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Development of Bacterial Biosensors and Human Stem Cell-Based *In Vitro* Assays for the Toxicological Profiling of Synthetic Nanoparticles

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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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Rekombinantsetel sensorbakteritel ja inimese tüvirakkudel põhinevate *in vitro* testide väljatöötamine sünteetiliste nanoosakeste toksikoloogiliseks uurimiseks

OLESJA BONDARENKO

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KIRJASTUS

To all good people surrounding me

TABLE OF CONTENTS

LIST OF ORIGINAL PUBLICATIONS

MAIN PUBLICATIONS

I Ivask A, **Bondarenko O**, Jepihhina N, Kahru A **(2010)** Profiling of the reactive oxygen species-related ecotoxicity of CuO, ZnO, TiO_2, silver and fullerene nanoparticles using a set of recombinant luminescent *Escherichia coli* strains: differentiating the impact of particles and solubilised metals. Analytical and Bioanalytical Chemistry, 398, 701 - 716

II Käkinen A, **Bondarenko O**, Ivask A, Kahru A **(2011)** The effect of composition of different ecotoxicological test media on free and bioavailable copper from $CuSO_4$ and CuO nanoparticles: comparative evidence from a Cu-selective electrode and a Cu-biosensor. Sensors, 11(11), 10502 – 10521

III **Bondarenko O**, Ivask A, Käkinen A, Kahru A **(2012)** Sub-toxic effects of CuO nanoparticles on bacteria: kinetics, role of Cu ions and possible mechanisms of action. Environmental Pollution 169, 81 - 89

IV Hoelting L, Scheinhardt B, **Bondarenko O**, Schildknecht S, Kapitza M, Tanavde V, Lee QY, Mecking S, Leist M, Kadereit S. A 3-dimensional human embryonic stem cell (hESC)-derived model to detect developmental neurotoxicity of nanoparticles (Nano-DNT**)**. Archives of Toxicology (to be accepted after minor revision)

AUTHORS CONTRIBUTION TO THE PUBLICATIONS

I Olesja Bondarenko (OB) participated in the study design, constructed the luminescent superoxide-deficient *Escherichia coli* strains and performed the assays with superoxide-anion biosensor *E. coli* K12::soxRSsodAlux. She interpreted the data and participated in the preparation of the manuscript.

II OB participated in the study design, performed the assays with *Pseudomonas fluorescens* OS8::KnCueRPcopAlux Cu-biosensor, interpreted the data and participated in the preparation of the manuscript.

III OB was responsible for the study design, constructed the hydrogen peroxide-specific biosensor strain *E. coli* K12::katGlux and the DNA damage-inducible strain *E. coli* MC1061(pDEWrecAlux). She performed all the assays except the characterization of CuO particles and EDTA chelation experiment. She was the major interpreter of data and corresponding author of the manuscript.

IV OB participated in the study design, performed the differentiation of hESC cells, performed a part of the toxicity assays with methylmercury and a part of the toxicity assays with polyethylene and CuO nanoparticles. She took the fluorescence microscopy images, isolated RNA and performed real-time PCR analysis for a couple of replicates. She interpreted the data and participated in the preparation of the manuscript.

Copies of the Publications **I-IV** are included into the thesis.

OTHER PUBLICATIONS IN PEER-REVIEWED JOURNALS

Ivask A, George S, **Bondarenko O**, Kahru A **(2012)** Metal-containing nano-antimicrobials: differentiating the impact of solubilized metals and particles. Nicola Cioffi (Eds). Springer, Nano-antimicrobials - Progress and Prospects, pp 253-290

Blinova I, Bityukova L, Kasemets K, Ivask A, Käkinen A, Kurvet I, **Bondarenko O**, Kanarbik L, Sihtmäe M, Aruoja V, Schvede H, Kahru A **(2012)** Environmental hazard of oil shale combustion fly ash. Journal of Hazardous Materials 229-230, 192-200

Kurvet I, Ivask A, **Bondarenko O,** Sihtmäe M, Kahru A **(2011)** *LuxCDABE* - transformed constitutively bioluminescent *Escherichia coli* for toxicity screening: comparison with naturally luminous *Vibrio fischeri*. Sensors, 11(8), 7865-7878

Bondarenko O, Rahman PKSM, Rahman TJ, Kahru A, Ivask A **(2010)** Effects of rhamnolipids from *Pseudomonas aeruginosa* DS10-129 on luminescent bacteria: toxicity and modulation of cadmium bioavailability. Microbial Ecology, 59(3), 588-600

Bondarenko O, Rõlova T, Kahru A, Ivask A **(2008)** Bioavailability of Cd, Zn and Hg in soil to nine recombinant luminescent metal sensor bacteria. Sensors, 8, 6899-6923

INTRODUCTION

According to the new EU chemicals policy more than 100,000 chemicals produced in the EU in quantities exceeding 1 t/year should be toxicologically characterized by the year 2018. This chemicals policy covers also synthetic nanoparticles − particles with at least one dimension between 1 and 100 nm − which are increasingly produced and already used in thousands of industrial and household products. However, the small size of nanoparticles that determines their novel economically beneficial physico-chemical properties may also increase environmental toxicity and adverse health effects. The main proposed toxicity mechanism of synthetic nanoparticles is the generation of reactive oxygen species, which may lead to the cellular oxidative stress. Due to the practically infinite number of different nanoparticles and their variations, the traditional toxicity testing strategies that involve large-scale low-throughput animal testing, are inefficient and ethically questionable. Thus, there is a need for a transition from traditional descriptive *in vivo* testing to the rapid and cost-effective high-throughput *in vitro* assays.

The main objective of the current study was to develop and apply a suite of *in vitro* bioassays for the mechanistic toxicological analysis of synthetic nanoparticles. The focus was set on construction and application of bioluminescent bacterial biosensors. For that, bacterial cells were genetically modified to produce and increase their bioluminescence in response to intracellular (i) reactive oxygen species, (ii) DNA damage and (iii) metal ions or (iv) decrease the bioluminescence upon cellular oxidative stress. The developed bacterial biosensors were evaluated using different positive and negative controls and applied for the elucidation of toxicity mechanisms of nanoparticles of C_{60}-fullerene, CuO, Ag, ZnO and TiO_2. In total, 16 different bioluminescent bacterial strains were used, 11 of which were constructed in this study.

In addition to the bacterial biosensors, human cells *in vitro* were used for toxicological characterization of CuO and polyethylene nanoparticles. Using three-dimensional *in vitro* test model, in which human embryonic stem cells were differentiated into neuronal precursor cells, the potential neurotoxic effects of CuO and polyethylene nanoparticles were demonstrated.

Testing approaches based on both prokaryotic and eukaryotic cells contribute to the EU strategy for development of novel *in vitro* test systems intending to reduce, replace and refine *in vivo* experiments. The developed test assays based on bacterial biosensors are being currently implemented into the EU FP7 project NanoValid, which aims to work out the reference methods and standardized test protocols for the toxicological profiling of nanoparticles.

ABBREVIATIONS

3.5-DCP	3.5-dichlorophenol
AAS	atomic absorption spectroscopy
CAS number	unique number given to chemicals by the Chemical Abstracts Service
Cu-ISE	Cu-ion selective electrode
DI	deionized water
DLS	dynamic light scattering
EC_{20}	effective concentration 20%; refers to the concentration of test substance that cause studied adverse effect to 20% of the test organisms within specified exposure time
EC_{50}	effective concentration 50%
EDTA	ethylenediaminetetraacetic acid
GFP	green fluorescent protein
hESC	human embryonic stem cells
HMM	heavy metal MOPS medium
ICP-MS	inductively coupled plasma mass spectrometry
ISE	ion-selective electrode
ISO	International Organization for Standardization
IUPAC	International Union of Pure and Applied Chemistry
LB	Luria-Bertani medium
LOD	limit of determination
MEFs	mouse embryonic fibroblasts
MMC	mitomycin C
MOPS	3-(N-morpholino)propanesulfonic acid
MSDS	material safety data sheet
NEPs	neuroepithelial cells
NP(s)	nanoparticle(s)
OD	optical density
OECD	Organization for Economic Cooperation and Development
REACH	EU chemical policy on Registration, Evaluation, Authorization and Restriction of Chemicals
ROS	reactive oxygen species
RT-PCR	real-time PCR
SEM	scanning electron microscopy
SOD	superoxide dismutase
TEM	transmission electron microscopy
TGF-β	transforming growth factor β

1. LITERATURE REVIEW

1.1. Synthetic nanoparticles and nanotoxicology

In 1959, the Nobel Laureate in physics Richard Feynman challenged the scientists to "Think small"- referring to what could be achieved if we were able to manipulate matter in nanoscale (Feynman, 1959). To many, this was the beginning of the concept of nanoscience. The practical development of nanoscience started in the early 1980s, facilitated by the advent of scanning tunneling microscopy (Hussain et al., 2006). Nowadays, nanoindustry is a billion-industry, being one of the fastest growing industries in the history of mankind (National Nanotechnology Initiative, 2012). Around 10,000 unique patents were applied in nanotechnology by the year 2008, e.g., for the military and aerospace needs (Dang et al., 2010), and several Nobel Prizes for the discoveries in nanotechnology have been awarded (e.g., in chemistry in 1996 for the discovery of fullerene and in physics in 2010 for experiments with graphene). Despite of the recent global recession in 2009, the world market for nanomaterials is undergoing rapid expansion, which highlights the high-value nature of nanotechnological products.

The principal components of nanotechnological products are synthetic nanoparticles (NPs) - particles with at least one of the dimensions between 1 and 100 nanometers (ISO, 2008). Natural and anthropogenic NPs have always existed in our environment (Klaine et al., 2008); synthetic NPs, however, have been manufactured only since 1990s either by "milling" of the bulk particles into smaller pieces or by controlled growth from supramolecular structures (Raab et al., 2011). As NPs are chemically identical to their bulk counterparts and have the same CAS numbers, they are not usually recognized by industry as a new class of chemicals. For this reason, NPs are produced without special restrictions and are already used in thousands of house-hold products (Cassee et al., 2011), e.g., antibacterial textiles (Cerkez et al., 2012), toothpaste, beauty products, sunscreens (Serpone et al., 2007) and a variety of electronic devices (Maynard, 2007). People and the environment are being exposed to these NPs-containing products on a daily basis without knowledge on NPs safety. However, there is increasing evidence that the unique desired physico-chemical properties of NPs, which make nanomaterials more efficient for industrial applications, turn these nanomaterials also more harmful to living organisms.

The research into toxicological effects of NPs serves two opposing goals: on the one hand, we need NPs that exert specific properties, e.g., are effective in killing antibiotic-resistant bacteria and cancer cells but on the other hand, we want these NPs to be safe for the environment and human health. Thus, an important challenge for nanotoxicologists is to decrease the environmental toxicity and health risks of NPs to human without compromising their intended function (Xia et al., 2011). For that, it is crucial (i) to address the toxicity of NPs and (ii) to understand the mechanism behind the observed toxicity.

1.1.1. Toxicity mechanisms of synthetic nanoparticles

The transition from bulk particles to NPs yields dramatic changes in their physico-chemical properties (Hussain et al., 2006). With the decreasing size of particles, the number of atoms on the particle surface increases, leading to increased specific surface area and thus, increased reactivity (Figure 1).

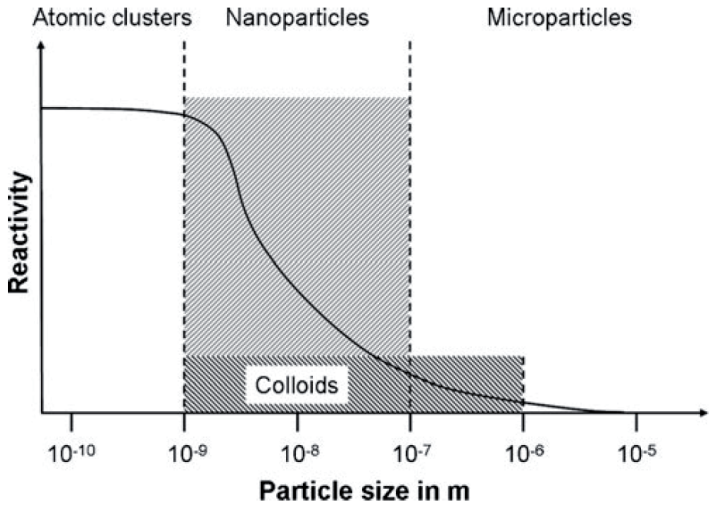

Figure 1. Increased reactivity of nanoparticles (NPs) compared to their micro-sized counterparts (Delay and Frimmel, 2012). Reprinted with the permission of Springer.

Since many important physico-chemical interactions depend on the surface area, NPs exhibit substantially different properties from their macro-scale counterparts of the same composition (Nel et al., 2006; Warheit, 2008). Increased reactivity of NPs enhances the interactions of NPs with the surrounding environment (cell membranes, proteins, DNA, oxygen) (Xia et al., 2006). The main consequence of these interactions is production of reactive oxygen species (ROS) - small active derivates of molecular oxygen, e.g., hydroxyl radical (·OH), hydrogen peroxide (H_2O_2), superoxide anion (O_2^-) and others. Although ROS are also formed as natural byproducts of normal oxygen metabolism and control some cellular processes (Droge, 2002), their levels in the cells should be carefully regulated. This is achieved by various non-enzymatic (α-tocopherol, ascorbic acid and glutathione) and enzymatic (superoxide dismutases, catalases, glutathione peroxidases and glutathione S-transferases) ROS defense systems. Any imbalance in the capacity of ROS defense systems to remove ROS either due to defects in ROS defence systems or due to excess of ROS produced results in a deleterious state called oxidative stress (Fridovich and Freeman, 1986). The tendency to induce oxidative stress is a common property of all NPs and is therefore currently the main paradigm for the explanation of their toxicity (Nel et al., 2006 and 2012). However, in the case of metal-containing NPs – the main objects of this study – oxidative damage that is caused by the particles' surface may be accompanied by toxic effects derived from dissolution (release of metal ions) (Choi and Hu, 2008; Puzyn et al., 2011). Thus, in the case of metal-containing NPs, the key toxicity descriptor ROS may originate from at least two sources:

(i) NPs dissolve and release toxic metal ions, which induce ROS-triggering redox-reactions (a metal-ion-specific effect)

(ii) the surface of the metal-containing NPs interacts with molecular oxygen or biomolecules, inducing ROS directly (a nanosize-specific effect)

Based on the current literature, it is still unclear which of these toxicity mechanisms prevails in the case of metal-containing nanoparticles (Cioffi et al., 2005; Heinlaan et al., 2010; Gunawan et al., 2011). From the scientific viewpoint, the differentiation between these two mechanisms is very important. The dissolution-related mechanism can be relatively easily described as the toxicology of metals has been thoroughly studied. However, the nanosize-specific effects may be unpredictable. Bacterial cells are especially relevant for the differentiation between dissolution and particle-specific effects, because bacteria have no endocytosis. Thus, bacteria are *a priori* protected against NPs entry by their rigid cell wall and can mostly be damaged by NPs that are located outside the cells or *via* dissolution of NPs. As exception, NPs less than 10 nm in size were shown to penetrate the cell membrane of bacteria (Morones et al., 2005). Differently from bacteria, most eukaryotic cells may internalize NPs relatively easily and, thus, respond to NPs differently (Arvizo et al., 2012).

Besides the size, chemical composition and cell model used, the toxicity mechanisms of NPs depend on their shape, surface charge, surface modifications (Kunzmann et al., 2011; Arvizo et al., 2012) and test environment, whereas the latter is probably the biggest reason of uncertainty and complexity. As amino acids, salts and other components that are commonly added to the toxicity test environment often destabilize NPs, leading to their aggregation or dissolution, the carefully chosen test environment and characterization of NPs in the test conditions is crucial for the interpretation of NPs toxicity data (Warheit, 2008). Obviously, the interactions of different types of NPs with test environment and their mechanisms of toxicity differ and should be investigated case-by-case. Next chapter summarizes the available toxicological data for selected metal-containing NPs of TiO_2, ZnO, Ag and CuO (the main focus of this study) and NPs of polyethylene and C_{60}-fullerene. All the selected metal-containing NPs except CuO belong to the representative list of 14 NPs compiled by the Organization for Economic Cooperation and Development (OECD) (report ENV/JM/MONO (2008)13/REV).

1.1.1.1. C_{60}-fullerene

A 60-carbon atom hollow sphere, also known as the buckyball fullerene, was the first type of nanoparticle manufactured (Kroto et al., 1985). Fullerene was set to be produced on an industrial scale due to its unique superconductor properties (King, 1999). The structure of a C_{60}-fullerene permits its active participation in many free-radical and electron-transfer processes, generating ROS (Sayes et al., 2004) or on the other hand, quenching the ROS (Johnston et al., 2010). The pioneering studies by Lovern and Klaper (2006) and Oberdörster et al. (2006) and

further studies by others demonstrated that fullerenes were toxic to different aquatic organisms. Interestingly, because of the high hydrophobicity of underivatized C_{60}-fullerene, most of these studies utilized organic solvents like tetrahydrofuran or toluene as a solubilizing vehicle for the toxicity tests, which led to several false conclusions on toxicity of fullerene. Namely, it was recently shown that tetrahydrofuran may be transformed into toxic byproducts during preparation and most of the toxicity of fullerene observed in the previous studies was due to toxicity of the solvent tetrahydrofuran (Kovochich et al., 2009). Indeed, the very recent study by Dai et al (2012) showed that fullerene dispersed in tetrahydrofuran was toxic to *Escherichia coli* cells, while fullerene dispersed in water or toluene had a cytoprotective role. In addition, some other studies have shown that fullerene had cytoprotective antioxidant properties in rodents *in vivo* (Gharbi et al., 2005). Thus, the toxicological information on fullerene remains controversial.

1.1.1.2. Nano-TiO_2

TiO_2 is one of the most widely used nanoscale material to date – the annual global production of TiO_2 NPs is around 10,000 tons (Piccinno et al., 2012) and has been predicted to increase to 260,000 tons by 2015 (Robichaud et al., 2009). TiO_2 NPs are incorporated into industrial products like paints, lacquers, plastics, papers and into consumer products such as toothpastes, dental implants, food additives (as whitener E171), sporting goods and sunscreens (as UV scatter) (Philips and Babrano, 1997; Schumacher et al., 2004; Mueller and Novack, 2008; Robichaud et al., 2009). Nano-TiO_2 is released into environment *via* landfills, sewage treatment and waste incineration plants and the predicted concentrations of TiO_2 in soils and sediments are the highest among other high-production NPs (Gottschalk et al., 2009). According to the estimations, predicted environmental concentrations of TiO_2 in surface waters are high enough (0.7-16 μg/l) to pose a threat to aquatic organisms (Mueller and Novack, 2008). The adverse effects of TiO_2 NPs are at least partly photocatalytic: TiO_2 NPs absorb UV-light, leading to the generation of ROS including $OH\cdot$ and O_2^- (Serpone et al., 2007). In acute toxicity experiments with bacteria, photoactivated TiO_2 NPs can kill both Gram-negative and Gram-positive bacteria including pathogens such as *E. coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* (Wei et al., 1994; Kühn et al., 2003; Chen et al., 2010a), but antimicrobial concentrations of TiO_2 NPs are quite high, varying usually between 100 and 1000 mg/l.

In *vitro* studies of TiO_2 NPs in mammalian cell cultures showed the uptake of NPs, which however, did not elicit any adverse effects in the dark (Xia et al., 2008). Also in fish cells *in vitro,* 50 mg/l TiO_2 NPs were not toxic nor genotoxic in dark, but induced the formation of ROS and oxidative DNA lesions under illumination (Vevers and Jha, 2008). Thus, the proposed mechanism of toxicity of TiO_2 *in vitro* has been considered oxidative stress as a result of photoactivity and redox properties of TiO_2 (Nel et al., 2009). Remarkably, *in vivo* oral and topical toxicity data on rodents suggest that TiO_2 NPs have low systemic toxicity and are well tolerated on the skin (Nohynek et al., 2007).

1.1.1.3. Nano-ZnO

The second highest production volume metal-containing NPs are nano-ZnO. Around 550 tons of ZnO NPs are produced annually (Piccinno et al., 2012). The applications of ZnO NPs include packaging materials in food industry (Tankhiwale and Bajpai, 2012), white paints and ceramics (ATSDR, 2005), antimicrobial wallpapers and textile (Ghule et al., 2006), UV-light scattering additives in cosmetics (Schumacher et al., 2004; Serpone et al., 2007), baby powders, antiseptic deodorants and soaps (Sweetman, 2005).

ZnO NPs are shown to be relatively toxic to bacterial cells: 5-h EC_{50} 100 mg/l (Zhang et al., 2007), 8-h EC_{50} 80 mg/l (Heinlaan et al., 2008); mammalian cell cultures: 6-h EC_{20} 50 mg/l (Xia et al., 2008), 18-h EC_{50} 80 mg/l (Karlsson et al., 2008) and highly toxic to aquatic organisms: 72-h EC_{50} 0.04 mg/l for algae (Aruoja et al., 2009), 24-h EC_{50} 2.6 mg/l for crustacean (Heinlaan et al., 2008). Also the material safety data sheet (MSDS) for ZnO NPs on Sigma-Aldrich webpage was recently updated for labeling ZnO NPs as "very toxic to aquatic life with long lasting effects" (Sigma-Aldrich, 2011).

In some studies the toxic effects of ZnO NPs for various types of aquatic organisms (crustaceans, algae, and bacteria) were explicable by NPs dissolution (Heinlaan et al., 2008; Aruoja et al., 2009; Wong et al., 2010; Publication **I**). However, there are other studies visualizing the morphological changes of bacterial cell membranes after their contact with ZnO NPs by transmission electron microscopy (TEM) (Brayner et al., 2006) and arguing that ZnO NPs may also have nanosize-specific effects (Jiang et al., 2009).

Studies on toxicity mechanisms of ZnO NPs in mammalian cells *in vitro* show that ZnO NPs are internalized by the cells and enter acidifying lysosomes, where they dissolve, leading to the generation of ROS, lysosomal damage, inflammation and cell death (Xia et al., 2008; Nel et al., 2009). Systemic *in vivo* toxicological studies have been mostly conducted using inhalation toxicity testing. *In vivo* inhalation of ultrafine ZnO particles at high dose (500 mg/m^3) for 2 hours did not induce acute systemic effects in humans (Beckett et al., 2005).

1.1.1.4. Nano-Ag

Similarly to ZnO, Ag NPs have been long known for their antibacterial properties. With the development of nanotechnology, the applications of silver have been extended further (Mueller and Nowack, 2008). Ag NPs are one of the first commercial nanoparticle-based products that prevent infection and enhance wound healing (in Acticoat wound dressings since 2001, NuCrust Pharmaceuticals). Currently, Ag NPs are used as antimicrobials in over 300 consumer products (The Project on Emerging Nanotechnologies, 2012) ranging from cosmetic applications, clothing, shoes, detergents, dietary supplements to surface coatings in respirators, water filters, phones, laptops, children's toys and commercial home water purification systems such as Aquapure, Kinetico and QSI-Nano (Marambio-Jones and Hoek 2010; Cerkez et al., 2012).

Ag NPs are inhibiting the growth of bacteria at remarkably low concentrations: 0.2 -60 mg/l (Gajjar et al., 2009; Vertelov et al., 2009). Ag nanoparticles proved their effectiveness against several bacterial pathogens such as *P. aeruginosa, S. aureus* and *Enterococcus faecalis* with the minimal inhibitory concentrations of 1.6-13.5 mg/l (Kvitek et al., 2008). Several authors have demonstrated that the bactericidal effect of silver nanoparticles increases as the size decreases (Panacek et al., 2006; Choi and Hu, 2008), probably because smaller particles have greater binding affinity for the cell membranes of bacteria (Morones et al., 2005). The smallest particles of 25 nm studied by Panacek et al. (2006) showed the highest bactericidal activity against Gram-positive and Gram-negative bacteria, including highly multiresistant strains such as methicillin-resistant *S. aureus*. In another study the inhibitory effects of Ag NPs to nitrifying bacteria correlated with the fraction of NPs less than 5 nm in size (Choi and Hu, 2008).

