of NPs induce oxidative stress. One of the important biological targets of oxidative stress in the cells are lipids, as lipid molecules make up approximately 30 to 80 % of biological membranes by mass (Harrison and Lunt, 1980). Lipid peroxides formed decompose rapidly to malondialdehyde (MDA), 4-hydroxy-2-nonenal and 4-hydroxy-2-hexenal, the main product of polyunsaturated fatty acid peroxidation being MDA (Catalá, 2009). Therefore, MDA is usually quantified from the cells as an indicator of peroxidized membrane fatty acids. In most of these assays MDA derivatized with thiobarbituric acid (TBA) to yield MDA-TBA adduct that can be measured colorimetrically or fluorimetrically. Several NPs have been shown to generate lipid peroxidation both in mammalian cell lines, for example CuO and TiO_2 NPs in human airway epithelial cells *in vitro* (Ahamed et al., 2010; Gurr et al., 2004), as well as in whole organisms, e.g. Ag NPs in zebrafish (Choi et al., 2010A) and Au NPs in mussel *Mytilus edulis* (Tedesco et al., 2010).

1.2.3.5. Membrane fluidity: implications to toxicity

Cell membranes are dynamic, fluid structures of lipids and proteins, and most of these molecules are able to move in the plane of the membrane. Fluidity is the quality of ease of movement and represents the reciprocal value of membrane viscosity. Fluid properties of biological membranes are essential for numerous cell functions. Even slight changes in membrane fluidity may cause aberrant function and pathological processes. Several evidences suggest that trace elements, e.g., iron, copper, zinc, selenium, chromium, cadmium, mercury and lead may influence membrane fluidity. The interaction of heavy metals with cellular membranes may contribute to explain, at least partially, the toxicity associated with these metals (Garcia et al., 2005). Changes in membrane fluidity have been detected also after exposure to engineered NPs. For instance, CuO NPs have been shown to decrease the cell stiffness, contraction and relaxation of human airway smooth muscle cells in vitro (Berntsen et al., 2010).

1.3. The need for high-throughput toxicity screening of engineered nanoparticles

In view of the growing production and application of eNPs, and concerns about nanomaterial safety there is an increasing need for the reliable and reproducible toxicity screening methods. Major factors contributing to the insufficient amount of safety data are the high cost and length of time to complete even a single toxicological screen through animal testing. Choi et al. (2009) calculated the costs for traditional testing of NP already on the market to be between $ 250 million and $ 1.2 billion and the time required at 34-53 years. In addition, some of the traditional toxicological assays are not suitable for the testing of NPs (Meng et al., 2009).

The number of different NP we might need to address is potentially extremely high, with various shapes, size distributions, and coatings for each material. This alone suggests the importance of using alternative methods, which often allow higher throughput, replicates, and parallel tests. Thus, the limit of test throughput is more relevant for NP (Hartung, 2010). In order to reduce animal testing, new testing strategies, involving toxicity assays performed, e.g. on bacteria and non-vertebrate animals, need to be developed. Therefore, over the last twenty five years, alternative, non-animal test systems (mainly eukaryotic cell cultures) have been introduced to supplement and, in some cases, to replace toxicity tests using animals (Carere et al., 2002) contributing to the 3R's concept (Replacement, Reduction, Refinement of test animals, Russell and Burch, 1959).

1.4. *Vibrio fischeri* – a prokaryotic model organism for the toxicity screening

For initial toxicity screening of chemicals, bacteria are an additional attractive alternative to eukaryotic organisms. The most well-known bacterial *in vitro* test is Ames assay with *Salmonella typhimurium* (Claxton et al., 2010), which may predict genotoxic effects of chemicals also to higher organisms (e.g. humans). One of the most widely used bacterial *in vitro* assays is also the Microtox® test, which uses the inhibition of bioluminescence of *Vibrio fischeri* NRRL-B-11177 as a toxicity endpoint (Bulich and Isenberg, 1981). *V. fischeri* (formerly *Photobacterium phosphoreum*) and recently classified as *Aliivibrio fischeri* (Urbanczyk et al., 2007) is a Gram-negative marine bacterium inhabiting the light organs of bobtail squids and monocentrid fishes. Luminescence in the fish or squid is thought to be involved in the attraction of prey or even as camouflage (Ruby, 1996). The bioluminescence of *V. fischeri* is a result of a complex chain of biochemical reactions, where reduced flavin mononucleotide ($FMNH_2$), molecular oxygen, a long-chain fatty acid aldehyde and luciferase are the key players:

$FMNH_2 + O_2 + RCHO \rightarrow FMN + RCOOH + H_2O + light$

For the regeneration of $FMNH_2$ cellular NADH is needed and due to that the bioluminescence of the bacteria is intrinsically tied to their central metabolism. Thus, any damage of cellular metabolism caused by the toxicity of a sample could be monitored by measuring the change of light output of bacteria, the degree of toxicity being proportional to the decrease in light output (Hastings, 1978; Bulich and Isenberg, 1981).

1.4.1. Application of Vibrio fischeri assay in aquatic toxicity testing

Several different luminescence inhibition tests of *V. fischeri* have been developed so far – most of them are designed for analysis of aqueous samples (Microtox®, Bio-Tox™, LUMIStox™, ToxAlert™ that all use the strain NRRL-B-11177). Slightly modified (kinetic) version of this test using the same *V. fischeri* strain has been standardized also for water and effluent samples (ISO 11348-3:1998).
The *V. fischeri* luminescence inhibition assays (such as Microtox® test) have been extensively used for aquatic toxicity testing as rapid, reproducible, and cost effective assays. In the test, the bacteria are exposed to a range of concentrations of the tested compound and the reduction in intensity of light emitted from the bacteria is measured along with standard solutions and control samples. The change in light output and concentration of the toxicant produce a dose/response relationship which enables to calculate the EC_{50} value (concentration producing a 50% reduction in light). The results of *V. fischeri* toxicity tests have been shown to correlate well with the data from the assays with higher aquatic organisms, e.g. algae, protozoa, crustaceans and fish (Kaiser, 1998). Analysis of the toxicity data for 47 MEIC (Multicenter Evaluation of *In Vitro* Cytotoxicity) reference chemicals showed that the EC_{50} values of *P. phosphoreum* bioluminescence inhibition assay correlated with literature data not only on acute toxicity data for daphnids and fish but also for animal and human cell lines, rodents, dog and man, whereas the log-log correlation coefficients (R^2) ranged between 0.20-0.79, depending on the data compared (Kahru, 2006).

1.4.2. Application of Vibrio fischeri assay in the toxicity testing of turbid and solid samples

Luminescent bacterial assays have been also used for the evaluation of the toxicity of polluted soils, sediments and solid wastes (Põllumaa et al., 2001; Lapa et al., 2002). The main approach in testing the solid samples used to be assaying the water or solvent extractable bioavailable toxicants in the extracts. However, testing the extracts might not always reflect the actual toxicity of the solid environmental sample, due to the low solubility of the toxicants or incorrect extracting procedures or the toxicity of solvents used in extraction

(Heinlaan et al., 2007). To overcome these problems, special modifications of the bacterial luminescence inhibition test have been developed. The assay with the luminescent bacterium *P. phosphoreum*, with direct contact between bacteria and sediment (Brouwer et al., 1990), involves low-speed centrifugation, whereas the Microtox® solid-phase toxicity test (Kwan & Dutka, 1992) uses filtration to separate bacteria and sediment particles prior to the measurement.

To overcome the problems arising from the sample extraction, but also correction for turbidity of the sample, a contact *V. fischeri* luminescence inhibition assay for solid, turbid and colored samples – Flash Assay – was developed by Lappalainen et al. (1999). In this assay the bacteria is in direct contact with the sample matrix and thus the presence and bioavailability of all the toxic compounds can be detected (Lappalainen et al., 1999). The assay format allows for the recording of the luminescence starting from the moment of adding the test bacteria to the sample (Fig. 1.5), thus it is a kinetic assay where each sample acts as a reference for itself, with no need for the color/turbidity correction.

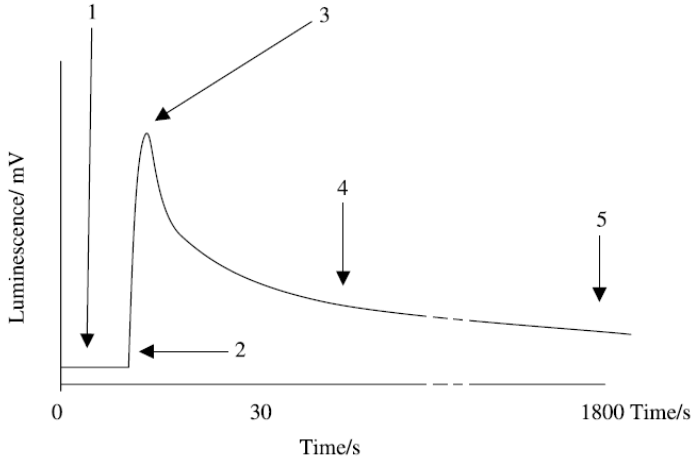

Figure 1.5. Scheme of the Flash Assay. 1 – start measuring and mixing; 2 – inject bacteria; 3 – record peak value from 0-5 s; 4 – record signal for 30 s; 5 – at 30 min, record signal. Modified from Lappalainen et al., 2001.

In 2007 an interlaboratory comparison of the *V. fischeri* kinetic assay was performed, with NICPB Group of *In Vitro* and Ecotoxicology being one of the 8 laboratories participating in the comparison. The Flash Assay method was standardized in 2010 (ISO 21338:2010(E)). In our laboratory, the Flash Assay has formerly been used for toxicity testing of suspensions of contaminated soils, sediments and solid wastes (Põllumaa et al., 2000; Heinlaan et al., 2007). As suspensions of NPs are often turbid due to insolubility and/ or aggregation of particles, Flash Assay format has been shown to be appropriate also for screening the toxicity of NPs. The *V. fischeri* kinetic assay performed in a tube

luminometer has been used for measuring the toxicity of metal oxide NPs (Heinlaan et al., 2008).

1.5. *Tetrahymena thermophila* – a unicellular model organism for studying the effects of engineered nanoparticles on eukaryotic cells

1.5.1. Phylogeny and biology of Tetrahymena

Tetrahymena are free-living ciliate protozoa, which are common in fresh-water. A phylogeny based on the comparative analysis of small-subunit ribosomal RNA places ciliates among the "crown group" of eukaryotes. Ciliates are one of the three major groups within a monophyletic group of alveolates that also includes the dinoflagellates and apicomplexans (Fig. 1.6.).

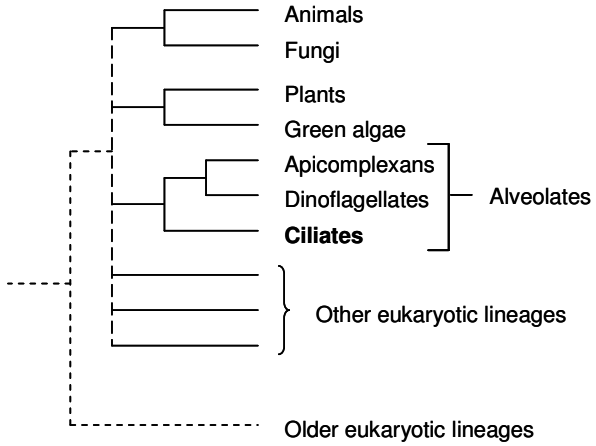

Figure 1.6. Phylogenetic tree showing the place of ciliates among the eukaryotes. Adopted from Frankel, 2000.

Two species of the genus *Tetrahymena* (*T. pyriformis* and *T. thermophila*) have been used as model eukaryotic cells since 1923, when Andre Michel Lwoff, a French microbiologist (a 1965 Nobel Prize Laureate in Physiology and Medicine, for discoveries concerning the genetic regulation of enzyme and virus synthesis), started to grow *Tetrahymena* in pure culture (Orias, 2002).

Within microorganisms, *Tetrahymena* is a large, motile, phagotrophic cell with dimensions of 50 μm in length and 20 μm in width. A distinctive feature of all ciliates, including *Tetrahymena*, is nuclear dualism: it possesses a diploid germinal micronucleus, which is capable of both mitosis and meiosis, and a somatic macronucleus that is made up of multiple copies of a rearranged micronuclear genome. This is the reason why *Tetrahymena* has been extensively used as a model organism in cell biology and genetics studies. Landmark discoveries made in *Tetrahymena* include the discovery of dynein motors, the 1989 Nobel Prize-winning discovery of RNA-mediated catalysis, the 2009

Nobel Prize-winning description of telomeres and telomerase, and the function of histone acetyltransferases in transcription regulation (reviewed in Turkewitz et al., 2002).

Another distinctive feature of ciliates is the arrangement of structures in the surface of the cell (Fig. 1.7). Underneath the plasma membrane are located 18 to 21 longitudinal microtubule bands with ciliary basal bodies aligned next to them. The cilia of *Tetrahymena* resemble those of other eukaryotes and possess nine peripheral microtubule doublets and one pair of central microtubules. Along the ciliary rows are docked the secretory granules, which discharge their contents upon stimulation. The cell of *Tetrahymena* exhibits extraordinary polarity: in the anterior end there is a complex oral apparatus made up of four compound ciliary elements (hence *Tetra-hymena*), the cell mouth or cytostome located at the posterior end of oral apparatus; the cell-anus or cytoproct is located at the posterior end of the cell. A single contractile vacuole, the osmoregulatory system, is located at the posterior end of the cell and it empties through two contractile vacuole pores. *Tertahymena* forms large food vacuoles (phagosomes) at the inner end of the oral apparatus and defecates their remaining contents at cytoproct. After formation phagosomes undergo a series of maturation steps, which includes acquiring the hydrolytic enzymes that participate in the phagolysosomal degradation of ingested particles (Jacobs et al., 2006). *Tetrahymena* cells carry out aerobic respiration and hence have numerous mitochondria, which are present throughout the cytoplasm but are concentrated in the cell cortex, i.e. directly underneath the cell membrane.

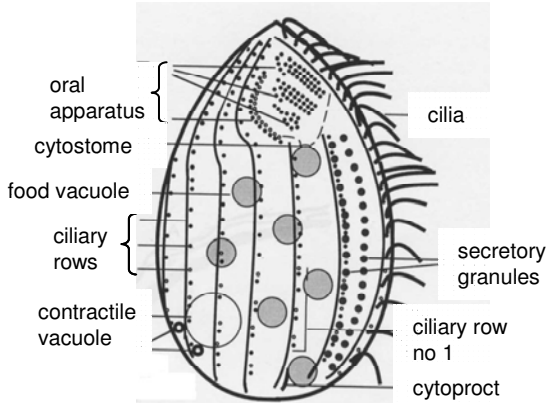

Figure 1.7. Diagram of the cytoplasmic organization of *Tetrahymena thermophila*. Adopted from Frankel, 2000.

1.5.2. Tetrahymena – a surrogate model for more complex organisms

Tetrahymena has a diverse sensory system. It is quickly becoming a model organism for studying neurobiology as it expresses genes that are homologs of many neurotransmitter receptors, which makes it a good surrogate animal

system, for instance in the study of analgesic and anti-inflammatory compounds. Pharmaceutical companies are already using *Tetrahymena* for drug development and testing (Cilian website). Readily visualized and quantifiable physiological endpoints allow for combined analysis of growth rate, phagocytosis rate, induced exocytosis, swimming speed and direction, chemotaxis, osmoregulation (contractile vacuole pulse rate; Hennessey and Kuruvilla, 1999). Hundreds of cilia provide large amount of plasma membrane for the high level expression of surface proteins, which are high priority targets for drug development by the pharmaceutical industry (Aldag et al., 2011). It is also an advantageous eukaryotic model system for mechanistic studies, as it contains many genes conserved in several eukaryotes (including humans), differently from other widely used unicellular model organisms. For example, more than 800 human genes have orthologs in *T. thermophila* but not in *S. cerevisiae*, and 58 of these genes are associated with human diseases (Eisen et al., 2006).

1.5.3. Tetrahymena – a surrogate model for toxicology

The ciliated protozoa *Tetrahymena* sp. has been used in toxicology for decades as a useful model organism for cellular and molecular biologists as well as for environmental research (Sauvant et al., 1999; Gutiérrez et al., 2003). Protozoa are ubiquitous in nature: they form an essential part in biological communities of the activated-sludge treatment processes (Esteban et al., 1991). Although protozoan tests are currently not standardized at OECD or ISO level, the commercially available 24-h growth inhibition test (Protoxkit F, 1998) is widely incorporated into test batteries for toxicity screening of pure compounds (Blinova et al., 2010; Sihtmäe et al., 2010) as well as natural waters (Mankiewicz-Boczek et al., 2008). In addition, toxicity data based on the growth inhibition of protozoa *Tetrahymena pyriformis* has been collected for 2,400 industrial organic compounds and incorporated in the TETRATOX database (Dimitrov et al., 2003). These data have been extensively used for quantitative structure–activity relationship (QSAR) analysis and currently there is almost as much of QSAR data available for *Tetrahymena* as for fish, although toxicity data for protozoa are not used for legislatory purposes in classification and labeling of chemicals for environmental hazard (Aruoja et al., 2011). *T. thermophila* could also be important for toxicogenomic studies since its macronuclear genome was sequenced in 2006 (Eisen et al., 2006).

1.5.4. Tetrahymena – a surrogate model for nanotoxicology

As protists have highly developed systems for internalization of nanoscale (100 nm or less) and microscale (100–100,000 nm) particles (Frankel, 2000), they are very good model organisms for nanotoxicology (Holbrook et al., 2008; Kahru et al., 2008). Despite of the favorable properties of protozoa there are only a few studies, where these organisms have been used for the evaluation of

biological effects of engineered nanoparticles, and the literature on *Tetrahymena* and NPs is scarce (Table 1.1). Protozoa *T. pyriformis* have been used in the toxicity studies of carbon nanotubes (Zhu et al., 2006) and fullerols (Zhao et al., 2006). *T. thermophila* has been shown to ingest single wall carbon nanotubes (SWNT) and bacteria with no apparent discrimination up to the SWNT concentration of 3.6 mg/l, where the bacterivory became inhibited. This indicates that SWNT may move up the food chain and CNTs may potentially disrupt the role of ciliates in regulating bacterial populations (Ghafari et al., 2008). Recently it was shown that the CdSe quantum dots accumulated in bacteria *Pseudomonas aeruginosa* were biomagnified in *T. thermophila* that preyed on the bacteria. Cadmium concentrations in the protozoa were five times higher than in bacterial prey, and as this had no toxic effect on protozoa, thus, the intact quantum dots were available to higher trophic levels (Werlin et al., 2011).

Table 1.1. Bibliometry of peer-reviewed papers published on effects of nanoparticles on different organisms according to Thomson Reuters ISI Web of Science for years 1980–2011. Search was performed on May 25, 2011. Timespan=All Years. Databases=SCI-EXPANDED, SSCI, A&HCI, CPCI-S, CPCI-SSH.

Keyword(s) used for the search (field: Topic)	**No. of papers retrieved by the respective keyword(s)**
nanoparticle*	***>100 000***
nanoparticle* AND *in vitro*	8805
nanoparticle* AND *in vivo*	7649
nanoparticle* AND bacteria	1664
nanoparticle* AND *Vibrio fischeri*	19
nanoparticle* AND rabbit*	428
nanoparticle* AND rodent*	105
nanoparticle* AND fish	185
nanoparticle* AND algae*	173
nanoparticle* AND *Daphnia*	132
nanoparticle* AND protozoa*	12
nanoparticle* AND *Tetrahymena*	8

AIMS OF THE STUDY

There is a severe shortage of scientific information on potential adverse effects of engineered nanoparticles (eNPs) on environmentally relevant test organisms. This knowledge is crucial for the environmental risk assessment of eNPs and nanomaterials, to ensure the sustainable development of nanotechnologies.

The general objective of the present study was to evaluate the hazard of selected inorganic and organic eNPs to two environmentally relevant aquatic model organisms: (i) prokaryotic unicellular organism – bacteria *Vibrio fischeri* – that *a priori* are not internalizing nanoparticles, and (ii) eukaryotic unicellular particle-ingesting organism – protozoa *Tetrahymena thermophila*. The focus was set on particle-ingesting *T. thermophila* as a relevant organism to mechanistic studies of nanotoxicology.

This was approached through the following specific aims:

- to adapt the acute kinetic *V. fischeri* bioluminescence inhibition test (Flash Assay) for the high-throughput and cost-efficient toxicity screening of eNPs (ZnO, CuO, PAMAM dendrimers G2 and G5 and polyethylenimine);

- to use the screening data of Flash Assay for the preliminary ranking of tested eNPs according to their toxic effects to *V. fischeri*;

- to evaluate the acute toxicity of two types of metal oxide NPs (ZnO and CuO) using *T. thermophila* as a eukaryotic model;
    - to find a testing approach, which would be an alternative to the growth inhibition assay and suitable for screening the toxicity of metal oxide NP suspensions to *T. thermophila*;

- to elucidate the mechanism of toxic action of CuO NPs to a eukaryotic particle-ingesting organism using *T. thermophila* as a model;
    - to elucidate the effects of CuO NPs on the fatty acid composition, lipid peroxidation and intracellular reactive oxygen species generation in *T. thermophila* to reveal the mechanisms of toxic action of CuO NPs.

2. EXPERIMENTAL

2.1. Physicochemical characterization of (nano)particles

The stock suspensions of metal oxides (40 g/l) were prepared in deionized water (MilliQ, Millipore), sonicated in ultrasonication bath (Techpan Type UM2-2, Poland) for 30 min and further stored at room temperature in the dark.

Scanning electron microscopy (SEM; Jeol, JSM-8404) from the suspensions of nano- and bulk CuO and ZnO and the analysis of specific surface area of the powders of the respective metal oxides by Brunauer-Emmet-Teller (BET) analysis (Sorptometer Kelvin 1042, Costech Instruments) were performed in Tallinn University of Technology, Center of Materials Research and Laboratory of Inorganic Materials.

The hydrodynamic diameter of nanoCuO in the exposure media was determined using dynamic light scattering (DLS) and the zeta potentials of nanoCuO and bulk CuO were measured using Zetasizer Nano ZS (Malvern Instruments). The hydrodynamic diameter and zeta potential of nanoCuO (100 mg/l) and the zeta potential of bulk CuO (2500 mg/l) were measured in MQ water and Osterhout's mineral medium.

2.2. Toxicity testing with *Vibrio fischeri*

The freeze-dried photobacteria *V. fischeri* (strain NRRL-B-11177, Aboatox, Turku, Finland) were rehydrated and stabilized first at 4 °C for 30 min and then at 20 °C for another 30 min. The testing was performed at 20 °C. For Flash Assay in cuvettes, 200 µl of sample was transferred into each cuvette and, inside the luminometer, 200 µl of bacterial suspension was automatically dispensed onto the sample. The luminescence was measured during 30 s under continuous mixing. After 30 min of incubation, the luminescence was measured again for 30 s under continuous mixing. In case of the Flash Assay in microplates 100 µl of test solution was pipetted into each well, which was supplemented with 100 µl of bacterial suspension by automatic dispensing in the luminometer testing chamber. The luminescence was recorded during the first 30 s after the dispensing of the bacteria in each well without additional mixing of the sample during the measurement. After 30-min incubation the light output was recorded again. BioOrbit 1251 luminometer was controlled by MultiUse 2.03 software and Fluoroskan Ascent FL plate luminometer by Ascent Software Version 2.4.1.

For comparison, conventional luminescence inhibition assay (Microtox® test) as ''gold standard" was performed with all compounds except for turbid suspensions of metal oxides. The testing was done using the 30-min exposure time essentially as described by Kahru (1993) with one exception: incubation/testing temperature was 20 °C.

2.3. Growth inhibition test with *Tetrahymena thermophila*

The commercially available *T. thermophila* growth inhibition test (Protoxkit F™) was used. The investigated compound and *T. thermophila* culture were added to the food substrate in the test medium that resulted in a turbid suspension. While normally growing (dividing) protozoan culture cleared the added substrate suspension in 24 h, inhibition of the growth of protozoa was reflected by residual turbidity of the food substrate and was measured by optical density (OD) of the tests samples at 440 nm. The incubation of the test organisms with metal oxides and food suspension was performed in 4-ml plastic cuvettes in the dark at constant temperature of 30°C. The toxicity was evaluated from 2 to 3 independent experiments, each in 2 replicates.

2.4. Cultivation of *Tetrahymena thermophila*

T. thermophila (strain BIII) was grown axenically in modified SSP medium (Gorovsky et al., 1975) 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). To prepare the cultures for toxicity testing, 1 ml of the stock culture was transferred to 9 ml of sterile modified SSP medium and grown for 24 h. 10 ml of the 24-h culture was transferred to 40 ml of sterile medium in a 250-ml Erlenmeyer flask and further cultivated for 18–24 h. The cultures were grown on an orbital shaker at 100 rpm, 30 °C. 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 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; Osterhout, 1906), pH 6.6, conductivity 170 µS/cm. Cell density was determined by counting the cells in haemocytometer (Neubauer Improved, bright line; Germany) after immobilizing 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).

2.5. Determination of cell viability of *Tetrahymena thermophila*

For the cell viability assays protozoan culture was prepared as described in section 2.4 and exposed to the toxicants at the following nominal concentrations: 0.3, 0.6, 1.2, 2.5, 5.0 mg Cu^{2+}/l of $CuSO_4$; 31.3, 62.5, 125, 250, 500 mg/l of nanoCuO; 500, 1000, 2000, 4000, 8000 mg/l of bulk CuO; 2.8, 5.7, 11.4, 22.8, 45.5 mg Zn^{2+}/l of $ZnSO_4\times7H_2O$; 1.9, 5.6, 8.3, 12.5, 25 mg/l of nano- and bulk ZnO. The testing was done as follows: 500 µl of the toxicant in Osterhout's medium was pipetted into the wells of 24-well polystyrene culture plates, each concentration in two replicates, and 500 µl of *T. thermophila* cells in Osterhout's

medium was added to the wells (final cell density 5×10^5 cells/ml). Osterhout's medium served as a control. In addition, a cell-free control was made, where 500 µl of Osterhout's medium was added to 500 µl of toxicant suspension/solution. The test plates with protozoa were incubated for 4 and 24 h at 25ºC in the dark, without shaking.

After 4 and 24 h of incubation of *T. thermophila* with or without toxicants, 100 µl was transferred from each well to 96-well black polypropylene microplate (Greiner Bio-One, Germany) for the viability testing with the fluorescent dye propidium iodide (PI, Fluka) and another 100 µl into a microcentrifuge tube for the ATP assay. The stock solution of PI was prepared in deionized water at a concentration of 1mg/ml. This was further diluted with deionized water to obtain the working solution of 100 µg/ml which was 10 times the final concentration of the viability assay. 10 µl of the PI working solution was pipetted directly into each well of 96-well microplate containing 100 µl of test medium and the microplates were further incubated for 15min at 25 °C in the dark. The fluorescence was quantified using the Fluoroskan Ascent FL microplate reader (Thermo Labsystems, Helsinki, Finland) at excitation and emission wavelengths of 530 and 590 nm, respectively.

ATP content of the cellular suspensions was measured using the luciferin–luciferase method essentially as described in Kahru et al. (1982). Briefly, for ATP extraction 100 µl of protozoa culture samples were rapidly mixed with an equal volume of ice-cold 10% trichloroacetic acid containing 4 mM ethylenediamine-tetraacetic acid (EDTA) in microcentrifuge tubes. The samples were stored at −18 °C till analysis. Prior to analysis the samples were thawed and diluted 50-fold with Tris–EDTA buffer (0.1 M Tris, 2 mM EDTA, adjusted to pH 7.75 with acetic acid). 200 µl of diluted sample was pipetted into the luminometer cuvette and first the background light emission ($RLU_{background}$) was measured. Then 20 µl of reconstituted ATP Monitoring Reagent from the ViaLight®HS Bioassay Kit (Lonza Rockland, USA) was added and the light emission of the sample (RLU_{sample}) was measured. For the internal calibration, 10 µl of ATP standard (1.65×10^{-6} M ATP) was added to the sample and light emission ($RLU_{internal\ standard}$) was measured again. The amount of the ATP in each well was calculated according to the following equation:

$$ATP,\ \mu mol = ((RLU_{sample} - RLU_{background})/RLU_{ATP\ standard}) \times ATP\ standard,\ \mu mol.$$

2.6. Exposure of *Tetrahymena thermophila* to toxicants for the fatty acid and lipid peroxidation analysis

T. thermophila was prepared as described in section 2.4 and exposed to toxicants at two concentrations: 24-h EC_{20} and EC_{50}, i.e. nominal concentrations of chemicals (mg/l) causing a 20% and 50% decrease in the viability of the protozoa, respectively. The viability was determined by measuring the ATP content as described in section 2.5. For exposures the following concentrations

were used: 1.3 and 1.6 mg Cu^{2+}/l of $CuSO_4$, 60 and 80 mg/l of nanoCuO, 1500 and 2500 mg/l of bulk CuO, 2.6 and 5.3 mg/l of 3,5-DCP, and 43 and 68 mg/l of H_2O_2.

For membrane fatty acid analysis 20 ml of *T. thermophila* (10^6 cells/ml) was added to 20 ml of nanoCuO or the control chemical solution/suspension (all in Osterhout's medium) in 100-ml beakers to yield a final cell density in the test of 5×10^5 cells/ml. The beakers were shaken at 80 rpm at 25ºC for 24 h. After exposure the cell suspensions were centrifuged at 300×g for 5 min, 4°C, washed once with Osterhout's medium, and the pellets were resuspended in sterile double-distilled water, frozen at –20°C, lyophilized and kept at 4°C until further analysis. The experiments were repeated for three days.

For lipid peroxidation analysis *T. thermophila* cells were exposed to nanoCuO and the corresponding controls in 24-well culture plates: 500 µl of *T. thermophila* in Osterhout's medium was pipetted into the wells containing 500 µl of the toxicant, each concentration in two replicates. Osterhout's medium was used as a diluent for chemicals and as a negative control. The test plates were incubated on a microplate shaker (Heidolph Titramax 1000, 300 rpm) at 25°C. After 2- and 24-h exposure 100 µl of cell suspension was sampled from each well, 5 µl of 1% BHT in methanol as antioxidant was added and the samples were kept at –80°C until the analysis. The experiments were repeated for three days.

2.7. Determination of cellular fatty acid composition of *Tetrahymena thermophila* by gas chromatography (GC)

Prior to analysis lipids were transesterified using the HCl/methanol procedure (Dionisi et al., 1999). Fatty acid methyl esters (FAMEs) were separated using Agilent 6890 series capillary gas chromatograph equipped with capillary column Omegawax TM 320 (30 m×0.32 mm ID×0.25 mm, Supelco) with polyethylene glycol as the stationary phase, and a flame-ionization detector. Helium was used as a carrier gas with a flow of 2.0 ml/min. The starting temperature for analysis was 185 °C and the end temperature was 215°C, with a temperature increase speed of 1 °C/min. The run time was 54 min and the volume of injection was 2 µl. The identification of FAMEs was done by retention time comparison and results were calculated using response factors derived from chromatographic standards of known composition (Nu Chek Prep). Relative proportions of fatty acids between C10:0 and C20:0 were calculated from peak areas. The results were analyzed using Agilent ChemStation Plus® software. The analysis of each sample was made in duplicate.

