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Ecotoxicological Impacts of Industrially Relevant Engineered Nanomaterials: Effects on *Tetrahymena thermophila*

KATRE JUGANSON

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TALLINN UNIVERSITY OF TECHNOLOGY
School of Science
Department of Chemistry and Biotechnology

NATIONAL INSTITUTE OF CHEMICAL PHYSICS AND BIOPHYSICS
Laboratory of Environmental Toxicology

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Supervisors: Dr. Monika Mortimer, Project Scientist, Bren School of Environmental Science & Management and Earth Research Institute, University of California, Santa Barbara, United States

Dr. Angela Ivask, Senior Research Scientist, Laboratory of Environmental Toxicology, National Institute of Chemical Physics and Biophysics, Estonia

Opponents: Prof. Dr. Bernd Nowack, Technology and Society Laboratory, Empa - Materials Science and Technology, Switzerland

Dr. Arvo Tuvikene, Institute of Agricultural and Environmental Sciences, Estonian University of Life Sciences, Estonia

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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 doctoral or equivalent academic degree.

Katre Juganson

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Tööstuslike nanomaterjalide keskkonnatoksilisuse hindamine: nanoosakeste mõju algloomale *Tetrahymena thermophila*

KATRE JUGANSON

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CONTENTS

LIST OF PUBLICATIONS

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

I Bondarenko, O., **Juganson, K.**, Ivask, A., Kasemets, K., Mortimer, M., Kahru, A. (2013). Toxicity of Ag, CuO and ZnO nanoparticles to selected environmentally relevant test organisms and mammalian cells *in vitro*: a critical review. *Archives of Toxicology 87*, 1181-1200.

II **Juganson, K.**, Ivask, A., Blinova, I., Mortimer, M., Kahru A. (2015). NanoE-Tox: New and in-depth database concerning ecotoxicity of nanomaterials. *Beilstein Journal of Nanotechnology 6,* 1788-1804.

III **Juganson, K.**, Mortimer, M., Ivask, A., Kasemets, K., Kahru, A. (2013). Extracellular conversion of silver ions into silver nanoparticles by protozoan *Tetrahymena thermophila*. *Environmental Science-Processes & Impacts 15*, 244-250.

IV **Juganson, K.**, Mortimer, M., Ivask, A., Pucciarelli, S., Miceli, C., Orupõld, K., Kahru, A. (2017). Mechanisms of toxic action of silver nanoparticles in the protozoan *Tetrahymena thermophila*: from gene expression to phenotypic events. *Environmental Pollution 225*, 481-489.

AUTHOR'S CONTRIBUTION TO THE PUBLICATIONS

I The author participated in planning and meta-analysis of the study, was responsible for gathering the information about the effects of Ag, CuO and ZnO nanoparticles and their respective soluble metal salts to algae and protozoa and participated in the preparation of the manuscript. Her contribution was equal to the first author.

II The author participated in the study design, was responsible for collecting ecotoxicological information on Ag, ZnO, CeO_2 and CuO nanoparticles, participated in collecting ecotoxicological information on TiO_2 nanoparticles, carbon nanotubes and fullerenes and uniformed the data collected by other contributors. She conducted the meta-analysis and was corresponding author of the manuscript.

III The author was responsible for the study design, performed all the assays except visualisation of the formed silver nanoparticles with scanning electron microscopy. She was the major interpreter of the data and participated in the preparation of the manuscript.

IV The author was responsible for the study design, performed all the assays except AAS analysis, interpreted the data and was corresponding author of the manuscript.

OTHER PUBLICATIONS IN RELATED FIELD

Kurvet, I., **Juganson, K.**, Vija, H., Sihtmäe, M., Blinova, I., Syvertsen-Wiig, G., Kahru, A. (2017). Toxicity of Nine (Doped) Rare Earth Metal Oxides and Respective Individual Metals to Aquatic Microorganisms *Vibrio fischeri* and *Tetrahymena thermophila, Materials* 10, Article ID 754.

Heinlaan, M., Muna, M., **Juganson, K.**, Oriekhova, O., Stoll, S., Kahru, A., Slaveykova, V.I. (2017). Exposure to sublethal concentrations of Co_3O_4 and Mn_2O_3 nanoparticles induced elevated metal body burden in *Daphnia magna*. *Aquatic Toxicology 189*, 123-133.

Jemec, A., Kahru, A., Potthoff, A., Drobne, D., Heinlaan, M., Böhme, S., Geppert, M., Novak, S., Schirmer, K., Rekulapally, R., Singh, S., Aruoja, V., Sihtmäe, M., **Juganson, K.**, Käkinen, A., Kühnel, D. (2016). An interlaboratory comparison of nanosilver characterisation and hazard identification: harmonising techniques for high quality data. *Environment International 87*, 20-32.

Joost, U., **Juganson, K.**, Visnapuu, M., Mortimer, M., Kahru, A., Nõmmiste, E., Joost, U., Kisand, V., Ivask, A. (2015). Photocatalytic antibacterial activity of nano-TiO2 (anatase)-based thin films: Effects on *Escherichia coli* cells and fatty acids. *Journal of Photochemistry and Photobiology B-Biology 142*, 178-185.

Ivask, A., **Juganson, K.**, Bondarenko, O., Mortimer, M., Aruoja, V., Kasemets, K., Blinova, I., Heinlaan, M., Slaveykova, V., Kahru, A. (2014). Mechanisms of toxic action of Ag, ZnO and CuO nanoparticles to selected ecotoxicological test organisms and mammalian cells *in vitro*: A comparative review. *Nanotoxicology 8*, 57-71.

Visnapuu, M., Joost, U., **Juganson, K.**, Künnis-Beres, K., Kahru, A., Kisand, V., Ivask, A. (2013). Dissolution of Silver Nanowires and Nanospheres Dictates Their Toxicity to *Escherichia coli. Biomed Research International*, Article ID 819252.

Klauson, D., Pilnik-Sudareva, J., Pronina, N., Budarnaja, O., Krichevskaya, M., Käkinen, A., **Juganson, K.**, Preis, S. (2013). Aqueous photocatalytic oxidation of prednisolone. *Central European Journal of Chemistry 11*, 1620-1633.

INTRODUCTION

Nanotechnology is believed to result in a paradigm shift in many fields. It is also considered to be the 21st century industrial revolution [1] as manipulating with materials in nanometre scale can result in lighter, stronger, more durable and more efficient materials. Sustainable nanotechnology aims to produce engineered nanomaterials (ENMs, particles with at least one dimension below 100 nm) that have the desired properties but do not trigger adverse environmental effects [2]. The design of environmentally safe ENMs has been hindered by the limited knowledge on the mechanisms of toxic action of ENMs. Moreover, ENM risk assessment and predictive modelling of ENM adverse outcomes need to overcome specific challenges posed by the variety of different sizes, shapes, crystallinities and coatings of ENMs, each driving a distinct toxicity profile. To facilitate ENM hazard characterisation, quantitative nanostructure-activity relationship (QNAR) models have been developed. However, the main drawback of such approach is the limited availability of data on ENM parameters, their fate and properties in the test environment and the details of the toxicity testing procedure because these are often not reported in the published literature [3].

Currently, silver nanoparticles (Ag NPs) that have antimicrobial properties are one of the most used ENMs in consumer products. Namely, 12-24% of all the nano-enabled products registered in different inventories contain Ag NPs [4-6]. Thus, it is likely that in the course of the product life-cycle, Ag NPs may be released into the environment and reach water bodies. Alarmingly, Ag NPs are very toxic to many aquatic organisms but, albeit extensively studied, the mechanism of toxic action of Ag NPs is still debatable [3]. Therefore, additional information on toxicity mechanisms of ENMs to various species is needed for realistic environmental risk assessment. Protozoa, unicellular eukaryote consumers, are abundant in aquatic habitats and crucial in nutrient recycling. As grazers of bacteria, protozoa play an important ecological role in mineralisation of organic matter making nutrients more available to primary producers. Owing to their natural abundance and important ecological role, protozoa are excellent models in ecotoxicology, including nanotoxicology. Ciliated protozoan *Tetrahymena thermophila*, a popular molecular biology model organism, is well-suited for studying the mechanisms of toxic action of ENMs due to its extensively characterised cell structure, metabolism and genome [7].

In the current thesis, existing literature on the effects of selected industrially relevant, generally biocidal metal-based ENMs towards organisms that they are meant to fight (i.e., “target” organisms) and other organisms (i.e., “non-target” organisms) was critically reviewed. Further, ecotoxicological data on eight industrially relevant ENMs was mapped, analysed and published as an open-access database. Finally, Ag toxicity, both in nanoparticulate (Ag NPs) and ionic (Ag^+) form, towards ciliate *Tetrahymena thermophila* was studied at gene transcription and physiological level to shed light on the detoxification and toxicity mechanisms of Ag in free-living phagotrophic protozoa.

ABBREVIATIONS

AAS	atomic absorption spectroscopy
ATP	adenosine 5'-triphosphate
BET	Brunauer-Emmet-Teller
CAT	catalase
CLP	Classification, Labelling and Packaging
CNT	carbon nanotubes
DCF	fluorescent 2',7'-dichlorofluorescein
DCFH	non-fluorescent 2',7'-dichlorofluorescein
DCFH-DA	2',7'-dichlorodihydrofluorescein-diacetate
DI	deionised water
DLS	dynamic light scattering
DNA	deoxyribonucleic acid
EC_{50}	the median effective concentration of the toxicant that induces a designated effect in 50% of the test organisms after a specified exposure time
ENMs	engineered nanomaterials
EU	European Union
GPX	glutathione peroxidase
GSR	glutathione reductase
HSP	heat shock protein
ISO	International Organisation for Standardization
MDA	malondialdehyde
MIC	minimal inhibitory concentration
MTT	metallothionein
NOEC	no observed effect concentration
NOM	natural organic matter
NP(s)	nanoparticle(s)
OD	optical density
OECD	Organization for Economic Co-operation and Development
PCR	polymerase chain reaction
PDI	polydispersity index
PS	polystyrene
PVP	polyvinylpyrrolidone
QDs	quantum dots
Q(N)SAR	quantitative (nano)structure-activity relationship
REACH	Registration, Evaluation, Authorisation and restriction of CHemicals
RFU	relative fluorescence unit
RLU	relative light unit
ROS	reactive oxygen species
SD	standard deviation
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEF	soluble extracellular fraction of *T. thermophila*

SEM	scanning electron microscopy
SOD	superoxide dismutase
SSA	specific surface area
TBA	thiobarbituric acid
TBARS	thiobarbituric acid reactive substances
TEM	transmission electron microscopy
UV	ultraviolet
WoS	Clarivate Analytics Web of Science

1. LITERATURE REVIEW

1.1 Nanomaterials – innovative materials with a wide range of applications

1.1.1 Nanomaterial definitions and sources

By most definitions, nanomaterials are materials that have at least one size dimension in the range of 1-100 nm. Thus, according to their shape, nanomaterials can be nanoparticles (all three dimensions in nanoscale), fibres and rods (two dimensions in nanoscale), or films and plates (one dimension in nanoscale) [8]. In order to ensure legal clarity, various organisations have developed their definition for "nanomaterial". European Commission recommended the following definition on October 18, 2011: *'Nanomaterial' means a natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50 % or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm-100 nm. In specific cases /…/ the number size distribution threshold of 50 % may be replaced by a threshold between 1 and 50 %. /…/ fullerenes, graphene flakes and single wall carbon nanotubes /…/ should be considered as nanomaterials* [9]. This definition is also implemented in the EU biocidal products regulation [10], however, earlier regulations, such as cosmetic products regulation [11] and food-related regulations [12, 13], include different definitions. Namely, according to cosmetics regulation, nanomaterial is defined as intentionally manufactured insoluble or biopersistent material [11], and in food industry, any intentionally produced material that is ≤ 100 nm or structures that are larger than 100 nm but retain nanoproperties, like large specific surface area and/or distinctive physico-chemical properties, is considered a nanomaterial [12, 13].

Throughout their evolution, humans and the environment have been exposed to natural airborne ultrafine nanoscale particles. For instance, fullerenes and other nanomaterials are released during natural combustion processes like volcano eruptions and forest fires. Furthermore, most of the biological macromolecules are also "nanosized materials". With industrialisation, anthropogenic nanoparticles, released from different sources of thermo-degradation, e.g., power plants, jet engines, and internal combustion engines, were added to the natural ones [14].

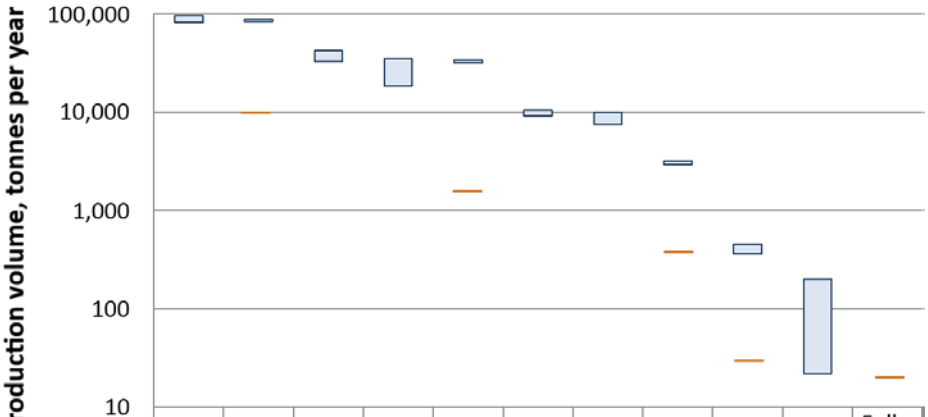
Nanomaterials tend to be more reactive compared to their bulk counterparts due to higher specific surface area which means that relatively large fraction of atoms is exposed on the particle surface. The latter is the main driver of nanomanufacturing and design of novel ENMs during recent decades. Some examples of ENM desirable properties include quantum effects, novel optical and electrical properties. ENMs can vary both in size and chemical composition – some of the ENMs can be based on only one material (e.g., carbon nanotubes (CNT), Ag NPs) or metal oxide (e.g., CuO NPs, ZnO NPs, TiO_2 NPs), others have complex structures like quantum dots (QDs) that have core shell organisation [8, 15].

In the beginning of 1990s, the term "green chemistry" was introduced in the field of chemistry, the goal of which was to reduce chemical waste and the use of toxic substances, introduce novel biodegradable materials and increase overall sustainability

[16]. More recently, green chemistry principles have been applied also in ENM synthesis by employing a natural process, biomineralisation, which occurs at ambient conditions. Naturally occurring mechanisms of mechanical protection (SiO_2 for nanosized spikes, stronger shells), detoxification of metals (e.g., Au, Ag, Cd), and navigation (magnetic Fe_3S_4 and Fe_3O_4 NPs) have been successfully used for ENM synthesis in the laboratories [17]. Biomineralisation of metal ions by enzymatically detoxifying or reducing them to zero-valent metals, that then form nanosized particles, has been shown to take place in many different organisms, including bacteria, algae, plants and fungi [18]. Moreover, also cell-free extracts containing proteins, peptides, polysaccharides and other reducing compounds can be used to produce "green" ENMs [17].

1.1.2 Worldwide production of nanomaterials

The available information on ENM worldwide production volumes is limited, but it is estimated that the global ENM production levels exceeded 340,000 tonnes in 2016 [19]. Half of the world's ENMs (50%) are produced in the United States followed by the EU (19%) and China (12%) [20]. Based on the estimated global production volumes, the following ten ENMs are being produced at volumes exceeding 100 t/yr: SiO_2, TiO_2, Fe, Al_2O_3, ZnO, nanoclays, CeO_2, CNT, Ag, and Cu (Fig. 1). According to Sun et al. [21], the ENM production estimates have increased in time and in 2014 the annual production in the European Union had reached about 10,000 tonnes (t) for TiO_2-NPs, 1,600 t for ZnO-NPs, 380 t for CNT, 30 t for Ag NPs, and 20 t for fullerenes.



	SiO_2	TiO_2	Fe	Al_2O_3	ZnO	nano-clays	CeO_2	CNT	Ag	Cu	Fulle renes
Global low	82,500	83,500	33,000	18,500	31,500	9,200	7,500	2,916	360	22	
Global high	95,000	88,000	42,000	35,000	34,000	10,400	10,000	3,200	450	200	
EU		10,000			1,600			380	30		20

Figure 1. Estimated annual production of engineered nanomaterials globally (blue columns, based on Keller and Lazareva [20]) and in the European Union (EU, orange dashes, based on Sun et al. [21]). The minimum (Global low) and maximum (Global high) values and the values reported for EU are shown in the table below the graph and are in tonnes per year.

In the EU, France is in the front rank of states collecting data about production and import of ENMs. The French Ministry of the Environment, Energy, and the Sea announced in

its 2017 annual report that in 2016, reported production and import volumes of ENMs in France were 304,282 and 120,041 t/yr, respectively. Remarkably, the volumes of different types of ENMs registered in France reflect the global trends – SiO_2, TiO_2, and nanoclays are produced or imported to France over 10,000 t/yr, Al_2O_3 1000-10,000 t/yr, Fe_2O_3, and CeO_2 100-1000 t/yr, ZnO 10-100 t/yr, Fe_3O_4 and 1-10 t/yr, Ag 1-10 kg/yr, and CuO 0.1-1 kg/yr. In addition, the combined production and import of nanosized $CaCO_3$, carbon black (including CNT), AlO(OH) and some organic carbon compounds to France exceeded 100 t/yr in 2016 [22].

1.1.3 Nanomaterial applications

Nanomaterials have been used since ancient times. For example, Lycurgus Cup from fourth century contains gold and silver NPs and can change its colour from green to red depending on the illumination. Similarly, gold, silver and copper NPs have been used in stained glass windows and ceramics [23]. Nowadays, nanotechnology is applied in diverse fields, e.g., drug-delivery systems [24], water-filtration [25], agriculture, feed and food [26], electronics, thin film coatings [15], and intelligent textiles [27].

Based on three inventories that list nanotechnology products [4-6], thousands of nano-enabled products are available on the market. Ag NPs have been incorporated into the highest number of products, followed by TiO_2 NPs and SiO_2 (Table 1). The latter two are also produced in the largest volumes (Fig. 1). In the EU market, more than half (55%) of the nano-enabled consumer products are used in the field of "health and fitness", 21% of the products belong to "home and garden" category, and the third major field is "automotive" (12%) [28]. Additionally, humans can be exposed to ENMs *via* nanomedicine. ENMs are already used as contrast agents (e.g., iron oxide, functionalised gold) and drug delivery agents (e.g., polymeric micelles, polymer-drug conjugates, liposomes), and the course has been set to nanostructured implants that are based on nano-hydroxyapatite and Ag NP-based wound management [29]. In the next paragraphs, the applications of major ENMs (according to Fig. 1) are introduced.

Table 1. Nano-enabled products in different inventories. Search was performed on June 25, 2017.

	Consumer Products Inventory [4]	**The Nanodatabase** [5]	**Nanotechnology Products Database** [6]
Total number of products	**1827**	**3005**	**7742**
CeO_2	2	1	1
Fe-containing	6	2	7
Fullerenes	7	10	36
Nanoclays	7	7	47
Al_2O_3	8	5	65
Cu-containing	10	9	25
ZnO	38	21	39
CNT	38	64	55
SiO_2	43	54	222
TiO_2	92	123	511
Ag	442	379	979

The widest applications for **CeO_2** have been in polishing applications and as an additive for diesel fuel to reduce soot [30]. In addition, CeO_2 NPs are used in electronic and optical devices, paints, metallurgy and as catalysts in petroleum refining [31]. Interestingly, CeO_2 could serve as anti-inflammatory material [30].

Due to their magnetic properties, **iron**-containing NPs have attracted interest in medicine as contrasting agents in magnetic resonance imaging and in drug delivery. In addition, Fe-containing ENMs can bind organic pollutants and heavy metals from the environment, and serve as an iron supplement for plants and animals [32]. Compared to bulk iron particles, nanosized Fe-compounds are better absorbed by farm animals, e.g., sheep and thus, serve as more efficient iron sources. Similar effects are expected also in humans where Fe-containing NPs could help to relieve iron deficiency [26].

Fullerenes are excellent radical scavengers and have been used as antioxidants in cosmetics and biological systems and to protect polymers. Fullerenes can also be used as electron acceptors in photovoltaics, and alkali metal doped fullerenes form superconducting crystals [33].

Nanoclays vary in composition and therefore properties, and include materials such as halloysite, smectites, and Laponite. Nanoclays are mainly used as fillers in polymers, toothpaste, cosmetics, coatings, and agriculture but also in medicine as drug carriers and as binders in catalysts. Due to porous nature nanoclays serve as adsorbents for various organic and inorganic pollutants [34].

Al_2O_3 has a wide range of useful properties such as high strength, good acid and alkali resistance, thermal conductivity and heat resistance, and high adsorption capacity. Thus, the applications of nano-alumina include catalysts, porous membranes, adsorbents for removing toxic contaminants, pigments, refractory materials, electrical insulators, thermometry sensors, abrasion resistant materials for surface protection and other fields [35, 36].

CuO has narrow band gap, low thermal emittance, high carrier concentration and good electrical properties. Nano-CuO applications range from catalysts, energy storage, sensors, photodetectors, cathodes or barrier layers in solar cells, and supercapacitors to absorbents for inorganic pollutants. CuO nanostructures are superhydrophobic which allows to use them in self-cleaning coatings, surface protection and microfluidics [37]. Since CuO NPs are antimicrobial, their potential applications include water treatment, and medical devices [38].

As opposed to CuO NPs, **ZnO** has wide band gap suitable for semiconductors, (UV-)shielding materials, sensors and filling materials [39]. Nano-ZnO is used in cosmetics, sunscreens and baby powders [40], and it is a promising material for remediation of hazardous waste [41]. In textile industry, ZnO whiskers have been used to enhance antistatic properties of synthetic fibres and render textiles UV-protective and antimicrobial [27]. ZnO may also be used in food packaging to protect food from pathogens [26].

CNTs have high aspect ratio, low density, high mechanical strength, and high electrical and thermal conductivity, rendering them suitable for electronics, sensors, energy storage devices, filters, automobiles and sporting goods [42]. CNT-coated cotton fibres can mimic lotus leaves. Additionally, CNT-polymer composites can decrease weight of textiles, improve their strength and toughness [27]. CNTs have potential

applications in medicine as implants, agents in tissue engineering and cell identification, and drug carriers [43].

Amorphous **silica** has been widely used as a stabiliser in medicine, food, and cosmetics. In addition, it is used as a filler, adsorbent, anti-caking agent and a carrier. Colloidal SiO_2 can also be used as a catalyst [44]. Furthermore, SiO_2 NPs have brought innovation to clothing industry as combining them with water-repellents results in hydrophobic textiles [27]. Porous hollow SiO_2 could potentially be used as a pesticide/herbicide carrier [26].

TiO_2 is a common food additive that is used as a pigment for its white colour. It has usage as an antimicrobial agent and TiO_2-based sensors can be used in food packaging to monitor changes occurring to the product [26]. In textiles, TiO_2 NPs have been applied to reduce static charge, block UV-light, and impart antimicrobial properties [27]. Other uses of nano-TiO_2 include sunscreens and other cosmetics, paints, self-cleaning and anti-fogging coatings, and solar cells [45, 46].

In the EU market, **silver** is ENM that is added to the largest variety of consumer products [28]. Ag NPs antimicrobial activity has been exploited in many applications, for example in textiles [27], food packaging, and as a replacement to conventional antibiotics in drinking water of chickens and pigs [26], in cosmetics, in medicine for antimicrobial catheters, scalpels and bandages, and in coatings of mobile phones and toothbrushes [47]. In medicine, Ag NPs have perspective as antiviral agents to fight diseases like HIV, hepatitis, influenza, and norovirus. Other potential medical applications of Ag NPs include photothermal therapy and cancer treatment [48]. In addition, Ag NPs possess unique catalytic, optical and sensing properties that make them useful as catalysts and sensors, and in applications such as nanophotonics, solar cells and Raman spectroscopy [47].

1.1.4 Nanomaterial release and fate in the environment

Due to the increasing production and use, ENMs may enter to the environment during their life cycle starting from ENM production, incorporation into products, the use and finally disposal. As ENM detection in the environment is a challenging task, most of the data on ENM release into the environment are based on modelling [8]. An EU scale modelling study performed by Sun et al. [21] suggested that due to their wide applications in cosmetics, TiO_2 and ZnO NPs are the most prominently released NPs into wastewater during the production, product manufacturing and consumption. As TiO_2 is also used as a pigment, the residues of nano-TiO_2 may end up in landfills. Among other types of NPs that are incorporated into consumer products, Ag NPs were found to be mainly released into wastewater or recycled and carbonaceous ENMs burned in waste incineration plants or recycled. The same study stated that the annual increment for TiO_2, ZnO and Ag NPs, CNTs and fullerenes in soils due to ENM release is in ng/kg scale and the concentrations in surface waters below 1 µg/L. Global-scale modelling performed by Liu et al. [19] found that ENMs are primarily released into water, followed by soil and air (Fig. 2).

In the environment, ENMs undergo different transformations that determine the environmental effects of these ENMs [49]. In aqueous environments, the stability, dissolution, surface reactions, and mobility of ENMs depend on both ENM surface properties and environmental conditions (pH, ionic strength, natural organic matter or

NOM). The main surface modifications of ENMs in the environment are adsorption/desorption of inorganic and organic compounds, chemical reduction, oxidation or sulphidation, and recrystallisation. As a result of these processes, the NP original pristine coating that usually provides suspension stability through steric or electric repulsions is replaced by NOM biocorona. It has been demonstrated with a variety of carbon-based, metallic, and metal-oxide NPs that NOM may either improve the stability of nanosuspensions or cause their coagulation. Interactions of NPs with inorganic ions may change the surface chemistry significantly and affect agglomeration processes. Chemical redox reactions occurring on the surface of metal-containing ENMs often lead to formation of core-shell structures. For instance, the surface of zero-valent iron NPs may corrode and form an iron oxide shell, Ag NPs may undergo complexation with chloride and sulphides and form AgCl or Ag_2S shell, Cu NPs may be oxidised to CuO_2 which results in the formation of copper oxide shell. Another type of transformation that certain metal-based NPs, such as Ag, ZnO, FeOx, CuO NPs, undergo is dissolution [50]. NP dissolution rate can vary in different environmental media and is an important characteristic of NPs that often contributes to NP toxicity. Similar transformation processes have also been described in soils; however, compared to aquatic environments, the prediction of ENMs' behaviour in soils is more complicated due to the complex nature of soil – solid matrix that may interact with NPs and aqueous phase containing high concentrations of natural particulate material [51].

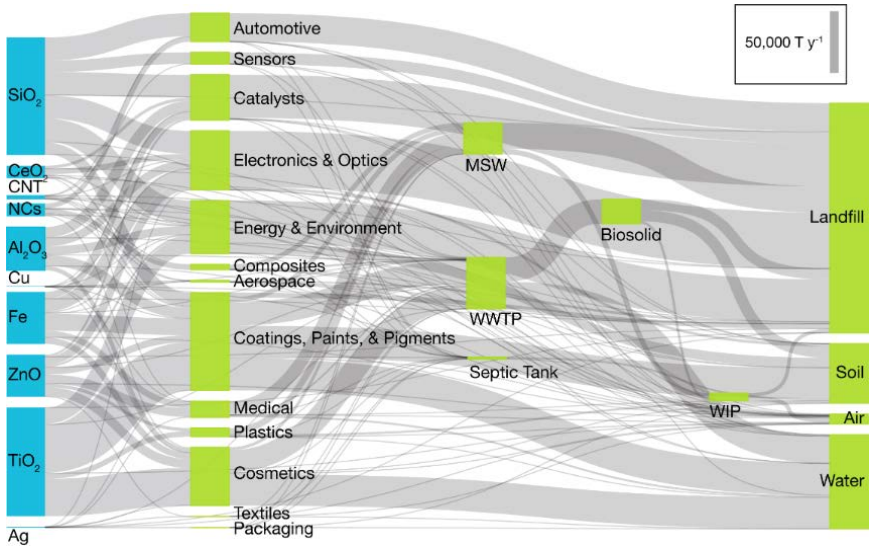


Figure 2. The flows of global production, use, disposal and release of industrially relevant nanoparticles. The vertical size indicates estimated volumes (tonnes per year). NCs – nanoclays, MSW – municipal solid waste, WWTP – wastewater treatment plant, WIP – waste incineration plant. Sankey diagram by Liu et al. [19] is licenced under CC BY 2.0.

While potential transformation processes and their mechanisms have been mostly studied at laboratory-scale and by changing one experimental condition at a time, in natural environments several processes could occur at once. For instance, in case of Ag

NPs, besides physical transformations where Ag NPs may form homo- or hetero-agglomerates [52], the particles may undergo simultaneous environment-dependent chemical transformations. In aerobic conditions, the first step of most chemical transformations is oxidative dissolution. Oxidative dissolution of Ag NPs, that in aerobic environment are usually coated with Ag_2O layer, has two stages: (i) the fast stage where the Ag_2O layer dissolves and Ag^+ ions are released from the NP surface, (ii) and the slow stage, where oxidation of NPs leads to the formation of Ag-core Ag_2O-shell structures [53]. The formed Ag_2O layer may again undergo fast dissolution; it might also protect the NPs from further oxidation. The dissolution rate is additionally controlled by the size and concentration of NPs – larger and highly concentrated particles tend to persist in the environment for longer time. Ag^+, formed during oxidative dissolution, may react with Cl^-, and the resulting AgCl may coat Ag NPs, hindering further dissolution. However, at high chloride concentrations (e.g., in seawater) the NP dissolution continues and species like $AgCl_2^-$, $AgCl_3^{2-}$, and $AgCl_4^{3-}$ may be released from the NPs. Simultaneously to chlorination, sulphidation of Ag NPs that have high affinity towards sulphur may occur leading to the formation of very stable Ag_2S. Remarkably, direct sulphidation of Ag NPs is also possible in anaerobic conditions (i.e. without prior Ag^+ release) in the presence of high concentrations of sulphides; such conditions prevail in anaerobic zones of the wastewater treatment plants [53, 54]. In the presence of sunlight and dissolved organic matter the released Ag^+ can be reduced to Ag^0 that may deposit on the surface of Ag NPs or form new NPs. Such simultaneous oxidation-reduction processes take place in all natural water bodies, making the elucidation of Ag NP fate, transport and mechanisms of toxic action in environmentally relevant conditions challenging [53].

1.1.5 Methodological considerations for physico-chemical characterisation of nanomaterials

In order to understand the environmental fate, transformations and toxicity mechanisms of ENMs, their physico-chemical properties need to be thoroughly characterised. At large, ENM properties can be divided into intrinsic and environment-dependent properties. The intrinsic properties of as-synthesised ENMs include chemical composition, crystallinity, purity, particle primary size, shape and surface properties (surface chemistry, charge and specific surface area). The environment-dependent properties reflect the changes that have occurred to ENMs during their storage, or during experiments. The latter include agglomeration, dissolution, change in the surface charge and biocorona formation [2].

Physico-chemical characterisation of ENMs usually involves the use of several different techniques, including (i) microscopic methods (atomic-force microscopy, scanning tunnelling microscopy, transmission, and scanning electron microscopy) to study the shape, size, surface texture, chemical composition and electrical conductivity; (ii) atomic spectrometry techniques, e.g., inductively coupled plasma mass spectrometry (ICP-MS) to determine the elemental composition of ENMs; (iii) X-ray techniques that provide detailed information on surface chemistry, crystallinity, and elemental composition; (iv) light scattering techniques for measuring ENM hydrodynamic size in aquatic environments; and (v) spectroscopic techniques that enable to monitor the stability of ENM suspensions [55, 56].

Generally, using a combination of different characterisation techniques is needed for sufficient physico-chemical characterisation of ENMs to enable interpretation of the experimental data and elucidate ENM mechanisms of toxic action. However, the existing methods also have several drawbacks. For instance, microscopic sample preparation may often introduce artefacts, imaging techniques can be time-consuming for quantitative analysis of ENMs, light scattering techniques are not suitable for ENM diameter measurements in samples with highly heterogeneous size distributions or non-spherical particles, and some techniques (like ICP-MS) fail to distinguish between metal-NP and respective ionic metal [55, 56]. Additionally, detection and accurate quantification of ENMs in the environment is currently still a challenge due to ENM low concentrations in the environment and high environmental backgrounds of natural nanoparticulate matter and colloids that have composition similar to the ENMs [8].

1.2 Ecotoxicity of engineered nanomaterials

1.2.1 Ecotoxicity testing and hazard assessment of engineered nanomaterials

In the early 2000s, the field of nanotoxicology emerged from the studies of airborne incidental particles with diameters <100 nm [29]. These studies outlined the importance of considering the unique properties of materials at nanoscale when evaluating the biological effects of such materials. Namely, it was argued that the ratio of surface atoms to total atoms determines the material reactivity and thus, nanosized particles were likely to have increased biological activity. In addition, the small size of ENMs that is in the same range of biological macromolecules facilitates cellular uptake and other ENM interactions with cells and subcellular structures [14].

According to EU Chemicals Regulation REACH, all substances that are manufactured in the EU or imported to the EU more than 1 t/yr have to be registered and characterised for their potential hazardous effects [57]. Although the term “nano” is not used in REACH, ENMs are included in “all substances”; thus, the same requirements for registration and hazard assessment apply to ENMs and other substances. In general, for hazard assessment, it is recommended to use more than one test species belonging to different trophic levels. For example, to estimate toxicity to aquatic food-web, usually one species of algae, crustaceans, and fish are used [58]. REACH regulation lists a variety of ecotoxicological assays that must be conducted along with assays that can be used to evaluate the toxicity towards humans. The exact requirements for ecotoxicological assays depend on the quantity of substance manufactured/imported, namely,

(i) acute effects on aquatic invertebrates (preferably *Daphnia* species) and the effect on growth of aquatic plants (preferably algae) must be studied for all substances manufactured/imported > 1 t/yr;
(ii) substances produced/imported > 10 t/yr must be additionally tested with fish (either short- or long-term) and activated sludge;
(iii) additional long-term tests with invertebrates (preferably *Daphnia* species) and fish (including various endpoints), and short-term tests with terrestrial invertebrates, plants and soil organisms are required for substances produced/imported > 100 t/yr;

(iv) and finally, long-term tests with terrestrial invertebrates and plants, sediment organisms, and birds must be additionally conducted if the production/import volume exceeds 1000 t/yr [57].

Based on the data obtained from these assays, acute and chronic hazard of a chemical (or an ENM) can be categorised according to EU's regulation on classification, labelling and packaging of substances and mixtures (CLP). Hazard evaluation is based on the half-effective or half-lethal concentration (i.e. the concentration that affects 50% of the population or tested organisms, $E(L)C_{50}$) of the substance in most sensitive organism, substance degradation rate and its bioconcentration factor. According to CLP, substances are classified as acutely very toxic to aquatic environment when $E(L)C_{50}$ for fish, crustaceans, algae or other aquatic plants is less than 1 mg/L. If the tested substance does not degrade rapidly or its bioconcentration factor exceeds 500, the substance could also pose long lasting effects to aquatic environment. In the latter case, the chronic hazard can be divided into 4 categories: $E(L)C_{50} \leq 1$mg/L – very toxic; $E(L)C_{50} > 1$ to ≤ 10 mg/L – toxic; $E(L)C_{50} > 10$ to ≤ 100 mg/L – harmful; and if $E(L)C_{50}$ > water solubility, the compound might cause long lasting harmful effects. [59]

On the other hand, the European Chemical Agency (ECHA) has admitted that due to the heterogeneous nature of ENMs, the implementation of REACH is challenging and resource intensive [60]. Moreover, Hjorth et al. [61] recently emphasised the importance of considering the dynamic properties of ENMs in the toxicity tests. The authors argued that there is a dilemma whether to use standard test guidelines for better comparability of the test results or modified test protocols for more realistic exposure conditions as standard guidelines often do not consider ENM-specific properties. Thus, alternative approaches for preliminary ENM hazard and risk evaluations are being developed to address the issue. For example, a recent review by Romero-Franco et al. [62] lists 18 existing risk assessment frameworks that could be applied for hazard identification or for risk assessment both in the natural and occupational environments. Among these, the most recent frameworks considering hazard evaluation of ENMs aim to divide ENMs into categories by applying either a road-map (NanoRiskCat, uses colour codes red-yellow-green-grey for high-medium-low-unknown hazard respectively), decision-making tiered framework (DF4nanoGrouping, groups ENMs as soluble, biopersistent, passive or active) [63], or by modifying existing chemical hazard assessment tools (e.g., GreenScreen where after inclusion of nanospecific parameters the hazard of ENMs can be compared to the hazard of the same substances in conventional form) [64]. Furthermore, it has been proposed that for more cost-effective environmental risk evaluations, the tests for exposure, fate, kinetics, and hazard of ENMs should be performed in tiered approach, starting from simple screening tools and moving to more complex and realistic conditions if necessary [49, 65]. In parallel with experimental approaches, several studies have developed Q(N)SAR models or read-across methods to link existing hazard information with intrinsic properties of ENMs and thereby be able to predict the hazard of novel ENMs [66]. However, only a few of these studies have concentrated on environmentally relevant organisms. Chen et al. [67] modelled the effect of metallic ENMs to widest range of organisms – zebrafish *Danio rerio*, crustacean *Daphnia magna*, algae *Raphidocelis subcapitata* (formerly *Selenastrum capricornutum* and *Pseudokirchneriella subcapitata*), and bacteria *Staphylococcus aureus*, and

demonstrated higher than 80% predictability of toxicity values in species-specific models. Other two environmentally relevant QNARs modelled the effect of various ENMs towards *D. rerio* embryos to estimate qualitatively whether an ENM is toxic or not (accuracy over 70%) [68], and whether it induces mortality at 24 or 120 h post fertilisation (hpf) or causes heart malformations at 120 hpf (accuracy over 70%) [69]. Read-across methods for the prediction of ENM-caused hazard have been used even less and mainly with *E. coli* [66]. For example, Gajewicz et al. [70] proposed a nano-read-across method that consisted of the following steps: (i) calculation of the structural similarities of ENMs, (ii) grouping of the ENMs by the similarities, and (iii) read-across analysis that enabled qualitative estimation of ENM hazard. Interestingly, they showed that enthalpy of formation of the metal cation in the gas phase could explain about 85% of the toxicity of metal oxide ENMs towards *Escherichia coli*. However, this ENM property was effective only with *E. coli* and metal oxide NPs; for other model systems the calculation may result in both false negative and false positive predictions and thus, other specific ENM properties may be needed for read across purposes [70].