In general, the mode of action of Ag NPs is thought to be broadly similar to that of silver ions (Pal et al., 2009): Ag NPs produce Ag ions, which move into the cell, bind SH groups of proteins and generate ROS (Hwang et al., 2008; Publication **I**). However, in some cases the nanoparticles were reported to be toxic to bacteria in significantly lower concentration than Ag ions (Lok et al., 2006). The fact the Ag NPs may induce membrane damage and subsequently, higher uptake of Ag ions, has been suggested as the main reason for this high toxicity of Ag NPs.

In human cells *in vitro* (lung fibroblast cells IMR-90 and human glioblastoma cells U251), Ag NPs reduced ATP contents, caused mitochondrial and DNA damage, chromosomal aberrations and induced ROS. TEM analysis indicated the presence of Ag NPs inside mitochondria and nucleus of human cells *in vitro*, implicating their direct involvement in mitochondrial toxicity and DNA damage (AshaRani et al., 2009). It is important to note that all these effects in human cells were observed in cell cultures and starting from around 200 mg/l of Ag NPs, which is most probably too high concentration, to be reached in the human organism *in vivo* during real exposure scenarios. Also, *in vivo* inhalation experiments with rats have established lung function changes and inflammation upon chronic (90-days 6 h/day) inhalation of Ag NPs, but only at the highest dose tested, i.e., 2.9 x 10^6 particles/cm^3 (Sung et al., 2008). Thus, it seems that Ag NPs are significantly less toxic in eukaryotic than in prokaryotic cells.

1.1.1.5. Nano-CuO

In contrast to the previously described metal-containing NPs, CuO NPs are manufactured and used at lower quantities and the potential hazardous effects of CuO NPs are poorly studied (Kahru and Dubourguier, 2010). CuO NPs are applied in antimicrobial textiles, hospital equipment, wood preservation and antifouling paints (Gabbay et al., 2006). The most important and unique application area of CuO NPs is probably technology (semiconductors, electronic chips, heat transfer nanofluids), as CuO has excellent thermophysical properties (Ebrahimnia-Bajestan et al., 2011). On the other hand, CuO has been shown to be toxic to aquatic organisms such as zebrafish, crustaceans *Daphnia magna* and

Thamnocephalus platyurus and algae *Pseudokirchneriella subcapitata* - already at remarkably low concentrations (24-72-h EC_{50} 1.5; 3.2; 0.18 and <1 mg/L, respectively) (Griffitt et al., 2007; Heinlaan et al., 2008; Aruoja et al., 2009). CuO NPs were also moderately toxic to mammalian cells in *vitro* with the 4-18 h EC_{50} of 20-80 mg/l (Karlsson et al., 2008; Midander et al., 2009). Among different metal-containing NPs tested by Karlsson et al. (2008) (CuO, TiO_2, ZnO and various Fe oxides), CuO NPs proved most toxic to the human lung epithelial cell line A549, whereas 40 mg/l CuO induced oxidative stress and DNA damage. In addition, *in vivo* studies with juvenile carp (*Cyprinus carpio*) showed that CuO NPs (100 mg/l) can translocate into the brain and inhibit cholinesterase (Zhao et al., 2011). Potential neurotoxicity of CuO NPs was also demonstrated *in vitro* in hippocampal CA1 neurons of rats, where CuO NPs inhibited the sodium and potassium voltage-gated currents (Xu et al., 2009; Liu et al., 2011). Also Cu ions released from CuO NPs may be involved in neurodegenerative diseases (Rossi et al, 2006).

1.1.1.6. Nano-polyethylene

Differently from the above described metal-containing NPs that are mostly used in different consumer applications, some types of NPs are purposely designed for targeting live cells and imaging of different organs, e. g. brain (Kircher et al., 2003; Corot et al., 2004; Cengelli et al., 2006). For brain-targeting, these NPs have to be designed to pass the blood-brain-barrier. These NPs carry covalently linked magnetic resonance imaging contrast agents such as fluorescent and visible dyes, which makes them suitable for the visualization of brain pathologies and theoretically usable in treatment of brain diseases as brain-targeting drug delivery systems (Kreuter, 1995; Roney et al., 2005). Polyethylene is of interest as a component for such particles as the simplest organic polymer in terms of molecular structure (Pecher et al., 2010). In addition, due to the inertness toward hydrolysis and other decomposition reactions, polyethylene is one of the most frequently used plastics and has been therefore investigated more intensely than other polymers (Yu and Mecking, 2009). The polyethylene NPs could be efficiently taken up by HeLa cells without any toxic effects (Pecher et al., 2010). However, as the final goal for these nanoparticles is brain targeting and these NPs are designed to be purposely directed into the brain, their potential toxicity to neuronal cells should be also elucidated.

1.2. Toxicological profiling systems relevant for nanoparticles

As already mentioned, according to the prevailing scientific opinion, almost all of the manufactured nanoparticles exert their toxicity at least partly *via* oxidative stress (Xia et al., 2006). In addition, metal-containing nanoparticles may also release toxic metal ions. Therefore, the proposed toxicity profiling assays for metal-containing NPs should elucidate both, the ROS-inducing potential as well as the dissolution potential of NPs.

1.2.1. Determination of reactive oxygen species

To detect ROS in biological samples, usually fluorescent dyes, which increase their fluorescence after intracellular oxidation (2,7-dichlorofluorescein and its various derivatives, calcein, hydroethidium) are used (Invitrogen, 2006; Karlsson et al., 2008; Gunawan et al., 2011). The drawback of the use of fluorescent dyes is that they usually have high background fluorescence, are susceptible to photo- and auto-oxidation and may be entrapped into cell membrane lipid bilayer and thus, not reach the cytosol (Chen et al., 2010b). In addition, at physiological pH, the fluorescence of these dyes can be quenched by metal ions (e. g., Fe^{3+} and Cu^{2+}) (Invitrogen, 2006), making these dyes not suitable for the detection of ROS produced by respective metal-containing NPs.

Another method for the detection of ROS in biological samples is monitoring of the levels of oxidative stress markers, e.g., antioxidative enzymes. As already mentioned, the major antioxidant enzymes in the cells are superoxide dismutases, catalases, glutathione S-transferases and glutathione peroxidases (Fridovich and Freeman, 1986). As the main function of antioxidative enzymes is neutralization of ROS, the genes of respective enzymes are mostly induced in response to certain ROS. By the measurement of increased enzymatic activity of antioxidant enzymes (Ahamed et al., 2010) or the induction of the promoters of respective genes (Choi et al., 2010), one can draw conclusions on the presence of ROS in the cells. One of the most often used methods for the monitoring the promoter activities is a real-time PCR (RT-PCR), which needs *prior* extraction of RNA from the samples and subsequent two DNA synthesis reactions. Yet, this analysis is relatively time-consuming and costly.

1.2.2. Analysis of dissolution of nanoparticles

Dissolution of NPs can be measured with reasonable accuracy by a combination of size fractionation methods: dialysis (Rice et al., 2009), ultracentrifugation (Midander et al., 2009), ultrafiltration or field flow fractionation (Lyven et al., 2003) followed by total metal analysis by inductively coupled plasma mass spectrometry (ICP-MS) or atomic absorption spectroscopy (AAS). However, these combined approaches are not routine, and become methodically very challenging especially in case of separation of NPs below 10 nm. In addition, at low concentrations, the sorptive loss of the metals to the filter membrane can become problematic (Handy et al., 2008). Another very important issue underlying these methods is the fact that not all separated and chemically quantified metals in filtrate or supernatant will be in the form of free metal ion – the active form of metal taken up by the cells (Rensing and Maier, 2003). Substantial fraction of metal may be in the form of soluble metal complexes (Cuppett et al., 2006), which are not bioavailable i.e. are not taken up by the cell and thus do not cause toxic effect. Therefore, according to chemical analysis the concentration of metal ions dissolved from NPs may be high, whereas actual active concentration of free metal ions causing biological effects may be low.

This may lead to the underestimation of the role of metal ions in the toxicity of metallic NPs.

One of the few tools currently available for the direct measurement of free metal ion concentration is the ion-selective electrode (ISE). Cu, Ag, Cd, Pb ion-selective electrodes are commercially available (Frant et al., 1994). As the determination limits for these electrodes are typically around 10^{-6} M (i.e., 0.06 mg/l for Cu ion and 0.1 mg/l for Ag ion), these electrodes are suitable only for the analysis of relatively high metal ion concentrations. Another common drawback of ISEs is the interference of the sample matrix (chlorides, sulfides and organics) with the measurement.

1.2.3. Toxicological profiling system proposed in the current PhD thesis

The current PhD thesis introduces a novel system which allows the simultaneous analysis of different toxicological effects of metal-containing NPs, including general toxicity, ROS-inducing properties and dissolution. This toxicity profiling system is based on a suite of bacterial whole-cell biosensors: recombinant *E. coli* strains capable to specifically recognize (i) superoxide anions, (ii) hydrogen peroxide, (iii) DNA damaging agents, (iv) intracellular metal ions and six constitutively bioluminescent *E. coli* strains with step-wise decreased capacity to cope with oxidative stress. The following chapters describe the working principles, construction and application of this bacterial biosensor panel in more detail.

1.3. Bacterial whole-cell biosensors

According to IUPAC definition a biosensor is defined as "a self-contained integrated device that is capable of providing specific quantitative or semi-quantitative analytical information using a biological recognition element (biochemical receptor) which is in direct spatial contact with a transduction element" (Turdean, 2011). In whole-cell biosensors, sensing and reporting occur inside a living cell. The sensing element is a regulator protein and its regulated gene promoter. The regulator protein is activated upon the changes in environmental conditions or by specific binding of the target analyte. When activated, regulator protein deactivates certain repressor protein or, alternatively, acts as an activator on its regulated promoter. As a result, the genes (in recombinant biosensor these are genes that encode for reporter element) that are regulated by that promoter will be expressed. Thus, the activity of a reporter element − a gene or a group of genes, which produce a measurable signal (e.g., luminescence, fluorescence) − is proportional to the activity of the promoter (Köhler et al, 2000). The choice of the sensing element determines the specificity of the biosensor, while the reporter element determines the system's sensitivity (Belkin et al., 2003).

1.3.1. Reporter elements in bacterial whole-cell biosensors

Several genes have been adapted as reporter elements suitable for bacterial whole-cell biosensors. The most frequently used reporter genes include different variants of green fluorescent protein (GFP)-encoding *gfp* gene, bacterial bioluminescence reaction-encoding *luxCDABE* genes, bacterial luciferase-encoding *luxAB* genes and eukaryotic luciferase-encoding *lucFF* gene (Purohit, 2003). Some researchers consider GFP as most suitable reporter system (Tsien et al., 1998). Indeed, expression of GFP does not require any additional substrate (the protein itself is self-fluorescent) and it also opens the possibility for microscopic visualization of the cells. GFP has been chosen as a reporter for the construction of *E. coli* promoter-GFP library consisting of 2000 fusions (Zaslaver et al., 2006) that may be used to study *E. coli* gene expression in different environments and in the presence of different chemicals. However, the main disadvantage of GFP is its low sensitivity: each molecule of fluorescent protein produces at most one fluorophore (Tsien, 1998). In addition, the autofluorescence of most biological systems, which interfere with the measurement of GFP fluorescence, is also one reason for the low sensitivity of GFP (Daunert et al., 2000). In contrast, enzyme-based (e.g., luciferases) reporters are much more sensitive due to the ability to utilize an indefinite number of substrate molecules, amplifying the signal (Hakkila et al., 2002). In contrast to GFP fluorescence, the signal produced by luciferases lacks the endogenous background in most of the biological systems (Lewis et al., 1998). However, the main drawback of the use of eukaryotic luciferase (the most widely used variant is luciferase from American firefly *Photinus pyralis,* encoded by *lucFF* gene) as a reporter gene is its requirement for the reaction substrate D-luciferin, which should be added exogenously after the lysis of cells. The reason for the exogenous addition of the substrate is that the pathway of D-luciferin biosynthesis is complex and not fully identified. Thus, respective genes cannot be cloned to provide autonomous bioluminescence system. In contrast, bacterial bioluminescence system includes all the components required for light production and thus, is a preferred reporter system in prokaryotic cells (Belkin et al., 2003).

1.3.1.1. Bioluminescence and bacterial bioluminescence system

Bioluminescence is a light produced by enzymes luciferases as a by-product of their oxidation reaction. Bioluminescence is characteristic to numerous marine and a few terrestrial organisms (about 666 genera from 13 *phyla*) extending from bacteria and dinoflagellates to fishes (Girotti et al., 2008). Despite of the common name, the bioluminescence reactions in different organisms are very different, using different substrates, different catalyzing enzymes (although commonly named as luciferases) and emitting bioluminescence at different wavelengths.

Bacterial luciferase is a heterodimeric enzyme that utilizes long chain aliphatic aldehyde, oxygen and reduced flavin mononucleotide ($FMNH_2$) as substrates. 490 nm light is a by-product of this reaction (Equation 1).

$FMNH_2 + RCHO + O_2 \rightarrow FMN + H_2O + RCOOH + hv\ 490\ nm$ (Equation 1)

The genes essential for bioluminescence of bacteria are arranged into a single operon, *luxCDABE*. *LuxA* and *luxB* genes encode for α- and β-subunit of luciferase, respectively. *LuxCDE* genes encode a fatty acid reductase complex involved in synthesis of the main substrate of luciferase - aliphatic aldehyde (RCHO) (Meighen, 1991). In recombinant constitutively luminescent bacteria, the *luxCDABE* genes are expressed from constitutive promoter and support bioluminescence constantly as long as bacteria are alive and metabolically active (Figure 2A). This is energetically very costly as bioluminescence consumes around 12-20% of the total cellular ATP (Lee et al., 1974). Any damage of cellular metabolism (decrease in ATP) of bacteria results in proportional decrease of bioluminescence. The decrease in the light output could be easily monitored by special instrumentation, enabling to utilize bioluminescence as a very convenient endpoint in general toxicity tests (Hastings, 1978; Bulich, 1981). This has led to the development of commercial toxicity tests such as Microtox®, BioTox™, LUMIStox™ and ToxAlert™ that use the inhibition of bioluminescence of naturally luminescent *Vibrio fischeri* as their toxicity endpoint (Parvez et al., 2006) (Figure 2A). The assay with bioluminescent *V. fischeri* has proven as a rapid, simple and sensitive method in toxicity testing of a wide spectrum of chemical substances and environmental samples including wastewater, solid waste and sludge extracts (Kahru and Põllumaa, 2006; Parvez et al., 2006). Recently a kinetic format using bioluminescent *V. fischeri* applicable also for sediments, solids and colored samples has been standardized (ISO, 2010).

In summary, the assays utilizing the inhibition of bioluminescence of bacteria enable rapid determination of the toxicity range of the chemicals or NPs (Belkin, 2003). However, the information on general toxicity of a compound is not enough to describe its mechanisms of toxic action and more specific test systems should be implemented for further characterization.

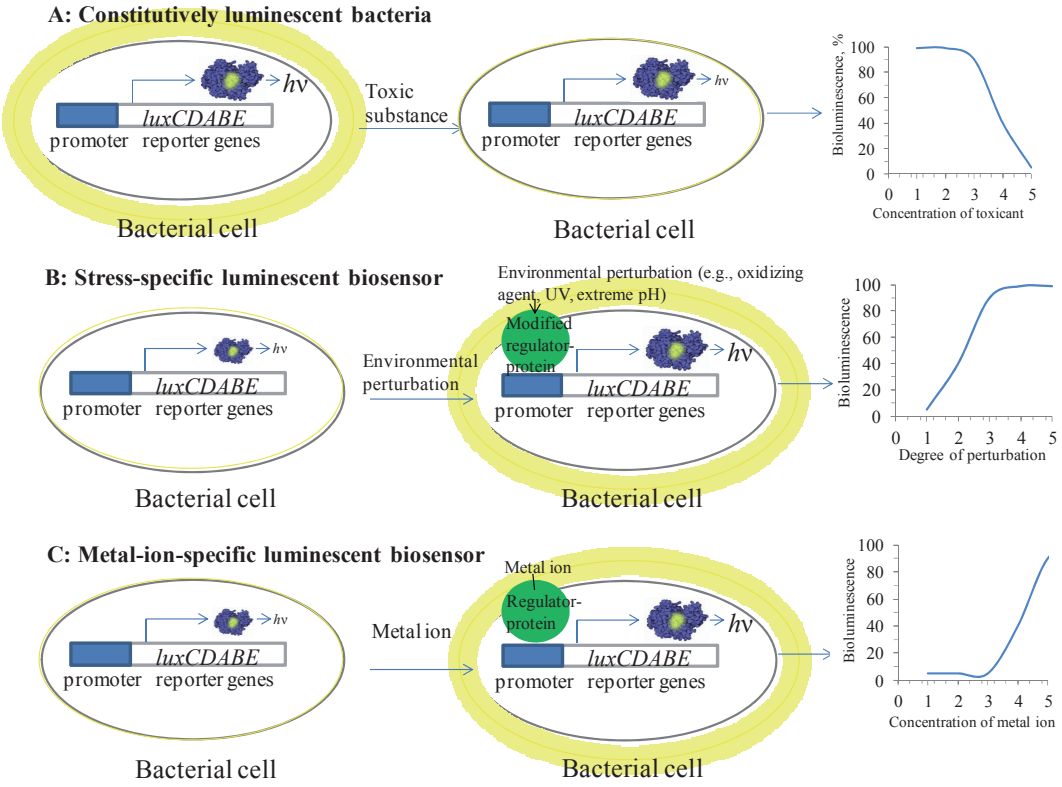

Figure 2. In constitutively bioluminescent bacteria the bioluminescence decreases due to the interference of toxicant with energy production of bacteria (inhibition of bioluminescence reaction) (A), in stress-specific biosensors the bioluminescence increases due to the induction of luciferase synthesis in response to stress (B) and in metal-ion-specific biosensors the bioluminescence increases in response to intracellular-bioavailable metal ion (C).

1.3.2. Stress-specific whole-cell biosensors

Differently from constitutively luminescent bacteria, stress-specific biosensors may be used to estimate the mechanism of toxicity of chemical (Figure 2B). All these biosensors are genetically modified (recombinant) and carry a sensing system, which is responsible for the activation of certain pathways, which help bacteria to adapt and survive in "stress conditions" (non-physiological osmolarity and temperature, oxidative stress, radiation, toxic chemicals, starvation etc.; Daunert et al., 2000). The most frequent sensing elements in stress-specific biosensors are regulatory proteins and their regulated promoters from global regulatory circuits (Köhler et al., 2000), which are expected to respond to a broad range of perturbations. The properties of the following stress-response systems were utilized for the construction of the stress-specific biosensors in the current PhD thesis:

(i) Oxidative stress response system

Here a response to superoxide anion and hydrogen peroxide may be distinguished.

Intracellular superoxide anions are kept under the control by superoxide dismutase (SOD) enzymes, which catalyze the dismutation of superoxide into hydrogen peroxide (Equation 2):

$$O_2^- + 2H^+ \rightarrow H_2O_2 \qquad \text{(Equation 2)}$$

There are three superoxide dismutase genes in *E. coli* cells: *sodA, sodB* and *sodC*. SodA and SodB are intracellular superoxide dismutases, whereas SodC locates in the periplasm of *E. coli*. Among all the three superoxide dismutase genes, only *sodA* is specifically inducible by superoxide anions (Hassan and Fridovich, 1977), which makes this promoter suitable for the construction of superoxide anion-biosensor. The activation of *sodA* promoter is mediated by the regulators SoxR and SoxS. SoxR is a transcriptional activator which induces the transcription of *soxS* gene after the oxidation by O_2^-. SoxS is another transcription regulator that directly activates the transcription of around 15 oxidative stress response genes, including *sodA* (Greenberg et al., 1990).

Another system that protects the *E. coli* cells against oxidative stress is controlled by *oxyRS* genes. After oxidation by H_2O_2 the regulator OxyR undergoes conformational change and becomes the transcriptional activator for *oxyS*. The product of *oxyS* is a small untranslated mRNA, which activates the transcription of 20-30 oxidative stress response genes, including *katG* (Christman et al., 1985). Bacterial KatG is a catalase-peroxidase, which converts hydrogen peroxide to water (Equation 3).

$$2H_2O_2 \rightarrow 2H_2O + O_2 \qquad \text{(Equation 3)}$$

(ii) SOS response system

Bacterial SOS response system is induced by DNA damage and activates the genes responsible for recombination, repair and error-prone replication of DNA and therefore, adaptation of the bacterial cell to the presence of DNA damaging agents. The repressor protein LexA and inducer protein RecA play a key role in the regulation of the SOS response. RecA, after recognizing and binding of the single-stranded DNA, mediates the autocatalytic cleavage of LexA. Inactivation of LexA repressor leads to the derepression of SOS regulon genes, which consists of around 40 genes, including *recA* gene itself (Little et al., 1983).

1.3.3. Metal-ion-specific whole-cell biosensors

Metal-ion-specific biosensors represent a class of recombinant bacterial biosensors that detect specifically intracellular-bioavailable metal ions. The sensing system in these biosensors usually consists of a protein that specifically binds to the metal ion and acts as a transcriptional activator on a specific promoter (Figure 2C). Most of the sensing elements of heavy metal biosensors originate from the heavy-metal-resistant bacteria and represent the promoters of genes the products of which are responsible for the efflux of excessive metal ions from the bacterial cell. In recombinant biosensor bacteria, these promoters are separated from the original gene and are genetically coupled to the reporter gene. The first such metal-ion-inducible biosensor was constructed in 1991 and its sensing system was based on *merB* promoter and Hg-binding transcriptional activator MerR (Geiselhart et al., 1991). Since then, biosensors for different heavy metals (Cd, Hg, Zn, Cu, As, Cr, Pb (Daunert et al., 2000; Köhler et al., 2000; Ivask et al., 2009)) have been constructed and applied mostly for the determination of bioavailability of heavy metals from soils and sediments (reviewed by Ivask et al., 2002). In the current work, heavy metal-specific biosensors were applied for the first time in a new context – for the determination of the dissolution of metal-containing nanoparticles. The details of this technique are described in Materials and Methods (section 2.2.4.2).

1.4. Toxicological profiling of nanoparticles using bacterial biosensors

Applications of whole-cell bacterial biosensors for mechanistic toxicological profiling of nanoparticles range from general toxicity assessment (constitutively luminescent bacteria) to the assessment of certain kind of physiological stress induced by NPs (stress-specific biosensors) and analysis of dissolution of metal-containing NPs *via* quantification of respective metal ions (metal-ion-specific biosensors). As mentioned in the section 1.3.1.1., the constitutively luminescent bacteria are integrating all the toxic effects, but are unable to define the exact mode of toxicity. However, the non-specific assay can be relatively easily modified by replacing the wild-type bacterial host by the bacterium with modified properties. For instance, certain genes of bacteria that are presumably important in toxic response to chemicals/NPs could be knocked out and the sensitivity of the knock-out bacterium to NPs can be compared with the wild-type strain. This phenotypic profiling can be performed as a comparison of the bacterial viability or growth rate and enables to identify the genes, the loss of which increases the sensitivity of bacteria to tested nanoparticles and thus, to identify the pathways of toxicity of nanoparticles. The described approach was used for example to determine the mechanisms of toxicity of polystyrene NPs in *E. coli* (Ivask et al., 2012) and also the mechanisms of action of metal-containing NPs in this PhD thesis (Publication **I**).

Specific classes of perturbations caused by NPs may be studied using stress-specific bacterial biosensors. By analyzing the changes in gene expression, it is possible to predict the mode of action of NPs. Such approach allows the detection

of perturbations caused by NPs already at sub-toxic concentrations and is orders of magnitude more sensitive compared to the general toxicity endpoints. Several panels of genetically engineered bacteria responding to the presence of different physiological stresses by synthesizing a reporter protein have already been developed (Belkin et al., 1997; Davidov et al., 2000; Van Dyk et al., 2000, Zaslaver, et al., 2006). Some potential applications of these stress-response systems for the classification of chemicals-induced cellular damage have also been presented (Dardenne et al., 2007; Park et al., 2009). However, the use of stress-specific biosensors for the elucidation of mechanisms of toxic action of synthetic nanoparticles has been described only in few recent papers (Hwang et al., 2008; Onnis-Hayden et al., 2009; Gou et al., 2010; Publication **I, III**).