2.8. Analysis of lipid peroxidation in *Tetrahymena thermophila* 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) 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 EC_{50} concentrations used in the cell expose experiments. No interference of the chemicals with the test results was detected at the relevant MDA concentrations.

2.9. Analysis of reactive oxygen species (ROS) in *Tetrahymena thermophila* 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 nanoCuO 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 under light microscope (Olympus CX41 equipped with a DP71 camera). Images were taken using software Cell B (Olympus).

2.10. Analysis of the dissolved fraction of metal oxides using the recombinant metal-sensing *Escherichia coli*.

The bioavailable metal ions present in the *T. thermophila* exposure medium (Osterhout's medium) were quantified using recombinant metal sensor bacteria in which bioluminescence is specifically induced by intracellular metal ions. The induction is mediated by a protein that recognizes the respective metal ions and regulates a promoter controlling the expression of the luminescence encoding gene cassette luxCDABE leading to increase in bioluminescence of sensor bacteria (Ivask et al., 2002). To determine the dissolved fraction of nano- and bulk ZnO and CuO at EC_{50} concentrations, the suspensions of metal oxides prepared in Osterhout's medium were incubated either with protozoa (cell density 5×10^5 cells/ml, Paper **III**) or without protozoa (Paper **IV**) in 24-well microplates (total volume: 1 ml per well) for 4 h at 25°C without shaking (as in the toxicity tests) and thereafter filtered through 0.1 μm filter (Sartorius). The filtrate was analyzed for bioavailable metal ions using recombinant luminescent Zn-sensor bacteria *Escherichia coli* MC1061 (pSLzntR/pDNPzntAlux) and Cu-sensor bacteria *E. coli* MC1061 (pSLcueR/pDNPcopAlux) (Ivask et al., 2009). In parallel to sensor bacteria, luminescent control strain *E. coli* MC1061 (pDNlux) (Leedjärv et al., 2006) with constitutively expressed luminescence was used to take into account the turbidity and possible toxic effects of the tested compounds. The test was conducted essentially as described in Ivask et al. (2009). Briefly, the sensor and control bacteria were pre-grown in Luria–Bertani medium till exponential phase, then harvested and washed twice with Osterhout's medium supplemented with casamino acids (1 g/l) and glucose (1 g/l) and diluted with washing medium until OD600 ~0.1. 100 μl of filtrate and 100 μl of sensor/control bacteria were pipetted into 96-well white microplates (Thermo Labsystems) and incubated for 2 h at 30 °C. The bioluminescence was registered with Fluoroskan Ascent FL microplate reader (Thermo Labsystems, Helsinki, Finland). $ZnSO_4 \times 7H_2O$ and $CuSO_4$ were used as 100% bioavailability controls, respectively.

3. RESULTS AND DISCUSSION

3.1. Selection and characterization of nanoparticles

To demonstrate that *V. fischeri* kinetic luminescence inhibition assay (Flash Assay) can be used to test the toxicity of various NPs (Paper **I**), two types of metal oxide NPs and three types of organic NPs were selected and analyzed using *V. fischeri* Flash Assay (Table 3.1) To evaluate the effect of particle size, also the bulk (i.e., micro-sized) forms of the respective metal oxides were studied in parallel. For solubility control of metal oxides, respective water-soluble metal salts were tested (Table 3.3). Both, zinc and copper oxide form turbid aqueous suspensions, which are white and black in color, respectively (Fig. 3.1, panel A). Polycationic polymers – PEI, PAMAM dendrimers G2 and G5 formed transparent aqueous solutions.

Table 3.1. Characteristics of nanoparticles analyzed for the toxicity screening in *Vibrio fischeri* kinetic luminescence inhibition assay (Flash Assay, Paper **I**)

Chemical	Form when purchased	Size[a], nm	SSA of powder[b], m^2/g	M_w	CAS no.
Metal oxides					
NanoZnO	white powder	50 - 70	12.9	–	1314-13-2
Bulk ZnO	white powder	-	7.16	–	1314-13-2
NanoCuO	black powder	30	25.5	–	1317-38-0
Bulk CuO	black powder	-	0.64	–	1317-38-0
Polycationic polymers					
Polyethyleneimine, branched	water-free gel	–	–	25,000	9002-98-6
Polyamidoamine dendrimer G2	20 wt % in methanol	–	–	3256	93376-66-0
Polyamidoamine dendrimer G5	5 wt % in methanol	–	–	28,825	163442-68-0

[a] according to the manufacturer (Sigma-Aldrich)
[b] specific surface area (SSA) measured by Brunauer-Emmet-Teller (BET) analysis

SEM images of CuO and ZnO (Fig. 3.1) show that nanosized oxides contained smaller particles than bulk oxides, but there were also <100 nm particles in the bulk preparation. The difference in size was further confirmed by BET analysis: nanoZnO had a 1.8-fold higher specific surface area than bulk ZnO and nanoCuO 39-fold higher specific surface area than bulk CuO (Table 3.1).

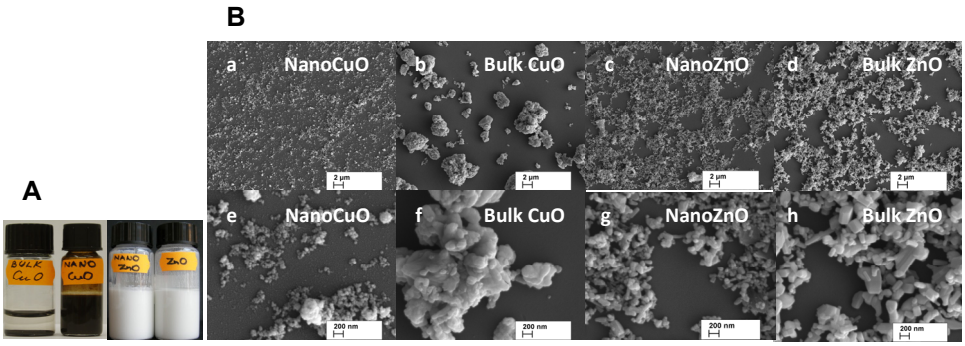

Figure 3.1. Stock solutions (A) and SEM images (B) of nano-and micro-sized CuO and ZnO. Panel A: stock solutions in MilliQ water at 40,000 mg/l. Panel B: images a, b, c and d: ×2000 magnification; e, f, g and h: ×20 000 magnification.

Table 3.2. Characteristics of nano- and microsized (bulk) CuO

Particle		**nanoCuO**	**bulk CuO**
Hydrodynamic diameter[c], nm	MQ water (pH 6.0)	209 ± 10	nd
	Osterhout's mineral medium (pH 6.0)	1230 ± 200	nd
Zeta potential[c], mV	MQ water (pH 6.0)	+37	+32
	Osterhout's mineral medium (pH 6.0)	+17	- 5

[c] measured at 100 mg/l (nanoCuO) and 2500 mg/l (bulk CuO) by dynamic light scattering (DLS); mean of three samples ± standard deviation

As NPs tend to aggregate in the aqueous media the size of CuO NPs was further analyzed in the aqueous suspensions using dynamic light scattering (DLS) technique (Paper **IV**). The average hydrodynamic diameter of nanoCuO, as measured by DLS, appeared around 200 nm in MQ water and even in micrometer scale (1,230 nm) in the Osterhout's medium (Table 3.2), while the size of CuO NPs in the powder form was 30 nm (Table 3.1). Although the concentration of mineral salts in Osterhout's medium is very low (conductivity 170 µS/cm), CuO NPs were observed to aggregate and sediment at much higher rate in Osterhout's medium (Paper **IV**, Fig. 2, B) than in MQ water (Paper **IV**, Fig. 2, A) after 3 h at 25 °C. The influence of the composition of mineral medium on particle aggregation and thus, higher instability of the suspensions, was also confirmed by the different zeta potential values of CuO measured in MQ water and Osterhout's mineral medium (Table 3.2). While the zeta potential values of both bulk and nanoCuO suspensions in MQ water exceeded +30 mV, which is considered an indication of a stable suspension, the respective values in the mineral medium were + 17 mV for nanoCuO and –5 mV for bulk CuO,

indicating higher propensity for aggregation of both CuO formulations in Osterhout's medium. However, when the hydrodynamic diameter was analyzed after removing the larger aggregates by a low speed centrifugation, it was evident that the suspension contained also smaller aggregates of nanoCuO with the average diameter of 295 nm (Fig. 3.2).

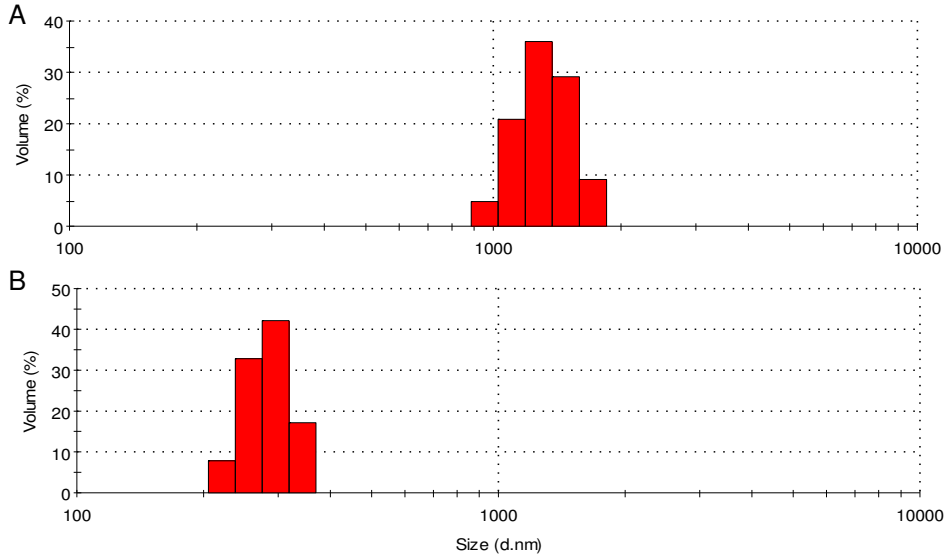

Figure 3.2. Size distribution of nanoCuO by dynamic light scattering. DLS measurement before (A) and after (B) removing the larger particles by centrifugation at 700×g for 5 min. The measurement was done at nanoCuO concentration of 100 mg/l in Osterhout's mineral medium.

The tendency of NPs to aggregate in aqueous systems is a well-known phenomenon, which has been observed in the case of different types of NPs (Griffitt et al., 2007; Karlsson et al., 2008); it has even been proposed that in ecotoxicology the definition of NPs should be extended to particles with a size up to 500 nm (Handy et al., 2008). Moreover, the micrometer sized aggregates of NPs are still readily ingestible for some particle-feeding organisms such as daphnids and protozoa, facilitating the possible bioaccumulation of NPs. Protozoa also contribute to the agglomeration of NPs by ingesting particles into food vacuoles, where the NPs are packed together. The packed NPs are either excreted from the cytoproct of the living cells or escape the intracellular space after the cell death, facilitating the sedimentation of NPs. As a result, the concentration of NPs in the water column is decreased and the exposure to other aquatic organisms is reduced, as was recently proposed in a study on fullerene exposed *Daphnia magna* (Tervonen at al., 2010). Consequently, this could increase the concentration of NPs in the sediments, where the aggregates of NPs could be redispersed and render toxic to benthic fauna; for example, it was recently demonstrated that nanoCuO had a significant inhibitory effect on the biogas production by anaerobic bacteria (Luna-delRisco et al., 2011).

3.2 Evaluation of the hazard of selected inorganic and organic eNPs (Paper I, II, III)

3.2.1. Toxicity screening of selected nanoparticles using the Vibrio fischeri kinetic luminescence inhibition assay in microplate format (Paper I)

The aim of the study was to demonstrate that Flash Assay (see section 1.4.2) can also be performed in a microplate format and used as a high throughput, cost-efficient and fast method for screening the toxicity (antibacterial properties) of various NP suspensions to *V. fischeri*. The toxicity of zinc and copper oxide NPs and polycationic polymers, along with the reference chemicals (Table 3.1. and Table 3.3), was comparatively screened using the *V. fischeri* kinetic luminescence inhibition assay in a conventional cuvette format and newly developed microplate format, and the results were compared to the traditional *V. fischeri* luminescence inhibition test Microtox®. The latter is considered as a 'gold standard' and was performed in parallel with the kinetic assay with all compounds except for turbid suspensions of metal oxides. The Microtox® test was done using the 30-min exposure time essentially as described by Kahru (1993). While in Microtox® assay the background luminescence value of bacteria is measured first and then the test sample is added to the bacteria, in the kinetic luminescence inhibition assay the test bacteria is added to the sample and the recording of the luminescence is started simultaneously, allowing for the sample to act as a reference for itself, with no need for the color/turbidity correction. Differently from the standard protocol of Microtox® assay, where the bacteria is kept at 15 °C during the testing, it is not possible in the Flash Assay due to the restrictions of the luminometers used for measuring. For this reason also the Microtox® assay was done at the incubation/testing temperature of 20°C, to allow comparison with Flash test format data. It is important to note that the Flash Assay (but not the Microtox® test format) is applicable also for microplate luminometers and high throughput analysis.

Despite of using higher temperature than recommended in the Microtox® assay there was no significant difference in the toxicity results of kinetic assay (both microplate and tube format) and Microtox® test, with the exception of PEI and PAMAM G5, which appeared more toxic in Microtox® assay (Table 3.3). The higher toxicity obtained in Microtox® assay may be explained by the differences in the test protocol. The comparison of EC_{50} values obtained in the cuvette and microplate format of the kinetic Flash Assay showed good correlation (log-log $R^2 = 0.98$; Paper **I**, Fig. 3). The only outlier in the current study nanoCuO, being the most turbid sample tested, was ~3 times less toxic in the kinetic assay performed in the microplates compared to the cuvette format. The underestimation of the toxicity of nanoCuO in microplate format could be attributed to the lack of continuous mixing of the sample, which is not possible in microplates. Without mixing the suspended solids and particles tend to settle

in time and this might influence the test results. The difficulty of obtaining repeatable results in testing the toxicity of particle-containing samples is also characterized by the high standard deviation values. Nonetheless, both formats of the Flash Assay were comparable in this aspect (Table 3.3). Moreover, the 30-min EC_{50} values obtained in both Flash Assay formats allowed the same classification of the chemicals, except for nanoCuO, according to the risk phrases of the EC Directive 93/67/EEC (Table 3.3).

In addition to estimating the toxicity of the chemicals the kinetic *V. fischeri* assay also allows for characterization of the mode of action of the chemicals. As it can be seen from the Table 3.3 and the Fig. 3.3, during the first 30 s of exposure of *V. fischeri* to copper and zinc compounds no inhibition of luminescence was observed, while a clear dose-effect pattern was established after 30 min. The increasing toxicity of heavy metal compounds upon prolonged exposure is well established in photobacteria (Kaiser and Devillers, 1994). In the contrary to the 'slow acting' metal compounds, the organic NPs PEI and PAMAM dendrimers exerted toxic effects already after first seconds of contact and by 30 s of exposure the luminescence signal already reached a plateau (Table 3.3 and Fig. 3.3). Thus, the 30-s and 30-min EC_{50} values calculated based on the kinetic assay did not differ significantly, indicating 'ultrafast' mode of action of these toxicants to the bacteria.

The antibacterial effects of PAMAM dendrimers have been previously studied on Gram-negative bacteria *Pseudomonas aeruginosa* and Gram-positive *Staphylococcus aureus* (Calabretta et al., 2007). In the latter study a 2-h incubation time followed by plating out the cells and standard colony counting after overnight incubation at 37°C was used to estimate the toxicity of PAMAM G5 dendrimer. The different endpoint and longer exposure time could explain much lower EC_{50} values: for *P. aeruginosa* 1.5 mg/l (corresponding to 0.05 μM) and for *S. aureus* 20.8 mg/l (corresponding to 0.7 μM), compared to the results of the current study (Table 3.3). Naha et al. (2009) studied the aquatic toxicity of three different generations of PAMAM dendrimers: G4, G5 and G6 among other test models also on *V. fischeri* using the standard Microtox® test. The toxicity of each dendrimer was found to increase linearly with increasing exposure time (5, 15 and 30 min), which is different from the current study, where no difference in 30-s and 30-min EC_{50} values was detected. Although the 30-min EC_{50} value of PAMAM G5 dendrimer was more than 10 times higher in this study compared to Naha et al. (2009), a similar trend concerning the increase in toxicity as a function of increasing generation was observed in both studies (G5 > G2 in this study and G6 > G5 > G4 in Naha et al., 2009). The increase in toxicity was attributed to the physicochemical properties of the PAMAM dendrimers as well as structure activity relationship, as in successive generations there is a systematic increase in molecular weight, number of surface amino groups and particle size.

Table 3.3. EC_{50} values of the tested chemicals in *Vibrio fischeri* Flash Assay performed in cuvette and microplate format and Microtox® test

Test substance	30 sec EC50				30 min EC50						Classification*
	cuvettes		microplates		cuvettes		microplates		Microtox®		
	mg/l	µM	mg/l	µM	mg/l	µM	mg/l	µM	mg/l	µM	
References											
3,5-dichlorophenol	6.2 ± 0.5	38 ± 3.1	5.0 ± 0.2	31 ± 1.2	3.1 ± 0.1	19 ± 0.6	2.4 ± 0.05	15 ± 0.3	2.0 ± 0.04	12 ± 0.2	toxic
Cr^{6+} (tested as $K_2Cr_2O_7$)	27 ± 1.9	90 ± 6.5	66 ± 11	224 ± 37	3.6 ± 0.5	12 ± 1.7	6.0 ± 0.2	20 ± 0.7	4.1 ± 0.02	14 ± 0.1	toxic
Zinc compounds											
nanoZnO	> 100	> 1228	> 100	> 1228	4.8 ± 1.1	59 ± 14	3.8 ± 0.7	47 ± 9	nd	nd	toxic
bulk ZnO	> 100	> 1228	> 100	> 1228	4.3 ± 1.7	53 ± 21	3.9 ± 1.8	48 ± 22	nd	nd	toxic
Zn^{2+} (tested as $ZnSO_4*7H_2O$)	> 20	> 306	> 20	> 306	3.8 ± 1.7	58 ± 26	3.2 ± 1.0	49 ± 15	3.5 ± 0.07	54 ± 1	toxic
Copper compounds											
nanoCuO	> 250	> 3143	> 250	> 3143	68 ± 4.3†	856 ± 54	204 ± 42†	2,564 ± 528	nd	nd	nc
bulk CuO	> 10,000	> 125,715	> 10,000	> 125,715	3894 ± 1871	48,953 ± 23,521	4,208 ± 2,471	52,900 ± 31,064	nd	nd	nc
Cu^{2+} (tested as $CuSO_4$)	> 1.6	> 25	> 1.6	> 25	0.8 ± 0.1	13 ± 1.6	0.8 ± 0.3	13 ± 4.7	0.3 ± 0.002	4.7 ± 0.03	very toxic
Polycationic polymers											
polyethylenimine	225 ± 162	9.0 ± 6.5	216 ± 117	8.6 ± 4.7	215 ± 139	8.6 ± 5.6	238 ± 123	9.5 ± 4.9	67.4 ± 1.1	2.7 ± 0.04	nc
polyamidoamine G2	603 ± 187	185 ± 57	559 ± 394	170 ± 120	424 ± 162	130 ± 50	631 ± 415	194 ± 128	nd	nd	nc
polyamidoamine G5	561 ± 303	20 ± 11	686 ± 342	24 ± 12	539 ± 235	19 ± 8.2	775 ± 374	27 ± 13	179 ± 7.9	6.2 ± 0.27	nc

EC_{50} numbers are the average values ± SD (standard deviation) of two testing on different days both performed in two parallels.

* Classification based on 30-min EC_{50} data obtained in Flash Assay microplate format according to the risk phrases for ranking toxicity of chemicals for aquatic organisms (EC Directive 93/67/EEC): $EC_{50} \leq 1$ mg/l: very toxic; 1 - 10 mg/l: toxic; 10 - 100 mg/l: harmful. EC50 values > 100 mg/l are designated as nc - not classified.

nd - not determined

† significant difference ($p < 0.05$).

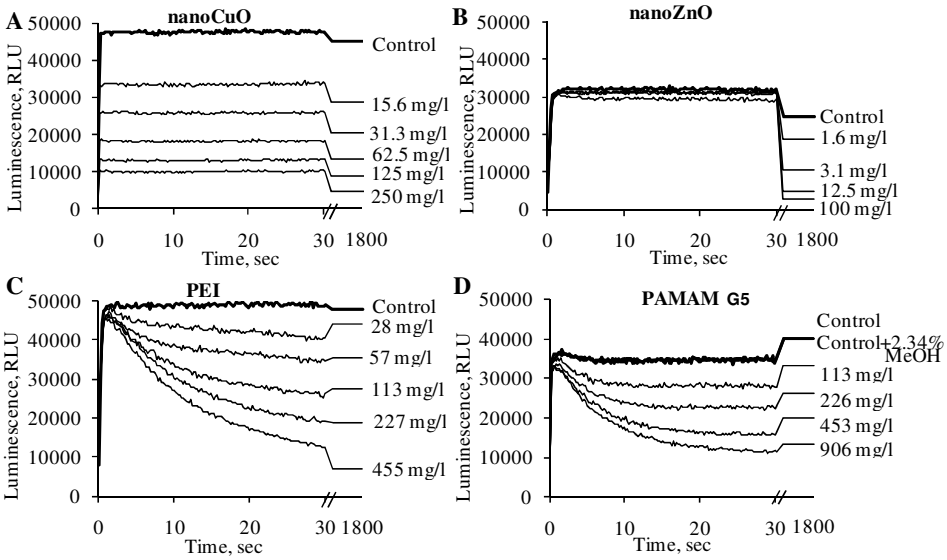

Figure 3.3. Kinetics of luminescence (relative light units, RLU) of *Vibrio fischeri* exposed to suspensions of nanoCuO (A), nanoZnO (B), polyethylenimine, PEI (C) and polyamidoamine dendrimer, PAMAM G5 (D) measured in microplate format of Flash Assay. 2% NaCl was used as a control and diluent for nanoCuO, nanoZnO and PEI, and 2% NaCl + 2.3% methanol served as a control and diluent for PAMAM dendrimer

The toxic effect of nanoZnO on bacteria *V. fischeri* did not differ from the effect of bulk ZnO nor ionic zinc based on the 30-min EC_{50} values. Similar toxicities of ZnO NPs and bulk formulation to *Escherichia coli* and *Bacillus subtilis* have previously been reported also by Adams et al. (2006), and to bacteria *E. coli* AB1157 by Ivask et al. (2010), although in a more recent study ZnO NPs were found to be more toxic than their bulk counterparts to *B. subtilis*, *E. coli* and *Pseudomonas fluorescens* (Jiang et al., 2009). In the latter study it was concluded that the toxicity of NPs was not only from the dissolved metal ions, but also from their greater tendency to attach to the cell walls than to aggregate together.

The toxicity of nanoCuO to *V. fischeri* appeared remarkably higher than the toxicity of bulk CuO in both Flash Assay formats in microplates and cuvettes (Table 3.3). The toxicity data for nanoCuO and bulk CuO tested in tube luminometer (68 and 3,894 mg/l; Table 3.3) were very close to our previous data (Heinlaan et al., 2008), where the same method was applied using *V. fischeri* preserved at –80°C in 10% glycerol instead of freeze-dried *V. fischeri* preparation as in the current study. In addition, the data were very close to the 30-min EC_{50} values of nano- and bulk CuO to *E. coli* AB1157 (50 and 3,746 mg/l, respectively, Ivask et al., 2010).

In a recent study, where among other NPs the toxic effects of CuO and ZnO NPs were studied in *E. coli*, *B. subtilis*, and *Streptococcus aureus* it was found that CuO NPs were the most toxic of the tested NPs, followed by the ZnO NPs

(Baek and An, 2011). In the same study the effects of dissolved metal ions in terms of NP toxicity were found to be negligible. Study of Ivask et al. (2010) on comparison of toxic effects of different NPs on *E. coli* AB1157 also showed that nanoCuO (2-h EC_{50} = 8.1 mg/l) was about 7-fold more toxic than nanoZnO (54 mg/l), whereas in both cases the solubilized ions largely explained the toxic effect.

3.2.2. Evaluation of the acute toxicity of ZnO and CuO nanoparticles to a eukaryotic model organism (Paper II, III)

It is widely accepted that as most of the standardized bioassays are conducted in environmentally non-relevant conditions they do not characterize accurately the potential impacts of hazardous chemicals on natural ecosystems, both aquatic and terrestrial ones, as previously demonstrated also by our group (Aruoja et al., 2004; Blinova, 2004; Kahru et al., 2005). Most of the ecotoxicity data on chemicals available for standard freshwater test organisms have been generated using so-called artificial freshwater (AFW). However, as bioavailability and toxic effect of a chemical depend on its speciation and hence, on water composition (Witters, 1998), the hydrochemical parameters of water used as test medium are very important. In Paper **II** the acute toxicity of CuO and ZnO NPs to particle ingesting aquatic species was determined comparatively in AFW and natural waters sampled from Estonian rivers. The test species battery also included the protozoa *T. thermophila*, which was used to evaluate the toxicity of CuO and ZnO in MilliQ water (a standard test environment for Protoxkit F™ test) and three river waters. In this study the commercially available *T. thermophila* growth inhibition test (Protoxkit F™) was used. Table 3.4 shows that the toxicity of $ZnSO_4$ to protozoa decreased in natural waters (from 7.1 to 21.1 mg Zn/l; $p < 0.05$), but for nanoZnO the statistically significant decrease was observed only in the water of river 5 (from 9.4 to 26.5 mg Zn/l; $p < 0.05$). In case of bulk ZnO, however, the toxicity in river waters even increased (from 27.1 to 12.0 mg Zn/l; $p < 0.05$). Analysis with recombinant Zn-sensing bacteria showed that at the concentrations and conditions used in the current study both nano- and bulk ZnO were almost fully dissolved. This would suggest that the toxicities of all the tested zinc compounds should be similar, which was also demonstrated for nanoZnO and $ZnSO_4$ (when expressed as mg Zn/ml; Table 3.4.). The significantly higher EC_{50} value obtained for bulk ZnO could be partly attributed to the test format used, which occasionally presents problems with the reproducibility of the test. The unsuitability of the Protoxkit F™ is further illustrated in case of the CuO samples, where no reproducible data was obtained (Table 3.4). Also, differently from other aquatic species used in this study, toxicity of $CuSO_4$ to protozoa in river waters 2 and 4 was not statistically different from EC_{50} values in standard test conditions, i. e. MilliQ water (Table 3.4). This result is not in accordance with the data obtained in crustacean biotests and recombinant sensor bacteria test, which both indicated that natural

waters have high mitigation effect for copper. The ability of natural organic matter (NOM) to complex copper and remarkably reduce the bioavailable copper ion concentrations is well known (Allen and Hansen, 1996). The possible explanation for the discrepancy in case of protozoa in this study could be the binding of copper ions by food particles, which are added to the test medium at the beginning of the assay, and the resulting masking of the mitigating effect of organic matter in river waters.

Table 3.4. Toxicity (24-h growth inhibition) of nanoCuO and nanoZnO, their bulk forms and respective soluble salts to *Tetrahymena thermophila*

	24-hour EC_{50} (mean ± SD, mg metal/l)					
Test media	**NanoCuO**	**Bulk CuO**	**$CuSO_4$**	**NanoZnO**	**Bulk ZnO**	**$ZnSO_4 \times 7H_2O$**
MilliQ water	ns	ns	0.40 ± 0.1	9.4 ± 3.0	27 ± 1.0	7.1 ± 0.6
River 2	ns	ns	0.46 ± 0.02	16 ± 0.4	17 ± 0.9	21 ± 1.3
River 4	ns	ns	0.27 ± 0.03	12 ± 0.8	12 ± 0.5	19 ± 0.6
River 5	ns	ns	< 0.2	27 ± 2.5	15 ± 1.1	> 22

ns – not shown as no reproducible data was obtained

Regardless of not being able to establish the EC_{50} values for nano and bulk CuO using the protozoan growth inhibition assay, because of the black color interfering with OD measurements, microscopic observations of the protozoa after exposure to the sub-toxic concentrations of nanoCuO showed clear accumulation of the CuO NPs in their food vacuoles (Fig. 3.4).

Figure 3.4. Protozoa *Tetrahymena thermophila* under light microscope after 4-h exposure to nanoCuO at 100 mg/l.

To overcome the interference of turbidity caused by insolubility and/or aggregation of NPs in the test suspensions, two alternative methods for assessing the toxic effects of NPs to *T. thermophila* were proposed and adopted for fast and robust viability measurements (Paper **III**). Propidium iodide (PI) staining and cellular ATP concentration that both correlated with the cell viability were used to assess the acute toxicity of two types of metal oxide NPs (ZnO and CuO) to *T. thermophila* at two exposure times (4 and 24 h). The test medium used in the protozoan toxicity assays was Osterhout's mineral medium, which contains very low amount of mineral salts (conductivity 170 µS/cm), and no food substrate was added to protozoa during the exposure, thus the interference of NOM with the test results was minimized. Moreover, both assays were successfully performed directly in the exposure medium (see Section 2.5), avoiding the time consuming intermediate step of removing the toxicant suspension/solution from the cells by centrifuging prior

to the viability measurement (Dayeh et al., 2005). As the toxicity tests were conducted in starving conditions, the cell number and size of protozoa in the control was slightly reduced during 24 h (by 15% and 30%, respectively), but protozoa remained active and motile, which was confirmed by visualizing the cells under the light microscope.