1.2.2 Existing test formats and their limitations in nanoecotoxicology

A number of guidelines are available for ecotoxicity tests with various aquatic, terrestrial and avian species that provide information about acute, chronic or specific effects, like reproduction, development, and respiration [71]. However, it is now widely recognised that, because of the possible interferences of ENMs with the assays, these protocols should be used and the results interpreted with reservations when testing ENMs [58]. For example, in aquatic assays, turbid ENM suspensions may cause shading of light, which in algal assays may lead to misinterpretation of the results. Moreover, some ENMs are known to cause cell agglomeration when in contact with test organisms (e.g., TiO_2 NPs entrap algae, ENM adsorption on crustaceans may immobilise or hinder the respiration of the organisms). Additionally, ENMs may interfere with toxicity assays by quenching the fluorescence of indicator dyes or interacting with other assay components. For instance, Comet assay, that is used to assess the extent of DNA damage, has been shown to indicate false positive results when used with ENMs [72, 73]. Another aspect of the existing standardised methods, that is considered problematic in ENM toxicity testing, is the flexibility in the selection of the test medium. Since various particle- and media-dependent changes occur to ENMs during the exposure, it is often difficult to attribute the observed toxic effects to pristine ENMs or their modifications. Thus, the use of different test media hinders the comparisons between the results obtained using ecotoxicological assays with the same organism [74]. Moreover, sometimes the effect of ENMs may be misinterpreted due to the study design – the effects of ENMs may be overestimated as some ENMs adsorb nutrients from the test media leading to nutrient depletion [72]. On the other hand, the effects of soluble ENMs might be underestimated due to unintentional use of chelators in the test media [74]. ENM testing in soils is even more complicated than in aqueous media because of the complexity of the soil matrix that makes defining “the representative soil” difficult. Another challenge in case of soil matrices is achieving homogeneous distribution of ENMs and ensuring equal ENM concentrations in each replicate [75].

Many of the aforementioned issues were addressed by Hund-Rinke et al. [75] who explored the applicability of eight OECD standard tests and proposed their modifications suitable for testing ENMs. For example, test guideline (TG) 201 for algal growth (*R. subcapitata*) was adapted for ENMs by removing a chelating agent from the medium and using chlorophyll A concentration measurements as a proxy for biomass instead of optical density that can easily be interfered with by the turbidity of ENMs. According to Hund-Rinke et al [62], ENM sedimentation was an issue in two tests: TG 202: "Acute immobilisation of *Daphnia magna*" and TG 210: "Fish early-life stage test". The proposed solution for TG202 was to use low ionic strength media, and for TG 210 to apply daily water changes. In the latter test, additional issue with the sensitivities of different developmental stages of the test organism to ENMs was noted – ENMs were more toxic to the hatched larvae than to fish embryos. As a solution, the authors proposed to employ the more sensitive developmental stage – larvae – in testing ENM toxicity to fish.

1.2.3 Ciliates as relevant and promising models for environmental hazard assessment

Ciliates are abundant in a range of different environments, including soil and aquatic ecosystems where they prey on bacteria and are a food source for larger organisms [76]. In addition, although the biological wastewater purification is primarily conducted with bacteria, organics- and bacteria-consuming protozoa are the second most abundant community in such systems where their main task is to clarify the effluent. Among the hundreds of different protozoan species identified in wastewater treatment plants, the ciliates are the most abundant group [77].

For environmental hazard assessment, majority of toxicological assays have been conducted with freshwater ciliates, including species from wastewater treatment plants [78]. The ciliate species most often used in toxicity testing belong to genera *Tetrahymena, Paramecium*, *Colpidium*, and *Euplotes* [76]. So far, *Tetrahymena* is the ciliate genera most widely employed in the toxicity assay development. TETRATOX assay that was developed by Schultz [79] has been used to test the toxicity of about 2400 industrial organic chemicals. The obtained dataset has been the basis for QSAR modelling in several reported studies [80, 81]. In addition, *T. thermophila* is the species used in the commercial protozoan toxicity test kit "Protoxkit F™" [82]. However, although widely employed by researchers, the ciliate toxicity tests have not been standardised at OECD or ISO level yet. On the other hand, in October 2017, OECD adopted its first guideline that involves ciliates – Protozoan Activated Sludge Inhibition Test (OECD 244). The test measures phagocytic activity of ciliates grazing on bacteria by comparing the turbidity decrease in the samples incubated with the test chemical and control samples without toxicants [83]. The results obtained with this method, however, may not be comparable across studies because of the varying composition of the inoculums collected from different sewage treatment plants.

1.2.3.1 *Tetrahymena thermophila* as a promising ciliate model

Tetrahymena – a unicellular eukaryotic organism

The unicellular freshwater ciliated protists belonging to genus *Tetrahymena* could have appeared on the Earth tens of millions of years ago [84]. Overall, there are more than 41 different named species and many unnamed species in the genus *Tetrahymena*, all

of which have oral apparatus comprised of four ciliated oral structures and were initially designated as *T. pyriformis* [85].

Natural habitat for *Tetrahymena* is a freshwater lake, a pond or a stream that has some vegetation where they prefer to stay at the bottom, near decaying vegetation and bacteria [86]. While different *Tetrahymena* species are present in waterbodies all over the world, *T. thermophila* has been found exclusively in the Eastern United States [84, 87]. *T. thermophila* prefers to live in small ponds and roadside ditches near the shore [86]. In addition, *T. thermophila* has a role in the activated sludge process as was shown by Esteban et al. [88] who studied a sewage-treatment plant close to Madrid, Spain. Based on the sampling data, *T. thermophila* could be found only in the ponds where the water temperature exceeded 13 °C, and it is unclear how it survives the winters with temperatures below that. The upper tolerable temperature limit for *T. thermophila* is about 41 °C [86].

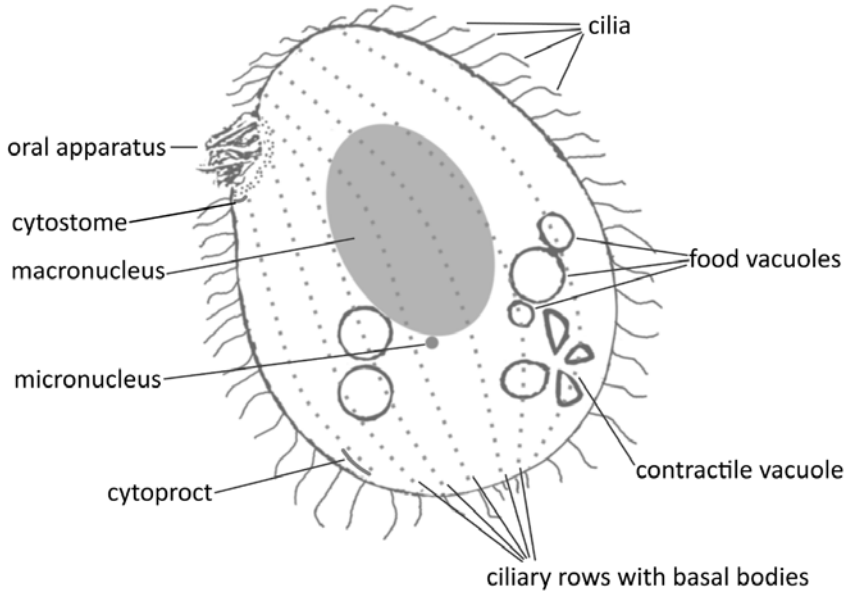


Figure 3. Schematic diagram of Tetrahymena thermophila. Illustration by the author of this thesis based on Fig. 3 by Frankel [95].

Compared to most eukaryotic cells, the size of *T. thermophila* is rather large – approximately 30 × 50 µm [89]. *T. thermophila* cell is covered by multi-layer cortex that is semi-rigid and arranged into 15-25 longitudinal rows (ciliary rows) of cortical units containing basal bodies mostly accompanied by the cilia (Fig. 3). The cilia enable directional motility of the cells; additionally, pinocytosis can occur at the anterior of each basal body. Altogether, there are about 150 oral basal bodies and 500-600 somatic basal bodies in *Tetrahymena* cell. The number of somatic basal bodies increases dramatically in starving cells enabling them to swim more rapidly. In addition to pinocytosis, nutrients are taken up *via* phagocytosis that is the main feeding mechanism of *Tetrahymena* and occurs in a funnel-like oral apparatus located near the anterior cell pole. During the first stage of phagocytosis, the synchronous beating of the cilia in the oral apparatus directs food particles into the cytostome. The cytostome

opens into a passage where the food vacuoles are formed. The digestion of the internalised food occurs during phagosome maturation. In the course of the maturation various vesicles delivering acidification machinery or hydrolytic enzymes fuse with the food vacuoles, and new vesicles removing selected compounds from phagosomes are released. Near the posterior end of the cell is located cytoproct where undigested food particles are excreted from the cell. Osmoregulation of *Tetrahymena* cell is controlled by an organelle named contractile vacuole that accumulates and releases collected fluid through contractile vacuole pores [90, 91].

Like other ciliates, *Tetrahymena* species have two nuclei: polyploid somatic macronucleus that is transcriptionally active during vegetative growth, and diploid, germline micronucleus that is used during mating [84]. In the beginning of 2000s, the macronuclear genome of *T. thermophila* was sequenced [84]. *T. thermophila* has estimably 24,725 protein-coding genes in its macronucleus [92], which is substantially more than in another common unicellular eukaryotic model - yeast *Saccharomyces cerevisiae* (6,000 protein-coding genes), and is comparable to mammals (~20,000 protein-coding genes in humans; 19,000 in dogs; 30,000 in mice) [93]. Though *T. thermophila* mostly grows vegetatively in the presence of food, starving cells are capable of sexual reproduction during which two cells from different mating types (*T. thermophila* has seven mating types) exchange their genetic information [94]. Over the years, many fundamental biological discoveries have been made in *T. thermophila* research; namely, self-splicing RNA, structure of telomeres, discovery of telomerase, and how transcription is regulated by histone acetylation [87].

As *T. thermophila* can be grown in axenic media, i.e. without other microorganisms as a food source, and it has a relatively short doubling time (approximately 2 h at 37 °C), it has been adopted as a model organism in biochemical and physiological research [84, 89]. In toxicology, the most common endpoint in tests with *T. thermophila* has been growth inhibition, which has been commonly monitored in the proteose peptone based rich growth medium by counting the cells or measuring the optical density of the cultures [96-98]. To conduct tests in the conditions that are similar to these in the natural environment, mineral medium [99-102] or spring water [99] has been used for exposing *Tetrahymena* to toxicants. In some of the latter studies protozoan viability has been used as an endpoint, measured with fluorescent dyes or by ATP content [101, 103]. Other, more sensitive endpoints measured include respiration activity (determined with oxygen sensor) [97], heat production (microcalorimetric measurements), cell membrane fluidity (fluorescence polarisation) [98], enzymatic activities [82], behavioural changes (change in swimming speed or locomotion) [82, 104].

The use of *Tetrahymena* in nanotoxicology

In their review in 2008, Kahru et al. suggested that for better ecotoxicological hazard assessment of ENMs a multitrophic test battery involving primary producers (algae), consumers (crustaceans and protozoa), and decomposers (bacteria) should be used [105]. Specifically, *Tetrahymena* species were suggested to be of interest in nanotoxicological research. Using keywords "*Tetrahymena* AND (nanotech* OR nanopart* OR nanomat* OR nanotube*)" in searches performed in WoS and Scopus databases on August 23, 2017, 71 and 62 abstracts were retrieved, respectively.

Tetrahymena was used to study ENM bioeffects in total of 48 research papers; out of these, 45 papers were included in WoS and 43 in Scopus (Fig. 4A). Thus, most of the published papers were included in both literature databases. Other search results were conference abstracts, reviews and research papers on other topics. From the available papers it was evident that although there are numerous species in *Tetrahymena* genus, nanotechnological research has been conducted with only two of them – *T. thermophila* and *T. pyriformis* (Fig. 4B). *Tetrahymena* had been used as a model for studying CNT bioeffects in total of 9 research papers (Fig. 4C). These included the first study about the effect of ENMs on *Tetrahymena*, published by Zhu et al. [106] in 2006. In the aforementioned research, it was found that multi-walled CNTs even at high

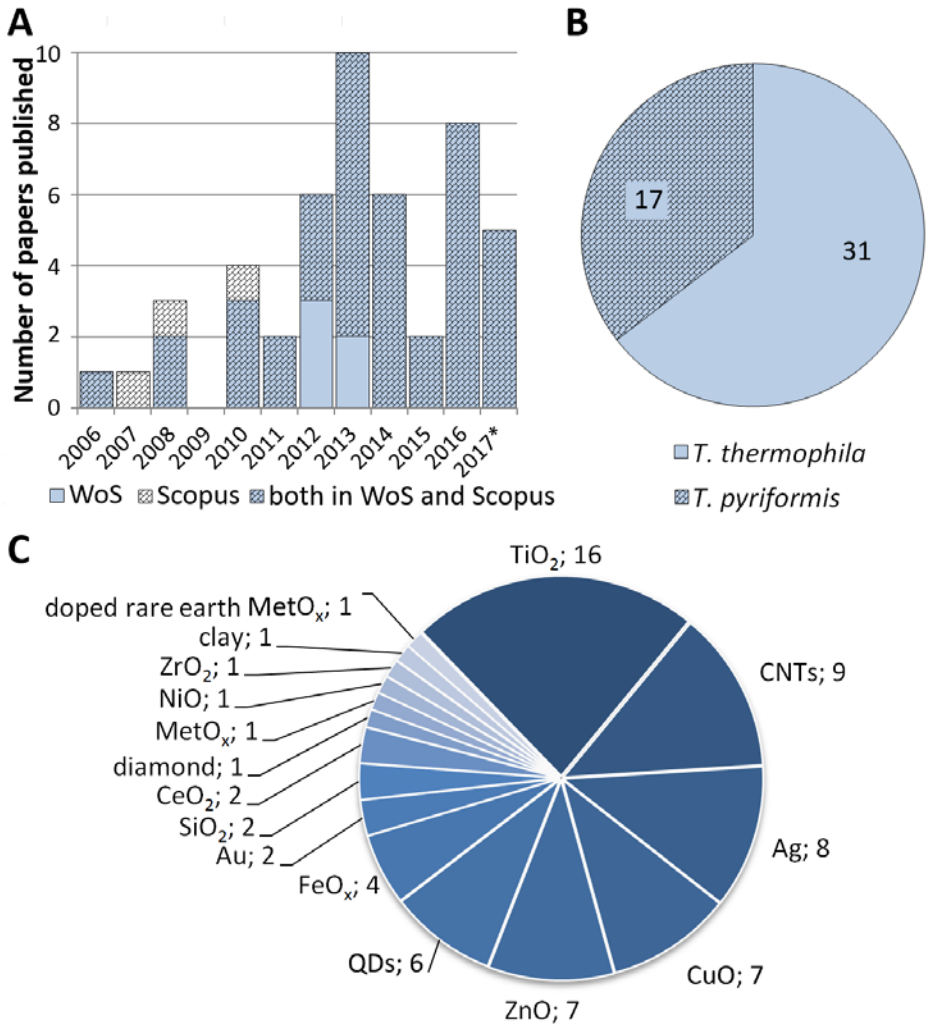


Figure 4. Number of papers concerning Tetrahymena and engineered nanomaterials in WoS and Scopus on August 23, 2017 (A). Share of papers according to tested Tetrahymena species (B). Number of studies involving Tetrahymena and different nanomaterials (C).

concentrations (100 mg/L) could stimulate the growth of *T. pyriformis* in rich proteose peptone yeast extract medium. However, CNTs appeared to be toxic to *Tetrahymena* in filtrated pond water where CNTs induced lipid peroxidation and depleted SOD activity in *T. pyriformis.* Growth-stimulating effects of multi-walled CNTs in *T. pyriformis* culture were also reported in another study by the same authors [107]. In a later study the same group showed that dose-dependent growth-stimulating effects existed for glucosamine-functionalised multi-walled CNTs whereas decylamine-functionalisation lead to inhibition of *T. pyriformis* growth [108]. Multi-walled CNTs at concentrations up to 100 mg/L were shown to be non-toxic also to *T. thermophila* [109], while on the contrary, single-walled CNTs caused cell death at concentrations exceeding 10 mg/L [110, 111]. Furthermore, it has been noted that single-walled CNTs at low doses inhibited bacterivory: CNT-exposed *T. thermophila* egested vesicles that contained viable bacteria instead of digested content [111, 112]. Considering other carbonaceous materials, carbon black was proved to be non-toxic towards *T. thermophila* at concentrations as high as 7 g/L [111].

The effect of TiO_2 NPs on *Tetrahymena* was studied in nearly one third of the retrieved papers (Fig. 4C). Rajapakse et al. showed that while there was no effect on *T. thermophila* cell membranes at 100 mg/L [113], and doses as high as 1000 mg/L did not affect *T. thermophila* viability nor produced ROS or caused lipid peroxidation, high nano-TiO_2 concentrations increased the cell membrane thickness and decreased cell fluidity [114]. In addition, although TiO_2-exposed *T. thermophila* may grow as fast as control cells, it might not reach as high cell densities [102]. Other studies have reported dose- and time-dependent decrease of *T. thermophila* cell reproduction and viability, but these effects were observed at doses higher than 100 mg/L [115, 116]. Additionally, it has been reported that due to photoactivation, TiO_2 NPs have more pronounced effects under illumination compared to the exposures in dark [116-118]. Zou et al. [118] observed that co-exposure to TiO_2 NP and light irradiation caused lipid peroxidation and protein degeneration in *T. pyriformis*. Similarly, although TiO_2 NPs did not affect *T. thermophila* viability at concentrations up to 100 mg/L, proteomic analysis indicated ENM-specific effects on lipid and fatty acid metabolism and ion-regulation [119]. Only one study has reported that TiO_2 NPs may be harmful to *T. thermophila*: viability-based 24 h EC_{50} value for bare TiO_2 NPs was 53 mg/L [120]. The latter effect was likely not caused by the test format as the same laboratory has found other TiO_2 NPs to be non-toxic to the same strain of *T. thermophila* [109]. Yang et al. [121] demonstrated the role of coating in nano-TiO_2 toxicity by determining that sodium polyacrylate (PAA)-functionalised TiO_2 NPs were more toxic compared to bare TiO_2 NPs. Recently, Fekete-Kertesz et al. [122, 123] introduced a more sensitive sub-lethal test method – phagocytic activity assay which showed that, compared to unexposed control cells, *T. pyriformis* exposed to 0.1 μg/L of TiO_2 NPs (size 89 nm) produced only half the number of phagosomes. Interestingly, an inverse dose response was shown: the inhibitory effect to phagocytosis was not noted at higher concentrations, i.e. cells exposed to 10 mg/L of TiO_2 NPs did not differ in the number of phagosomes from the unexposed control cells. Such relationship was not observed for smaller TiO_2 NPs in sizes 16 nm and 36 nm [123].

The effects of ZnO and CuO NPs on *T. thermophila* have been reported in 7 papers for each of the NPs (Fig. 4C). ZnO NPs were toxic to *T. thermophila* in mineral media and

deionised water with 24 h EC_{50} values in the range of 1-10 mg/L [101, 109, 120, 124] and the observed effect was caused by the released Zn^{2+} ions. Decrease in ZnO NP toxicity was observed in metal complexing media such as river water, suggesting the major role of Zn^{2+} in ZnO NP toxicity [124]. In rich medium, Chen et al. [125] observed dose-dependent *T. thermophila* growth stimulating effect of ZnO NPs at concentrations up to 100 mg/L, while Nalecz-Jawecki [126], who employed the commercial Protoxkit F, found growth inhibitory effect with 24 h EC_{50} values 11.5-13.5 mg/L. Gupta et al. [127] reported that in aquatic environments ZnO NPs and clay particles heteroagglomerated which, despite decreasing the NP-suspension stability, increased the bioavailability and toxicity of NPs to *T. pyriformis*. Similarly to ZnO NPs, Mortimer et al. [101], proposed that dissolution was a trigger for CuO NP toxicity. A follow-up study indicated that Cu^{2+} ions had only a partial role in CuO NP toxic effects to *Tetrahymena*: NPs caused higher oxidative stress and lower fluidity of the cell membranes than Cu^{2+} [128]. In addition, compared to ZnO NPs, there is a higher variation in the reported EC_{50} values of CuO NP (from 2.0 mg/L to over 100 mg/L) and this variability can be attributed to the nature of test media. This in turn may indicate higher variability in CuO NP solubilisation rates in different media [101, 109, 120].

QD effects to *Tetrahymena* have been studied in total of 6 papers (Fig. 4C). Holbrook et al. [129] showed that bioaccumulation and biopersistence of CdSe/ZnS QDs depended on the functionalisation – while both carboxylated QDs (initial concentration $2.58*10^{12}$ QDs/mL) and biotinylated QDs (initial concentration $1.63*10^{12}$ QDs/mL) accumulated in time-dependent manner, the latter were retained in *T. pyriformis* for longer. The reported effects for QDs vary and seem to depend, among other parameters, also on capping, i.e. functionalisation of QDs with biomolecules might change the biological outcome. Namely, approximately 2.7 nM of biotinylated CdSe/ZnS QDs and up to 10 nM of carboxyl functionalised CdSe/ZnS QDs did not affect the ciliates [129, 130] while some capping ligands were shown to promote the growth of *Tetrahymena*. For instance, 0.5 nM of adenosine 5'-monophosphate (AMP)-capped CdSe/ZnS QDs stimulated the growth of *T. thermophila* [131], and even 56 nM of AMP-CdSe/ZnS QDs slightly increased *T. thermophila* growth rate compared to the control [132]. On the other hand, as low as 0.5 nM concentration of mercaptoacetic acid (MAA)-capped CdSe/ZnS QDs decreased *T. thermophila* metabolism [131]; moreover, 9.04 μM of MAA-CdSe/ZnS QDs resulted in 80.6% inhibition of *T. thermophila* growth [132]. In addition, ZnS shell rendered CdSe QDs less toxic to *T. thermophila* [132].

The effect of Ag NPs to *Tetrahymena* has been studied in 8 papers (Fig. 4C). Shi et al. [133] demonstrated that Ag NPs are toxic to *T. pyriformis* with 24-h IC_{50} of 1.46 mg/L in the dark. They showed that, compared to dark conditions, less Ag ions were released under light irradiation resulting in decrease of Ag NP toxicity [134, 135]. The authors also reported a correlation between the size and toxicity of Ag NPs: smaller Ag NPs released higher amounts of Ag ions than larger Ag NPs, resulting in increased toxicity of the smaller NPs [134]. For *T. thermophila* the EC_{50} values of Ag NPs range from 3 to nearly 300 mg/L depending on the particle tested [109, 136-138]. The effects of silver to *T. thermophila* have been discussed in detail in the "Results and discussion" section.

Regarding the effects of other ENMs to *Tetrahymena*, there are mostly one or two papers per ENM with the exception of FeO_x which was studied in 4 reports (Fig. 4C). Species-specific toxicity has been reported for Fe_3O_4 NPs: while Zou et al. [139] found

that Fe_3O_4 NPs have growth-inducing effect in *T. pyriformis* even at the highest concentration tested (17 mg/L), Fe_3O_4 NPs decreased *T. thermophila* viability with 24-h EC_{50} being 26 mg/L [120]. NiO NPs proved toxic to *T. thermophila* (EC_{50} of 0.58 mg/L), probably due to released ions [140]. Harmful effects on *T. thermophila* growth were reported also for ZrO_2 NPs (24-h EC_{50} 12.8 mg/L) [141], and nanodiamonds at concentration of 20 µg/mL with 5 nm nanodiamonds being more toxic than 100 nm [142]. Other tested ENMs have not been shown to exert significant effects on *Tetrahymena* viability. CeO_2 NPs did not affect the growth rate of *T. pyriformis* in experiments lasting up to 64 days, however, the cells exposed to higher CeO_2 NP concentrations (200 mg/L) did not reach as high cell densities as control cultures [143]. Similarly, CeO_2 NPs and other doped rare earth metal oxides did not have effects on *T. thermophila* viability [144]. No harmful effects on *T. thermophila* viability were reported for SiO_2 NPs [109, 120], Al_2O_3, Co_3O_4, MgO, Mn_3O_4, Sb_2O_3, WO_3 and Pd NPs at concentrations up to 100 mg/L [120], and Au NPs at concentration up to 30 mg/L [109].

Only one study has reported the effects of *Tetrahymena* co-exposure to two different ENMs. Namely, TiO_2 NPs were found to reduce the toxicity of Ag NPs in the dark and in natural light. However, the two ENMs had a slight but non-significant synergistic toxic effect under continuous illumination due to increased oxidative stress which was confirmed by measuring the decrease in catalase activity [135].

Most studies have observed dose- and time-dependent phagocytic uptake of ENMs by *Tetrahymena* [101, 102, 110, 111, 114, 115, 117, 120-125, 127-130, 135, 139, 142-145]. Mortimer et al. [146] showed that maximum percentage of ENMs in *T. thermophila* population was reached faster with higher ENM concentrations, but the highest multi-walled CNT mass per one cell was measured at 2 h after the beginning of the exposure, independent of the dose. Similar observations were made with carboxyl functionalised CdSe/ZnS QDs for which the maximal uptake based on fluorescence values was after 2-h exposure and decreased after 24-h exposure [130]. Other ENMs like Au, Ag, CuO, and TiO_2 have also been shown to follow the same temporal pattern of NP uptake and depuration [147]. In addition, a study with differently capped CdSe/ZnS QDs showed that *T. thermophila* might preferably ingest QDs that have capping with nutritional value like adenosine 5'-monophosphate [131]. Mortimer et al. [147] have reported that the uptake rate of ENMs is independent of primary particle size for Au and Ag NPs. The fate of excreted ENMs might depend on *Tetrahymena* exposure medium and food source – in proteose peptone based medium the faecal pellets can be small enough to be reingested but pellets from bacteria-based diet tend to form larger agglomerates [146]. However, the detection and quantification of ENM uptake into cells is often challenging at lower ENM concentrations and when bacteria serve as a food source to *Tetrahymena.* In such cases, elemental analysis has been employed. For instance, Angerer et al. [148] used time-of-flight secondary ion mass spectrometry (ToF-SIMS) to detect TiO_2 signals inside *T. pyriformis* and were able to generate 3D image of the cell to confirm that ENMs were localised in the food vacuoles. Another approach that has been used is labelling ENMs with isotopes that are not abundant in the environment such as ^{14}C for labelling multi-walled CNTs [149].

In addition, a few studies assess the bioaccumulation and trophic transfer potential of ENMs. Mielke et al. [102] found that TiO_2 NPs adsorbed to *Pseudomonas aeruginosa* and were transferred to *T. thermophila* by phagocytosis of TiO_2-encrusted bacteria.

TiO_2 NPs were shown not to biomagnify but reduced *Tetrahymena* population yield. Similarly, Mortimer et al. [146] have shown that multi-walled CNTs attached to *P. aeruginosa* accumulated in the digestive system of *T. thermophila* but did not biomagnify. Contrarily, Holbrook et al. [129] did not observe trophic transfer of CdSe/ZnS QDs from *E.* coli to *T. pyriformis* because the QDs were associated only with the surface of aggregated *E. coli* cells (which were too large for ingestion) but not individual cells. However, the latter is not attributable to all QDs as Werlin et al. [145] showed that *T. thermophila* could ingest *P. aeruginosa* that had accumulated 25% of Cd from CdSe QDs (initial concentration 75 mg Cd/L). Remarkably, *T. thermophila* did not recover from QD-poisoning after it was transferred to rich growth media without added Cd (only a few ciliates were motile) [145]. In addition, Mortimer et al. [130] found that 20-h incubation of *T. thermophila* in QD-free buffer did not lead to complete clearance of QD-s. It was found that QDs may persist in *T. thermophila* at least for two generations after the cells were transferred to QD-free rich medium. Thus, a transfer potential of these ENMs to higher trophic levels was suggested [130].

Some research papers report on method development for nanotoxicological research in *T. thermophila*. For instance, Mortimer et al. [149] have developed gradient centrifugation technique to separate ciliates and bacteria from multi-walled CNTs that could be used to quantify bioaccumulation and trophic transfer of ENMs. In addition, hyperspectral imaging microscopy has been shown to be a promising tool for semi-quantitative analysis of ENM uptake in *T. thermophila* [147]. A few papers study the potential applications of *Tetrahymena* in nanotechnology. Kim et al. [150] introduced magnetite particles to *T. pyriformis* cells in order to create magnetotaxis and enable the use of these ciliates as magnet-controlled microrobots [151]. Another recent study showed that selenite could be reduced with the aid of glutathione into amorphous protein-bound selenium NPs with a diameter of 50-500 nm and length of up to 1 µm inside *T. thermophila* cells [152].

1.3 Nanoparticle-triggered toxicity mechanisms

The mechanisms of toxic action of ENMs have been studied already in thousands and reviewed in hundreds of papers. Despite, for most ENMs the proposed toxicity mechanisms are still not conclusive [153]. A brief overview of the major suggested toxicity pathways is presented in the following sections.

1.3.1 Nano-bio interactions

One possible toxicity pathway for ENMs is through direct interactions with organisms [154]. For instance, ENMs could adsorb onto various aquatic organisms and inhibit their mobility, filtering and respiration efficiency [155]. Furthermore, it has been shown that cell-ENM contact is often needed to trigger or enhance the toxicity of ENMs [153]. For instance, positively charged chitosan particles could interact with cell membranes that have natural negative charge due to fatty acid chains. Such interactions increase membrane permeability and cause leakages of the cell. In addition, other positively charged particles (e.g. cationic polymer coated metal ENMs like Ag, ZnO) adsorbed to the cells and caused alterations of membrane properties [3, 156, 157]. In addition to electrostatic forces, ENMs may interact with cells by hydrophobic interactions, van der Waals forces or by interacting with receptors located on the surface of the cell.

The latter mechanism where ENM acts as a ligand could trigger various signalling cascades that may result in unexpected and adverse outcomes [157].

As ENMs are in the similar size range to proteins and biocorona formation renders NPs with similar properties to biomolecules, ENMs can be recognised by specific receptors in the cell membranes and transported into cells [158, 159]. Inside a cell, ENMs may interact with organelles and enzymes reducing or causing loss of their activity [157]. Among single-celled species, ENM interactions with intracellular machinery are more likely in phagocytosing organisms due to active uptake of ENMs into the cells [3].

Nano-bio interactions can also have indirect effects on the viability of the organisms. Namely, as ENMs have high affinity towards various biomolecules, nutrients may adsorb to the surface of ENMs that could significantly reduce the bioavailability of nutrients and consequently affect organisms in natural waters [72].

1.3.2 Dissolution of metal-containing nanomaterials

Many studies have shown that the toxic effects of partially soluble metal-containing ENMs can be often attributed to the respective metal ions that are released from the ENMs [153]. ENM dissolution depends strongly on particle size: smaller particles tend to dissolve faster than larger ones. Additionally, other ENM intrinsic properties like shape and surface coating may influence the solubility [160]. Direct link between solubility and toxicity has been drawn for instance for ZnO NPs which were less toxic when doped with iron that reduced the dissolution of NPs [161]. Some studies have demonstrated that ZnO NP toxicity is fully explainable by NP dissolution [162-164]. Other studies have shown that for some target organisms ZnO NP dissolution explains toxicity only partially and additional NP-specific mechanisms contribute to the NP adverse outcome [153]. The latter has been proven for both Ag and CuO NPs with various methods; however, for these NPs their dissolution strongly depends on the environment and could be altered by cell-NP contact [3].

1.3.3 Reactive oxygen species generation

Similar to airborne particles out of which the field of nanotoxicology emerged, generation of reactive oxygen species (ROS) is often associated with the toxic effects of ENMs. In fact, in their review Djurišić et al. claimed that only very few studies propose non-ROS mediated toxicity mechanisms for ENMs [153]. ROS-generation potency of ENM has been related to the presence of defects in its crystal structure. However, the exact mechanism of ROS generation and nature of oxygen species generated depends on the type of the ENM. For example, ZnO and TiO_2 NPs can be activated with illumination (commonly in the UV-region) that generates electron-hole pairs due to the wide band gap of these materials and results in the formation of superoxide anions (O_2^-) and hydroxyl radicals ($^{\bullet}OH$) [153]. Contrarily, CuO NPs produce free radicals by dissolution as dissolved Cu ions participate in Fenton reaction [3]. Like solubility, ROS production depends on particle size (smaller particles are more reactive), their crystal structure (TiO_2 anatase phase produces more ROS than rutile phase), and coating [160]. Generated ROS can react with various biomolecules, including DNA, thus ENMs may cause genotoxicity *via* ROS [165]. Interestingly, although many studies have observed ROS-driven toxicity mechanisms in cells, the causal link between ENM properties, their ROS-generation potency and resulting toxicity is yet to be discovered [166].

1.3.4 Other mechanisms of action

In addition to previously mentioned mechanisms, there are some specific mechanisms of action that depend on ENM properties and can be organism-specific. For example, due to their relatively small diameters and high aspect ratio, CNTs could cause physical damage by "piercing" the cells [156]. Similar effects have been shown for 2D graphene sheets that could act as blades and cut the lipid membrane of the cell [157]. ENMs could also inhibit the growth of algal cells by shading of the light, and hence inhibit photosynthesis [72].

1.4 Current efforts to gather and analyse the existing nanoecotoxicological knowledge

Since the beginning of 2000s, when the concern over the potential novel risks of ENMs to humans and the environment emerged, several countries and organisations have allocated funds for nanosafety research. For example, in the Framework Programmes 7 and Horizon 2020, the EU invested 332,000,000 euros into nanotechnology risk-related research between 2006 and 2016. The respective amount in the US was 830,000,000 USD between 2006 and 2015. The increase in funding has resulted in increased number of publications addressing the toxicity of ENMs [167]. Despite of that, in 2012 it was pointed out that there were very little data available on ENMs in environmental, health and safety databases [168]. However, collection, analysis and processing of the existing data are of great importance. Also, annexes VII-X of the REACH directive [57] state that "*Before new tests are carried out /…/ all available in vitro data, in vivo data, historical human data, data from valid (Q)SARs and data from structurally related substances (read-across approach) shall be assessed first."*

Since 2012 several projects have concentrated on developing databases for ENM hazard assessment [169]. Some of the most recent and largest ENM databases are discussed in the following paragraphs.

The Nanodatabase (http://nanodb.dk/) is an inventory of consumer products available in the EU that according to manufacturers' claims contain ENMs. Besides the information about the product and the ENM incorporated into it, the Nanodatabase includes safety evaluation for each product. However, as in nearly 60% of the products the ENMs have not been identified by the manufacturer, the database provides only an initial overview about the European ENM market along with some exposure and hazard evaluation data [28]. The hazard identification roadmap NanoRiskCat used for categorisation of data in the Nanodatabase is useful for qualitative hazard assessment but lacks quantitative data for specific products [62].

DaNa$^{2.0}$ (https://www.nanopartikel.info/en/) enables to search ENM by commercially available applications. The database lists various inorganic ENMs and includes also their properties, toxicity to humans and environmental organisms. The articles on specific ENMs are compiled into different levels of complexity based on the background and interest of the potential user. The most complex level includes also original references used to generate the articles [170].

eNanoMapper (https://enanomapper.net/) intends to link the existing databases relevant for ENM toxicity assessment. It aims to integrate physical and chemical identities of ENMs in great detail (including particle size distributions, differences in

surface modification, manufacturing conditions, etc.) together with biological outcomes [169].

Recently, in June 2017 the European Union launched Observatory for Nanomaterials (EUON, https://euon.echa.europa.eu/) that is hosted by the European Chemical Agency (ECHA). The intent for this observatory is to provide reliable and neutral information on ENMs available on the EU market and their safety. The development of this data collection is still in progress and is planned to continue at least until 2020 [171].

AIMS OF THE STUDY

This thesis addresses the aspects related to hazard and risk assessment of industrially relevant ENMs that are essential for sustainable development of nanotechnology. The specific aims of the thesis were:

- to collect, analyse and critically evaluate existing literature data on ecotoxicology of industrially relevant engineered nanomaterials in order to (i) improve the understanding of ENM mechanisms of toxic action and (ii) identify the data gaps to guide further experimental work;

- to assess the hazard and determine the mechanisms of toxic action of nanosized silver – a widely applied, industrially relevant ENM with known antimicrobial properties – using the protozoan *Tetrahymena thermophila* as a model organism.

2. MATERIALS AND METHODS

2.1 Literature search and data analysis

The literature searches for both Papers **I** and **II** were performed in WoS to ensure the comparability of these two studies. The main differences in conducting the data search were:

- in Paper **I** only data relevant for Ag, CuO and ZnO ENMs and their respective ionic constituents was included; in Paper **II** all industrially relevant ENMs were studied;
- in Paper **I** the search was conducted organism-wise by introducing keywords related to specific organism groups; in Paper **II** the search results were narrowed down by introducing truncated keyword ecotoxic* that enabled to give equal weight to all organisms;
- in Paper **I** only E(L,I)C_{50} values and minimal inhibitory concentrations (MIC) were considered, and information was collected on ENM primary size, coating, and the test medium (mineral, complex, natural, pure water); in Paper **II** all endpoints and various characteristics of pristine ENMs, their behaviour in test media, toxicity test conditions and outcomes were included.