1.5. Toxicological profiling of nanoparticles using human cell-based assays

In addition to bacterial cells, human cell cultures are another attractive model for studying the toxicological effects of NPs. While bacterial tests enable simple and rapid pre-screening of the NPs, the results from these assays are difficult (or rather impossible) to extrapolate to humans. Therefore, to describe the possible toxic effects of NPs to human health, the human cell based assays are more relevant test systems.

A considerable number of the nanotoxicological studies conducted to date in human cells *in vitro* have been performed using transformed cell lines (Kunzmann et al., 2011). However, it is more relevant to study NPs in primary cell cultures and other *in vitro* model systems, which are closer to the in *vivo* situation. One of the most relevant eukaryotic cell types for the elucidation of toxicity mechanisms of NPs are naturally phagocytic cells such as pulmonary macrophages. Moreover, lungs are also the primary targets of NPs toxicity *in vivo* (Renwick et al., 2004; Chen and Schluesener, 2008; Cho et al., 2012). Macrophages can easily internalize NPs, whereas the uptake pathway depends on the type and coating of NPs and internalization efficiency on size of NPs, being, in general, the highest for 40–50 nm particles (Arvizo et al., 2012). Using macrophages it has been demonstrated that after internalization of NPs into a lysosome, the particles will be released to the intracellular environment and in the case of metal-containing NPs, dissolved in the acidic environment of the lysosome inducing different toxicological effects like oxidative stress, energy depletion and inflammatory responses (Nel et al., 2012). Interestingly, NPs can escape from normal phagocytic clearance in the respiratory system and gain access to the systemic circulation and as a result, even translocate into the brain (Medina et al., 2007; Simkó and Mattsson, 2010). In addition, NPs may be taken up by nerve endings of the olfactory bulb after inhalation from the air (Oberdörster et al., 2004). Reaching the brain, NPs may induce several neural disorders including disruption of voltage-gated currents in neurons (Xu et al., 2009; Liu et al., 2011), depletion of dopaminergic systems, induction of oxidative stress and inflammation responses (Wang et al., 2009) as shown in rodents. As the *in vivo* toxic effects of NPs in brain have been mostly studied in rodents, the extrapolation of these data

to humans remains a big challenge (Hu and Gao, 2010). One of the few reports investigating the effects of NPs in human brain (autopsy of brain samples of residents of polluted cities and small cities with low air pollution) suggest that chronic exposure of humans to NPs is an important factor in the pathogenesis of neurodegenerative diseases (Peters et al., 2006). As neural system has been proposed to be one of the potential targets for NPs and the developing human brain is much more susceptible to toxicants than is the brain of an adult (Dobbing et al., 1968), it is reasonable to hypothesize that NPs may modulate also the neural development of fetus, i.e. exert developmental neurotoxicity.

1.5.1. Toxicological profiling of nanoparticles using developing human neuronal cells in vitro

The use of embryonic stem cells is currently considered the most promising approach to assess developmental toxicity *in vitro* (Adler et al., 2008). However, only two human embryonic stem cell-based *in vitro* systems have been proposed so far to estimate the developmental neurotoxicity in humans (Moors et al., 2009; Stummann et al., 2009). None of them has been validated or used for the profiling of neurotoxicity of synthetic NPs (Crofton et al., 2011).

In this PhD thesis, an *in vitro* assay for human neurotoxicity assessment was used to study the effects of NPs on the developing neuronal cells. In this assay, neural progenitor cells that were differentiated from human embryonic stem cells (hESC) were used as an *in vitro* test model. hESC are self-renewing pluripotent cells derived from the inner cell mass of blastocyst of the developing embryo (Thomson, et al. 1998). *In vitro*, hESC have an indefinite proliferation potential while maintaining the capability to differentiate into theoretically any cell type in the body (Amit, 2000). Several steps are required to induce hESC to differentiate into neuronal cells. First, the formation of mesoderm and endoderm should be inhibited and the formation of neuroectoderm should be induced *via* several neural inducing factors, e.g., noggin, follistatin and chordin. A key function of these factors is to antagonize the transforming growth factor β (TGF-β) signaling. This is achieved *via* the inhibition of downstream target of TGF-β receptor: SMAD signaling. Prevention of SMAD signaling leads to the inhibition of epidermis formation and induction of neuroectoderm (McMahon et al., 1998). In human *in vivo,* the process of neural induction results the formation of neural plate which further develops to the neural groove. Approximately on week 4 postfertilization, the neural groove closes and forms the neural tube which consists of a layer of neuroepithelial progenitor cells (NEPs). At the end of neurulation process, these cells give rise to the central nervous system (Colas and Schoenwolf, 2001).

According to Conti and Cattaneo (2010), *in vitro* differentiation of hESC to neural progenitor cells mimics *in vivo* situation and thus, this system can serve as a model of neurogenesis (Figure 3).

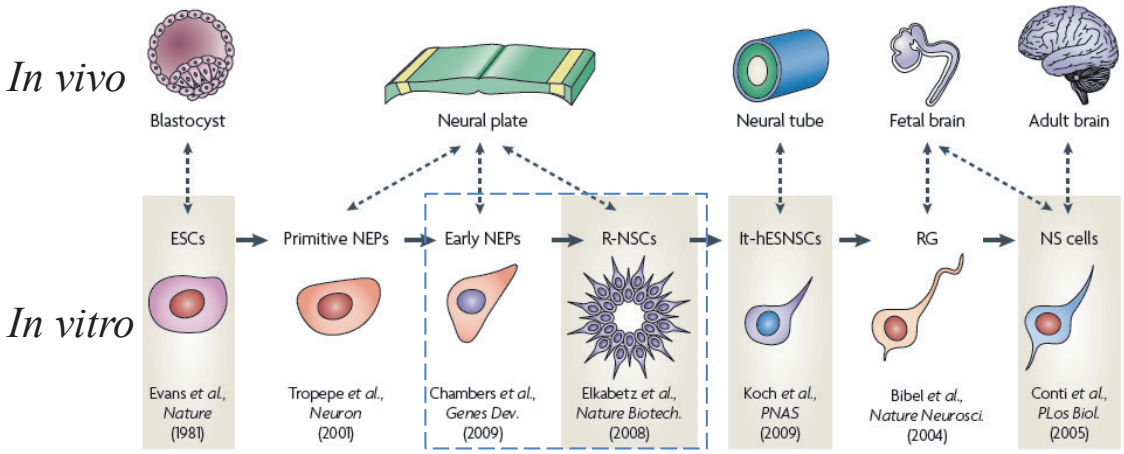

Figure 3. Schematic representation of the development of neural progenitor cells (NEPs) from human embryonic stem cells (hESC) *in vitro* with the corresponding *in vivo* developmental stage (Conti and Cattaneo, 2010). The dotted line highlights the type of cells that was used as a model for the neurotoxicological studies described in this PhD thesis. Reprinted with the permission of Nature Publishing Group.

During neural development, different populations of neural progenitor cells are characterized by distinct marker expression, which could be monitored to evaluate the progress of differentiation. Before the induction of differentiation the pluripotency-associated genes such as Nanog, Lefty1 and Pou5f1 are highly expressed in the cells (Chambers et al., 2009). With the progress of differentiation, the expression of the earliest neural stem cell markers such as Epha4, Cd113, Pax6 and Nes could be detected, preceding the induction of neuronal precursor cell markers (Neurod1, Ascl1, Dcx) and neuronal cell markers (Tubb3, Kcnj6, Slc17a6). Even small disruptions in differentiation and patterning of neuronal (precursor) cells may result in severe neurological impairments. Therefore, tracking the expression of the neuronal differentiation markers could be a very sensitive endpoint for the evaluation of the neurotoxic potential of NPs.

AIMS OF THE STUDY

The main objective of this study was to develop a suite of *in vitro* tests (with the focus on genetically engineered bacterial biosensors) for the toxicological profiling of synthetic nanoparticles.

Given the nanoparticles are thought to exert toxicity *via* production of reactive oxygen species and metal-containing nanoparticles may also release toxic metal ions, the main objective was approached through the following specific sub-tasks:

1. Construction and evaluation of a suite of constitutively bioluminescent *Escherichia coli* strains with step-wise decreased capacity to resist reactive oxygen species.

2. Construction and evaluation of new stress-specific bioluminescent *E. coli* biosensors for the detection and quantification of low sub-toxic levels of (i) superoxide anions, (ii) hydrogen peroxide and (iii) DNA damaging agents.

3. Physico-chemical characterization of synthetic nanoparticles in powder and in test media used for the bioanalysis.

4. Refinement of protocols for bioanalysis of nanoparticles using (i) developed bacterial biosensors (ii) differentiating human embryonic stem cells *in vitro*.

5. Mechanistic profiling of the toxic effects (e.g., general toxicity, dissolution, production of reactive oxygen species, DNA damage, neurotoxicity) of selected NPs of CuO, ZnO, TiO_2, Ag, C_{60}-fullerene and polyethylene with bacterial biosensors and differentiating human embryonic stem cells *in vitro*.

2. MATERIALS AND METHODS

2.1. Nanoparticles

2.1.1. Preparation of nanoparticles

Nano-sized CuO, ZnO, TiO_2, Ag (manufacturer- advertised primary sizes 30, 25–70, 50–70 and <100 nm, respectively) and C_{60}-fullerene were purchased from Sigma-Aldrich. If available, soluble metal salts (as $CuSO_4 \cdot 5H_2O$, $ZnSO_4 \cdot 7H_2O$ and $AgNO_3$) and micro-sized particles (CuO, ZnO, TiO_2) were used in parallel to NPs to control the effects of dissolution and size, respectively. Stock solutions of NPs and micro-particles were sonicated in ultrasonication bath for 30 minutes and stored in the dark at +4°C. For the tests, stock solutions were diluted in 0.9% NaCl (Publication **I**), in deionized water (DI) (Publication **III**) or in the test media (Publications **II**, **IV**). Suspensions of micro-TiO_2 and nano-TiO_2 were illuminated (if indicated; 290-400 nm, 400 W) for 30 min *prior* the test.

Polyethylene nanoparticles were synthesized *via* aqueous microemulsion polymerization in-house as described in Publication **IV**. A perylene diimide dye was used as a fluorescence marker to label these NPs. For reduction of the surfactant (sodium dodecyl sulfate) necessary for the synthesis, the as-obtained polymer dispersions were dialyzed after synthesis against DI water to yield dispersions with a surfactant and polymer content of <0.2% and >1%, respectively.

2.1.2. Characterization of nanoparticles

Scanning electron microscopy (SEM, Jeol, JSM-8404) imaging and measurement of specific surface area (Sorptometer Kelvin 1042; Costech Instruments) of NPs and their micro-sized references were performed in Tallinn University of Technology, Center of Materials Research and Laboratory of Inorganic Materials. The hydrodynamic size of nanoparticles in DI water and in test media was measured using dynamic light scattering (DLS) (Malvern Zetasizer Nano-ZS, Malvern Instruments). UV-visible wavelength absorption spectra (UV-Vis) of nanoparticles in DI water were obtained using a Thermo Multiscan spectrophotometer (Thermo Electron).

2.2. Luminescent bacteria

Luminescent bacterial strains constructed and/or used in this study are shown in Table 1. In total, 16 different luminescent bacterial strains were used, whereas eleven strains were constructed as a part of this PhD study (Publications **I**, **III**). Five strains were constructed earlier and described by Ivask et al. (2009).

Table 1. Bioluminescent bacteria used in this study.

Strain	Description	Purpose in this PhD thesis (publication)	Reference (publication)
Constitutively luminescent bacteria			
E. coli AB1157 (pSLlux)	Parental strain for *sod*-defective strains, *luxCDABE* genes in a high-copy plasmid pSLlux	Profiling of ROS-inducing potential of NPs (I)	**I**
E. coli JI130 (pSLlux)	same as AB1157 but *sodA* defective (*sodA*$^-$)	Profiling of ROS-inducing potential of NPs (I)	**I**
E. coli JI131 (pSLlux)	same as AB1157 but *sodB* defective (*sodB*$^-$)	Profiling of ROS-inducing potential of NPs (I)	**I**
E. coli AS393 (pSLlux)	same as AB1157 but *sodC* defective (*sodC*$^-$)	Profiling of ROS-inducing potential of NPs (I)	**I**
E. coli JI132 (pSLlux)	same as AB1157 but *sodAB* defective (*sodAB*$^-$)	Profiling of ROS-inducing potential of NPs (I)	**I**
E. coli AS391 (pSLlux)	same as AB1157 but *sodABC* defective (*sodABC*$^-$)	Profiling of ROS-inducing potential of NPs (I)	**I**
E. coli K12::lux	chromosomal insertion of *luxCDABE* genes	Control for stress-specific biosensors (I, III)	**I**
E. coli MC1061 (pDEW201)	*luxCDABE* genes in a medium-copy plasmid pDEW201	Control for DNA-damage specific biosensor (III)	**III**
E. coli MC1061 (pDNlux)	*luxCDABE* in a medium-copy plasmid pDNlux	Control for metal-ion-specific biosensors (I, III)	Ivask et al., 2009
P. fluorescens OS8::lux	chromosomal insertion of *luxCDABE* genes	Control for Cu-ion-specific biosensor (II, III)	Ivask et al., 2009
Stress-specific luminescent biosensors			
E. coli K12:: soxRSsodAlux	chromosomal insertion of *luxCDABE* genes under the control of *sodA* (superoxide dismutase) promoter and its regulators *soxRS*	Profiling of superoxide anion-inducing potential of NPs (I)	**I**
E. coli K12::katGlux	chromosomal insertion of *luxCDABE* genes under the control of H_2O_2-inducible promoter *katG* (catalase-peroxidase)	Profiling of hydrogen peroxide-inducing potential of NPs (III)	**III**
E. coli MC1061 (pDEWrecAlux)	*luxCDABE* genes in a medium-copy plasmid pDEW201 under DNA-damage-inducible promoter *recA* (recombinase A)	Profiling of DNA damage-inducing potential of NPs (III)	**III**

Table 1 is continued on the next page

Metal-ion-specific luminescent biosensors			
E. coli MC1061 (pSLzntR/pDNP zntAlux)	*luxCDABE* genes under control of *zntA* promoter (Zn/Cd/Pb/Hg-inducible) and its regulator *zntR*	Dissolution of ZnO nano- and micro-particles (I)	Ivask et al., 2009
P. fluorescens OS8:: CueRPcopAlux	chromosomal insertion of *luxCDABE* genes under the control of *copA* (Cu/Ag-inducible) promoter and its regulator *cueR*	Dissolution of CuO nano- and micro-particles (II, III)	Ivask et al., 2009
E. coli MC1061 (pSLcueR/ pDNPcopAlux)	*luxCDABE* genes under control of *copA* promoter (Cu/Ag-inducible) and its regulator *zntR*	Dissolution of CuO nano- and micro-particles (II, III)	Ivask et al., 2009

2.2.1. Construction of luminescent bacteria

All bioluminescent bacteria constructed and/or used in this study, carried the bioluminescence encoding reporter genes *luxCDABE* from naturally bioluminescent bacterium *Photorhabdus luminescens.* The eleven biosensor strains developed in this study are described below.

2.2.1.1. Construction of constitutively luminescent bacteria

Tn5 mini-transposon mutagenesis method (de Lorenzo and Timmis, 1994) and suicide plasmid pTCRKnlux (Ivask et al., 2009) were used to construct *E. coli* K12::lux carrying a single chromosomal insertion of *luxCDABE* (Publication **I**). To transform pSLlux plasmid to *E. coli* and its *sod*-deficient mutant strains (Publication **I**) and pDEW201 plasmid to *E. coli* MC1061 (Publication **III**), a standard electroporation protocol (Sambrook et al., 1989) was used. All *sod*-deficient *E. coli* strains and their wild-type analogue were kindly provided by Prof. J. Imlay (University of Illinois, Urbana, USA).

2.2.1.2. Construction of stress-specific biosensors

The superoxide anion-inducible strain *E. coli* K12::soxRSsodAlux and hydrogen peroxide-inducible strain *E. coli* K12::katGlux were constructed by inserting a single copy of *luxCDABE* from *P. luminescens* under the control of respective sensor element(s) (Table 1) into the *E. coli* chromosome using *Tn5* mini-transposon mutagenesis method (de Lorenzo and Timmis, 1994) (Publications **I**, **III**). For the construction of *E. coli* K12::soxRSsodAlux, a 308-bp *sodA* and 894-bp *soxRS* genes amplified from the genomic DNA of *E. coli* K12 (primers sodA_sense, sodA_antis and soxRS_sense, soxRS_antis,Table 2) were used as a sensor elements (Publication **I**).

Table 2. Primers used for the construction of stress-specific luminescent biosensors.

Name	Sequence	Publication
sodA_sense	5′ ATATCCTAGGTCTTCTTATCCTCATCATTTTTC 3′	**I**
sodA_antis	5′ AATTGGATCCCATATTCATCTCCAGTATTG 3′	**I**
soxRS_sense	5′ ATATACTAGTTTACAGGCGGTGGCGATAAT 3′	**I**
soxRS_antis	5′ AATTCCTAGGGCTTTAGTTTTGTTCATCTTCCA 3′	**I**
katG_sense	5′ TATACCTAGGAATGAGGCGGGAAAATAAGGT 3′	**III**
katG_antis	5′ TTAAGGATCCCATCAATGTGCTCCCCACTA 3′	**III**
recA_sense	5′ ATATGAATTCCATGCCGGGTAATACCGGATA 3′	**III**
recA_antis	5′ AATTGGATCCACCGTGATGCGGTGCGTCGTC 3′	**III**

For the construction of the H_2O_2-inducible strain *E. coli* K12::katGlux a 283-bp *katG* promoter from the genomic DNA of *E. coli* K12 was amplified (primers katG_sense and katG_antis; Table 2) and used as a sensor element (Publication **III**).

E. coli MC1061(pDEWrecAlux) was constructed by inserting the 164-bp *recA* promoter (primers recA_sense and recA_antis) in the front of *luxCDABE* genes in plasmid pDEW201 (LaRossa et al., 1998) (Publication **III**). The plasmid pDEW201 was kindly donated by Prof. S. Belkin (Hebrew University of Jerusalem, Israel).

2.2.2. Cultivation of luminescent bacteria and test conditions

All 16 bioluminescent bacteria were cultivated freshly by growing the cultures overnight in 3 ml of Luria-Bertani (LB) medium (Sambrook et al., 1989) supplemented with appropriate antibiotics (Publications I-III). The overnight culture was diluted 1:50 with LB medium and grown until OD_{600} of ~0.6. Cell pellet was washed, resuspended in test medium and diluted to OD_{600} of 0.1 (10^6−10^7 cells/ml). The test medium was either heavy metal MOPS medium (HMM) (LaRossa et al., 1995), 0.9% NaCl or 0.9% NaCl supplemented with 0.1% glucose and cas-amino acids.

2.2.3. Profiling of nanoparticles with constitutively luminescent bacteria

The bioluminescence inhibition assay was performed on 96-well microplates according to the ISO standard for *V. fischeri* (ISO, 2010) with minor modifications: test temperature 30°C instead of 20°C and 0.9% NaCl instead of 2% NaCl as test environment.

2.2.4. Profiling of nanoparticles with biosensors

2.2.4.1. Determination of ROS and DNA-damaging potential of nanoparticles

ROS-generation potential of chemicals and NPs was analyzed by H_2O_2 biosensor *E. coli* K12::katGlux and O_2^- -biosensor *E. coli* K12::soxRSsodAlux. DNA damaging potential was determined with *E. coli* MC1061(pDEWrecAlux). In case

of turbid metal oxides, potential quenching of bacterial bioluminescence was taken into account by parallel use of constitutively luminescent (control) bacteria, not inducible by the target chemicals: *E. coli* K12::lux, *E. coli* MC1061(pDEW201), *E. coli* MC1061(pDNlux) or *P. fluorescens* OS8::lux (Publications **I-III**). Fold induction of bioluminescence of recombinant bacterial sensors was calculated as follows:

$$\text{Fold induction of bioluminescence} = \frac{\text{SLs}}{\text{CLs}} \times \text{CF},$$

where SLs was the luminescence of the biosensor strain after its exposure to chemical/particles, CLs was the luminescence of the same strain in control solution and CF was the correction factor. CF was calculated:

$$\text{CF} = \frac{\text{CLc}}{\text{SLc}},$$

where CLc was the luminescence of the control strain in a chemical-free control solution and SLc was the luminescence of that strain after its exposure to chemical or particle. Limit of determination (LOD) of the sensor bacteria for the tested chemical was set to fold induction of bioluminescence=2.

2.2.4.2. Determination of dissolution of nanoparticles with bacterial biosensors

E. coli MC1061(pSLcueR/pDNPcopAlux) and *P. fluorescens* OS8::KncueR-PcopAlux were used to determine the dissolution of CuO and Ag NPs and *E. coli* MC1061 (pSLzntR/pDNPzntAlux) to determine the dissolution of ZnO particles. The concentration of heavy metal ions dissolved from (nano) particles was quantified by comparing the sub-toxic linear part of concentration-response curves of biosensors to particles and to the respective heavy metal's soluble salt, assuming 100% relative bioavailability of the salt. An example of using Cu-ion specific biosensor to determine the dissolution of nano- and micro-sized CuO particles is shown in Figure 4. For that, Cu-ion biosensor was exposed in parallel to Cu ion (in this example from soluble Cu salt $CuSO_4*7H_2O$) and to different concentrations of CuO nano- and microparticles. As Cu-ion biosensor is responding to intracellular Cu ions, the increase in bioluminescence of biosensor upon incubation with CuO showed the dissolution of these nanoparticles and internalization of dissolved Cu ions. The dissolution of CuO was quantified by linear regression from concentration-response curves, i.e. by comparing the response of biosensor to $CuSO_4$ and to CuO. In the shown example, the dissolution rate of nano-CuO was around 35% and the dissolution of micro-CuO ~0.2%. Using this approach, dissolution of CuO NPs was calculated in Publications **I**, **III**.

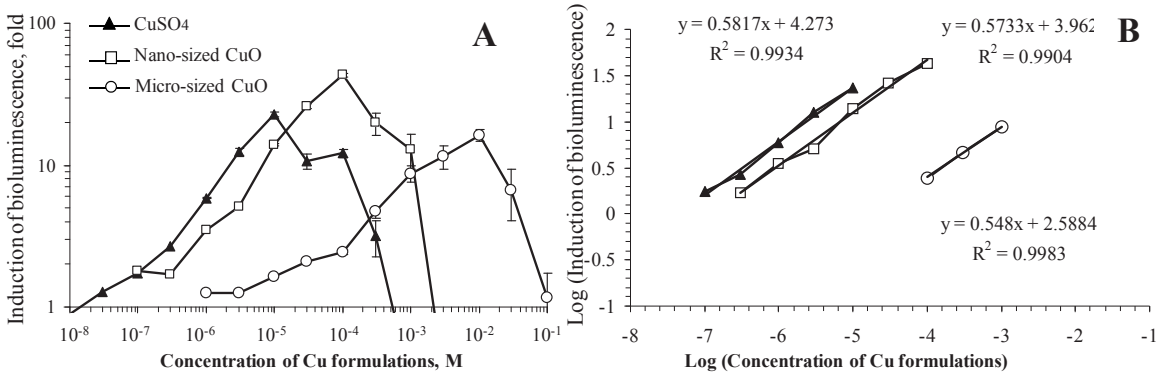

Figure 4. A: Concentration-response curves of Cu-biosensor *Escherichia coli* MC1061 (pSLcueR/pDNPcopAlux) for $CuSO_4$ (▲), nano-sized CuO (□) and micro-sized CuO (○) after 2-h exposure **B:** Log-log regressions of the sub-toxic linear part of the concentration-response curves, regression equations and R^2 values are shown.

2.3. Determination of dissolution of nanoparticles with chemical analysis

In parallel to the metal-ion biosensor analysis, the Cu-ion-specific electrode (Cu-ISE, Orion Research) was used to measure the free metal ions directly from NPs dispersions (Publication **II)**.

In addition, soluble fraction of heavy metals was determined by atomic absorption spectroscopy (AAS) from particle-free supernatants (ultracentrifugation at 30,000 g for 30 min). AAS analysis was made in a certified laboratory of Tallinn University of Technology, Estonia, using standard procedures EVS-EN ISO/IEC 17025:2005.