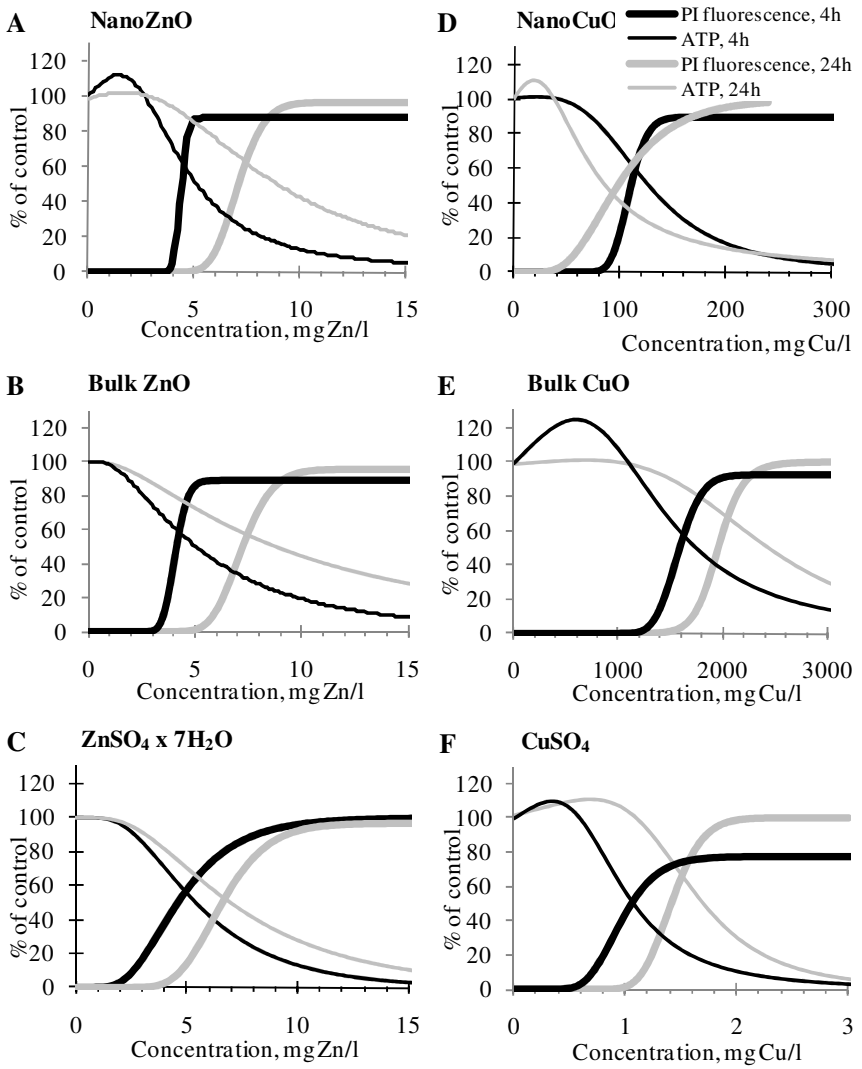

Figure 3.5. Dose-response curves obtained upon exposure of *Tetrahymena thermophila* to zinc (A, B,C) and copper (D, E, F) oxide (nano)particles and the respective metal salts for 4 and 24 h. The results of the assay on ATP content are expressed as a percentage compared to non-exposed controls (100% viability), the results of the propidium iodide (PI) fluorescence assay are calculated as a proportion of the maximum fluorescence value (100% cytotoxicity). The dose-response curves were calculated based on the data from three independent experiments, using REGTOX software. Figure modified from Paper **III**.

Dose-dependent decrease in the cellular content of ATP (indicating the decrease in viable cells) and an increase in fluorescence of PI (indicating the increase in dead cells) were observed in *T. thermophila* after exposure to NPs as well as to the reference compounds (Fig. 3.5). At the lowest (subtoxic) concentrations tested, most of the zinc and copper compounds had a stimulatory effect on ATP concentration of *T. thermophila* (about 10% increase compared to the non-exposed control). The stimulating effect of zinc and copper to *Tetrahymena* has been reported previously (Nilsson, 1981, 2003) and it is not surprising, as both metals are needed as microelements for normal functioning of the cells (Valko et al., 2005). Two different endpoints (relative decrease in ATP content and relative increase in PI fluorescence) used to measure the dose-response of *T. thermophila* to CuO and ZnO were the 'mirror images' of the cell viability and correlated well (Fig. 3.6). The correlation between the cellular ATP content and PI staining method was better at 4-h exposure ($R^2 = 0.8957$) compared to 24-h exposure ($R^2 = 0.7691$).

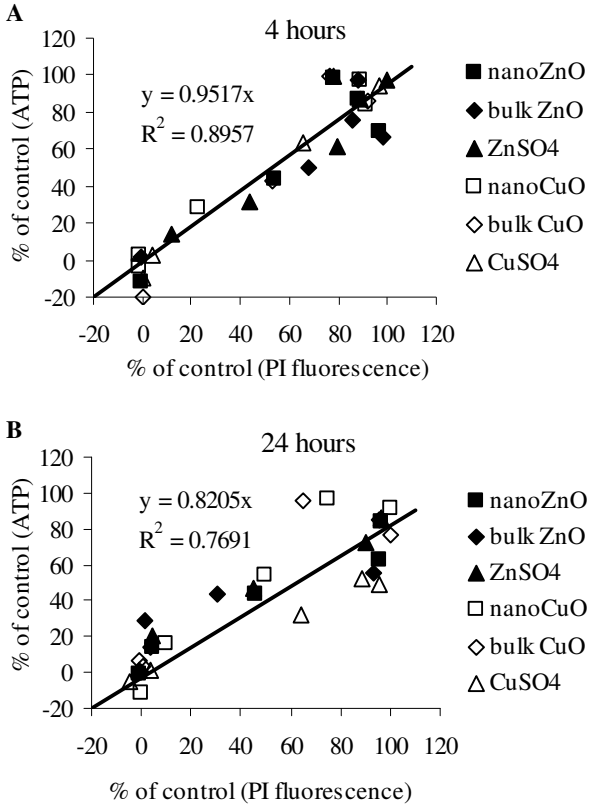

Figure 3.6. Cellular ATP content (percentage compared to non-exposed controls, i. e. 100% viability) *versus* propidium iodide (PI) fluorescence of the cells (proportion of the maximum fluorescence value, i. e. 100% cytotoxicity) upon exposure of *Tetrahymena thermophila* to zinc and copper oxide (nano)particles and the respective metal salts for 4 h (A) and 24 h (B). Data are plotted from Figure 3.5.

As it can be seen in the left panel of Fig. 3.5 (A–B), all the tested zinc compounds had toxic effects to *T. thermophila* in the same concentration range (from ~2–15 mg Zn/l). Similar tendencies of toxicity in case of nano and bulk ZnO and $ZnSO_4 \times 7H_2O$ were also seen in bacteria *V. fischeri* (Paper **I**) and have previously been shown also for other aquatic organisms (Franklin et al., 2007; Heinlaan et al., 2008; Aruoja et al., 2009; Kasemets et al., 2009), indicating that the toxicity of ZnO was most likely caused by the dissolved zinc. This hypothesis was further supported by the results of the dissolution studies of ZnO: using the recombinant zinc-sensing bacteria we demonstrated that at the respective EC_{50} concentrations to protozoa approximately 80% of zinc in nano and bulk ZnO in the protozoan test medium was in dissolved form (Fig. 3.7). Also, both, nano and bulk ZnO suspended at up to 10 mg/l in two different artificial freshwaters used for crustacean tests were almost fully dissolved and bioavailable for bacterial sensors (Paper **II**). Kasemets et al. (2009) showed that about 80% of nano and bulk ZnO was dissolved in malt extract medium used for the cultivation of yeasts even at a concentration of ~100 mg ZnO/l.

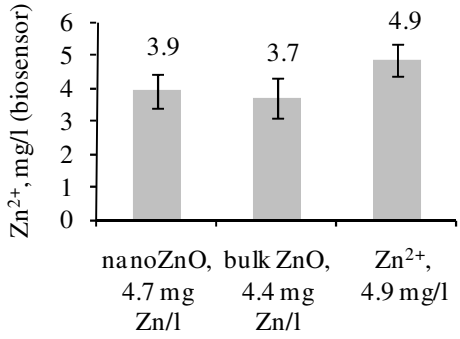

Figure 3.7. Bioavailable zinc, mg/l, measured with recombinant zinc-sensing bacteria in the suspensions/solutions of nano and bulk ZnO and $ZnSO_4 \times 7H_2O$ after incubation with *T. thermophila* for 4 h in Osterhout's mineral medium at 25°C. Concentrations on x-axis refer to the respective 4-h EC_{50} nominal concentrations used in the exposures. $ZnSO_4 \times 7H_2O$ was considered 100% bioavailable to the sensor bacteria. Data are the means of three independent experiments ± SD. Figure is modified from Paper **III**.

Differently from nanoZnO, which had similar toxicity to bulk ZnO and zinc ions, nanoCuO was one order of magnitude more toxic than bulk CuO and two orders of magnitude less toxic than $CuSO_4$ (Fig. 3.5, D–F). Higher toxicity of nanoCuO compared to its bulk formulation has been previously shown for algae *Pseudokirchneriella subcapitata* (Aruoja et al., 2009), yeast *S. cerevisiae* (Kasemets et al., 2009) and duckweeds *Landoltia punctata* (Shi et al., 2011). The quantification of dissolved copper in the test medium with the copper-sensor bacteria, after incubation of *T. thermophila* in equitoxic CuO suspensions, showed that nanoCuO (160 mg/l) was remarkably more soluble than bulk CuO (2134 mg/l): after 4-h exposure 2% of nanoCuO and only 0.12% of bulk CuO

was dissolved (i.e., at these nominal equitoxic concentrations nanoCuO was 16-fold more soluble than bulk CuO, Fig. 3.8, A). Strangely, the amount of dissolved copper detected by sensor bacteria in the suspensions of both CuO formulations exceeded the estimated EC_{50} concentrations of copper ions (1.1 mg Cu^{2+}/l, Fig. 3.8, A). The analysis of the dissolved fraction of CuO was designed to be as relevant to the actual toxicity testing conditions as possible, therefore the copper-sensor bacteria assay was performed after incubating *T. thermophila* in CuO suspension and removing the protozoan cells by filtering, before adding copper-sensing bacteria to the test medium. In our following studies it was found that the methodical approach overestimated the solubilization of CuO due to the interference of the protozoan cell residues in the test medium with the luminescence signal of sensor bacteria. Therefore, in our further studies analysis of the solubilization of CuO was done without the protozoan cells, but maintaining all the other test conditions unchanged (Paper **IV**). As a result, the concentration of copper ions in the suspension of nanoCuO was shown to be significantly lower, 0.5 mg/l, than in the equitoxic solution/suspension of $CuSO_4$ and bulk CuO (Fig. 3.8 B, C). At the same time, the difference between the solubility percentages of nanoCuO (0.5%) and bulk CuO (0.05%) was similar to the result of the previous test design (10 times *versus* 16 times). No significant time-dependence in the solubility of CuO was detected, so the test results of the recent study, where 2 and 24-h testing times were used (Paper **IV**) most likely apply also for the previous study of 4-h incubation time (Paper **III**).

Both, PI staining and ATP method yielded similar EC_{50} values (Table 1, Paper **III**), further supporting the reliability of the test results. In Table 3.5 the average EC_{50} values of PI fluorescence and ATP measurements from the acute *T. thermophila* assay and the EC_{50} values from the *T. thermophila* growth inhibition assay are comparatively presented. Based on the EC_{50} values from the acute assay both, bulk and nanosized ZnO as well as $ZnSO_4$ had similar toxic effects on *T. thermophila*, causing 50% loss in viability at around 4–5 mg Zn/l after 4-h exposure and around 7–8 mg Zn/l after 24 h. Comparing the 24-h EC_{50} values from the acute assay and the growth inhibition test showed no significant difference in case of nanoZnO and $ZnSO_4$. Among the copper compounds $CuSO_4$ was found to be the most toxic (4- and 24-h EC_{50} in the acute assay 1.1–1.7 mg Cu/l and 24-h EC_{50} in the growth inhibition assay 0.4 mg Cu/l; Table 3.5), being about 120 times more toxic than nanoCuO and about 1500 times more toxic than bulk CuO in the acute assay. The growth inhibition assay was not suitable for determining the EC_{50} values of nano and bulk CuO as described previously (Paper **II**). After 4-h exposure, nanoCuO was about 13 times more toxic than bulk CuO and after 24 h the difference in toxicities increased even to 18 times. The slightly reduced toxicities of metal compounds, except for nanoCuO, to *T. thermophila* after 24 h compared to 4-h exposure could be explained by the adaptation of the cells to metals (Fig. 3.5, Table 3.5). It has been shown that protozoa can sequester metals by ingesting into the food

vacuoles or accumulating excessive metal ions in cytoplasmic dense granules (Nilsson, 2003). The increase in toxicity of nanoCuO in time instead of decrease observed in case of the rest of the tested compounds could indicate a different toxicity mechanism for nanoCuO compared to bulk CuO and $CuSO_4$ as well as all the zinc compounds tested. To test this hypothesis, further studies were carried out (Paper **IV**).

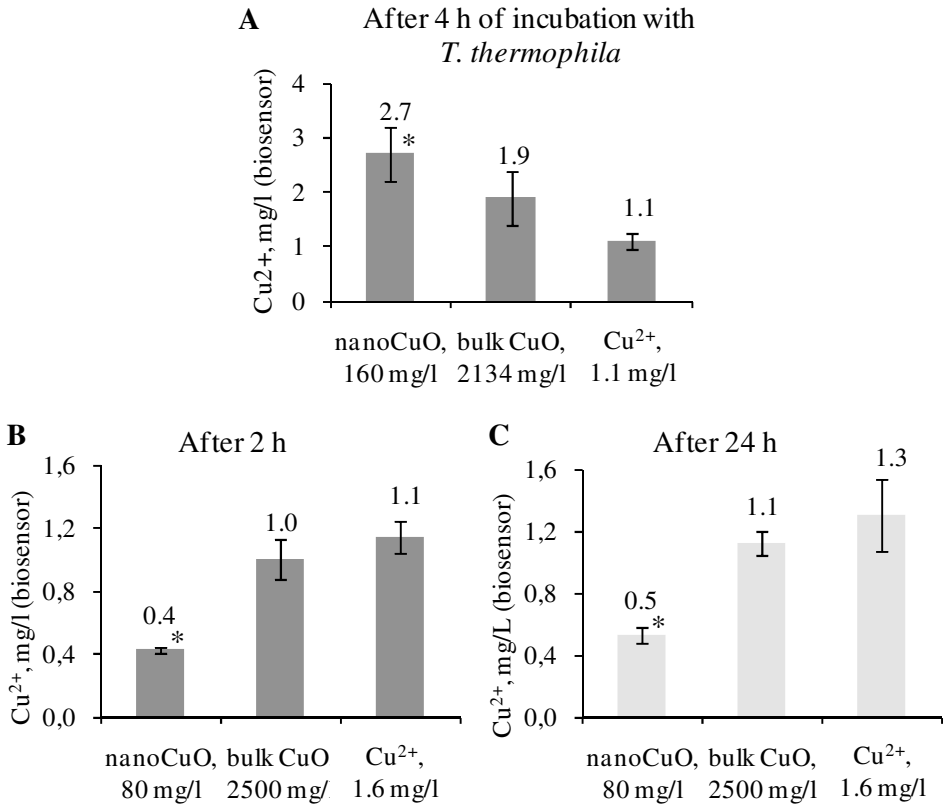

Figure 3.8. Bioavailable copper, mg/l measured with recombinant copper-sensing bacteria in the nominal equitoxic suspensions/solutions of nano and bulk CuO and $CuSO_4$ after incubation with *Tetrahymena thermophila* for 4 h in Osterhout's mineral medium at 25°C (A), and after 2 h (B) and 24 h (B) in Osterhout's mineral medium at 25°C without prior incubation with *T. thermophila*. Concentrations on x-axis refer to the respective 4-h EC_{50} concentrations (A) or to the respective 24-h EC_{50} concentrations (B, C) used in the exposures of *T. thermophila* (see Materials and methods). Data are presented as means of three independent experiments ± SD. *significant difference from Cu^{2+} (A) or from Cu^{2+} and bulk CuO (B, C), $p < 0.05$. Figure is modified from Papers **III** and **IV**.

Table 3.5. EC_{50} values from the *Tetrahymena thermophila* acute toxicity assay (4-h and 24-h exposures, mean values calculated from nominal concentration-effect data based on PI-fluorescence and ATP measurements, Paper **III** and Fig. 3.5) and growth inhibition assay (24-h exposure, Paper **II** and Table 3.4).

		EC_{50} (mean ± SD, mg metal/l)					
Assay	**Exposure time**	**Nano-ZnO**	**Bulk ZnO**	**$ZnSO_4 \times 7H_2O$**	**Nano-CuO**	**Bulk CuO**	**$CuSO_4$**
T. thermophila acute toxicity assay (Paper **III**)	4 h	4.7 ± 0.7[a]	4.4 ± 0.9[a]	4.9 ± 0.6[a]	128 ± 14	1705 ± 568	1.1 ± 0.2[a]
	24 h	7.7 ± 1.1	8.1 ± 1.4	6.9 ± 0.7	116 ± 41	2132 ± 295	1.7 ± 0.3
T. thermophila growth inhibition assay (Paper **II**)	24 h	9.4 ± 3.0	27 ± 1.0[b]	7.1 ± 0.6	ns	ns	0.4 ± 0.1[b]

[a]significantly different from the 24-h EC_{50} values for the respective compound;
[b]significantly different from the EC_{50} values of the acute assay for the respective compound; ns – not shown as no reproducible data was obtained.

3.3. Evaluation of the mechanisms of toxic effects of CuO nanoparticles to protozoa *Tetrahymena thermophila* (Paper IV)

The toxicity mechanism of nanoCuO to *T. thermophila* was studied, focusing on changes in membranes – a barrier between biotic and abiotic compartments. The results of this study are described in detail in Paper **IV** (Appendix I, manuscript). Briefly, changes in fatty acid profile, lipid peroxidation metabolites and reactive oxygen species (ROS) were measured. 24-h exposure to 80 mg/l of nanoCuO (EC_{50}) decreased the proportion of two major unsaturated fatty acids (UFA, C18:3 cis-6, 9, 12 and C18:2 cis-9, 12) and increased the relative amount of two saturated fatty acids (SFA, C18:0 and C16:0) in *T. thermophila*. As the reactive oxygen species generation and fatty acid modifying profiles of nanoCuO and $CuSO_4$ were different, and the content of dissolved copper in nanoCuO suspension was significantly lower than in equitoxic $CuSO_4$ solution (Fig. 3.8, C), it was concluded that the toxic effect of nanoCuO was not solely caused by the dissolved fraction of the NPs. No change in the ratio of UFA and SFA upon exposure of protozoa to nanoCuO was detected at 2-h exposure and no simultaneous dose- or time-dependent lipid peroxidation occurred, thus it is likely that one of the adaptation mechanisms of protozoa to nanoCuO was lowering the membrane fluidity by the inhibition of *de novo* synthesis of fatty acid desaturases.

4. CONCLUSIONS

- The acute kinetic *Vibrio fischeri* bioluminescence inhibition test (Flash Assay) was adapted for high-throughput and cost-efficient toxicity screening of eNPs on 96-well microplates. For that, the toxic effects of selected inorganic and organic eNPs (ZnO, CuO, PAMAM dendrimers G2 and G5 and polyethylenimine) were analyzed. The comparison of the new microplate format with the conventional Flash Assay performed in cuvettes and the conventional Microtox® test (a low-throughput *V. fischeri* luminescence inhibition assay) showed good correlation (Paper **I**).
- The screening data on toxicity obtained with *V. fischeri* using both Flash Assay formats (cuvettes *vs* microplates) after 30-min exposures classified all analyzed chemicals, except nanoCuO (that was more toxic in cuvette format), analogously when compared to the risk phrases of the EC Directive 93/67/EEC for ranking toxicity of chemicals for aquatic organisms (Paper **I**).
- For *Tetrahymena thermophila* alternative acute toxicity tests (measurement of cellular ATP content as a viability endpoint and propidium iodide (PI) - fluorescence as a mortality endpoint) to the commercial *T. thermophila* growth inhibition assay (Protox™ test) were adapted, which were more suitable for screening the toxicity of metal oxide NP suspensions to *T. thermophila* (Paper **III**).
- For the first time the toxicity of ZnO and CuO NPs was determined for the unicellular ciliated protozoa *T. thermophila*. It was shown that bulk and nanosized ZnO as well as Zn^{2+} were of comparable toxicity (4-h EC_{50} values 4.7 and 4.4 mg Zn/l, and 4.9 mg Zn^{2+}/l, respectively). Using recombinant Zn-sensing bacteria we showed that at these low concentrations ZnO was practically dissolved in the test conditions applied. Thus, the toxicity of ZnO NPs to protozoa was caused by the dissolved Zn-ions. CuO NPs were 10–20 times more toxic than bulk CuO (Paper **III**).
- The effect of nanoCuO on membranes of *T. thermophila* was studied. This was the first study on the effects of eNPs on the fatty acid composition of membranes. It was shown that 24-h exposure to 80 mg/l of nanoCuO (EC_{50}) decreased the proportion of two major unsaturated fatty acids (C18:3 cis-6, 9, 12 and C18:2 cis-9, 12) and increased the relative amount of two saturated fatty acids (C18:0 and C16:0) in *T. thermophila*, indicating adaptive lowering of the membrane fluidity. Also, as the reactive oxygen species generation and fatty acid modifying profiles of nanoCuO and $CuSO_4$ were different, and the content of dissolved copper in nanoCuO suspension was significantly lower than in equitoxic $CuSO_4$ solution, it was concluded that the toxic effect of nanoCuO was not solely caused by the dissolved fraction of the NPs, but had a distinctive toxicity pattern (Paper **IV**).

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ABSTRACT

The growing production and application of engineered nanomaterials increases the possibility of accidental release of these materials to the environment. Therefore, the studies on effects of engineered nanoparticles (eNPs) on environmentally relevant organisms are crucial for the hazard evaluation of nanotechnologies. In addition, eNPs are increasingly used in consumer products, e.g. in cosmetics and disinfectants, and have to be evaluated for their adverse biological effects. As toxicity assays on laboratory animals are expensive, time consuming and ethically questionable, new *in vitro* testing strategies involving bacteria and non-vertebrate animals are developed. Using one of the most widely used prokaryotic test organism in ecotoxicology – naturally bioluminescent bacteria *Vibrio fischeri* – we showed that *V. fischeri* kinetic luminescence inhibition test (Flash Assay) can be successfully used also for the toxicological screening of turbid suspensions of eNPs. Both, metal-containing (ZnO and CuO) and organic NPs (polyamidoamine dendrimers G2 and G5 and polyethylenimine) were comparatively tested in cuvette and microplate formats of Flash Assay establishing an excellent correlation ($r = 0.98$). Thus, the Flash Assay is a promising tool for high-throughput, cost-efficient and fast screening of antibacterial effects of various eNPs.

The toxicity of ZnO and CuO NPs was further evaluated on a eukaryotic unicellular model organism *Tetrahymena thermophila*. We showed that ZnO NPs were remarkably more toxic than CuO NPs with respective 4-h EC_{50} values of 5 and 128 mg metal/l. Bulk and nanosized ZnO as well as Zn^{2+} were of comparable toxicity (4-h EC_{50} values 4.7 and 4.4 mg Zn/l, and 4.9 mg Zn^{2+}/l, respectively). Using recombinant Zn-sensing bacteria we showed that at these low concentrations ZnO was practically dissolved in the test conditions applied. Thus, the toxicity of ZnO NPs to protozoa was caused by the dissolved Zn-ions. CuO NPs were 10–20 times more toxic than bulk CuO.

To elucidate the mechanism of toxicity of CuO NPs to *T. thermophila* its effects on the composition of protozoan fatty acids – crucial components of the cell membranes – was studied. Bulk CuO and $CuSO_4$ served as controls for size and solubility and 3,5-dichorophenol (3,5-DCP) as a control for a chemical known to directly affect the membrane. 24-h exposure of protozoa to 80 mg/l of nanoCuO (EC_{50}) significantly decreased the proportion of two major unsaturated fatty acids (UFA, C18:3 cis-6,9,12; C18:2 cis-9,12), while the relative amount of two saturated fatty acids (SFA, C18:0; C16:0) increased. The latter effect was not observed when protozoa were exposed to equitoxic suspensions of bulk CuO, Cu-ions or 3,5-DCP. Exposure to all copper compounds induced the generation of ROS, whereas nanoCuO was most potent. Analysis of dissolved copper in nanoCuO suspensions showed that toxic effect of nanoCuO to *T. thermophila* was not solely caused by the dissolved fraction of CuO NPs, but rather displays a distinctive toxicity pattern reflected also by changes of UFA:SFA ratios which modulate membrane fluidity.

KOKKUVÕTE

Sünteetilisi nanoosakesi toodetakse juba tööstuslikes mahtudes ja kasutatakse paljudes tarbekaupades, kuid andmed nanosuuruses ainete mõjude kohta inimesele ja keskkonnale on puudulikud. Kuna katseloomadega tehtavad toksilisuse testid on kulukad, aeganõudvad ja eetiliselt küsitavad, on tekkinud vajadus uute, *in vitro* testimisstrateegiate rakendamiseks. Kasutades ühte levinumat bakteritesti – *Vibrio fischeri* luminestsentsi inhibeerimise testi – näitasime, et kineetilist Flash-testi on võimalik teostada ka mikroplaadil ja seega kasutada nanoosakeste toksilisuse efektiivseks, kulutõhusaks ja kiireks hindamiseks. Kokku testiti *V. fischeri* Flash-testi küveti- ja mikroplaadiformaadis võrdlevalt anorgaanilisi (ZnO ja CuO) ja orgaanilisi nanoosakesi (polüamidoamiindendrimeerid ja polüetüleenimiin) ning tuvastati väga hea korrelatsioon kahe testformaadi tulemuste vahel.

ZnO ja CuO nanoosakeste toksilisust uuriti ka lihtsaimal eukarüootsel ainuraksel mudelorganismil *Tetrahymena thermophila*. Tulemused näitasid, et nanoZnO osutus oluliselt toksilisemaks kui nanoCuO (EC_{50} väärtused 5 mg *versus* 128 mg metalli/l). Mikro- ja nanosuuruses ZnO ning Zn^{2+} avaldasid *T. thermophila* rakkudele võrdset toksilist toimet (EC_{50} väärtused pärast 4-h ekspositsiooni vastavalt 4,7 ja 4,4 mg Zn/l ja 4,9 mg Zn^{2+}/l). Zn-sensorbakeri abil näitasime, et nimetatud kontsentratsioonide juures oli ligi 100% ZnO testikeskkonnas lahustunud. Seega võib järeldada, et nii mikro- kui ka nanosuuruses ZnO toksilisus algloomale oli tingitud metallioksiidi lahustunud ioonidest. CuO nanoosakesed olid *T. thermophila*le 10–20 korda toksilisemad kui mikrosuuruses CuO.

Nanosuuruses CuO toimemehhanismi selgitamiseks uuriti ka CuO nanoosakeste võimalikku mõju *T. thermophila* rakumembraanide rasvhappelisele koostisele. Mikrosuuruses CuO kasutati osakese suuruse ja $CuSO_4$ ioonse kontrollina ning 3,5-diklorofenooli (3,5-DCP) otseselt membraane mõjutava kontrollkemikaalina. *T. thermophila* 24-h eksponeerimine 80 mg/l nanoCuO-le (EC_{50}) vähendas oluliselt kahe peamise küllastamata rasvhappe (C18:3 cis-6,9,12; C18:2 cis-9,12) ja suurendas kahe küllastatud rasvhappe (C18:0; C16:0) suhtelist osakaalu rakkudes. Sarnast efekti ei täheldatud, kui algloома rakke eksponeeriti kontrollkemikaalidele (mikrosuuruses CuO, $CuSO_4$ ja 3,5-DCP) samaväärset toksilisust põhjustavate kontsentratsioonide juures. Kõik vaseühendid indutseerisid *T. thermophila* rakkudes ülemäärast reaktiivsete hapnikuühendite (ROS) teket, kusjuures kõige kõrgemat ROS taset põhjustas nanoCuO. NanoCuO suspensioonis määratud vaseioonide hulk näitas, et nanoCuO toksiline toime *T. thermophila*le ei ole põhjustatud ainult lahustunud vaseioonidest, vaid toimib erineva toksilisuse mehhanismi teel, mida tõestas ka CuO nanoosakeste mõju rakkude rasvhappelisele koostisele.

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High throughput kinetic *Vibrio fischeri* bioluminescence inhibition assay for study of toxic effects of nanoparticles

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Abstract

Despite of the growing production and use of nanoparticles (NPs) in various applications, current regulations, including EC new chemical policy REACH, fail to address the environmental, health, and safety risks posed by NPs. This paper shows that kinetic *Vibrio fischeri* luminescence inhibition test – Flash Assay – that up to now was mainly used for toxicity analysis of solid and colored environmental samples (e.g. sediments, soil suspensions), is a powerful tool for screening the toxic properties of NPs. To demonstrate that Flash Assay (initially designed for a tube luminometer) can also be adapted to a microplate format for high throughput toxicity screening of NPs, altogether 11 chemicals were comparatively analyzed. The studied chemicals included bulk and nanosized CuO and ZnO, polyethylenimine (PEI) and polyamidoamine dendrimer generations 2 and 5 (PAMAM G2 and G5). The results showed that EC_{50} values of 30-min Flash Assay in tube and microplate formats were practically similar and correlated very well ($\log - \log R^2 = 0.98$), classifying all analyzed chemicals, except nano CuO (that was more toxic in cuvette format), analogously when compared to the risk phrases of the EC Directive 93/67/EEC for ranking toxicity of chemicals for aquatic organisms. The 30-min EC_{50} values of nanoscale organic cationic polymers (PEI and dendrimers) ranged from 215 to 775 mg/l. Thirty-minute EC_{50} values of metal oxides varied largely, ranging from ~4 mg/l (bulk and nano ZnO) to ~100 mg/l (nano CuO) and ~4000 mg/l (bulk CuO). Thus, considering an excellent correlation between both formats, 96-well microplate Flash Assay can be successfully used for high throughput evaluation of harmful properties of chemicals (including organic and inorganic NPs) to bacteria.

Keywords: Flash Assay; Microplate luminometer; *Vibrio fischeri*; Nanoparticles; Heavy metals; PEI; PAMAM dendrimers

1. Introduction

During the recent decade nanotechnology and production of nanoparticles (NPs) has developed rapidly. Regardless of deficient data on their potential hazard (Nel et al., 2006), NPs are increasingly included in various consumer products. For example, ZnO NPs are added to modern sunscreens, because they reflect/scatter ultraviolet light more efficiently than larger particles, and are considered safe to humans (Nohynek et al., 2007). ZnO is also increasingly used in antibacterial applications as bacteriostatic activity of ZnO powders towards both Gram-negative and Gram-positive bacteria has been shown to increase with decreasing particle size (100–800 nm; Yamamoto, 2001).

In addition to metal containing NPs, organic NPs are gaining interest. For instance, nanoscale cationic polymers polyethylenimine (PEI) and polyamidoamine (PAMAM) dendrimers are attractive for different biomedical applications such as drug delivery, gene transfection and bioimaging (Godbey et al., 1999; Svenson and Tomalia, 2005). Both PEI and PAMAM dendrimers have been shown to

Abbreviations: NPs, nanoparticles; PEI, polyethylenimine; PAMAM G5 and G2, polyamidoamine generations 5 and 2, respectively; 3,5-DCP, 3,5-dichlorophenol; MeOH, methanol; EC_{50}, mean effective concentration.

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cause toxic effects in animal cell lines by forming nanoscale holes in cellular membrane and enhancing its permeability (Hong et al., 2006). Permeabilizing effects of PEI and PAMAM dendrimers have also been shown in Gram-negative bacteria *Escherichia coli, Salmonella typhimurium* and *Pseudomonas aeruginosa* (Helander et al., 1997; Calabretta et al., 2007).