Meta-analysis for both Papers was carried out in Microsoft Excel, and the data collected in the frame of Paper **II** was compiled into Excel-format database NanoE-Tox, available as a supporting information of Paper **II**. More details about literature studies can be found in the respective Papers (**I, II**).

2.2 Experimental studies

The effects of two different commercial Ag NPs: Sigma-Ag (Paper **III**) and Collargol (Paper **IV**), $AgNO_3$ (Papers **III, IV**), and polystyrene NPs (Paper **IV**) on *T. thermophila* strains BIII (Paper **III**), CU427 and CU428 (Paper **IV**) were studied. All the experiments were conducted according to the previously established methods; exact details can be found in the publications included in this thesis. The main methods, differences between the two Papers and innovations are discussed below.

2.2.1 Methods for NP characterisation

- UV-Vis spectroscopy was used for the measurement of Ag NP absorbance spectra (Paper **III**);
- dynamic light scattering (DLS) technique was used to measure hydrodynamic size (Papers **III, IV**);
- electrophoretic light scattering (ELS) technique was used to address the particle zeta potential (Paper **III**);
- scanning electon microscopy (SEM) was used to visualise particles and their primary size (Paper **III**);
- sodium dodecyl sulphate gel electrophoresis (SDS-Page) with silver staining was used for protein corona assessment (Paper **III**);

- Ag/S-ion selective electrode was used to measure free Ag ion concentration (Paper **III**);
- atomic absorption spectroscopy (AAS) was used to measure Ag NP dissolution (Paper **IV**);
- fluorescence based methods were used to determine the potential of NPs to generate reactive oxygen species (ROS) (Paper **IV**).

A novel method for Ag NP biosynthesis in the soluble extracellular fraction (SEF) of *T. thermophila* was developed in Paper **III**.

2.2.2 Methods for studying Ag NP effects on *Tetrahymena thermophila*

Prior to experiments with NPs, *T. thermophila* culture was grown in SSP medium (2% proteose peptone, 0.1% yeast extract and 0.2% glucose, supplemented with 250 µg/mL each of streptomycin sulphate and penicillin G) overnight at 30 °C.

- In Paper **III** the cultivation was carried out in an Erlenmeyer flask with continuous shaking (100 rpm) to supply enough oxygen, at ambient light conditions.
- In Paper **IV** the cells were cultivated in a Petri dish where large surface area and low height of media column provided the oxygen needed for the cells. Thus, the Petri dishes were not shaken and the culture was grown in the dark. In addition, Fe-EDTA and fungicide amphotericin B were added to SSP medium to avoid nutrient depletion and fungal growth.

For toxicity tests, the test format described by Mortimer et al. [101] was modified in both Paper **III** and **IV** to avoid Ag speciation in the mineral test medium by transferring *T. thermophila* culture into deionised water where *Tetrahymena* could survive for at least a week [172]. In order to reduce the time needed for culture density adjustment, the cell number counted in a haemocytometer was correlated with optical density at 600 nm ($5*10^5$ cells/mL corresponded to OD_{600}=1, published in Jemec et al. [137]). All the exposures were conducted in polystyrene vessels (96-well plate, 24-well plate, Petri dish with 4 sections, depending on the amount of sample needed for further analysis).

Viability of the *T. thermophila* cells was determined with ATP-assay. In Paper **IV**, an optimised ATP measurement protocol (developed by the author of this thesis, published in Jemec et al. [137]) for plate luminometer using 96-well white polypropylene plates was employed.

To assess **morphological changes** in *Tetrahymena*, the cells were visualised and imaged using a light microscope with a digital camera (Papers **III, IV**).

Real-time PCR (Paper **IV**) was used to study the changes in the **expression of** selected **genes** after exposure to sub-lethal concentrations of the tested substances. Oxidative stress related **physiological changes** at the same toxicant concentrations were monitored using fluorescence assays (DCFH-DA, TBARS), and enzymatic assays (superoxide dismutase and catalase activities).

2.2.3 Data analysis

For viability analysis, ATP concentration-based dose-response curves and EC_{50} values (with 95% confidence intervals) were constructed using the REGTOX software for Microsoft Excel. Differences in EC_{50} values were considered statistically significant if the 95% confidence intervals did not overlap.

Protein gel images (Paper **III**) were analysed with TotalLabQuant software. Statistically significant differences ($p < 0.05$) of the results of gene expression and physiological assays obtained with untreated control cells and *T. thermophila* treated with sub-lethal concentrations of substances were assessed in R using ANOVA followed by Tukey post-hoc test (Paper **IV**).

3. RESULTS AND DISCUSSION

3.1 Synthesis of existing data on ecotoxicological effects of engineered nanomaterials (Papers I-II)

In order to provide thorough overview about ENM ecotoxic potential and their mechanisms of toxic action, two individual studies were performed. First, a review was conducted on published data regarding metal-containing ENMs that are known for their antimicrobial potential mainly because of their potential to release biocidal metal ions but that could also pose a threat to environmental organisms. Data on the effects of ENMs and their respective ions on the viability of selected organisms (MIC for bacteria, EC_{50} for other organisms) were collected and analysed to identify correlations between ENM dissolution and toxicity (Paper **I**). Next, guided by the results of the review, in Paper **II** data were collected and analysed for a wider selection of ENMs that were considered industrially relevant. To enable further analysis of the collected data and make the information available for public use, the parameters considered relevant to ENMs and test conditions were organised into the database NanoE-Tox (Paper **II**).

3.1.1 Comparison of toxicity of Ag, CuO and ZnO nanoparticles to selected organisms and *in vitro* test systems (Paper I)

According to the literature, both bulk- and nanosized Ag, CuO and ZnO are applied as antimicrobial agents to avoid unwanted growth of bacteria, fungi or algae (i.e., "target" organisms) [58]. Like pesticides, these ENMs could also cause adverse effects in other, i.e., "non-target" organisms that include various aquatic and terrestrial species. In order to weigh beneficial effects of ENMs against their potential harmful outcomes, toxicity data for bacterial and fungal species was compared to toxicity data for aquatic crustaceans, algae, fish, protozoa, the symbiotic bacterium *Vibrio fischeri* (a model for non-pathogenic bacterium), soil nematode, and mammalian cells *in vitro*. The ENMs selected for this study were all metal-containing, with negative surface charge at physiological pH, and soluble in aqueous media to certain extent.

The number of toxicity values ($E(L)C_{50}$ or MIC) reported in the literature for the three ENMs varied greatly by ENM and by test organism. Namely, while a relatively large number of studies could be found on toxicity of Ag NPs (166 values), there was substantially less information on ZnO NP toxicity (85 values) and the data on CuO NP toxicity (62 values) was rather scarce. On the other hand, data on soluble metal salts indicated that Cu-salts appeared to be studied the most (101 toxicity values), followed by Ag-salts (81 values) and Zn-salts (61 values) (Table 2, Fig. 5 in Paper **I**). Analysis of the toxicity values reported for the major organism groups showed that among crustaceans, most studies were conducted with *D. magna*, and among algae the most often used species was *R. subcapitata* (Table S2 in Paper **I**), both of which are widely acknowledged aquatic model organisms in regulatory toxicology [57, 58]. Further, *Caenorhabditis elegans* was most prominent among nematodes, *E. coli* among bacteria and *S. cerevisiae* among yeasts, all of which are considered as traditional model organisms in biology [173]. While data for crustaceans, algae and fish were mostly obtained with standard protocols, the test formats for other organisms varied to a great extent.

*Table 2. Median L(E,I)C_{50} values[†] (mg/L) and number of entries[‡] for all compounds included in Papers **I** and **II**.*

	Ag NPs		Ag ions	CuO NPs		Cu ions	ZnO NPs		Zn ions	CeO_2 NPs	CNTs	FeOx NPs	Fullerenes	TiO_2 NPs
	Paper **I**	Paper **II**	Paper **I**	Paper **I**	Paper **II**	Paper **I**	Paper **I**	Paper **II**	Paper **I**	Paper **II**	Paper **II**	Paper **II**	Paper **II**	Paper **II**
Algae	**0.36** [17] (0.005-21.2)	**0.2** [4] (0.03-2.12)	**0.008** [10] (0.001-0.59)	**2.8** [5] (0.68-47)	**25.7** [4] (2.8-57)	**0.07** [20] (0.004-13)	**0.08** [5] (0.05-4.56)	**0.05** [5] (0.05-0.07)	**0.09** [8] (0.04-3.48)	**8.5** [21] (0.01-100)	**12.6** [14] (0.1-41)	-	-	**18.7** [18] (1.76-241)
Bacteria[§]	**7.13** [46] (0.5-250)	**5.8** [37] (0.26-571)	**3.25** [27] (0.004-108)	**200** [13] (20-280)	**12.2** [17] (2.53-250)	**32** [13] (0.4-640)	**500** [15] (50-1000)	**73.5** [26] (0.1-234)	**30** [9] (12.5-1430)	**46.6** [21] (0.27-100)	**4.5** [3] (4.5-100)	**240** [4] (52-1000)	-	**589** [18] (0.62-66820)
Crustaceans	**0.01** [17] (0.001-0.04)	**0.02** [105] (0.0004-295)	**0.0009** [8] (0.0005-0.008)	**2.1** [8] (0.08-12.3)	**5.9** [29] (0.05-224)	**0.02** [8] (0.004-0.07)	**2.35** [10] (0.62-22)	**2.25** [28] (0.05-100)	**1.33** [6] (0.41-1.8)	**25.4** [17] (0.01-270)	**9.8** [20] (0.4-100)	**0.23** [1] (0.23-0.23)	**10.5** [3] (9.34-100)	**58.5** [68] (0.03-20000)
Echinoderms	#N/A	-	#N/A	#N/A	-	#N/A	#N/A	**0.1** [1] (0.1-0.1)	#N/A	-	-	-	-	-
Fish	**1.36** [17] (0.03-12.6)	**0.13** [29] (0.03-10.6)	**0.06** [4] (0.005-0.15)	**100** [1] (100-100)	-	**0.28** [19] (0.01-7.5)	**3.02** [4] (1.79-4.92)	**2.07** [3] (1.79-3.97)	**7.48** [3] (2.54-8.06)	-	-	**44.7** [2] (36.1-53.4)	**1.5** [1] (1.5-1.5)	**30** [7] (2.46-500)
Insects	#N/A	**0.95** [4] (0.59-20)	#N/A	#N/A	**569** [1] (569-569)	#N/A	#N/A	**3376** [2] (3159-3593)	#N/A	-	-	-	-	**6.83** [2] (6.56-7.09)
Mussels	#N/A	-	#N/A	#N/A	-	#N/A	#N/A	-	#N/A	-	-	-	-	**27.5** [2] (16.4-38.6)
Nematodes	**3.34** [21] (0.1-55)	-	**4.77** [4] (0.06-22)	-	-	**19.4** [6] (1.27-101)	**39.2** [6] (2.2-982)	**2.2** [1] (2.2-2.2)	**49.1** [6] (1.39-884)	-	-	-	-	**79.9** [1] (79.9-79.9)
Plants	#N/A	**12.5** [4] (9.36-20)	#N/A	#N/A	**13.7** [2] (0.46-26.8)	#N/A	#N/A	**46.5** [3] (10.8-64)	#N/A	-	-	-	-	-
Protozoa	**38** [7] (1.46-286)	**246** [2] (205-286)	**1.5** [3] (1.46-1.8)	**124** [6] (0.98-161)	**80** [1] (80-80)	**0.43** [14] (0.01-1.7)	**11.7** [9] (5.35-574)	**14.4** [4] (9.4-26.5)	**7** [9] (3.58-175)	-	-	-	-	-
Rotifers	#N/A	-	#N/A	#N/A	**0.32** [2] (0.24-0.39)	#N/A	#N/A	-	#N/A	-	-	-	-	**10.4** [3] (5.37-267)
Snails	#N/A	**0.009** [2] (0.002-0.02)	#N/A	#N/A	-	#N/A	#N/A	**0.15** [1] (0.15-0.15)	#N/A	-	-	-	-	**201** [2] (56.9-346)
Worms	#N/A	**100** [2] (83-146)	#N/A	#N/A	-	#N/A	#N/A	-	#N/A	-	**338** [2] (176-500)	-	-	-
Yeasts	**7.9*** [14] (0.88-48.5)	-	**2.16*** [5] (0.22-10)	**17.1** [4] (4.8-643)	-	**11.1** [4] (0.82-516)	**121** [7] (40-488)	-	**78.2** [2] (75.3-81.2)	-	-	-	-	-
Mammalian cells *in vitro*	**11.3** [25] (0.51-140)	#N/A	**2** [18] (0.62-83)	**25** [21] (13-100)	#N/A	**53** [10] (5.56-80)	**43.4** [25] (4.48-75)	#N/A	**9.8** [11] (5.88-15.7)	#N/A	#N/A	#N/A	#N/A	#N/A
Lowest median L(E)C_{50} and organisms used	**0.01** Crustaceans	**0.009** Snails	**0.0009** Crustaceans	**2.1** Crustaceans	**0.32** Rotifers	**0.02** Crustaceans	**0.08** Algae	**0.05** Algae	**0.09** Algae	**8.5** Algae	**4.5** Bacteria	**0.23** Crustaceans	**1.5** Fish	**6.83** Insects
Minimal L(E)C_{50} and for which organism	0.001 Crustaceans	0.0004 Crustaceans	0.0005 Crustaceans	0.08 Crustaceans	0.05 Crustaceans	0.004 Crustaceans	0.05 Algae	0.05 Algae	0.04 Algae	0.01 Crustaceans	0.1 Algae	0.23 Crustaceans	1.5 Fish	0.03 Crustaceans

[†] **median half-lethal(effective, inhibitory) concentration** [number of entries] (minimum-maximum value).

[‡] the differences in number of entries will be explained in section 3.1.2.1

[§] median minimal inhibitory concentration (MIC) in Paper **I**, median half-lethal(effective, inhibitory) concentration in Paper **II**.

* median of any reported effect for Ag NP or Ag ion exposed yeast.

#N/A - not assessed in the study; - - no respective L(E,I)C_{50} values obtained with the selected search criteria.

L(E,I)C_{50}/MIC < 1 mg/L

L(E,I)C_{50}/MIC 1…10 mg/L

L(E,I)C_{50}/MIC 10…100 mg/L

L(E,I)C_{50}/MIC >100 mg/L

Based on median L(E,I)C_{50} values for the most sensitive organism, the toxicity order of ENMs studied in Paper **I** was: Ag NPs > ZnO NPs > CuO NPs. The most susceptible organisms to Ag- and Cu-compounds were crustaceans that are an important link in aquatic food webs. On the other hand, the most sensitive organisms to Zn-compounds were algae that could be considered as targeted organisms e.g., through algaecidal ship paints; however, as primary producers, algal biomass is also crucial in energy and oxygen production, requiring protection from harmful effects of ENMs as non-target species [105]. In order to assess the potential hazard of studied compounds, the substances were ranked in Paper **I**; however, it was performed according to previous EU directive that did not take into account the degradation rate and bioconcentration factor of a substance. Nevertheless, the current CLP legislation [59] that is described in section 1.2.1 enables similar classification ranking of Ag NPs, Ag ions, Cu ions, ZnO NPs and Zn ions as acutely very toxic to aquatic organisms with potential chronic effects, and Cu NPs as potentially chronically toxic to aquatic organisms (Table 2, Fig. 6 in Paper **I**). Although one of the main targets of antimicrobial agents are bacteria, the median MIC values of metal NPs and their ionic forms were more similar to the median L(E,I)C_{50} values of the respective compounds measured in mammalian cell cultures, and 1-2 orders of magnitude higher than the median L(E,I)C_{50} values determined for non-target organisms, indicating that application of Ag, CuO and ZnO NPs as antimicrobial agents could potentially be a threat to non-target aquatic organisms.

The comparison of toxicity values of NPs and their respective ions provided some insight into the potential toxicity mechanism of ENMs. Namely, for Ag- and Zn-compounds the sensitivity patterns of NPs and respective ions were highly similar, and the toxicity values correlated well as R^2 coefficient of determination was above 0.8 (Fig. 7 in Paper **I**). Moreover, for Zn-compounds the toxicity values nearly overlapped. The latter indicated that for ZnO NPs the toxicity could be caused by releasing toxic metal ions. While for ZnO NPs the dissolution seemed to be the only trigger of toxicity, it was not so obvious for CuO NPs that reportedly tend to form various complexes with organic media components [174]. Furthermore, as CuO NPs proved more toxic than Cu ions towards yeast and mammalian cells *in vitro*, CuO NPs appeared to possess intrinsic toxic properties that manifest in specific models. These observations are in accordance with a recent review on the potential ENM toxicity mechanisms by Djurišić et al. [153]. Another noteworthy finding of the study was that the toxicity values for CuO and ZnO NPs were less variable than the Ag NP toxicity values. Such difference was attributed to the range of different coatings used for stabilizing Ag NPs (e.g., PVP, citrate, peptides) while all CuO NPs and, with one exception, all ZnO NPs were uncoated (Table 2, Tables S3, S5, and S7 in Paper **I**).

3.1.2 NanoE-Tox database – a collection of ecotoxicity data of industrially relevant engineered nanomaterials

The ever-growing number of nanotoxicity-related scientific papers has recently inspired several review articles that have summarised specific aspects of the field [1, 3, 31, 58, 153, 175-179]. However, as mentioned in section 1.4 of this thesis, to our knowledge there was no comprehensive database that would include all parameters of ENMs that affect their potential toxicity, conditions for performed toxicity tests and detailed outcomes of the toxicity tests. To improve and assist predictive modelling of the

mechanisms of action of novel ENMs, [153], systematic approach to map and organise published nanoecotoxicity data into a database was implemented in Paper **II**.

Considering that thousands of papers have been published about (eco)toxicity of ENMs, Paper **II** focused on industrially relevant ENMs, i.e. ENMs with large estimated production values (Fig. 1). Accordingly, the initial list included SiO_2, TiO_2, Fe, Al_2O_3, ZnO, nanoclay, CeO_2, CNT, Ag, and Cu NPs and fullerenes. However, the list of ENMs was modified based on the results of the preliminary data collection. Specifically, SiO_2 and Al_2O_3 NPs and nanoclays were not included in the final database for the following reasons. Although SiO_2 has the highest estimated production volume, studies have reported that it is a biocompatible and non-toxic material that is generally safe [180]. Furthermore, silica is not biopersistent and does not bioaccumulate [44]. Similarly, nanoclay particles are promising drug delivery candidates, owing to their high absorptive capacity, chemical inertness and low toxicity [181]. During the literature search for Paper **II**, the data about ecotoxicity of Al_2O_3 NPs was limited and contradictory. Namely, while concentrations up to 1 g/L did not affect hatching rate of zebrafish embryos [182] and Al_2O_3 NPs had very low toxicity towards crustacean *Ceriodaphnia dubia* [183], reported 24-h LC_{50} for nematode was 81.6 mg/L [184], chronic exposure to Al_2O_3 could induce oxidative stress in nematodes [185], and concentration of 1 mg/L was shown to reduce the viability of freshwater bacteria *Bacillus licheniformis* [186, 187]. Thus, these three ENMs were omitted from the further study.

Currently available QNAR modelling studies are applicable only to a small range of ENMs and limited number of organisms as they are developed using small experimental ENM libraries [188]. Modelling the relationship between biological activities and physico-chemical parameters of ENMs (i.e. development of QNARs) on a larger scale requires thorough characterisation of ENMs [1, 55, 58, 189-192] which was also pointed out in section 1.1.5 of this thesis. Therefore, several intrinsic (Fig. 5A) and test-environment-specific (Fig. 5B) parameters of ENMs were collected together with detailed descriptions of toxicity test conditions and outcomes (Fig. 5C) from the analysed papers and current knowledge on the development of *in silico* models in predicting and classifying the hazard of metallic ENMs, identified a list of ENM parameters including type, composition, size distribution, coating, purity, crystallinity, surface area, surface charge, shape, agglomeration, stability in the test medium, zeta potential, that were found to be essential for computational toxicology of ENMs. Moreover, Chen et al. suggested that ENM toxicity reports should include, at a minimum, details of the tested organisms (taxonomy, name of species, exposure route, life-stage, and strain), experimental conditions (test guideline, modifications, medium, pH, irradiation, and time), and endpoints (effects, endpoint type, value, and unit) [66]. The same data on organisms, test conditions and endpoints is also required by REACH Regulation [193]. Additional parameters specified in REACH Regulation, like dissolved oxygen levels, dissolved organic carbon, hardness and salinity of the test medium and other relevant parameters (e.g., total organic carbon) were also included in NanoE-Tox under the categories of “test media” and “other important conditions”. Since the list of the parameters recorded in NanoE-Tox overlaps with the list suggested by Chen et al. and the regulatory requirements, the database developed in this study

contains valuable information for the development of *in silico* approaches for ENM risk modelling and hazard assessment.

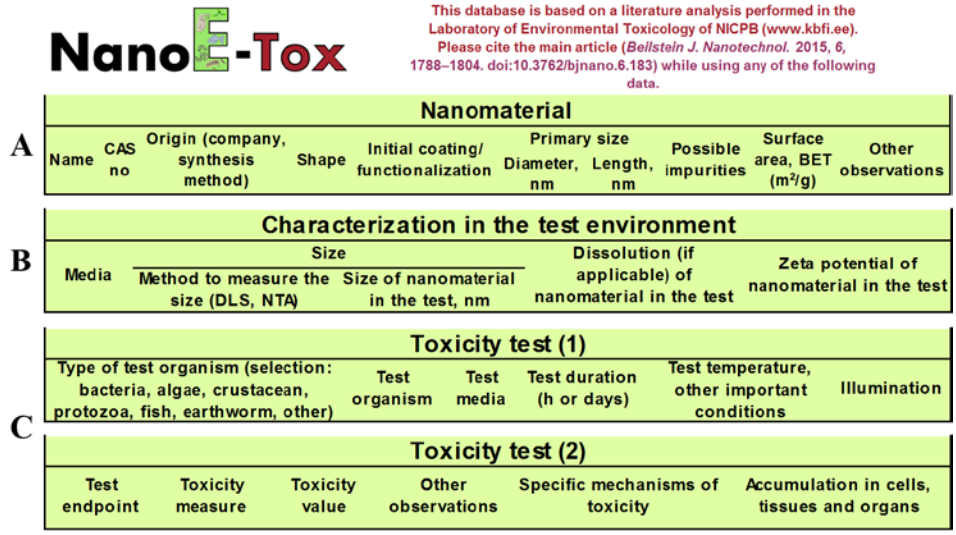


*Figure 5. Headers of columns in NanoE-Tox database describing (A) the pristine particle parameters, (B) environment-related parameters of the particles, and (C) toxicity test conditions and results (SI File 2 of Paper **II**).*

3.1.2.1 Relevance and distribution of data, observations on keyword selection in literature search

Altogether 1,518 toxicity values for all the selected NPs were organised into the database NanoE-Tox. The collected data was analysed for the number of papers published on ecotoxicity of each ENMs (Fig. 6A) and for the number of entries (number of toxicity values reported) for each ENM (Fig. 6B). Interestingly, the number of papers on a specific ENM did not reflect the amount of information available for the toxicity of the same ENM. The most papers were published on the toxicity of TiO_2 NPs (80 papers, 36% of the papers) which was also one of the first ENMs studied for its ecotoxicity, followed by Ag NPs (71 papers, 32%), and ZnO NPs (35 papers, 16%) (Fig. 6A). The most toxicity values (entries) in the database were for Ag NPs (528 entries, 35%) followed by TiO_2 NPs (332 entries, 22%), and CeO_2 NPs (197 entries, 13%) (Fig. 6B). According to this study (Paper **II**), the least information is published about fullerenes (16 papers, 57 entries), CuO NPs (15 papers, 87 entries), and FeO_x NPs (9 papers, 24 entries) (Fig. 6).

Compared to Paper **I** where more than 300 toxicity values were collected for Ag, ZnO and CuO NPs, nearly 2.5 times more values were collected for these NPs in Paper **II.** This difference was partly due to the increase in published papers (Fig. 6A), and partly due to the keywords used in Paper **II** which enabled to include a wider range of organisms. However, the main reason for the higher number of toxicity values in Paper **II** was the difference of the set goals – in Paper **I**, the aim was to include ten most recent papers per ENM per organism but in Paper **II**, all relevant papers retrieved with the selected search terms were included.

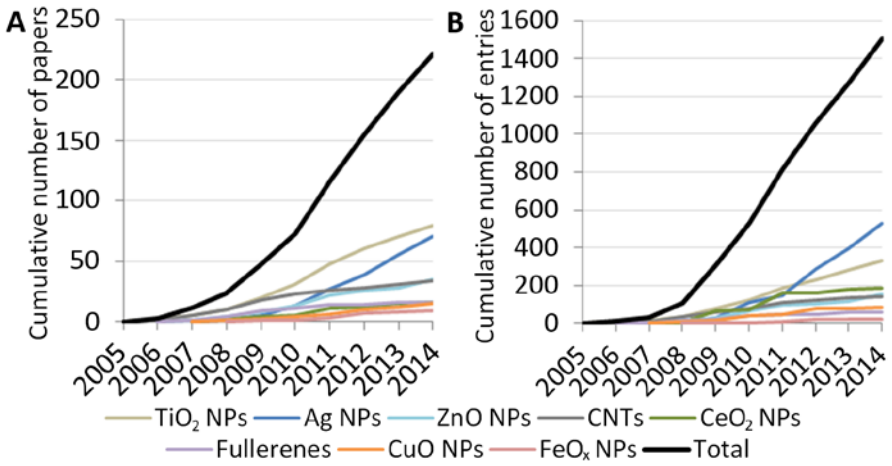


*Figure 6. Evolution of information on ecotoxicity of selected nanomaterials according to (A) the number of papers and (B) the number of entries in NanoE-Tox database (SI File 2 of Paper **II**).*

As the outcome of bibliographic search relies largely on the selection of keywords, the following points outline some of the observations made in the course of the literature search (Paper **II**).

- Although the keywords that were selected for the search were rather specific and involved truncated terms “nano*”, “ecotoxic*” and truncated names, molecular formulas or common abbreviations of NPs of interest, only 224 out of the nearly 500 retrieved individual papers were research papers on the topic, i.e. about ENMs of interest.
- The importance of including synonyms in the list of keywords when performing a bibliographic search was noted. This is illustrated by the following example: while search with the keyword “cerium *oxide” resulted in the list of 30 papers, the search with the keyword “CeO2” resulted in 34 papers, whereas only 20 of these papers overlapped. Hence, for NanoE-Tox, data search was performed using truncated names, molecular formulas and abbreviations of the selected ENMs (Table 1 in Paper **II**).
- Including keywords such as “ecotoxic*” can result in incomplete list of relevant papers because not all ecotoxicity studies define the research as such. For example, in chronologically earlier Paper **I** where the search was organism specific (i.e., not confined to ecotoxicology), 17 EC_{50} values were retrieved for Ag NPs towards algae. In Paper **II**, only 4 EC_{50} values for Ag NP algal toxicity were obtained when including the keyword “ecotoxic*” (Table 2). This indicated that there could have been studies on Ag NP toxicity to algae and studies about other relevant ENMs and organisms that were not included in data synthesis in Paper **II**. However, by including the keyword “ecotoxic*” equal weight was given to all organisms in Paper **II**.

3.1.2.2 Analysis of data on physico-chemical characterisation of ENMs

To evaluate whether existing papers could be useful for modelling purposes, the coverage of available data on physico-chemical characterisation of ENMs used in the studies included in NanoE-Tox was analysed (Fig. 7). Although not an ENM-specific parameter *per se*, the origin of ENMs is important as it enables to identify whether NPs used in different studies were of the same origin and, therefore, comparable. The origin of NPs was reported in case of 99% of the entries in NanoE-Tox and 80% of the studied NPs were of commercial origin (Fig. 7A). Among the ENM-specific intrinsic parameters, size of the NPs was the most often reported parameter. Moreover, it was the only pristine particle-specific property that was reported in case of more than 50% of the entries (precisely for 93% of the entries). The latter is in agreement with the fact that the novelty of ENMs comes from their small dimensions and many authors have hypothesised that ENMs have size-dependent effects [194, 195]. Yet, it has been stated that only NPs with a diameter smaller than 20-30 nm have "true nano-effects" [196]. Indeed, Ivask et al. [197] have shown that for many different organism groups the toxicity of 20, 40, 60 and 80 nm monodisperse citrate-coated Ag NPs depends entirely on their dissolution but 10 nm Ag NPs seemed to act *via* an additional toxicity mechanism. The reported ENM size was below 30 nm in case of 62% of the entries (Fig. 7A).

In decreasing order of number of entries, the other reported intrinsic parameters were coating (44% of all the entries), specific surface area (37%), impurities (34%), shape (33%), and other observations like crystal structure, density, absorbance, etc. (33%, Fig. 7A). The relatively small proportion of entries where these parameters were reported is alarming as coating and/or surface functionalisation determines both the stability and surface chemistry of NPs and consequently also NP interactions with biomolecules and cells/organisms [159]. For instance, positively charged ENMs have been shown to interact with negatively charged cell membranes via electrostatic interactions, causing damage to the cell membranes [153, 198]. In some cases, the impurities from the synthesis process, such as chemical vapour deposition, may contribute to NP toxicity [199]. The fact that NP shape was reported in only approximately one third of the analysed papers and that most of these studies were conducted with spherical NPs, suggests that shape-related effects of NPs are understudied, despite of a few reports on higher toxicity of rod-shaped and triangular NPs compared to spherical ones [200-202].

The test environment could alter both the size (by causing agglomeration or dissolution of NPs) and surface charge (by ion sorption or biocorona formation on NPs) of NPs. Thus, NP characterisation in the ecotoxicological media before and during the test is essential for the interpretation of the test results. However, the analysis of the published literature showed that, compared to the intrinsic NP properties, the environment-specific properties of ENMs were reported less often (Fig. 7B). Hydrodynamic size was reported for 59% of all the entries; 69% of the reported diameters were larger than 100 nm indicating NP agglomeration in the aquatic media. Zeta potential is often used as an indicator of NP surface charge and stability. Namely, while some neutral NPs could be stabilised sterically by covalently bound biomolecules, other NPs are considered to form stable aqueous suspensions if their zeta potential is either greater than 30 mV or smaller

than -30 mV [203]. The information about zeta potential was available for 40% of the entries and stable positively charged NPs (i.e., NPs that are likely to interact, *via* electrostatic interactions, with cell membranes that have an overall negative surface charge) were used only in 1% of the studies. The least reported environment-dependent parameter was the dissolution of particles. While no dissolution data for TiO_2 NPs, CNTs and fullerenes was expected, only half (51%) of the studies about NPs that could potentially release toxic ions (Ag, ZnO, CuO, CeO_2 and FeO_x NPs) reported the solubility of NPs (Fig. 7B).

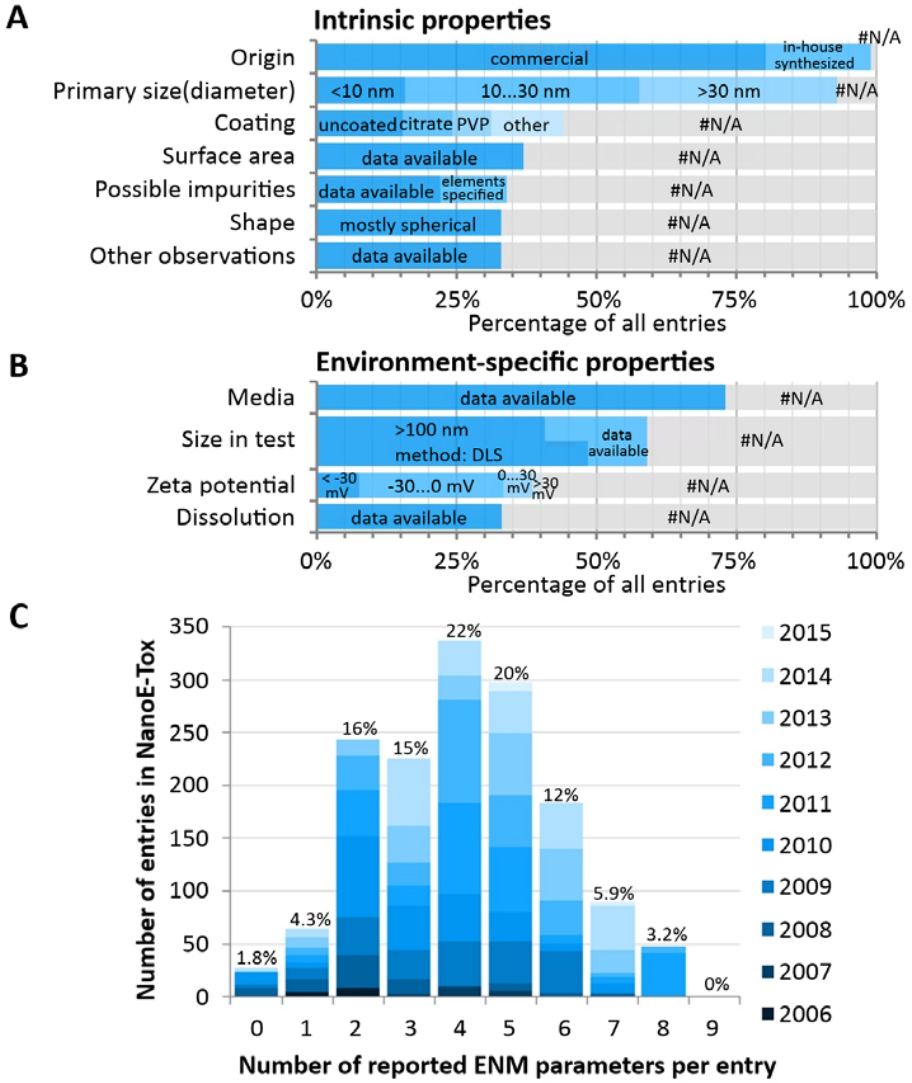


Figure 7. Coverage of available data on characterisation of ENMs in NanoE-Tox. Percentages of reported (A) intrinsic and (B) environment-specific parameters of all entries (1,518) in the database. (C) Number of reported ENM parameters (primary size, coating, surface area, possible impurities, shape, other observations, size in the test, zeta potential, dissolution) and percentage of all entries by publication year (lighter colour indicates more recent year). #N/A – data not available. Figure is modified from Paper ***II****.*

Finally, the data compiled in NanoE-Tox database was assessed from the perspective of its applicability for QNAR modelling. The analysis showed that the vast majority of the studies (85%) had reported 2-6 NP-specific parameters (Fig. 7C). One of the conclusions that can be drawn from the data analysis is that despite of the wide scientific discussion about the minimum set of parameters that should be included in a nanotoxicological report [66], there is still room for improvement. For instance, from all 224 individual studies none reported all parameters included in NanoE-Tox database and minority (9%) of the papers reported 7-8 parameters (Fig. 7C). However, considering that the majority of the studies reported ENM primary size and hydrodynamic diameter in the respective test medium, these data may be applicable for modelling of size-dependent biological effects.

3.1.2.3 Analysis of nanoecotoxicological data

Considering the increasing production volumes of ENMs, there is a need for nanoecotoxicological data for the hazard assessment in the framework for risk characterisation. Expectedly, more than half of the entries in NanoE-Tox involved organisms that are used in the standard aquatic toxicity testing – crustaceans, algae and fish (Fig. 8, Fig. 5 in Paper **II**). As in Paper **I**, *D. magna* was the most prominent species among crustaceans, *R. subcapitata* among algae, *E. coli* among bacteria, and *C. elegans* among nematodes. Including also fish *D. rerio* and naturally luminescent bacterium *V. fischeri*, the mentioned six species constituted nearly half (47%) of all the entries in NanoE-Tox database. Overall, nanoecotoxicological studies were performed using a range of different types of organisms – the database includes 116 different species from the following groups: plants, yeasts, protists, amphibians, bivalves, cnidarians, echinoderms, insects, nematodes, rotifers, snails and worms (Table S5 in Paper **II**). However, the distribution of the toxicity data on ENMs among different organisms was uneven (Fig. 8). For instance, using the selected keywords, no information was found about the effects of fullerenes and FeO_x NPs on algae and only one study was found for CuO NP effects in algae (Table 2, Fig. 8). While this outcome could be influenced by the choice of keywords, overall, there appear to be data gaps in algal and fish nanotoxicity studies (Table 2).

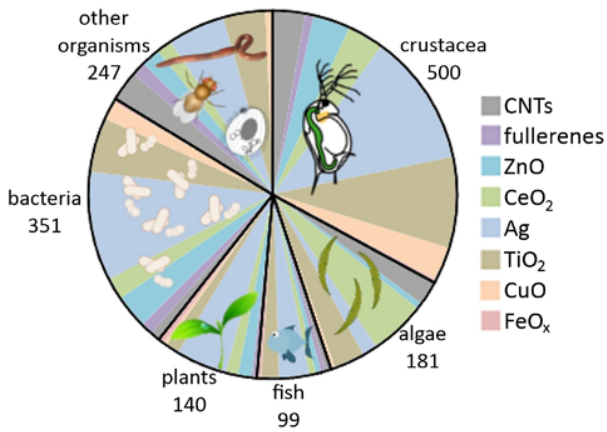


Figure 8. Distribution of test organisms and tested ENMs by number of entries in NanoE-Tox database.