2.4. Profiling of nanoparticles with human cell-based assays

2.4.1. Human embryonic stem cells differentiation and exposure to methylmercury and CuO nanoparticles

The hESC (line H9, WA09, WiCell) were maintained on a feeder layer of mitomycin C inactivated mouse embryonic fibroblasts (MEFs).
hESC cells were first differentiated to neuronal progenitor cells (NEPs) in adherent cell-culture (on matrigel) using dual inhibition of SMAD signaling by neural inducing factors i.e., noggin, dorsomorphin and SB431542 essentially as described by Chambers et al. (2009). On day 8 of differentiation, the cells were replated onto low-adhesion plates, where they formed neurospheres, offering the microenvironment for the further proliferation and maturation of NEPs to early neuronal (precursor) cells (Figure 5).

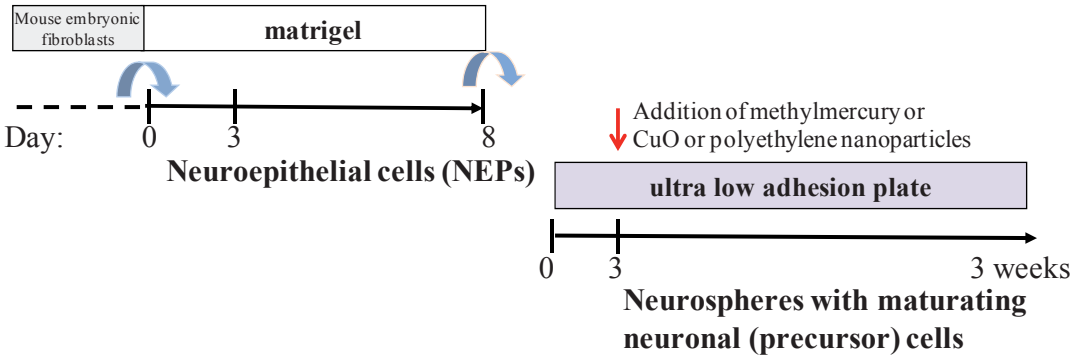

Figure 5. Schematic representation of the differentiation of human embryonic stem cells (hESC) towards neuronal cells. First, hESC cells were differentiated towards neuroepithelial progenitors (NEPs) essentially as described in Chambers et al. (2009). Blue arrows indicate re-plating of the cells. At day 8, NEPs were plated into suspension for the formation of the neurospheres, which provided the environment for the further maturation of NEPs to more mature neuronal cells. Starting from day 3 (red arrow), neurospheres were exposed to toxicant.

The developed system was evaluated (measurement of expression of neural and neuronal markers during differentiation in adherent phase and within neurospheres) by transcriptome analysis and verified by RT-PCR. The markers which are normally expressed during the differentiating of hESC into neural cells are shown in Figure 6.

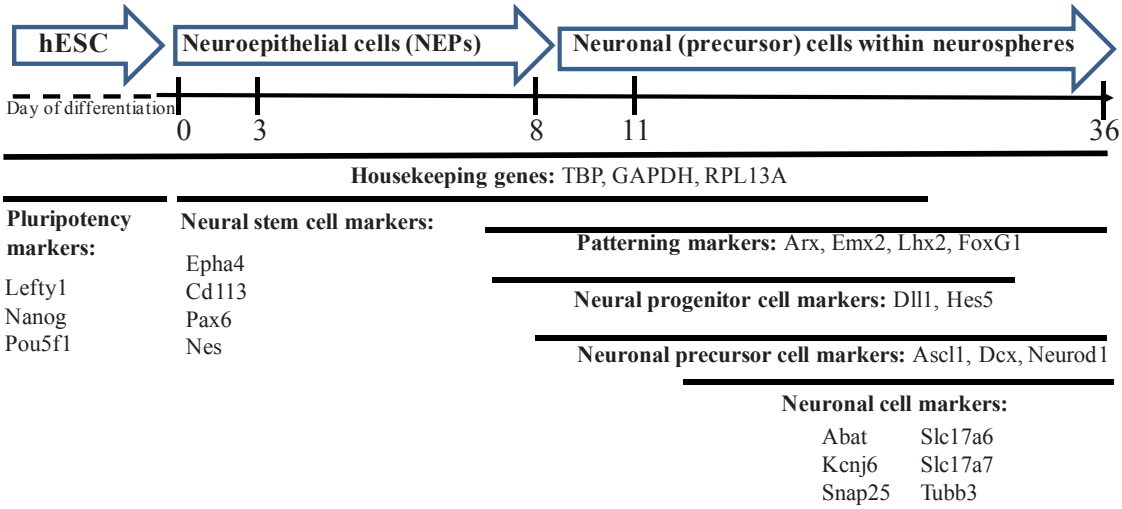

Figure 6. Schematic representation of the expression of marker genes during differentiation of human embryonic stem cells (hESC) into neuronal cells.

Starting from differentiation day 11 (day-3 neurospheres), neurospheres were exposed to well-known neurodevelopmental toxicant methylmercury (a positive control) or to CuO or polyethylene nanoparticles for 2 days (acute exposure) and 18 days (chronic exposure). The viability of cells was monitored using resazurin assay (Zimmer et al., 2011) or ATP measurement. The morphology of neurospheres and the internalization of polyethylene nanoparticles were monitored using phase-contrast and fluorescence microscopy. After the exposure, the cells containing non-toxic concentrations of toxicants as well control samples

were used for the isolation of RNA and synthesis of cDNA. The sub-toxic effects of methylmercury or nanoparticles were estimated after measurement of expression of neural and neuronal markers by RT-PCR (housekeeping genes Rpl13A and Gapdh were used for normalization) and comparing the gene expression with un-affected control. The details of the experiments and the list of used specific primers are described in Publication **IV**.

3. RESULTS AND DISCUSSION

3.1. Construction and characterization of constitutively luminescent bacteria

Due to the fact that oxidative stress induction has been considered as one of the main mechanisms of toxicity of nanoparticles, a set of constitutively luminescent recombinant *E. coli* strains with step-wise altered capacity to fight ROS was constructed (Publication **I**). For that, superoxide dismutase (*sod*)-deficient mutant strains either defective in one of the cytoplasmic superoxide dismutases (*sodA*− or *sodB*−*)*, periplasmic superoxide dismutase (*sodC*−), both cytoplasmic superoxide dismutases (*sodAB*−) or all the three *E. coli* superoxide dismutases (*sodABC*−) were transformed with a plasmid carrying constitutively expressed *luxCDABE* genes from *Photorhabdus luminescens*. It was hypothesized that if the test chemical/nanoparticles induce the formation of superoxide anions, then their toxicity to *sod*-deficient strains should be higher than to the non-mutated *E. coli* strain with functional superoxide dismutases. This hypothesis was tested using (i) paraquat – superoxide-inducing chemical and (ii) hydrogen peroxide - a chemical known to induce oxidative stress, but not specifically superoxide anions. In addition, 3.5-dichlorophenol (3.5-DCP) not involved in oxidative stress and acting *via* polar narcosis (Dimitrov et al., 2003) was used as a negative control. In all these toxicity assays, effects on mutated strains were compared with the effects on the wild type, i.e. non-mutated *E. coli* strain.

As shown in Figure 7A, the wild-type strain and single *sod*-deficient mutants were significantly less sensitive to superoxide anions inducing chemical paraquat than *sod*-deficient double and triple mutants. Interestingly, also hydrogen peroxide was more toxic to *sod*-deficient mutants than to wild-type bacteria (Figure 7B). This was somewhat surprising, as *sod*-deficient mutants were expected to be sensitive specifically to superoxide anion-producing compounds. However, analogous results were previously obtained by Imlay and Linn (1987) who showed that *sod* mutants were especially sensitive to H_2O_2 and concluded that superoxide anions contribute to the bactericidal effects of hydrogen peroxide. As expected, the toxicity of 3.5-DCP to wild-type bacteria and *sod*-deficient mutants was comparable (Figure 7C). Thus, the assay with *sod*-deficient strains was considered as specific to ROS in general and not only to superoxide anions.

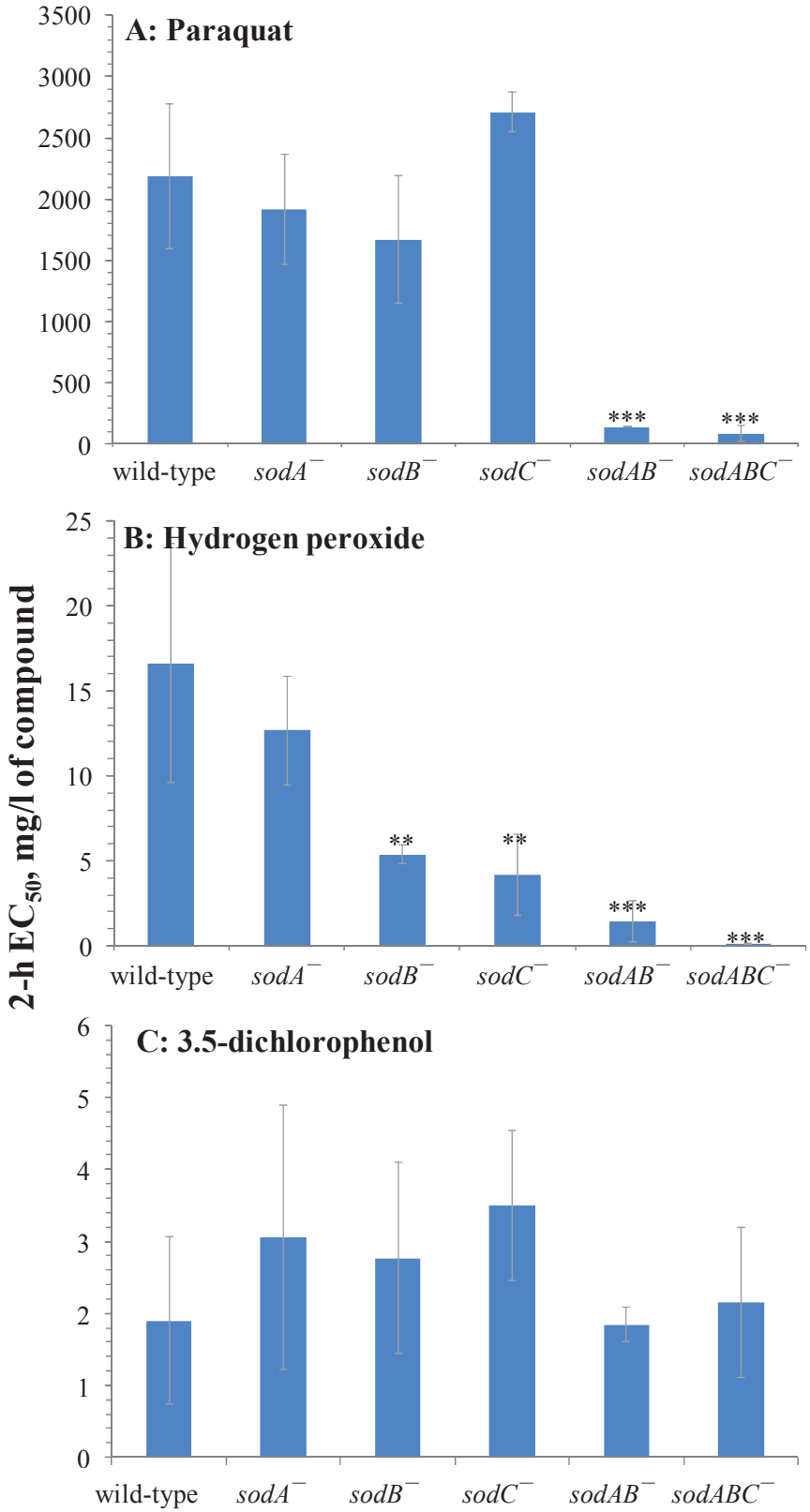

Figure 7. Toxicity (2-h EC_{50}) of positive and negative control-chemicals to superoxide dismutase (*sod*)-wild-type (non-mutated) *Escherichia coli* strain and *sod*-defective strains after 2-h exposure (***($p \geq 99\%$) and ** ($p \geq 95\%$) indicate that *sod*-defective strain is different from the wild-type strain).

3.2. Construction and characterization of luminescent biosensors

All metal-ion-specific biosensors used in the current PhD thesis were constructed and described previously (Ivask et al., 2009). All the stress-specific biosensors (Table 1) were developed during this PhD study. Two new ROS biosensors were constructed to recognize (i) superoxide anions (*E. coli* K12::soxRSsodAlux, Publication **I**) and (ii) hydrogen peroxide (*E. coli* K12::katGlux, Publication **III**). Third stress biosensor was constructed to recognize DNA damage either due to direct genotoxic effects of NPs or due to oxidative effects of ROS on genetic material (*E. coli* MC1061(pDEWrecAlux), Publication **III**).

The performance and sensitivity of the newly constructed stress-specific biosensors was first characterized using positive and negative control-chemicals: superoxide anion-inducing paraquat, strong mutagen mitomycin C (MMC), H_2O_2 and membrane-permeabilizer 3.5-DCP. After 2 hours of exposure, all the biosensors were induced by their positive control-chemicals and none of the biosensors was induced by the negative control 3.5-DCP (Figure 8). The superoxide anion biosensor K12::soxRSsodAlux was specifically induced by paraquat starting from 0.01 mg/l and no induction was observed with H_2O_2, showing the specificity of biosensor to superoxide anions (Figure 8A).

The H_2O_2-biosensor strain *E. coli* K12::katGlux was induced by H_2O_2 starting from 0.1 mg/l (2-h exposure). Paraquat practically did not induce this biosensor (Figure 8B) after 2-h exposure but showed slight response (around 4-fold induction) after 8-h exposure (Publication **III**). Due to the fact that the response of this biosensor to superoxide anions was extremely weak and it occurred only after 8 hours of exposure, this biosensor was considered as specific to H_2O_2.

The DNA damage biosensor *E. coli* MC1061(pDEWrecAlux) was induced by both positive controls, mitomycin C and H_2O_2 (Figure 8C). The strong mutagen MMC that acts by direct binding to DNA induced the biosensor starting from 0.003 mg/l. H_2O_2 (an unspecific mutagen) induced the biosensor starting from 3 mg/l. Thus, the DNA damage sensor showed slower response and lower sensitivity (higher LOD) towards H_2O_2 than the H_2O_2-biosensor *E. coli* K12::katGlux. This was also expected because in the bacterial cells the catalases represent the first line of defense towards peroxides whereas DNA damage is a secondary unspecific effect.

Taken together, all the constructed biosensors showed expected specificity: the biosensors were inducible with positive controls and were not induced by the negative control 3.5-DCP.

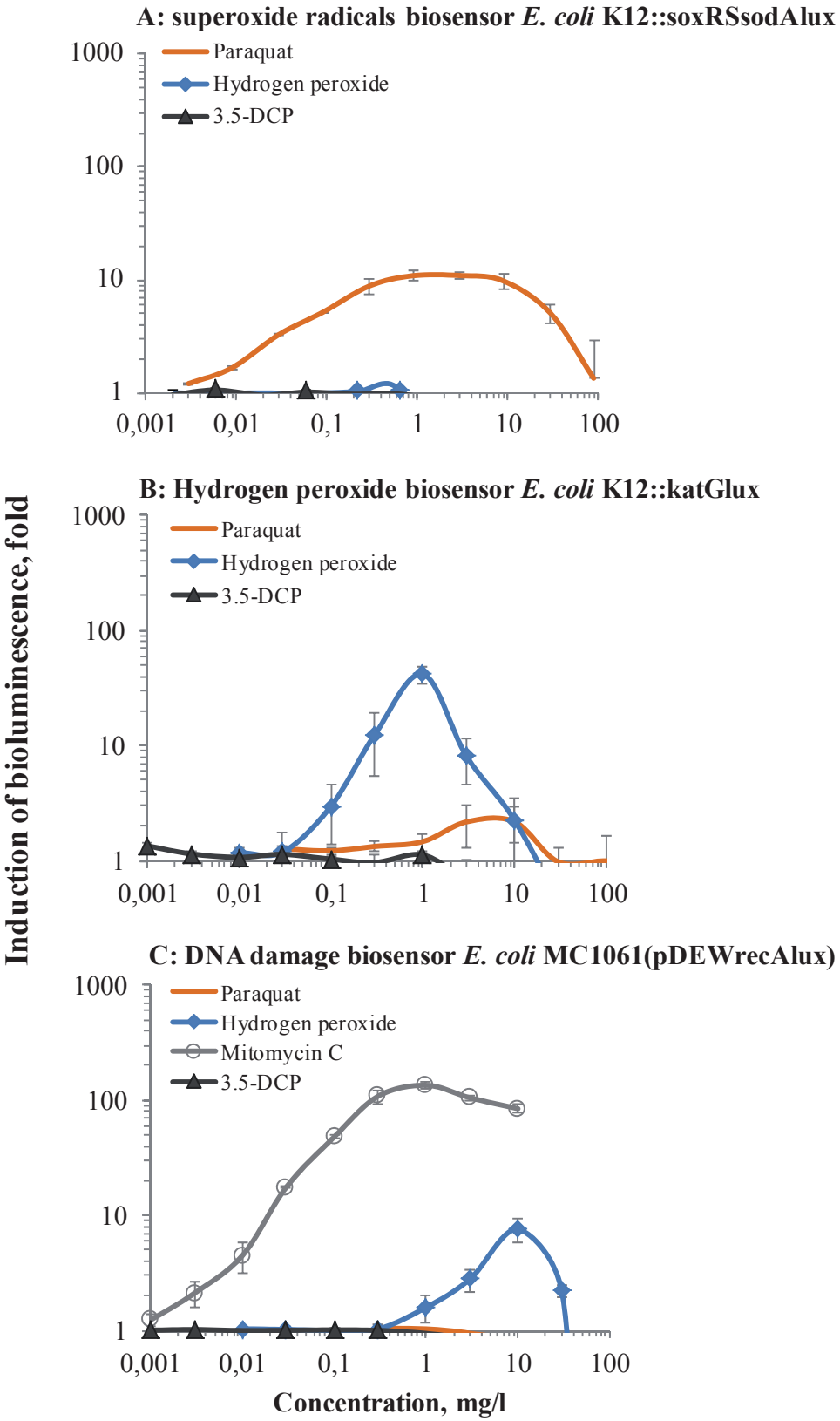

Figure 8. Induction of bioluminescence in stress-specific *Escherichia coli* biosensors after 2-h exposure to positive and negative control-chemicals.

3.3. Characterization of nanoparticles

The nanoparticles were selected based on their current use in consumer products (nano-ZnO, nano-Ag, nano-TiO_2) and/or high toxicity to aquatic organisms (nano-ZnO, nano-Ag, nano-CuO and C_{60}-fullerene). When possible, the chemically identical micro-sized particles were used in parallel to investigate the effect of size and the respective metal ions were used to investigate the effects of dissolution (Table 3). The micro-sized particles were clearly distinct from nano-sized particles, as they had lower specific surface areas (Table 3).

Table 3. Nanoparticles and reference chemicals used. Table is modified from Publication **I**.

Nanoparticle		**Reference for size**		**Reference for dissolution**
Name (size[1])	Surface area[2] (m^2/g)	Name	Surface area[2] (m^2/g)	
nano-ZnO (25-70 nm)	12.9	**micro-ZnO**	7.2	**$ZnSO_4 \cdot 7H_2O$**
nano-CuO (30 nm)	25.5	**micro-CuO**	0.64	**$CuSO_4 \cdot 5H_2O$**
nano-TiO_2 (50-70 nm)	24.8	**micro-TiO_2**	8.5	**no**
nano-Ag (<100 nm)	n.d.	**no**	no	**$AgNO_3$**
Fullerene	n.d.	**no**	no	**no**

[1]Manufacturer-advertised primary size; [2]Measured by BET.

The SEM figures showed that nano-sized particles were indeed substantially smaller than micro-sized particles and that the primary size of nanoparticles was under 100 nm (Figure 9) confirming the information provided by the manufacturer.

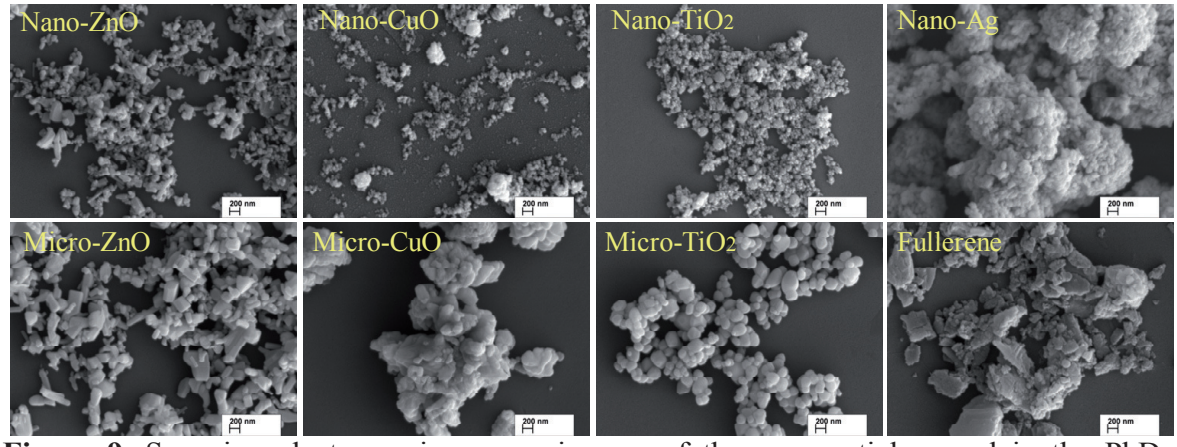

Figure 9. Scanning electron microscopy images of the nanoparticles used in the PhD study. Figure is modified from Publication **I**. Scale bar: 200 nm.

3.3.1. Characterization of nanoparticles in deionized water and various (eco)toxicological test media

Expectedly, the hydrodynamic size of NPs (Figure 10) was larger than their primary size (Figure 9). For example, when the mean primary size of the nano-CuO particles in powder was 30 nm (Table 3, Figure 9), the mean hydrodynamic size of these particles in DI water was 190 nm (Figure 10A). The difference in the primary size of NPs measured by SEM and the hydrodynamic size of NPs measured by DLS is a well-known phenomenon and can be explained by the different measurement principles of these two techniques. When SEM measures the primary size of dry powder of particles, the DLS measures the Brownian motion in the aqueous solution of particles and gives the hydrodynamic diameter, which in addition to the primary size includes also a few solvent layers (Kumar et al., 2011). In addition, in the aqueous solution the nanoparticles tend to aggregate, thereby giving the mean size of clustered particles. The particles aggregate even more when dispersed in media with a high content of mineral salts and organic components. This was evident when the hydrodynamic diameter of nano-CuO was compared in DI water and in toxicity test media − 0.1% amino acids-supplemented HMM medium. The average particle size in DI water was 190 nm and in the HMM media 385 nm (Figure 10).

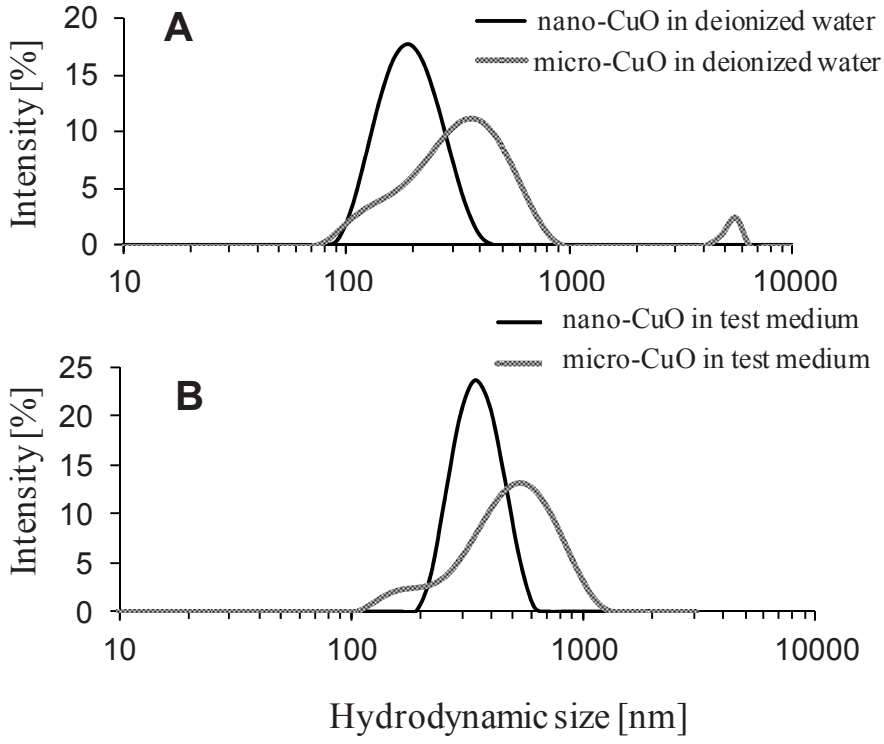

Figure 10. Hydrodynamic diameter of nano-CuO (6.35 mg Cu/l) and micro-CuO (635 mg Cu/l) in deionized water (A) and heavy metal MOPS medium (B) as measured by dynamic light scattering. Modified from the Supplementary Figure 1 of Publication **III**.