Considering the increasing application of nanomaterials, e.g. in consumer products, there is an urgent need for information on safety and potential hazards of manufactured NPs (Nel et al., 2006). Toxicity assays on laboratory animals are expensive, time consuming and ethically questionable. The application of EU new chemical policy REACH (EC, 2006) is prognosed to demand additional 2.6 million vertebrate animals (Coecke et al., 2006). Currently, the European legislation, including REACH, does not address the risks caused by NPs, but this issue is actively debated (Franco et al., 2007). In order to reduce animal testing to a minimum, new testing strategies (involving toxicity assays performed, e.g. on bacteria and non-vertebrate animals) are needed. One of the widely used tests in ecotoxicology, but also quite well predicting the toxicity of chemicals to other *in vitro* systems (Kahru, 2006), is the *Vibrio fischeri* luminescence inhibition assay. The bioluminescence of the Gram-negative marine bacterium *V. fischeri* is a result of a complex chain of biochemical reactions, where reduced flavin mononucleotide ($FMNH_2$), a long-chain fatty acid aldehyde and luciferase are the key players. This special pathway uses NADH as a cofactor and is intrinsically linked to the central metabolism of the microorganism (Hastings et al., 1987). Thus, the reduction of light output is a reflection of inhibition in bacterial metabolic activity and proportional to the toxicity of test sample (Bulich, 1982).

Several different luminescence inhibition tests of *V. fischeri* have been developed so far – most of them are designed for analysis of aqueous samples (Microtox®, BioTox™, LUMIStox™, ToxAlert™), while one of the test protocols – Flash Assay, can successfully be used for analysis of suspensions, turbid and colored samples: in this kinetic assay each sample acts as a reference for itself (Lappalainen et al., 1999). In our laboratory, the Flash Assay has formerly been used for toxicity testing of suspensions of contaminated soils, sediments and solid wastes (Põllumaa et al., 2000; Heinlaan et al., 2007). Considering, that suspensions of NPs are often turbid due to insolubility and/or aggregation of particles, we assumed that differently from conventional *V. fischeri* luminescence inhibition test Microtox®, Flash Assay format could be appropriate for screening the toxicity of NPs. In our previous studies on toxicity of metal oxide NPs (Heinlaan et al., 2008) a tube luminometer was used for Flash Assay. The aim of the current study was to demonstrate that Flash Assay can also be performed in microplate format and used as high throughput, cost-efficient and fast method for screening the toxicity (antibacterial properties) of various NP suspensions to *V. fischeri*. Eleven chemicals with different properties, including two types of nanoparticles (metal-containing and organic ones), were comparatively tested in cuvette and microplate formats of Flash Assay and also in conventional Microtox® assay (a "gold standard" of photobacterial luminescence inhibition assay). To our knowledge, this is the first study on using the microplate format of *V. fischeri* Flash Assay for toxicity screening of NPs.

2. Materials and methods

2.1. Chemicals and samples

Altogether 11 chemicals (all of analytical grade) were studied for toxicity. 3,5-Dichlorophenol (3,5-DCP, Riedel-de Haen) and $K_2Cr_2O_7$ (Sigma–Aldrich) were used as general controls for test bacteria performance. $ZnSO_4 \cdot 7H_2O$ and $CuSO_4$ (both from Alfa Aesar) served as ionic controls for ZnO and CuO. Nanosized ZnO (advertised particle size 50–70 nm) and CuO (30 nm) were purchased from Sigma–Aldrich and analyzed in parallel with bulk ZnO (Fluka) and CuO (Alfa Aesar). PEI (branched, average M_w 25,000) and PAMAM generation 2 (G2) and 5 (G5) dendrimers (advertised M_w 3256 and 28,825, respectively) were purchased from Sigma–Aldrich. PAMAM G2 and G5 dendrimers were in methanol solution (20% w/w and 5% w/w, respectively). The stock solutions of tested chemicals were prepared in deionized water (MilliQ, Millipore); 2% w/v NaCl served as a control and diluent solution for all samples except for PAMAM dendrimers. For dendrimers 2% NaCl + 2.3% methanol (MeOH) served as control and diluent. Methanol (2.3%) is not toxic to *V. fischeri* (Kahru et al., 1996).

2.2. Luminometers

Two luminometers were used for Flash Assay. BioOrbit 1251 tube luminometer (Turku, Finland) allows dispensing of bacterial reagent, continuous mixing of the sample and simultaneous measuring of the luminescence. Altogether 25 test tubes, that are subsequently measured, can be loaded into this luminometer. Four millilitre polypropylene cuvettes were used. For microplate assay format, the Fluoroskan Ascent FL plate luminometer (ThermoLabsystems, Helsinki, Finland), which allows simultaneous dispensing of bacterial reagent and measuring of the luminescence, but does not have the mixing function while the luminescence is recorded, was used. The testing was performed in 96-well white polypropylene microplates. The conventional *V. fischeri* luminescence inhibition assay – the Microtox® test, was performed using 1253 luminometer (ThermoLabsystems, Helsinki, Finland).

2.3. Toxicity testing

The freeze-dried photobacteria *V. fischeri* (strain NRRL-B-11177, Aboatox, Turku, Finland) were rehydrated and stabilized first at 4 °C for 30 min and then at

20 °C for another 30 min. The testing was performed at 20 °C. For Flash Assay in cuvettes, 200 µl of sample was transferred into each cuvette and, inside the luminometer, 200 µl of bacterial suspension was automatically dispensed onto the sample. The luminescence was measured during 30 s under continuous mixing. After 30 min of incubation, the luminescence was measured again for 30 s under continuous mixing. In case of the Flash Assay in microplates 100 µl of test solution was pipetted into each well, which was supplemented with 100 µl of bacterial suspension by automatic dispensing in the luminometer testing chamber. The luminescence was recorded during the first 30 s after the dispensing of the bacteria in each well without additional mixing of the sample during the measurement. After 30 min-incubation the light output was recorded again. BioOrbit 1251 luminometer was controlled by MultiUse 2.03 software and Fluoroskan Ascent FL plate luminometer by Ascent Software Version 2.4.1.

The Flash Assay in cuvette and microplate format was performed simultaneously using the same batch of rehydrated test bacteria. All the chemicals were tested at least on two different days, each time in two parallels. The chemicals were screened for the background values of luminescence without the bacteria and the measurement results for all of the compounds showed no difference from the 2% NaCl solution used as a control.

For comparison, conventional luminescence inhibition assay (Microtox® test) as "gold standard" was performed with all compounds except for turbid suspensions of metal oxides. The testing was done using the 30-min exposure time essentially as described by Kahru (1993) with one exception: incubation/testing temperature was 20 °C.

2.4. Data analysis

The inhibitory effect of chemicals (INH%) on bacterial luminescence was calculated as described in Lappalainen et al. (2001). Thirty-second and 30-min EC_{50} values (the concentration of chemical which reduces the light output of bacteria by 50% after contact time of 30 s or 30 min, respectively) were determined from concentration *versus* INH% curves by means of standard least-squares statistics. EC_{50} values obtained in different Flash Assay formats were compared using a two-tailed unpaired *t*-test.

3. Results and discussion

To test the performance of the bacteria, the toxicity of two reference compounds (3,5-DCP, $K_2Cr_2O_7$) and two ionic controls ($CuSO_4$, $ZnSO_4 \cdot 7H_2O$) was comparatively measured in plate and cuvette format Flash Assay as well as in Microtox® assay (Table 1): toxicity data obtained for above mentioned chemicals were comparable with previously published data (Kaiser and Devillers, 1994; Lappalainen et al., 2001).

3.1. Comparative testing of ZnO and CuO bulk and nano formulations

The Flash Assay performed in microplate format showed that during the first 30 s of exposure of *V. fischeri* to all Cu and Zn compounds no inhibition of luminescence was observed (Fig. 1). However, after 30 min of exposure, a clear dose–effect pattern was established. The increase of toxicity of heavy metal-containing compounds with

Table 1
EC_{50} values of tested substances in *Vibrio fischeri* Flash Assay performed in cuvette and microplate format and Microtox® test

Test substance	30 s EC_{50} (mg/l)			30 min EC_{50} (mg/l)				
	Cuvettes	Microplates	Ratio[a]	Cuvettes	Microplates	Ratio[b]	Microtox®	Classification[c]
References								
3,5-Dichlorophenol	6.2 ± 0.5	5.0 ± 0.2	1.24	3.1 ± 0.1	2.4 ± 0.05	1.29	2.0 ± 0.04	Toxic
Cr^{6+} (tested as $K_2Cr_2O_7$)	26.5 ± 1.9	66.2 ± 11	0.40	3.6 ± 0.49	6.0 ± 0.2	0.60	4.1 ± 0.02	Toxic
Ionic controls								
Zn^{2+} (tested as $ZnSO_4 \cdot 7H_2O$)	nd	nd	–	3.8 ± 1.7	3.2 ± 1.0	1.19	3.5 ± 0.07	Toxic
Cu^{2+} (tested as $CuSO_4$)	nd	nd	–	0.8 ± 0.1	0.8 ± 0.3	1.00	0.3 ± 0.002	Very toxic
Metal oxides								
BulkZnO	nd	nd	–	4.3 ± 1.7	3.9 ± 1.8	1.10	nd	Toxic
NanoZnO	nd	nd	–	4.8 ± 1.1	3.8 ± 0.7	1.26	nd	Toxic
BulkCuO	nd	nd	–	3894 ± 1871	4208 ± 2471	0.93	nd	nc
NanoCuO	nd	nd	–	68.1 ± 4.3*	204 ± 42*	0.33	nd	nc
Nanoscale cationic polymers								
Polyethylenimine	225 ± 162	216 ± 117	1.04	215 ± 139	238 ± 123	0.90	67.4 ± 1.1	nc
Polyamidoamine G2	603 ± 187	559 ± 394	1.08	424 ± 162	631 ± 415	0.67	nd	nc
Polyamidoamine G5	561 ± 303	686 ± 342	0.82	539 ± 235	775 ± 374	0.70	179 ± 7.9	nc

EC_{50} numbers are the average values ± SD (standard deviation) of two testing on different days both performed in two parallels.

[a] Ratio of 30-s EC_{50} values in cuvettes and microplates.

[b] Ratio of 30-min EC_{50} values in cuvettes and microplates.

[c] Classification based on 30-min EC_{50} data obtained in Flash Assay microplate format according to the risk phrases for ranking toxicity of chemicals for aquatic organisms (EC Directive 93/67/EEC): $EC_{50} < 1$ mg/l: very toxic; 1–10 mg/l: toxic; 10–100 mg/l: harmful. EC_{50} values > 100 mg/l are designated as nc – not classified.

nd – not determined, no toxic effect or low inhibition (%) at the maximum concentration tested.

* Significant difference ($p < 0.05$).

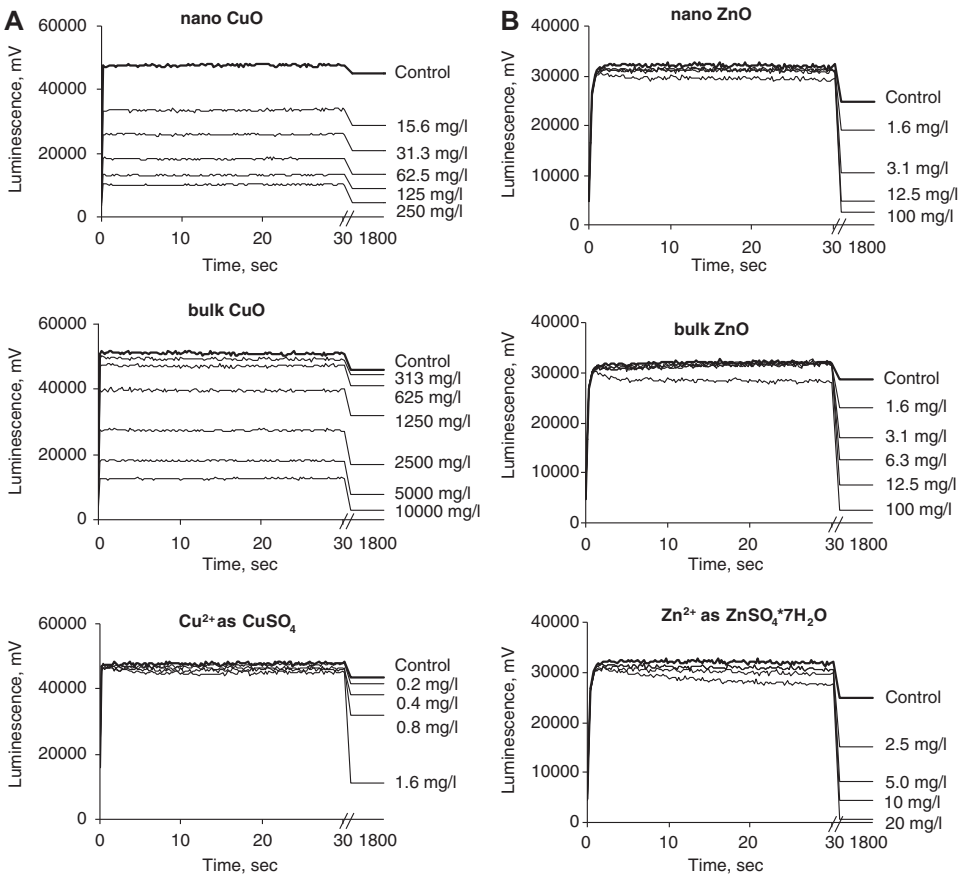

Fig. 1. Kinetics of luminescence of *Vibrio fischeri* exposed to suspensions of nano and bulk CuO and $CuSO_4$ (A), and nano and bulk ZnO and $ZnSO_4 \cdot 7H_2O$ (B) measured in microplate format of Flash Assay. NaCl (2%) served as a control and diluent. Concentrations of nano and bulk metal oxides are nominal.

prolonged exposure time is coherent with previous toxicity data of heavy metals for photobacteria (Kaiser and Devillers, 1994; Lappalainen et al., 2001). Due to the significant turbidity of both tested CuO formulations, the luminescence values of test solutions were different at time zero, and depended on concentration (Fig. 1A). However, as mentioned above, the interference of particle-related turbidity with the luminescence signal does not present a problem in the Flash Assay as each sample acts as a reference to itself (Lappalainen et al., 1999).

In both Flash Assay formats toxicity of nano CuO was remarkably higher than toxicity of bulk CuO (20-fold in microplate and 60-fold in cuvette format). In microplates, the 30-min EC_{50} for bulk CuO was 4208 mg/l and for nano CuO 204 mg/l. The respective toxicities obtained in tube luminometer were 3894 mg/l and 68 mg/l. The toxicity data for Cu oxides tested in tube luminometer (Table 1) were very close to our previous data (Heinlaan et al., 2008), where *V. fischeri* Flash Assay in cuvettes was one of the tests of the ecotoxicological test battery for evaluation of toxicity of various metal oxide (nano)particles, and where instead of freeze-dried *V. fischeri* preparation, bacteria preserved at −80 °C in 10% glycerol were used.

In the current study, differently from Cu oxides, bulk and nano ZnO showed considerably higher toxicity (30-min EC_{50} values in microplate Flash Assay being approximately 3.9 mg/l and in tube format ~4.5 mg/l), whereas there was no difference between bulk and nano formulations. These EC_{50} values are ~2.5-fold higher than in Heinlaan et al. (2008) and could be explained by different *V. fischeri* preparations used (see above).

3.2. Comparative testing of organic nanoparticles

The 30-s kinetic curves of microplate format Flash Assay in Fig. 2 show that differently from metal compounds (Fig. 1), PEI and both PAMAM dendrimers were toxic to *V. fischeri* already during the first seconds

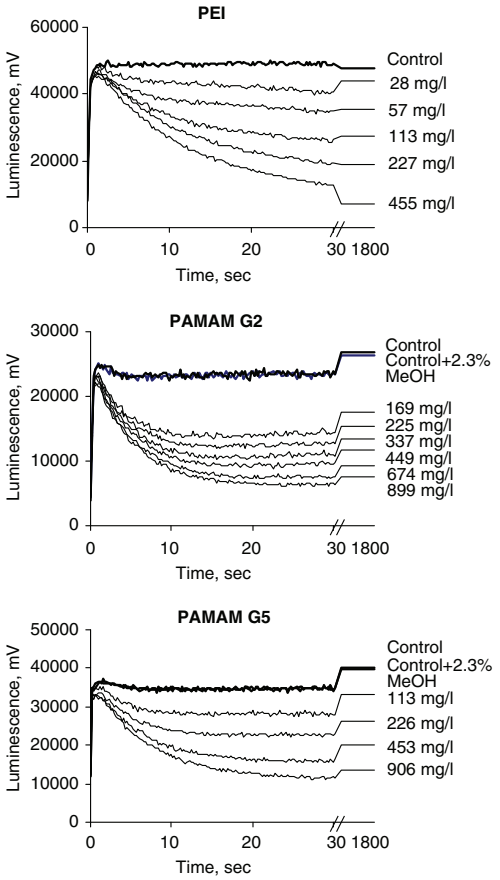

Fig. 2. Kinetics of luminescence of *Vibrio fischeri* exposed to suspensions of PEI, PAMAM G2 and G5 measured in microplate format of Flash Assay. NaCl (2%) served as a control and diluent for PEI and 2% NaCl + 2.3% methanol served as a control and diluent for PAMAM dendrimers.

of exposure, indicating that these compounds are fast-acting toxicants to bacteria. Thus, no significant difference in the 30-s and 30-min EC_{50} values was observed. In microplate format of Flash Assay, the 30-min EC_{50} value for PEI was 238 mg/l, for PAMAM G2 631 mg/l and PAMAM G5 775 mg/l. In tube format of Flash Assay the toxicities were practically similar to these obtained in microplate test (Table 1). Notably, the conventional Microtox® test yielded ~3-fold higher toxicities compared to Flash Assay (Table 1), which could be explained by the differences in the test protocols.

The antibacterial properties of PAMAM G5 dendrimers have been studied by Calabretta et al. (2007) by using a standard colony count assay: after 2 h exposure time, the EC_{50} value for Gram-negative bacteria was 1.5 mg/l and for Gram-positive bacteria 20.8 mg/l. The approximately 500-fold higher EC_{50} value obtained in our study (Table 1) could be explained by the shorter exposure time (30 min) and the different endpoint used.

3.3. Comparison of the data obtained in different test formats

Table 1 shows that for chemicals comparatively tested in Flash Assay (both, microplate and tube format) and Microtox®, there was no significant difference in toxicity results, with the exception of PEI and PAMAM G5 dendrimer that were more toxic in conventional Microtox® assay.

As suspended solids and particles tend to settle, the continuous mixing of the sample (which is not possible in the microplate format of Flash Assay) during testing might influence the test results. Indeed, nano CuO (most turbid sample tested; Fig. 1A) was ~3-fold less toxic in microplate format (Table 1). However, for other compounds tested the ratios of the EC_{50} values obtained in cuvette and microplate formats of the Flash Assay were close to one and correlated very well ($\log - \log R^2 = 0.98$; Fig. 3). The standard deviation values were quite remarkable for parti-

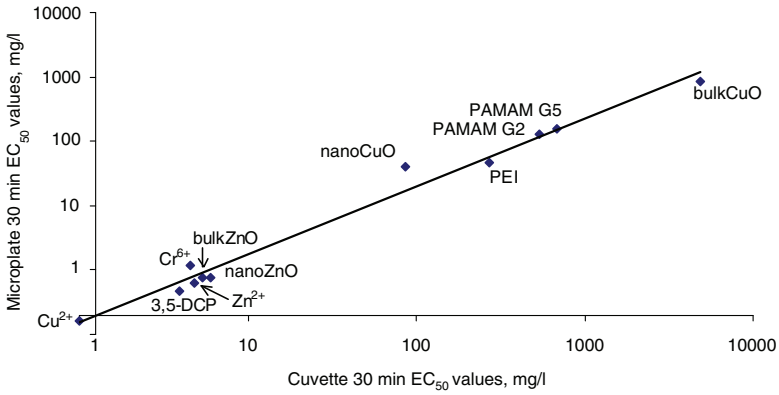

Fig. 3. 30-min EC_{50} values obtained in *Vibrio fischeri* Flash Assay: cuvette *versus* microplate format. Equation for the log–log regression line: $y = 1.05x + 0.007$ and log–log R^2 value 0.98.

cle-containing samples, but nevertheless comparable for both Flash Assay formats used (Table 1).

The data on toxicity obtained with both Flash Assay formats after 30-min exposures classified all analyzed chemicals, except nano CuO (that was more toxic in cuvette format), analogously when compared to the risk phrases of the EC Directive 93/67/EEC for ranking toxicity of chemicals for aquatic organisms (Table 1). Thus, considering an excellent correlation between both formats, 96-well microplate format of Flash Assay can be successfully used for high throughput evaluation of harmful properties of chemicals (including organic and inorganic NPs) to bacteria.

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Ecotoxicity of nanoparticles of CuO and ZnO in natural water

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Natural waters remarkably reduced the toxicity of nanoCuO but not nanoZnO.

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ABSTRACT

The acute toxicity of CuO and ZnO nanoparticles in artificial freshwater (AFW) and in natural waters to crustaceans *Daphnia magna* and *Thamnocephalus platyurus* and protozoan *Tetrahymena thermophila* was compared. The L(E)C_{50} values of nanoCuO for both crustaceans in natural water ranged from 90 to 224 mg Cu/l and were about 10-fold lower than L(E)C_{50} values of bulk CuO. In all test media, the L(E)C_{50} values for both bulk and nanoZnO (1.1–16 mg Zn/l) were considerably lower than those of nanoCuO. The natural waters remarkably (up to 140-fold) decreased the toxicity of nanoCuO (but not that of nanoZnO) to crustaceans depending mainly on the concentration of dissolved organic carbon (DOC). The toxicity of both nanoCuO and nanoZnO was mostly due to the solubilised ions as determined by specific metal-sensing bacteria.

1. Introduction

The last decade is distinguished by the drastic growth of production and use of manufactured nanoparticles (NPs). NPs of metal oxides such as ZnO and TiO_2 are already widely used in personal care products (e.g., sunscreens), coatings and paints; CuO is used in gas sensors, photovoltaic cells, in catalyst applications and in heat transfer nanofluids. Subsequently, the risk of natural water contamination by synthetic NPs continuously increases (Klaine et al., 2008).

It should be stressed that natural NPs, including nano-sized particles of metal oxides, exist in all ecosystems and play important role in biogeochemical processes (Wigginton et al., 2007). During the evolution living organisms have adapted to the presence of natural NPs in the environment. For synthetic NPs, however, it is recognized that their potential harmful properties on ecosystems have to be evaluated (Handy et al., 2008; Nowack, 2009). Despite the rapidly increasing amount of nanotoxicological peer-reviewed papers (Medina et al., 2007) data on ecotoxicity of synthetic NPs (Baun et al., 2008; Handy et al., 2008) and especially on metal oxide NPs, except nanoTiO_2, are rare (Kahru et al., 2008). As water is an essential compartment in ecosystems and natural vehicle for pollutant migration, the data on fate and behavior of synthetic NPs in different types of natural waters as well as their potential ecotoxic effects are essential for evaluation of the environmental risks of nanotechnologies (Nowack and Bucheli, 2007).

The main goal of evaluation of ecotoxicological properties of chemicals is to prevent the hazard to the ecosystems via establishing respective environmental standards, which guarantee the absence of negative effects of those compounds on living organisms. At the same time, it is widely accepted and also shown in our earlier works (Aruoja et al., 2004; Blinova, 2004; Kahru et al., 2005) that due to the environmentally non-relevant conditions used in regulatory testing, most of the standardized bioassays do not appropriately characterize the potential impacts of hazardous substances on the environment, in particular, on water ecosystems (Allen and Hansen, 1996; Hyung et al., 2007; Lewis, 1995). Most of the ecotoxicity data on chemicals available for standard freshwater test organisms such as crustaceans, algae and fish have been generated using so-called artificial freshwater (AFW), which composition differs from natural waters. However, as bioavailability and toxic effect of a chemical depend on its speciation and hence, on water composition (Witters, 1998), the hydrochemical parameters of water used as test medium are very important.

Till now, the impact of the composition of natural water on fate and biological effects of chemicals in the aquatic ecosystems has not been adequately explored. For example, in spite of the intensive investigation of the effects of natural water composition on bioavailability of heavy metals during the last decades leading even to the elaboration of several models, which are used for the prediction of metal toxicity

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in natural water (Jager et al., 2006; Kim et al., 1999; Long et al., 2004; McGeer et al., 2002; Niyogi and Wood, 2004; Pagenkopf, 1983, etc.), understanding of the behavior and biological effects of trace metals in natural waters is still limited (Borgmann, 2000; Handy et al., 2008; Town and Filella, 2002; Van Leeuwen et al., 2005). For synthetic NPs, it is known that the type and amount of natural organic matter in the water affects their stability and bioavailability (Giasuddin et al., 2007; Hyung et al., 2007; Wigginton et al., 2007), and may strongly influence their behavior in surface waters (Klaine et al., 2008; Lead and Wilkinson, 2006). However, the effect of organic ligands as well as of other hydrochemical parameters (pH, hardness, ionic strength, etc.) on the bioavailability of NPs to aquatic organisms is still inadequately investigated. The latter decreases the practical value of ecotoxicity test results obtained with AFW (Handy et al., 2008; Nanotechnology, 2006; Velzeboer et al., 2008).

In order to be relevant for the use in the risk assessment and establishment of environmental quality standards, ecotoxicity tests should give information on a chemical's bioavailability and toxicity in the given environment (McGeer et al., 2002; Van Assche et al., 2002). Additional knowledge required to extrapolate laboratory test results to field populations may be received by extending the standard protocols of ecotoxicological testing (Jager et al., 2006), for example, by replacing the AFW with a natural one.

The aim of the present study was to compare the acute toxicity of CuO and ZnO NPs towards particle-ingesting aquatic species (two crustaceans and one protozoan) in AFW and in six natural waters with different hydrochemical characteristics. Bulk CuO and ZnO and the respective soluble salts ($CuSO_4$ and $ZnSO_4 \cdot 7H_2O$) were used as controls for size-dependent and solubility effects. Bioavailability of Cu and Zn as well as the solubilisation of metal oxides in natural waters was studied by recombinant sensor bacteria.

2. Materials and methods

2.1. Natural water

Natural water samples were taken during November–December 2007 from six Estonian rivers with different hydrochemical characteristics. Sampling places were chosen according to the data of the national monitoring program. The chemical analysis of water samples (Table 1) was performed in a certified laboratory using the following standard analytical methods: EN 1899-1:1998 for biochemical oxygen demand (BOD_7), ISO 9963-1:1994 for alkalinity, ISO 11905-1:1997 for total nitrogen (N_{tot}), ISO 6878:1998 for total phosphorus (P_{tot}), ISO 8245:1999 for dissolved organic carbon (DOC), ISO 17294-2:2003 for Zn and Cu, ISO 10304-1:1992 for sulphate, ISO 10304-1:1992 for chloride, ISO 6058:1984 for calcium, SFS 3032 (1976) for ammonium. Before the biotesting, suspended solids and plankton were separated from the water samples by filtration through a 0.45 μm pore size standard filter (Millipore).

Table 1
Characterization of natural waters used as test media.

Parameter	Unit	River 1	River 2	River 3	River 4	River 5	River 6
pH		8.2	8.2	7.9	8.1	7.5	8.1
DOC[a]	mg C/l	13.3	13.2	25.9	29.2	34.5	31.5
BOD_7[b]	mg O_2/l	1.1	1.5	1.5	1.3	1.1	1.9
N_{tot}[c]	mg/l	7.6	3.2	9	10.1	4.7	5.2
P_{tot}[d]	mg/l	0.039	0.045	0.037	0.028	0.052	0.015
Alkalinity	meq/l	4.4	5.6	4.1	3.9	3.8	2.7
Ca^{2+}	mg/l	122	124	106	111	82.0	58.0
Cl^-	mg/l	15.4	17	4.6	13.5	9.2	7.7
SO_4^{2-}	mg/l	96.1	69.1	55.6	76.1	20.9	14.9
Zn_{tot}[e]	μg/l	2.6	2.6	1.4	2.2	1.5	3.1
Cu_{tot}[f]	μg/l	2.0	3.0	4.6	21.3	2.0	11
NH_4^-	mg N/l	0.06	0.15	0.11	0.09	0.53	0.19

[a] DOC – dissolved organic carbon.
[b] BOD_7 – biochemical oxygen demand.
[c] N_{tot} – total nitrogen.
[d] P_{tot} – total phosphorus.
[e] Zn_{tot} – total zinc.
[f] Cu_{tot} – total copper.

2.2. Chemicals

NanoCuO (advertised particle size 30 nm), nanoZnO (advertised particle size 70 nm) and $ZnSO_4 \cdot 7H_2O$ were purchased from Sigma–Aldrich, the bulk form of ZnO from Fluka, the bulk CuO and $CuSO_4$ from Alfa Aesar. The stock solutions of metal salts and suspensions of metal oxides were prepared in MilliQ water. The suspensions of metal oxides (40 g/l) were sonicated for 30 min and stored in the dark at +4 °C.

2.3. Electron microscopy imaging

The aqueous suspensions of the studied metal oxides (both nano- and bulk formulations) have been previously characterized by scanning electron microscopy (SEM): despite of agglomeration, individual nanoscale particles were present in nanoZnO and nanoCuO suspensions (Kahru et al., 2008). For the current study, size distribution of CuO NPs (40 mg CuO/l) was analysed by transmission electron microscopy in *Daphnia magna* test medium (AFW) using a JEOL 1230 TEM at 120 kV. For the characterization of NPs after ingestion by *D. magna*, organisms were exposed to nanoCuO (4 mg/l) and live crustaceans were fixed for TEM observations using a JEOL 1011 TEM at 100 kV. TEM photographs were taken from the gut after thin-sectioning. Individual particles were sized (based on 200 measurements) from TEM photographs using the freeware ImageJ (NIH, USA).

2.4. Aquatic bioassays

The crustacean *D. magna* acute immobilisation assay (Daphtoxkit F™) adhering to OECD 202 guidelines, crustacean *Thamnocephalus platyurus* acute mortality test (Thamnotoxkit F™) and ciliate protozoan *Tetrahymena thermophila* growth inhibition test (Protoxkit F™) were used. The Toxkits were purchased from MicroBioTests, Inc. (Nazareth, Belgium). In both crustacean assays viable and dead organisms were counted under dissection microscope after 24 h (*T. platyurus*) or 48 h (*D. magna*) of exposure. For protozoan growth inhibition test, the investigated compound and *T. thermophila* culture were added to the food substrate suspension in test medium. While normal proliferating protozoan culture clears the substrate suspension in 24 h, inhibition of the growth of protozoa is reflected by residual turbidity of the food substrate measured by optical density (OD) of the tests samples at 440 nm. All the tests were performed in the dark at constant temperature (20 °C for *D. magna*, 25 °C for *T. platyurus* and 30 °C for *T. thermophila*) according to the respective guidelines of the Toxkits.

The test organisms were exposed to different concentrations of CuO and ZnO (both, nano- and bulk forms), $CuSO_4$ and $ZnSO_4 \cdot 7H_2O$. The filtered river waters (Table 1) were used as basic test medium in the tests, i.e. for the dilution of studied compounds and as a control. Evaluation of the toxicity was performed in two steps: i) determination of 0–100% tolerance range of the test species to the respective compound and ii) determination of the 50% effect values $L(E)C_{50}$. The toxicity was evaluated from 2 to 3 independent experiments, each in several replicates (four for *D. magna*, three for *T. platyurus* and two for *T. thermophila*).