Contrary to ENM parameters that were not fully described in many papers, the toxicity test conditions were generally well reported: the test environment and duration were mentioned in nearly all the studies, the temperature was reported in more than 90% of the entries and illumination conditions in 75% of the entries. Further analysis of the data showed that 79% of the studies were conducted in artificial media that could complicate the knowledge transfer to natural conditions. The limited number of studies conducted under natural conditions has been also outlined in other papers [124, 204, 205]. The most commonly used toxicity endpoint was viability (77% of the entries) that was mainly reported as a half-effective concentration (EC_{50}, 28% of the entries), half-lethal concentration (LC_{50}, 10% of the entries) or no observed effect concentration (NOEC, 20% of the entries). Other reported endpoints included NP effects on reproduction or NP-triggered malformations. Several studies had tested only one or two NP concentrations that did not allow for the construction of a dose-effect curve, and thus, calculation of half-effective or half-lethal concentrations needed for the risk evaluation. Another aspect that may compromise the suitability of some of the reported test results for hazard assessment is the use of modified standard test protocols. Namely, many of the results were obtained with standard test species, however, the protocols were often modified, e.g., the test duration was altered compared to the standard protocols.

3.1.2.4 Effects and mechanisms of action of selected ENMs based on the analysis of NanoE-Tox data

For modelling purposes, the most commonly used toxicity values are median E(L,I)C_{50} values. In order to compare the toxic effects of different ENMs and to identify the most sensitive organisms, E(L,I)C_{50} values reported for species that belonged to the same organism group were pooled together. Altogether 47 different median E(L,I)C_{50} values were obtained. However, only 18 of these were based on data from three or more papers, 10 median values were calculated based on data from two papers and 19 median values were each based on data from only one paper (Fig. 6 in Paper **II**). Interestingly, E(L,I)C_{50} values for ZnO NPs were derived from the tests with the widest selection of organisms (10 organism groups), followed by Ag and TiO_2 NPs (both values for 9 organism groups) and CuO NPs (values for 7 organism groups). For fullerenes, however, EC_{50} values were derived from only two organism groups, even though the number of papers in NanoE-Tox was the same for fullerenes and CuO NPs (Table 2, Fig. 6 in Paper **II**). In addition, the analysis by organism groups indicated that most of the nanotoxicity studies have been conducted with crustaceans and bacteria, while other types of organisms were relatively less studied.

Remarkably, the median E(L,I)C_{50} values of Ag, ZnO and CuO NPs by organism groups in NanoE-Tox were comparable i.e. in the same order of magnitude to those collected in Paper **I** (Table 2). In addition, the median E(L,I)C_{50} were in agreement with the values reported in other reviews [31, 58]. The most toxic were Ag NPs, followed by other ENMs in decreasing order of toxicity: Ag > ZnO > CuO > CeO_2 > CNTs > TiO_2 > FeO_x (Table 2, Fig. 6 in Paper **II)**. Fullerenes were omitted from the analysis because of the limited toxicity data.

The most susceptible organisms determined based on the lowest median E(L,I)C_{50} values, were crustaceans, algae and fish (Table 2). For some ENMs the derived median

$E(L,I)C_{50}$ values were lower in organisms that belonged to other groups, for instance, the lowest $E(L,I)C_{50}$ value for Ag NPs was reported with snails, for CuO with rotifers, for CNTs with bacteria and for TiO_2 with insects. Although these values were calculated based on less than three papers, this may suggest the potential for the emerging new data to alter the current knowledge on the most sensitive species. Still, the single lowest reported $E(L,I)C_{50}$ values, i.e., minimal instead of median values, for each ENM were obtained with crustaceans, algae or fish, reinforcing the suitability of the established standard organisms [58] for the risk assessment of ENMs.

Majority of the studies included in NanoE-Tox did not provide information about toxicity mechanisms, but some papers suggested that the toxic effect might be at least partially NP-specific, i.e., not only caused by the released toxic ions. Admittedly, such results were mostly reported for insoluble NPs tested at high particle concentrations. The mechanism of toxic action of partially soluble NPs, such as Ag, CuO and ZnO NPs, was often explained by the release of respective metal ions. Namely, such mechanism was proposed in 53% of the papers observing the effect of CuO NPs, 17% ZnO NPs, and 14% Ag NPs (Table S2 in Paper **II**). As the distinction between the mechanisms triggered by the released ions and NPs is difficult [206], several modes of action that may be caused by both, ions and particles, have been reported. For instance, Ag NPs have shown to induce oxidative stress in the cells [207-213], cause destabilisation or mechanical damage of cell membranes [212-215], and DNA damage/genotoxicity [216-218]. Due to high affinity of Ag to S, Ag NPs may also bind to sulfhydryl groups of various proteins and disrupt their functions [219]. Effects on membranes, oxidative stress, and genotoxic effects were reported also for ZnO NPs [220-225]. Substantially less studies were performed to assess the toxic mechanisms of other ENMs, known to be practically insoluble (CeO_2, CNTs, and TiO_2). Still, when reported, the main mechanisms were membrane damage and oxidative stress (Table S2 in Paper **II**). 18 studies showed that NPs were ingested by various organisms and accumulated in the organisms (34 studies) or on their surface (15 studies) (Table S3 in Paper **II**).

In summary, the analyses performed in Papers **I** and **II** indicated that although the nanoecotoxicological data is emerging and a lot is already known about the toxicity of ENMs towards environmentally relevant organisms, there are still several data gaps, such as information on some standard and most non-standard test species and lack of detailed characterisation of tested ENMs. Similar conclusions were drawn in a recent review by Chen et al. [66] who outlined the limited existing data on nanotoxicity and incomplete reporting of the ENM parameters and test conditions. According to our knowledge, NanoE-Tox is currently the largest in-depth online-available database on ENM ecotoxicity. It enables comparison of both the effects of different ENMs towards single species and effects of specific ENMs towards multiple species which could be valuable for computational toxicology and hazard assessment.

3.2 Effects of nanosilver on *Tetrahymena thermophila* (Papers III, IV)

According to the estimations on production volumes, Ag NPs are not among the top three ENMs (Fig. 1); however, Ag NPs are the most prevalent in consumer products. Namely, Ag NPs are included in 12-24% of all the nano-enabled products listed in the

inventories (Table 1) [4-6]. Thus, as considerable amounts of Ag NPs could reach water bodies (Fig. 2) it is important to assess its effect on different levels of the aquatic food web. The toxicity of Ag NPs has been studied in many species (Papers **I**, **II**) but prior to the current study there were only two papers on nanosilver effects on ciliates [133, 226] and no reports on the mechanism of action of Ag NPs in ciliates. Furthermore, despite the extensive efforts to elucidate the mechanisms of toxicity of Ag NPs in aquatic organisms, there is still no consensus on that matter. It has been proposed that Ag NP toxicity results from the effects of both silver ions and particles and depends on the environment [53]. For these reasons, following parts of this thesis focus on evaluating silver effects on the protozoan *T. thermophila*.

3.2.1 Toxicity of silver compounds to *Tetrahymena thermophila*

3.2.1.1 Characterisation of Ag nanoparticles

Two commercially available silver NPs were tested to elucidate their toxic effect on *T. thermophila* (Table 3). Ag NPs purchased from Sigma-Aldrich (Sigma-Ag NPs) were noncoated and have been tested in other nanotoxicological studies previously [208, 209, 227-233]. The second type of Ag NPs included in this study were medically relevant colloidal Ag NPs (Col-Ag NPs). Such colloidal Ag solutions have been used as antibacterial agents for more than hundred years and are available in the pharmacies also today [234]. The two tested Ag NPs differed in several parameters. Namely, compared to casein-coated Col-Ag NPs, Sigma-Ag NPs were larger, more heterogeneous and less soluble (Table 3). Literature data suggests that in moderately hard water the hydrodynamic diameter of Sigma-Ag NPs could be even larger than 147 nm which was reported in Paper **III** [228, 230]. Ag NP dissolution in deionised water was in similar range as reported previously for Sigma-Ag NPs [229] and Col-Ag NPs [235, 236].

Table 3. Physico-chemical properties of tested Ag NPs.

Parameter	**Sigma-Ag NPs (Paper III)**	**Col-Ag NPs (Paper IV)**
Primary size	<100 nm	14.6 ± 4.7 nm
Coating	Noncoated	Casein (30% of total mass)
Shape according to TEM	#N/A	spherical
Hydrodynamic diameter[†]	147 nm	44 nm
Polydispersity index[†]	0.48	0.2
Zeta potential[†]	-51 mV	-42.7 mV
Solubility[†]	0.2% at 205 mg/L in 0 h 0.3% at 205 mg/L in 2 h 0.42% at 205 mg/L in 24 h	3.8% at 20 mg/L in 2 h 2.3% at 100 mg/L in 2 h 6.6% at 20 mg/L in 24 h 3.0% at 100 mg/L in 24 h

[†] measured in DI water
#N/A – not assessed

As Col-Ag NPs primary size was below 20 nm, they were more likely to act *via* nano-effects compared to agglomeration-prone Sigma-Ag NPs. Indeed, Col-Ag NPs generated

time- and dose-dependent ROS in deionised water (Fig. S5A in Paper **IV**). Abiotic ROS generation has also been reported in case of citrate-coated Ag NPs with diameters up to 20 nm [197]. The observed ROS are likely the intermediate products of the oxidative dissolution of Col-Ag NPs [53, 237].

3.2.1.2 Effects of silver compounds on *Tetrahymena thermophila* viability

Toxicity testing of Ag compounds was conducted in two separate studies (Paper **III** and Paper **IV**) using three *T. thermophila* wild strains (BIII, CU427, CU428). Overall, no significant differences in sensitivities to Ag compounds were expected between the strains because nearly equal susceptibility to a heterogeneous set of over 50 chemicals has been reported in two different species of *Tetrahymena* – *T. thermophila* and *T. pyriformis* [238]. Indeed, EC_{50} values of $AgNO_3$ were in the same order of magnitude in case of all three *T. thermophila* strains, suggesting that the effects of Ag compounds on *T. thermophila* viability may be compared across the strains (Fig. 9). Thus, among the Ag compounds tested in this study, Sigma-Ag NPs were regarded as the least toxic with EC_{50} values ranging from 205 to 286 mg/L, tested with strain BIII, a strain commonly used in ecotoxicity testing and included in the commercially available Protoxkit [238] (Fig. 9). These EC_{50} values were higher than the values reported for other aquatic organisms, such as the crustacean *D. magna* (24-h immobilisation-based EC_{50} 1-4 mg/L) [230] and bacteria (2-h luminescence inhibition-based EC_{50} 45.9 mg/L) [208]. Compared to Sigma-Ag NPs, Col-Ag NPs were more toxic to *T. thermophila*, tested in strains CU427 and CU428, with EC_{50} values ranging from 72 to 100 mg Ag/L (Fig. 9). Interestingly, while the difference in Col-Ag NP toxicity between strains CU427 and CU428 that have almost identical genetic backgrounds was not large (strain CU428 was 20-30% more sensitive compared to strain CU427), it was statistically significant and could indicate that the mechanisms of action could slightly differ.

In order to analyse, whether the effect of NPs could be triggered by their dissolution, the effect of respective metal ions has to be known. In general, Ag ions have been shown to be very toxic to many aquatic species; the median EC_{50} value was as low as 0.0009 mg/L in crustaceans (Paper **I)**. In *T. pyriformis*, $AgNO_3$ caused about 60% growth inhibition at 1.5 mg Ag/L, however no half-effective concentration was reported [133]. Comparison of EC_{50} values obtained with three different *T. thermophila* strains: BIII (Paper **III**), CU427, and CU428 (Paper **IV**) indicated, as mentioned above, that the susceptibility of these strains to $AgNO_3$ was similar as all EC_{50} values were in the range of 1.5 to 2.8 mg Ag/L, and the toxicity did not change significantly in time (Fig. 9). Interestingly, slight recovery of the culture was observed after 24 h of exposure which was also supported by statistically significant increase in EC_{50} values for strains CU427 and CU428 (Fig. S6 in Paper **IV**). Therefore, compared to other aquatic organisms like crustaceans, algae or fish (Paper **I**), *T. thermophila* had relatively high tolerance to $AgNO_3$ which was in agreement with the results reported by Shi et al. [133] for *T. pyriformis*. One explanation for such phenomenon is that to survive in heavily polluted environments like wastewater treatment plants, *T. thermophila* has acquired resistance to various drugs and toxins *via* ABC transporters [239].

The solubility-based calculated toxicity of the Col-Ag NPs resulted in EC_{50} values similar to respective EC_{50} values of $AgNO_3$ (Fig. S7 in Paper **IV**) which indicates the importance of released Ag ions in Ag NP toxicity. Indeed, as the toxicity of $AgNO_3$ for all

three *T. thermophila* strains was similar (Fig. 9), the difference in toxicity of Sigma-Ag NPs and Col-Ag NPs could be explained by different dissolution rates of the particles. Moreover, another type of Ag NPs which were coated with PVP and had higher share of free Ag ions were much more toxic to *T. thermophila* strain BIII (EC_{50} values ranging from 3.2 to 3.9 mg Ag/L) [137]. In summary, the effect of Ag NPs on the viability of *T. thermophila* appears to depend on NP physico-chemical parameters (including hydrodynamic diameter, stability and solubility) and the EC_{50} values can vary in the range of orders of magnitudes even in the same test environment.

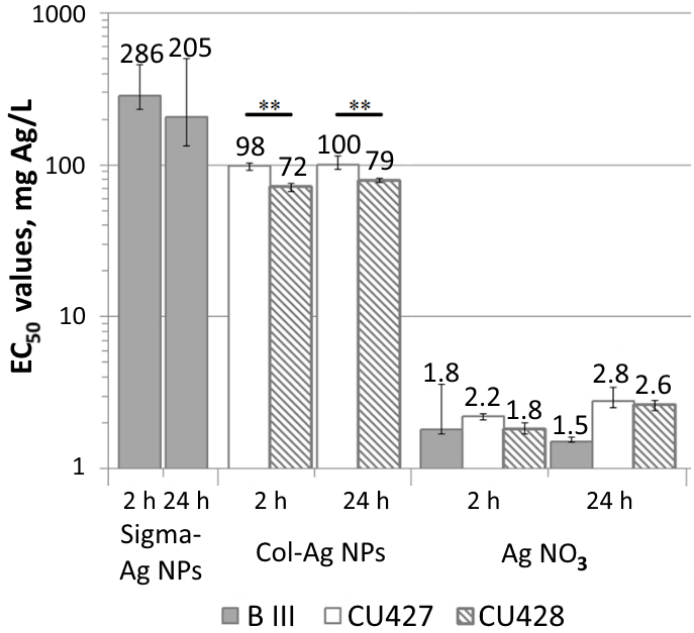


Figure 9. The toxicity (EC_{50}, mg Ag/L) of tested Ag-compounds to different strains of Tetrahymena thermophila after 2- and 24 h exposure in DI water. The data are the average values of at least three independent assays and error bars indicate 95% confidence intervals. The asterisks (**) mark highly significant difference ($p < 0.01$) among tested strains.

3.2.2 Silver nitrate biomineralisation by *Tetrahymena thermophila*

In recent years, green synthesis of ENMs has gained popularity; metal ENM biosynthesis from the respective metal ions has been demonstrated using extracts or cultures of a range of different organisms, e.g., plants, bacteria, and fungi [18]. Such biological reduction of metal ions has been suggested to act as a mechanism of detoxification of toxic metal species [17]. Microorganisms are known to secrete secondary metabolites and extracellular polymeric substances that enable cell-cell communication, facilitate nutrient acquisition and protect from toxicants. *T. thermophila* was reported to secrete ~30 different proteins into the extracellular medium [240]. To study if Ag ions, e.g., those released from Ag NPs could be detoxified by *T. thermophila via* biomineralisation, experiments were conducted using *T. thermophila* extracellular substances (Paper **III**). Indeed, when $AgNO_3$ was added to DI water that contained *T. thermophila* soluble

extracellular fraction (SEF) and incubated under illumination, a colour change of the solution from colourless to maroon was observed (Fig. 1 in Paper **III**). Light has been shown to be essential for (photo)reduction of Ag ions into Ag NPs in other biological systems [241, 242]. The formation of Ag NPs in the SEF water solution was confirmed using UV-Vis spectroscopy and the particles were characterised as described below.

3.2.2.1 Characterisation of formed silver nanoparticles

Ag NPs are known to have plasmon resonance peak in UV-Vis absorbance spectra at 400-450 nm [243, 244]. Aside from visual change, the peak at 420-450 nm in absorbance spectra (Fig. 1 in Paper **III**) together with decrease in soluble Ag content (Fig. 2B in Paper **III**), and particle identification with SEM (Fig. S1 in Paper **III**) confirmed the formation of Ag NPs in SEF. The NP dispersion was relatively heterogeneous as indicated by the high polydispersity index value (0.45-0.59) and SEM images. The particle size increased over time which was reflected by increase in average hydrodynamic diameter: 70 nm after 2-h incubation, 105 nm after 24-h incubation, and over 10 µm after 7 days of incubation (Fig. 2A in Paper **III**). The increase in particle size was also supported by visual examination as after 1 week of incubation the formed particles had settled to the bottom of the test tubes (Fig. 1 in Paper **III**). According to our knowledge this was the first demonstration of metal ion reduction and formation of nanosized metallic particles by the extracellular substances of ciliates. Later, *T. thermophila* SB210 has been shown to synthesise Se NPs [152].

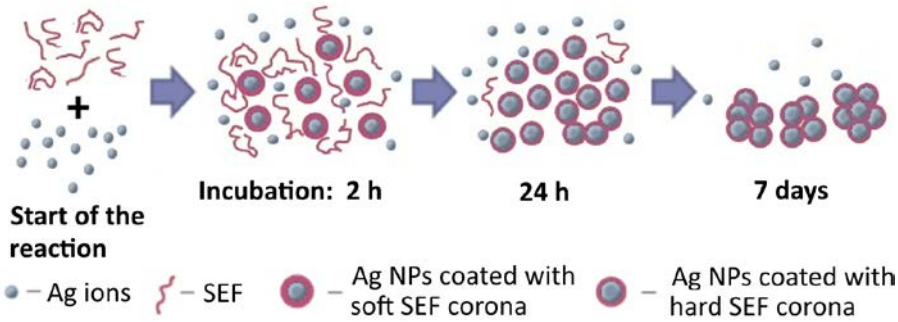


*Figure 10. Proposed mechanism for the formation of Ag nanoparticles from Ag ions in deionized water containing soluble extracellular fraction (SEF) of Tetrahymena thermophila. Soft SEF corona – proteins are weakly bound to nanoparticles; hard SEF corona – proteins are strongly bound to nanoparticles. Modified from Paper **III**.*

It is generally recognised that biomineralisation occurs *via* reduction of metal ions and precipitation of the formed insoluble nontoxic metal-containing ENMs; however, the detailed mechanism varies from species to species [245]. *T. thermophila* SEF contains various peptides and proteins (e.g., acid hydrolases [246] and other proteases [240]), which may contribute to the reduction of Ag ions [241]. In addition, NPs tend to adsorb proteins onto their surface to form protein corona [247] which helps to stabilise NPs in the aqueous media [248]. Indeed, protein analysis of the Ag-containing protozoan SEF indicated time-dependent decrease in the intensity of the most prevalent protein bands of 22-34 kDa size range (Fig. 3 in Paper **III**). Moreover, longer incubation resulted in decreased intensity of other protein bands, suggesting that Ag biomineralisation was limited by the SEF protein concentration. Following the

reduction of Ag ions and formation of NPs, proteins probably started to form soft corona around the particles. As the reduction of Ag ions continued (confirmed by UV-Vis, Fig. 1 in Paper **III**), NP concentration and diameters increased, absorbing more proteins from the solution. The reduction in protein concentration resulted in the decrease of the corona thickness, dissociation of weakly bound proteins (soft corona) and formation of hard corona consisting of strongly adsorbed proteins (i.e. hard corona). Due to the depletion of free proteins in SEF, NPs formed at later stages remained uncoated and agglomerated, forming a precipitate (Fig. 10).

3.2.2.2 Toxicity of extracellularly formed silver nanoparticles to *Tetrahymena thermophila*

When *T. thermophila* was exposed to Ag-SEF mixture, dark aggregates were observed in the phagosomes of *T. thermophila* (Fig. 5 in Paper **III**), that further confirmed the formation of Ag NPs in SEF and their uptake by phagocytosis in the ciliates. The results of the viability assay showed that the incubation of $AgNO_3$ in SEF reduced Ag toxicity to *T. thermophila* time-dependently – the EC_{50} values were approximately 2.3 times higher upon exposure to 7-day Ag-SEF mixture than to pure $AgNO_3$ (Table 1 and Fig. 4 in Paper **III**). Thus, the formation of Ag NPs could render Ag less bioavailable to *T. thermophila* meaning that SEF has a role in adaptation to environments containing toxic metal ions. The latter is supported by the work of Prasad et al. [245] who suggested that NPs are by-products of microbial resistance mechanisms developed during evolution to cope with high environmental metal concentrations. Additionally, NP formation and agglomeration in the phagosomes could further reduce Ag bioavailability. Indeed, ingestion of ENMs and releasing larger agglomerates has been proposed as a detoxification mechanism also for TiO_2 NPs in *T. thermophila* [115].

3.2.3 Sub-lethal effects of silver compounds on *Tetrahymena thermophila*

Although all Ag compounds were toxic to *T. thermophila* at mg per L range which is much higher than the estimated concentration of silver in the nature (ng to µg per L range) [21], harmful local concentrations may occur as a consequence of accidents. In addition, organisms may experience stress also at sub-lethal concentrations [249]. Moreover, as *T. thermophila* ingests NPs, it could concentrate the particles in its phagosomes and thus introduce higher local concentrations to its predators. Indeed, Ag NPs were in *T. thermophila* food vacuoles as evidenced by dark agglomerates in the cells visualised using bright field microscopy (Fig. 11).

Still, the literature reports about the mechanism of action of Ag NPs are controversial. Namely, gene expression studies often report up-regulation of oxidative stress related genes and propose that Ag NPs generate oxidative stress [213, 250, 251]. Contrarily, the results from bioassays are often inconsistent and both increase and decrease of intracellular ROS levels, superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX) activities [252-254] have been reported. Thus, effects of Ag NPs at sub-lethal concentrations (20 mg Ag/L) on the gene transcription and physiology of *T. thermophila* were studied in parallel to elucidate the mode of toxicity of Ag NPs in the ciliate. As Sigma-Ag NPs were relatively large and tended to agglomerate in the aquatic media, manifestation of nano-specific effects was unlikely for these NPs. Consequently, the following experiments were conducted with Col-Ag NPs that were stabilised with casein coating and formed a stable dispersion in

DI water. $AgNO_3$ was included as an ionic Ag control and polystyrene NPs of similar size range as Col-Ag NPs as a control for nano-specific effects.

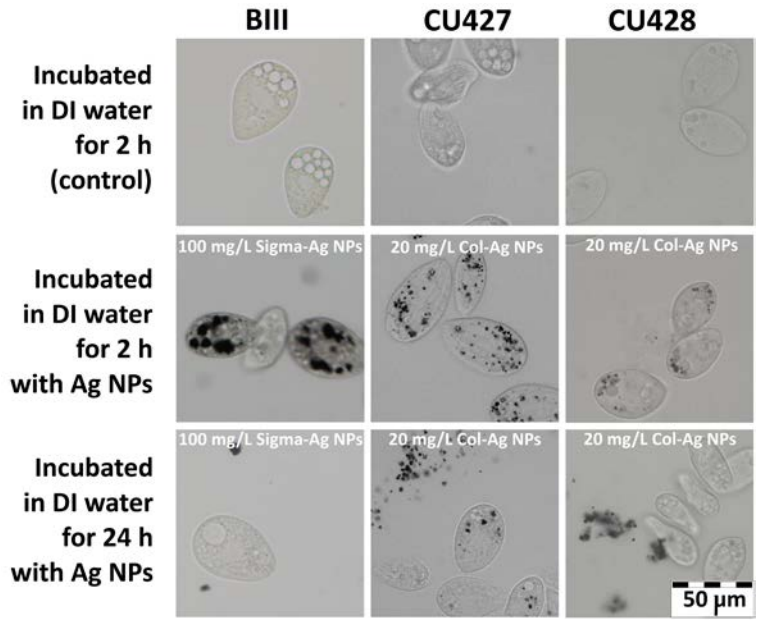


Figure 11. Bright field images of three different T. thermophila strains (BIII, CU427, CU428) after 2-h incubation in DI water, and after 2- and 24-h exposure to Ag NPs. Strain BIII was exposed to 100 mg/L of Sigma-Ag NPs (noncoated) and strains CU427 and CU428 to 20 mg/L of Col-Ag NPs (casein-coated).

3.2.3.1 Effects on gene expression

Changes at gene expression level are often related to physiological outcomes, e.g., elevated heat shock protein (HSP) genes could reflect that the organism is under stress [255]. As Ag NPs were toxic to *T. thermophila* (Fig. 9), sub-lethal concentrations of Ag NPs were expected to cause cellular stress responses in the protozoan. Additionally, Col-Ag NPs generated abiotic ROS (Fig. S5 in Paper **IV**) and released Ag species (Table 3) that could be bound by specific proteins present in cells. Thus, the genes selected for real-time PCR study had three main functions: coping with general stress, coping with oxidative stress and binding metal ions. Based on the comparison between EC_{50} values of $AgNO_3$ and Col-Ag NPs, calculated based on dissolved Ag ions (Fig. S7 in Paper **IV**), the dissolution of Col-Ag NPs seemed to explain their toxicity, however, the gene expression profiles in protozoa exposed to 20 mg Ag/L of Col-Ag NPs or 1.5 mg Ag/L of $AgNO_3$ (i.e. at solubility-based equitoxic concentrations) were different (Fig. 12). As in general polystyrene NPs did not affect the expression of selected genes (Fig. 1 in Paper **IV**), these effects could not be attributed to NPs in general but were (Col)-Ag NP-specific. Thus, (Col)-Ag NP-induced changes in *T. thermophila* gene expression levels were either caused by the specific properties of Ag NPs, casein coating or the combination of both. Furthermore, although the toxicity of $AgNO_3$ did not differ statistically between strains CU427 and CU428, exposure to $AgNO_3$ at the sub-toxic concentration triggered different responses in these strains.

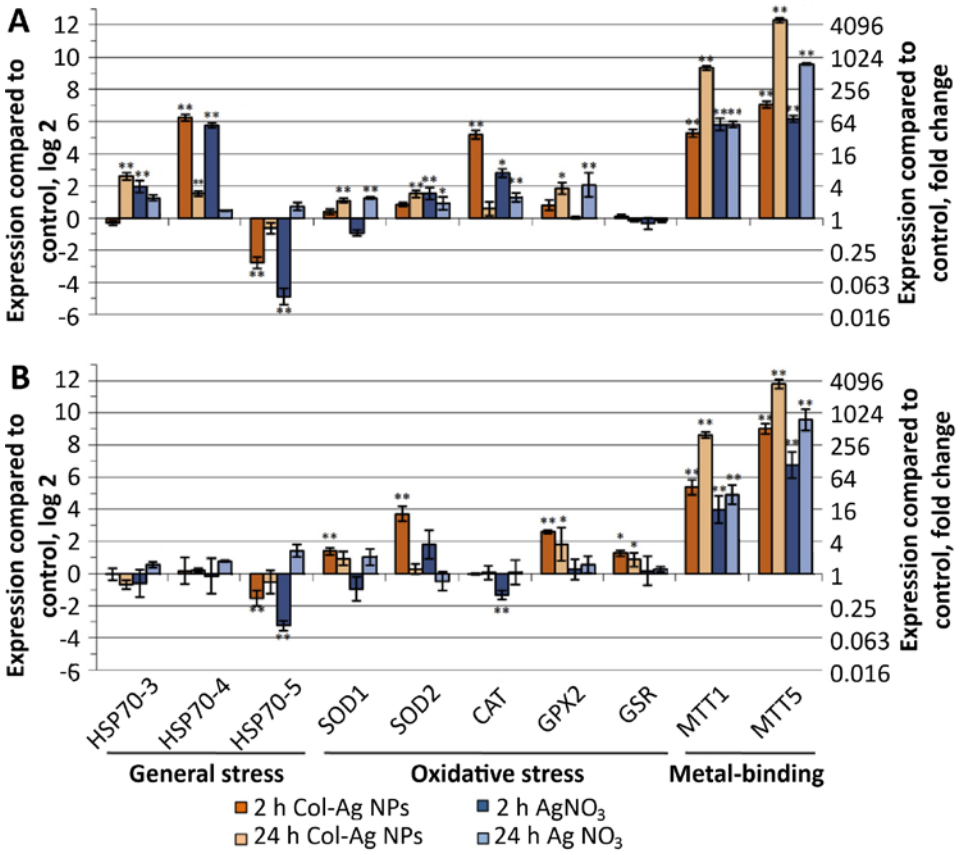


Figure 12. Expression of selected genes in two different Tetrahymena thermophila strains CU427 (A) and CU428 (B) upon exposure to sub-lethal concentrations of Col-Ag NPs and $AgNO_3$ for 2 h and 24 h at 25 °C in the dark. The asterisk (*) marks significant difference ($p < 0.05$) and (**) marks highly significant difference ($p < 0.01$) from the expression levels in the respective strain of Tetrahymena incubated in DI water. HSP – heat shock protein, SOD – superoxide dismutase, CAT – catalase, GPX – glutathione peroxidase, GSR - thioredoxin and glutathione reductase, MTT – metallothionein. Figure is modified from Paper ***IV****.*

The expression levels of HSP-s can be indicators of environmental stress [255] and have been linked to Ag NP induced stress in *C. elegans* [233]. Interestingly, two out of three studied *HSP* genes (*HSP70-3* and *HSP70-4*) were up-regulated upon exposure to Col-Ag NPs (*HSP70-3* up to 6-fold and *HSP70-4* up to 77-fold) and $AgNO_3$ (*HSP70-3* up to 4-fold and *HSP70-4* up to 55-fold) only in strain CU427 (Fig. 12A), and no change in the expression of these genes was detected in strain CU428 (Fig. 12B). Due to the involvement of HSP-s in biological processes like protein folding [256], the upregulation of *HSP* genes should give an advantage to strain CU427 in rapid stress management. This may explain the results of the viability test where Ag compounds were less toxic to strain CU427 than CU428 (Fig. 9). On the other hand, the third *HSP* gene, *HSP70-5*, was down-regulated in both strains (Fig. 12) after 2-h exposure to Ag compounds. Fukuda et al. [256] have shown that, in case of heat-stress, HSP70-5 may be compensated by

other proteins and *HSP70-5* is not essential during starvation [257]. Here, the down-regulation of *HSP70-5* likely indicated that protozoa redirected their resources to Ag stress management.

Although hydroxyl radicals (•OH) are the most reactive ROS in the nature, biological systems do not possess enzymes to scavenge •OH [258]. Hence, the detection of •OH at gene expression level is not possible. Instead we focused on the expression of other oxidative stress related enzymes present in *T. thermophila*. Namely, the mitochondrial Mn-SOD (*SOD1*), a cytosolic Cu/Zn-SOD (*SOD2*) that had been previously shown to have an important role in combating oxidative stress [259], the *CAT* gene, and a GPX (*GPX2*) and a glutathione reductase (*GSR*) both having uniform expression in starving *T. thermophila* were selected for the study. Like with *HSP* genes, the expression of oxidative stress related genes varied in different strains and indicated that Ag compounds may induce mild oxidative stress in *T. thermophila* (Fig. 12).

Generally, in strain CU427 both Col-Ag NPs and $AgNO_3$ induced gene expression in a similar manner and the expression of several genes was up-regulated compared to the control after 24-h exposure (Fig. 12A). Specifically, the highest up-regulation (36- and 7-fold upon exposure to Col-Ag NPs and $AgNO_3$, respectively) was observed with *CAT* after 2-h exposure to Ag compounds. Similarly, up-regulation of *CAT* was observed in the midge *Chironymus riparius* upon exposure to sub-lethal concentrations of Ag NPs [209]. These observations could indicate that excess H_2O_2 was generated by SOD in the process of disproportionation of superoxide radicals ($O_2^{\bullet-}$) into oxygen and hydrogen peroxide, and to manage this stress, the cells produced CAT which is responsible for dismutation of H_2O_2 into water and oxygen [258]. Interestingly, after 24-h exposure to Ag compounds *GPX2* showed the highest expression (about 4-fold increase) among oxidative stress related genes in strain CU427 which could indicate that CAT was replaced by GPX2 having a similar function (GPX2 reduces H_2O_2 to water and lipid hydroperoxides to alcohols). Slight but significant up-regulation (up to 3-fold) of mitochondrial *SOD1* and cytosolic *SOD2* was also observed after 24-h exposure of strain CU427 to both Col-Ag NPs and $AgNO_3$ and only *GSR* expression was not affected at all by Ag compounds (Fig. 12A).

Contrarily, in strain CU428 the gene expression indicated mechanisms specific to only Ag NPs as $AgNO_3$ did not significantly up-regulate any of the selected oxidative stress related genes. Moreover, in contrast with strain CU427, the highest expression rates compared to the control were reached at the earlier time point (i.e. after 2-h exposure, Fig. 12B). The latter could reflect different metabolic rates of the two strains because it has been shown that, upon osmotic shock, strain CU428 maturated faster than CU427 [260]. Surprisingly, in strain CU428, the highest up-regulation (nearly 14-fold) was observed with cytosolic *SOD2* upon 2-h exposure to Col-Ag NPs. Although after 2-h exposure to Col-Ag NPs the mitochondrial *SOD1* expression was only twice higher than in control of strain CU428, the difference was significant and in accordance with the results obtained with *C. elegans* whose mitochondrial *SOD* was up-regulated by Ag NPs but not Ag ions [233]. Unlike in strain CU427, *CAT* expression in strain CU428 was not affected by Ag NPs and was even down-regulated after 2-h expression to $AgNO_3$. *GPX2* expression in strain CU428 was elevated after both 2- and 24-h exposure to Col-Ag NPs. This may indicate that GPX2 compensated for the absence of CAT,

similarly to strain CU427 after 24-h exposure. *GSR* which reduces oxidised glutathione was up-regulated in strain CU428 up to 2-fold in the similar manner to *GPX2* (Fig. 12B).

Finally, metallothioneins (*MTT*s) *MTT1* and *MTT5*, both of which are mainly induced by Cd and respond to wide variety of stressors [261], were up-regulated (*MTT1* up to 650-fold and *MTT5* up to 5000 fold) in both strains by all Ag compounds and at all time points (Fig. 12). This was expected as MTTs are known to bind toxic metals [262] and could also remove ROS [263]. Moreover, *MTT* expression has been shown to increase upon exposure to Ag NPs in other models like zebrafish [264]. As the *MTT* expression in *T. thermophila* exposed to Col-Ag NPs increased in time (Fig. 12), it is likely that Ag NPs released Ag ions that were transported to cytosol turning NPs into "Trojan horses" [265].

3.2.3.2 Effects on physiological responses

As Ag NP specific effects were observed in the expression of oxidative stress related genes, levels of intracellular ROS, lipid peroxidation, and activity of ROS scavenging enzymes SOD and CAT were determined to evaluate whether these biomarkers correlated with gene expression levels. In spite of several Ag NP and strain-specific differences observed at gene expression level, the only indicator of oxidative stress at the physiological level was lipid peroxidation in Ag-exposed *T. thermophila* (Fig. 3 in paper **IV**). However, this was likely due to the presence of Ag ions as only $AgNO_3$ (1.5 mg Ag/L) and not Col-Ag NPs (20 mg/L) induced lipid peroxidation in strain CU428. Surprisingly, no excess ROS generation, nor increase in the activity of antioxidant enzymes SOD and CAT was found in *T. thermophila* exposed to Ag compounds (Fig. 3E-H in paper **IV**). Although the latter seemed to be a contradicting result, the up-regulation of *SOD* and *CAT* might be necessary to maintain the balance of these enzymes in the cell to counteract the inhibition of enzymes by Ag. Namely, Ag has a high affinity towards sulfhydryl groups of the cysteine residues [266] that may inhibit enzyme activities. Indeed, Zhang et al. [267] demonstrated that Ag NPs interacted with Cu/Zn-SOD. Here, the activity of CAT was inhibited by Col-Ag NPs (Supplementary of paper **IV**). Moreover, the up-regulation of *HSP* genes in strain CU427 could also be a result of Ag compounds interacting with vital proteins as it has been proposed that in bacteria treated with Ag NPs *hsp* is up-regulated to restore the protein structures [268].

Consequently, Ag ions were likely the main cause of Col-Ag NP toxicity as the EC_{50} values could be explained by the dissolution of the particles and exposure of *T. thermophila* with Ag NPs and Ag ions resulted in dose-response curves with similar slopes. Deeper insight into mechanism of action confirmed the initial results as the expression of metallothionein genes was elevated the most (up to 5000-fold) compared to other selected genes upon exposure to both Col-Ag NPs and $AgNO_3$, and both Ag compounds had similar effects on the selected *T. thermophila* biomarkers. Nevertheless, mechanistic studies provided valuable information that explained the difference in the susceptibility of strains CU427 and CU428 to Ag compounds.