As mentioned in the theoretical part, the size of nanoparticles in toxicological assays is very important, because it determines the specific surface area of the NPs and therefore the intensity of their interactions with the surrounding environment. NPs may interact with each other, leading to aggregation, with the test media components, leading to the aggregation/agglomeration or dissolution (in case of metal-containing NPs) and with test organism, leading to the toxic effects. This complex interplay of several factors determines the mechanisms of toxicity of NPs, which should be therefore elucidated only in the context of the specific test environment (Warheit, 2008). To understand the behavior of the metal-containing NPs in different test environments, a comprehensive study analyzing the dissolution of nano-CuO in different test media was undertaken (Publication **II**). Hypothesizing that nano-CuO exerts toxicity at least partly *via* release of Cu ions, the effects of the test media on speciation of Cu ions were studied in parallel. Two techniques – a Cu-ion selective electrode (Cu-ISE) and a recombinant luminescent Cu-ion specific bacterial biosensor *P. fluorescens* OS8::CueRPcopAlux – that enable the quantification of free and bioavailable (intracellular) Cu, respectively, were applied.

Analysis of 17 different ecotoxicological and microbiological test media with gradually increasing content of organics (modeled by different concentration of amino acids) showed that organics in the test media was effectively complexing the dissolved Cu ions from $CuSO_4$ (Table 2 in Publication **II**). This was especially evident in case of 0.9% NaCl, where the gradual addition of amino acids was accompanied by a delayed response of Cu-ion biosensor as well as of the Cu-ISE (Figure 1E,F in Publication **II**). The maximum tested concentration of amino acids (0.5% by weight) decreased the LOD of bacterial Cu-ion biosensor and Cu-ISE to Cu ions by 90- and 2000-fold, respectively (Table 2 in Publication **II**).

Analogously to $CuSO_4$, the fraction of free Cu dissolved from nano-CuO ions mostly decreased when nano-CuO was introduced to various laboratory test media. However, there were some significant exceptions: in the test media with high content of organic, the fraction of free Cu from nano-CuO was significantly higher than could be predicted from the results of $CuSO_4$. This indicated that in the presence of organic matter, the dissolution of nano-CuO increased. This is a relatively unknown phenomenon, which may be explained by ligand-enhanced dissolution, when the complexation of dissolved metal ions pushes the equilibrium of metal-containing NPs towards their further dissolution. Similar phenomenon was observed for nano-ZnO exposed to metal ion-complexing citrate (Li et al., 2011) and also for nano-CuO exposed to ethylenediaminetetraacetic acid (EDTA) in our later studies (Publication **III**).

Among the media suitable for the cultivation of bacteria the limits of determination for Cu ions dissolved from nano-CuO were the lowest in amino acids-supplemented 0.9% NaCl and in HMM medium (Table 3 in Publication **II**). For this reason, these test media were selected as the test environments for all further toxicological studies.

3.4. Profiling of nanoparticles using luminescent bacteria

Combination of constitutively bioluminescent *sod*-deficient *E. coli* strains, metal-ion-specific *E. coli* and *P. fluorescens* biosensors and superoxide anions-inducible *E. coli* strain was used to study the toxicological effects of five types of nanoparticles − nano-CuO, nano-ZnO, nano-Ag, nano-TiO_2 and fullerene (Publication **I**). In addition, the sub-toxic effects of nano-CuO were also analyzed with hydrogen peroxide and DNA damage inducible *E. coli* strains (Publication **III**). It was hypothesized that if the tested NPs would induce ROS, then (i) the *sod*-deficient strains would show remarkably higher sensitivity compared to the respective non-mutated strain and (ii) induction of ROS-inducible *E. coli* strains would be observed. If the toxicological effects of nanoparticles would be driven by dissolved metals, the metal-ion specific biosensors would show this process.

As an example, the toxicological profiling of nano-CuO is described here in more detail. When CuO NPs in parallel to CuO microparticles and $CuSO_4$ were exposed to the combination of different bacterial biosensors, it was evident that all the copper compounds induced oxidative damage in *E. coli* cells, showing higher toxicity to *sod* double and triple mutants than to *sod* single mutants and the wild-type strain (Figure 11).

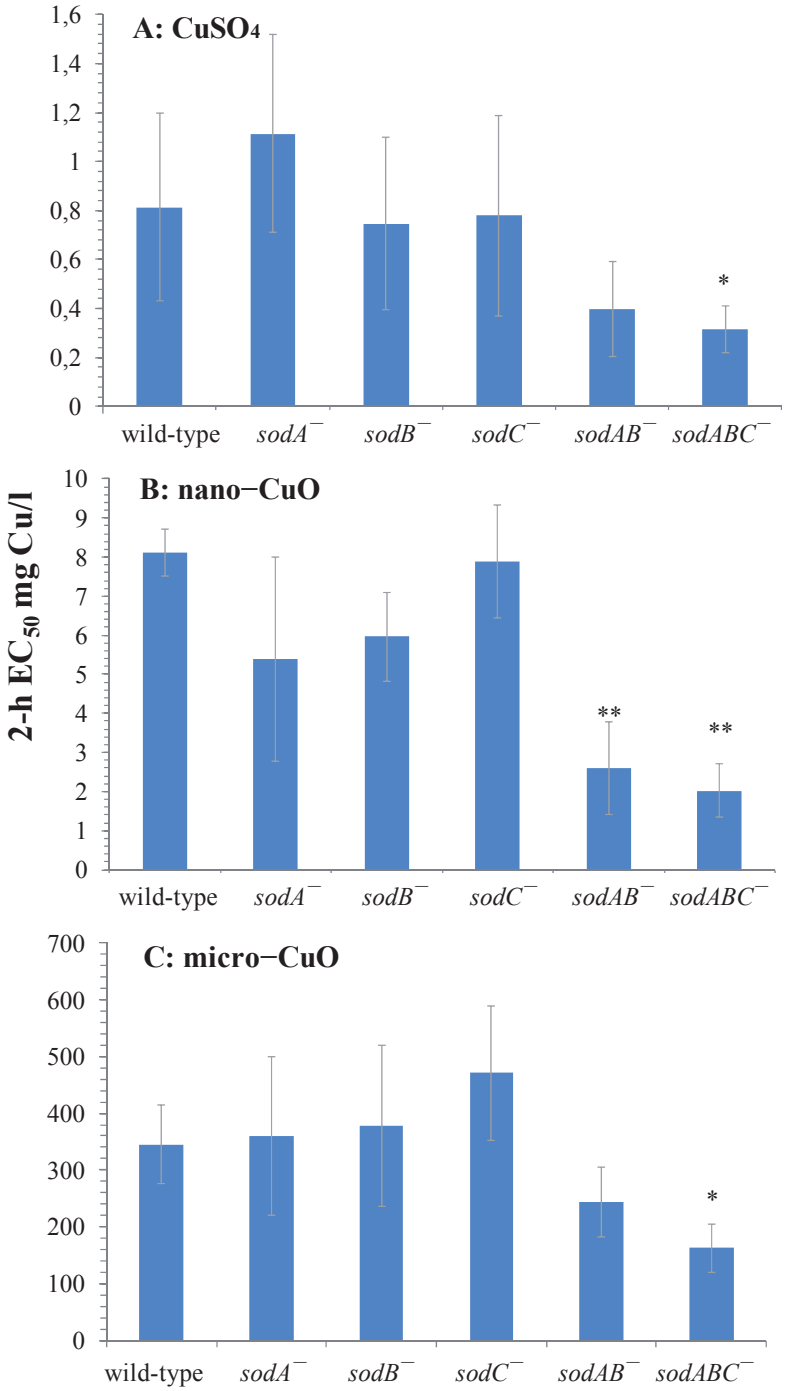

Figure 11. Toxicity (2-h EC_{50}, mg Cu/l) of Cu formulations to superoxide dismutase (*sod*)-wild-type *Escherichia coli* strain and *sod*-defective strains after 2-h exposure (***(p≥99%) and ** (p≥95%) indicate that *sod*-defective strain is different from the wild-type strain).

This was also confirmed by stress-inducible biosensors, which showed dose-dependent production of superoxide radical and hydrogen peroxide by all the Cu formulations (Figure 12B,C). In addition, DNA-damage specific biosensor revealed that similarly to $CuSO_4$, CuO particles induced single-stranded DNA breaks (Figure 12D).

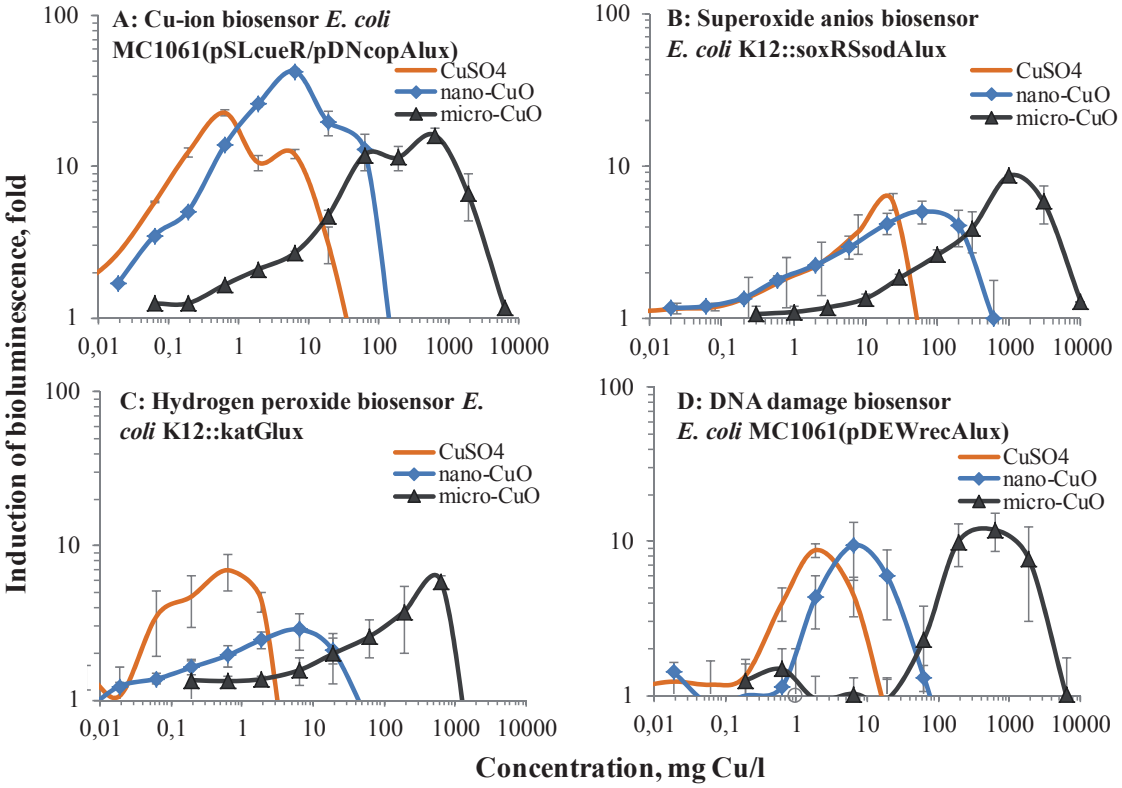

Figure 12. Induction of bioluminescence in *Escherichia coli* biosensors after 5-h (Cu-ion, superoxide anions and hydrogen peroxide biosensors) and 8-h (DNA damage biosensor) exposure to Cu formulations. All concentrations are nominal and presented on Cu basis.

Interestingly, the concentration-response pattern of ROS- and DNA damage-specific biosensors (Figure 12B-D) to different Cu formulations was similar to that of Cu ions biosensor (Figure 12A). Particularly, all the biosensors were most sensitive to $CuSO_4$ followed by nano-CuO and micro-CuO. In addition, the fold induction values of the three stress-specific biosensors and the Cu-ion biosensor to the tested Cu formulations were similar (Figure 12). Taking into consideration that Cu ion biosensor is responding to intracellular Cu ions (Rensing and Maier, 2003; Ivask et al., 2009), this observation allowed us to conclude that the ROS and DNA damage responses were triggered by Cu ions solubilized from CuO particles. This was also confirmed by chemical analysis of dissolved Cu from the ultracentrifuged supernatants of $CuSO_4$, nano-CuO and micro-CuO (Figure 4A in Publication **III**). In addition, chelation of Cu ions by metal ion chelator EDTA abolished the response of ROS and DNA damage biosensors to $CuSO_4$, CuO nano -and microparticles (Figure 5 in Publication **III**), showing that only Cu ions and not particles themselves triggered the response of these biosensors.

Similar toxicological profiling was also performed for other NPs and the results are summarized in Table 4.

Table 4. Summary on toxic effects of nanoparticles to recombinant *Escherichia coli* biosensors according to Publications **I** and **III**.

Name	**2-h EC_{50} (inhibition of bioluminescence), mg/l of metal**		**Induction[1] of ROS in ROS biosensor, mg/l of metal (time needed for induction)**		**Impact of dissolution on toxicity**
	wild-type *E. coli*	Δ*sodABC* *E. coli*	O_2^-	H_2O_2	
nano-CuO	8.1	2.0	6.4 (4-h)	1.9 (5-h)	100%
nano-ZnO	54	4.5	not induced	n.t[2]	100%
nano-Ag	46	3.1	0.081 (4-h)	n.t[2]	not clear
nano-TiO_2[3]	>12,000	>12,000	1800 (7-h)	n.t[2]	n.t[2]
Fullerene	>20,800	3,882	not induced	n.t[2]	n.t[2]

[1] Limit of determination of the bacterial biosensor defined as 2-fold increase in bioluminescence is indicated; [2] Not tested ; [3] Illuminated *prior* the test.

The ROS-inducing concentrations of all NPs except of **nano-CuO** were substantially lower than respective 2-h EC_{50} values. In case of nano-CuO, the concentration which induced ROS was close to the 2-h EC_{50} value (Table 4). This shows that probably ROS was not the primary toxicity mechanism but rather a consequence of toxicity of nano-CuO *via* an alternative toxicity pathway as also briefly discussed in Publication **III**. Nevertheless, the most important conclusion was that nano-CuO had no nanosize-specific antibacterial effects and its toxicity to bacteria can be fully explained through the toxicity of Cu ions.

Nano-ZnO was about 10-fold more toxic to *sodABC*-mutant than to the wild-type *E. coli*, showing that oxidative stress might be important in acute toxicity of nano-ZnO. However, superoxide anions-biosensor was not induced with sub-toxic concentrations of nano-ZnO, suggesting that the acute oxidative injury induced by nano-ZnO was independent from the overproduction of superoxide anions. In addition, Zn-inducible biosensor showed that already after 30-min exposure, nano-ZnO was fully dissolved (Figure 2 in Publication **I**). Interestingly, the dissolution of nano-ZnO was systematically overestimated by Zn-biosensor, suggesting that Zn ions from the nano-ZnO were more bioavailable to Zn-biosensor than Zn ions applied as $ZnSO_4$. The expression of 2-h EC_{50} values of nano-ZnO, micro-ZnO and $ZnSO_4$ on bioavailable Zn ions (Table 4 in Publication **I**) showed that the toxicity of nano-ZnO to bacteria was apparently mediated by Zn ions.

Despite of the huge number of articles on the antibacterial effects of Ag ions and **nano-Ag**, the mechanism of toxicity of both is not jet fully understood. Also the results of this study were controversial: when the quantification of bioavailable Ag ions revealed that the general toxicity of nano-Ag to *E. coli sod*-mutants and wild-type was mediated *via* Ag ions, the results from superoxide anions-inducible strain revealed opposite (Table 4 in Publication **I**). Similarly, in our on-going studies we showed that despite that the antibacterial effects of various Ag NPs were mediated by intracellular Ag ions, the direct contact of bacteria with Ag particles increased the efficiency of transportation of Ag ions into bacterial cells (Bondarenko, unpublished data). The reason for that may be the fact that Ag NPs are mainly located on the surface of the bacteria as shown

previously by TEM (Morones et al., 2005). The local release of Ag ions in the vicinity of the bacterial cell may perforate the bacterial cell wall (Lok et al., 2006), disturb the permeability of the membranes and the functionality of Ag ion efflux systems (Ruparelia et al., 2008). Therefore, when the Ag ions will be exposed to bacteria on the surface of NPs, they will be more bioavailable to the cells due to the membrane damage and decreased ability of bacterial cells to export Ag ions. These results suggest a complex mechanism of toxicity of Ag NPs, occurring both inside and outside the bacterial cell and the need for the further investigation into the detailed mechanism of toxicity of nano-Ag.

Nano-TiO_2 was not toxic to the bacterial cells even at concentrations 12,000 mg/l. In addition, superoxide anions in *E. coli* cells were induced only by remarkably high concentrations (1,800 mg Ti/l) of illuminated TiO_2 and only after 7 h. This indicates that TiO_2, at least the nanoparticles purchased from Sigma-Aldrich (CAS number 13463-67-7) and used in this study, were ineffective antimicrobials. The toxicity of nano-TiO_2 that has been shown earlier by other researchers, depended greatly on the size of the used NPs, their crystalline form (rutile or anatase) and the intensity and wavelength of the light used for photoactivation (Wei et al., 1994; Hu et al., 2009). Generally, anatase was considered to be the photoactive form of TiO_2 that killed microbes in minutes (Wei et al., 1994), while rutile was considered to have lower photocatalytic and hence, antimicrobial, activity (Rincón and Pulgarín, 2003). The TiO_2 used in our study was most probably in rutile form as it did not shown any antibacterial effects nor was active against eukaryotic cells (Heinlaan et al., 2008; Kasemets et al., 2009). In addition, the fact that only illuminated nano-TiO_2 induced ROS in superoxide anions biosensor highlighted once more the importance of photocatalytic effects in toxicity of nano-TiO_2.

Similarly, also **C_{60}-fullerene** was not toxic to wild-type *E. coli* cells even at 20,800 mg/l. At the same time the 2-h EC_{50} of fullerene to *E. coli sodABC* triple mutant was 3,882 mg/l. Despite the fact that an EC_{50} value could be derived for this mutant, the concentration was extremely high. Also, taken into account that the superoxide anion-biosensor *E. coli* was not induced by fullerene, we concluded that fullerene used in this study did not produce significant levels of superoxide anions. This result supports the hypothesis that water-dispersed C_{60}-fullerene is not toxic to the cells and the toxicity of these NPs observed in early studies was due to the toxic solvent tetrahydrofuran.

In summary, two of tested metal-containing NPs – nano-CuO and nano-ZnO – showed dissolution-dependent antibacterial efficiency, suggesting no nanosize-specific toxic effects. Interestingly, most publications by others showed that in eukaryotic cells such as protozoa, yeast and human cells *in vitro* the toxic effects of these particles cannot be explained only by dissolution (Karlsson et al., 2008; Xia et al., 2008; Kasemets et al., 2009; Mortimer et al., 2011). However, no direct comparison on toxicity of metal-containing NPs to eukaryotic and prokaryotic cells has been performed to date. Therefore, a study intending to compare the role of Cu ions in toxicity of nano-CuO to eukaryotic and prokaryotic cells was undertaken. To adequately compare the effects of nano-CuO in pro- and eukaryotic cells, the same batch and stock of nano-CuO, which was used for

toxicity profiling with bacterial biosensors, was also used in the study with eukaryotic cells.

3.5. Profiling of nanoparticles using human cell-based assays

3.5.1. Preparation and characterization of neuronal (precursor) cells

The human embryonic stem cells (hESC) were used as a model system for the toxicological profiling of nano-CuO in eukaryotic cells. Before exposure, the hESC cells were differentiated to neural progenitor cells (NEPs) and further inside the neurospheres to more mature neuronal precursor cells as described in the Materials and Methods (Figure 5) and Publication **IV**. The development of cells inside the neurospheres mimicked the basic processes of brain development, such as proliferation, differentiation, migration, apoptosis, and each of these processes may be modulated by developmental neurotoxicants (Moors et al., 2009). Analysis of expression of marker genes showed that during the differentiation of hESC the expression of pluripotency markers such as Nanog, Lefty and Oct4 decreased and the expression of neural stem cell markers (such as Pax6) and neuronal markers such as β-tubulin III (Tubb3) and Slc17a6 increased (Figure 2 in Publication **IV**). Proper development of NEPs was also proven by very low expression of endoderm marker FoxA1 and mesoderm marker Brachyury (data not shown). All the previous data showed the efficient differentiation of hESC into neural cells.

3.5.2. Evaluation of neurospheres assay with methylmercury

The potential of neurospheres-system to detect neurodevelopmental toxicity was first evaluated using methylmercury. Methylmercury is the most toxic form of mercury and one of the few chemicals, which have been established as developmental neurotoxicants in human (Mergler et al., 2007). The developmental toxicity of methylmercury became evident in the 1960s in Minamata (Japan), where epidemic blindness and mental disorders were observed in children born from mothers who consumed fish from contaminated water (Harada, 1995). The extremely high toxicity of methylmercury is due to its ability to easily penetrate biological barriers, including blood-brain barrier. Intracellular mercury has been shown to damage DNA (Sager et al., 1984), disrupt neuronal migration, to inhibit microtubule formation and proteins synthesis in neurons (Goyer, 1996).

Figure 13 represents the morphology of neurospheres with developing neuronal cells exposed to methylmercury for 18 days. Exposure to the highest concentration of methylmercury (11 mg/l, $5x10^{-5}$ M) changed the morphology of neurospheres. Neurospheres lost their characteristic round shape with defined boarders and acquired more undefined structure (shown by black arrows). According to resazurin assay, the viability of cells at the highest tested concentration of methylmercury was around 10%, showing that the morphology of neurospheres was reasonably good predictor of cellular viability (Figure 3A in Publication **IV**).

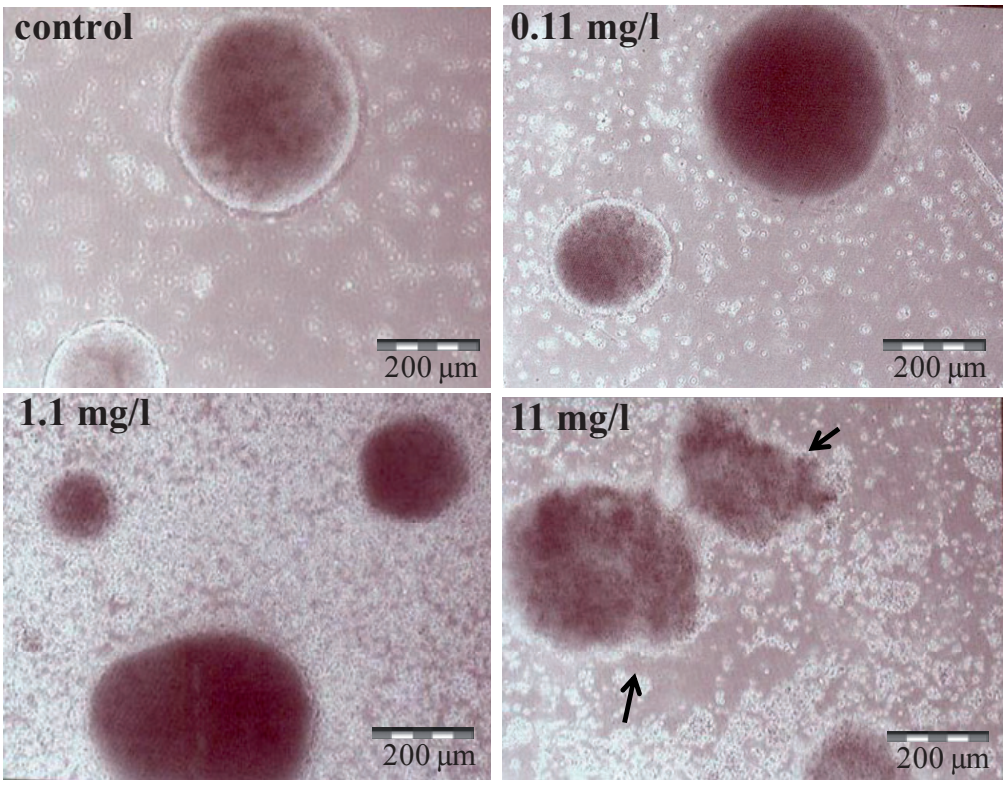

Figure 13. Phase contrast microscopy images of neurospheres after 18-day exposure to different concentrations of methylmercury. Black arrows indicate the alterations in the shape of the neurospheres.