The AFW (test medium used in the standard test procedure) for crustaceans has following composition (mg/l): for *D. magna* – $CaCl_2 \cdot 2H_2O$ – 294, $MgSO_4 \cdot 7H_2O$ – 123.25, $NaHCO_3$ – 64.75, KCl – 5.75, pH −7.8 ± 0.2 and for *T. platyurus* – $CaSO_4 \cdot 2H_2O$ – 60, $MgSO_4 \cdot 7H_2O$ – 123, $NaHCO_3$ – 96, KCl – 4 mg/l; pH – 7.8 ± 0.2 dissolved in MilliQ water, i.e. AFW does not contain organic compounds. MilliQ water was used as standard test medium for *T. thermophila*.

2.5. Bacterial metal-specific biosensors

In parallel to the aquatic biotests, dissolved bioavailable Zn^{2+} and Cu^{2+} in the solutions/suspensions of tested compounds were quantified using recombinant bioluminescent Zn-sensor bacteria *Escherichia coli* MC1061(pSLzntR/pDNPzntAlux) and Cu-sensor bacteria *E. coli* MC1061(pSLcueR/pDNPcopAlux), respectively (Ivask et al., 2009). Bioluminescence of those sensor bacteria increases proportionally with the concentration of bioavailable Cu^{2+} (Cu-sensor) or Zn^{2+} (Zn-sensor) in the test medium (Ivask et al., 2002). A constitutively luminescent control strain *E. coli* MC1061(pDNlux) (Leedjärv et al., 2006) not induced by heavy metals, but otherwise similar to sensor strains, was used to take into account the potential quenching of bacterial bioluminescence by the turbid suspensions of metal oxides. 100 μl of the suspension of Zn- or Cu-sensor bacteria or the constitutively luminescent control bacteria in 9 g/l of NaCl supplemented with 1 g/l of cas-aminoacids (acid hydrolysate of casein, LabM) and 0.9 g/l of glucose was mixed with 100 μl of the solution or suspension of studied metal compound diluted either in MilliQ, AFW for *D. magna* or *T. platyurus* or in the natural river waters and incubated for 2 h at 30 °C as described previously (Heinlaan et al., 2008). The amount of bioavailable Cu and Zn was quantified assuming that $CuSO_4$ and $ZnSO_4 \cdot 7H_2O$ were 100% bioavailable to the sensor bacteria, when compounds were diluted in MilliQ. Detection limits of this method were 2 μg Cu^{2+}/l and 20 μg Zn^{2+}/l.

2.6. Statistical analysis

One-way analysis of variance (ANOVA) followed by *t*-tests was used to determine statistical significance of the differences between toxic effects of the compounds in different test media. The differences were considered significant, when $p < 0.05$.

3. Results and discussion

3.1. Chemistry of natural water samples

The main water quality parameters, which may affect bioavailability of studied compounds, are presented in Table 1. As considerable part of Estonian rivers and lakes, similarly to typical boreal ones, contain relatively high concentration of dissolved organic matter (DOM), this study was mainly focused on the effect of DOM on bioavailability and toxicity of CuO and ZnO NPs. Table 1 shows that the content of DOM (expressed as dissolved organic carbon, DOC) varied to a great extent: from 13.2 to 34.5 mg C/l. Also, the level of nutrients (phosphorous and nitrogen) in the water samples was relatively high. The values of biochemical oxygen demand (BOD_7), however, were comparable to unpolluted rivers. The pH in the water samples was slightly above neutral, varying between 7.5 and 8.2, and remained within the same range during the toxicity testing. The background values of total copper and zinc in river waters (Table 1) were negligible compared to respective $L(E)C_{50}$ values of $CuSO_4$ and $ZnSO_4 \cdot 7H_2O$ (Table 2). Also, the bacterial Cu- and Zn-sensors did not detect bioavailable Cu and Zn in any of the natural water samples.

3.2. Bioavailability of Zn and Cu to recombinant sensor bacteria

At the low concentrations used for the toxicity testing (up to 10 mg/l) ZnO (bulk and nano) was almost fully dissolved and bioavailable for bacterial biosensors in all test media (Fig. 1). Differently from both ZnO formulations, nanoCuO and especially bulk CuO were of remarkably lower solubility: only about 12% of copper from nanoCuO (the tested concentration range was 0.006–20 mg/l) and about 0.3% of copper from bulk CuO suspensions (the tested concentration range was 0.2–2000 mg/l) proved bioavailable to sensor bacteria in AFW (Fig. 1). Thus, in AFW the solubility of nanoCuO exceeded the solubility of bulk CuO about 40-fold. Analogously, the toxicity of nanoCuO in AFW was about 50-fold (*D. magna*) and 45-fold (*T. platyurus*) higher than the toxicity of bulk CuO (Table 2) proving again that the toxicity of copper oxides for crustaceans are due to solubilised Cu-ions as also previously shown for bacteria (Heinlaan et al., 2008) and algae *Pseudokirchneriella subcapitata* (Aruoja et al., 2009). In comparison with AFW, the natural waters reduced the bioavailability of all studied Cu-compounds to recombinant sensor bacteria by 89–99% (calculated from data presented in Fig. 1), whereas the effect was most remarkable in case of nanoCuO. Analogous remarkable reduction in bioavailability was not observed for Zn compounds (Fig. 1).

3.3. Toxicity of copper compounds in natural waters to crustaceans

The effect of the natural water composition (especially DOM) on toxicity of the studied copper compounds towards *D. magna* and *T. platyurus* was analysed by comparison of the toxicity results in river water (this work) and AFW (Heinlaan et al., 2008). In case of both crustaceans there was a significant decrease in the toxicity of both forms of CuO as well as $CuSO_4$ in natural water samples as compared with the AFW (Table 2) and the decrease was most remarkable for nanoCuO. Indeed, the *D. magna* EC_{50} values for nanoCuO, bulk CuO and $CuSO_4$ increased by 50–140, 6.5–13 and 3–13-fold, and *T. platyurus* EC_{50} values by 55–130, 7.3–21 and 10–100-fold, respectively, depending on river water used for testing (Table 2). Thus, the data obtained by biotests (Table 2) as well as recombinant sensor bacteria (Fig. 1) both showed that natural waters have higher mitigation effect for nanoCuO than bulk CuO.

It is well known that natural organic matter (mainly humic and fulvic acids) is strongly complexing copper and reducing the bioavailable copper ion concentrations (Allen and Hansen, 1996). The relationship between dissolved organic matter in test media and bioavailability of copper to aquatic organisms has been shown previously by many investigators (De Schamphelaere et al., 2004a; Kim et al., 1999; Kramer et al., 2004; Oikari et al., 1992; Hyne et al., 2005; Santos et al., 2008). The data of the current study (Fig. 2A) are coherent with data previously reported by Kramer et al. (2004) and De Schamphelaere et al. (2004a). Statistical analysis of the data on $CuSO_4$ toxicity for *D. magna* in river waters (Tables 1 and 2) revealed significant differences between rivers with relatively low (rivers 1, 2) and high DOC levels (rivers 4, 5 and 6). Also, the differences between *D. magna* EC_{50} values for nanoCuO obtained for rivers 1 and 2 (lower DOC content) and rivers 5 and 6 (high DOC content) were statistically significant ($p < 0.05$). A good correlation

Table 2
Toxicity of nanoCuO and nanoZnO, their bulk forms and respective soluble salts to aquatic organisms.

Test media	NanoCuO	Bulk CuO	$CuSO_4$	NanoZnO	Bulk ZnO	$ZnSO_4 \cdot 7H_2O$
Crustacean *Daphnia magna*, 48 h EC_{50} (mean ± SD, mg metal/l)						
AFW[a]	2.6 ± 1.3	132 ± 19.7	0.07 ± 0.01	2.6 ± 1.04	7.1 ± 1.1	1.4 ± 0.24
River 1	92.7 ± 12.4	844 ± 14.4	0.24 ± 0.03	3.3 ± 1.15	9.5 ± 1.8	1.8 ± 0.32
River 2	149 ± 16.6	799 ± 19.2	0.38 ± 0.04	9.0 ± 0.28	12.0 ± 2.1	2.0 ± 0.19
River 3	160 ± 28.4	>1500	0.58 ± 0.18	1.7 ± 0.27	6.9 ± 0.55	1.6 ± 0.14
River 4	200 ± 17.5	1566 ± 34.0	0.81 ± 0.06	3.5 ± 0.30	>5.0	2.5 ± 0.18
River 5	224 ± 15.9	1737 ± 0	0.84 ± 0.03	2.8 ± 0.39	16.2 ± 2.3	1.4 ± 0.18
River 6	>200	>1500	0.92 ± 0.04	3.4 ± 1.56	10.8 ± 1.4	1.7 ± 0.13
Crustacean *Thamnocephalus platyurus*, 24 h LC_{50} (mean ± SD, mg metal/l)						
AFW[a]	1.7 ± 0.4	75.6 ± 4.5	0.04 ± 0.02	0.14 ± 0.02	0.19 ± 0.03	0.22 ± 0.06
River 1	152 ± 22.7	593 ± 94.0	0.41 ± 0.06	1.1 ± 0.23	>4	0.92 ± 0.11
River 2	217. ± 19.3	874 ± 25.2	0.60 ± 0.06	6.0 ± 0.71	1.9 ± 0.46	1.6 ± 0.2
River 3	92.7 ± 8.8	1054 ± 42.8	2.1 ± 0.28	3.6 ± 0.74	3.0 ± 0.37	0.61 ± 0.26
River 4	112 ± 0	1518 ± 49.9	2.1 ± 0.32	1.5 ± 0.32	<0.5	0.75 ± 0.06
River 5	129 ± 23.7	1550 ± 42.6	1.1 ± 0.05	5.3 ± 0.50	2.1 ± 1.9	1.1 ± 0.40
River 6	90.3 ± 12.5	546 ± 108.7	4.6 ± 0.18	1.4 ± 0.35	1.2 ± 0.16	1.7 ± 0.88
Protozoa *Tetrahymena thermophila*, 24 h EC_{50} (mean ± SD, mg metal/l)						
AFW	nd[b]	nd	0.40 ± 0.12	9.4 ± 3.0	27.1 ± 1.0	7.1 ± 0.66
River 2	nd	nd	0.46 ± 0.02	16.4 ± 0.41	16.6 ± 0.91	21.1 ± 1.37
River 4	nd	nd	0.27 ± 0.03	12.4 ± 0.75	12.0 ± 0.45	18.6 ± 0.59
River 5	nd	nd	<0.2	26.5 ± 2.52	14.5 ± 1.09	>22.0

[a] $L(E)C_{50}$ values in artificial freshwater from Heinlaan et al. (2008).
[b] nd – not determined.

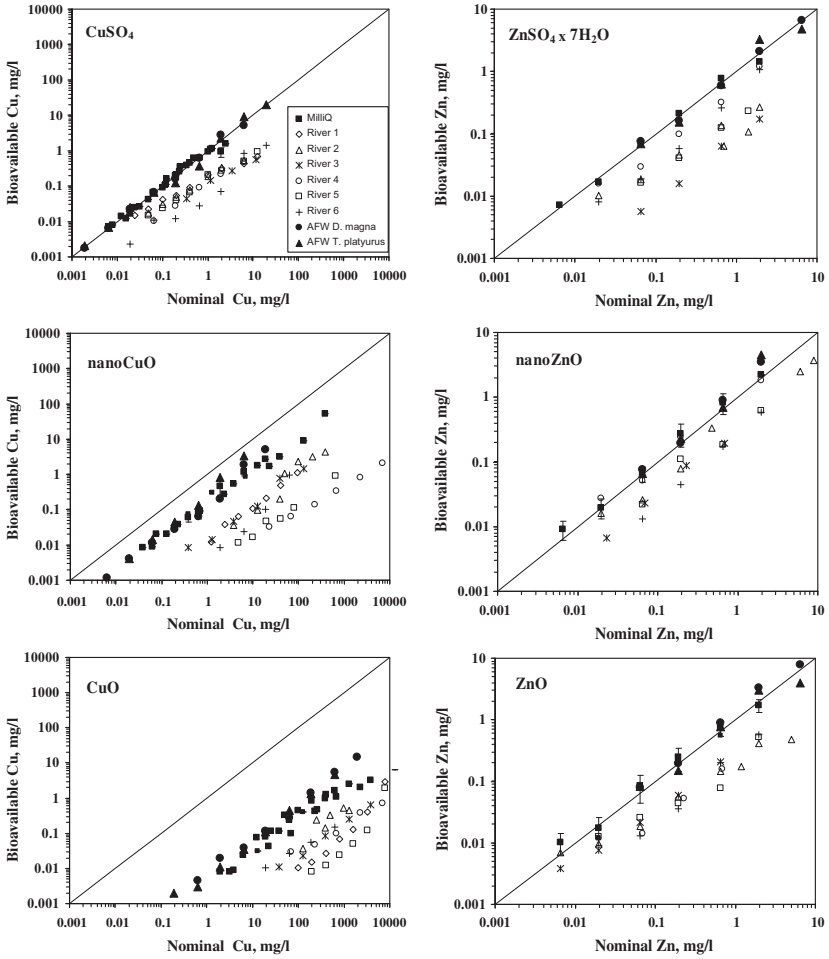

Fig. 1. Nominal (*x*-axis) *versus* bioavailable Cu and Zn ions (*y*-axis) in six natural waters and artificial freshwater (AFW). Dissolved bioavailable metals were quantified by recombinant *Escherichia coli* Cu-sensor (left panel) and Zn-sensor (right panel).

($r^2 = 0.81$) between the toxicity of nanoCuO to *D. magna* and DOC in natural waters used as testing medium (Fig. 2B) is in agreement with the assumption that toxicity of CuO NPs was mostly caused by copper ions.

In all natural waters at EC_{50} values of $CuSO_4$ for *D. magna* the concentration of copper bioavailable to sensor bacteria was 0.07 ± 0.03 mg Cu/l, corresponding to the EC_{50} value of $CuSO_4$ in AFW (Table 2). However, not all copper available to sensor bacteria from

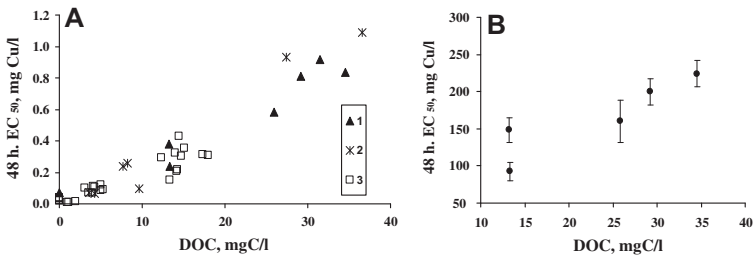

Fig. 2. Effect of dissolved organic carbon (DOC) concentration in natural waters on toxicity of copper salts (A) and nanoCuO (B) to *Daphnia magna*. (A): 1 – this study; 2 – De Schamphelaere et al. (2004a); 3 – Kramer et al. (2004). The natural waters were spiked with different copper salts (1 – $CuSO_4$; 2 – $CuCl_2$; 3 – $CuCl_2 \cdot 2H_2O$).

bulk and nanoCuO proved bioavailable to crustacean: the concentrations of bioavailable copper ions in bacterial assay exceeded significantly the measured EC_{50} in tests with *D. magna* (data not shown). Several factors may be involved in this apparent discrepancy. For example, assays were done in optimal respective temperature conditions, i.e. sensor assay at 30 °C and crustacean tests at 20° or 25 °C. It is also possible that some of the copper complexes formed with DOM might be bioavailable to the bacteria but not for crustaceans (Apte et al., 2005). In addition, the ingestion and excretion of CuO by crustaceans during the test may modulate the bioavailable fraction of CuO. Indeed, the copper oxide accumulation in the gut (one of the most crucial exposure routes for a particle-feeding organism) of both crustaceans was clearly visible under the microscope as black gut content (*T. platyurus* as an example; Fig. 3). Interestingly, the gut of daphnids exposed either to sub-toxic concentrations of CuO (i.e. no mortality observed) or toxic concentrations (>50% mortality) were filled with visible CuO. This gives additional evidence that dissolved CuO (copper ions) and not the gut-accumulated particles are main contributors to the toxicity of nano and bulk CuO.

TEM analysis performed in AFW as well as in the gut of *D. magna* clearly showed that differently from bulk CuO (data not shown), no clear agglomerates of nanoCuO were observed in the gut lumen (Fig. 4). Average nanoCuO particle size in the gut of *D. magna* – 31 ± 12.8 nm remains similar with that in AFW – 30 ± 10 nm (Fig. 4).

The test results of *T. platyurus* for $CuSO_4$ in natural water showed (Table 2) analogous trends as in the test with *D. magna* with the exception of river 5 (discussed below). However, in contrast to *D. magna*, dependence between toxicity of nanoCuO towards crustacean *T. platyurus* and DOC concentrations in test water was not revealed (the differences between EC_{50} values for all rivers except for river 2 were not statistically significant). It should be noted that though both *D. magna* and *T. platyurus* belong to planktonic crustaceans, their sensitivity towards the same substances may differ (Blinova, 2004). For example, *T. platyurus* was much more sensitive than *D. magna* towards ammonium ions (I. Blinova, unpublished data). Also, Koivisto (1995) and Shaw et al. (2006) have shown that *D. magna* is more tolerant to copper and zinc than other cladocerans. A greater ability of *D. magna* to adapt to toxic stress may be linked to the fact that differently from other cladocerans *D. magna* has adapted to withstand wider fluctuations in environmental conditions, e.g., hypoxia, turbidity, ionic strength (Koivisto et al., 1992). Furthermore, the body size (surface area/weigh ratio) is also important, since one of the uptake routes of soluble metals by crustaceans is directly from water through the body surface (Krantzberg and Stokes, 1988; Robinson et al., 2003). In case of NPs, the toxic effect may also depend on the mechanical adhesion of NPs on the organism's surface, which in turn is related to the body size and the structure of exoskeleton. Indeed, adhesion of aggregates of NPs to the exoskeleton of crustaceans has been noted by Baun et al. (2008) and also observed (under the microscope) in our experiments. As a result, the combination of abovementioned factors leads to different probability of survival of *D. magna* and *T. platyurus* in the same conditions. This can explain the lack of relationship between DOC concentration and toxicity of nanoCuO to *T. platyurus* and comparatively high toxicity of $CuSO_4$ in river 5 (high ammonium concentration).

3.4. Toxicity of zinc compounds in natural waters to crustaceans

Differently from Cu compounds, the change in toxicity of Zn compounds in natural waters as compared with AFW was less remarkable (Table 2). The *D. magna* EC_{50} for nanoZnO, bulk ZnO and $ZnSO_4$ increased only by 1–1.75, 1–2.3 and 0.7–3.6-fold, respectively and in most cases the changes were not statistically significant. These results are in accordance with the data obtained with Zn-sensor bacteria showing only small differences in bioavailable Zn-ion concentrations in AFW and river waters (Fig. 1). Thus, it can be concluded that the toxicity of nano and bulk ZnO in natural waters was due to the solubilised Zn-ions.

The other crustacean (*T. platyurus*) was in general more than 10 times more sensitive than *D. magna* towards Zn compounds in AFW but the difference between L(E)50 values was not so remarkable in natural waters (Table 2). To explain the reasons for higher mitigating effect of natural waters for *T. platyurus* than for *D. magna* further studies are needed.

It is obvious that natural waters used in the current study remarkably affected the bioavailability of copper but not zinc. Also, there was no clear relationship between DOC and toxicity of Zn compounds (Tables 1 and 2). The results published by other authors also indicate that in modulation of the toxicity of zinc ions the DOM is not so important, as, for example, calcium (Clifford and McGeer, 2009; De Schamphelaere et al., 2004b; Hyne et al., 2005). However, we have not revealed any relationship between toxicity of Zn compounds and Ca, probably due to the masking effect by other water components.

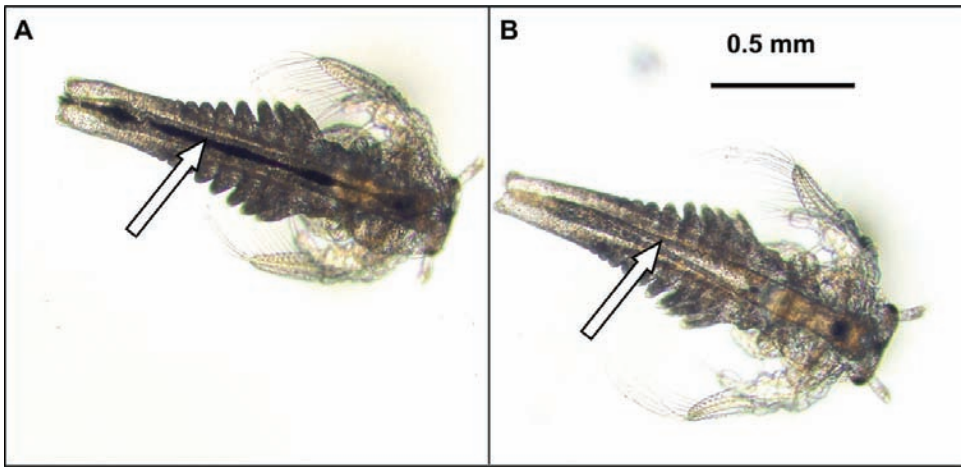

Fig. 3. Crustacean *Thamnocephalus platyurus* under the light microscope. Arrows mark the presence (A: exposed organism) or absence (B: control) of black CuO nanoparticles in the gut.

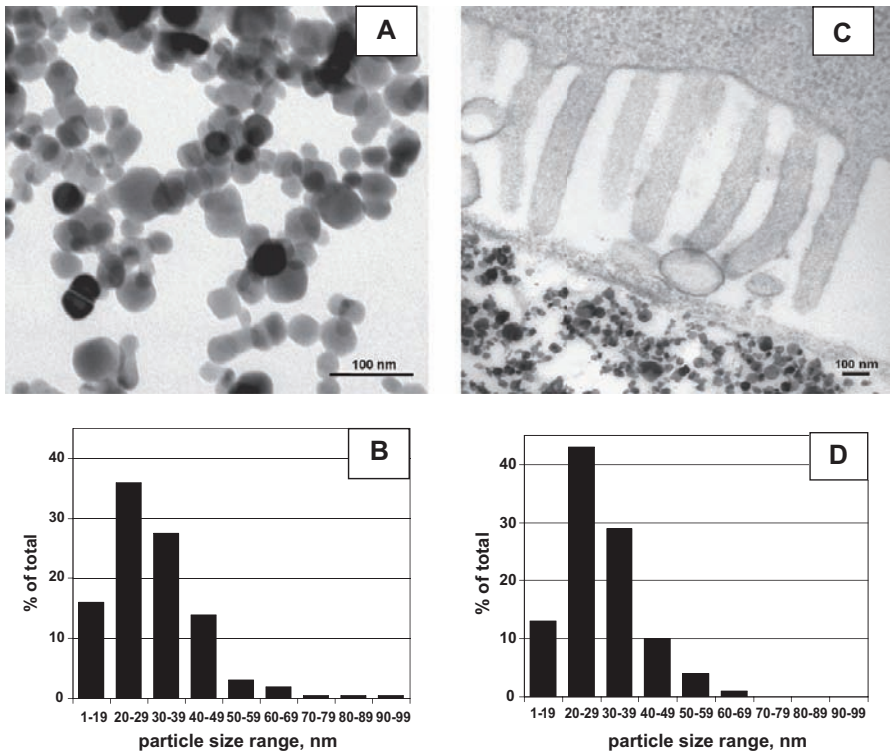

Fig. 4. Nanoparticles of CuO in artificial freshwater (A: TEM; B: particle size distribution) and in the gut of *Daphnia magna* (C: TEM; D: particle size distribution).

3.5. Toxicity of Zn and Cu compounds to T. thermophila

Toxicity of nano- and bulk ZnO formulations, $ZnSO_4 \cdot 7H_2O$ and $CuSO_4$ to *T. thermophila* in standard test conditions and three river waters was compared (Table 2). Toxicity of $ZnSO_4 \cdot 7H_2O$ decreased in natural waters ($p < 0.05$), but for nanoZnO the statistically significant decrease was observed only in the water of river 5. In case of bulk ZnO, however, the toxicity in river waters even increased ($p < 0.05$).

Differently from crustaceans, toxicity of $CuSO_4$ to protozoa in river waters 2 and 4 was not statistically different from EC_{50} values in standard test conditions (Table 2). It could be assumed that binding of copper ions by food particles (added to test medium at the beginning of the assay) was masking the mitigating effect of organic matter in river waters.

In case of both CuO formulations, their black color was interfering with OD measurements impairing the reliability of EC_{50} determination. Therefore, no EC_{50} values for copper oxides are presented in Table 2. However, visual microscopic observations of the protozoa after exposure to the sub-toxic concentrations of nanoCuO showed clear accumulation of the CuO NPs in their food vacuoles. The latter is important for investigation of the fate of nanoCuO in the aquatic ecosystems.

4. Conclusions

This paper shows that ecotoxicological tests are indispensable tools for hazard evaluation of synthetic NPs as they integrate both the harmful and mitigating effects and show the net influence of the tested compounds in the given experimental conditions. The use of natural water as test medium in ecotoxicological assays can increase the predictive power of these tests for the environmental risk assessment.

This study showed the remarkable potential of natural water to mitigate the toxic effects of CuO NPs but not ZnO NPs. In addition, as in standard test conditions, the toxic effect of CuO and ZnO NPs in natural waters was mainly due to dissolved metal ions. Thus, to understand the mechanisms of ecotoxicological action of metal oxides NPs and its ecological consequences, solubility and speciation are the crucial aspects contrarily, for example, to manufactured carbon NPs, where size and aggregation seem to be the key factors.

Lastly, this study confirms that biology and physiology of the target organisms may be of primary importance in transfer of synthetic NPs along the food-web and environmental compartments and, thus, also important in risk assessment. For example, interaction and adhesion of NPs on the surface of living organisms and accumulation in particle-ingesting organisms should be taken into account, when behavior and transfer of NPs in the water ecosystems is investigated.

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Toxicity of ZnO and CuO nanoparticles to ciliated protozoa *Tetrahymena thermophila*

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Toxicity

ABSTRACT

The toxic effects of nanoparticles (NPs) of ZnO and CuO to particle-ingesting model organism protozoa *Tetrahymena thermophila* were evaluated. Nano-ZnO was remarkably more toxic than nano-CuO (EC_{50} values ~5 mg metal/l *versus* 128 mg metal/l). Toxic effect of CuO depended on particle size: nano-CuO was about 10–20 times more toxic than bulk CuO. However, when calculated on basis of bioavailable copper (quantified using recombinant Cu-sensor bacteria) the 4-h EC_{50} values of nano- and bulk formulations were comparable (2.7 and 1.9 mg bioavailable Cu/l, respectively), and statistically different from the EC_{50} value of Cu^{2+} (1.1 mg/l). Differently from CuO particles, bulk and nanosized ZnO as well as Zn^{2+} were of similar toxicity (4-h EC_{50} values 3.7 and 3.9 mg bioavailable Zn/l, respectively, and 4.9 mg Zn^{2+}/l). Thus, the toxic effect of both, CuO and ZnO (nano)particles to protozoa was caused by their solubilised fraction. The toxic effects of the copper compounds were not dependent on exposure time (4 and 24 h), whereas the toxicity of zinc compounds was about 1.5 times lower after 24 h of exposure than after 4 h, probably due to adaptation. In summary, we recommend *T. thermophila* as a simple eukaryotic particle-ingesting model organism for the toxicity screening of NPs. For the high throughput testing we suggest to use the 4-h assay on microplates using ATP and/or propidium iodide for the evaluation of cell viability.

1. Introduction

Metal oxide-based nanoparticles (NPs) are increasingly used in applications such as fillers, opacifiers, catalysts, semiconductors, cosmetics, and microelectronics (Nel et al., 2006; Reijnders, 2006). ZnO NPs are included in personal care products—toothpaste, beauty products and sunscreens (Serpone et al., 2007), as well as in textiles (Becheri et al., 2008). Nano-CuO has potential wide industrial use in applications such as gas sensors and catalytic processes (Carnes and Klabunde, 2003; Dutta et al., 2003).

Although some NPs are already produced in industrial amounts, and thus, may pose hazard to humans and environment, ecotoxicity data for NPs are just emerging (see review by Kahru et al., 2008). Concerning ZnO NPs, there are ecotoxicity data available for bacteria (Adams et al., 2006; Heinlaan et al., 2008; Huang et al., 2008; Mortimer et al., 2008), algae (Franklin et al., 2007; Aruoja et al., 2009), crustaceans (Heinlaan et al., 2008) and nematodes (Wang et al., 2009). In some studies the bacteria have been studied comparatively with eukaryotic cell lines (Reddy et al., 2007; Nair et al., 2008). The effects of ZnO nano- and bulk formulations have also been studied on yeast *Saccharomyces cerevisiae* (Kasemets et al., 2009). Differently from nano-ZnO, the toxicity data regarding nanosized CuO are rare and restricted mostly to ecotoxicological effects towards bacteria and crustaceans (Heinlaan et al., 2008), and algae (Aruoja et al., 2009).

The ciliated protozoa *Tetrahymena* sp. has been used in toxicology for decades as a useful model organism for cellular and molecular biologists as well as for environmental research (Sauvant et al., 1999; Gutiérrez et al., 2003). It is also an advantageous eukaryotic model system for mechanistic studies, as it contains many genes conserved in several eukaryotes (including humans), differently from other widely used unicellular model organisms. For example, more than 800 human genes have orthologs in *Tetrahymena thermophila* but not in *S. cerevisiae*, and 58 of these genes are associated with human diseases (Eisen et al., 2006). Lastly, as protists have highly developed systems for internalisation of nanoscale (100 nm or less) and microscale (100–100,000 nm) particles (Frankel, 2000), they are very good model organisms for nanotoxicology (Holbrook et al., 2008; Kahru et al., 2008).

Both, zinc and copper are essential trace elements for the living organisms, but in high concentrations can produce cellular damage (Goyer and Clarkson, 2001). The influence of copper and zinc on *Tetrahymena* has been formerly studied using several endpoints of physiological response: mortality, cell proliferation, rate of endocytosis, cell membrane integrity, grazing capacity, metabolic activity, lysosomal function (Nicolau et al., 1999; Dias and Lima,

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2002; Nilsson, 2003; Dayeh et al., 2005a,b; Gallego et al., 2007). In general, it has been shown that copper is more toxic than zinc for protozoa (Gallego et al., 2007), although in some studies the opposite effect has been demonstrated (Nicolau et al., 1999, 2004). Rico et al. (2009) have shown that there exists remarkable variability in biological responses to heavy metals among different protozoa species, which is further increased by the diversity of experimental conditions used (Gallego et al., 2007). Indeed, it is known that the toxic effects of metals on protozoa are influenced by cell density and factors that modify speciation and bioavailability of metals (pH, content of organic matter, etc.) (Gutiérrez et al., 2003). In addition, metal solubility and speciation may be changed also by organisms; for instance, in case of bacteria it has been shown that initially insoluble forms of heavy metals in soil–water suspensions may become bioavailable due to the direct contact between bacteria and soil particles (Kahru et al., 2005). Variation of the test results may also be related to different toxicity endpoints and assay protocols. One of the assays commonly used for measuring the viability of *Tetrahymena* involves various fluorescent indicator dyes, for instance, neutral red, alamar blue, 5-carboxyfluorescein diacetate acetoxymethyl ester, propidium iodide (Dayeh et al., 2005a,b; Gallego et al., 2007). In addition, quantification of adenosine-5′-triphosphate (ATP) has been used as a marker for general energetic state of protozoa after exposure to toxicants, including zinc and copper (Nicolau et al., 2004).