CONCLUSIONS

- Standard aquatic test organisms – crustaceans, algae and fish - were shown to be more susceptible to Ag, CuO and ZnO NPs than bacteria who are often the main target organisms of these NPs used frequently for biocidal purposes. Overall, the toxicity ranges of the three metal NPs, often applied as biocidal agents, overlapped for target and non-target organisms, which indicates that environmental release of these NPs may pose a threat to aquatic biota (Paper **I**).
- The toxicity of industrially relevant ENMs according to most sensitive organism (mostly crustaceans or algae) decreased in the order of Ag (acutely very toxic, potentially chronically very toxic) > ZnO (acutely very toxic, potentially chronically very toxic) > CuO (potentially chronically toxic) > CeO_2 (potentially chronically toxic) > CNTs (potentially chronically toxic) > TiO_2 (potentially chronically harmful) > FeO_x (potentially chronically harmful, not enough data) (Papers **I**, **II**).
- An online-available database NanoE-Tox with detailed information on 8 industrially relevant ENMs and their toxicity (1,518 toxicity values) was compiled using 224 peer-reviewed papers published before 2015. NanoE-Tox enabled to identify several data gaps in the existing nanotoxicity data: (i) NPs are often characterised insufficiently, (ii) tests with standard models like algae and fish are often performed using modified protocols, and (iii) the data on toxicity of industrially relevant ENMs to organisms other than standard models are scarce (Paper **II**).
- *T. thermophila* has a relatively high tolerability of Ag compounds with EC_{50} of noncoated Ag NPs (Sigma-Ag NPs) in the range of 205-286 mg Ag/L, EC_{50} of casein-coated Ag NPs (Col-Ag NPs) in the range of 72-100 mg Ag/L, and EC_{50} of $AgNO_3$ in the range of 1.5-2.8 mg Ag/L (Papers **III**, **IV**).
- The main mechanism of toxic action of Sigma-Ag NPs and Col-Ag NPs in *T. thermophila* was through the release of toxic Ag ions (Papers **III**, **IV**).
- It was demonstrated that Ag ions were reduced into Ag NPs in the soluble extracellular fraction (SEF) of the protozoan *Tetrahymena thermophila*. The biomineralisation process was a result of the interactions between Ag and SEF proteins that likely play a role in detoxification of Ag ions and the process is likely one of the reasons for low toxicity of Ag compounds to the protozoan (Paper **III**).
- The parallel study of two wild type strains of *T. thermophila* indicated that although the protozoan has a low sensitivity towards Ag NPs, strain to strain variability in cellular responses may occur. 20-30% higher susceptibility of *T. thermophila* strain CU428 to Col-Ag NPs was accompanied with Ag NP specific gene expression, differently from the responses in strain CU427 (Paper **IV**).
- Although overexpression of oxidative stress related genes was observed in Ag NP-exposed *T. thermophila*, this was not manifested in the increased levels of intracellular ROS and the activity of antioxidant enzymes SOD and CAT. This was likely due to the effective antioxidant defence mechanisms in the ciliate (Paper **IV**).

LIST OF FIGURES

LIST OF TABLES

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ABSTRACT

In recent decades, nanotechnology has developed rapidly – the estimated global production volumes of engineered nanomaterials (ENMs) are in hundreds of thousands of tonnes, and thousands of nano-enabled products are already available on the market. Thus, it is likely that environmental concentrations of ENMs with novel properties will increase, but the specific mechanisms of ENM toxicity to environmental organisms are not well understood. The diversity of ENMs renders their risk assessment with standard methods challenging and resource intensive. Therefore, great efforts have been made to develop alternative methods, e.g., risk assessment frameworks, quantitative (nano)structure-activity relationships, and read-across methods. These methods rely on good-quality experimental toxicity data. However, while numerous papers have been published in the field of nanoecotoxicology, the results are often contradictory and no comprehensive overview of the toxicity data was available.

The current thesis aimed to synthesise published ecotoxicity data of industrially relevant ENMs and assess toxicity mechanisms of Ag NPs in the protozoan *Tetrahymena thermophila*. First, the effects of CuO, ZnO and Ag nanoparticles (NPs), often used as biocides, to "target" organisms (bacteria, fungi and algae), were compared to their effects to "non-target" organisms (symbiotic bacteria, eukaryotic microbes, aquatic and terrestrial invertebrates, fish and mammalian cells *in vitro*) based on literature data. Meta-analysis showed that the median minimal inhibitory concentration (MIC) values of these NPs for bacteria were one to two orders of magnitude higher than the median $L(E,I)C_{50}$ values for the most susceptible "non-target" organisms – crustaceans, algae and fish. Thus, environmental release of these NPs may pose a threat to aquatic biota. Secondly, the existing knowledge on ecotoxicity of industrially relevant ENMs (Ag, CeO_2, CNTs, CuO, FeO_x, fullerenes, TiO_2, and ZnO) was compiled into a database. Published data about test conditions, ENM physico-chemical parameters and reported toxicity mechanisms were included in the created database NanoE-Tox. The database consolidated 1,518 toxicity values from 224 peer-reviewed papers published before 2015. The analysis of the data indicated that (i) NPs were often characterised insufficiently, (ii) standard protocols were often modified, obstructing the use of obtained results in risk assessment, and (iii) the information on ecotoxicity of these ENMs to many naturally abundant species was limited. Still, synthesis of the data in the two literature-based studies enabled to rank the industrially relevant ENMs in order of decreasing toxicity to the most susceptible organisms as follows: Ag > ZnO > CuO > CeO_2 > CNTs > TiO_2 > FeO_x.

In the experimental part of the thesis, the effects of Ag NPs with different primary sizes and surface properties (Sigma-Ag NPs and Col-Ag NPs) on the viability of ciliate *T. thermophila* were studied. It was found that *T. thermophila* was relatively resistant to Ag compounds (EC_{50} of Ag NPs > 70 mg Ag/L and of $AgNO_3$ 1.5-2.8 mg Ag/L). The toxicity of Ag NPs was likely triggered by NP dissolution, which was supported by both solubility measurements and significant overexpression of metal-binding metallothionein genes upon exposure of *T. thermophila* to Ag compounds. Additionally, it was found that cell-free exudates of *T. thermophila* reduced Ag ions into less toxic Ag NPs which could be one of the reasons for the low toxicity of Ag compounds to the protozoan. Interestingly, in the transcriptomics study, where two wild type

T. thermophila strains were compared, strain to strain variabilities were observed in the susceptibility of the ciliate to Col-Ag NPs both in viability and in the expression profiles of oxidative stress related genes. While overexpression of some oxidative stress related genes occurred in both studied *T. thermophila* strains exposed to sub-lethal concentrations of Ag NPs, the intracellular ROS and the activity of antioxidant enzymes SOD and CAT did not increase. The latter was likely due to the efficient antioxidant defence mechanisms in *T. thermophila*.

In summary, this thesis provides valuable information on ecotoxicity of industrially relevant ENMs and systematically collected, analysed and arranged data which could be used for modelling the hazard of novel ENMs. Mechanistic study of the effects of Ag NPs to ciliate *T. thermophila* advanced nanoecotoxicological knowledge on environmentally relevant and abundant organisms.

KOKKUVÕTE

Sünteetilisi nanomaterjale, mille vähemalt üks mõõde jääb suurusvahemikku 1-100 nm, rakendatakse nanotehnoloogias kergemate, tugevamate, vastupidavamate ja tõhusamate materjalide tootmiseks. Nanomaterjalide unikaalsed optilised, elektrilised ja magnetilised omadused on tingitud nende suurest eripinnast (osakese pinnal olevate aatomite osakaal on seda suurem, mida väiksemate mõõtmetega on osake). Sünteetiliste nanomaterjalide hinnangulised tootmismahud 2016. aastal ulatusid sadadesse tuhandetesse tonnidesse ning enim toodeti SiO_2, TiO_2, Fe, Al_2O_3, ZnO, nanosavide, CeO_2, süsiniknanotorude (CNT), Ag, ja Cu-põhiseid materjale. Taolisi nanostruktuure kasutatakse tuhandete toodete valmistamisel, kusjuures tarbekaupades on enim esindatud Ag, TiO_2 ja SiO_2-põhised nanomaterjalid. Kiiresti arenevas nanomeditsiinis kasutatakse nanomaterjale kontrastainete ja ravimikandjatena ning suund on võetud nanostruktuursete implantaatide ja antimikroobsete katete valmistamisele.

Nanomaterjalid ja neid sisaldavad tooted satuvad jäätmetena või heitvetega suure tõenäosusega keskkonda, kus need võivad kujutada ohtu sealsetele organismidele. Ainete mõju hindamiseks on välja töötatud mitmesuguseid juhiseid, kuid kõikide turul olevate nanomaterjalide ohutuse hindamine oleks väga aja- ja ressursimahukas. Seda peamiselt seetõttu, et lisaks keemilisele koostisele võivad nanomaterjalid erineda kristallstruktuuri, osakese suuruse, kuju, pinnaomaduste ning mitmete teiste parameetrite poolest, mis võivad mõjutada nanomaterjalide toksilisust. Oluline on ka keskkond, kuhu nanomaterjalid jõuavad, sest interaktsioonid keskkonnas leiduvate ainetega võivad muuta nende omadusi, peamiselt osakeste suurust ja pinnaomadusi. Ühe lahendusena nanomaterjalide riski hindamisel nähakse alternatiivsete meetodite väljatöötamist: arendatakse riski hindamise raamistikke, struktuuri-aktiivsuse kvantitatiivse seose mudeleid ning analoogmeetodeid toksilisuse ennustamist keemiliselt ja struktuurselt sarnaste nanomaterjalide toime põhjal.

Ainete, sealhulgas nanomaterjalide, mõju hindamiseks vesikeskkonnale kasutatakse enim Majandusliku Koostöö ja Arengu Organisatsiooni (OECD) ning Rahvusvahelise Standardiorganisatsiooni (ISO) väljatöötatud teste vesikirpude, vetikate ja kaladega. Samas on vesikeskkonnas teisigi laialtlevinud organisme, näiteks ripsloomi, kes toituvad bakteritest ning on omakorda saakloomadeks suurematele organismidele. Lisaks on ripsloomadel tähtis roll reovee puhastamisel. Erinevate ainete mõju hindamiseks reovee puhastusprotsessile juurutas OECD 2017. aasta oktoobris esimese juhise, kus kasutatakse testorganismina algloomi. Teadustöödes on ainete mõju hindamiseks enim kasutatud magevees elavaid *Tetrahymena* perekonda kuuluvaid ripsloomi.

T. thermophila rakk on võrdlemisi suur (30 × 50 µm) ning seda katvad ripsed võimaldavad rakul liikuda. *T. thermophila* toitub lisaks pinotsütoosile ka fagotsütoosi teel, osmootset rõhku rakus reguleerib *T. thermophila* pulseeriva vakuooliga, mis võimaldab tal mõnda aega elus püsida ka destilleeritud vees. *T. thermophila*l on kaks tuuma: diploidne mikrotuum ja polüploidne somaatiline makrotuum. 2000. aastate alguses tehti kindlaks *T. thermophila* makrotuuma genoomi järjestus, millest selgus, et ripslooma genoomis on hinnanguliselt 24 725 valku kodeerivat geeni. *Tetrahymena* on sobiv nanotoksikoloogia mudelorganism, kuna fagotsütoosi teel toituva algloomа toiduvakuoolidesse satuvad ka keskkonnas olevad sünteetilised nanoosakesed, mis

võimaldab uurida rakku sisenenud nanomaterjalide toksilisuse mehhanisme. 2017. aasta augustis leidus teaduskirjanduse andmebaasides ligi 50 artiklit, kus uuriti nanomaterjalide mõju kas *T. thermophila*le või *T. thermophila*ga sarnasele ripsloomale *T. pyriformis*. Nende artiklite põhjal on enim uuritud TiO_2 ja CNT nanostruktuuride mõju *Tetrahymena*le.

Lisaks toksilisuse määrale (näiteks kui suur kogus nanomaterjali mõjub surmavalt teatud hulgale testorganismidele) on oluline mõista ka mehhanisme, mis ühe või teise aine või nanomaterjali organismidele toksiliseks muudavad. On välja selgitatud, et sünteetilised nanomaterjalid võivad sõltuvalt oma keemilistest ja füüsikalistest omadustest avaldada kahjulikku mõju näiteks mehaanilise kokkupuute teel organismidega, kõrge afiinsuse tõttu biomolekulide suhtes, mürgiste metalliioonide vabanemise tõttu metallipõhiste nanomaterjalide lahustumise käigus ja reaktiivsete hapnikuühendite (ROS) tekitamise tõttu. Sageli põhjustab üks nanomaterjal toksilisust mitme mehhanismi koosmõjul, paljude nanomaterjalide toksilisuse mehhanismid on aga lõpuni välja selgitamata.

Käesoleva doktoritöö eesmärgiks oli nanomaterjalide keskkonnaohu hindamise kitsaskohtade vähendamine, mis aitab kaasa nanotehnoloogia jätkusuutlikule arengule. Selleks koguti süstemaatiliselt teaduskirjandusest maailmas olulistes mahtudes toodetavate nanomaterjalide keskkonnatoksilisuse andmeid, analüüsiti ja koostati saadud andmete põhjal andmebaas. Tulemused aitavad anda senisest parema ülevaate nanomaterjalide toksilisuse mehhanismidest ja selgitada välja kitsaskohad, mida edasiste katsete kavandamisel arvesse võtta. Töö eksperimentaalses osas uuriti nanohõbeda mõju ja toksilisuse mehhanisme algloomas *T. thermophila*.

Kirjandusandmeid koguti teaduskirjanduse andmebaasist *Web of Science*, kus otsingute teostamisel kasutati kahe uuringu puhul erinevaid strateegiaid. Esimeses uuringus otsiti andmeid sageli antimikroobse toime tõttu kasutatavate Ag, CuO ja ZnO nanoosakeste mõju kohta erinevatele organismidele. Seetõttu kasutati otsingusõnadena lisaks nanoosakese nimele organismi üldist või ladinakeelset nimetust või rakukultuuri nime. Andmeid koguti ainult ainete poolefektiivsete/-inhibitoorsete/-letaalsete ($E(L,I)C_{50}$) väärtuste või minimaalsete inhibitoorsete kontsentratsioonide (MIC), osakeste suuruse, pinnakatte ning testikeskkonna kohta. Leiti, et erinevate nanomaterjalide kohta avaldatud infohulk varieerub suuresti: enim leidus andmeid nanohõbeda kohta, mis oli kogutud andmete alusel ka mürgiseim nanomaterjal. Täheldati, et kõige tundlikumad organismid nimetatud kolme nanomaterjali suhtes olid vesikirbud, vetikad ja kalad, kusjuures bakterite, kelle vastu uuritud nanomaterjalid sageli suunatud on, MIC oli üldjuhul eelmainitud organismide $E(L,I)C_{50}$ väärtustest 1-2 suurusjärku kõrgem. Tulemusest võib järeldada, et biotsiidsete nanomaterjalide kasutamisel tuleb tähelepanu pöörata nende võimalikele keskkonnamõjudele.

Teise kirjandusuuringu eesmärgiks oli nanomaterjalide keskkonnatoksilisuse teabe hetkeseisu kohta ülevaate koostamine, mistõttu koguti infot maailmas olulistes mahtudes toodetavate nanomaterjalide (Ag, CeO_2, CNT, CuO, FeO_x, fullereenid, TiO_2, and ZnO) kohta ning otsingutes ei kasutatud organismide nimetusi, vaid kasutati üldterminit keskkonnatoksiline. Antud uuringu põhjal koostati nanomaterjalide keskkonnaohtlikkuse andmebaas NanoE-Tox, kuhu koondati detailsed andmed nanomaterjalide füüsikalis-keemiliste omaduste kohta nii enne kui ka pärast testikeskkonda (näiteks söötmesse või puhvrisse) lisamist ning üksikasjalik toksilisuse

katse kirjeldus koos saadud tulemuste ning väljapakutud toksilisuse mehhanismidega. Ühtekokku koguti NanoE-Tox andmebaasi nanomaterjalide mõju kohta 1518 väärtust 224 artiklist. Kui enim artikleid oli avaldatud nano-TiO_2 kohta, siis enim toksilisuse väärtusi leiti nanohõbeda kohta. Andmebaas võimaldas tuvastada järgmised olemasolevas teabes esinevad kitsaskohad: (i) nanomaterjalide füüsikalis-keemiliste parameetrite kajastamine erines analüüsitud artiklites suurel määral, (ii) standardiseeritud juhendeid oli sageli muudetud, mistõttu on raskendatud nende kasutamine keskkonnariski hindamisel ning (iii) ligi pool (47%) leitud toksilisuse väärtustest põhinesid testidel, milles kasutati kuute levinud mudelorganismi: vesikirp *Daphnia magna,* vetikas *Raphidocelis subcapitata,* kala *Danio rerio,* nematood *Caenorhabditis elegans* ning bakterid *Escherichia coli* ja *Vibrio fischeri*. Nanomaterjalide füüsikalis-keemiliste omaduste kohta kogutud andmeid analüüsides leiti, et kui osakeste suurus oli märgitud peaaegu kõikides artiklites (93%) ning hüdrodünaamiline diameeter (nanoosakese suurus vesikeskkonnas) oli mõõdetud rohkem kui pooltes töödes (59%), siis ülejäänud omadusi kirjeldati tunduvalt vähem – valdavas osas artiklitest (85%) oli esitatud 2-6 nanomaterjali iseloomustavat parameetrit ning kõigest 9% töödest sisaldas põhjalikumat füüsikalis-keemiliste omaduste kirjeldust, s.t. 7-8 parameetrit. Tulemusest võib järeldada, et seniavaldatud andmed võimaldavad modelleerida peamiselt nanomaterjalide suurusest tingitud mõjusid. Kuigi antud uuringus ei teostatud organismi- ja liigispetsiifilist otsingut, leiti andmeid nanomaterjalide mõju kohta kokku 116 erinevale liigile. Ligi pooled leitud toksilisuse väärtustest olid saadud testidega, milles kasutati kuute levinud mudelorganismi, mistõttu võib järeldada, et nanomaterjalide mõju on uuritud peamiselt kitsa organismide ringiga. Kuna ligi 40% kogutud toksilisuse väärtustest olid $E(L,I)C_{50}$ väärtused, oli võimalik uuritud nanomaterjalid järjestada nende toksilisuse alusel. Nanomaterjalide toksilisus kahanevas järjekorras oli järgmine: Ag > ZnO > CuO > CeO_2 > CNT > TiO_2 > FeO_x. Kõige tundlikemaks organismideks osutusid sarnaselt esimesele uuringule vesikirbud, vetikad ja kalad. Toksilisuse mehhanismide osas leiti mõnedes artiklites, et vees vähelahustuvate metalliliste nanomaterjalide toksilisus ei ole seletatav vabanenud ioonidega, vaid esinevad ka nano-spetsiifilised mehhanismid. Osaliselt veeslahustuvate nanomaterjalide (Ag, CuO, ZnO) toksilisust põhjendati see-eest peamiselt mürgiste metalliioonide toimega. Vähemal määral mainiti erinevate osakeste toksilisuse mehhanismidena ka oksüdatiivse stressi põhjustamist, rakumembraanide destabiliseerimist, DNA kahjustuste tekitamist ja muud.

Vaatamata sellele, et nanohõbe on tarbekaupades laialt levinud ja kirjanduse andmete põhjal väga mürgine, oli selle mõju ripsloomadele vähe uuritud. Seetõttu valiti eksperimentaalseks uurimuseks kaks erinevat nanohõbedat: <100 nm pinnakatteta Sigma-Ag osakesed ja 14.6 ± 4.7 nm kaseiiniga kaetud Col-Ag osakesed, mida kasutatakse meditsiinis. Nanoosakeste iseloomustamiseks kasutati UV-nähtava valguse spektroskoopiat, dünaamilise valguse hajutamise meetodit, elektroforeetilise valguse hajutamise meetodit, skaneerivat elektronmikroskoopiat, Ag/S selektiivset elektroodi ja aatomabsorptsioon-spektroskoopiat. Osakesi ümbritsevat valgupärga uuriti SDS-polüakrüülamiid geelelektroforeesiga ning tuvastamaks osakeste võimet tekitada ROS-e abiootilistes tingimustes kasutati fluorestsentsipõhiseid meetodeid. Nanohõbeda mõju kolme erineva *T. thermophila* tüve – BIII, CU427 ja CU428 – elulevusele määrati rakkude ATP sisalduse kaudu ja rakke visualiseeriti valgusmikroskoobiga. Nanohõbedast

lahustunud hõbeda-ioonide mõju kontrolliks kasutati $AgNO_3$. Leiti, et *T. thermophila* on võimeline mõlemat tüüpi nanohõbedat fagotsüteerima ning Sigma-Ag osakesed olid algloomale vähem mürgised ($EC_{50,\ BIII}$ vahemikus 205-286 mg Ag/l) kui Col-Ag osakesed ($EC_{50,\ CU427\ ja\ CU428}$ vahemikus 72-100 mg Ag/l). Kuigi Sigma-Ag ja Col-Ag osakeste mõju uuriti erinevate *T. thermophila* tüvedega, tulenes erinevus osakeste mürgisuses suure tõenäosusega vastavate osakeste erinevast lahustuvusest, kuna $AgNO_3$ mõju erinevatele tüvedele oli sarnane (EC_{50} kõikidel tüvedel vahemikus 1,5-2,8 mg Ag/l). Teisalt oli tüvi CU428 Col-Ag osakestele 20-30% tundlikum kui tüvi CU427. Võrreldes *T. thermophila* tundlikkust hõbedale vesikeskkonnas levinud mudelorganismide – vesikirbu, vetika ja kala – tundlikkusega nähtub, et *T. thermophila* talub küllaltki kõrgeid hõbedakontsentratsioone, mis võib olla kohastumus kõrgema saastumusega elukeskkonnale. Kirjanduse andmetel biomineraliseerivad mitmed organismid mürgiseid metalli-ioone vastavateks nanoosakesteks, vähendades seeläbi ioonide mürgist mõju. Käesolevas töös näidati, et hõbeda-ioone on võimalik redutseerida hõbeda nanoosakesteks ka *T. thermophila* rakuvabas fraktsioonis ehk *T. thermophila* rakuväliste valkude kaasabil. Lisaks märgati, et rakuvabas fraktsioonis inkubeeritud hõbeda mürgisus *T. thermophila*le vähenes inkubeerimisaja pikenemisel. Täiendavalt uuriti Ag nanoosakeste toksilisuse mehhanisme sub-letaalsete kontsentratsioonide juures: mõõdeti üldise ja oksüdatiivse stressiga seotud geenide ekspressiooni taset (kvantitatiivse polümeraasi ahelreaktsiooniga) ja oksüdatiivse stressi markereid algloomas (fluorestsentsipõhiste ja ensümaatiliste meetoditega). Huvitaval kombel täheldati üldise ning oksüdatiivse stressiga seotud geenide avaldumises tüvespetsiifilisi mustreid. Kui tüves CU427 mõjutasid Col-Ag osakesed ja $AgNO_3$ geenide avaldumist sarnaselt, siis tüves CU428 nähti muutusi ainult Col-Ag osakestega kokkupuutunud ripsloomade oksüdatiivse stressiga seotud geenides. Metalle siduvad metallotioneiini geenid avaldusid mõlemas tüves hõbedaühenditega kokkupuute järel kümneid kuni tuhandeid kordi kõrgemal tasemel kui hõbedaga mitte kokku puutunud algloomades. See viitab, et nanohõbeda toksilisus algloomas *T. thermophila* on tingitud peamiselt hõbeda-ioonidest. Füsioloogilisel tasemel nanohõbeda-spetsiifilisi efekte ei tuvastatud ja geenide tasemel ülesreguleeritud superoksiidi dismutaasi ning katalaasi aktiivsused hõbedale eksponeeritud rakkudes ei erinenud kontrollrakkude vastavatest tasemetest, viidates *T. thermophila* tõhusatele antioksüdatiivsetele kaitsemehhanismidele.

Kokkuvõttes annab käesolev doktoritöö põhjaliku ülevaate maailmas olulistes mahtudes toodetavate nanomaterjalide keskkonnatoksilisusest. Doktoritöö raames kogutud andmeid saab rakendada uudsete nanomaterjalide võimalike ohtude modelleerimiseks, mis kiirendab nende materjalide riskihindamist. Nanohõbeda tokslisuse mehhanismide uuringutega panustati nanomaterjalide keskkonnatoksikoloogia alastesse teadmistesse, mis aitavad paremini mõista antimikroobsete toodete võimalikke mõjusid zooplanktonile.

PUBLICATION I

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REVIEW ARTICLE

Toxicity of Ag, CuO and ZnO nanoparticles to selected environmentally relevant test organisms and mammalian cells in vitro: a critical review

Olesja Bondarenko · Katre Juganson · Angela Ivask · Kaja Kasemets · Monika Mortimer · Anne Kahru

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Abstract Nanoparticles (NPs) of copper oxide (CuO), zinc oxide (ZnO) and especially nanosilver are intentionally used to fight the undesirable growth of bacteria, fungi and algae. Release of these NPs from consumer and household products into waste streams and further into the environment may, however, pose threat to the 'non-target' organisms, such as natural microbes and aquatic organisms. This review summarizes the recent research on (eco)toxicity of silver (Ag), CuO and ZnO NPs. Organism-wise it focuses on key test species used for the analysis of ecotoxicological hazard. For comparison, the toxic effects of studied NPs toward mammalian cells in vitro were addressed. Altogether 317 L(E)C50 or minimal inhibitory concentrations (MIC) values were obtained for algae, crustaceans, fish, bacteria, yeast, nematodes, protozoa and mammalian cell lines. As a rule, crustaceans, algae and fish proved most sensitive to the studied NPs. The median L(E)C50 values of Ag NPs, CuO NPs and ZnO NPs (mg/L) were 0.01, 2.1 and 2.3 for crustaceans; 0.36, 2.8 and 0.08 for algae; and 1.36, 100 and 3.0 for fish, respectively. Surprisingly, the NPs were less toxic to bacteria than to aquatic organisms: the median MIC values for bacteria were 7.1, 200 and 500 mg/L for Ag, CuO and ZnO NPs, respectively. In comparison, the respective median L(E)C50 values for mammalian cells were 11.3, 25 and 43 mg/L. Thus, the toxic range of all the three metal-containing NPs to target- and non-target organisms overlaps, indicating that the leaching of biocidal NPs from consumer products should be addressed.

Keywords Risk assessment · In vitro toxicology · Antimicrobials · Mechanism of action · REACH · QSARs

Olesja Bondarenko and Katre Juganson have contributed equally to this work.

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O. Bondarenko · K. Juganson · A. Ivask · K. Kasemets · M. Mortimer · A. Kahru (✉)
Laboratory of Environmental Toxicology, National Institute of Chemical Physics and Biophysics, Akadeemia tee 23, 12618 Tallinn, Estonia
e-mail: anne.kahru@kbfi.ee

K. Juganson
Department of Chemistry, Tallinn University of Technology, Ehitajate tee 5, 19086 Tallinn, Estonia

M. Mortimer
Aquatic Biogeochemistry and Ecotoxicology, Institute F.-A. Forel, Faculty of Sciences, University of Geneva, 10 route de Suisse, 1290 Versoix, Switzerland

Introduction

Nanoindustry is one of the fastest growing industries in the history of mankind and has been referred to as the next industrial revolution (Lux Research 2008). The first national nanotechnology program—the National Nanotechnology Initiative—was launched in USA in 2000. Since then, more than 60 nations have established similar programs. In 2010, worldwide annual public and private sector funding for nanotechnologies was 17.8 billion dollars in total (Sargent 2012). As a result, the global socio-economic value of nanotechnologies is steadily increasing, and currently, nanoscale particles have significant impacts on almost all industries and all areas of society.

According to the recent review issued by the European Commission (2013), nanomaterial is defined as 'a natural, incidental or manufactured material containing particles, in

an unbound state or as an aggregate or as an agglomerate and where, for 50 % or more of the particles in the number size distribution, one or more external dimensions is in the size range 1–100 nm. In specific cases and where warranted by concerns for the environment, health, safety or competitiveness the number size distribution threshold of 50 % may be replaced by a threshold between 1 and 50 %.' In scientific literature engineered (or manufactured or synthetic or man-made) nanoparticles (NPs) are usually defined as particles with at least one dimension between 1 and 100 nm.

At nanoscale materials have different or enhanced properties compared with their conventional 'bulk' (microsize) counterparts, due to an increased relative surface area that translates into higher reactivity (Nel et al. 2006). While in bulk materials the surface atoms constitute only a few percent of the total number of atoms, in NPs most of the atoms lay close to or at the surface (Casals et al. 2012). There is increasing evidence that the unique desired physico-chemical properties of NPs, which make nanomaterials more efficient in industrial applications, render these materials also more harmful to living organisms. Due to increasing production volumes of NPs and growing likelihood of occupational and environmental exposure to nanomaterials, the legislative bodies in both EU and USA have currently focused their activities on assessing health and environmental risks of nanotechnology.

As shown in Fig. 1, this review aims to provide a critical summary of recent scientific literature on potential hazardous effects of three types of engineered metal-containing NPs—zinc oxide (ZnO), copper oxide (CuO) and silver (Ag). All these compounds (either in the bulk or nanoform) have been historically used as biocides, that is, for avoiding or stopping the growth of microorganisms and algae (Kahru and Dubourguier 2010). Therefore, similarly to pesticides, these nanomaterials should be monitored for their toxic action also toward non-target species, including humans. In the context of the current review, 'target organism' is defined as an organism for which the biocidal NPs were designed for (e.g., bacteria and fungi as target organisms of all three NPs and algae as target organisms of CuO and Ag NPs) and 'non-target organism' is an organism which will be exposed to NPs after their incidental release into the environment. To gain a better understanding whether the accidental release of metal-containing NPs may pose a threat to non-target species, we collected toxicity data on these NPs for algae, crustaceans, fish, bacteria, yeast, nematodes, protozoa and mammalian cell lines and compared the toxicity values of NPs to target- and non-target organisms. In addition, we analyzed the collected data with respect to the correlation between the dissolution, size and coating of NPs and their toxicity to different organism groups. Finally, we classified the studied NPs into different hazard categories. However, the proposed hazard categories are rather general and could only be applied for the initial hazard identification. For complete risk assessment, further data on realistic environmental exposure scenarios for these NPs are required. Also, in case of mammalian cell lines, we do not discuss the transferability of collected in vitro data to in vivo situation.

Production and application of Ag, CuO and ZnO (nano)particles

Estimated global production of NPs is shown in Fig. 2a (adapted from Piccinno et al. 2012). Although SiO_2 NPs are produced at the highest production volume (Fig. 2), Ag NPs are the ones most used in consumer products. According to the Woodrow Wilson Database (Wilson 2012), there were more than 1,300 nanotechnological consumer products on the market in March 2011, and 313 of them contained nanosilver. In consumer products, NPs are either added to the bulk material to reinforce the physical properties of the material or applied on the surface of the product to provide enhanced surface features such as scratch resistance, water repellency, reflectivity and photo activity. As the number of published articles can be considered as an early indicator of the future use of NPs, ISI Web of Science (ISI WoS) was used to gather data on the current and potential applications of Ag, ZnO and CuO NPs (Table S1 and in Fig. 2). The analysis of the collected data showed that the majority of articles concerned the applications of Ag NPs (7,699 papers, 59 %), followed by ZnO (4,640 papers, 36 %) and finally CuO NPs (690 papers, 5 %). Interestingly, the most prominent application area of all these three NPs was sensors, sensing devices and catalysis (Fig. 2b–d). Moreover, as silver is the best conductor among the metals (Ren et al. 2005) and Ag NPs have favorable chemical and physical properties such as biocompatibility, unique electronic and catalytic properties, Ag NP-based electrochemical (bio)sensing systems have been developed (Lian et al. 2013) that enable enhancing electron transfer between biomolecules (e.g., proteins) and electrode surfaces. As expected, a considerable share (19 %) of all the fields of application of Ag NPs concerned antimicrobial usage. In case of CuO NPs and ZnO NPs, this share was much lower, 4 and 2.6 %, respectively.

Ag nanoparticles

Silver has been used to fight infections as far back as the days of ancient Greece and Egypt. In World War I, before the advent of antibiotics, silver compounds were used to prevent and treat infections. Currently, Ag NPs are the most widely commercialized NPs that are used as antimicrobials in various consumer products ranging from cosmetics,

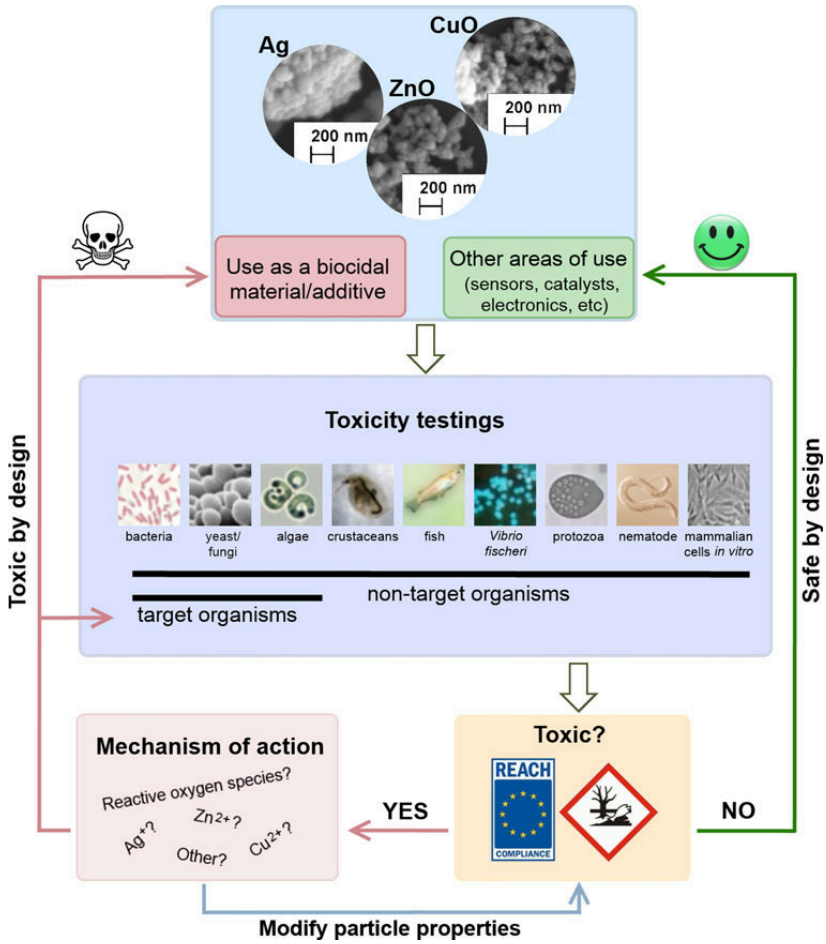

Fig. 1 Schematic representation of the scope of the current review

clothing, shoes, detergents, dietary supplements to surface coatings in respirators, water filters, phones, laptops, toys and commercial home water purification systems such as Aquapure, Kinetico and QSI-Nano (Bystrzejewska-Piotrowska et al. 2009; Marambio-Jones and Hoek 2010; Cerkez et al. 2012). In addition to antibacterial, antiviral and antifungal properties (for the review and references therein, see Ivask et al. 2012), nanosilver has also been shown to facilitate wound healing (Nair and Laurencin 2007). Estimated global annual production of Ag NPs is ~55 tons (a median value; Piccinno et al. 2012; Fig. 2a).

ZnO nanoparticles

According to different sources, the worldwide annual production of ZnO NPs is estimated to be between 550 (Piccinno et al. 2012; Fig. 2d) and 33,400 tons (Research and Markets 2012). Thus, among metal-containing NPs, ZnO NPs have the third highest global production volume after SiO_2 and TiO_2 NPs (5,500 and 3,000 tons annually, respectively) (Piccinno et al. 2012; Fig. 2a). ZnO NPs are mostly used as a UV light scattering additive in cosmetics such as sunscreens, toothpastes and beauty products (Serpone et al. 2007). ZnO NPs are widely used in rubber manufacture, production of solar cells and LCDs, pigments (as a whitener), chemical fibers, electronics and textiles (Dastjerdi and Montazer 2010; Song et al. 2010). In addition, ZnO is an essential ingredient in almost all types of antifouling paints (IPPIC 2012), and recently bulk ZnO has been increasingly replaced by ZnO NPs because of their enhanced antibacterial properties (Padmavathy and Vijayaraghavan 2008).