The 18-day exposure of neurospheres to sub-toxic concentration of methylmercury (11 µg/l, 50 nM) induced significant down-regulation of neuronal precursor gene Notch1 and its down-stream target Neurod1 as detected by RT-PCR (Figure 3C in Publication **IV**). Also the markers of more mature cell phenotypes (Tubb3, Kcnj6 and Scl17a6) were down-regulated upon exposure to 50 nM methylmercury, showing that neurospheres system was sensitive enough to predict the potential neurodevelopmental effects of sub-toxic concentrations of methylmercury.

3.5.3. Toxicity of nano-CuO to neurospheres

Scientific publications have shown that CuO NPs have multiple targets in the brain, including ion channels (Liu et al., 2011), key protein (ά-synuclein) in Parkinson disease (Wang et al., 2009) and modulations of neurotransmitter levels (Boyes et al., 2012).

In acute toxicity assay the 48-h EC_{50} of nano-CuO was 35 mg/l (~26 mg Cu/l of nano-CuO) (Supplementary Figure 7 in Publication **IV**). However, much lower concentrations of CuO NPs were required for chronic toxicity: the chronic exposure (18 days) of neurospheres already to 0.5 mg Cu/l of nano-CuO resulted in morphological disorders (Figure 14). While in the absence and at low sub-toxic concentration (0.01 mg Cu/l) of CuO NPs, neurospheres were round and well-defined, at 0.5 mg Cu/l nano-CuO the density of neurospheres near to its borders

decreased probably because of the death of the cells. At 10 mg Cu/l CuO NPs, the regular structure of neurospheres was lost (Figure 14) and the shape of neurospheres was characteristic to what was observed when the neurospheres were exposed to toxic concentrations of methylmercury (Figure 13).

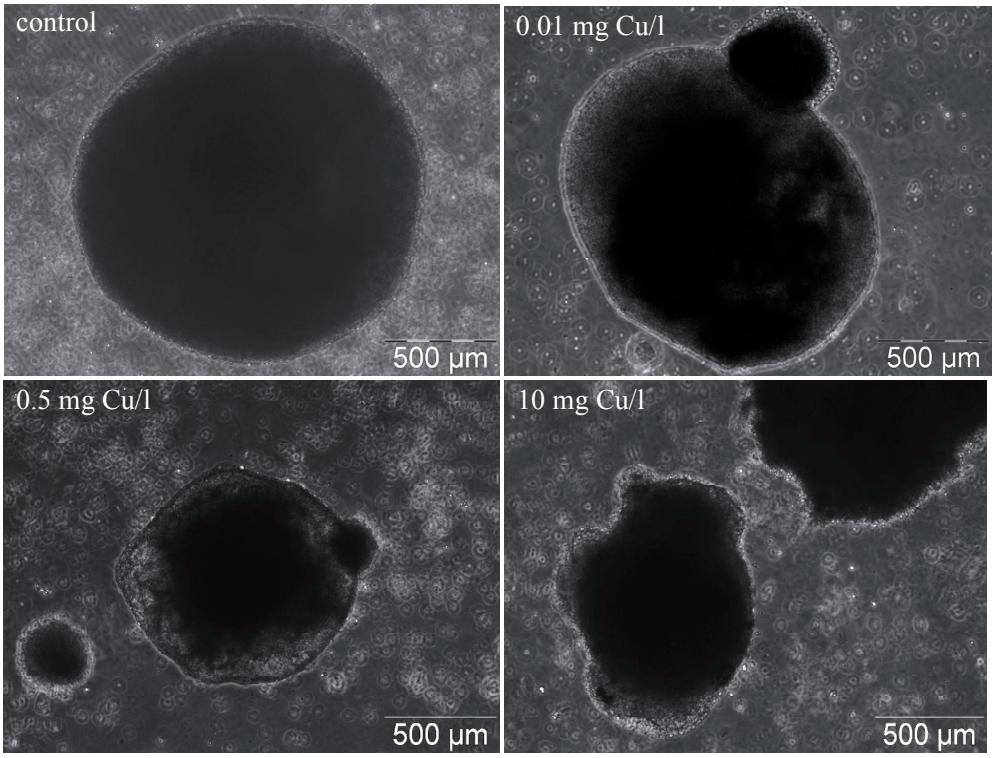

Figure 14. Phase contrast microscopy images of neurospheres after 18-day exposure to different concentrations of nano-CuO. Concentrations are presented on Cu basis.

The cell viability (resazurin) assay showed that 18-day EC_{50} of nano CuO was 0.5 mg Cu/l (Figure 15).

Interestingly, when the EC_{50} of methylmercury in acute and chronic toxicity experiment was comparable (Figure 3A,B in Publication **IV**), the EC_{50} of CuO NPs was remarkably lower in the chronic test (chronic 18-day EC_{50}=0.5 mg Cu/l of nano-CuO (Figure 15) *vs* acute 48-h EC_{50}, ~26 mg Cu/l of nano-CuO) (Supplementary Figure 7 in Publication **IV**). This shows potential long-term toxic effects of nano-CuO on developing neuronal cells. Besides, CuO NPs were about one order of magnitude more toxic than equimolar ionic Cu (applied as $CuCl_2$*$2H_2O$) (Figure 15). This indicated that the toxic effects of CuO NPs were not only due to their dissolution into ionic copper. Most probably, the toxicity of CuO NPs in the neurospheres was accomplished by a combination of the dissolution and nanosize-specific properties, which was not the case in prokaryotic cells (Table 4; Publications **I**, **III**).

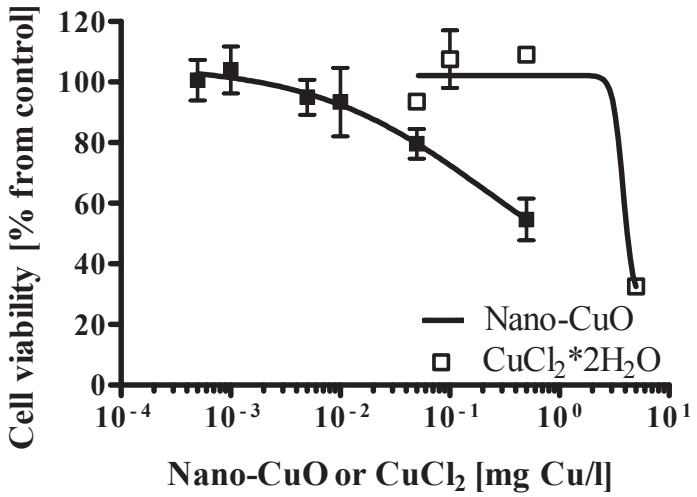

Figure 15. Chronic toxicity of CuO nanoparticles and $CuCl_2*2H_2O$ to neurospheres. Day11 neurospheres were exposed to Cu compounds for 18 days, cell viability was assessed by resazurin assay and expressed as percentage from un-affected control. Concentrations are presented on Cu basis. Mean of 3 (Nano-CuO) or 2 ($CuCl_2$) individual experiments ± standard error of the mean is shown.

The fact that only the metal ions affected prokaryotic cells whereas also particles as such had adverse effects on eukaryotic cells may be because of the ability of the latters to internalize NPs. The metal-containing nanoparticles therefore serve as Trojan-horse type carriers (Limbach et al., 2007) enabling the transport of high levels of metals into the cells. Once inside the cells, the metal ions start to release from metal-containing nanoparticles and may cause significant cellular damage. This means that intracellularization of metal-containing NPs may induce additional toxicity to eukaryotic cells. On the other hand, this kind of effect cannot be observed with bacterial cells that do not *a priori* intracellularize nanoparticles. Thus, before the use of metal-containing nanoparticles e.g., in antimicrobial applications, their potential side-effects to humans should be carefully characterized.

All these observations directed us to more detailed studies about the specific effects of nanosize in eukaryotic cells. Since this task was complicated using NPs of CuO that tended to dissolve during the test, NPs of polyethylene were selected as model for the further investigation. Polyethylene NPs were 33 nm in diameter, chemically inert, surfactant-stabilized and labeled with fluorescent chromophore, which allowed us to study the internalization of these NPs into human neurospheres.

3.5.4. Toxicity of nano-polyethylene to neurospheres

The time- and dose-dependent incorporation of fluorescent nano-polyethylene into neurospheres was observed as indicated by the dose-dependent increase in fluorescence (Figure 6A in Publication **IV**). Indeed, neurospheres that had been exposed to 180 mg/l nano-polyethylene for 18 days were fluorescent upon

excitation at 555 nm indicating efficient internalization of NPs. At the same time, no fluorescence was observed in control (non-exposed) neurospheres. Thus, using labeled nano-polyethylene as a fluorescent marker, it was shown that NPs of polyethylene and possibly other NPs may be internalized into the neurospheres (Figure 16). In addition to efficient internalization, we observed that polyethylene NPs exhibited also toxicological effects. After 18-day chronic exposure to 180 mg/l nano-polyethylene, the round-shaped structure and viability (ATP content) of neurospheres was altered (Figure 16; Figure 6C in Publication **IV**).

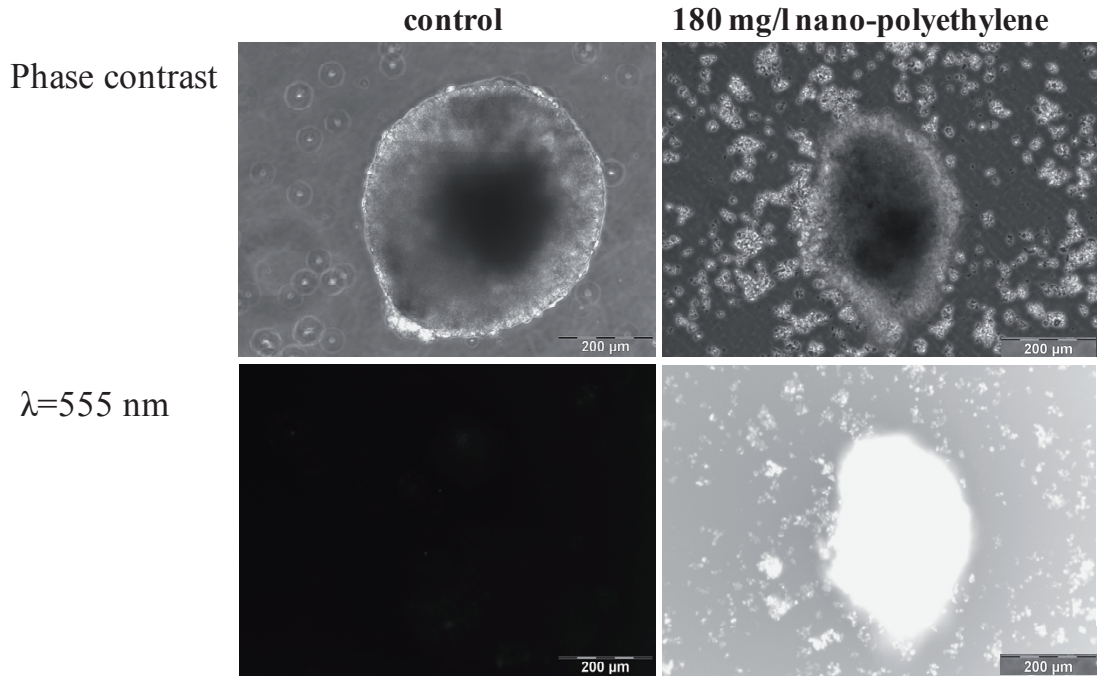

Figure 16. Phase contrast microscopy and fluorescence microscopy (at excitation wavelength λ=555, excitation time 2 s) images of unexposed neurospheres (control) and neurospheres that were exposed to 180 mg/l of polyethylene nanoparticles for 18-days.

Although the morphological effects of polyethylene were observed at relatively high concentrations (180 mg/l), already low sub-toxic concentration (22.5 mg/l, 18-day exposure) of nano-polyethylene altered the expression of several neuronal precursor and neuronal markers (Figure 6D in Publication **IV**). Interestingly, the genes which expression was down-regulated were similar to those that were affected by methylmercury and belonged to the NOTCH pathway: Notch1 receptor and Notch targets Hes5, Neurod1 and Ascl1. Notably, the activation of NOTCH pathway promotes the survival of neural stem cells (Androutsellis-Theotokis et al., 2006) and plays a key role in embryonic neural development (Lasky and Wu 2005). Premature inhibition of NOTCH pathway by nano-polyethylene may induce precocious maturation and depletion of neural stem cells, leading to several disorders (Bolos et al., 2007).

All these observations indicated that in human cells *in vitro* NPs may show nanosize-specific effects which would be impossible to predict using non-human cell-based test systems. Using nano-CuO as an example we showed that

surprisingly, metal-containing nanoparticles that are often proposed for antimicrobial applications, intending to replace the respective ions-based antimicrobial formulations, may exert nanosize-dependent undesired toxicity in human cells *in vitro* and not in bacterial cells. Due to the scarcity of relevant exposure studies (long term-exposure to low concentrations of NPs) and relevant non-cancerous human cell-based models, the potential nanosize-specific adverse effects in mammalian cells may be currently underestimated and should be definitely addressed further, especially *in vivo*.

3.6. Possibilities and limitations of the developed assays

Table 5 summarizes the relevance, cost-efficiency and applicability of the developed *in vitro* assays for the profiling of chemicals, e.g., synthetic nanoparticles.

Table 5. Characteristics of the assays developed in this PhD thesis.

Assay	Relevance	Simplicity and cost-efficiency	Information obtained
Bioluminescent *sod*-deficient *E. coli* strains	Highly relevant for bacteria. Low relevance for humans	Very simple Inexpensive	General toxicity to bacteria; potential to induce oxidative stress
Metal-ion- and stress-inducible *E. coli* strains	Highly relevant for bacteria. Low-to-medium relevance for humans	Relatively simple Inexpensive	Solubilization of metal-containing (nano)particles; potential to induce certain reactive oxygen species and DNA damage
Human neural cells *in vitro* differentiated from stem cells	Relatively high relevance for humans	Technically challenging Very expensive	General toxicity and neurotoxicity potential to humans

In general, the developed bacterial assays are powerful tools for the routine large-scale bioanalysis of chemicals, including nanoparticles. This may be very useful for the industry to create preliminary safety information, which due to the current chemicals regulation REACH will be soon needed for all chemicals produced in EU in quantities higher than 1 t/year. However, these biosensors were developed mostly bearing in mind the oxidative stress as a mechanism of toxic action, i.e. the biosensors were mainly intended to "catch" the chemicals with oxidative stress-inducing properties. For detailed mechanistic analysis, however, the use of large knock-outs libraries or "omics"-based approaches would be more appropriate choice.

The main advantages of the described human neural cell-based *in vitro* assay are the use of untransformed human cells and possibility to conduct long-term chronic toxicity assay. In general, long-term toxicity assays are not possible in *in vitro* cell cultures, as the dividing cells need passaging and therefore cannot be exposed to the chemicals during several generations. Our 3-dimensional neurospheres-based system enables to avoid this problem and is relatively unique

in addressing long-term neurotoxic potential of chemicals in unmodified human neuronal cells. This is much more close to the real *in vivo* situation than many other currently used models that use cancerous cells and irrelevantly high concentrations of chemicals for demonstration of biological effects. Still, despite of these advantages, the proposed model is based on *in vitro* cell culture, which does not take into account the biological complexity of living organism. The biggest challenge would be the further development of human stem cell-based 3-dimensional scaffolds, which mimic human tissues and would be most probably the most relevant systems for human toxicity assessment.

4. CONCLUSIONS

- A suite of 16 bacterial biosensors that enable to profile the key toxicity descriptors of synthetic metal-containing nanoparticles (general toxicity, generation of reactive oxygen species (ROS) and dissolution) was developed and consisted of:

  - newly constructed and evaluated bioluminescent *Escherichia coli* strains defective in reactive oxygen species inactivating enzymes (Publication **I**)

  - newly constructed and evaluated bioluminescent *E. coli* biosensors recognizing intracellular (i) superoxide anions, (ii) hydrogen peroxide and (iii) single-stranded DNA breaks (Publications **I** and **III**)

  - bioluminescent *E. coli* metal-ion biosensors recognizing intracellular Ag/Cu/Zn ions (Publications **I**–**III**)

- The physico-chemical properties of NPs of Ag, CuO, ZnO, TiO_2 and C_{60}-fullerene were characterized and test protocols for bioanalysis were developed. As one of the most important properties of metal-containing nanoparticles, their dissolution (release of metal ion) (Publications **I**, **III**) and speciation of the released metal ions (using Cu ions as example) in different toxicity test media (Publication **II**) were analyzed.

- Using developed bioluminescent biosensors, it was shown that Ag and CuO NPs induced the formation of superoxide anions in bacterial cells already at low sub-toxic concentrations (~0.1 and ~1 mg/l, respectively). TiO_2 NPs induced superoxide anions at 1,800 mg/l only in case of pre-illumination (photoactivation) and ZnO NPs showed the potential to generate ROS, but not superoxide anions (Publication **I**).

- CuO NPs also induced the formation of H_2O_2 and DNA damage in bacterial cells. The Cu ion-specific biosensor in combination with chemical analysis of Cu ion revealed that dissolution of CuO particles was the key factor triggering the ROS and DNA damage responses in bacteria (Publication **III**).

- In human neuronal cells differentiated from human stem cells *in vitro* CuO NPs exerted also additional, most likely size-related toxicity that was independent on dissolution.

- Exposure of human neuronal cells to fluorescently labeled nano-polyethylene showed efficient uptake of NPs by the cells and down-regulation of several genes (Notch1, Hes5, Neurod1, Ascl1) associated with the survival of neuronal cells in embryonic neural development (Publication **IV**).

REFERENCES

Adler S, Pellizzer C, Hareng L, Hartung T, Bremer S (2008) First steps in establishing a developmental toxicity test method based on human embryonic stem cells. Toxicol In Vitro (1):200-11

Ahamed M, Siddiqui MA, Akhtar MJ, Ahmad I, Pant AB, Alhadlaq HA (2010) Genotoxic potential of copper oxide nanoparticles in human lung epithelial cells. Biochem Biophys Res Commun 396:578−83

Amit M, Carpenter MK, Inokuma MS, Chiu CP, Harris HP, Waknitz MA, Itskovitz- Eldor J et al. (2000) Clonally derived human embryonic stem cell lines maintain pluri-potency and proliferative potential for prolonged periods of culture. Dev Biol 227: 271-8

Androutsellis-Theotokis A, Leker RR, Soldner F, Hoeppner DJ, Ravin R, Poser, Rueger MA, Bae SK, Kittappa R, McKay RD (2006) Notch signalling regulates stem cell numbers in vitro and in vivo. Nature 442(7104): 823-826

Aruoja V, Dubourguier HC, Kasemets K, Kahru A (2009) Toxicity of nanoparticles of CuO, ZnO and TiO_2 to microalgae *Pseudokirchneriella subcapitata*. Sci Total Environ 407(4), 1461 - 1468.

Arvizo RR, Bhattacharyya S, Kudgus RA, Giri K, Bhattacharya R, Mukherjee P (2012) Intrinsic therapeutic applications of noble metal nanoparticles: past, present and future. Chem Soc Rev 41(7): 2943-2970

AshaRani PV, Low Kah Mun G, Hande MP, Valiyaveettil S (2009) Cytotoxicity and genotoxicity of silver nanoparticles in human cells. ACS Nano 3(2):279-290

ATSDR (2005) Toxicological profile for zinc. US Department of Health and Human Services, Public Health Service, Agency for Toxic Substances and Disease Registry

Beckett WS, Chalupa DF, Pauly-Brown A, Speers DM, Stewart JC, Frampton MW, Utell MJ et al (2005) Comparing inhaled ultrafine versus fine zinc oxide particles in healthy adults: a human inhalation study. Am J Respir Crit Care Med 171:1129–1135

Belkin S, Smulski DR, Dadon S, Vollmer AC, Van Dyk TK, LaRossa RA (1997) A panel of stress-responsive luminous bacteria for toxicity detection. Wat Res 31:3009-3016

Belkin S (2003) Microbial whole-cell sensing systems of environmental pollutants. Curr Opin Microbiol 6:206-12

Bolos V, Grego-Bessa J, de la Pompa JL (2007) Notch signaling in development and cancer. Endocr Rev 28:339–363

Boyes WK, Chen R, Chen C, Yokel RA (2012) The neurotoxic potential of engineered nanomaterials. Neurotoxicology, In press

Brayner R, Ferrari-Iliou R, Brivois N, Djediat S, Bebedetti MF, Fievet F (2006). Toxicological impact studies based on *Escherichia coli* bacteria in ultrafine ZnO nanoparticles colloidal medium. Nano Letters 6(4): 866-870

Bulich AA, Isenberg DL (1981) Use of the luminescent bacterial system for the rapid assessmentb of aquatic toxicity. ISA Transactions 20, 29–33

Cassee FR, van Balen EC, Singh C, Green D, Muijser H, Weinstein J, Dreher K (2011) Exposure, health and ecological effects review of engineered nanoscale cerium and cerium oxide associated with its use as a fuel additive. Crit Rev Toxicol 41(3):213-229

Cengelli F, Maysinger D, Tschudi-Monnet F, Montet X, Corot C, Petri-Fink A, Hofmann H, Juillerat-Jeanneret L (2006) Interaction of functionalized superparamagnetic iron oxide nanoparticles with brain structures. J Pharmacol Exp Ther 318:108–116

Cerkez I, Kocer HB, Worley SD, Broughton RM, Huang TS (2012) Multifunctional cotton fabric: Antimicrobial and durable press. Journal of applied polymer science 124(5):4230-38

Chambers SM, Fasano CA, Papapetrou EP, Tomishima M, Sadelain M, Studer L (2009) Highly efficient neural conversion of human ES and iPS cells by dual inhibition of SMAD signaling. Nat Biotechnol 27:275-80

Chen X, Schluesener HJ (2008) Nanosilver: A nanoproduct in medical application. Toxicology Letters 186:1–12

Chen CY, Wu LC, Chen HY, Chung YC (2010a) Inactivation of *Staphylococcus aureus* and *Escherichia coli* in water using photocatalysis with fixed TiO(2). Water Air and Soil Pollution, 212(1-4):231-238

Chen X, Zhong Z, Xu Z, Chen L, Wang Y (2010b) 2′,7′- Dichlorodihydrofluorescein as a fluorescent probe for reactive oxygen species measurement: Forty years of application and controversy. Free Radical Res, 44(6):587-604

Cho WS, Duffin R, Poland CA, Duschl A, Oostingh GJ, Macnee W, Bradley M, Megson IL, Donaldson K (2012) Differential pro- inflammatory effects of metal oxide nanoparticles and their soluble ions *in vitro* and *in vivo*; zinc and copper nanoparticles, but not their ions, recruit eosinophils to the lungs. Nanotoxicol 6(1):22-35

Choi O, Hu Z (2008) Size dependent and reactive oxygen species related nanosilver toxicity to nitrifying bacteria. Environm Sci Technol 42(12): 4583-4588

Choi JE, Kim S, Ahn JH, Youn P, Kang JS, Park K, Yi J, Ryu DY (2010) Induction of oxidative stress and apoptosis by silver nanoparticles in the liver of adult zebrafish. Aquat Toxicol 100(2):151-9

Christman MF, Morgan RW, Jacobson FS, Ames BN (1985) Positive control of a regulon for defenses against oxidative stress and some heat-shock proteins in *Salmonella typhimurium.* Cell 41:753–62

Cioffi N, Torsi L, Ditaranto N, Tantillo GL, Ghibelli L, Sabbatini T, Bleve-Zacheo M, D'Alessio P, Zambonin G, Traversa E (2005) Copper nanoparticle/polymer composites with antifungal and bacteriostatic properties Chemistry of Materials 17: 5255-5262

Colas JF, Schoenwolf GC (2001) Towards a cellular and molecular understanding of neurulation. Dev Dyn 221:117-45

Conti L, Cattaneo E (2010) Neural stem cell systems: physiological players or *in vitro* entities? Nat Rev Neurosci 11:176-87

Corot C, Petry KG, Trivedi R, Saleh A, Jonkmanns C, Le Bas JF, Blezer E, Rausch M et al. (2004) Macrophage imaging in central nervous system and in carotid atherosclerotic plaque using ultrasmall superparamagnetic iron oxide in magnetic resonance imaging. Invest Radiol 39:619–625

Crofton MK, Mundy WR, Lein PJ, Bal-Price A, Coecke S, Seiler AE, Knaut H, Buzanska L, Goldberg A (2011) Developmental neurotoxicity testing: recommendations for developing alternative methods for the screening andprioritization of chemicals. ALTEX 28: 9-15

Cuppett JD, Susan E, Duncan SE, Dietrich AM (2006) Evaluation of copper speciation and water quality factors that affect aqueous copper tasting response. Chem. Senses 31, 689– 697

Dai J, Wang C, Shang C, Graham N, Chen GH (2012) Comparison of the cytotoxic responses of *Escherichia coli (E. coli)* AMC 198 to different fullerene suspensions (nC60). Chemosphere. 87(4):362-8

Dang Y, Zhang Y, Fan L, Chen H, Roco MC (2010) Trends in worldwide nanotechnology patent applications: 1991 to 2008. J Nanopart Res. 12(3):687-706

Dardenne F, Nobels I, De Coen W, Blust R. 2007. Dose–response relationships and statistical performance of a battery of bacterial gene profiling assays. Appl Microbiol Biotechnol 75:223-234

Daunert S, Barrett G, Feliciano JS, Shetty RS, Shrestha S, Smith-Spencer W (2000) Genetically engineered whole-cell sensing systems: coupling biological recognition with reporter genes. Chem. Rev. 100, 2705–2738

Davidov Y, Rozen R, Smulski D R, Van Dyk T K, Vollmer A C, Elsemore D A, LaRossa R A, Belkin S. Improved bacterial SOS promoter::*lux* fusions for genotoxicity detection. Mutat Res. 2000; 46:97–107

Delay M, Frimmel FH (2012) Nanoparticles in aquatic systems. Anal Bioanal Chem. 402(2):583-92

Dimitrov SD, Mekenyan OG, Sinks GD, Schultz TW (2003) Global modeling of narcotic chemicals: ciliate and fish toxicity J. Mol. Struct. 622, 63– 70

Dobbing J (1968) Vulnerable periods in developing brain. Davison AN, Dobbing J (Eds.), Appl Neurochem, Davis, Philadelphia, pp. 287–316

Droge W (2002) Free radicals in the physiological control of cell function. Physiol Rev 82:47 – 95

Ebrahimnia-Bajestan E, Niazmand H, Duangthongsuk W, Wongwises S (2011) Numerical investigation of effective parameters in convective heat transfer of nanofluids flowing under a laminar flow regime. Int J Heat Mass Transfer 54, 4376-4388

Feynman R (1959) There's plenty of room at the bottom. A talk at the annual meeting of the American Physical Society at the California Institute of Technology.