The aim of the present study was to evaluate the toxicity of two types of metal oxide NPs (ZnO and CuO) to ciliated protozoa *T. thermophila*. It has been shown previously that in case of unicellular organisms (algae, bacteria, yeasts), which *a priori* do not internalise particles, the toxicity of above mentioned NPs was mostly explained by solubilised metal ions and thus correlated with the toxicity of Cu^{2+} and Zn^{2+} to those organisms (Heinlaan et al., 2008; Aruoja et al., 2009; Kasemets et al., 2009). *Tetrahymena*, however, is a particle-ingesting organism and thus, the toxicity mechanism of metal oxide NPs may be different. In addition, it is important to investigate the effects of NPs on protozoa as they are crucial members of the aquatic food chain as well as an important part of activated sludge biological consortium involved in the treatment of wastewaters. Indeed, sooner or later wastewater treatment plants face NPs as many of them (nano-ZnO, nano-TiO_2 and nano-silver) are already produced in high tonnages and used in various consumer products.

Dose–response effects of ZnO and CuO nanoparticles to protozoa were evaluated at two different exposure times (4 and 24 h). To study the effect of particle size, respective bulk formulations were studied in parallel. $ZnSO_4$ and $CuSO_4$ served as ionic controls for evaluating the toxic effect of solubilised metals. Two different endpoints were used to evaluate the toxic effects of nanoparticles: (i) propidium iodide (PI) staining that gives information on cells with disrupted membranes and (ii) amount of cellular adenosine-5′-triphosphate (ATP) that reflects the number of viable cells. PI is a DNA intercalating dye, which is generally excluded from viable cells, thus the fluorescence detected is proportional to the number of membrane-damaged or dead cells (Dayeh et al., 2004). ATP is the major energy currency molecule of the cell that can be formed either in photosynthesis, fermentation or aerobic respiration, depending on organism, and consumed by many enzymes and cellular processes including biosynthetic reactions, motility and cell division (Prescott et al., 1999). ATP is ubiquitously distributed in any biological material and can be easily extracted from the cells and assayed (Lundin and Thore, 1975). To quantify the bioavailable fraction of metals released from the metal oxide particles during the test, the recombinant metal-sensing bacteria were used. To our knowledge, this is the first study on effects of metal oxide NPs to ciliated protozoa *T. thermophila*.

2. Materials and methods

2.1. Cell culture

T. thermophila (strain BIII) was grown axenically in modified SSP medium (Gorovsky et al., 1975) 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). To prepare the cultures for toxicity testing, 1 ml of the stock culture was transferred to 9 ml of sterile modified SSP medium and grown for 24 h. 10 ml of the 24-h culture was transferred to 40 ml of sterile medium in a 250 ml Erlenmeyer flask and further cultivated for 18–24 h. The cultures were grown on an orbital shaker at 100 rpm, 30 °C. During the exponential growth phase (at the cell density of 5×10^5 cells/ml) the cells were harvested by centrifugation at $300 \times g$ for 5 min 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, pH 6.6; Osterhout, 1906; Society of Protozoologists, 1958). Cell density was determined by counting the cells in haemocytometer (Neubauer Improved, bright line; Germany). To allow the counting, cells were first immobilised in 5% formalin. For toxicity analysis, the density of cells in Osterhout's medium was adjusted to 10^6 cells/ml (twice the final cell density used in the testing).

2.2. Nanoparticles, reference compounds and exposure of T. thermophila

Nano-ZnO (advertised particle size 50–70 nm) and nano-CuO (30 nm) were purchased from Sigma–Aldrich and analysed in parallel with bulk ZnO (Fluka) and CuO (Alfa Aesar). $ZnSO_4 \times 7H_2O$ and $CuSO_4$ (both from Alfa Aesar) served as ionic controls for ZnO and CuO, respectively. The stock suspensions/solutions of the tested chemicals were prepared in deionised water (MilliQ, Millipore). The stock suspensions of metal oxides (40 g/l) were sonicated for 30 min, stored in the dark at +4 °C and used for testing within 2 months. Stock solutions of $ZnSO_4 \times 7H_2O$ and $CuSO_4$ were prepared analogously, but were not sonicated. Before toxicity testing, stocks were vigorously vortexed. The aqueous suspensions of the studied metal oxides (both nano- and bulk formulations) have been previously characterized by scanning electron microscopy (SEM): despite of agglomeration, individual nanoscale particles were present in nano-ZnO and nano-CuO suspensions (Kahru et al., 2008). Osterhout's medium was used throughout the experiments as a diluent and a control. The following nominal concentrations (chosen according to pre-screening results) were used for the toxicity testing: 0.31, 0.62, 1.24, 2.48, 4.97 mg Cu^{2+}/l of $CuSO_4$; 31.25, 62.5, 125, 250, 500 mg/l of nano-CuO; 500, 1000, 2000, 4000, 8000 mg/l of bulk CuO; 2.84, 5.69, 11.37, 22.75, 45.49 mg Zn^{2+}/l of $ZnSO_4 \times 7H_2O$; 1.85, 5.55, 8.33, 12.5, 25 mg/l of nano- and bulk ZnO. The toxicity testing was conducted as follows: 500 μl of the toxicant in Osterhout's medium was pipetted into the wells of 24-well polystyrene culture plates (Falcon), each concentration in two replicates, and 500 μl of *T. thermophila* cells in Osterhout's medium (10^6 cells/ml) was added to the wells (final cell density in the test medium was 5×10^5 cells/ml). Osterhout's medium served as a control. In addition, a cell-free control was made, where 500 μl of Osterhout's medium was added to 500 μl of toxicant suspension/solution. The test plates with protozoa were incubated for 4 and 24 h at 25 °C in the dark, without shaking. The pH of *T. thermophila* control culture in Osterhout's medium was 6.5. The pH of nano- and bulk ZnO solutions containing *T. thermophila* increased slightly with increasing concentration of metal oxide, being 6.9 at the highest concentration tested, while the pH of nano- and bulk CuO suspensions was 6.6 at all the tested concentrations. The pH of the exposure medium increased on average by 0.4 units during the 24-h exposure time in all the experiments.

2.3. Cell viability assays

After 4 and 24 h of incubation of *T. thermophila* with or without toxicants, 100 μl was transferred from each well to 96-well black polypropylene microplate (Greiner Bio-One, Germany) for the viability testing with the fluorescent dye propidium iodide (PI, Fluka) and another 100 μl into a microcentrifuge tube for the ATP assay. The stock solution of PI was prepared in deionised water at a concentration of 1 mg/ml. This was further diluted with deionised water to obtain the working solution of 100 μg/ml, which was 10 times the final concentration in the viability assay. 10 μl of the PI working solution was pipetted directly into each well of 96-well microplate containing 100 μl of exposure medium and the microplates were further incubated for 15 min at 25 °C in the dark. The fluorescence was quantified using the Fluoroskan Ascent FL microplate reader (Thermo Labsystems, Helsinki, Finland) at excitation and emission wavelengths of 530 and 590 nm, respectively.

ATP content of the cellular suspensions was measured using the luciferin–luciferase method essentially as described in Kahru et al. (1982). Briefly, for ATP extraction 100 μl of protozoa culture samples were rapidly mixed with an equal volume of ice-cold 10% trichloroacetic acid containing 4 mM ethylenediamine-tetraacetic acid (EDTA) in microcentrifuge tubes. The fixed samples were stored at −18 °C till analysis. Prior to analysis the samples were thawed and diluted 50-fold with Tris–EDTA buffer (0.1 M Tris, 2 mM EDTA, adjusted to pH 7.75 with acetic acid). 200 μl of diluted sample was pipetted into the luminometer cuvette and first the background light emission ($RLU_{background}$) was measured. Then 20 μl of reconstituted ATP Monitoring Reagent from the ViaLight®HS Bioassay Kit (Lonza Rockland, USA) was added and the light emission of the sample (RLU_{sample})

was measured. For the internal calibration, 10 μl of ATP standard (1.65×10^{-6} M ATP) was added to the sample and light emission ($RLU_{internal\ standard}$) was measured again. The amount of the ATP in each well was calculated according to the following equation:

$$ATP, \mu mol = ((RLU_{sample} - RLU_{background})/RLU_{ATP\ standard}) \times ATP\ standard, \mu mol.$$

Cells were visualized with light and fluorescence microscope Olympus CX41 equipped with DP71 camera. Images were taken and the cell size was measured using software Cell B (Olympus).

2.4. Determination of dissolved fraction of metal oxides with recombinant sensor bacteria

The bioavailable metal ions present in the *T. thermophila* exposure medium (Osterhout's medium) were quantified using recombinant metal sensor bacteria in which bioluminescence is specifically induced by intracellular metal ions. The induction is mediated by a protein that recognizes the respective metal ions and regulates a promoter controlling the expression of the luminescence encoding gene cassette *luxCDABE* leading to increase in bioluminescence of sensor bacteria (Ivask et al., 2002). To determine the dissolved fraction of nano- and bulk ZnO and CuO at each of the tested concentrations (see Section 2.2), the suspensions of metal oxides prepared in Osterhout's medium were first inoculated with protozoa (cell density 5×10^5 cells/ml) in 24-well microplates (total volume: 1 ml per well) and incubated for 4 h at 25 °C without shaking (as in the toxicity tests) and thereafter filtered through 0.1 μm filter (Sartorius). The filtrate was analysed for bioavailable metal ions using recombinant luminescent Zn-sensor bacteria *Escherichia coli* MC1061 (pSLzntR/pDNPzntAlux) and Cu-sensor bacteria *E. coli* MC1061 (pSLcueR/pDNPcopAlux) (Ivask et al., 2009). In parallel to sensor bacteria, luminescent control strain *E. coli* MC1061 (pDNlux) (Leedjärv et al., 2006) with constitutively expressed luminescence was used to take into account the turbidity and possible toxic effects of the tested compounds. The test was conducted

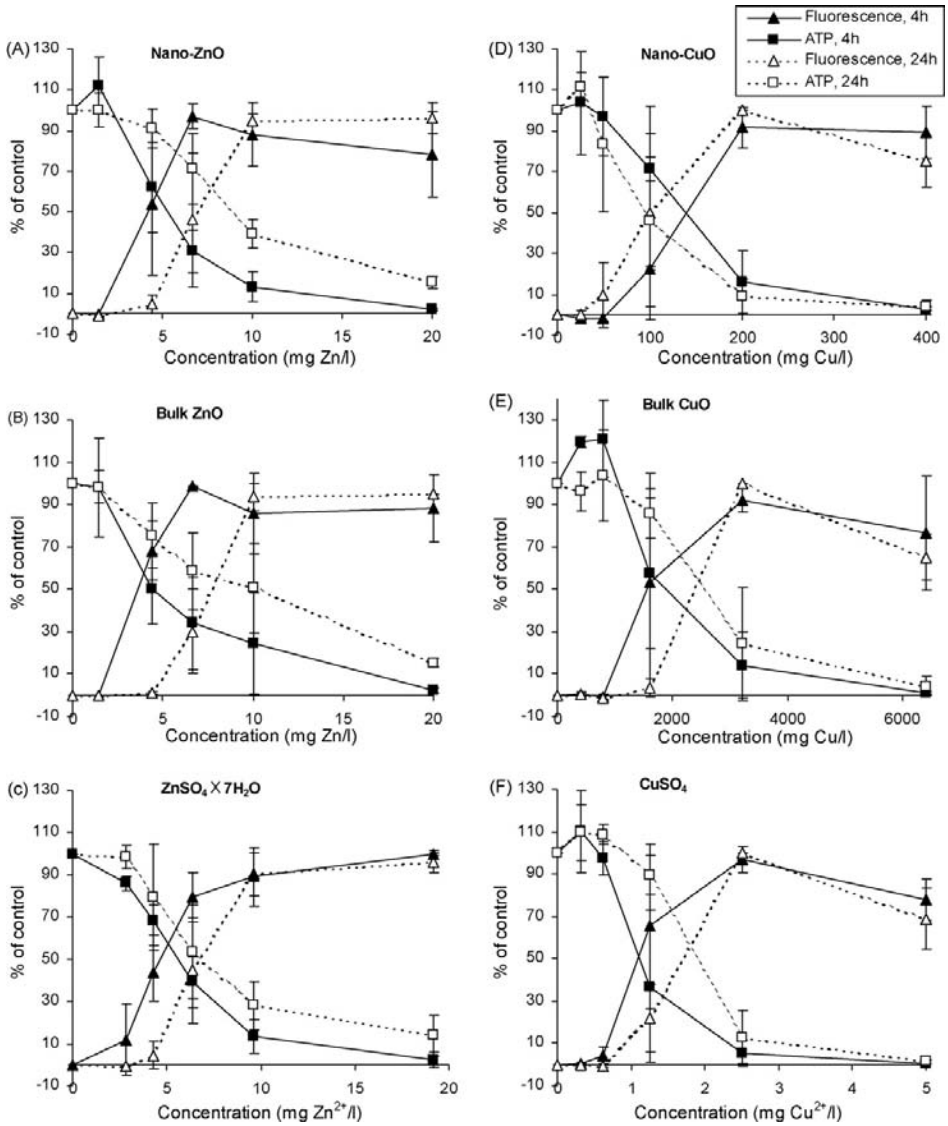

Fig. 1. Dose–response of *Tetrahymena thermophila* upon exposure to Zn and Cu oxide (nano)particles and respective metal salts for 4 and 24 h. The results of the assay on ATP content (4 h: ■; 24 h: □) are expressed as a percentage compared to non-exposed controls (100% viability), the results of the propidium iodide (PI) fluorescence assay (4 h: ▲; 24 h: △) are calculated as a proportion of the maximum fluorescence value (100% cytotoxicity). Data points represent the mean of three independent experiments ± standard deviation.

Table 1
EC_{50} values from two different cell viability assays (measurement of fluorescence and ATP) after 4- and 24-h exposure time.

Test substance	4-h EC_{50}[a] (mg metal/l)		24-h EC_{50}[a] (mg metal/l)	
	Fluorescence[b]	ATP	Fluorescence[b]	ATP
Nano-ZnO	4.3 (3.9–5.1)[c]	5.0 (4.8–5.8)[d]	6.8 (6.4–7.3)[c]	8.3 (7.7–9.4)[d]
Bulk ZnO	3.9 (3.5–4.4)[e]	4.9 (4.2–6.2)[f]	7.4 (6.9–8.0)[e]	8.1 (7.5–10.9)[f]
Zn^{2+} (tested as $ZnSO_4 \times 7H_2O$)	4.5 (4.1–5.0)[g]	5.2 (4.6–5.9)	6.7 (6.3–7.1)[g]	7.0 (6.3–8.1)
Nano-CuO	127 (124–144)	129 (111–149)	97.9 (80.4–138)	101 (91.1–190)
Bulk CuO	1580 (1321–2262)	1829 (1629–2883)	1966 (1874–2014)	2314 (1975–2651)
Cu^{2+} (tested as $CuSO_4$)	1.1 (0.98–1.4)	1.1 (1.0–1.3)[h]	1.4 (1.4–2.0)	1.7 (1.6–1.9)[h]

The superscripts of same letters (c–h) indicate significant difference between the two values (4-h *versus* 24-h exposure).
[a] EC_{50} numbers are the average values (95% confidence intervals) of two independent assays.
[b] The fluorescence of propidium iodide.

essentially as described in Heinlaan et al. (2008). Briefly, the sensor and control bacteria were pre-grown in Luria–Bertani medium till exponential phase, then harvested and washed twice with Osterhout's medium supplemented with casamino acids (1 g/l) and glucose (1 g/l) and diluted with washing medium until $OD_{600} \sim 0.1$. 100 μl of filtrate and 100 μl of sensor/control bacteria were pipetted into 96-well white microplates (Thermo Labsystems) and incubated for 2 h at 30 °C. The bioluminescence was registered with Fluoroskan Ascent FL microplate reader (Thermo Labsystems, Helsinki, Finland). $ZnSO_4 \times 7H_2O$ and $CuSO_4$ were used as 100% bioavailability controls, respectively.

2.5. Data analysis

The relative fluorescence unit (RFU) values measured in microplate wells containing *T. thermophila* cells in different concentrations of toxicant suspensions/solutions were corrected for background fluorescence by subtracting the corresponding RFU values obtained from the wells with the same toxicant concentrations without protozoa. The RFU values of protozoa-free suspensions/solutions were constant in case of all toxicant concentrations tested except for nano-CuO, where a slight decrease in background fluorescence occurred at the highest metal oxide content. The RFU values as well as ATP concentrations in the samples were both expressed as percentages of the non-treated controls. In PI assay, the concentration, which induced the maximum RFU value, was considered 100% cytotoxic. The concentration-effect curves used for the EC_{50} calculations were fitted with REGTOX software for Microsoft Excel™ (Vindimian, 2005) by the log-normal model and the EC_{50} values (effective concentration leading to a 50% cell death) were calculated with their 95% confidence interval. One-way analysis of variance (ANOVA) was used to determine statistical significance of the differences between values, whereas the level of significance was accepted at $p < 0.05$.

3. Results

Two different endpoints at two exposure times (4 and 24 h) were used to evaluate the toxic effects of nanoparticles to protozoa: propidium iodide (PI) staining and cellular ATP concentration that are both correlated to the cell viability. The state of the control culture during the test period was also assessed by visualizing the cells under light microscope to observe the size, number and motility of the protozoa. Although the cell number in the control culture was slightly reduced (by 15% during 24 h), the cells remained active and motile. Thus, the results of the experiments could be considered reliable. Interestingly, we observed a slight reduction in cell size in the control culture during the test period. The length and width of the cells decreased after 4 h by 15% and 20%, respectively and after 24 h by 20% and 30%, respectively (data not shown). Although the cells were smaller, no dividing cells in the 24-h controls were observed. Decrease in the cell size could probably be attributed to the starvation due to the lack of nutrients as reported already by Hellung-Larsen and Andersen (1989). The latter was also confirmed by our experiments: the ATP content in the 24-h control culture decreased by 25% compared to 4-h exposure (data not shown).

Dose–response of *T. thermophila* to nano- and bulk ZnO and CuO as well as soluble salts is shown in Fig. 1. The respective EC_{50} values (concentrations causing a 50% decrease in viability) calculated from these data using REGTOX software are presented in Table 1. Dose-dependent toxic effects (decrease in the content of ATP and an increase in fluorescence of PI) were observed for NPs as well as for the reference compounds. Interestingly, at the lowest (subtoxic) concentrations tested, most of the Zn and Cu compounds had a stimulatory effect on ATP concentration of *T. thermophila* (about 10% increase compared to the non-exposed control). The toxic effect of the tested compounds was slightly reduced after 24 h of exposure compared to 4-h exposure time (Fig. 1A–C, E, F), except for nano-CuO, which showed slightly higher, but not significant ($p > 0.05$), toxicity (Fig. 1D). However, when both endpoints (fluorescence and ATP) were considered, the significant reduction of the toxic effect after 24-h exposure was proved only for Zn compounds.

According to the EC_{50} values, both, bulk and nanosized ZnO as well as $ZnSO_4$ had similar toxic effects on *T. thermophila*, causing 50% of loss in viability at around 4–5 mg Zn/l after 4-h exposure and around 7 mg Zn/l after 24 h. The most toxic Cu compound among the tested ones was $CuSO_4$ (4- and 24-h EC_{50} 1.1–1.7 mg Cu^{2+}/l; Table 1), being about 120 times more toxic than nano-CuO and about 1500 times more toxic than bulk CuO (expressed as nominal concentra-

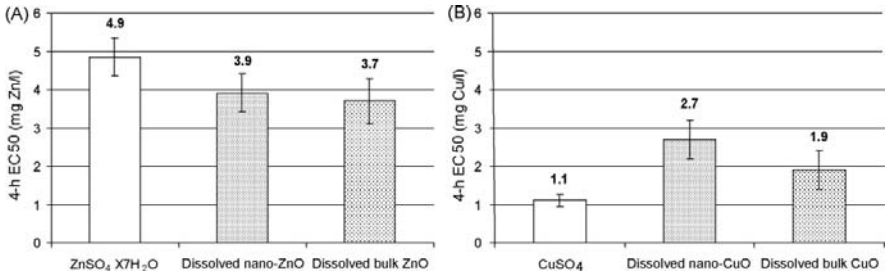

Fig. 2. 4-h EC_{50} values of $ZnSO_4 \times 7H_2O$, nano-ZnO, bulk ZnO (A) and $CuSO_4$, nano-CuO and bulk CuO (B) based on bioavailable Zn^{2+} and Cu^{2+} ions, respectively. The bioavailable ions in Osterhout's medium were quantified using luminescent recombinant sensor bacteria as described in Section 2.4. $ZnSO_4 \times 7H_2O$ and $CuSO_4$ were considered 100% bioavailable. 4-h EC_{50} values of $ZnSO_4 \times 7H_2O$ and $CuSO_4$ are the average of the results of propidium iodide fluorescence assay and the ATP assay (Table 1). Values indicate the mean of three independent experiments ± standard deviation.

tions on metal basis). After 4 h of exposure, nano-CuO was about 10 times more toxic than bulk CuO and after 24 h the difference in toxicities increased even to 20 times (Table 1). The EC_{50} values calculated from the measurements of ATP content were slightly higher than the values from PI fluorescence measurements, however the difference between these two endpoints (assays) was not significant ($p > 0.05$).

The solubilised fraction of zinc and copper oxides in the test medium was determined using the recombinant bacterial Zn- and Cu-sensors, respectively. The Zn-sensor analysis showed that at the 4-h EC_{50} level of nano-ZnO (4.7 mg Zn/l) and bulk ZnO (4.4 mg Zn/l), about 80% of ZnO was dissolved (3.9 and 3.4 mg Zn^{2+}/l, respectively) (Fig. 2A). Differently from ZnO, the solubility of both CuO preparations in the test medium was remarkably lower: at 4-h EC_{50} level of nano-CuO (128 mg Cu/l) and bulk CuO (1705 mg Cu/l) the concentration of dissolved copper was 2.7 and 1.9 mg Cu^{2+}/l, respectively (Fig. 2B). The solubility of nano-CuO exceeded the solubility of bulk CuO about 16 times (2% *versus* 0.12%).

4. Discussion

Usually, the toxic effects of chemicals on *Tetrahymena* have been evaluated by the reduction of the growth followed by optical density of the culture (Schultz, 1997). As test suspensions of NPs are often turbid due to insolubility and/or aggregation of particles, optical density measurement is not a suitable parameter for the quantification of the biomass of protozoa in this test environment. Thus, in the current study, the toxic effects of NPs on *T. thermophila* were assessed by PI staining and ATP measurement, to avoid the potential interference of the assay methods with the test results. Moreover, we succeeded to perform both assays directly in the exposure medium (see Section 2.3), avoiding the time consuming intermediate step of removing the toxicant suspension/solution from the cells by centrifuging prior to the viability measurement (Dayeh et al., 2005a). Both, PI staining and ATP method yielded practically similar EC_{50} values (Table 1), supporting the reliability of the test results. At the same time, the two assays also yielded complementary information, especially in case of the lowest and highest toxicant concentrations used (Fig. 1). For example, the stimulatory effect (hormesis) caused by the lowest doses of zinc and copper compounds (0.31 mg Cu^{2+}/l of $CuSO_4$, 31 mg/l of nano-CuO, 500 mg/l of bulk CuO, 2.84 mg Zn^{2+}/l of $ZnSO_4 \times 7H_2O$ and 1.85 mg/l of nano- and bulk ZnO) were detected only by the ATP measurements and not in the PI staining assay (Fig. 1). Also, when the PI-stained samples were visualized under conventional light microscope after the fluorescence measurements, the peak fluorescence value detected did not always coincide with 100% mortality in the sample, i.e. varying numbers of motile cells were seen in these samples in independent experiments. This phenomenon was also evident from the results of the ATP assay: at the toxicant concentrations, where the maximum fluorescence value was reached (apparent 100% cytotoxicity), the ATP content measured in the sample was still around 10–30% (and not zero) compared to the control (Fig. 1). This discrepancy could result from the fact that PI does not only enter the dead cells, but passes also the membranes of damaged cells, which could still contain ATP (Ataullakhanov and Vitvitsky, 2002). The stimulating effect of zinc and copper to *Tetrahymena* has been also reported previously (Nilsson, 1981, 2003; Nicolau et al., 1999, 2004) and it is not surprising, as both metals are needed as microelements for normal functioning of the cells (Valko et al., 2005). For example, addition of up to 100 mg/l copper or 50 mg/l zinc to the 2% proteose peptone stimulated phagocytosis in *Tetrahymena* (Nilsson, 1981, 2003). Also, copper (at concentrations of 30 and 65 mg/l) has been found to stimulate grazing activity (Nicolau et al., 1999) and digestive function of *Tetrahymena* (Nicolau et al., 2004). The remarkably higher stimulatory concentrations of zinc and copper reported in the literature, compared to our data, can be explained by the different test media: Osterhout's medium has very low complexing activity. Indeed, the concentrations of free Cu^{2+} ions in Osterhout's medium were similar to the respective values in distilled water, when measured by copper ion selective electrode (ORION 96-29 ionplus® electrode, data not shown).

The slightly reduced toxicities of metal compounds to *T. thermophila* after 24 h compared to 4-h exposure, which were more pronounced in case of zinc compounds (Fig. 1), could be explained by the adaptation of the cells to metals. It has been shown that protozoa can sequester the metals by ingesting it into the food vacuoles or accumulating excessive metal ions in cytoplasmic dense granules (Nilsson, 2003; Martín-González et al., 2005). As zinc and copper are both essential trace elements, the cells possess the mechanisms for regulating the intracellular concentration of these metals. Two main types of metal sequestration have been proposed (Vijver et al., 2004). The first one involves the compartmentalization of metals in cytoplasmic granules or membrane-bound vesicles. In *Tetrahymena*, the formation of small refractive granules upon exposure to Zn^{2+} and Pb^{2+} has been reported by several authors (Nilsson, 2003). The second mechanism is mediated by metal-binding proteins – metallothioneins – low molecular weight cysteine-rich proteins that bind mainly Cd, Zn and Cu ions (Diaz et al., 2007). It has been reported that metallothioneins in *Tetrahymena* as in other organisms are multi-stress inducible, being induced in addition to high metal concentrations also by the starvation stress after 24-h exposure (Amaro et al., 2008). Considering that we also used the mineral medium, the latter could be one explanation to the adaptation of the cells to the metals during 24-h exposure.

The 24-h EC_{50} values for Zn^{2+} to *Tetrahymena* found in the literature range from 3.58 to 196 mg Zn^{2+}/l, and the respective values for Cu^{2+} from 0.47–200 mg Cu^{2+}/l, whereas the higher values are usually obtained in the tests conducted in rich organic growth media (Nicolau et al., 1999; Rico et al., 2009) than in minimal salt medium or inorganic buffer solutions (Dayeh et al., 2005b; Gallego et al., 2007). The results of the current study are comparable with the results of Dayeh et al. (2005b), where the 24-h EC_{50} value upon exposure of *T. thermophila* to Cu^{2+} in Osterhout's medium was 1.41 mg/l, and Gallego et al. (2007), where the respective values in the toxicity tests performed in 10 mM Tris–HCl buffer (pH 6.8) were 3.58 mg/l for Zn^{2+} and 0.47 mg/l for Cu^{2+}. It has been reported previously that *Tetrahymena* is more resistant to zinc and copper in organic growth media than in mineral solutions (Nilsson, 1989). For example, Nicolau et al. (2004), who used proteose peptone yeast extract medium in testing the toxicity of water-soluble metal salts to protozoa *T. pyriformis*, showed that, depending on concentration tested, about 30–66% of zinc and 60–73% of copper proved to be bound to the dissolved organic matter.

We showed that the toxicities of nano- and bulk ZnO to *T. thermophila* did not differ (Table 1), which corresponds to the results of the earlier studies on other organisms (Franklin et al., 2007; Heinlaan et al., 2008; Mortimer et al., 2008; Aruoja et al., 2009; Kasemets et al., 2009; Wang et al., 2009). As the EC_{50} numbers of both ZnO formulations and $ZnSO_4$ were similar, when calculated on metal basis, the toxicity of ZnO was most probably caused by the dissolved zinc. Indeed, the quantification of solubilised zinc with recombinant Zn-sensor bacteria showed that ~80% of Zn in nano- and bulk ZnO suspensions was in dissolved form at the concentrations of their EC_{50} level (Fig. 2A). The similar toxicities of nanosized and bulk ZnO to *T. thermophila* could therefore be contributed to the same rate of dissolution of both formulations in the conditions of the current study. Recently, Franklin et al. (2007) also showed that the solubility of ZnO was similar for bulk and nanoparticulate ZnO. In the aforementioned study, where equilibrium dialysis was used, the solubility of ZnO at concentration of 100 mg/l in 0.01 M

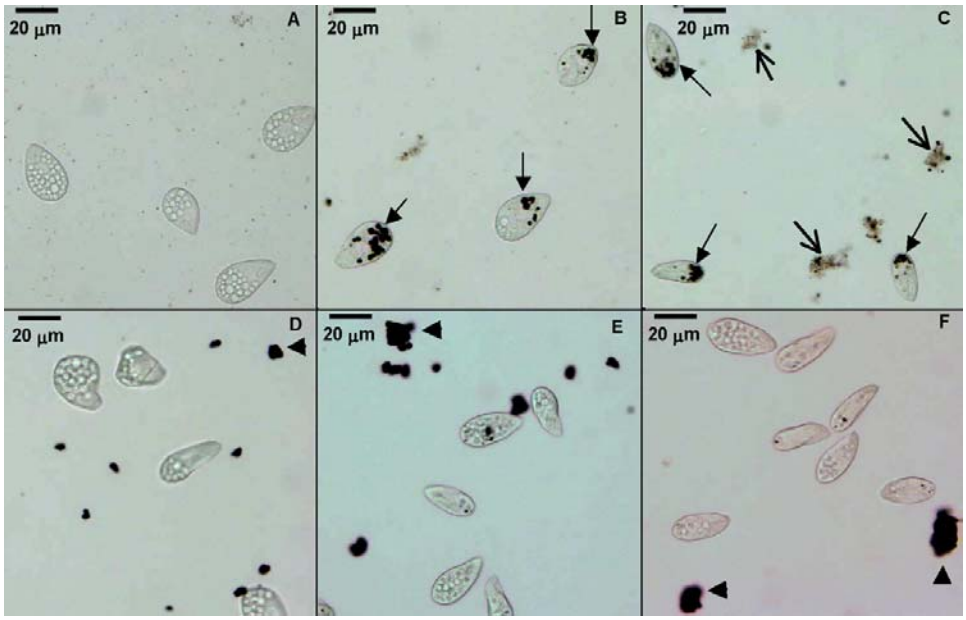

Fig. 3. *Tetrahymena thermophila* in the suspension of 125 mg/l of nano-CuO at 0 h (A), after 2 h (B) and 4 h (C) of exposure, and in suspension of 2000 mg/l of bulk CuO at 0 h (D), after 2 h (E) and 4 h (F) of exposure. The cells were exposed at concentrations of approximate respective 4-h EC_{50} values (Table 1). Open arrows indicate aggregates of nano-CuO attached to the cell debris, filled arrows indicate food vacuoles filled with nano-CuO and filled arrowheads indicate aggregates of bulk CuO.