CuO nanoparticles

In contrast to Ag and ZnO NPs, we were not able to retrieve data on the current production volumes of CuO

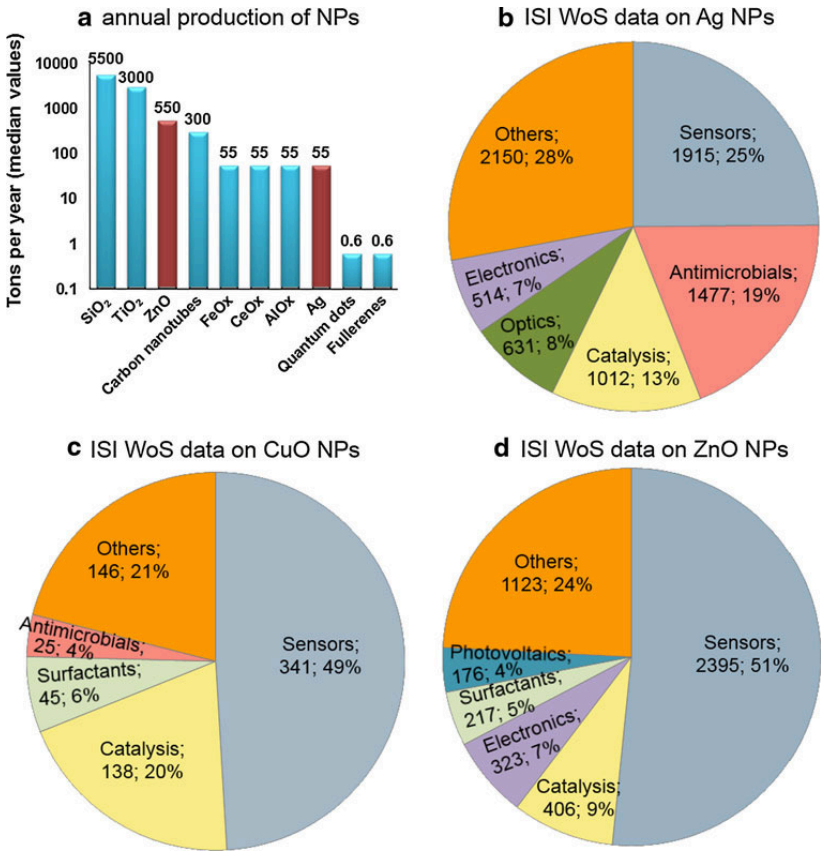

Fig. 2 **a** Annual production volumes of nanomaterials (data are adapted from Piccinno et al. 2012). **b–d** Fields of application of Ag (**b**), CuO (**c**) and ZnO (**d**) nanoparticles based on the publications indexed by Thomson Reuters ISI Web of Science. Search was done in March 2013. The following search terms were used: 'silver' OR 'CuO' OR 'ZnO' AND 'nano*' AND 'application category' (indicated in the figure). Numbers next to each application category indicate the number of articles retrieved and their respective percent share. The numerical data are presented in Supplementary Table S1

NPs. As these NPs are used in lower quantities and compared to other NPs the potential hazardous effects of CuO NPs are poorly studied (Kahru and Savolainen 2010), it is reasonable to conclude that they are also manufactured in lower amounts compared to other NPs. As reflected by Fig. 2c, the most important and unique application area of CuO NPs is electronics and technology (semiconductors, electronic chips, heat transfer nanofluids), as CuO has excellent thermophysical properties (Ebrahimnia-Bajestan et al. 2011). Also other applications such as gas sensors (Li et al. 2007), catalytic processes (Carnes and Klabunde 2003), solar cells and lithium batteries (Guo et al. 2009; Sau et al. 2010) have been suggested for CuO NPs. CuO NPs have been shown to inhibit the growth of microorganisms and exert antiviral properties (Borkow and Gabbay 2004; Gabbay et al. 2006). For these reasons, CuO NPs have been used in face masks, wound dressings and socks to give them biocidal properties (Borkow et al. 2009, 2010a, b).

The need for toxicity data on ZnO, CuO and Ag (nano)particles

Toxicity data and data quality gaps for nanoparticles

The scientific information on potential harmful effects of NPs severely lags behind the development of nanotechnologies (Shvedova et al. 2010; Kahru and Ivask 2013). In addition, the available nanotoxicity data are inconsistent because experimental approaches vary from article to article making it impossible to compare results (Schrurs and Lison 2012). To overcome these problems, nanotoxicology community has recently started a discussion about the implementation of general guidelines for nanotoxicology research and establishment of common parameters that should be addressed in all nanotoxicological articles (Nature Nanotech Editorial 2012).

Legislation gaps for nanoparticles

Currently, the production and use of nanoparticle-containing products is not internationally regulated by any distinct safety regulation (EC 2008). Compared to bulk materials, NPs have unique physico-chemical properties such as higher stability in the aquatic environment (Fig. 3b), decreased size (Fig. 3c) and increased specific surface area (SSA), and thus enhanced reactivity. These properties make NPs more efficient and interesting for different industrial applications but at the same time make them more harmful to living organisms. Thus, theoretically a special guidance should be considered for NPs. Yet, as NPs are chemically identical to their bulk counterparts and thus have the same CAS number (Fig. 3a), they are not recognized by industry as a new class of chemicals. As a result, the production and use of metal-containing NPs are subject to analogous regulation as the conventional bulk chemical compounds regulated in Europe by EU chemical safety policy REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals). The REACH regulation states that when chemicals/NPs are produced in a volume of more than one ton per year and sold at the European market, they must be characterized for their potential impact on aquatic ecosystems (European Parliament 2006). The data provided by the producer/importer should include short-term (48 h) toxicity testing on crustaceans (preferred species *Daphnia magna*, OECD 2004) and 72h growth inhibition of aquatic plants (preferably algae, OECD 2011). In addition, short-term (96 h) toxicity testing on fish (OECD 1992) is required at the next annual tonnage level (>10 tons per year). As shown in Crane et al. (2008), Kahru et al. (2008) and Kahru and Dubourguier (2010), the types of test species and biological endpoints used within standard environmental hazard assessment frameworks are generally appropriate also for nanoecotoxicological purposes. The additional specific requirements for NP studies are the dispersion conditions and characterization of the particles in the test environment as well as careful consideration of test conditions for potential artifacts that can arise due to the color of NPs or their sorptive properties (Handy et al. 2012; Schrurs and Lison 2012; Bayat et al. 2013). Analogously to the rest of the chemical compounds, NPs are classified with respect to their environmental toxicity according to the response of the most sensitive of the three test organisms: algae, crustaceans and fish (European Union 2011).

Specific physico-chemical properties of metal-containing nanoparticles

In order to understand the mechanisms behind the toxicity of NPs, the physico-chemical properties of the particles should be thoroughly analyzed in relevant test environments. Recent review by Bandyopadhyay et al. (2012) gives an in-depth overview of the methods that can be applied to characterize NPs size, shape, crystal structure, aggregation, chemical composition, surface properties (surface charge, area, chemistry), solubility and porosity. Since detailed reviews about characterization of the NPs can be found elsewhere, the following paragraphs of this review focus on joint nominators and differences in the physico-chemical characteristics of Ag, CuO and ZnO NPs.

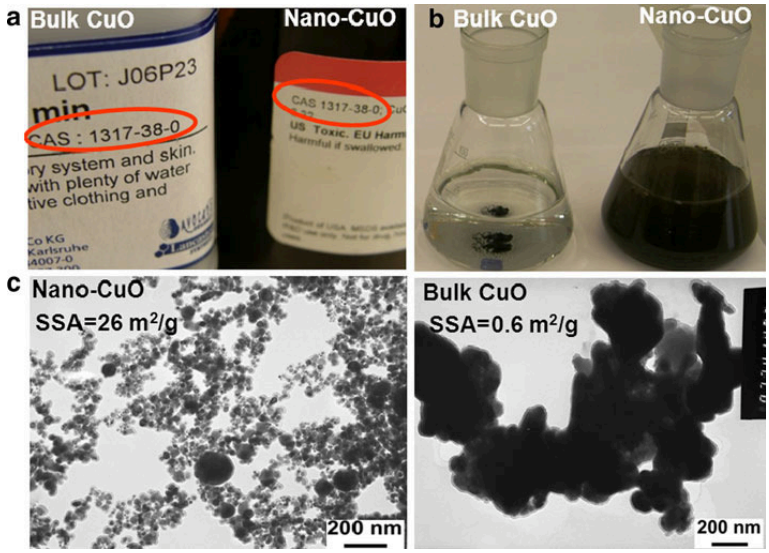

Fig. 3 **a** Labels of bulk CuO and nanosized CuO. Note the same CAS number. **b** 200 mg/L stock suspensions of CuO. **c** TEM image of nano CuO and bulk CuO. Note 43-fold difference in the SSAs of bulk CuO and nanosized CuO

Joint nominators for Ag, CuO and ZnO nanoparticles

Considering the joint nominators for Ag, CuO and ZnO NPs, the first to notice is the metallic elemental composition of all the three selected particles. Secondly, all the three NPs are applied to fight the undesirable growth of microorganisms. Although among the three nanomaterials, silver NPs are used most widely as antimicrobials, also CuO and ZnO NPs have been successfully used as biocides (Fig. 2c–d). The third joint nominator for the three NPs is their negative surface charge, which results from oxygen atoms in CuO and ZnO (Xu et al. 2012). Though Ag NPs do not initially contain oxygen, the surface of metallic Ag NPs is oxidized under most environmental conditions (aerobic) and negatively charged hydroxo and oxo groups cause the negative surface charge of the particle (Levard et al. 2012). The fourth and toxicologically perhaps the most important joint property is that all the three NPs are soluble to some extent in aqueous media. We have previously shown that the solubility of CuO and ZnO NPs is the key issue in the toxicity of metal-containing (nano)particles and stressed that the solubility data reported as N/A (not available or not applicable) in Material Safety Data Sheet (MSDS) of ZnO and CuO NPs should be addressed (Aruoja et al. 2009; Ivask et al. 2010; Bondarenko et al. 2012). It has been also emphasized that aqueous solubility of NPs has to be incorporated into the environmental risk assessment models of NPs in addition to other key physico-chemical characteristics relevant to NPs (European Commission 2007). Solubility of NPs and the behavior of released metal ions, that is, the proportion of intact particles, metal ions and metal complexes, depend greatly on the properties of the test environment (for a review and references therein, see Casals et al. 2012). The most important parameters of the test environment are pH, dissolved organic carbon content and water hardness (Wiench et al. 2009; Fabrega et al. 2011). For instance, the solubility of all the three selected particles is enhanced at more acidic pH (Dimkpa, et al. 2011; Fabrega et al. 2011; Levard et al. 2012; Ma et al. 2013). Also, the solubility of the aforementioned NPs depends on their interactions with organic material in the test environment (proteins, amino acids, natural organic matter, humic substances) that may coat and disperse NPs or complex metal ions. For example, reduced solubility and toxicity toward crustaceans has been observed in natural waters for Ag NPs (Gao et al. 2009) and CuO NPs (Blinova et al. 2010).

Figure 4 illustrates the behavior of NPs in various test environments: in all test media *coated* Ag NPs are remarkably more stable than the *uncoated* NPs. That is coherent with the results by Fabrega et al. (2011) showing that in high ionic strength suspensions uncoated Ag NPs tend to precipitate and sediment within a few hours after the start of the toxicity assay. Also, CuO and ZnO NPs were remarkably unstable and tended to sediment. Figure 4 also shows that the agglomeration/sedimentation of CuO and ZnO was especially high in mineral media—media that are used for key regulatory ecotoxicological assays (crustaceans, algae) described above. In contrast, the components of the complex test media (defined here as the test environment with organic components) dispersed NPs and prevented their sedimentation. In addition, the complex media may promote dissolution of NPs (Käkinen et al. 2011; Kasemets et al. 2013).

In summary, as also underlined in the recent paper by Casals et al. (2012), it is extremely important to assess the physico-chemical properties of NPs in the media where the biological toxicity tests are performed. As dissolution is one of the main contributors to the toxicity of Ag, CuO and ZnO NPs, in this review their toxicity is discussed in parallel with the toxic effects of the respective ions.

Differences between Ag, CuO and ZnO nanoparticles

In addition to the above-described *joint nominators*, there are also *differences* between the three NPs selected for this study. To begin with, their chemical composition is different; thus, in similar particle size their toxicity is likely different (Sharifi et al. 2012). In addition, copper is a redox element having common valences of $+2$ or $+1$. Thus, differently from zinc and silver, redox-active Cu ions may also be involved in electron-transfer processes. Third, the surface of Ag NPs but not CuO and ZnO NPs is frequently functionalized with different coatings, polyvinylpyrrolidone (PVP) and citrate being the most widely used. Last but not least, copper and zinc (but not silver) are necessary trace elements for almost all types of living cells, while silver has no known function in the living organisms (Sandstead 1995).

Toxicity of Ag, CuO and ZnO nanoparticles to target and non-target organisms

The review by Crane et al. (2008) summarizes various OECD assays that can be applied for the toxicity testing of NPs. Assessment of the environmental hazard of NPs under REACH regulation requires that at least two OECD tests with algae (OECD201) and crustacean *D. magna* (OECD202) should be used. In this review, we collected, analyzed and summarized the toxicity data (including but not limited to the key OECD test species) from the published literature on ZnO, CuO and Ag NPs.

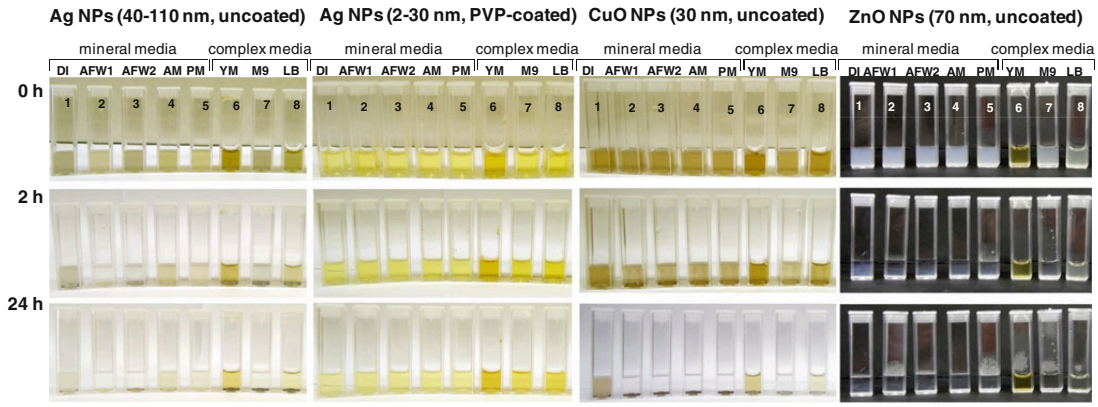

Fig. 4 Uncoated Ag (50 mg/L), PVP-coated Ag (50 mg/L), uncoated CuO (50 mg/L) and ZnO NPs (200 mg/L) after 0, 2 and 24 h incubation in different (eco)toxicological test environments: *1* deionized water; *2* artificial freshwater for the tests with *Daphnia* sp. (OECD 202); *3* AFW for *Thamnocephalus* sp. (Thamnotoxkit F^{TM} 1995); *4* algal growth medium (OECD 201); *5* protozoan mineral test medium (Osterhout's); *6* yeast extract peptone dextrose medium; *7* bacterial M9 medium supplemented with 0.1 % glucose and 0.5 % amino acids; *8* bacterial LB medium containing tryptone and yeast extract. Detailed composition of test media is given in Käkinen et al. (2011)

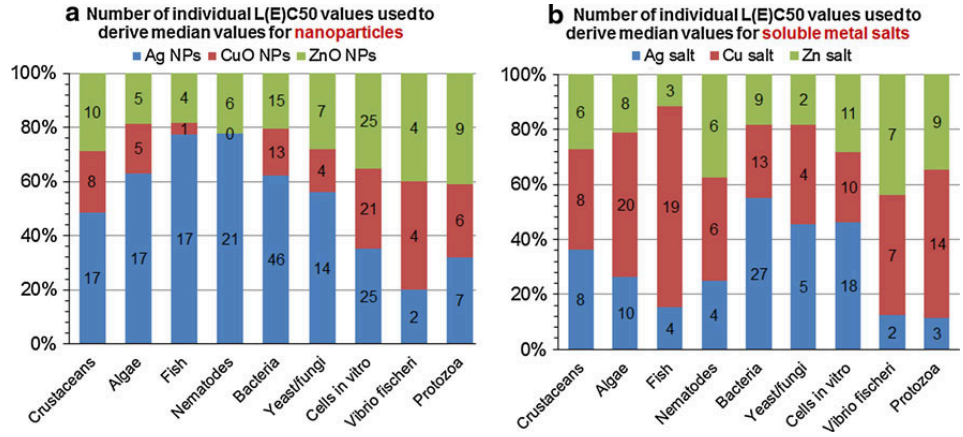

Fig. 5 Number and share of individual L(E)C50 or MIC values used to derive the median L(E)C50 or MIC for nanoparticles (**a**) and metal salts (**b**). Total number of individual values: 317

Characterization of retrieved toxicity data set

When collecting the toxicity data for Ag, CuO and ZnO NPs, we relied on recent nano(eco)toxicological peer-reviewed literature that preferably contained data not only on toxicity of NPs but also physico-chemical characteristics of the studied NPs prior to and during toxicity testing. Our goal was to find at least 10 quantitative toxicity values (EC50, LC50, MIC) per organism and NP type. In parallel, we collected toxicity data for metal ions to assess the impact of dissolution on toxicity of NPs. Organism-wise we focused on bacteria, crustaceans, algae, fish, nematodes, yeasts, protozoa as well as on mammalian cell lines.

Figure 5 shows the availability of the toxicity data in ISI WoS. As can be seen, relatively large amount of data was available on toxicity of Ag NPs, whereas less information was published on toxicity of ZnO NPs and the data on CuO were especially scarce. At the same time, there was a lot of data on the toxicity of both Cu and Ag ions, while less information was available on the toxicity of Zn ions.

Table S2 presents data on the test organisms that were used most often for determining the L(E)C50 and MIC values in the analyzed literature. As shown in Table S2, the

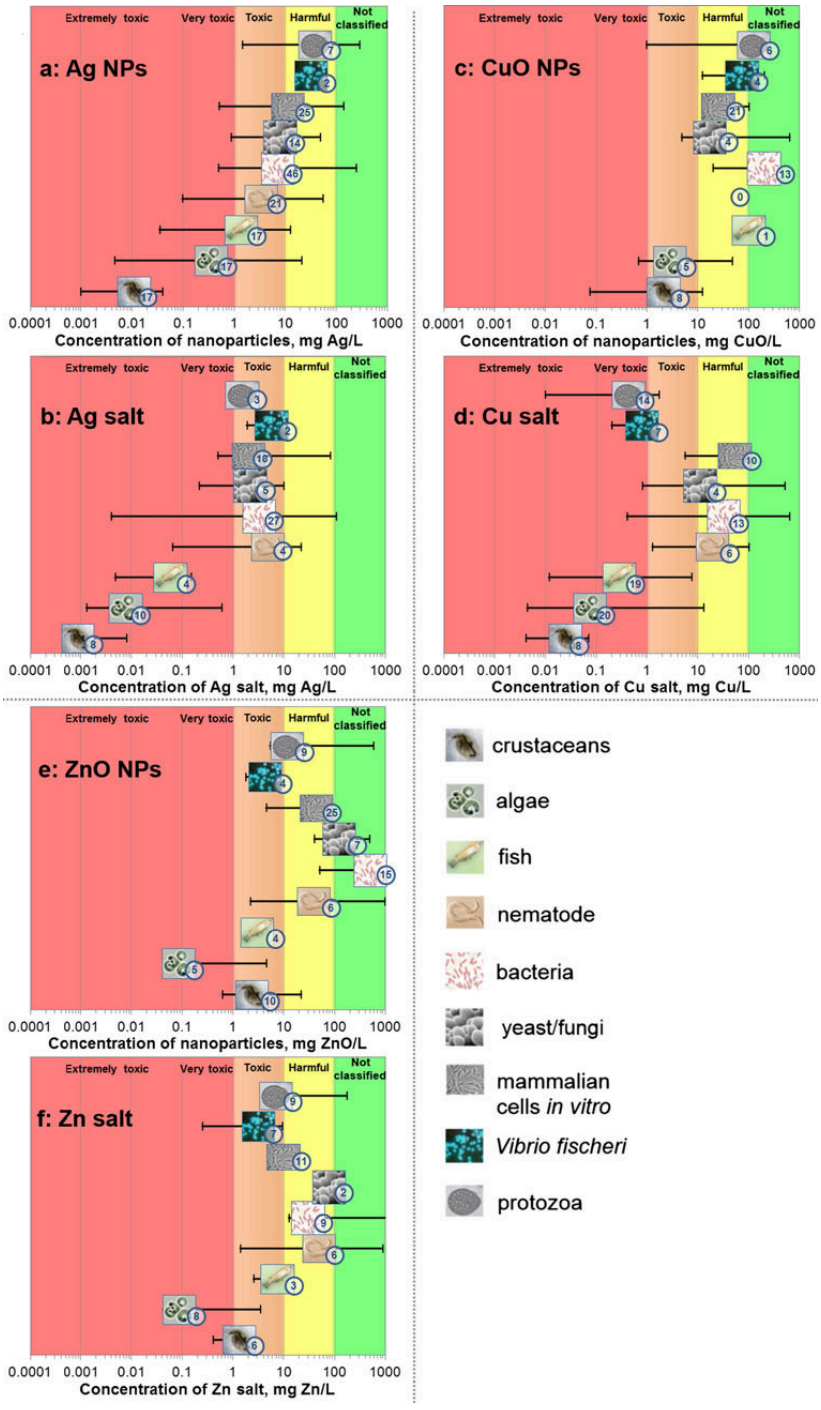

Fig. 6 Toxicity of CuO, ZnO and Ag nanoparticles to different organisms. Median L(E)C50 values for all other organisms except bacteria and MIC for bacteria ± minimum and maximum values are presented. Different organisms/cells are shown by respective pictograms and the number on the pictogram indicates the number of L(E)C50 values used to derive the median value. Note the logarithmic scale of *x*-axis and that L(E)C50 and MIC values of NPs reflect nominal concentrations. The classification to hazard categories is explained in Table 1

Table 1 Median L(E)C50 values for all organisms except bacteria and median MIC for bacteria for Ag, CuO and ZnO nanoparticles (NPs) and the respective metal salts

Group of organisms	Median L(E)C50 or MIC, on compound basis, mg/L (number of data)*			Median L(E)C50 or MIC, on metal basis, mg metal/L (number of data)*		
	Ag NPs	CuO NPs	ZnO NPs	Ag salt	Cu salt	Zn salt
Crustaceans	0.01 (17)	2.1 (8)	2.3 (10)	0.00085 (8)	0.024 (8)	1.3 (6)
Algae	0.36 (17)	2.8 (5)	0.08 (5)	0.0076 (10)	0.07 (20)	0.09 (8)
Fish	1.36 (17)	100 (1)	3.0 (4)	0.058 (4)	0.28 (19)	7.5 (3)
Nematodes	3.34 (21)	Not found (0)	39 (6)	4.8 (4)	19.4 (6)	49 (6)
Bacteria	7.10 (46)	200 (13)	500 (15)	3.3 (27)	32 (13)	30 (9)
Yeast	7.90 (14)	17 (4)	121 (7)	2.16 (5)	11.1 (4)	78 (2)
Mammalian cells in vitro	11.3 (25)	25 (21)	43 (25)	2 (18)	53 (10)	9.8 (11)
V. fischeri[a]	32 (2)	73.6 (4)	4.3 (4)	5.7 (2)	0.78 (7)	3.2 (7)
Protozoa	38 (7)	124 (6)	11.7 (9)	1.5 (3)	0.43 (14)	7 (9)
Lowest L(E)C50, MIC	0.01	2.1	0.08	0.00085	0.024	0.09
Most sensitive organisms	Crustaceans	Crustaceans	Algae	Crustaceans	Crustaceans	Algae
Classification (EU-Directive 93/67/EEC (CEC 1996)[b]	Very toxic	Toxic	Very toxic	Very toxic	Very toxic	Very toxic
Classification (Sanderson et al. 2003; Blaise et al. 2008)[c]	Extremely toxic	Toxic	Extremely toxic	Extremely toxic	Extremely toxic	Extremely toxic

* In the brackets next to the median value, the number of data used to derive the median value is presented

Data are summarized from Supplementary Tables S3–S8 and are arranged throughout according to the decreasing sensitivity (increasing median L(E)C50 values) of test organisms to silver nanoparticles. The L(E)C50 and MIC numbers are from the following articles: Borovanský and Riley (1989), Ershov et al. (1997), McCloskey et al. (1996), Lin et al. (1996), Zhao et al. (1998), Mobley et al. (1999), Mastin and Rodgers (2000), Grass and Rensing (2001), Franklin et al. (2002), Graff et al. (2003), Harmon et al. (2003), Teitzel and Parsek (2003), Yilmaz (2003), De Boeck et al. (2004), Hsieh et al. (2004), Jonker et al. (2004), de Oliveira-Filho et al. (2004), Shakibaie and Harati (2004), Apte et al. (2005), Cho et al. (2005), Heijerick et al. (2005), Lee et al. (2005), Chen et al. (2006), Hiriart-Baer et al. (2006), Jeng and Swanson (2006), Kungolos et al. (2006), Madoni and Romeo (2006), Panáček et al. (2006), Dechsakulthorn et al. (2007), Franklin et al. (2007), Gallego et al. (2007), Zhang et al. (2007), Calafato et al. (2008), Griffitt et al. (2008), Heinlaan et al. (2008), Hernández-Sierra et al. (2008), Jin et al. (2008), Karlsson et al. (2008), Kim et al. (2008), Martínez-Castanón et al. (2008), Mortimer et al. (2008), Navarro et al. (2008), Padmavathy and Vijayaraghavan (2008), Ruparelia et al. (2008), Zhu et al. (2008), Aruoja et al. (2009), Chae et al. (2009), Foldbjerg et al. (2009), Jain et al. (2009), Kasemets et al. (2009), Kim et al. 2009a, b, Kvitek et al. (2009), Lewis and Keller (2009), Lin et al. (2009), Liu et al. (2009), Ma et al. (2009), Oliva et al. (2009), Park and Heo (2009), Pavlica et al. (2009), Sovova et al. (2009), Teodorovic et al. (2009), Wang et al. (2009), Zhu et al. (2009), Ahamed et al. (2010), Baker et al. (2010), Blinova et al. (2010), Chen et al. (2010), Contreras et al. (2010), Ebrahimpour et al. (2010), Kennedy et al. (2010), Kim et al. (2010), Laban et al. (2010), Liu et al. (2010), Meyer et al. (2010), Miao et al. (2010), Mortimer et al. (2010), Nowrouzi et al. (2010), Panjehpour et al. (2010), Song et al. (2010), Suresh et al. (2010), Wang and Guan (2010), Wong et al. (2010), Alsop and Wood (2011), Bao et al. (2011), Dua et al. (2011), Emami-Karvani and Chehrazi (2011), Foldbjerg et al. (2011), He et al. (2011), Kim et al. (2011), Kurvet et al. (2011), Lipovsky et al. (2011), Ma et al. (2011), Majzlik et al. (2011), McLaughlin and Bonzongo (2011), Mortimer et al. (2011), Murphy et al. (2011), Naddafi et al. (2011), Niazi et al. (2011), Poynton et al. (2011), Xie et al. (2011), Xiong et al. (2011), Yu et al. (2011), Zhao et al. (2011), Albers et al. (2012), Ansari et al. (2012), Binaeian et al. (2012), Blinova et al. (2012), Brandt et al. (2012), Böhmert et al. (2012), Cao et al. (2012), Ellegaard-Jensen et al. (2012), Govindasamy and Rahuman (2012), Greulich et al. (2012), Haase et al. (2012), Harrington et al. (2012), Hassan et al. (2012), He et al. (2012), Hoheisel et al. (2012), Jo et al. (2012), Kashiwada et al. (2012), Kennedy et al. (2012), Kim et al. (2012), Kwok et al. (2012), Li et al. (2012a, b) Lim et al. (2012), Little et al. (2012), Manusadžianas et al. (2012), Monteiro et al. (2012), Oukarroum et al. (2012), Patra et al. (2012), Perreault et al. (2012), Piret et al. 2012a, b, Poynton et al. (2012), Rallo et al. (2012), Seiffert et al. (2012), Shaw et al. (2012), Shi et al. (2012), Unger and Lück (2012), Vargas-Reus et al. (2012), Wang et al. (2012a,b), Wu et al. (2012), Yang et al. (2012), Zhang et al. (2012a, b), Zhao et al. (2012), Zhao and Wang (2012), Debabrata and Giasuddin (2013), Juganson et al. (2013), Kasemets et al. (2013), Wu and Zhou (2013)

[a] *V. fischeri* data were retrieved separately from other bacteria, because *V. fischeri* (also an ISO (2010) test organism) was considered as non-target aquatic species

[b] Classification of NPs and their soluble salts to hazard categories adheres to EU-Directive 93/67/EEC (CEC 1996) and is based on the lowest median L(E)C50 value of the three key environmental organisms: algae, crustaceans and fish. <1 mg/L = very toxic to aquatic organisms; 1–10 mg/L = toxic to aquatic organisms; 10–100 mg/L = harmful to aquatic organisms; >100 mg/L = not classified

[c] Analogous to classification of CEC (1996) except that one category is added: <0.1 mg/L = extremely toxic to aquatic organisms

main representative species among crustaceans was *D. magna*, among algae *Pseudokirchneriella subcapitata,* among nematodes *Caenorhabditis elegans*, among bacteria *Escherichia coli* and among yeasts *Saccharomyces cerevisiae*. In all other groups, the dominant organism/cell type varied depending on NP type.

Altogether 317 L(E)C50 or minimal inhibitory concentrations (MIC) values for studied NPs were retrieved. Most of the data on crustaceans, algae and fish were obtained using standardized test methods. However, the protocols of bacterial, yeast, nematode and mammalian cell assays varied considerably. Most of the retrieved data represented EC/LC_{50} values except for bacteria where MIC values were collected as more relevant for indicating the antimicrobial properties of NPs.

Analysis of retrieved toxicity data set

Figure 6 depicts the median L(E)C50 or MIC values and the respective variation scale for the selected NPs and the respective soluble metal salts toward different groups of organisms/cells. Table 1 provides numerical median L(E)C50 values and the number of individual values used to derive the median value. The individual L(E)C50 values are shown in Supplementary Tables S3–S8.

Classification of NPs and soluble metal salts to different hazard categories was performed according to EU-Directive 93/67/EEC. This classification scheme is based on the lowest median L(E)C50 value of the three key environmental organisms: algae, crustaceans and fish (CEC 1996). The lowest median L(E)C50 value <1 mg/L classifies chemical as very toxic to aquatic organisms; 1–10 mg/L = toxic to aquatic organisms; 10–100 mg/L = harmful to aquatic organisms; >100 mg/L = not classified (CEC 1996). An additional category 'extremely toxic' applied by Sanderson et al. (2003) and Blaise et al. (2008) was also employed in the current review. Note that according to EU-Directive 93/67/EEC, the lowest EC50 value obtained either in tests with crustaceans, algae or fish will determine the final hazard class of the chemical compound (Table 1).

Ag NPs exhibited the highest toxicity to the crustaceans with median L(E)C50 value of 0.01 mg/L, that is, according to the most sensitive organism of the test battery crustaceans–algae–fish, Ag NPs should be classified as 'very toxic' to aquatic organisms (CEC 1996). The toxicity of Ag NPs to algae was slightly lower (median L(E)C50 = 0.36 mg/L), followed by fish, nematodes, bacteria, yeast, various mammalian cells, *Vibrio fischeri* and protozoa (Fig. 6a; Table 1). Thus, Ag NPs that are mostly used in antimicrobials and in algaecides (Nowack et al. 2011) were the most toxic toward non-target aqueous organisms—the crustaceans that are crucial components of the aquatic food web. Toxicity data of Ag NPs on bacteria, aquatic organisms and eukaryotic cells in vitro was also recently summarized by Chernousova and Epple (2013). Similarly to our findings (Table 1), these authors showed that the MIC values of Ag NPs to bacteria were in the range of 0.1–20 mg/L and to eukaryotic cells in vitro in the range of 10–100 mg/L.

It is noteworthy that the sensitivity pattern of different organisms to studied metal-containing NPs largely followed the pattern of their sensitivity to the respective metal ions. For instance, similarly to the tendency noted with Ag NPs, crustaceans, algae and fish proved the most sensitive organisms also to Ag ions (Fig. 6b; Table 1). As a rule, the difference between the L(E)C50 values of Ag NPs and Ag ions was 10–15 times (Fig. 7a), with the exception of nematode *C. elegans* for which the toxicity of Ag NPs and Ag ions, was nearly the same. However, most of the toxicity data on Ag NPs to *C. elegans* originate from the study of Yang et al. (2012), who utilized a set of toxic Ag NPs that were prepared in-house. Thus, it is difficult to conclude whether increased toxicity of Ag NPs compared to Ag ions was determined by the specific properties of Ag NPs prepared by Yang et al. (2012) or whether Ag NPs in general have more prominent particle-specific effects in *C. elegans*.

Similarly to Ag NPs, also CuO NPs were the most toxic to crustaceans and algae, but at a slightly higher level: median L(E)C50 values were around 2–3 mg CuO/L

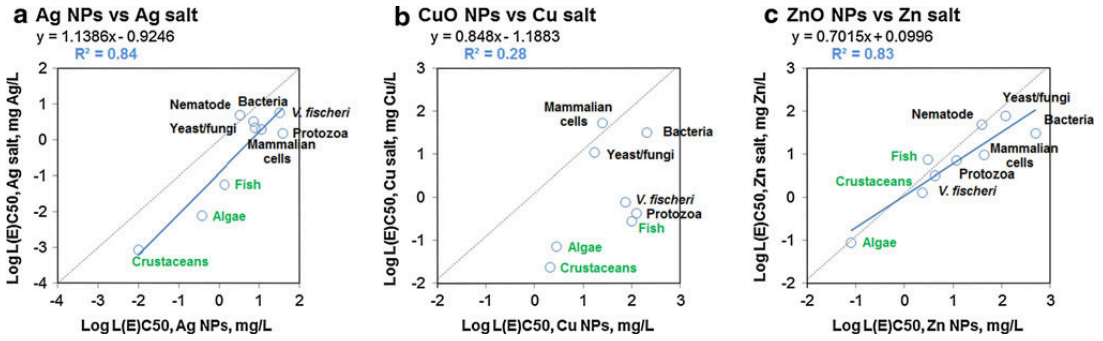

Fig. 7 Plots of the median L(E)C50 values of Ag, CuO and ZnO NPs versus the median L(E)C50 values of the respective soluble metal salts to different organism groups. Data are plotted from Table 1

(Fig. 6c; Table 1). Thus, according to the most sensitive organism of the test battery crustaceans–algae–fish, CuO NPs should be classified as 'toxic' to aquatic organisms (CEC 1996). As a rule, in all other ecotoxicological organisms, CuO NPs exerted toxicity at relatively high nominal concentrations (L(E)C50 > 100 mg/L). As CuO NPs are also used as antibacterials (Fig. 2c), it is interesting to note that bacteria proved not sensitive toward CuO NPs (MIC > 250 mg/L). On one hand, the insensitivity of bacteria toward CuO NPs may be explained by the differences in the test media and toxicity endpoints used. Indeed, in the toxicity assays with crustaceans and algae a mineral medium with low potential for complexing of Cu ions was utilized, whereas the bacterial inhibition assays (for MIC calculation) were mostly performed in organic media with high potential for complexing of Cu ions. On the other hand, the bacterial MIC values were very similar to EC50 values collected for bioluminescent aquatic bacterium *V. fischeri* where the assay was performed in 2 % NaCl (ISO 2010). Thus, apparently CuO NPs are indeed substantially more toxic to crustaceans and algae than to bacteria, and their use as antimicrobials should be perhaps re-considered due to the ecotoxicological concerns during the 'life cycle' of CuO NP-containing products.

Cu ions were more toxic than CuO NPs to all organisms except for yeast and mammalian cells in vitro (Figs. 6d, 7b). This is an important finding showing that in mammalian cells in vitro, CuO NPs may have an additional particle-specific intrinsic toxicity that is hard to predict using non-mammalian cell models. One may hypothesize that the particles are endocytosed (a Trojan horse model) and when already inside the cell their solubilization cannot be controlled by the mechanisms used to regulate the concentration of Cu ions in the cell. On the other hand, the toxicity assays with mammalian cells in vitro use serum that may disperse and coat NPs (Zook et al. 2012) increasing their bioavailability to the cells. For yeast *S. cerevisiae*, it was shown that while the toxicity tests were done in protein-rich medium, CuO NPs enhanced the Cu-ion-associated stress assumingly due to the stronger sorption of protein-coated NPs onto the cell surface that was suggested to facilitate the dissolution of CuO in the close vicinity of the yeast cell wall. Interestingly, this effect was prominent in complex organic medium, but not in distilled water (Kasemets et al. 2013).

As in case of Ag and CuO NPs, the toxicity of ZnO NPs to algae (median L(E)C50 = 0.08 mg/L) crustaceans (L(E)C50 = 2.3 mg/L) and fish (median L(E)C50 = 3.0 mg/L) was remarkably higher than to bacteria (MIC 622 mg/L). Thus, according to the most sensitive organism of the test battery crustaceans–algae–fish, ZnO NPs should be classified as 'very toxic' to aquatic organisms (CEC 1996).

The toxicity of ZnO NPs and Zn ions to different organisms was stunningly similar (Figs. 6e–f, 7c; Table 1), indicating that the toxicity of ZnO NPs is largely caused by dissolved Zn. To further illustrate the role of dissolution in the toxicity of studied NPs, the toxicity of NPs to various organisms was plotted against the toxicity of the respective metal ions. As shown in Fig. 7, the L(E)C50 values of Ag and ZnO NPs correlated well with the respective values of the soluble salts ($R^2 = 0.84$ and 0.85, respectively). However, the plot of the L(E)C50 values of CuO NPs and Cu ions formed two clusters, distinguishing mammalian cells, yeast and bacterial cells from all other organisms. As discussed above, this was most probably caused by the test

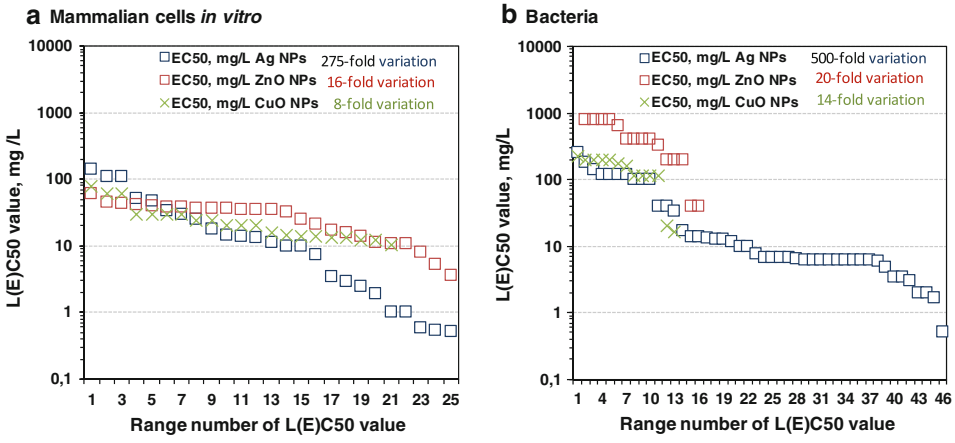

Fig. 8 Variation in individual L(E)C50 or MIC values used to derive the median L(E)C50 or MIC value for mammalian cells in vitro (**a**) and bacteria (**b**)

environment rich in organic compounds, where organic matter enhanced dispersion of CuO NPs and increased their bioavailability to the cells.