Frant MS (1994) Historical perspective. History of the early commercialization of ion-selective electrodes. Analyst, 119:2293

Fridovich I, Freeman B (1986) Antioxidant defenses in the lung. Annu Rev Physiol. 48:693-702

Gabbay J, Borkow G, Mishal J, Magen E, Zatcoff R, Shemer-Avni Y (2006) Copper oxide impregnated textiles with potent biocidal activities. J. Ind. Text. 35:323– 335

Gajjar P, Pettee B, Britt DW, Huang W, Johnson WP, Anderson AJ (2009) Antimicrobial activities of commercial nanoparticles against an environmental soil microbe, *Pseudomonas putida* KT2440. Journal of Biological Engineering 3:9

Geiselhart L, Osgood M, Holmes DS (1991) Construction and evaluation of a self-luminescent biosensor. Ann N Y Acad Sci 646:53-60

Gharbi N, Pressac M, Hadchouel M, Szwarc H, Wilson SR, Moussa F (2005) Fullerene is a powerful antioxidant *in vivo* with no acute or subacute toxicity Nano Lett 5(12):2578–85

Ghule K, Ghule AV, Chen BJ, Ling LY (2006) Preparation and characterization of ZnO Nanoparticles coated paper and its antibacterial activity study. Green Chem 8:1034–1041

Girotti S, Ferri EN, Fumo MG, Maiolini E (2008) Monitoring of environmental pollutants by bioluminescent bacteria. Analytica Chimica Acta 608: 2-29

Gottschalk F, Sonderer T, Scholz RW, Nowack B (2009) Modelled environmental concentrations of engineered nanomaterials (TiO_2, ZnO, Ag, CNT, fullerenes) for different regions. Environ Sci Technol 43:9216–9222

Gou N, Onnis-Hayden A, Gu AZ (2010) Mechanistic Toxicity Assessment of Nanomaterials by Whole-Cell-Array Stress Genes Expression Analysis. Environ Sci Technol 44(15):5964-5970

Goyer RA (1996) Toxic effects of metals. In: Klaassen CD, editor. Casseret and Doull's Toxicology. The Basic science of poisons. 5th ed. New York: McGraw-Hill. p 691–736

Greenberg JT, Monach P, Chou JH, Josephy PD, Demple B (1990) Positive control of a global antioxidant defense regulon activated by superoxide-generating agents in *Escherichia coli.* PNAS 87, 6181–6185

Griffitt RJ, Weil R, Hyndman KA, Denslow ND, Powers K, Taylor D, Barber DS (2007) Exposure to copper nanoparticles causes gill injury and acute lethality to zebrafish (*Danio rerio*). Environ. Sci. Technol. 41(23):8178–86

Gunawan C, Teoh WY, Marguis CP, Amal R (2011) Cytotoxic origin of copper(II) oxide nanoparticles: Comparative studies with micron-sized particles, leachate, and metal salts. ACS Nano 27:7214-7225

Hakkila K, Maksimow M, Karp M, Virta M (2002) Reporter genes *lucFF*, *luxCDABE, gfp*, and *dsred* have different characteristics in whole-cell bacterial sensors. Analytical Biochemistry 301, 235–242

Handy RD, von der Kammer F, Lead JR, Hassellöv M, Owen R, Crane M (2008) The ecotoxicology and chemistry of manufactured nanoparticles. Ecotoxicology 17:287-314

Harada M (1995) Minamata disease: methylmercury poisoning in Japan caused by environmental pollution. Crit Rev Toxicol, 25: 1–24

Hassan HM, Fridovich I (1977) Enzymatic defences against the toxicity of oxygen and of streptonigrin in *Escherichia coli*. J Bacteriol 129:1574–1583

Hastings JW (1978) Bacterial bioluminescence: An Overview. In: *Methods in Enzymology,* 57 (ed. M.A. DeLuca), pp. 125-135. New York: Academic Press

Heinlaan M, Ivask A, Blinova I, Dubourguier HC, Kahru A (2008)Toxicity of nanosized and bulk ZnO, CuO and TiO_2 to bacteria *Vibrio fischeri* and crustaceans *Daphnia magna* and *Thamnocephalus platyurus*. Chemosphere 71(7):1308–16

Heinlaan M, Kahru A, Kasemets K, Arbeille B, Prensier G, Dubourguier HC (2010) Changes in the Daphnia magna midgut upon ingestion of copper oxide nanoparticles: A transmission electron microscopy study. Water Res 45(1):179-190.

Hu X, Cook S, Wang P, Hwang H (2009) *In vitro* evaluation of cytotoxicity of engineered metal oxide nanoparticles. Science of the Total Environment,407:3070–3072

Hu YL, Gao JQ (2010) Potential neurotoxicity of nanoparticles. Int J Pharm 394(1–2):115

Hussain F, Hojjati M, Okamoto M, Gorga RE (2006) Review article: Polymer- matrix nanocomposites, processing, manufacturing, and application: an overview. J Compos Mater 40: 1511–1575

Hwang ET, Lee JH, Chae,YJ, Kim YS, Kim BC, Sang BI, Gu MB (2008) Analysis of the toxic mode of action of silver nanoparticles using stress-specific bioluminescent bacteria. Small. *4,* 746-750

Imlay JA, Linn S (1987) Mutagenesis and stress responses induced in *Escherichia coli* by hydrogen peroxide. J Bacteriol 169:2967–2976

Invitrogen (2006) http://probes.invitrogen.com/media/pis/mp36103.pdf

ISO 27687:2008, Nanotechnologies - Terminology and definitions for nano-objects - Nanoparticle, nanofibre and nanoplate

ISO 21338:2010, Water quality - Kinetic determination of the inhibitory effects of 412 sediment, other solids and coloured samples on the light emission of *Vibrio fischeri* 413 (kinetic luminescent bacteria test)

Ivask A, Virta M, Kahru A (2002) Construction and use of specific luminescent recombinant bacterial sensors for the assessment of bioavailable fraction of cadmium, zinc, mercury and chromium in the soil. Soil Biol. Biochem. 34:1439-1447

Ivask A, Rõlova T, Kahru A (2009) A suite of recombinant luminescent bacterial strains for the quantification of bioavailable heavy metals and toxicity testing. BMC Biotech 9:41

Ivask A, Suarez E, Patel,T, Boren D, Ji Z; Holde P, Telesca D, Damoiseaux R et al. (2012) Genome-wide bacterial toxicity screening uncovers the mechanisms of toxicity of cationic polystyrene nanomaterials. Environ Sci Technol 46 (4): 2398–2405

Jiang W, Mashayekhi H (2009) Bacterial toxicity comparison between nano- and micro-scaled oxide particles. Environmental Pollution 157(5): 1619-1625

Johnston HJ, Hutchison GR, Christensen FM, Aschberger Karin, Stone V (2010) The biological mechanisms and physicochemical characteristics responsible for driving fullerene toxicity. Toxicol Sci 114(2): 162–182

Kahru A, Põllumaa L (2006) Environmental hazard of the waste streams of the Estonian oil-shale industry: an ecotoxicological review. Oil Shale, 23(1):53-93

Kahru A, Dubourguier HC (2010) From ecotoxicology to nanoecotoxicology, Toxicology 269 105-19

Karlsson HL, Cronholm P, Gustafsson J, Möller L (2008) Copper oxide nanoparticles are highly toxic: a comparison between metal oxide nanoparticles and carbon nanotubes. Chem Res Toxicol 21(9):1726–32

Kasemets K, Ivask A, Dubourguier HC, Kahru A (2009) Toxicity of nanoparticles of ZnO, CuO and TiO_2 to yeast *Saccharomyces cerevisiae*. Toxicol Vitro 23 1116–22

King RB (1999) Chemical structure and superconductivity. J Chem Inf Compos Sci 39 180–191

Kircher MF, Mahmood U, King RS, Weissleder R, Josephson L (2003) A multimodal nanoparticle for preoperative magnetic resonance imaging and intraoperative optical brain tumor delineation. Cancer Res. 63, 8122–8125

Klaine SJ, Alvarez PJ, Batley GE, Fernandes TF, Handy RD, Lyon DY, Mahendra S, McLaughlin MJ et al. (2008) Nanomaterials in the environment: behavior, fate, bioavailability, and effects. Environ Toxicol Chem 27(9):1825-51

Köhler S, Belkin S, Schmid RD (2000) Reporter gene bioassays in environmental analysis. Fresenius Journal of Analytical Chemistry 366: 769 – 779

Kovochich M, Espinasse B, Auffan M, Hotze EM, Wessel L, Xia T, Nel AE, Wiesner MR (2009) Comparative toxicity of C-60 aggregates toward mammalian cells: role of tetrahydrofuran (THF) decomposition. Environ Sci Technol 43(16):6378-84

Kreuter J, Alyautdin RN, Kharkevich DA, Ivanov AA (1995) Passage of peptides through the blood-brain barrier with colloidal polymer particles (nanoparticles). Brain Res 674:171-4

Kroto HW, Heath JR, O'Brien SC, Curl RF, Smalley RE. (1985) C60: Buckminsterfullerene. Nature 318:162

Kühn K.P, Chaberny Ir.F., Massholder K., Stickler M., Benz V.W., Sonntag H.-G, Erdinger L (2003) Disinfection of surfaces by photocatalytic oxidation with titanium dioxide and UVA light. Chemosphere, 53:71–77

Kumar A, Pandey AK, Singh SS, Shanker R, Dhawan A (2011) A flow cytometric method to assess nanoparticle uptake in bacteria. Cytometry A. 79(9):707-12

Kunzmann A, Andersson B, Thurnherr T, Krug H, Scheynius A, Fadeel B (2011) Toxicology of engineered nanomaterials: Focus on biocompatibility, biodistribution and biodegradation. Biochim Biophys Acta 1810:361-73

Kvitek L, Panacek A, Soukupova J, Kolar M, Vecerova R, Prucek R (2008) Effect of surfactants and polymers on stability and antibacterial activity of silver nanoparticles (NPs). J Phys Chem C 112(15): 5825-5834

LaRossa R, Smulski D, Van Dyk T (1995) Interaction of lead nitrate and cadmium chloride with *Escherichia coli* K-12 and *Salmonella typhimurium* global regulatory mutants. J. Ind. Microbiol. 14, 252-258

LaRossa R, Van Dyk T, Rosson RA (1998) Bioluminescence methods and protocols: *Photorhabdus luminescens luxCDABE* promoter probe vectors. Humana Press Inc 102:85-90

Lasky JL, Wu H (2005) Notch signaling, brain development, and human disease. Pediatr Res 57(5 Pt 2):104R-109R

Lee J, Murphy CL, Faini GJ, Baucom. TL (1974) Bacterial bioluminescence and its application to analytical procedures. In: Liquid Scintillation Counting: Recent Developments (P. E. Stanley and B. A. Scoggins, eds.), Academic Press, New York pp. 403-420

Lewis JC, Feltus A, Ensor CM, Ramanathan S, Daunert S (1998) Applications of reporter genes. Anal Chem, 70 (17)579A–585A

Li M, Zhu L, Lin D (2011) Toxicity of ZnO nanoparticles to *Escherichia coli*: Mechanism and the influence of medium components. Environ Sci Technol 45(5):1977-83

Limbach LK, Wick P, Manser P, Grass RN, Bruinink A, Stark WJ (2007) Exposure of engineered nanoparticles to human lung epithelial cells: influence of chemical composition and catalytic activity on oxidative stress. Environ Sci Technol 41 (11) 4158– 4163

Little JW (1983) The SOS regulatory system: control of its state by the level of *recA* protease. J Mol Biol 167:791–808

Liu Z, Liu S, Ren G, Zhang T, Yang Z (2011) Nano-CuO inhibited voltage-gated sodium current of hippocampal CA1 neurons via reactive oxygen species but independent from G-proteins pathway. Journal of Applied Toxicology 31(5): 439–445

Lok CN, Ho CM, Chen R, He QY, Yu WY, Sun H, Tam PK, Chiu JF, Che CM. 2006. Proteomic analysis of the mode of antibacterial action of silver nanoparticles. J Proteome. Res 5:916–924

de Lorenzo V, Timmis KN (1994) Analysis and construction of stable phenotypes in gram-negative bacteria with Tn5- and Tn10- derived minitransposons. Methods Enzymol 235:386–405

Lovern SB, Klaper R (2006) *Daphnia magna* mortality when exposed to titanium dioxide and fullerene (C-60) nanoparticles. Environ Toxic Chem 25:1132–1137

Lyven B, Hassellov M, Turner DR, Haraldsson C, Andersson K (2003) Competition between iron- and carbon-based colloidal carriers for trace metals in a freshwater assessed using flow fieldflow fractionation coupled to ICPMS. Geochim Cosmochim Acta 67:3791–3802

Marambio-Jones C, Hoek EMV (2010) A review of the antibacterial effects of silver nanomaterials and potential implications for human health and the environment. Journal of Nanoparticle Research 12: 1531–1551

Maynard AD (2007) Nanotechnology – toxicological issues and environmental safety. NATO Security through Science Series, 1-14

McMahon JA, Takada S, Zimmerman LB, Fan CM, Harland RM, McMahon AP (1998) Noggin-mediated antagonism of BMP signaling is required for growth and patterning of the neural tube and somite. Genes Dev 12 (10): 1438–52

Medina C, Santos-Martinez MJ, Radomski A, Corrigan OI, Radomski MW (2007) Nanoparticles: pharmacological and toxicological significance. Br J Pharmacol.150, 552–58

Meighen EA (1991) Molecular biology of bacterial bioluminescence. Microbiol Rev 55, 123-142

Mergler HA, Anderson LMC, Hing KR, Mahaffey M, Murray M, Sakamoto AH, Stern AH (2007) Methylmercury exposure and health effects in humans: a worldwide concern Ambio, 36: 3–11

Midander K, Cronholm P, Karlsson HL, Elihn K, Möller L, Leygraf C, Wallinder IO (2009) Surface characteristics, copper release, and toxicity of nano- and micrometer-sized copper and copper(II) oxide particles: a cross-disciplinary study. Small 5, 389-399

Moors M, Rockel TD, Abel J, Cline JE, Gassmann K, Schreiber T, Schuwald J, Fritsche E (2009) Human neurospheres as three-dimensional cellular systems for developmental neurotoxicity testing. Environ Health Perspect 117:1131-8

Morones JR, Elechiguerra JL, Camacho A, Holt K, Kouri JB, Ramírez JT, Yacaman,MJ (2005) The bactericidal effect of silver nanoparticles, Nanotechnology 16: 2346–2353

Mortimer M, Kasemets K, Vodovnik M, Marins□ek-Logar R, Kahru A (2011) Exposure to CuO nanoparticles changes the fatty acid composition of protozoa *Tetrahymena thermophila*. Env Sci Tech 45 (15), 6617-6624

Mueller NC, Nowack B (2008) Exposure modeling of engineered nanoparticles in the environment. Environ Sci Technol 42:4447–4453

Nel A, Xia T, Mädler L, Li N (2006) Toxic potential of materials at nanolevel. Science 311: 622–627

Nel AE, Madler L, Velegol D, Xia T, Hoek EM, Somasundaran P, Klaessig F, Castranova V, Thompson M (2009) Understanding biophysicochemical interactions at the nano-bio interface. Nat Mater 8:543-557

Nel A, Xia T, Meng H, Wang X, Lin S, Ji Z, Zhang H (2012) Nanomaterial toxicity testing in the 21st century: use of a predictive toxicological approach and high-throughput screening. Acc Chem Res DOI: 10.1021/ar300022h

Nohynek GJ, Lademann J, Ribaud C, Roberts MS (2007) Grey goo on the skin? Nanotechnology, cosmetic and sunscreen safety. Crit Rev Toxicol 37(3):251-77

NuCrust Pharmaceuticals, http://www.nucryst.com/background_history.htm

Oberdörster G, Sharp Z, Atudorei V, Elder A, Gelein R, Kreyling W, Cox C (2004) Translocation of inhaled ultrafine particles to the brain. Inhal Toxicol. 16:437–445

Oberdörster E, Zhu S, Blickley T, McClellan-Green P, M. Haasch (2006) Ecotoxicology of carbon-based engineered nanoparticles: effects on aquatic organisms Carbon, 44:1112–1120

Onnis-Hayden A, Weng H, He M, Hansen S, Ilyin V, Lewis K, Gu AZ (2009) Prokaryotic real-time gene expression profiling for toxicity assessment. Environ Sci Technol 43(12): 4574-81

Pal S, Tak YK, Song JM (2007) Does the antibacterial activity of silver nanoparticles depend on the shape of the nanoparticle? A study of the gram-negative bacterium *Escherichia coli.* Appl Environ Microbiol 73:1712–1720

Panacek A, Kvitek R, Prucek M, Kolar R, Vecerova N, Pizurova VK, Sharma T et al (2006) Silver colloid nanoparticles: synthesis, characterization, and their antibacterial activity. J Phys Chem B 110:16248–53

Park HJ, Kim JY, Kim J, Lee JH, Hahn JS, Gu MB, Yoon J (2009) Silver-ion-mediated reactive oxygen species generation affecting bactericidal activity. Water Research 43(4): 1027-1032

Parvez S, Venkataraman C, Mukherji S (2006) A review on advantages of implementing luminescence inhibition test (*Vibrio fischeri*) for acute toxicity prediction of chemicals. Environment International 2006, 32:265-268

Pecher J, Huber J, Winterhalder M, Zumbusch A, Mecking S (2010) Tailor-made conjugated polymer nanoparticles for multicolor and multiphoton cell imaging. Biomacromolecules. 11(10):2776-80

Peters A, Veronesi B, Calderon-Garciduenas L, Gehr P, Chen LC, Geiser M, Reed W et al. (2006) Translocation and potential neurological effects of fine and ultrafine particles a critical update. Part Fibre Toxicol 3:13

Philips LG, Babrano DM (1997) The influence of fat substitutes based on protein and titanium dioxide on the sensory properties of lowfat milk. J. Dairy Sci. 80, 2726–2731

Piccinno F, Gottschalk F, Seeger S, Nowack B (2012) Industrial production quantities and uses of ten engineered nanomaterials for Europe and the world. J Nanopart Res 14: 1109–1120

Purohit HJ (2003) Biosensors as molecular tools for use in bioremediation. J. Clean. Prod., 11, 293–301

Puzyn T, Rasulev B, Gajewicz A, Hu X, Dasari TP, Michalkova A, Hwang HM (2011) Using nano-QSAR to predict the cytotoxicity of metal oxide nanoparticles. Nat Nano 6(3): 175-178

Raab C, Simkó M, Fiedeler U, Nentwich M, Gazsó A (2011) Production of nanoparticles and nanomaterials. NanoTrust Dossier No. 006; http://epub.oeaw.ac.at/ita/nanotrust-dossiers/dossier006en.pdf

Rensing C, Maier M (2003) Issues underlying use of biosensors to measure metal bioavailability. Ecotoxicol. Environ. Saf. 56, 140-147

Renwick LC, Brown D, Clouter A, Donaldson K (2004) Increased inflammation and altered macrophage chemotactic responses caused by two ultrafine particle types Occup. Environ. Med., 61, 442–447

Rice RH, Vidrio EA, Kumfer BM, Qin Q, Willits NH, Kennedy IM, Anastasio C (2009) Generation of oxidant response to copper and iron nanoparticles and salts: Stimulation by ascorbate. Chem Biol Interact 181(3):359-65

Rincón AG, Pulgarín C (2003) Photocatalytical inactivation of *E. coli* effect of (continuous- intermitent) light intensity and of (suspended-fixed) TiO_2 concentration. Appl Catal B 44:263–284

Robichaud CO, Uyar AE, Darby MR, Zucker LG, Wiesner MR (2009) Estimates of upper bounds and trends in nano-TiO_2 production as a basis for exposure assessment. Environm Sci Techn 43(12): 4227-33

Roney C, Kulkarni P, Arora V, Antich P, Bonte F, Wu A, Mallikarjuana NN, Manohar S, Liang HF, Kulkarni AR, Sung HW, Sairam M, Aminabhavi TM (2005) Targeted nanoparticles for drug delivery through the blood–brain barrier for Alzheimer's disease. J. Control Release 108, 193–214

Rossi L, Arciello M, Capo C, Rotilio G (2006) Copper imbalance and oxidative stress in neurodegeneration. Ital J Biochem 55: 212-221

Ruparelia JP, Chatterjee AK, Duttagupta SP, Mukherji S (2008). Strain specificity in antimicrobial activity of silver and copper nanoparticles. Acta Biomaterialia 4(3): 707-716

Sager PR, Aschner M, Roder PM (1984) Persistent differential alterations in developing cerebellar cortex of male and female mice after methylmercury exposure. Dev Brain Res 12:1–11

Sambrook J, Fritsch EF, Maniatis T (1989) *Molecular Cloning: A Laboratory Manual*, 2nd ed.; Cold Spring Harbor Laboratory Press: Plainview, NY, USA