$Ca(NO_3)_2$/PIPES buffer at pH 7.6 reached equilibrium at 16 mg/l. Thus, it can be assumed that ZnO suspensions at the concentrations less than 16 mg/l should be 100% dissolved. The fact that less than 100% of dissolved zinc was detected with Zn-sensor bacteria at nano- and bulk ZnO concentrations of ~4.5 mg Zn/l could be explained by the feeding pattern of *Tetrahymena*: the cells internalised part of ZnO into their food vacuoles from the test medium during 4 h exposure leaving less metal oxide in the surrounding medium to be dissolved before the bacterial sensor assay (see also Section 2.4).

Differently from ZnO, which toxicity in the current study was not dependent on particle size, nano-CuO was 10–20 times (after 4- and 24-h exposure, respectively) more toxic to *T. thermophila* than bulk CuO. Comparable results were obtained by Aruoja et al. (2009) for algae *Pseudokirchneriella subcapitata* (nano-CuO was 16-fold more toxic after 72-h exposure), and for *S. cerevisiae* even 60-fold difference in toxicities was reported (Kasemets et al., 2009). The quantification of dissolved copper in the test medium with the Cu-sensor bacteria showed that nano-CuO was remarkably more soluble than bulk CuO: after 4-h exposure 2% of nano-CuO and only 0.12% of bulk CuO was solubilised at the level of their EC_{50} values (128 and 1705 mg Cu/l, respectively; Fig. 2B and Table 1). Thus, nanoparticulate CuO was 16 times more soluble than bulk CuO. In a previous study even 141-fold difference in solubility of nano- and bulk CuO was shown in algal growth medium (Aruoja et al., 2009). This apparent discrepancy could not be attributed only to different experimental conditions (concentration of metal oxides, test medium, pH, incubation/solubilisation time), but also to the feeding pattern of *Tetrahymena* efficiently ingesting CuO particles into the food vacuoles (Fig. 3). As the solubility of bulk CuO (0.12%) was analogous with the results of Aruoja et al. (2009), but the proportion of dissolved nano-CuO was significantly lower in the current study (2% *versus* 25%), it could be assumed that nano-sized CuO particles were more readily ingested by *T. thermophila* than bulk CuO particles and thereby more rapidly removed from the medium (see also Fig. 3), leaving less metal oxide in the surrounding medium to be solubilised. *Tetrahymena* ingests particles by a special structure called cytostome, where the food vacuole or phagosome is formed and directed towards the posterior of the cell (Frankel, 2000). After formation phagosomes undergo a series of maturation steps, which includes acquiring the hydrolytic enzymes that participate in the phagolysosomal degradation of ingested particles (Jacobs et al., 2006). Considering that the pH in the lumen of food vacuoles becomes acidic (pH < 4) in 1 h after vacuole formation (Nilsson, 1977), which should facilitate the dissolution of metal oxide, it is interesting that *T. thermophila* is capable to function after ingesting so much CuO NPs that it is full of dark phagosomes (Fig. 3, closed arrows). It has been shown previously that the acidic environment of food vacuoles had no degrading effect on carboxylated and biotinylated quantum dots with CdSe core (Holbrook et al., 2008). Also, the retention of lead within the digestive vacuoles and accumulation of lead within the small refractile granules has been demonstrated in *Tetrahymena* exposed to lead acetate (Nilsson, 1979). The concentrations of solubilised Cu ions from both CuO formulations at the EC_{50} level were slightly but still significantly higher ($p < 0.05$) than the EC_{50} value of $CuSO_4$ (1.1 mg Cu^{2+}/l, Table 1 and Fig. 2B). Interestingly, the sensor-analysed concentrations were about twice as high as the observed toxic effects of Cu^{2+}. Further research is needed to explain these results. Nevertheless, it is evident that the toxicity of bulk and nano-CuO was caused by the dissolved fraction of these metal oxides.

5. Concluding remarks

To our knowledge, this is the first paper on toxicity of ZnO and CuO NPs to protozoa. One may think that particle-feeding organism should be more susceptible to toxic effects of particulate compounds than organisms not internalising particles. On the contrary,

this study showed that, even though intensively accumulated in the food vacuoles, the toxicity of nanoparticles of ZnO and CuO to *T. thermophila* was due to dissolved metal ions, analogously to bacteria, algae and crustaceans. We also demonstrated that higher toxicity of nano-CuO compared to its bulk formulation was due to increased solubilisation.

Lastly, we recommend *T. thermophila* as a simple eukaryotic particle-ingesting model organism for the high throughput toxicity screening of NPs, whereas ATP level and membrane integrity (PI-staining) could be both used as toxicity endpoints. As the genome of *T. thermophila* is sequenced, this organism may provide also new nanotoxicogenomic results.

Conflict of interest

The authors declare that they do not have conflicts of interest.

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APPENDIX I

Manuscript

Article

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

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

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ABSTRACT: In the current study, the toxicity mechanism of nanosized CuO (nCuO) to the freshwater ciliated protozoa *Tetrahymena thermophila* was studied. Changes in fatty acid profile, lipid peroxidation metabolites and reactive oxygen species (ROS) were measured. Bulk CuO and $CuSO_4$ served as controls for size and solubility and 3,5-dichorophenol (3,5-DCP) as a control for a chemical known to directly affect the membrane composition. Exposure to all copper compounds induced the generation of ROS, whereas nCuO was most potent. The latter effect was not solely explained by solubilized Cu-ions and was apparently particle-related. 24-h exposure of protozoa to 80 mg/L of nCuO (EC50) significantly decreased the proportion of two major unsaturated fatty acids (UFA) (C18:3 cis-6,9,12, C18:2 cis-9,12), while it increased the relative amount of two saturated fatty acids (SFA) (C18:0, C16:0). Analogous effect was not observed when protozoa were exposed to equitoxic suspensions of bulk CuO, Cu-ions or

3,5-DCP. As changes in the UFA:SFA upon exposure of protozoa to nCuO were not detected at 2-h exposure and no simultaneous dose- or time-dependent lipid peroxidation occurred, it is likely that one of the adaptation mechanisms of protozoa to nCuO was lowering membrane fluidity by the inhibition of *de novo* synthesis of fatty acid desaturases. This is the first study of the effects of nanoparticles on the membrane fatty acid composition.

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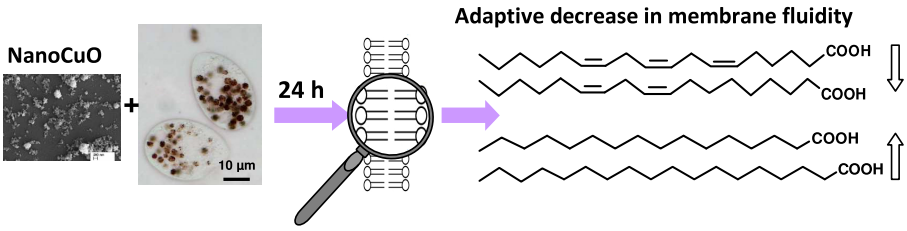


INTRODUCTION

Copper is an essential trace element in living organisms which plays a vital role in the function of proteins, but can also exert toxic effects to cells due to its redox activity and affinity for the binding sites of other metals.[1] The biocidal properties of copper have long been known and, during the last decades, technologies have been developed to incorporate copper nanoparticles (NPs) into textile fibers, latex and other polymers to give them biocidal properties.[2] As a result of the growing application of nanosized copper oxide (nCuO), there is an increasing risk of its accidental release to the environment leading to possible adsorption to aquatic organisms or organic matter or uptake by filter feeders and phagocytotic organisms.[3] Compared to some other metal oxide NPs (TiO_2 and ZnO), the potential hazardous effects of nCuO to environmentally relevant test organisms are poorly studied and warrant reconsideration since copper is highly toxic to aquatic organisms.[4] To what extent copper ions may contribute to the toxicity of nCuO is not fully understood, although it is generally accepted that one of the factors in exerting toxic effects of metal-containing NPs is the release of their metal ions.[5-8] Once liberated, redox-active metal ions may in turn yield free radicals via the Fenton-type reaction to elicit intracellular oxidative stress.[9]

One of the common paradigms proposed for the toxic mechanism of both metallic and carbon-based NPs is the generation of reactive oxygen species (ROS).[10] Indeed, in *in vitro* studies using human lung epithelial cells A549 nCuO has been shown to cause oxidative lesions, induce the accumulation of intracellular ROS[11], and show genotoxic potential probably also mediated through oxidative stress.[12] However, it has also been reported that the toxic effects of nCuO are unlikely mediated by particle-generated ROS.[13] The controversial data on the apparent mechanisms of NP toxicity may be partly caused by the complexity of NP behavior (aggregation, stability, mobility) in aqueous environment. The intricacy of the interactions of NPs with aquatic organisms is further increased by the presence of natural organic matter (NOM). For example, the remarkable surface-modifying properties of NOM render intrinsically hydrophobic fullerenes water soluble, mobile, and bioavailable.[14]

In addition to ROS-based mechanism of toxicity the importance of NP-biological interfaces has been stressed. Once the NPs encounter the cell membrane, the possible ways of interaction include specific or nonspecific forces, receptor–ligand binding interactions or membrane wrapping of the NPs.[10] During this process the NPs may have a direct damaging effect on the membrane composition, they could disrupt the intracellular balance after internalization[10], or the discharge of NPs by the cell through exocytosis or leakage could impact the membrane composition and fluidity[15]. For example, CuO NPs have been shown to decrease the cell stiffness, contraction and relaxation of human airway smooth muscle cells *in vitro*.[13]

Protozoa are the simplest unicellular eukaryotic organisms. Feeding by phagocytosis, *Tetrahymena* constantly forms extensive membranous structures in the cell, hence is a good model for studying the effects of NPs on the cell membranes. Also, compared to other aquatic organisms, ciliates show relatively high tolerance towards certain chemicals, including heavy metals and metal oxides,[16] which contribute to their habitation in waste water streams and biopurification systems. Investigations into the chemical resistance of *Tetrahymena* could offer new insight into the mechanisms of aquatic species adaptation to toxicants, including NPs. In addition, toxicity data based on the growth inhibition of protozoa *T. pyriformis* has been collected for 2,400 industrial organic compounds and incorporated in

the TETRATOX database, which is extensively used in quantitative structure-activity relationships (QSAR) studies.[17] As the genome of *T. thermophila* is sequenced, our results are expected to pave the way also for relevant nanotoxicogenomic studies.

In the current study, a free-living freshwater ciliate *Tetrahymena thermophila* was used to assess the toxic effects of nCuO on the cell membrane fatty acid composition. Bulk CuO (bCuO) was used as a control for particle size, $CuSO_4$ as a control for the effect of dissolved Cu-ions and 3,5-dichlorophenol as a control with known mechanism of action (polar narcosis).[18] In parallel to fatty acid profiling, ROS levels and lipid peroxidation in *T. thermophila* upon exposure to nCuO were studied, to elucidate the potential mechanism of toxic action.

MATERIALS AND METHODS

Reagents. CuO NPs (advertised particle size 30 nm) were purchased from Sigma-Aldrich, bCuO and $CuSO_4$ from Alfa Aesar, 3,5-dichlorophenol (3,5-DCP) from Pestanal, H_2O_2 (30%) from Merck, 2',7'-dichlorofluorescein-diacetate (H_2DCFDA), 2-thiobarbituric acid (TBA), malondialdehyde bis(dimethyl acetal) and butylated hydroxytoluene (BHT) from Sigma-Aldrich. All the chemicals were of analytical grade. The stock solutions of nCuO (40 g/L) and bCuO (100 g/L) were prepared in deionized water (MilliQ, Millipore), sonicated in ultrasonication bath (Techpan Type UM2-2, Poland) for 30 min and further stored at room temperature in the dark.

Characterization of CuO (nano)particles. The hydrodynamic diameter of nCuO in the exposure media was determined using dynamic light scattering (DLS) and the zeta potentials of nCuO and bCuO were measured using Zetasizer Nano ZS (Malvern Instruments). The hydrodynamic diameter and zeta potential of nCuO (100 mg/L) and the zeta potential of bCuO (2500 mg/L) were measured in MQ water and Osterhout's mineral medium.

Scanning electron microscopy (SEM; Jeol, JSM-8404) from the nCuO and bCuO suspensions and the analysis of specific surface area of the powders of nCuO and bCuO by Brunauer-Emmet-Teller (BET)

analysis (Sorptometer Kelvin 1042, Costech Instruments) were performed in Tallinn University of Technology, Center of Materials Research and Laboratory of Inorganic Materials.

Exposure of *Tetrahymena thermophila* to toxicants. Protozoan culture was prepared as described in the Supporting Information (SI) and exposed to the toxicants at two concentrations: 24-h EC20 and EC50, i.e. nominal concentrations of chemicals (mg/L) causing a 20% and 50% decrease in the viability of the protozoa, respectively. The viability was determined by measuring the ATP content using the luciferin–luciferase method essentially as described previously.[19] For exposures the following concentrations were used: 1.3 and 1.6 mg Cu^{2+}/L of $CuSO_4$, 60 and 80 mg/L of nCuO, 1500 and 2500 mg/L of bCuO, 2.6 and 5.3 mg/L of 3,5-DCP, and 43 and 68 mg/L of H_2O_2.

For membrane fatty acid analysis 20 mL of *T. thermophila* (10^6 cells/mL) was added to 20 mL of nCuO or the control chemical solution/suspension (all in Osterhout's medium) in 100-mL flasks to yield a final cell density in the test of 5×10^5 cells/mL. The flasks were shaken at 80 rpm at 25ºC for 24 h. After exposure the cell suspensions were centrifuged at 300×g for 5 min, 4°C, washed once with Osterhout's medium, and the pellets were resuspended in sterile double-distilled water, frozen at –20°C, lyophilized and kept at 4°C until further analysis. Separate experiments were conducted on three days.

For lipid peroxidation analysis *T. thermophila* cells were exposed to nCuO and the corresponding controls in 24-well culture plates: 500 µL of *T. thermophila* in Osterhout's medium was pipetted into the wells containing 500 µL of the toxicant, each concentration in two replicates. Osterhout's medium was used as a diluent for chemicals and as a negative control. The test plates were incubated on a microplate shaker (Heidolph Titramax 1000, 300 rpm) at 25°C. After 2- and 24-h exposure 100 µL of cell suspension was sampled from each well, 5 µL of 1% BHT in methanol as antioxidant was added and the samples were kept at –80°C until the analysis. Separate experiments were conducted on three days.

Determination of cellular fatty acid composition by gas chromatography (GC). Prior to analysis lipids were transesterified using the HCl/methanol procedure.[20] Fatty acid methyl esters (FAMEs) were separated using Agilent 6890 series capillary gas chromatograph equipped with capillary column Omegawax TM 320 (30 m×0.32 mm ID×0.25 mm, Supelco) with polyethylene glycol as the stationary

phase, and a flame-ionization detector. Helium was used as a carrier gas with a flow of 2.0 mL/min. The starting temperature for analysis was 185°C and the end temperature was 215°C, with a temperature increase speed of 1°C/min. The run time was 54 min and the volume of injection was 2 µL. The identification of FAMEs was done by retention time comparison and results were calculated using response factors derived from chromatographic standards of known composition (Nu Chek Prep). Relative proportions of fatty acids between C10:0 and C20:0 were calculated from peak areas. The results were analyzed using Agilent ChemStation Plus® software. The analysis of each sample was made in duplicate.

Analysis of lipid peroxidation and reactive oxygen species (ROS). Lipid peroxidation upon exposure of protozoa to toxicants was measured by determining the generation of thiobarbituric acid reactive substances (TBARS) by means of quantifying the malondialdehyde (MDA) content, and generation of ROS in the cells was measured using ROS-sensitive fluorescent probe 2',7'-dichlorofluorescein-diacetate (H_2DCFDA). See the Supporting Information for description of the assays.

Analysis of the dissolved fraction of CuO using the recombinant copper-sensing *Escherichia coli*. To determine the dissolved fraction of nCuO and bCuO at EC50 concentrations, the suspensions of metal oxides prepared in Osterhout's medium were incubated in 24-well microplates (1 mL per well) for 24 h at 25°C without shaking in the dark and thereafter filtered through sterile Minisart® 0.1 µm filter (Sartorius). The filtrate was analyzed for dissolved copper ions using recombinant luminescent copper-sensing bacteria *E. coli* MC1061 (pSLcueR/pDNPcopAlux).[21] In parallel to sensor bacteria, constitutively luminescent control strain *E. coli* MC1061 (pDNlux)[22] was used to take into account the possible toxic effects of the tested compounds. $CuSO_4$ solutions in MQ water were used as a 100% bioavailability control. The test with sensor bacteria was conducted as described previously.[19]

Data analysis. One-way analysis of variance (ANOVA) was used to determine the significant differences between values.

RESULTS AND DISCUSSION

Size and aggregation pattern of nCuO in the test medium. SEM images show that nCuO contained smaller particles than bCuO, but there were also <100 nm particles in the heterogeneous-sized bCuO preparation (Figure 1). The primary particle size of nCuO has been determined in artificial fresh water by transmission electron microscopy (TEM) in our previous study[17], and it corresponded to the size indicated by the manufacturer, i. e. 30 nm (Table 1). The difference in size of nCuO and bCuO was further confirmed by BET analysis: nCuO had a 39-fold higher specific surface area than bCuO (Table 1). The average hydrodynamic diameter of nCuO, as measured by DLS, appeared around 200 nm in MQ water and even in micrometer scale (1,230 ± 200 nm) in the Osterhout's medium (Table 1). Although the concentration of mineral salts in Osterhout's medium is very low (conductivity 170 µS/cm), CuO NPs were observed to aggregate and sediment at much higher rate in Osterhout's medium (Figure 2b) than in MQ water (Figure 2a) after 3 h at 25°C. The influence of the composition of mineral medium on particle aggregation and thus, higher instability of the suspensions, was also confirmed by the different zeta potential values of CuO measured in MQ water and Osterhout's mineral medium (Table 1). While the zeta potential values of both bulk and nCuO suspensions in MQ water exceeded +30 mV, which is considered an indication of a stable suspension, the respective values in the mineral medium were + 17 ± 4 mV for nCuO and – 5 ± 5 mV for bCuO, indicating higher propensity for aggregation of both CuO formulations in Osterhout's medium, which was also observed in the wells of the test plate for which shaking was applied during the exposure (Figure S1 in the Supporting Information). However, as *T. thermophila* is a ciliated protozoan, the cells were moving around in the test wells and thus were exposed to both nCuO and bCuO to a similar extent, which is also indicated by the food vacuoles filled with dark CuO particles after 2 h exposure (Figure S2 b, c in the Supporting Information). When the hydrodynamic diameter was analyzed after removing the larger aggregates by a low speed centrifugation (700×g for 5 min), it was evident that the suspension contained also smaller aggregates of nCuO with the average diameter of 295 nm (blue line in Figure S4a in the Supporting Information), which increased to 531 nm in 2 h (blue line in Figure S4b in the Supporting Information).

The tendency of NPs to aggregate in aqueous systems is a well-known phenomenon, which has been observed in the case of different types of NPs;[5,11] it has even been proposed that in ecotoxicology the definition of NPs should be extended to particles with a size up to 500 nm.[23] Moreover, the micrometer sized aggregates of NPs are still readily ingestible for some particle-feeding organisms such as daphnids and protozoa, facilitating the possible bioaccumulation of NPs. Protozoa also contribute to the agglomeration of NPs by ingesting particles into food vacuoles, where the NPs are packed together. The packed NPs are either excreted from the cytoproct of the living cells or escape the intracellular space after the cell death, facilitating the sedimentation of NPs. As a result, the concentration of NPs in the water column is decreased and the exposure to other aquatic organisms is reduced, as was recently proposed in a study on fullerene exposed *Daphnia magna*.[24]

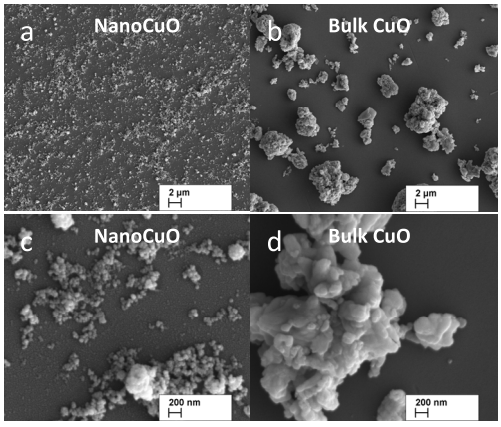


Figure 1. SEM images of nano- and micro-sized CuO. Panels a and b: ×2000 magnification; panels c and d: ×20 000 magnification.

Table 1. Characteristics of nano- and micro-sized CuO

Particle		nCuO	bCuO
Particle size in powder[a], nm		30	nd
Specific surface area of powder[b], m^2/g		25.5 ± 0.8	0.64± 0.02
Hydrodynamic diameter[c], nm	MQ water (pH 6.0)	209 ± 10	nd
	Osterhout's mineral medium (pH 6.0)	1230 ± 200	nd
Zeta potential[c], mV	MQ water (pH 6.0)	+37 ± 6	+32 ± 8
	Osterhout's mineral medium (pH 6.0)	+17 ± 4	- 5 ± 5

[a] According to the manufacturer.

[b] Measured by Brunauer-Emmet-Teller (BET) analysis.

[c] Measured at 100 mg/L (nCuO) and 2500 mg/L (bCuO), mean of three samples ± standard deviation.

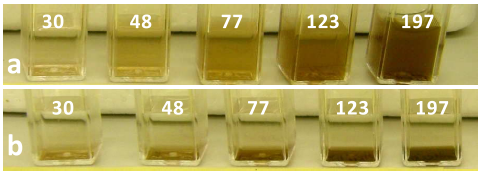
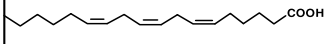
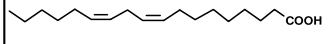
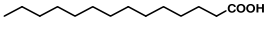
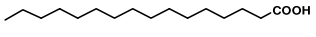
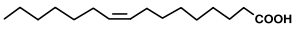
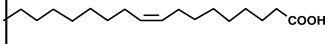
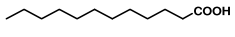
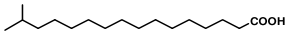
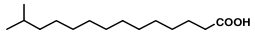
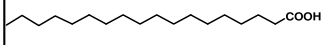
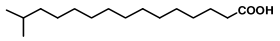
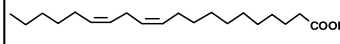
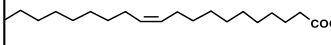
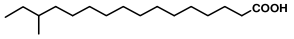
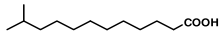
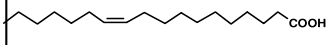
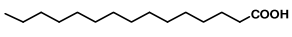
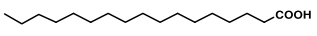
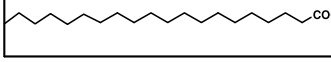


Figure 2. Effect of test medium composition on the agglomeration of CuO nanoparticles. Suspensions of nCuO in MQ water (a) and Osterhout's mineral medium (b) after 3 h at room temperature. The numbers refer to the nominal concentrations of nCuO (mg/L).

Effect of nCuO on the fatty acid composition of *Tetrahymena thermophila* membranes. *T. thermophila* was exposed to nCuO and the control chemicals (bCuO, $CuSO_4$ and 3,5-DCP) in Osterhout's mineral medium, i. e., in starving conditions for 24 h, hence some changes in the fatty acid composition occurred also in the non-exposed control culture. According to our data (Table 2), the most abundant fatty acid was γ-linolenic acid (C18:3), constituting approximately 30% of all fatty acids of *T. thermophila*, followed by linoleic acid (C18:2), approximately 10% of total fatty acids. The determined proportions of fatty acids were in good correlation with previously published data for *T. pyriformis*[25] and *T. thermophila*.[26] The fatty acid profiling of *T. thermophila* cells after incubation with nCuO and the control chemicals at 24-h EC20 and EC50 concentrations for 2 and 24 h revealed that nCuO had a different effect on fatty acid composition as opposed to the control and the other tested chemicals (Figure 3). In particular, 24-h exposure to the EC50 concentration of nCuO caused a significant increase in the relative amount of two saturated fatty acids, SFA (stearic and palmitic acid; C18:0 and C16:0), and a decrease in the proportions of two major unsaturated fatty acids, UFA (γ-linolenic and linoleic acid; C18:3 and C18:2); such increases in SFA, coupled with the decreases in UFA, reduced the area per lipid molecule resulting in the decrease of membrane fluidity. This effect is further illustrated by the ratios of SFA and UFA (Table 3): at 2 h in starving conditions, without (control) or with the added toxicants, the ratio of SFA to UFA remained at 0.6 and after 24 h the respective value was 0.5 in all cases except for nCuO, which increased the ratio dose dependently (to 0.7 at EC20 and 1.0 at EC50 concentration). Hence, in the latter case the amount of SFA and UFA in protozoa was equal, which was remarkably different compared to the non-exposed control cultures, or the cultures exposed to control chemicals at their EC50 level, where the proportion of UFA exceeded the relative amount of SFA about two-fold (Table 3). It has been shown that in response to a decrease in growth temperature, the level of UFA in *Tetrahymena* membrane phospholipids increases to maintain the optimal physical state (fluidity) of the membranes.[27] We observed a similar tendency, i.e. a significant increase in γ-linolenic acid (C18:3) in 3,5-DCP exposed cells after 24-h exposure to EC50 concentration (Figure 3d). Here 3,5-DCP was used as a positive control exerting toxic effect through polar narcosis, i.e. interfering with

membranes.[18] Different from the action of 3,5-DCP, the effect of nCuO manifested phenotypically as a decrease in the membrane fluidity. It has been shown that the alteration of membrane fluidity in *Tetrahymena* coincided with the proportional changes in the levels of UFA and SFA. This adaptive regulation has been attributed to the change in the activity of Δ^9-desaturase (EC 1.14.99.5), which catalyses the formation of the first double bond in C16:0 or C18:0 acids.[28] Thus, our data revealed that nCuO could inhibit Δ^9-desaturase. Furthermore, as we did not observe such effect at 2 h, it seems to be due to the inhibition of *de novo* synthesis of this enzyme. It should be mentioned that, in general, *de novo* synthesis of enzymes has been shown to take place in non-growing (starved) cultures of protozoa,[27] i.e., in the test conditions applied also in the current study. The adaptive elevation of the proportion of SFA has also been observed in *T. pyriformis* in response to the rise in growth temperature[29] and this was explained by the reduced activity of desturases and enhanced synthesis of SFA. Analogous changes in the ratios of SFA and UFA as observed after exposure to nCuO in *Tetrahymena*, were reported for green microalgae upon their exposure to sublethal concentration (0.5 mg/L) of $NiCl_2$, where a remarkable increase in C14:0, C18:0; C20:0 and C22:0 and simultaneous decrease in the proportions of C16:1, C16:4, C18:3 and C24:1 occurred. Nickel treatment was suggested as a cause for the altered fatty acid desaturation processes.[30]

Table 2. The fatty acid composition of *Tetrahymena thermopila* after 2 and 24 h incubation in Osterhout's medium (no toxicants added; starving conditions).

Fatty acid		% from total fatty acids[a]	
Structure	Name	After 2 h	After 24 h
	γ-linolenic acid, C18:3 cis-6, 9, 12	27.2 ± 2.4	30.4 ± 2.5
	linoleic acid, C18:2 cis-9, 12	10.3 ± 1.2	11.9 ± 0.7
	myristic acid, C14:0	7.8 ± 0.7	5.8 ± 0.3
	palmitic acid, C16:0	7.6 ± 1.2	8.5 ± 0.6
	palmitoleic acid, C16:1 cis-9	7.2 ± 1.0	4.6 ± 0.4
	oleic acid, C18:1 cis-9	5.2 ± 2.3	4.6 ± 2.0
	lauric acid, C12:0	2.8 ± 0.2	2.4 ± 0.1
	iso-C17:0	2.7 ± 0.7	2.2 ± 0.5
	iso-15:0	2.5 ± 0.6	1.7 ± 0.3
	stearic acid, C18:0	1.7 ± 0.5	2.3 ± 0.8
	iso-16:0	1.5 ±0.4	2.0 ± 0.3
	C20:2 cis-11, 14	1.4 ± 0.3	1.7 ± 0.2
	C20:1 cis-11	1.0 ± 0.7	1.1 ± 0.5
	anteiso-C17:0	1.0 ± 0.2	0.7 ± 0.1
	iso-C13:0	0.9 ± 0.3	0.6 ± 0.2
	C18:1, cis-11	0.6 ± 0.3	1.2 ± 0.5
	C15:0	0.4 ± 0.1	0.6 ± 0.1
	margaric acid, C17:0	0.2 ± 0.1	0.6 ± 0.1
	arachidic acid, C20:0	0.2 ± 0.2	0.4 ± 0.2

[a] mean values of three independent experiments ± standard deviation.