Variability of the retrieved toxicity data

Finally, we analyzed the obtained toxicity data with respect to the size and coating of NPs. As most of the literature data were available for bacterial cells (74 MIC values were retrieved, Fig. 6) and mammalian cells in vitro (71 EC50 values were retrieved, Fig. 6), the comparative analysis of particle size, coating and toxicity to these two cell types was performed. In addition, the toxicity mechanisms of NPs to these cell types are supposedly different, because mammalian cells internalize NPs and bacteria are more 'resistant' to the intracellularization of NPs, although some researchers have reported the penetration of NPs also into bacterial cells (Morones et al. 2005; McQuillan et al. 2012). The toxicity data of NPs to both mammalian and bacterial cells were supposed to vary because of the heterogeneity of bacterial strains and cell lines used (Table S2).

Surprisingly, we observed that the toxicity data of CuO and ZnO NPs to both groups, mammalian and bacterial cells, varied in quite narrow range: 16-fold and 20-fold for ZnO NPs and 8-fold and 14-fold for CuO NPs, respectively (Fig. 8).

Table 2 Characterization of sizes of NPs of Ag, CuO and ZnO used to derive the median MIC values in bacterial studies or L(E)C50 values in mammalian cell in vitro studies

	Mammalian cells in vitro			Bacteria		
	Ag	CuO	ZnO	Ag	CuO	ZnO
Nr of data	28	22	25	46	13	15
Maximum size, nm	69	55	1000	89	30	125
Median size, nm	20	50	55	20	9.2	20
Minimum size, nm	5	12	20	3.3	6	3
Average size, nm	29.3	44	145.2	20	15.4	31.7

In contrast, the toxicity values of Ag NPs varied greatly: 275-fold for mammalian cells in vitro and 500-fold for bacteria. Assumingly, the differential toxicity of nanosilver was due to different coatings that were often applied on the surface of Ag nanoparticles to stabilize them. Indeed, all used ZnO and CuO NPs were uncoated (Tables S5 and S7) but 60 % of Ag NPs used in studies with bacterial cells and 89 % of Ag NPs used in studies with mammalian cells were coated (Table S3). In case of mammalian cells, 55 % of studied Ag NPs had PVP coating, 24 % had peptide coating, and 11 % was uncoated. In case of bacterial cells PVP, mono- and disaccharides and biogenic coatings were reported. Interestingly, the uncoated Ag NPs were remarkably less inhibitory to bacteria than coated NPs. Specifically, to various bacterial strains 14 least inhibitory Ag NPs (MIC values >17 mg/L) were all uncoated. Within 32 Ag NPs that were inhibitory to bacteria at lower than 14 mg/L concentrations 28 were coated and only 4 uncoated, whereas the type of the coating seemed to play no role (Table S3). In case of mammalian cells in vitro we did not observe analogous effect of coating (Table S3).

Finally, we analyzed the obtained toxicity data with respect to the size of NPs. Information on size of NPs for which mammalian cell and bacterial toxicity data (Tables S3, S5 and S7) were collected is shown in Table 2. The median sizes of Ag, CuO and ZnO were 20, 50 and 55 nm, respectively, for mammalian cells in vitro and 20, 9.2 and 20 nm, respectively, for bacterial cells.

Example on correlation between toxicity of Ag NPs to mammalian cells in vitro and the NPs primary size is given in Fig. 9a. To avoid the interference of coating in Ag NPs' toxicity, only PVP-coated NPs were used. When all the retrieved L(E)C50 values of PVP-coated Ag NPs to mammalian cells were plotted against the primary size on these NPs, no correlation was observed ($R^2 = 0.1$) (Fig. 9a). At the same time, higher correlation ($R^2 = 0.4$) was observed when the toxicity data from one single article was used (Liu et al. 2010). Finally, when the toxicity data

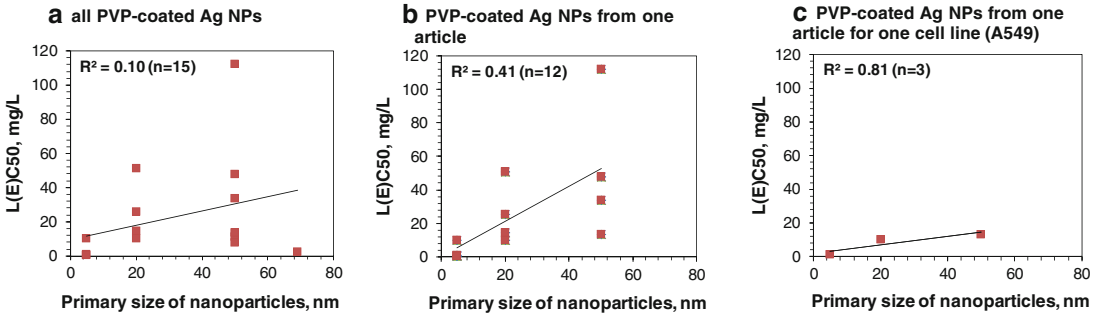

Fig. 9 L(E)C50 values of PVP-coated Ag NPs to mammalian cells versus size of nanoparticles. **a** All collected data were used; **b** data from one article (Liu et al. 2010) were used; **c** data from one article for one cell type were used (Liu et al. 2010)

for one cell line from one article was used, clear correlation was observed between the size and the toxicity of NPs ($R^2 = 0.81$, Fig. 9c). Similar observations were done for other articles that presented the toxic effects of a library of differently sized well-characterized NPs for various organism groups (Martínez-Castanón et al. 2008; Hoheisel et al. 2012; Wang et al. 2012a). These findings show clearly that the interlaboratory variations in preparation of NP suspensions and toxicity testing conditions make it difficult to draw general conclusions regarding the toxicity of NPs. At a single laboratory level, this problem may be resolved by using well-characterized monodisperse libraries of NPs. At the level of the whole nanotoxicology community, it is very important to proceed with the implementation of the general guidelines for nanotoxicology research to end up with the parameters that should be addressed in every nanotoxicological work, for example sufficient characterization of NPs and utilization of technically suitable toxicity tests and reference materials (Nature Nanotech Editorial 2012).

Conclusions

Our analysis of the literature data showed that:

1. The most toxic out of the three studied NPs was nanosilver. The L(E)C50 values of Ag NPs for the studied organisms/cells spanned nearly 4 orders of magnitude, from 0.01 mg/L for crustaceans to 38 mg/L for protozoa. For most of the species studied, the L(E)C50 values were below 10 mg/L, showing the hazardous properties of nanosilver compounds.
2. The $L(E)C_{50}$ values of CuO NPs ranged from 2 to 3 mg/L for crustaceans and algae, to >100 mg/L for protozoa and bacteria, and were in the range of 10–100 mg/L for most of the organisms studied.
3. ZnO NPs were the most toxic to algae (<0.1 mg/L), followed by crustaceans, fish, bacteria *V. fischeri* and protozoa. The L(E)C50 values of ZnO NPs were between 10 and 100 mg/L for nematodes, yeast and mammalian cells. Interestingly, ZnO NPs were not toxic to bacteria (median MIC 622 mg/L).
4. The toxic effect of Ag NPs and ZnO NPs (but not CuO NPs) was seemingly explained by solubilized ions. The intraspecies differences in toxicity seem to be at least partially explained by the composition of the test medium that affects the solubilization of metal-containing NPs and speciation of released metal ions.
5. Although bacterial cells are one of the target groups for all the studied nanoparticles, bacteria were among the least sensitive organisms. Instead, all the studied nanoparticles were remarkably more toxic to crustaceans, algae and fish.
6. Notably, one group of aquatic organisms most affected by the studied NPs was algae. This observation is noteworthy because planktonic microalgae as primary producers are the key component of food chain in aquatic ecosystems. Also, many algal species serve directly as a food source for zooplankton, which is subsequently consumed by other invertebrates or fish. Changes in the structure and productivity of the algal community may induce direct structural changes in the rest of the ecosystem and/or indirectly affect the ecosystem by affecting water quality (Nyholm and Petersen 1997).

Outlook

Crustaceans, algae and fish—the aquatic test organisms proposed for the classification and labeling of chemicals by EU REACH regulation—proved the most sensitive groups of organisms with respect to the toxic action of all three analyzed metal-containing NPs. Unexpectedly, the analysis of the published data on toxic effects of Ag, ZnO and CuO NPs showed that these three biocidal NPs were inhibitory to bacteria at considerably higher level than to non-target environmental organisms. Our observation is coherent with the recent statement of Chernousova and Epple (2013) on nanosilver: 'After analyzing a multitude of single studies, it can be concluded that the effect of silver towards bacteria is typically overestimated, and towards (eukaryotic) cells it is typically underestimated. Therefore, the application of silver in consumer products, cosmetics, and medical products should be critically assessed.'

To address the environmental impact of biocidal nanomaterials, we would like additionally to emphasize the following aspect of the species sensitivity pattern toward nanomaterials: As the toxicity range for all the three metal-containing NPs to non-target aquatic organisms and target organisms (bacteria, fungi, algae) warningly overlapped, the discharge or leaching of biocidal nanomaterials to surface waters may pose threat to aquatic species. This aspect of life cycle of nanomaterials could be controlled either at the level of 'safe by design' or, if applicable, by regulated discharge/disposal.

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BEILSTEIN JOURNAL OF NANOTECHNOLOGY

NanoE-Tox: New and in-depth database concerning ecotoxicity of nanomaterials

Katre Juganson*[1,2], Angela Ivask[1,3], Irina Blinova[1], Monika Mortimer[1,4] and Anne Kahru[1]

Full Research Paper

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Address:
[1]Laboratory of Environmental Toxicology, National Institute of Chemical Physics and Biophysics, Akadeemia tee 23, 12618 Tallinn, Estonia, [2]Department of Chemistry, Tallinn University of Technology, Akadeemia tee 15, 12618 Tallinn, Estonia, [3]Mawson Institute, University of South Australia, Mawson Lakes, 5095 South Australia, Australia and [4]Bren School of Environmental Science & Management, University of California Santa Barbara, Santa Barbara, California 93106-5131, United States

Email:
Katre Juganson* - katre.juganson@kbfi.ee

* Corresponding author

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Abstract

The increasing production and use of engineered nanomaterials (ENMs) inevitably results in their higher concentrations in the environment. This may lead to undesirable environmental effects and thus warrants risk assessment. The ecotoxicity testing of a wide variety of ENMs rapidly evolving in the market is costly but also ethically questionable when bioassays with vertebrates are conducted. Therefore, alternative methods, e.g., models for predicting toxicity mechanisms of ENMs based on their physico-chemical properties (e.g., quantitative (nano)structure-activity relationships, QSARs/QNARs), should be developed. While the development of such models relies on good-quality experimental toxicity data, most of the available data in the literature even for the same test species are highly variable. In order to map and analyse the state of the art of the existing nanoecotoxicological information suitable for QNARs, we created a database NanoE-Tox that is available as Supporting Information File 2. The database is based on existing literature on ecotoxicology of eight ENMs with different chemical composition: carbon nanotubes (CNTs), fullerenes, silver (Ag), titanium dioxide (TiO_2), zinc oxide (ZnO), cerium dioxide (CeO_2), copper oxide (CuO), and iron oxide (FeO_x; Fe_2O_3, Fe_3O_4). Altogether, NanoE-Tox database consolidates data from 224 articles and lists altogether 1,518 toxicity values (EC_{50}/LC_{50}/NOEC) with corresponding test conditions and physico-chemical parameters of the ENMs as well as reported toxicity mechanisms and uptake of ENMs in the organisms. 35% of the data in NanoE-Tox concerns ecotoxicity of Ag NPs, followed by TiO_2 (22%), CeO_2 (13%), and ZnO (10%). Most of the data originates from studies with crustaceans (26%), bacteria (17%), fish (13%), and algae (11%). Based on the median toxicity values of the most sensitive organism (data derived from three or more articles) the toxicity order was as follows: Ag > ZnO > CuO > CeO_2 > CNTs > TiO_2 > FeO_x. We believe NanoE-Tox database contains valuable information for ENM environmental hazard estimation and development of models for predicting toxic potential of ENMs.

Introduction

The production and use of engineered nanomaterials (ENMs) in consumer products is increasing rapidly [1]. As of March 20, 2015 there were more than 1,800 products listed in Consumer Products Inventory [2]. According to this inventory, the most abundant ENMs used in consumer products are silver (438 products), titanium (107), carbon (90), silica (81), zinc (38) and gold (24) with the main applications in antimicrobial protection (381 products), coatings (188) and health products (142). The number of published articles could serve as a good indicator of the potential future use of ENMs. A search performed on March 19, 2015 in Thomson Reuters Web of Science (WoS) with the keywords chosen based on Aitken et al. [3] and Bondarenko et al. [4] and listed in Table S1 (Supporting Information File 1) revealed that the majority of the papers concerned the applications of carbon nanotubes (36,609 papers, 40%), followed by Ag nanoparticles (NPs; 16,970, 19%), TiO_2 NPs (11,802, 13%), and iron oxide NPs (10,479, 11%) while the most common fields of application were sensors (28,027 papers, 31%), catalysis (10,435, 11%) and drug delivery (8,838, 10%) (Figure 1, Table S1, Supporting Information File 1). However, the exact production volumes of ENMs are not publicly available [4]. Piccinno et al. estimated based on a survey sent to companies producing and using ENMs that the most produced ENMs were TiO_2 (550–5,500 t/year), SiO_2 (55–55,000 t/year), AlO_x (55–5,500 t/year), ZnO (55–550 t/year), carbon nanotubes (CNT; 55–550 t/year), FeO_x (5.5–5,500 t/year), CeO_x and Ag (both 5.5–550 t/year), fullerenes and quantum dots (both 0.6-5.5 t/year) [5]. Warningly, the increasing production and use of ENMs leads inevitably to their higher concentrations in the environment. Thus, the risks caused by ENMs both to humans and the environment need to be assessed [6].

Risk assessment of all the ENMs in the market would require the sacrifice of enormous amounts of test organisms of diverse range [7]. Therefore, there is a need to refine, reduce or replace (3R's) animal testing and develop alternative risk evaluation methods [7,8]. Recently, the categorisation of ENMs based on their physico-chemical properties, exposure and use scenarios and biological effects was suggested as a strategy to facilitate regulatory decision making while minimising time-consuming and costly in vivo studies [9]. In addition to high-throughput screening tests, modelling can provide information for rapid assessment of the toxicity mechanisms of ENMs [10]. For instance, models based on dynamic energy budget (DEB) theory have been developed for predicting toxicity mechanisms of ENMs [11]. Also, quantitative (nano)structure-activity relationship (QSARs/QNARs) models have great potential for predicting the harmful effects of ENMs from their physical, chemical, and morphological properties that can be measured

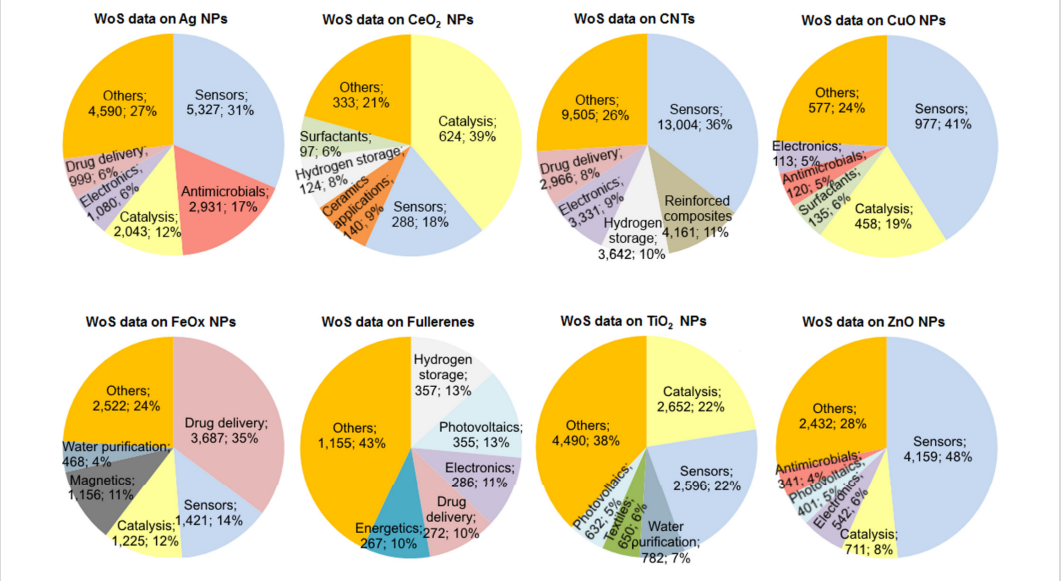

Figure 1: Proposed fields of application of engineered nanomaterials (ENMs) according to the publications in Thomson Reuters WoS. Keywords were selected from the review by Bondarenko et al. [4]. Numbers below each application category indicate the number and share of papers retrieved. The numerical data are presented in Table S1 (Supporting Information File 1). The bibliometric data search was performed in Thomson Reuters WoS on March 19, 2015.

experimentally or computed based on the ENMs structure [12]. Development of in silico methods relies on good-quality experimental data on ENM toxicity as the set of parameters which determine the toxic potential of each type of ENMs in specific test species/taxa is largely unknown [13].

In order to relate the toxic effects of ENMs to their physico-chemical properties and reveal the data gaps, the existing data have to be carefully collected and analysed. One increasingly popular approach in systematically collecting and organising available data on nanomaterials is creating databases. In 2012, Hristozov et al. emphasised that the available data on nanomaterials in environmental, health and safety databases and online chemical databases were very scarce [14]. Recently, a databases working group was established in the framework of European Union NanoSafety Cluster [15] which highlights the importance of development of in-depth databases on ENMs. In addition, nanotoxicity-related databases are developed and supported at national level in EU. For instance, in Germany an application-based nanomaterial database, which includes information on potential toxicological effects of ENMs, has been created in the DaNa project [16,17]. In Denmark, a database that focuses on potential risks of ENM containing products, "The Nanodatabase", has been developed [18]. The latter lists currently 1,425 products and introduces NanoRiskCat that evaluates ENMs risk according to potential exposure and hazard potential of these ENMs to humans and environment [19]. However, the risk estimations are derived from the available literature on the effects of nanomaterials but not on the actual risk assessment of the specific ENM-containing products. Therefore, the risk levels reported in the database do not account for concentrations or the physico-chemical properties of the specific ENMs used in the products. Independent online databases containing nanotoxicological information have also been created in other countries outside Europe. For instance, NanoToxdb: A database on Nanomaterial Toxicity [20] that is by description a comprehensive database containing information on nanomaterials toxicity to *Daphnia magna.* However, it contains altogether only 32 EC_{50} values for 10 different ENMs and contains no references for the toxicity data. Moreover, no information on physico-chemical properties of ENMs except primary particle size has been included in the database and regarding testing conditions, only the test duration is reported in a few cases. As a different approach, some databases, e.g., NHECD (Knowledge on the Health, Safety and Environmental Impact of Nanoparticles) [21] and Hazardous Substances Data Bank [22] comprise nanotoxicological papers.

In this communication we present a nanoecotoxicological database based on existing literature data on ecotoxicity of selected ENMs. In addition to quantitative toxicity data (e.g., EC_{50} values) information on physico-chemical properties of ENMs and testing conditions as well as on reported mechanisms and uptake of ENMs in the organisms was compiled. All the collected data were analysed to give an overview of ENM toxicity across different studied species. The following ENMs based on production volumes, application in consumer products and technological potential were included in the database: carbon nanotubes (CNTs), fullerenes, silver (Ag), titanium dioxide (TiO_2), zinc oxide (ZnO), cerium dioxide (CeO_2), copper oxide (CuO), and iron oxide (FeO_x; Fe_2O_3, Fe_3O_4). Furthermore, all these ENMs, except CuO, are listed by the Organisation for Economic Co-operation and Development (OECD) Working Party on Manufactured Nanomaterials as 'commercially relevant' representative manufactured nanomaterials to be investigated under the OECD sponsorship programme [23]. We believe the database presented in this paper contains valuable information for ENM environmental hazard estimation and development of models, including valid QSAR models, for predicting toxic potential of ENMs.

Methodology

The process of creating the nanoecotoxicological database can be roughly divided into three steps: selecting keywords for literature search, performing the literature search in Thomson Reuters WoS, collecting and classification of information from retrieved papers into a database. As the selection of keywords is critical in this type of data collection, all the keywords used in this study are listed in Table 1. To find different possible types

Table 1: Keywords used for bibliometric data search in Thomson Reuters WoS database.

ENM	Keywords
Ag	(nano* AND ecotoxic* AND silver) OR (nano* AND ecotoxic* AND Ag)
CeO_2	(nano* AND ecotoxic* AND cerium *oxide) OR (nano* AND ecotoxic* AND ceria) OR (nano* AND ecotoxic* AND CeO2)
CNT	(nano* AND ecotoxic* AND carbon nanotu*) OR (nano* AND ecotoxic* AND CNT) OR (nano* AND ecotoxic* AND *CNT)
CuO	(nano* AND ecotoxic* AND copper oxide) OR (nano* AND ecotoxic* AND CuO)
FeO_x	(nano* AND ecotoxic* AND iron *oxide) OR (nano* AND ecotoxic* AND Fe3O4) OR (nano* AND ecotoxic* AND Fe2O3)
fullerene	(nano* AND ecotoxic* AND fulleren*)
TiO_2	(nano* AND ecotoxic* AND titanium *oxide) OR (nano* AND ecotoxic* AND titania) OR (nano* AND ecotoxic* AND TiO2)
ZnO	(nano* AND ecotoxic* AND zinc oxide) OR (nano* AND ecotoxic* AND ZnO)

of 'nano' materials, i.e., nanoparticles, nanomaterials, nanotubes, a truncated search term "nano*" was selected. In order to give equal weight to all ecotoxicological test species, the restricting keyword "ecotoxic*" was used instead of organism-specific keywords. Thus, inevitably some of the ecotoxicological data on ENMs has been unintentionally excluded from the database because not all articles reporting studies on nanotoxicity to environmentally relevant organisms necessarily use terms "ecotoxic", "ecotoxicity" or "ecotoxicology". When performing the search, truncated names, molecular formulas and/or common abbreviations of the 8 NPs were used (Table 1).

Thomson Reuters WoS database – one of the largest international and multidisciplinary databases available, covering the most comprehensive list of journals published in English – was used for the bibliometric data search. Using WoS (all databases, all years) for the keyword searches enabled us to compare the data collected into NanoE-Tox with analyses performed in our previous reviews [4,8,24,25]. The search was performed on a regular basis from October 2012 to January 6, 2015. From each paper that was retrieved using the keywords specified in Table 1, maximum available information on physico-chemical properties of ENMs and the toxicity data were extracted and tabulated. It is important to note that in the earlier papers dating back 10 years from now, the NPs characterisation was often limited to their primary size. In more recent nanotoxicological articles, set of parameters required for characterisation of ENMs generally include chemical composition, purity, primary particle size, shape, surface area, coating, agglomeration and/or aggregation, hydrodynamic size in the aqueous test medium, surface charge, stability and solubility of ENMs. For the current NanoE-Tox database (Supporting Information File 2) we collected the following properties of the pristine NPs: chemical composition, origin (producer/in-house synthesised), shape, coating, primary size (diameter and length if applicable), impurities, surface area, and other reported observations. For the characterisation of ENMs in the test environment the following information was registered: test medium, hydrodynamic size of NPs in the test environment (including the method used for analysis), dissolution (if applicable), and surface charge (ζ-potential). Concerning the toxicity testing, we tabulated the following information: test organism, test medium, test duration, temperature, illumination and other reported conditions, toxicity endpoint/measure (e.g., EC_{50}, LC_{50}, NOEC), obtained toxicity value, and other reported observations. In addition, each paper was analysed to find information concerning (i) specific mechanism of toxicity of the studied ENM (Table S2, Supporting Information File 1) (ii) uptake in the organisms, and (iii) accumulation in cells, tissues and organs (Table S3, Supporting Information File 1). All the collected data were compiled into a Microsoft Excel spreadsheet which was used for creating a database on ecotoxicology of engineered nanomaterials, NanoE-Tox (Supporting Information File 2).

Results and Discussion

During the recent years, the number of peer-reviewed papers related to nanoecotoxicology has increased exponentially. According to Thomson Reuters WoS, 770 nanoecotoxicological peer-reviewed papers that corresponded to keywords "nano* AND ecotoxic*" were published between 2006 and March 2015. The rapidly increasing number of scientific publications on ecotoxicity of ENMs over the past decade, has inspired several review articles summarising the existing data in the field [4,8,13,24-31]. However, each review has focused on specific aspects and parameters of ENMs testing; therefore, it is difficult to get an overview of all the factors (and their values) that might influence the toxicity of ENMs. We have previously collected and analysed ecotoxicological data for seven different NPs (TiO_2, ZnO, CuO, Ag, SWCNTs, MWCNTs and C_{60} fullerenes) and seven organism groups representing different trophic levels (bacteria, algae, crustaceans, ciliates, fish, yeasts and nematodes). Altogether 77 toxicity values were analysed [24]. In our recent review [4], we summarised the recent research on toxicological and ecotoxicological findings for Ag, CuO and ZnO NPs including more than 300 toxicity values. In addition to ecotoxicological test species the toxic effects of studied NPs toward mammalian cells in vitro were reviewed [4]. The bibliographic search performed in the current study by using keywords listed in Table 1 resulted in nearly 500 individual papers. All the papers were thoroughly studied for ecotoxicity data. Unfortunately, many of the retrieved papers either did not concern the NP of interest or were review articles. In addition, the importance of including synonyms in keywords to increase the number of relevant articles in search results was apparent (Table 1). For example, the search using keywords "nano* AND ecotoxic* AND cerium *oxide" resulted in 30 papers, whereas "nano* AND ecotoxic* AND CeO2" resulted in 34 papers; remarkably, only 20 papers overlapped. The latter example was also true for other ENMs.

Analysis of the database: general overview of the sources and contents of the papers

The search in Thomson Reuters WoS using the time span of "all years" indicated that all the papers about ecotoxicity of ENMs have been published within the last ten years. Almost half of the papers retrieved from the initial bibliographic search, 224 of 500 articles from 66 journals, contained relevant nanotoxicological information and were included in NanoE-Tox database (Supporting Information File 2). From these studies 1,518 toxicity values were recorded with test conditions on toxicity testing and physico-chemical parameters of NPs linked to the toxicity data (further designated as 'database entry'). Out of 224

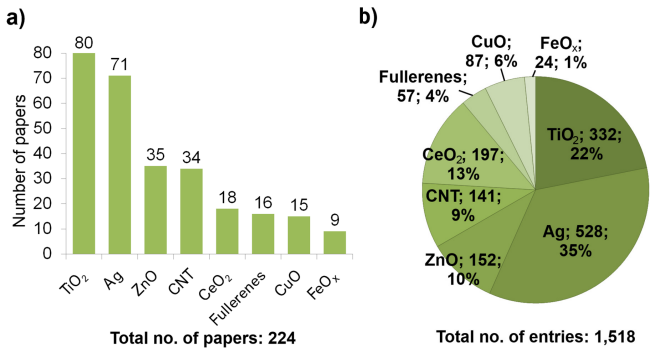

Figure 2: Information in the NanoE-Tox database for different types of engineered nanomaterials (ENMs): (a) number of scientific papers in the database and (b) number and share of entries for each of the tested nanoparticles (ENM; number of entries; share of entries). One entry equals one line that includes all the ENM parameters and toxicity test details. The database entries were selected based on bibliometric data search in Thomson Reuters WoS using the keywords as indicated in Table 1 as of January 6, 2015.

scientific papers that were selected for the database the largest number of papers concerned TiO_2 and Ag (80 and 71, respectively) followed by ZnO and CNTs (35 and 34 papers). For CeO_2, fullerenes and CuO, 15–18 papers were found and the lowest number of papers was retrieved for FeO_x (Figure 2a). From the 1,518 toxicity values (entries) in the database, the highest percentage (35%) concerned Ag followed by TiO_2 (22%), CeO_2 (13%), ZnO (10%), CNTs (9%), CuO (6%), fullerenes (4%) and FeO_x (1%) (Figure 2b).

Chronologically, the first nanoecotoxicological studies included in the database were published in 2006 and concerned TiO_2 NPs and CNTs (Figure 3). The first papers on ecotoxicity of fullerenes and ZnO NPs were published in 2007 followed by CeO_2, CuO and Ag NPs at 2008. While ecotoxicological effects of TiO_2 are still extensively studied, the interest in ecotoxicology of CNTs has slightly decreased. Notably, the most rapid increase rate appears to be in the number of published papers about nanosilver (Figure 3). The information on ecotoxicity of FeO_x particles started to emerge in 2009, i.e., later than for the other selected NPs (Figure 3). These findings are coherent with the literature survey by Kahru and Ivask [8] who showed that according to the citation pattern, the focus of the environment-related research shifted towards nanotoxicology by 2005 and the 'pioneering' NPs in environmental safety studies were CNTs, fullerenes, TiO_2, SiO_2 and ZnO. The analysis of the journals that contributed to the database revealed that more than half of the relevant papers originated from seven journals: Environmental Toxicology and Chemistry (29 papers), Environmental Science & Technology (25), Chemosphere (18), Environmental Pollution (12), Aquatic Toxicology (12), Science of the Total Environment (11), and Journal of Hazardous Materials (10 papers) (Table S4, Supporting Information File 1).

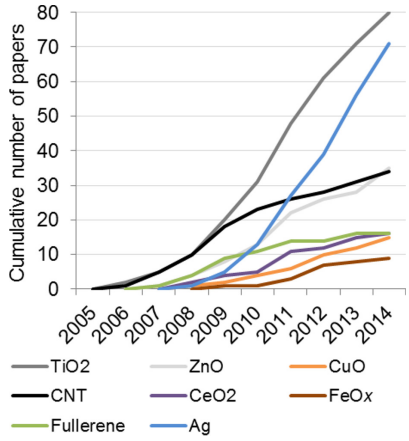

Figure 3: Evolution of nanoecotoxicological information about eight different nanomaterials according to the number of papers in NanoE-Tox database. The database entries were selected based on bibliometric data search in Thomson Reuters WoS using the keywords as indicated in Table 1 as of January 6, 2015.

Analysis of the database: physico-chemical characterisation of nanomaterials

The physico-chemical characteristics of ENMs included in the NanoE-tox database can be divided to intrinsic properties and properties that are specific to the test environment. The intrinsic characteristics are: name, CAS number, origin, shape, initial coating or functionalization, primary size, possible impurities, surface area and other observations, and the test environment-specific characteristics are: media, size, dissolution and zeta potential (Supporting Information File 2). Figure 4 illustrates the distribution of the data on ENM characteristics in NanoE-

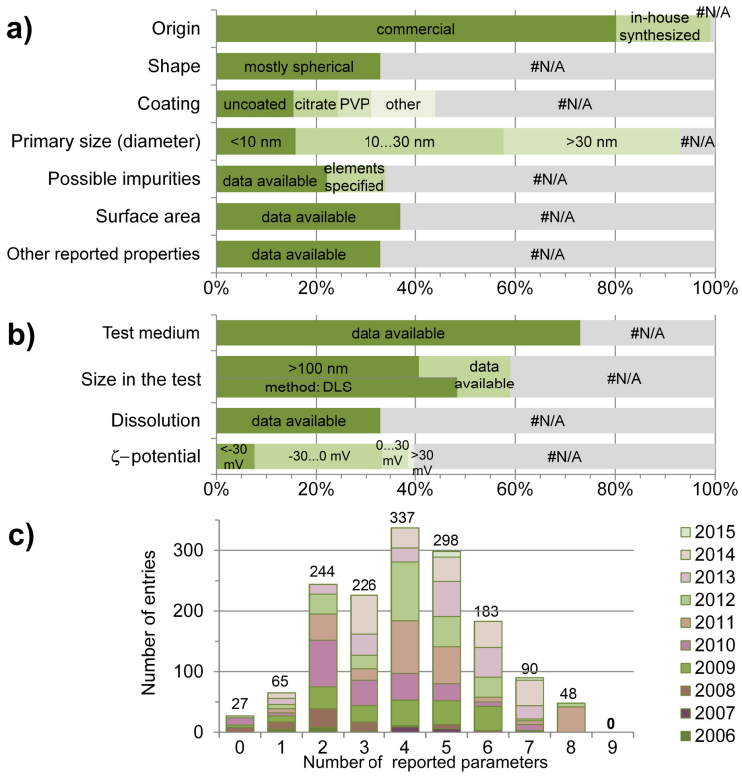

Figure 4: NanoE-Tox database: available data on characterisation of ENMs. Pristine (a) and environment-specific (b) properties as a percentage of all entries (1,518) in the database. Number of ENM parameters (shape, coating, primary size, impurities, surface area, other reported observations, size in the test, dissolution, ζ-potential) by number of entry and by publication year (c) #N/A - data not available. The database entries were selected based on bibliometric data search in Thomson Reuters WoS using the keywords as indicated in Table 1 as of January 6, 2015.

Tox database. Analysis of the papers revealed that in 99% of the entries the origin of the ENMs was known and 80% of the nanomaterials were obtained from commercial sources (Figure 4a). The most common source for all ENMs was Sigma Aldrich, 40% of all commercial particles were obtained from there. TiO_2 particles were mostly purchased from Evonik Industries (former Evonik-Degussa).

Many authors have emphasised that understanding the real risks of ENMs is a challenging task as there are several parameters that might have an influence on the biological effects of ENM [8,24,32-35]. Besides the chemical composition, the most important parameter determining the toxicity of NPs is their small size and size-dependent toxicity has been hypothesised in various papers [36,37]. Indeed, particle size has been considered as one of the most important physico-chemical parameter also in the papers collected in this study as this parameter was reported for 93% of the entries in the database. For all rod-shaped particles, also their length was reported. However, the results showed that most of the particles that were used in the 224 selected papers, were rather heterogeneous as in many cases the primary size was reported as a size range. According to Burello and Worth [38] ENMs with a diameter larger than 20–30 nm act often as bulk materials; thus, the "true nano-effects" are attributable to ENMs with smaller size. Indeed, in a recent paper on toxicity of different sizes of Ag NPs to bacteria, yeast, algae, crustaceans and mammalian cells in vitro Ivask et al. [39] showed that the toxicity of 20, 40, 60 and 80 nm monodisperse citrate-coated Ag NPs could fully be explained by released Ag ions whereas 10 nm Ag NPs proved more toxic than predicted. Analysis of the data in NanoE-Tox database revealed that the particles were smaller than 10 nm in 17% of the entries and in the size range of 10–30 nm in 45% of the entries (Figure 4a). Therefore, more than half of the studies have been performed using ENMs that should have size-dependent nanoeffects but as in most cases the NPs were polydis-

perse (i.e., had a broad size range) these effects were not often observed. Specific surface area that is closely related to the size of ENMs was reported in 37% of the entries (Figure 4a).

Another parameter that has been hypothesised to affect NP toxicity is morphology. For instance, some studies have shown that rod-shaped ENMs or triangular nanoplates could be more toxic than spherical ones [40-42]. However, the shape of ENMs was mentioned only in 33% of the entries and most of the experiments in the collected articles were performed with spherical particles (Figure 4a).

In addition to particle size and morphology, surface coating and/or functionalisation has been considered as an important parameter determining the biological effects of ENMs. For example, it has been discussed that coating on nanosilver plays an important role in Ag NPs toxicity [4,43,44]. However, information on initial coating or functionalisation of NPs was provided only in less than half of the entries. This is alarming because the surface chemistry of ENMs dictates their interactions with biological molecules and cells [45]. Altogether, 44% of the entries in the database contained information on NP coating: 29% of these were coated and 15% uncoated. ENMs were most often modified with citrate (31% of all coatings) and polyvinylpyrrolidone (PVP; 24% of all coatings) (Figure 4a). The high percentage of coated NPs in the database can be explained by the fact that nanosilver which constituted 35% of the database entries is frequently functionalised with different coatings, polyvinylpyrrolidone (PVP) and citrate being the most widely used.

A parameter closely related to NP surface properties is surface charge. It has been shown that positively charged ENMs tend to attach to the cellular surface that is negatively charged and these interactions may cause cell membrane damage [13,46]. In most studies ζ-potential is used as an indication of the surface charge of ENMs and NPs are considered to be stable in aqueous suspension if the ζ-potential is greater than ±30 mV [47]. In NanoE-Tox database, ζ-potential was reported in 40% of the entries. Most of the studies were performed with negatively charged ENMs (8% less than −30 mV, 25% −30…0 mV), 5% of the experiments were done with ENMs that had ζ-potential in the range of 0…+30 mV, and only 1% of the studies used stable positively charged ENMs (greater than +30 mV) (Figure 4b).

Another important parameter affecting toxicity of ENMs is the presence of impurities, for example presence of ‘seeding metals’ (catalysts) in CNTs that may count for observed toxic effects [48]. Purity of ENMs was reported in 34% of the entries; 65% of these cases mentioned purity as a percentage and 35% of the entries identified residual elements. Other reported observations, the most common parameters being crystal structure, density, and absorbance, were specified in 33% of the entries (Figure 4a).