Sayes CM, Fortner JD, Guo W, Lyon D, Boyd AM, Ausman KD, Tao YJ, Sitharaman B et al (2004) The differential cytotoxicity of water-soluble fullerenes. Nano Lett 4:1881–7

Schumacher KS, Hasenzahl, M. Moerters (2004) Powder mixture consisting of titanium dioxide, zinc oxide and zinc/titanium mixed oxide. Patent WO2004056706

Serpone N, Dondi D, Albini A (2007) Inorganic and organic UV filters: their role and efficacy in sunscreens and suncare products. Inorg Chim Acta 360:794–802

Sigma-Aldrich (2011) Material safety data sheet for <100 nm zinc oxide nanopowder, http://www.sigmaaldrich.com/catalog/search/ProductDetail/ALDRICH/544906

Simkó M, Mattsson MO (2010) Risks from accidental exposures to engineered nanoparticles and neurological health effects: a critical review. Part Fibre Toxicol 7:42

Stummann TC, Hareng L, Bremer S (2009) Hazard assessment of methylmercury toxicity to neuronal induction in embryogenesis using human embryonic stem cells. Toxicology 257: 117-26

Sung JH, Ji JH, Yoon JU, Kim DS, Song MY, Jeong J, Han BS, Han JH, Chung YH, Kim, J et al (2008) Lung function changes in sprague-dawley rats arter prolonged inhalation exposure to silver nanoparticles. Inhal. Toxicol. 20, 567–574

Sweetman SC (ed.) (2005) Martindale, The Complete Drug Reference, p. 1163, 34th Ed., Pharmaceutical Press, London, UK

Tankhiwale R, Bajpai SK (2012) Preparation, characterization and antibacterial applications of ZnO-nanoparticles coated polyethylene films for food packaging. Colloids and Surfaces B-Biointerfaces 90: 16-20

Thomson JA, Itskovitz- Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, Jones JM (1998) Embryonic stem cell lines derived from human blastocysts. Science 282: 1145-7

Tsien RY (1998) Green fluorescent protein. Annu Rev Biochem. 67:509–44

Turdean GL (2011) Design and development of biosensors for the detection of heavy metal toxicity. Intern J Electrochem doi:10.4061/2011/343125

Van Dyk TK, Belkin S, Vollmer AC, Smulski DR, Reed TR, LaRossa RA (2000) A panel of bioluminescent biosensors for characterization of chemically-induced bacterial stress responses. Chemical and Biological Sensors for Environmental Monitoring, American Chemical Society. 762: 167-184

Vertelov GK, Krutyakov TA, Eremenkova OV, Olenin AY, Lisichkin, GV (2008) A versatile synthesis of highly bactericidal Myramistin® stabilized silver nanoparticles. Nanotechnology 19(35)

Vevers WF, Jha AN (2008) Genotoxic and cytotoxic potential of titanium dioxide (TiO_2) nanoparticles on fish cells in vitro. Ecotoxicology 17, 410–420

Wang J, Rahman MF, Duhart HM, Newport GD, Patterson TA, Murdock RC, Hussain SM, Schlager J, Ali SF (2009) Expression changes of dopaminergic system-related genes in PC12 cells induced by manganese, silver, or copper nanoparticles. Neurotoxicology 30:926–933

Warheit DB (2008) How meaningful are the results of nanotoxicity studies in the absence of adequate material characterization? Toxicol Sci 101 183–185

Wei C, Lin WY, Zainal Z, Williams NE, Zhu K, Kruzic AP, Smith RL (1994) Bactericidal activity of TiO_2 photocatalyst in aqueous-media: toward a solar-assisted water disinfection system. Environm SciTech 28(5): 934-38

Wong SWY, Leung PTY, Djurisic AB, Leung,K,M,Y (2010) Toxicities of nano zinc oxide to five marine organisms: influences of aggregate size and ion solubility. Anal Bioanal Chem.396, 609–618

Xia T, Kovochich M, Brant J, Hotze M, Sempf J, Oberley T, Sioutas C, Yeh JI,Wiesner MR, Nel AE (2006) Comparison of the abilities of ambient and manufactured nanoparticles to induce cellular toxicity according to an oxidative stress paradigm. Nano Lett 6:1794–1807

Xia T, Kovochich M, Liong L, Madler B, Gilbert H, Shi JI, Yeh JI, Zink JI, Nel AE (2008) Comparison of the mechanism of toxicity of zinc oxide and cerium oxide nanoparticles based on dissolution and oxidative stress properties. ACS nano. 2:2121–2134

Xia T, Zhao Y, Sager T, George S, Pokhrel S, Li N, Schoenfeld D, Meng H et al (2011) Decreased dissolution of ZnO by iron doping yields nanoparticles with reduced toxicity in the rodent lung and zebrafish embryos. ACS Nano. 22;5(2):1223-35

Xu LJ, Zhao JX, Zhang T, Ren GG, Yang Z (2009) *In vitro* study on influence of nano particles of CuO on CA1 pyramidal neurons of rat hippocampus potassium currents. Environ. Toxicol. 24, 211–217

Yu SM, Mecking SJ (2009) Variable crystallinity polyethylene nanoparticles. Macromolecules, 42, 3669

Zaslaver A , Bren A, Ronen M, Itzkovitz S, Kikoin I, Shavit S, Liebermeister W, Surette MG, Alon U (2006) A comprehensive library of fluorescent transcriptional reporters for *Escherichia coli*. Nat Methods 3:623-628

Zhao J, Wang Z, Liu X, Xie X, Zhang K, Xing B (2011) Distribution of CuO nanoparticles in juvenile carp (*Cyprinus carpio*) and their potential toxicity. J Hazard Mater. 15(197)304-10

Zimmer B, Schildknecht S, Kuegler PB, Tanavde V, Kadereit S, Leist M (2011) Sensitivity of dopaminergic neuron differentiation from stem cells to chronic low-dose methylmercury exposure. Toxicol Sci 121(2):357-67

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ABSTRACT

The ever increasing amount of produced nanoparticles (NPs, particles with at least one dimension 1-100 nm) and their release to the environment requires more efficient, rapid and cost-effective strategies for testing their potential adverse effects. Besides well-established general toxicity tests the development of more specific assays that would efficiently elucidate the mechanisms of action behind the observed toxic effects, is needed. The main proposed mechanism of toxicity of synthetic NPs to date is the generation of reactive oxygen species (ROS), which may lead to the oxidative stress in the cells.

In this PhD thesis a set of biological *in vitro* assays, which enable to reveal the mechanisms of toxic action of metal-containing NPs, was developed. Developed assays were mainly based on bioluminescent bacterial biosensors that were genetically modified to recognize ROS (superoxide anion or hydrogen peroxide), DNA damage or metal ions dissolved from NPs. In total, 16 different bioluminescent bacterial strains were used, 11 of which were constructed in this PhD thesis. Using bioluminescent intracellular metal-ion sensing biosensors in combination with ROS-sensitive bacteria, this work showed that the dissolution (release of metal ions) of metal-containing NPs of CuO and ZnO was the key factor determining their oxidative stress-inducing properties. In case of CuO NPs, the dissolved Cu ions triggered a cascade of downstream adverse effects, including generation of ROS and DNA damage. Nano-Ag was also very toxic to bacterial cells and induced ROS already at 0.1 mg/l, but the quantitative role of Ag ions in bacterial toxicity of nano-Ag remains to be elucidated. At the same time, the TiO_2 NPs and C_{60}-fullerene were not toxic to the bacterial cells.

The mechanisms of action of nano-CuO were also studied using human neuronal cells differentiated from embryonic stem cells *in vitro*. The results revealed that in human cells CuO NPs were remarkably more toxic than in bacterial cells and their toxic effects were not explained by dissolution. This showed that the results on mechanisms of toxicity of CuO and possibly other nanoparticles cannot be translated from prokaryotic cells that are *a priori* protected against the NPs entry by the cell wall to the eukaryotic cells that can internalize NPs *via* endocytosis. Indeed, effective internalization of NPs by neuronal cells was shown using fluorescent chromophore-labeled 33 nm nano-polyethylene particles. Surprisingly, nano-polyethylene showed long-term adverse effects in developing human neuronal cells. The chronic exposure (18 days) of neuronal cells to 22.5 mg/l nano-polyethylene caused down-regulation of NOTCH pathway genes Notch1, Hes5, Neurod1 and Ascl1 that are associated with the impairments in embryonic neural development. The genes that were down-regulated during the chronic exposure to polyethylene nanoparticles had several overlapping with the genes which were down-regulated by well-known neurotoxicant methylmercury. However, before making the statement on developmental neurotoxicity of NPs, *in vivo* experiments should be carried out and the real exposure levels should be estimated.

KOKKUVÕTE

Sünteetilised ehk sihipäraselt toodetud nanoosaked on osakesed, mille vähemalt üks mõõde on vahemikus 1-100 nm. Sünteetilisi nanoosakesi toodetakse juba praegu tööstuslikes kogustes ning kasutatakse laialdaselt tarbekaupades, sh kosmeetikas, toidulisandites, värvides ja koduelektroonikas. Samal ajal ilmub üha rohkem teaduspublikatsioone, mis näitavad, et nanoosakeste uudsed füüsikalis-keemilised omadused muudavad need elusorganismidele ohtlikeks. On näidatud, et nanoosakeste toksilisus elusorganismidele korreleerub eelkõige nende võimega tekitada reaktiivseid hapnikuühendeid (*reactive oxygen species*, ROS, nt vesinikperoksiid ja superoksiidradikaal), mis metalliliste nanoosakeste puhul võivad olla põhjustatud lahustunud metalliioonidest. Standardmeetodid, mida saaks kasutada nanoosakeste toksilisuse mehhanismide, s.h üldtoksilisuse, ROS tekitamisvõime ja lahustuvuse uurimiseks, paraku veel puuduvad.

Antud doktoritöös töötati välja mitu elusrakkudel põhinevat *in vitro* testsüsteemi, mida kasutati nanoosakeste toksilisuse mehhanismide uurimiseks. Väljatöötatud testid põhinesid enamasti bakterirakkudel, mis olid geneetiliselt modifitseeritud tootma luminestsentsvalgust vastusena (i) superoksiidradi-kaalidele, (ii) vesinikperoksiidile, (iii) DNA kahjustustele või (iv) nanoosakestelt lahustunud Ag/Cu/Zn ioonidele. Töö tulemusena leiti, et CuO ja ZnO nano-osakestest lahustunud metalliioonid olid nende oksüdatiivse toime peapõhjuseks. Kõikidest testitutest olid Ag nanoosakesed bakteritele kõige toksilisemad ja tekitasid ROS juba alates 0.1 mg/l, samas jäi lahtiseks Ag-iooni panus hõbedaosakeste antibakteriaalsesse toimesse. TiO_2 osakestel ja fullereenidel antibakteriaalset toimet ei täheldatud.

CuO nanoosakeste toksilisuse mehhanisme uuriti lisaks bakterirakkudele ka inimese koekultuuri rakkudes. Selleks kasutati inimeste arenevaid neuronaalseid rakke, mida diferentseeriti *in vitro* embrüonaalsetest tüvirakkudest. Jälgiti nii rakkude elumust kui ka diferentseerumisega seotud markergeenide taset. Arenevate neuronaalsete rakkude eksponeerimisel CuO nanoosakestele täheldati rakkudes morfoloogilisi muutusi, mis olid sarnased tuntud neurotoksikandi – metüülelavhõbeda – mõjule. Erinevalt bakterirakkudest oli CuO nanoosakestel inimese koekultuuri rakkudes peale lahustuvuse veel teisigi toksilisuse mehhanisme, mis olid ilmselt tingitud eukarüootsete rakkude võimest nanoosakesi sisse võtta. Kasutades inertseid fluorestseeruvaid polüetüleennanoosakesi, näidati, et nanoosakesed tõepoolest sisenesid arenevatesse neuronaalsetesse rakkudesse. 18-päevase ekspositsiooni järel nanopolüetüleenile tuvastati neuronaalsetes rakkudes ka geeniekspressiooni muutusi, s.h NOTCH-raja geenide Notch1, Hes5, Neurod1 ja Ascl1 ekspressiooni vähenemist. NOTCH-rada on oluline, s.h emb-rüonaalses aju arengus, ning selle inhibeerimine võib aeglustada neuraalsete tüvirakkude jagunemist ning soodustada nende enneaegset küpsemist, viies aju arengu häireteni. Samas, põhjapanevate järelduste tegemiseks nanoosakeste neurotoksilisuse kohta peaks uurima ka nanoosakestele mõjusud *in vivo* testsüsteemidel, kasutades nanoosakeste realistlikke ekpositsioonitasemeid.

PUBLICATION I

Ivask A, **Bondarenko O**, Jepihhina N, Kahru A **(2010)** Profiling of the reactive oxygen species-related ecotoxicity of CuO, ZnO, TiO_2, silver and fullerene nanoparticles using a set of recombinant luminescent *Escherichia coli* strains: differentiating the impact of particles and solubilised metals. Analytical and Bioanalytical Chemistry, 398, 701–716

PUBLICATION II

Käkinen A, **Bondarenko O**, Ivask A, Kahru A **(2011)** The effect of composition of different ecotoxicological test media on free and bioavailable copper from $CuSO_4$ and CuO nanoparticles: comparative evidence from a Cu-selective electrode and a Cu-biosensor. Sensors, 11(11), 10502–10521

PUBLICATION III

Bondarenko O, Ivask A, Käkinen A, Kahru A **(2012)** Sub-toxic effects of CuO nanoparticles on bacteria: kinetics, role of Cu ions and possible mechanisms of action. Environmental Pollution 169, 81–89

PUBLICATION IV (MANUSCRIPT)

Hoelting L, Scheinhardt B, **Bondarenko O**, Schildknecht S, Kapitza M, Tanavde V, Lee QY, Mecking S, Leist M, Kadereit S. A 3-dimensional human embryonic stem cell (hESC)-derived model to detect developmental neurotoxicity of nanoparticles (Nano-DNT**)**. Archives of Toxicology (to be accepted after minor revision)

CURRICULUM VITAE

1. Personal data

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2. Contact information

Address	National Institute of Chemical Physics and Biophysics, Akadeemia tee 23, Tallinn 12618
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3. Education

2008–...	Tallinn University of Technology, Faculty of Science, PhD student in gene technology
2006–2008	Tallinn University of Technology, Faculty of Science (MSc degree in gene technology, *cum laude*)
2003–2006	University of Tartu, Faculty of Biology and Geography, Institute of Molecular and Cell Biology (BSc in gene technology)

4. Employment

2006–...	National Institute of Chemical Physics and Biophysics, Laboratory of molecular genetics, engineer and MSc-PhD student
2005–2006	University of Tartu, BSc student

5. Additional training

2011	Laboratory work in the University of Konstanz (Germany, 4.04–4.09.2011) in *In vitro* toxicology and biomedicine group (group leader Prof. Marcel Leist; supervisor Dr. S. Kadereit). Topics: 1. Nanotoxicology using differentiating neural progenitor cells from human embryonic stem cells. 2. Expression of highly sensitive and autonomous bacterial reporter gene cassette in mammalian cells.
2010–2011	Analysis of experimental data (30 academic hours) and Biostatistics (32 academic hours), Tallinn University of Technology, Estonia
2010	EUROTOX advanced toxicology course (EU REACH, Risk assessment, (Q)SAR), 8.04–13.04.2010, Bulgaria

6. Organization of courses

2011	Organization of course "Introduction to toxicology" for German and Estonian PhD students, 30/05-02/06, Tallinn
2009...	Co-organizer of Graduate school in Molecular Biology
2007...	Organization of practical course and lecturer in Genetics of Bacteria (YTG008, master student's course in TUT)

7. Supervised students

Aleksandr Käkinen, Master's Degree, 2010 (The mobility and bioavailability of heavy metals in the oil shale combustion fly ashes of Narva power plants and surrounding topsoils), Faculty of Chemical and Materials Technolog,y Tallinn University of Technology, Estonia

8. Honours & Awards

2012 TUT Development Fund, OÜ Bruker Baltics Scholarship

2010 Young Scientist Award - best poster presentation recognition on XII International Congress of Toxicology, Spain

2010 Stipend from Estonian Society of Toxicology for attending the EUROTOX Congress

2009 Grantee of Doctoral Studies and Internationalisation Programme "DoRa 3" (Strengthening research cooperation between universities and businesses)

2008 Estonian Academy of Sciences Students' research I prize

9. Publications in peer-reviewed journals

1. A 3-dimensional human embryonic stem cell (hESC)-derived model to detect developmental neurotoxicity of nanoparticles (Nano-DNT). (Hoelting L, Scheinhardt B, Bondarenko O, Schildknecht S, Kapitza M, Tanavde V, Lee QY, Kahru A, Mecking S, Leist M, Kadereit S) Submitted to Arch Toxicol

2. Sub-toxic effects of CuO nanoparticles on bacteria: kinetics, role of Cu ions and possible mechanisms of action (Bondarenko O, Ivask A, Käkinen A, Anne Kahru A), **2012,** Env Pol, 169, 81 - 89

3. Environmental hazard of oil shale combustion fly ash (Blinova I, Bityukova L, Kasemets K, Ivask A, Käkinen A, Kurvet I, Bondarenko O, Kanarbik L, Sihtmäe M, Aruoja V, Schvede H, Kahru A) **2012,** J Haz Mat 229-30:192 - 200

4. The effect of composition of different ecotoxicological test media on free and bioavailable copper from $CuSO_4$ and CuO nanoparticles: comparative evidence from a Cu-selective electrode and a Cu-biosensor (Käkinen A, Bondarenko O, Ivask A, Kahru A) Sensors, **2011**, 11(11), 10502 - 10521

5. *LuxCDABE* - transformed constitutively bioluminescent *Escherichia coli* for toxicity screening: comparison with naturally luminous *Vibrio fischeri* (Kurvet I, Ivask A, Bondarenko O, Sihtmäe M, Kahru A), Sensors, **2011**, 11(8), 7865 - 7878

6. Profiling of the reactive oxygen species related toxicity of CuO, ZnO, TiO_2, silver and fullerene nanoparticles using a set of recombinant luminescent *Escherichia coli* strains: differentiating the impact of particles and solubilised metals (Ivask A, Bondarenko O, Jepihhina N, Kahru A), Anal Bioanal Chem, **2010,** 398(2):701-16

7. Effects of rhamnolipids from *Pseudomonas aeruginosa* DS10-129 on luminescent bacteria: toxicity and modulation of cadmium bioavailability (Bondarenko O, Rahman P, Rahman TJ, Kahru A, Ivask A), Microbial Ecol **2010,** 59(3), 588-600

8. Bioavailability of Cd, Zn and Hg in soil to nine recombinant luminescent metal sensor bacteria (Bondarenko O, Rõlova T, Kahru A, Ivask A), Sensors **2008**, 8, 6899 – 6923

ELULOOKIRJELDUS

1. Isikuandmed

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3. Hariduskäik

2008–...	Tallinna Tehnikaülikool, Matemaatika-loodusteaduskond (geenitehnoloogia doktorant)
2006–2008	Tallinna Tehnikaülikool, Matemaatika-loodusteaduskond (magistrikraad geenitehnoloogias, *cum laude*)
2003–2006	Tartu Ülikool, Bioloogia-geograafiateaduskond, Molekulaar- ja Rakubioloogia Instituut (bakalaureusekraad geenitehnoloogias)

4. Teenistuskäik

2006–...	Keemilise ja Bioloogilise Füüsika Instituut, Molekulaargeneetika labor, insener ja magistrant/doktorant
2005–2006	Tartu Ülikool, Molekulaar- ja Rakubioloogia Instituut, bakalaureus

5. Tähtsaimad täienduskoolitused

2011	Välispraktikum Konstanzi Ülikooli *in vitro* toksikoloogia ja biomeditsiini rühmas (juht prof. M. Leist, juhendaja dr. S. Kadereit). Teemad: 1. Diferentseeruvatel inimeste embrüonaalsetel tüvirakkudel põhinevad mudelid nanoosakeste bioloogiliste mõjude uurimiseks. 2. Bakteriaalsete reportergeenide ekspresseerimine eukarüootsetes rakkudes; 4.04–4.09.2011, Saksamaa
2010–2011	Katseandmete analüüs (kemomeetria, statistika, 30 akadeemilist tundi) ning Biostatistika (32 akadeemilist tundi), Tallinna Tehnikaülikool
2010	EUROTOX korraldatud toksikoloogia kursus edasijõudnutele (EU REACH, 3R, riski hindamine, (Q)SARs), 8.04–13.04.2010, Bulgaaria

6. Kursuste korraldamine

2011	Doktorantide kursuse "Introduction to toxicology" (Tallinn-Konstanz) organiseerimine ja läbiviimine, 30.05–02.06, Tallinn
2009...	Molekulaarbioloogia doktorikooli organiseerimine
2007...	TTÜ magistriõppe aine Bakterigeneetika (YTG0080) loengute ja praktikumide läbiviimine

7. Juhendatud väitekirjad

Aleksandr Käkinen, magistrikraad, 2010, Raskmetallide liikuvus ja biosaadavus Narva elektrijaamade lendtuhkades ning neist mõjustatud pinnastes, Tallinna Tehnikaülikool, Keemia ja materjalitehnoloogia teaduskond

8. Teaduspreemiad ja -tunnustused

2012 TTÜ Arengufond, OÜ Bruker Baltics doktoriõppe stipendium
2010 Parima posteri auhind noorte arvestuses konverentsil XII International Congress of Toxicology, Hispaania
2010 Eesti Toksikoloogia Seltsi 2010. aasta stipendium EUROTOX aasta-kongressil osalemiseks
2009 Stipendiaat SA Archimedes programmis "DoRa" (tegevus 3 "Teadusalase koostöö tugevdamine ülikoolide ja ettevõtete vahel")
2008 Eesti Teaduste Akadeemia üliõpilastööde I auhind

9. Publikatsioonid eelretsenseeritud ajakirjades

1. A 3-dimensional human embryonic stem cell (hESC)-derived model to detect developmental neurotoxicity of nanoparticles (Nano-DNT). (Hoelting L, Scheinhardt B, Bondarenko O, Schildknecht S, Kapitza M, Tanavde V, Lee QY, Kahru A, Mecking S, Leist M, Kadereit S) Submitted to Arch Toxicol.

2. Sub-toxic effects of CuO nanoparticles on bacteria: kinetics, role of Cu ions and possible mechanisms of action (Bondarenko O, Ivask A, Käkinen A, Anne Kahru A), **2012,** Env Pol, 169, 81 - 89.

3. Environmental hazard of oil shale combustion fly ash (Blinova I, Bityukova L, Kasemets K, Ivask A, Käkinen A, Kurvet I, Bondarenko O, Kanarbik L, Sihtmäe M, Aruoja V, Schvede H, Kahru A) **2012,** J Haz Mat 229-30:192– 200

4. The effect of composition of different ecotoxicological test media on free and bioavailable copper from $CuSO_4$ and CuO nanoparticles: comparative evidence from a Cu-selective electrode and a Cu-biosensor (Käkinen A, Bondarenko O, Ivask A, Kahru A) Sensors, **2011**, 11(11), 10502 - 10521.

5. *LuxCDABE* - transformed constitutively bioluminescent *Escherichia coli* for toxicity screening: comparison with naturally luminous *Vibrio fischeri* (Kurvet I, Ivask A, Bondarenko O, Sihtmäe M, Kahru A), Sensors, **2011**, 11(8), 7865 - 7878.

6. Profiling of the reactive oxygen species related toxicity of CuO, ZnO, TiO_2, silver and fullerene nanoparticles using a set of recombinant luminescent *Escherichia coli* strains: differentiating the impact of particles and solubilised metals (Ivask A, Bondarenko O, Jepihhina N, Kahru A), Anal Bioanal Chem, **2010,** 398(2):701-16

7. Effects of rhamnolipids from *Pseudomonas aeruginosa* DS10-129 on luminescent bacteria: toxicity and modulation of cadmium bioavailability (Bondarenko O, Rahman P, Rahman TJ, Kahru A, Ivask A), Microbial Ecol **2010,** 59(3), 588-600

8. Bioavailability of Cd, Zn and Hg in soil to nine recombinant luminescent metal sensor bacteria (Bondarenko O, Rõlova T, Kahru A, Ivask A), Sensors **2008,** 8, 6899 – 6923

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

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