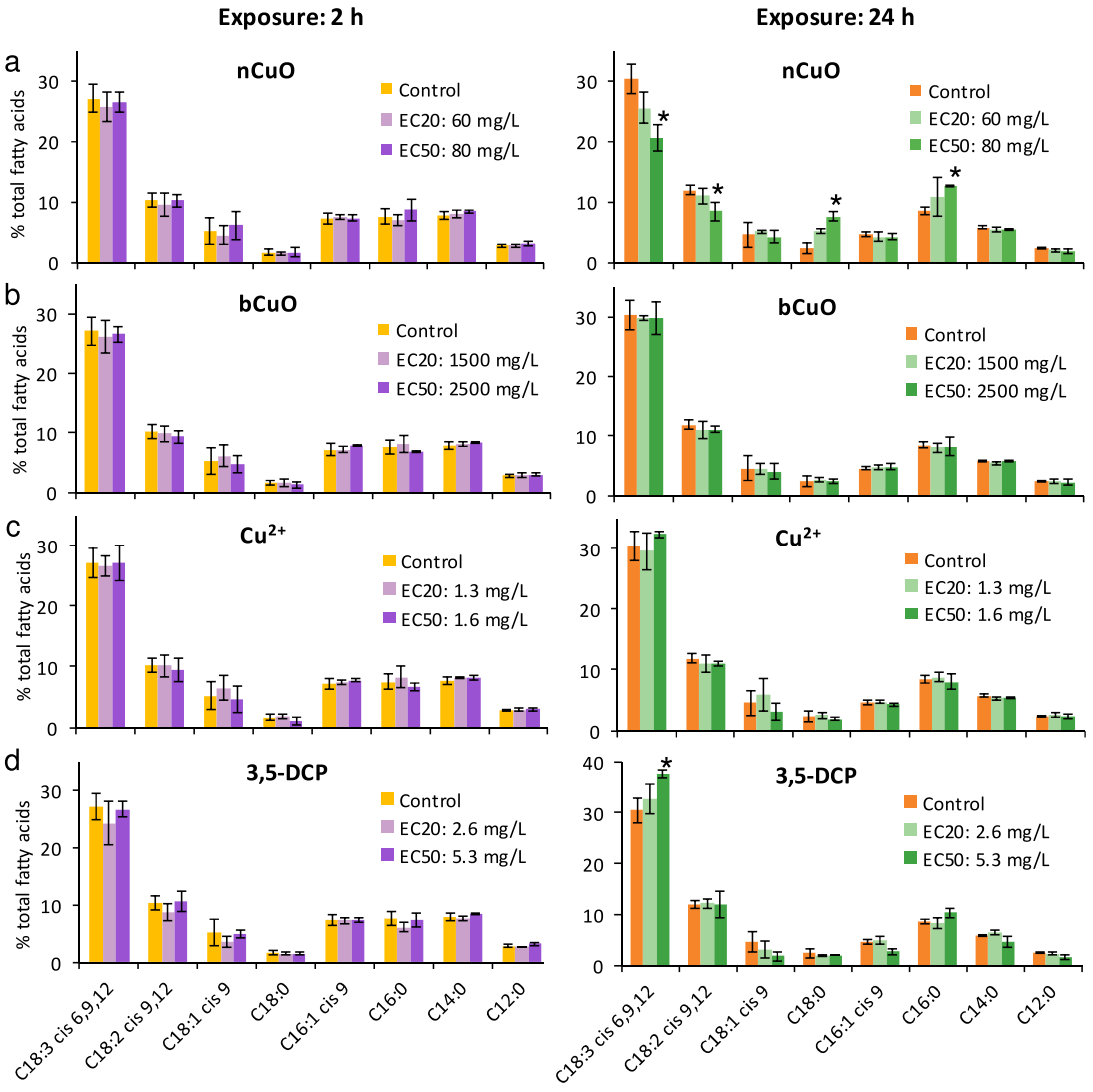


Figure 3. Percent distribution of fatty acids of *Tetrahymena thermophila* after 2- and 24-h exposure (left and right panel, respectively) to nCuO (a), bulk CuO (b), $CuSO_4$ (c) and 3,5-dichorophenol (d) at 24-h EC20 and EC50 concentrations. Incubation was done in Osterhout's medium (starving conditions) at 25 °C. Columns represent mean values of at least three independent experiments ± standard deviation. Significant differences from control are indicated by an asterisk (*), $p < 0.05$.

Table 3. Percentages of saturated and unsaturated fatty acids in *Tetrahymena thermophila*.

Exposures		Saturated fatty acids, %	Unsaturated fatty acids, %	Ratio[a]
Control				
2 h		30.3 ± 1.3	54.1 ± 3.6	0.6
24 h		29.1 ± 1.34	56.9 ± 1.7	0.5
nCuO				
2 h	EC20	30.4 ± 1.3	51.2 ± 7.4	0.6
	EC50	32.9 ± 0.9*	54.2 ± 3.5	0.6
24 h	EC20	34.6 ± 3.8	51.4 ± 3.9	0.7
	EC50	**42.2 ± 1.3***	**42.3 ± 4.2***	**1.0**
bCuO				
2 h	EC20	31.9 ± 1.8	53.2 ± 2.1	0.6
	EC50	31.0 ± 1.9	52.4 ± 4.3	0.6
24 h	EC20	30.2 ± 1.3	55.3 ± 2.8	0.5
	EC50	29.5 ± 2.3	54.9 ± 5.1	0.5
$CuSO_4$				
2 h	EC20	33.8 ± 2.1	55.0 ± 3.1	0.6
	EC50	31.2 ± 0.4	53.0 ± 8.8	0.6
24 h	EC20	29.8 ± 1.7	57.4 ± 2.3	0.5
	EC50	27.5 ± 2.4	55.8 ± 3.0	0.5
3,5-DCP				
2 h	EC20	28.5 ± 1.6	46.4 ± 7.2	0.6
	EC50	32.4 ± 1.8	53.5 ± 4.0	0.6
24 h	EC20	29.1 ± 1.8	57.0 ± 2.0	0.5
	EC50	27.6 ± 2.2	58.2 ± 3.3	0.5

The percentages are the means of 3 independent experiments ± standard deviation.

[a] Ratio of 14 saturated and 12 unsaturated fatty acids.

* Significantly different ($p < 0.01$) from the control at the respective time point

Role of copper ions in toxicity of nCuO. Analysis with copper-sensing bacteria showed that the equitoxic suspensions of bCuO and $CuSO_4$ contained essentially similar amounts of ionic copper, 1.1 ± 0.1 *versus* 1.3 ± 0.2 mg/L, respectively (Figure 4), indicating that the toxicity of bCuO was caused by the solubilized copper ions. In contrast, in the equitoxic suspension of nCuO the concentration of copper ions was significantly lower, 0.5 ± 0.05 mg/L, suggesting that copper ions contributed to the toxic effects of nCuO only partially. This has been previously demonstrated for other types of organisms, for example, yeast,[31] algae,[32] *Daphnia magna*,[33] and duckweed.[34] Although it has been demonstrated that the toxic concentrations of copper ions induce changes in the proportions of SFA and

UFA in plasma membranes of wheat roots[35] as well as in earthworms,[36] it has also been noted in some cases that high extracellular copper concentrations do not affect the membrane fatty acid balance nor cause peroxidation of polyunsaturated fatty acids in, for example, amphipods.[37] It has been suggested that in amphipods and other crustaceans the survival in high copper-containing environments is mediated through efficient storage and regulation/detoxification mechanisms such as binding trace metals to intracellular metallothioneins and production of insoluble intracellular granules.[37] Similar mechanisms involving the formation of refractile granules, which represent a copper ion regulating system, have been proposed also for *Tetrahymena*.[38] Furthermore, in a copper toxicity study on rat liver subcellular membrane fractions it was demonstrated that copper-related toxicity may be caused by indiscriminate binding of the metal to proteins, rather than generation of excess ROS and lipid peroxidation.[39] This could explain the results of the current study, where no changes in fatty acid composition were induced by $CuSO_4$ and bCuO (Figure 3b, c), and no excess TBARS were detected after exposure to $CuSO_4$ in *Tetrahymena* cells (Figure 5a).

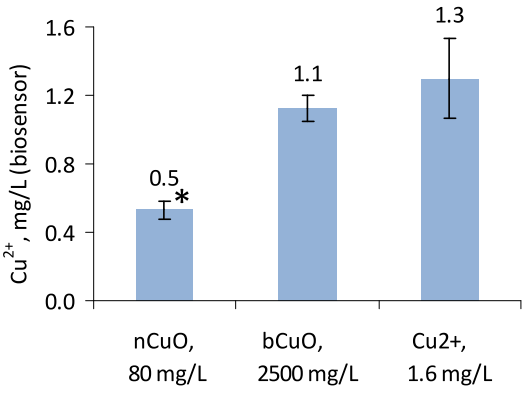


Figure 4. Bioavailable copper concentrations (measured by recombinant bacterial copper-sensor assay) in the suspensions/solutions of nano and bulk CuO and $CuSO_4$ after 24 h in Osterhout's mineral medium at 25°C. Concentrations on x-axis refer to the respective 24-h EC50 nominal concentrations used in the exposures of *T. thermophila* (see Materials and methods). Data are presented as means of three independent experiments ± SD. *significant difference from Cu^{2+} and bulk CuO, $p < 0.05$.

Role of lipid peroxidation and ROS generation in toxicity of nCuO. The TBARS levels generated by nano and bulk CuO were neither time nor concentration dependent (Figure 5a). The EC20 and EC50 concentrations of both, nano and bulk CuO, induced about four times higher levels of TBARS than in the control culture, while the positive control H_2O_2 generated a dose- (EC20 *vs* EC50) and time-dependent (2 h *vs* 24 h) increase in TBARS in *T. thermophila* cells (Figure 5a). This suggests that lipid peroxidation *per se* is not the main mechanism of toxicity for the tested copper compounds. All the tested copper compounds generated excess ROS levels at EC50 concentrations after 24-h exposure (Figure 5b and Figure S3 in the Supporting Information). This is in agreement with the previously published data, where copper has been shown to induce the generation of both hydrogen peroxide and superoxide anions in *Tetrahymena*.[40] In our study nCuO generated significantly higher levels of ROS compared to bCuO and $CuSO_4$ after 24 h exposure at EC50 concentration and nCuO was the only tested copper compound to induce ROS generation in *Tetrahymena* after 2-h exposure (Figure 5b). The higher tolerance to oxidative stress in nCuO exposed cells might be associated with the changes in the fatty acid composition of these cells. Specifically, it has been shown that the changes in lipid biosynthesis, including decrease in unsaturated fatty acids, increase stress resistance and longevity of *C. elegans*.[41] Relatively low ROS levels detected after exposure to EC20 concentration of H_2O_2 indicate further the remarkable ability of *Tetrahymena* to scavenge ROS and possibly decomposed H_2O_2.[42]

Considering the differential lipid peroxidation, ROS generation and fatty acid modifying profiles of nCuO and $CuSO_4$, in addition to the significantly lower dissolved copper content in equitoxic nCuO suspension than in the solution of $CuSO_4$, it can be concluded that the toxic effect of nCuO in *T. thermophila* is not solely caused by the dissolved fraction of the NPs, but rather displays a distinctive toxicity pattern. One of the key factors in the adaptation mechanism to nCuO could be the regulation of specific desaturase activity, thereby lowering membrane fluidity.

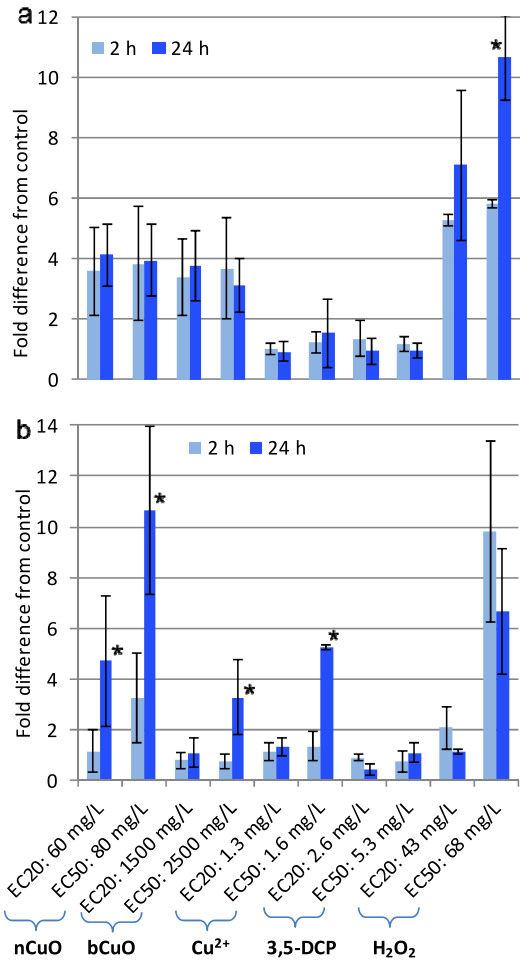


Figure 5. The potential of nCuO to generate lipid peroxidation (a) and ROS (b) in comparison to bulk CuO, $CuSO_4$, 3,5-dichlorophenol (3,5-DCP) and H_2O_2 in *T. thermophila* at the respective EC20 and EC50 concentrations. After 2- and 24-h exposure to the toxicants in Osterhout's medium at 25°C the lipid peroxidation in *T. thermophila* was measured by quantification of TBARS and ROS by DCF fluorescence. The results are presented as fold difference from the non-exposed (control) culture. Data are presented as means of at least three independent experiments ± SD. The asterisk (*) marks significant difference ($p < 0.05$) from the rest of the values (panel a) and from the respective 2-h value and the 24-h EC50 value of nCuO (panel b).

ACKNOWLEDGMENT

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ASSOCIATED CONTENT

Supporting Information. Methods and data on characterization of the size and aggregation of nCuO after exposure to *T. thermophila*, cultivation of protozoa, exposure of *T. thermophila* to nano and bulk CuO, analysis of lipid peroxidation and ROS. This material is available free of charge via the Internet at http://pubs.acs.org.

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SUPPORTING INFORMATION

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

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Summary

Number of pages: 6

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.

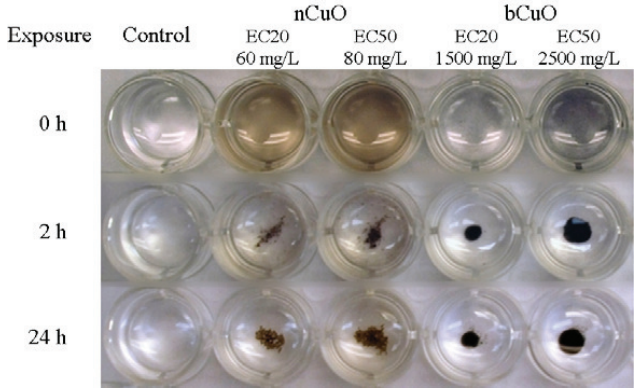

Figure S1. Agglomeration of nano- and bulk CuO in Osterhout's medium during the exposure of *Tetrahymen thermophila*. 500 µL of *T. thermophila* in Osterhout's medium was pipetted into the wells containing 500 µL of nano- or bulk CuO at the respective EC20 and EC50 concentrations. Control: *T. thermophila*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

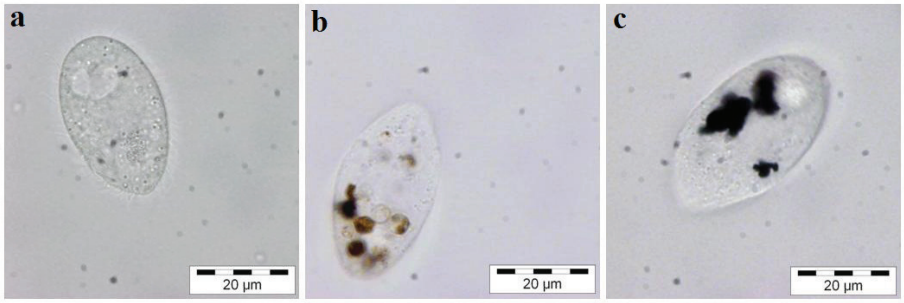

Figure S2. Bright field images of live *Tetrahymena thermophila* cells. Cells were exposed to 80 mg/L nCuO (b) and 2500 mg/L bCuO (c) (the respective EC50 concentrations) in Osterhout's mineral medium for 2 h. Panel a: control, i. e. *T. thermophila* cell after 2 h in Osterhout's medium.

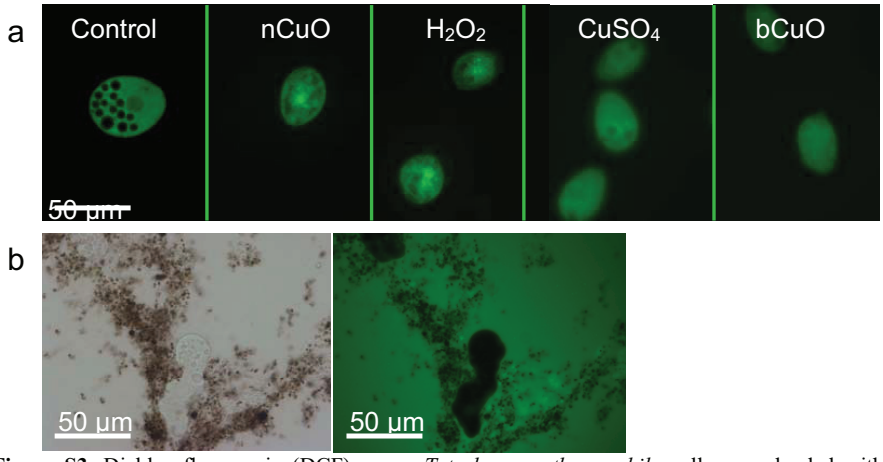

Figure S3. Dichlorofluorescein (DCF) assay. *Tetrahymena thermophila* cells were loaded with H_2DCF-DA, washed and exposed to nCuO and the respective control chemicals for 2 h and for 24 h. Panel a: fluorescence images of *T. thermophila* cells after 2-h exposure to the EC50 concentrations of the chemicals. 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.

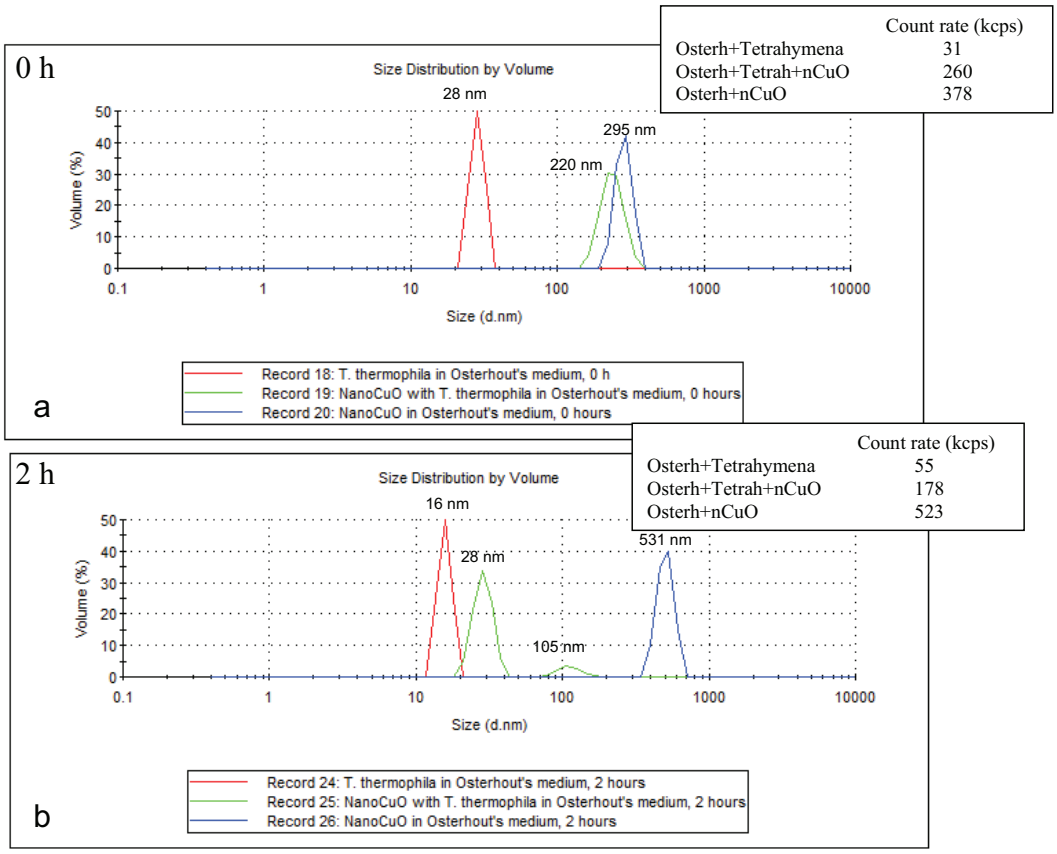

Figure S4. The effect of direct contact of *Tetrahymena thermophila* cells on the hydrodynamic diameter of nCuO in Osterhout's mineral medium, measured by DLS. Panel a: at the beginning of incubation (0 h); panel b: after 2-h incubation at 25 °C. Before the DLS analysis the incubation mixture was centrifuged (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.

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CURRICULUM VITAE

1. Personal data

Name	Monika Mortimer
Date and place of birth	20. 05. 1976, Tallinn, Estonia

2. Contact information

Address	Akadeemia tee 23, 12618, Tallinn, Estonia
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3. Education

2006 – 2010	Tallinn University of Technology, PhD student of Chemistry and Gene Technology
2004 – 2006	Tallinn University of Technology, Master of Science in Natural Sciences
2000 – 2004	Tallinn University of Technology, Bachelor of Science in Natural Sciences
2003 – 2004	Tallinn University, Translation and Interpreting
1994 – 1996	University of Tartu, Biology

4. Language competence/skills (fluent; average, basic skills)

Estonian, English (fluent)

Finnish, Russian (basic)

5. Special Courses

Aug. 21-22, 2010	Clemson University, SC, USA, short course "Nanomaterials in the Environment"
Jan. 4-30, 2010	Slovenia, University of Ljubljana, Biotechnical Faculty, fatty acid methyl ester analysis by gas chromatography
Nov. 16-20, 2009	University Ca'Foscari of Venice, First Nanosafety Autumn School, (Eco)toxicology of Nanoparticles: from Characterization to Risk Assessment
Feb. 11-12, 2008	Estonian Research Institute of Agriculture, course on "Methodology of Research"
Nov. 8-9, 2007	University of Tampere, Medical School, course "Cell Culture Techniques and Alternative Methods in Toxicology"

6. Professional Employment

2007 - ... National Institute of Chemical Physics and Biophysics; Researcher
2006 – 2007 National Institute of Chemical Physics and Biophysics; Engineer
2002 – 2006 Tallinn University of Technology, Faculty of Science, Department of Chemistry; Technician

7. Defended theses

2006 Master's thesis: „Catalytic activity and oxygenation specificity of 11*R*-lipoxygenase", supervisor Dr. Ivar Järving, Tallinn University of Technology, Department of Chemistry, Chair of Bioorganic Chemistry.
2004 Bachelor's thesis: „11*R*-lipoxygenase from the coral *Gersemia fruticosa*", supervisors Dr. Reet Järving and Dr. Ivar Järving, Tallinn University of Technology, Department of Chemistry, Chair of Bioorganic Chemistry.

8. Main areas of scientific work/Current research topics

Determining the potentially harmful effects of engineered nanoparticles at different levels of biological organization (cells, organelles, enzymes).

7. Publications

1) Mortimer, M., Kasemets, K., Vodovnik, M., Marinšek Logar, R., Kahru, A. (2011). Exposure to CuO nanoparticles changes the fatty acid composition of protozoa *Tetrahymena thermophila*. Environmental Science and Technology. Accepted.
2) Sihtmäe, M., Mortimer, M., Kahru, A., Blinova, I. (2010). Toxicity of five anilines to crustaceans, protozoa and bacteria. Journal of the Serbian Chemical Society, 75, 1291 – 1302.
3) Blinova, I., Ivask, A., Heinlaan, M., Mortimer, M., Kahru, A. (2010). Ecotoxicity of nanoparticles of CuO and ZnO in natural water. Environmental Pollution, 158, 41 - 47.
4) Mortimer, M., Kasemets, K., Kahru, A. (2010). Toxicity of ZnO and CuO nanoparticles to ciliated protozoa *Tetrahymena thermophila*. Toxicology, 269, 182 - 189.
5) Mortimer, M., Kasemets, K., Heinlaan, M., Kurvet, I., Kahru, A. (2008). High throughput kinetic *Vibrio fischeri* bioluminescence inhibition assay for study of toxic effects of nanoparticles. Toxicology in Vitro, 22, 1412 – 1417.
6) Mortimer, M., Järving, R., Brash, A. R., Samel, N., Järving, I. (2006). Identification and characterization of an arachidonate 11*R*-lipoxygenase. Archives of Biochemistry and Biophysics, 445, 147 – 155.

ELULOOKIRJELDUS

1. Isikuandmed

Ees- ja perekonnanimi	Monika Mortimer
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2. Kontaktandmed

Aadress	Akadeemia tee 23, 12618, Tallinn
Telefon	+327 6398361
E-posti aadress	monika.mortimer@kbfi.ee

3. Hariduskäik

2006 – 2010	Tallinna Tehnikaülikool, keemia- ja geenitehnoloogia doktorant
2004 – 2006	Tallinna Tehnikaülikool, loodusteaduste magister
2000 – 2004	Tallinna Tehnikaülikool, loodusteaduste bakalaureus keemia- ja materjaliteaduse erialal
2003 – 2004	Tallinna Ülikool, lisaeriala suuline ja kirjalik tõlkimine
1994 – 1996	Tartu Ülikool, bioloogia

4. Keelteoskus (alg-, kesk- või kõrgtase)

eesti, inglise (kõrgtase)
soome, vene (algtase)

5. Täiendusõpe

21.-22. aug. 2010	Clemsoni Ülikool, South-Carolina, USA, lühikursus: "*Nanomaterials in the Environment*"
4.-30. jaan. 2010	Sloveenia Ljubljana Ülikooli biotehnoloogia õppetool, rasvhapete metüülestrite gaaskromatograafiline analüüs
16.-20. nov. 2009	Veneetsia Ca'Foscari Ülikool, esimene nanoohutuse sügiskool, *(Eco)toxicology of Nanoparticles: from Characterization to Risk Assessment*
11.-12. veebr. 2008	Eesti Maaviljeluse Instituut, intensiivkursus "*Methodology of Research*"
8.- 9. nov. 2007	Tampere Ülikool, kursus "*Cell Culture Techniques and Alternative Methods in Toxicology*"

6. Teenistuskäik

2007 - ...	Keemilise ja Bioloogilise Füüsika Instituut; teadur
2006 – 2007	Keemilise ja Bioloogilise Füüsika Instituut; insener

2002 – 2006 Tallinna Tehnikaülikool, Matemaatika-loodusteaduskond, Keemiainstituut; laborant

7. Kaitstud lõputööd

2006 Magistritöö „11*R*-lipoksügenaasi katalüütiline aktiivsus ja oksüdatsioonispetsiifilisus“, juhendaja vanemteadur Ivar Järving, Tallinna Tehnikaülikool, Keemiainstituut, Bioorgaanilise keemia õppetool.

2004 Bakalaureusetöö: „11*R*-lipoksügenaas korallist *Gersemia fruticosa*“, juhendajad vanemteadurid Reet Järving and Ivar Järving, Tallinna Tehnikaülikool, Keemiainstituut, Bioorgaanilise keemia õppetool.

8. Teadustöö põhisuunad

Nanoosakeste potentsiaalselt kahjulike efektide kindlakstegemine erinevatel bioloogilise organisasiooni tasemetel (rakud, organellid, ensüümid).

DISSERTATIONS DEFENDED AT TALLINN UNIVERSITY OF TECHNOLOGY ON *NATURAL AND EXACT SCIENCES*

1. **Olav Kongas**. Nonlinear Dynamics in Modeling Cardiac Arrhytmias. 1998.

2. **Kalju Vanatalu**. Optimization of Processes of Microbial Biosynthesis of Isotopically Labeled Biomolecules and Their Complexes. 1999.

3. **Ahto Buldas**. An Algebraic Approach to the Structure of Graphs. 1999.

4. **Monika Drews**. A Metabolic Study of Insect Cells in Batch and Continuous Culture: Application of Chemostat and Turbidostat to the Production of Recombinant Proteins. 1999.

5. **Eola Valdre**. Endothelial-Specific Regulation of Vessel Formation: Role of Receptor Tyrosine Kinases. 2000.

6. **Kalju Lott**. Doping and Defect Thermodynamic Equilibrium in ZnS. 2000.

7. **Reet Koljak**. Novel Fatty Acid Dioxygenases from the Corals *Plexaura homomalla* and *Gersemia fruticosa.* 2001.

8. **Anne Paju**. Asymmetric oxidation of Prochiral and Racemic Ketones by Using Sharpless Catalyst. 2001.

9. **Marko Vendelin**. Cardiac Mechanoenergetics *in silico*. 2001.

10. **Pearu Peterson**. Multi-Soliton Interactions and the Inverse Problem of Wave Crest. 2001.

11. **Anne Menert**. Microcalorimetry of Anaerobic Digestion. 2001.

12. **Toomas Tiivel**. The Role of the Mitochondrial Outer Membrane in *in vivo* Regulation of Respiration in Normal Heart and Skeletal Muscle Cell. 2002.

13. **Olle Hints**. Ordovician Scolecodonts of Estonia and Neighbouring Areas: Taxonomy, Distribution, Palaeoecology, and Application. 2002.

14. **Jaak Nõlvak**. Chitinozoan Biostratigrapy in the Ordovician of Baltoscandia. 2002.

15. **Liivi Kluge**. On Algebraic Structure of Pre-Operad. 2002.

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17. **Janek Peterson**. Synthesis, Structural Characterization and Modification of PAMAM Dendrimers. 2002.

18. **Merike Vaher**. Room Temperature Ionic Liquids as Background Electrolyte Additives in Capillary Electrophoresis. 2002.

19. **Valdek Mikli**. Electron Microscopy and Image Analysis Study of Powdered Hardmetal Materials and Optoelectronic Thin Films. 2003.

20. **Mart Viljus**. The Microstructure and Properties of Fine-Grained Cermets. 2003.

21. **Signe Kask**. Identification and Characterization of Dairy-Related *Lactobacillus.* 2003.

22. **Tiiu-Mai Laht**. Influence of Microstructure of the Curd on Enzymatic and Microbiological Processes in Swiss-Type Cheese. 2003.

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43. **Igor Golovtsov**. Modification of Conductive Properties and Processability of Polyparaphenylene, Polypyrrole and polyaniline. 2005.

44. **Katrin Laos**. Interaction Between Furcellaran and the Globular Proteins (Bovine Serum Albumin β-Lactoglobulin). 2005.

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50. **Merle Uudsemaa**. Quantum-Chemical Modeling of Solvated First Row Transition Metal Ions. 2006.

51. **Tagli Pitsi**. Nutrition Situation of Pre-School Children in Estonia from 1995 to 2004. 2006.

52. **Angela Ivask**. Luminescent Recombinant Sensor Bacteria for the Analysis of Bioavailable Heavy Metals. 2006.

53. **Tiina Lõugas**. Study on Physico-Chemical Properties and Some Bioactive Compounds of Sea Buckthorn (*Hippophae rhamnoides* L.). 2006.

54. **Kaja Kasemets**. Effect of Changing Environmental Conditions on the Fermentative Growth of *Saccharomyces cerevisae* S288C: Auxo-accelerostat Study. 2006.

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58. **Tõnu Martma**. Application of Carbon Isotopes to the Study of the Ordovician and Silurian of the Baltic. 2006.

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89. **Merike Sõmera**. Sobemoviruses: Genomic Organization, Potential for Recombination and Necessity of P1 in Systemic Infection. 2010.

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91. **Kristin Lippur**. Asymmetric Synthesis of 2,2'-Bimorpholine and its 5,5'-Substituted Derivatives. 2010.

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94. **Tamara Aid-Pavlidis**. Structure and Regulation of BDNF Gene. 2010.

95. **Olga Bragina**. The Role of Sonic Hedgehog Pathway in Neuro- and Tumorigenesis. 2010.

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99. **Alla Maloverjan**. Vertebrate Homologues of Drosophila Fused Kinase and Their Role in Sonic Hedgehog Signalling Pathway. 2010.

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102. **Atanas Katerski**. Chemical Composition of Sprayed Copper Indium Disulfide Films for Nanostructured Solar Cells. 2011.

103. **Kristi Timmo.** Formation of Properties of $CuInSe_2$ and $Cu_2ZnSn(S,Se)_4$ Monograin Powders Synthesized in Molten KI. 2011.

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112. **Natalja Makarytsheva**. Analysis of Organic Species in Sediments and Soil by High Performance Separation Methods. 2011.