Both in toxicological tests as well as in natural environments, the bioavailability and toxicity of ENMs depends on their fate in respective conditions [24,49]. In aquatic environment, ENMs tend to form agglomerates that might lead to their precipitation from the water phase; on the other hand, metal-based ENMs can release potentially toxic metal ions due to dissolution [50]. Cu^{2+}, Zn^{2+} and Ag^{+}, which can easily be released from respective ENMs are very toxic to a variety of aquatic organisms already at concentrations of milligrams and even micrograms per litre [4]. Analysis of the database entries (Figure 4b) showed that the most often reported ENM characteristic in the toxicity tests was hydrodynamic size (59% of all the entries) that usually (in 82% of the entries) was measured using dynamic light scattering (DLS) method. The data on hydrodynamic sizes indicated that ENMs tend to agglomerate in test conditions as 69% of the reported sizes were larger than 100 nm (in comparison, nearly all respective primary sizes were less than 100 nm). Dissolution of ENMs in toxicity tests was reported in 33% of all the entries. From all the studies using potentially soluble NPs (Ag, ZnO, CuO, CeO_2 and FeO_x) only half (51%) had measured the solubility of the particles.

As emphasised above, one of the goals of generating experimental nanotoxicological data is to apply them in model development that would allow for the comparison of physico-chemical properties of ENMs with their biological effects (QNAR models). It has been proposed that the QNAR models may even partially replace the expensive animal tests for evaluation of ENM related hazards [13]. Currently, there are a few QNAR modelling studies available for NPs [51]. However, these studies are based on relatively limited set of experimental data and therefore, applicable only for a small range of ENMs and organisms. Thus, in order to create a model with reasonable predictive power, several physico-chemical properties as well as data on a variety of NPs have to be included into the modelling to correlate the properties with toxic effects [25]. To evaluate whether the data in NanoE-Tox database might be suitable for (QNAR-)modelling, we analysed how many physico-chemical parameters of ENMs that could later be compared with the toxicological data were reported in each study. Nine physico-chemical parameters—shape, coating, primary size, impurities, surface area, other reported observations, size in the test, dissolution, surface charge (ζ-potential)—were analysed for the rate of being measured, i.e., how many of these were reported in one entry. In most of the studies, 2–6 of these parameters were reported (Figure 4c). Analysis of the data by year of publication revealed that despite of increasing number of nanotoxico-

logical articles being published each year, some of these still report only up to three parameters of ENM. On the other hand, there were no studies where all nine selected physico-chemical properties were explored, and in only 9% of the studies 7–8 parameters were reported. Hence, although the ecotoxicological data on NPs are rapidly increasing, there is still a shortage of accompanying information concerning physico-chemical properties of ENMs that may limit the use of nano(eco)toxicological data for QNARs.

Analysis of the database: ecotoxicological data

According to the European Union (EU) regulation on Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), the potential ecotoxicological effect of all chemical substances (including ENMs) that are produced in a volume of more than one tonne per year and sold in the EU must be evaluated. The amount of tests required depends on the production volume. If it exceeds 1 t/year, short-term tests with aquatic invertebrates (preferred species is *Daphnia*) and plants (algae is preferred) must be conducted. In case of the production volume over 10 t/year additional short-term tests with fish and studies of activated sludge respiration must be performed. Aforementioned aquatic studies must be performed also as long-term experiments for substances produced over 100 t/year; in addition, early life stage toxicity tests on fish, short-term toxicity tests on fish embryo and sac-fry stages and juvenile growth tests on fish must be carried out. With production over 100 t/year also terrestrial tests, short-term toxicity to invertebrates and plants and effects on soil microorganisms, must be performed. Finally, if the production volume for a certain substance exceeds 1,000 t/year, long-term terrestrial toxicity tests must be performed with invertebrates, plants, sediment organisms and birds in addition to all the previously mentioned aquatic and terrestrial studies [52].

To evaluate the compatibility of the toxicological data collected to NanoE-Tox database with the regulatory requirements, we collected the following data: type of test organism, test media, test duration and temperature, illumination conditions, test endpoint, toxicity measure and value. Also specific mechanisms of toxicity and accumulation of NPs in the cells, tissues or organs, and other observations were noted.

Organisms used for evaluation of biological effects of ENMs

Though the exact production volumes of ENMs are unknown, the estimated production of several ENMs exceeds the set 1 t/year limit [5]. Thus, according to legislation, several tests have to be conducted to bring these ENMs to the market. Organism-wise analysis of NanoE-Tox database revealed that information about effects of selected ENMs is available for 116 different test species (Table S5). Most of the experiments have been performed with water flea *Daphnia magna* (337 entries), followed by bacterium *Escherichia coli* (120 entries), unicellular alga *Pseudokirchneriella subcapitata* (107 entries), fish *Danio rerio* (66 entries), naturally luminescent bacterium *Vibrio fischeri* (44 entries), and nematode *Caenorhabditis elegans* (41 entries). In summary, by far the most often used test organisms were crustaceans constituting approximately one third (500/1,518) of all the tested species (Figure 5, Table S5, Supporting Information File 1). The abundance of toxicity data in crustaceans is likely derived from the mandatory reporting of these data according to REACH legislation as stated above. On the other hand, the amount of information about the effects of ENMs on algae – another mandatory test for REACH – is much more limited. With the keywords used in this study (Table 1), no information was found on algal toxicity of fullerenes and iron oxide and only one study evaluated the effect of CuO NPs on algae (Figure 5). The latter indicates that even if there are more publications on algal toxicity of ENMs, which were not retrieved in this study, the effects of ENMs on algae have been poorly studied. The same applies also to articles on effects of ENMs on fish. In NanoE-Tox database, there are no studies on the effect of CuO NPs on fish and only one study reported the effect of CeO_2 NPs and two studies showed the effect of fullerenes and FeO_x NPs to fish. Interestingly, toxicity tests with plants have been conducted with all 8 NPs. While relatively many studies have been performed with bacteria, the majority of them consider the effects towards potentially pathogenic bacterial strains, e.g., *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* (Table S5, Supporting Information File 1), which is likely driven by the important application area of some types of ENMs (TiO_2, ZnO, CuO, Ag) as antimicrobials [4,53]. About 16% of the entries in the database regard test organisms other than crustaceans, algae, fish, plants and bacteria. Those organisms included yeasts, protists, amphibians, bivalves, cnidarians, echinoderms, insects, nematodes, rotifers, snails and worms (Table S5, Supporting Information File 1). Hence, quite a wide range of test organisms has already been included in the evaluation of biological effects of ENMs. This certainly increases environmental relevance of these studies and the NanoE-Tox database.

Environmentally relevant test conditions

Recently, it has been highlighted that though most of the ENMs end up in the environment, relatively small amount of studies have been conducted in conditions relevant to the nature [54-56]. This was also reflected by the data collected into NanoE-Tox: 79% of the studies were performed in various artificial test media and only 15% in natural waters and 5% in soils, sludge or

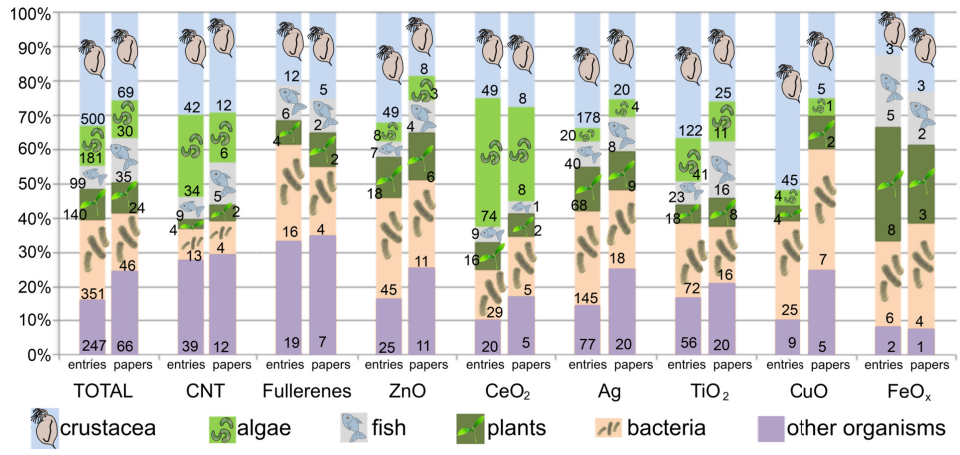

Figure 5: Types of test organisms used for evaluation of biological effects of selected ENMs in NanoE-Tox database. For each ENM, the left column represents the number of entries and right column represents the number of respective publications in the database. The database entries were selected based on bibliometric data search in Thomson Reuters WoS using the keywords as indicated in Table 1 as of January 6, 2015. The number of papers and entries for different ENMs is also presented in Figure 2.

sediments. Generally, the test conditions were relatively well reported in the majority of the analysed papers: the time of exposure (test duration) was reported in nearly all cases, while the test temperature was documented in more than 90% of the entries and information about illumination (illumination conditions/dark) was mentioned in 75% of the entries.

Toxicity endpoints used

The toxicity values for ENMs, irrespective of the endpoint, were based on nominal concentrations of ENMs. As expected, in most of the studies (77% of the entries) the toxicological endpoint was viability (e.g., mortality, immobilisation, growth inhibition, luminescence/fluorescence inhibition) while the effects on viability were classically expressed as half-effective (EC_{50}), half-inhibitory (IC_{50}), or half-lethal (LC_{50}) concentrations. 28% of the entries reported EC_{50} values, 10% LC_{50} values, 20% of the studies reported the concentration that did not exhibit any effect to the test organisms, i.e., NOEC (no observed effect concentration) values. However, some studies did not report any classical toxicity values because only one or two concentrations of NPs were tested by the authors; that did not allow for the establishment of a dose–response curve and, thus, calculations of E(L)C values. In addition, some papers considered the effect of ENMs on reproduction or studied possible malformations caused by ENMs that would be difficult to use for modelling purposes. As a result, the data that could be used as comparative inputs for models to evaluate the ecotoxicologial effects of ENMs is fairly limited in the database.

Analysis of the data consolidated into NanoE-Tox

Nano(eco)toxicological studies have usually two main aims: (i) the assessment of the toxic potential of ENMs, and (ii) the elucidation of the mechanism of toxic action [4,25]. In the following sections we will describe how NanoE-Tox database addresses these aims.

Toxicity of engineered nanomaterials

According to EU's regulation on classification, labelling and packaging of substances and mixtures (CLP) [57], chemical substances can be categorised as acutely or chronically toxic based on the results of standardised toxicity tests (reviewed by Crane et al. [58]) with fish (96 h), crustaceans (48 h) or algae (72 or 96 h). While by legislation acute toxicity has only one category ($E(L)C_{50}$ of the most sensitive organism ≤ 1 mg/L), chronic toxicity can be divided into four sub-categories ($E(L)C_{50}$ ≤1 mg/L; $E(L)C_{50}$ >1 to ≤10 mg/L; $E(L)C_{50}$ > 10 to ≤100 mg/L; $E(L)C_{50}$ > water solubility) that incorporate the degradation rate and bioconcentration factor of the chemical substance. Unfortunately, the latter two are not commonly determined in ecotoxicological studies; thus, in NanoE-Tox database bioconcentration factor has been reported only for FeO_x in fish larvae [59] and TiO_2 in coral tissue [60] and in crustaceans [61]. In order to give an overview of the ecotoxicity data collected for NanoE-Tox database (Figure 6), the hazard classification of ENMs was adjusted accordingly: acutely very toxic and potentially chronically very toxic ($E(L)C_{50}$ ≤ 1 mg/L), potentially chronically toxic ($E(L)C_{50}$ >1 to ≤10 mg/L), potentially chronically harmful ($E(L)C_{50}$ > 10 to

≤100 mg/L) and not classified (E(L)C_{50} > 100). Figure 6 depicts median values of all EC_{50}, LC_{50} and IC_{50} values with minimum and maximum values from NanoE-Tox database. Median EC_{50} values were calculated because these are the most precise estimates derived from the concentration–effect curve [62] and also, median EC_{50} values are often used in the QSAR analysis [63]. Analysis of the sources of the median values showed that most of the data in one data point originated from one (red frame, 19 points) or two (orange frame, 10 points) papers, only 18 median values were derived from 3 or more papers (green frame).

Based on the median toxicity values of the most sensitive organisms (i.e., theoretically representing the weakest link in the ecosystem), the toxicity of selected ENMs decreased in the order Ag > ZnO > FeO_x > CuO > fullerenes > CNTs > TiO_2 > CeO_2. However, when toxicity values that were derived from three or more papers were considered, the order slightly changed: Ag > ZnO > CuO > CeO_2 > CNTs > TiO_2 > FeO_x. The median values reported here are in general agreement with those published previously [4,24,26] (Table 2). However, such evaluation where the median values are derived across all different test conditions and test species is not in accordance with the current legislation. In order to be coherent with legislation, we next analysed the toxicity data obtained in standard tests with fish (96 h), daphnids (48 h) and algae (72 or 96 h) (Figure 7), i.e., the mandatory tests required under CLP [57] for classification of substances, and applied the same hazard ranking criteria as was used in Figure 6. This analysis showed that the most toxic ENM was Ag that could be classified as "acutely very toxic" and "potentially chronically very toxic". ZnO and FeO_x were also ranked as "acutely very toxic" and "potentially chronically very toxic" although less toxic than Ag. It is worth mentioning that the classification of FeO_x NPs was based on only one study (entry in the database), warranting further research of FeO_x NPs for more accurate ecotoxicity evaluation. According to median E(L)C_{50} values from the standard toxicity tests, CuO and CeO_2 NPs, CNTs and fullerenes

Table 2: Comparison of the median E(L,I)C_{50} values for different species in NanoE-Tox database and previous reviews [4,24,26].

ENM	E(L,I)C_{50} range in NanoE-Tox	E(L,I)C_{50} range in other reviews
Ag	0.01–245 mg/L	0.01–38 mg/L [4] 0.04–39 mg/L [24]
CeO_2	8.5–46.6 mg/L	0.1–100 mg/L [26]
CNTs	4.5–338 mg/L	1.0–500 mg/L [24]
CuO	0.32–569 mg/L	2.1–100 mg/L [4] 0.71–127 mg/L [24]
FeO_x	0.23–240 mg/L	#N/A[a]
fullerenes	1.5–11 mg/L	0.25–100 mg/L [24]
TiO_2	6.8–589 mg/L	39–11987 mg/L [24]
ZnO	0.05–3376 mg/L	0.08–121 mg/L [4] 0.055–97.4 mg/L [24]

[a] #N/A: not applicable.

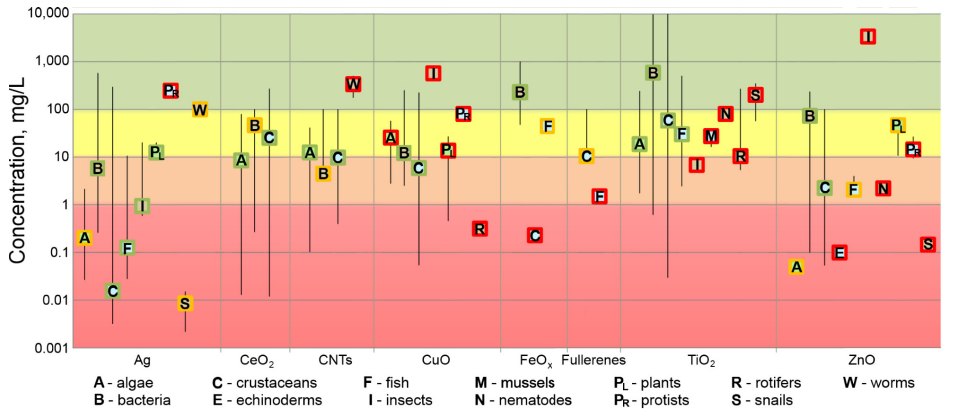

Figure 6: NanoE-Tox database: toxicity of selected nanoparticles to different organisms (data filtered by keyword ecotoxic*). Median E(L,I)C_{50} values ± minimum and maximum values. Colours of the frames surrounding the letters indicate the number of papers from which the respective data originates: red = 1 paper, orange = 2 papers, green ≥ 3 papers. The whiskers indicate the variability of the data. Note the logarithmic scale of y-axis. The E(L,I)C_{50} values used to derive the median values are from 113 papers and usually based on nominal concentration of the compound [44,55,56,61,64-172]. The toxicity ranking is indicated with the coloured background: E(L)C_{50} ≤ 1 mg/L – acutely very toxic, potentially chronically very toxic (red); E(L)C_{50} >1 to ≤10 mg/L – potentially chronically toxic (orange); E(L)C_{50} > 10 to ≤100 mg/L – potentially chronically harmful (yellow); E(L)C_{50} > 100 – not classified (green). The database entries were selected based on bibliometric data search in Thomson Reuters WoS™ using the keywords as indicated in Table 1 as of January 6, 2015.

fell into the category of "potentially chronically toxic" and TiO_2 NPs were ranked as "potentially chronically harmful".

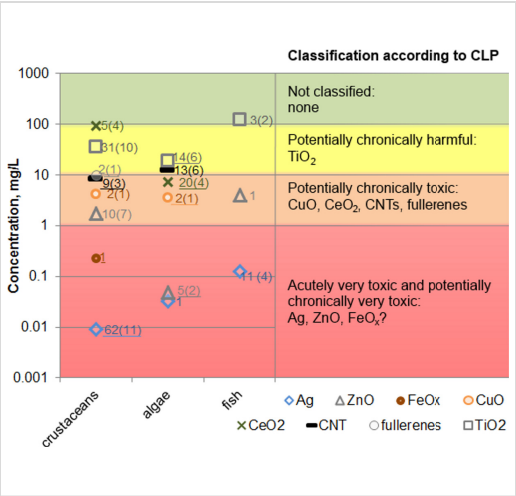

Figure 7: Classification of selected nanoparticles according to European Union CLP legislation based on their toxicity to fish (96 h), daphnids (48 h) and algae (72 or 96 h). Toxicity values were extracted from Figure 6. Classification of NPs is based on the most sensitive organism as described in CLP [57]. The number next to the symbol indicates the number of $E(L,I)C_{50}$ values used to derive the median value and the number in the parenthesis indicates the number of papers from which the respective data originates. Underlined numbers indicate the datapoints (lowest $E(L,I)C_{50}$ value for this ENM) used for classification. Note the logarithmic scale of the y-axis.

Mechanism of toxic action

While after a decade-long research the exact mechanisms of toxic action of ENMs are still debated, the main proposed mechanisms can be outlined as follows: (i) physical interactions of ENMs with cells or cellular components, (ii) production of reactive oxygen species and resulting induction of oxidative stress, and (iii) toxic effect of released ions from metal/metal oxide ENMs [13,25,28]. Analyses of the information in NanoE-Tox database (Table S2, Supporting Information File 1) revealed that the most often reported potential mechanism of toxic action for ZnO [128-132,173], Ag [44,64-73,174-177], and CuO [55,64,73,126-129,173] NPs was the release of metal ions. On the other hand, some studies have also proposed that the toxicity of these ENMs might be at least partially caused by the NPs themselves [73-84,178-181]. However, most of the studies reporting NP-specific effects of Ag, CuO and ZnO used insoluble particles and tested them in higher concentrations compared to the ones commonly reported as toxic. Thus, it can be concluded, in accordance with some previous studies [4,25], that in most cases the observed toxicity of these three ENMs was triggered by toxic metal ions. Other modes of toxic action reported for Ag NPs included destabilisation of cell membranes/mechanical membrane damage [89,175,182,183], oxidative stress [71,73,89,175,176,184,185], DNA damage/genotoxicity [102,186,187], and binding to sulfhydryl groups [100]. Similar effects were also demonstrated in case of ZnO NPs [84-86,188-190]. The mechanism of toxic action of insoluble ENMs like CeO_2 [109,110], CNTs [116,133,191] and TiO_2 [153-156,192] was usually reported as particle-driven mechanical membrane damage. NanoE-Tox database contains only one study suggesting the mechanism of toxicity of fullerenes (oxidative stress) [193] and there are no data about possible mechanism of action of FeO_x NPs.

Additionally, the information collected to the NanoE-Tox database indicated that ENMs were readily ingested by different organisms [55,72,77,119-123,192,194-202] and tended to accumulate in them [55,59,60,69-71,84,122-126,159,176-179,187,189,192,201-214] or on their surface [79,117-119,126,136-140,196,215-218] (Table S3, Supporting Information File 1). Similar findings have been reported in previous studies [24-26,29].

Conclusion

NanoE-Tox database that is available as Supporting Information File 2 of this paper is the first online-available database that contains in-depth nanoecotoxicological information on eight ENMs accompanied by considerable amount of information on ENM physico-chemical properties, testing conditions and, to some extent, also on mechanisms of toxic action. Hence, NanoE-Tox enables the comparison of toxicity of ENMs across different test species and, in addition, could provide valuable input for computational toxicity modeling (e.g., QSARs) and risk assessment.

The analysis of the database entries resulted in coherent data with previously published studies: the most toxic of the selected ENMs were Ag NPs followed by ZnO and CuO NPs and the toxicity of these ENMs was largely triggered by their solubility. Additionally, systematic collection of the data revealed several gaps in the current knowledge about ENM ecotoxicity: (i) in most cases the physico-chemical properties of the investigated NPs were described insufficiently, (ii) relatively few experiments have been performed with algae and fish, and (iii) ecotoxicity tests with standard test organisms were often performed with modified protocols (i.e., duration of the test was either shorter or longer than required by the OECD or ISO standards). Although the NanoE-Tox database is limited to a selected range of articles entered in the Thomson Reuters WoS database by January 6, 2015 and retrieved by using specific keywords, it provides a good overview of the existing ecotoxicological information about Ag, CeO_2, CuO, FeO_x, TiO_2 and ZnO NPs, carbon nanotubes and fullerenes.

Supporting Information

Supporting Information File 1

Supplementary tables.
[http://www.beilstein-journals.org/bjnano/content/supplementary/2190-4286-6-183-S1.pdf]

Supporting Information File 2

NanoE-Tox database.
[http://www.beilstein-journals.org/bjnano/content/supplementary/2190-4286-6-183-S2.xls]

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Supporting Information

for

NanoE-Tox: New and in-depth database concerning ecotoxicity of nanomaterials

Katre Juganson[1,2*], Angela Ivask[1,3], Irina Blinova[1], Monika Mortimer[1,4], and Anne Kahru[1]

Address: [1]Laboratory of Environmental Toxicology, National Institute of Chemical Physics and Biophysics, Akadeemia tee 23, 12618 Tallinn, Estonia; [2]Department of Chemistry, Tallinn University of Technology, Akadeemia tee 15, 12618 Tallinn, Estonia; [3]Mawson Institute, University of South Australia, Mawson Lakes, 5095 South Australia, Australia and [4]Bren School of Environmental Science & Management, University of California Santa Barbara, Santa Barbara, California 93106-5131, United States

Email: Katre Juganson* - katre.juganson@kbfi.ee

* Corresponding autor:

Supplementary tables

Table S1: Total number of publications in Thomson Reuters Web of Science™ for different applications of eight selected nanomaterials. Search was performed combining the keywords listed in the table with light grey background. The search was done on March 19th, 2015.

nano* AND	Ag NPs	CeO_2 NPs	CNTs	CuO NPs	FeO_x NPs	Fullerenes	TiO_2 NPs	ZnO NPs	Total
Fields of application	silver OR Ag	cerium *oxide OR ceria OR CeO2	carbon nanotu* OR CNT OR *CNT	copper oxide OR CuO	iron *oxide OR Fe3O4 OR Fe2O3	fulleren*	titanium *oxide OR titania OR TiO2	zinc oxide OR ZnO	
Hydrogen storage	189	124	3642	64	195	357	314	110	**1040**
Environmental remediation	110	16	155	36	219	23	303	74	**655**
Catalysis	2043	624	2553	458	1225	169	2652	711	**5215**
Drug delivery	999	55	2966	60	3687	272	515	284	**4818**
Medical imaging	97	7	151	4	292	19	32	21	**368**
Photovoltaics	299	3	368	41	14	355	632	401	**1443**
Textiles	568	11	482	44	77	14	650	305	**1090**
Therapeutics	109	16	324	8	322	35	32	17	**414**
Reinforced composites	172	37	4161	53	83	91	260	102	**589**
Electronics	1080	16	3331	113	116	286	278	542	**1335**
Optics	987	20	638	35	120	89	249	256	**749**
Coatings and pigments	19	12	10	8	36	1	143	53	**241**
Cosmetics	133	24	95	8	36	28	265	148	**485**
Ceramics applications	186	140	413	53	132	17	578	202	**982**
Anti-oxidants	10	13	14	4	4	7	3	5	**23**
Lubrication	78	10	181	26	37	115	55	35	**268**
Sensors	5327	288	13004	977	1421	255	2596	4159	**9408**
Absorbents	37	9	158	10	77	4	45	35	**171**
Energetics	136	18	801	11	38	267	218	54	**588**
Magnetics	84	3	154	29	1156	13	51	53	**1302**
Water purification	304	33	905	56	468	60	782	142	**1508**
Air emissions reduction	18	9	23	7	9	3	19	23	**61**
Natural and green products	36	1	11	2	10	0	6	6	**24**
Quantum computing	31	5	282	1	52	49	26	20	**148**
Masonry and building materials	1	0	0	0	0	0	1	0	**1**
Photonics	297	2	322	17	31	31	87	152	**318**
Surfactants	689	97	1166	135	468	80	506	335	**1524**
Antimicrobials	2931	13	299	120	154	52	504	341	**1171**
Total	**16970**	**1606**	**36609**	**2380**	**10479**	**2692**	**11802**	**8586**	**35939**

Table S2: Mechanisms of toxic action of selected ENMs in different organisms based on information in NanoE-Tox database.

	Ag	CeO_2	CNTs	CuO	Fullerenes	TiO_2	ZnO
Released ions	bacteria [1-5] bivalves [6] crustaceans [7-9] earthworms [10] plants [11-13] protists [14] yeasts [15]			bacteria [5,16-19] crustaceans [8,20] insects [21]			bacteria [16-18,22] crustaceans [23] echinoderms [24]
Effect of NPs/ their primary size	bacteria [25] crustaceans [26,27]			algae [28] plants [29]		algae [30] crustaceans [30,31] nematodes [32] rotifers [30]	plants [33]
Ions + NPs	algae [34] crustaceans [35] fish [36,37] nematodes [38]			protists [39] snails [40]			bacteria [5] crustaceans [41] fish [42]
Destabilization of cell membranes/ mechanical membrane damage	bacteria [43,44] earthworms [45] yeasts [15]	algae [46,47]	algae [48] bacteria [49] crustaceans [50]	protists [39]		bacteria [51] fish [52]	algae [53] fish [42]
Oxidative stress	bacteria [5,43,54] insects [55] bivalves [6] plants [12] yeasts [15]	nematodes [56]	algae [48,57] crustaceans [50]		crustaceans [58]	bacteria [43,51] fish [52,59] nematodes [32]	bacteria [60] earthworms [61] fish [62] snails [63]
DNA damage/ genotoxic	crustaceans [64] insects [65] plants [66]						bacteria [60] earthworms [61]
Disturbing ATP production			algae [57]				
Shading effect		algae [46]	algae [48,67]				
Effect of accumulation on the organisms	fish [36]	algae [46] crustaceans [68,69]	algae [67]			crustaceans [70]	fish [42]
Binding to -SH groups	fish [71]						

*There was no information about mechanisms of toxic action of FeO_x in the NanoE-Tox database

Table S3: Accumulation and uptake of selected ENMs in different organisms based on information in NanoE-Tox database.

	Ag	CeO_2	CNTs	CuO	FeO_x	Fullerenes	TiO_2	ZnO
Accumulated in organisms	bacterial biofilm [72] bivalves [6] crustaceans [73] earthworms [10] plants [11-13,66,74]		amphibians [75] bivalves [76] crustaceans [77,78]	bivalves [79] crustaceans [20] insects [21] snails [40] plant roots [29]	crustaceans [80] fish [81]	blackworms [82] crustaceans [83,84]	corals [85] crustaceans [86] fish [87-90] nematodes [32] plants [91,92]	earthworms [61] fish [42]
Accumulated on surface of organisms	crustaceans [93] fish eggs [37]	crustaceans [69] plant roots [94]	algae [67] bivalves [95] crustaceans [95-97] plant roots [98]	insects [21]			bacteria [99] crustaceans [70] plants [100]	algae [101] plant roots [102]
Ingested by organisms	crustaceans [26] protists [14]	nematodes [56]	amphibians [103] bivalves [76,95] crustaceans [77,84,95,96,104,105] protists [106]	crustaceans [20]	crustaceans [80]	crustaceans [84]	crustaceans [84,107,108] lugworms [109] nematodes [32]	crustaceans [84]
Translocation seed → plant (yes/no)			no [98]					
Translocation root → shoot (yes/no)		no/very low [94]		limited [29]		yes [98]	yes if size is less than 36 nm [92]	very low [102]
Other observations					BCF in fish larvae 0.04…0.14 [81]		Significant uptake in *E. coli; [60]* Dose-dependent increase in internalization in bacteria; [110] BCF in coral tissue 2…62, in posterior mixture 238…594; [85] BCF in crustaceans under illumination 502, in dark 318 [111]	Significant uptake in *E. coli; [60]* Dose-dependent increase in internalization in bacteria [110]

Table S4: List of journals in NanoE-Tox.

	Journal title	No. of papers	No. of entries	5-year impact factor
1	ENVIRONMENTAL TOXICOLOGY AND CHEMISTRY	29	156	3.282
2	ENVIRONMENTAL SCIENCE & TECHNOLOGY	25	170	6.277
3	CHEMOSPHERE	18	129	3.897
4	ENVIRONMENTAL POLLUTION	12	107	4.306
5	AQUATIC TOXICOLOGY	12	31	3.948
6	SCIENCE OF THE TOTAL ENVIRONMENT	11	85	3.906
7	JOURNAL OF HAZARDOUS MATERIALS	10	79	5.123
8	PloS one	7	34	4.015
9	ECOTOXICOLOGY AND ENVIRONMENTAL SAFETY	6	73	2.715
10	NANOTOXICOLOGY	5	23	7.766
11	ENVIRONMENTAL SCIENCE AND POLLUTION RESEARCH	5	62	2.951
12	ECOTOXICOLOGY	4	16	3.191
13	JOURNAL OF NANOPARTICLE RESEARCH	4	31	2.927
14	ENVIRONMENTAL TOXICOLOGY	4	50	2.371
15	JOURNAL OF ENVIRONMENTAL MONITORING	4	10	2.133
16	ENVIRONMENTAL TOXICOLOGY AND PHARMACOLOGY	4	28	2.093
17	ACS Nano	3	26	13.774
18	NANOMEDICINE	3	26	5.966
19	TOXICOLOGICAL SCIENCES	3	38	4.855
20	JOURNAL OF NANOBIOTECHNOLOGY	3	19	#N/A
21	ANALYTICAL AND BIOANALYTICAL CHEMISTRY	2	65	3.744
22	DESALINATION	2	29	3.481
23	MARINE ENVIRONMENTAL RESEARCH	2	10	2.525
24	JOURNAL OF ENVIRONMENTAL SCIENCES-CHINA	2	3	2.465
25	ARCHIVES OF ENVIRONMENTAL CONTAMINATION AND TOXICOLOGY	2	6	2.135
26	TOXICOLOGICAL AND ENVIRONMENTAL CHEMISTRY	2	24	#N/A
27	NATURE NANOTECHNOLOGY	1	5	38.586
28	SMALL	1	4	8.416
29	CARBON	1	7	6.638
30	WATER RESEARCH	1	6	6.092
31	FREE RADICAL BIOLOGY AND MEDICINE	1	2	5.983
32	SOIL BIOLOGY & BIOCHEMISTRY	1	1	4.785
33	LANGMUIR	1	6	4.489
34	COLLOIDS AND SURFACES B-BIOINTERFACES	1	14	4.226
35	APPLIED MICROBIOLOGY AND BIOTECHNOLOGY	1	12	4.138
36	ENVIRONMENTAL RESEARCH	1	4	4.033
37	TOXICOLOGY	1	9	3.884
38	TOXICOLOGY LETTERS	1	4	3.706
39	ENVIRONMENTAL MICROBIOLOGY REPORTS	1	3	3.556
40	ECOLOGICAL ENGINEERING	1	2	3.479
41	CYTOMETRY PART A	1	2	3.306
42	FOOD AND CHEMICAL TOXICOLOGY	1	1	3.21
43	PROCESS BIOCHEMISTRY	1	2	2.922
44	MUTATION RESEARCH-GENETIC TOXICOLOGY AND ENVIRONMENTAL MUTAGENESIS	1	4	2.716
45	COLLOIDS AND SURFACES A-PHYSICOCHEMICAL AND ENGINEERING ASPECTS	1	10	2.494
46	HYDROBIOLOGIA	1	3	2.35

	Journal title	No. of papers	No. of entries	5-year impact factor
47	PARASITOLOGY RESEARCH	1	2	2.286
48	APPLIED BIOCHEMISTRY AND BIOTECHNOLOGY	1	1	1.994
49	WATER AIR AND SOIL POLLUTION	1	1	1.943
50	ANALYTICAL METHODS	1	1	1.913
51	JOURNAL OF TOXICOLOGY AND ENVIRONMENTAL HEALTH-PART A-CURRENT ISSUES	1	2	1.868
52	BIOLOGICAL TRACE ELEMENT RESEARCH	1	1	1.656
53	JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY	1	2	1.484
54	SCIENTIFIC WORLD JOURNAL	1	12	1.3
55	JOURNAL OF ENVIRONMENTAL SCIENCE AND HEALTH PART A-TOXIC/HAZARDOUS SUBSTANCES & ENVIRONMENTAL ENGINEERING	1	4	1.233
56	BULLETIN OF THE KOREAN CHEMICAL SOCIETY	1	2	0.797
57	ASIAN PACIFIC JOURNAL OF TROPICAL MEDICINE	1	4	0.665
58	ARCHIVES OF BIOLOGICAL SCIENCES	1	4	0.606
59	INLAND WATER BIOLOGY	1	17	0.321
60	ENVIRONMENTAL ENGINEERING RESEARCH	1	3	#N/A
61	JOURNAL OF ENVIRONMENTAL HEALTH SCIENCES	1	16	#N/A
62	ACS SUSTAINABLE CHEMISTRY & ENGINEERING	1	3	#N/A
63	ENVIRONMENTAL HEALTH AND TOXICOLOGY	1	3	#N/A
64	ENVIRONMENTAL SCIENCE-PROCESSES & IMPACTS	1	2	#N/A
65	NANOCON 2009, CONFERENCE PROCEEDINGS	1	6	#N/A
66	NANOSAFE 2012: INTERNATIONAL CONFERENCES ON SAFE PRODUCTION AND USE OF NANOMATERIALS	1	1	#N/A
	TOTAL	224	1518	

Table S5: Organism-wise distribution of data in NanoE-Tox. Number in parenthesis indicates number of entries in the database.

		CNT	Fullerenes	ZnO	CeO2	Ag	TiO2	CuO	FeOx
Algae	Green algae	*Chlorella sp. (3)* *Chlorella vulgaris (18)* *Dunaliella tertiolecta (2)* *Pseudokirchneriella subcapitata (7)*		*Pseudokirchneriella subcapitata (5)*	*Chlamydomonas reinhardtii (4)* *Pseudokirchneriella subcapitata (68)*	*Chlorella vulgaris (2)* *Dunaliella tertiolecta (2)* *Pseudokirchneriella subcapitata (4)*	*Chlamydomonas moewusii (1)* *Chlamydomonas reinhardtii (1)* *Chlorella sp (2)* *Chlorella vulgaris (2)* *Desmodesmus subspicatus (4)* *Phaeodactylum tricomutum (3)* *Pseudokirchneriella subcapitata (23)* *Scenedesmus sp (2)* *Scenedesmus obliquus (2)*	*Chlorella spp (2)* *Nitellopsis obtusa (2)*	
	Red algae	*Thalassiosira pseudonana (4)*		*Thalassiosira weissflogii (3)*	*Nitzschia palea (2)*	*Ceramium tenuicorne (12)*	*Scenedesmus quadricauda (1)*		
Amphibians		*Ambystoma mexicanum (1)* *Xenopus laevis (13)*			*Pleurodeles waltl (2)* *Xenopus laevis (3)*				
Bacteria		*Bacillus subtilis (2)* *Cupriavididus metallidurans (1)* *Escherichia coli (4)* *Pseudomonas aeruginosa (2)* *Staphylococcus aureus (2)* *Vibrio fischeri (1)*	*Bacillus stearothermophilus (1)* *Escherichia coli (12)* *Vibrio fischeri (2)*	*Anabaena flos-aquae (3)* *Escherichia coli (24)* *Pseudomonas putida (7)* *Vibrio fischeri (6)*	*Anabaena (24)* *Escherichia coli (1)* *Vibrio fischeri (3)*	*Arthrobacter globiformis (2)* *Bacillus aquimaris (12)* *Bacillus thuringiensis (12)* *Enterococcus faecalis (1)* *Escherichia coli (27)* *Klebsiella pneumoniae (1)* *Nitrosomonas europaea (2)* *Pseudomonas aeruginosa (2)* *Pseudomonas chlororaphis (10)* *Pseudomonas putida (16)* *Staphylococcus aureus (2)* *Staphylococcus epidermidis (1)* *Vibrio fischeri (4)*	*Anabaena variabilis (1)* *Cupriavididus metallidurans (6)* *Escherichia coli (37)* *Pseudomonas putida (2)* *Vibrio fischeri (18)*	*Escherichia coli (13)* *Vibrio fischeri (7)*	*Escherichia coli (2)* *Vibrio fischeri (3)*
Bivalves	Clams					*Macoma balthica (2)*		*Macoma balthica (1)*	
	Mussels	*Mytilus galloprovincialis (2)* *Villosa iris (2)*	*Mytilus galloprovincialis (2)*			*Elliptio complanata (2)*	*Dreissena polymorpha (1)* *Mytilus galloprovincialis (4)*		
Cnidarians	Corals					*Acropora japonica (8)*	*Montastraea faveolata (2)*		
	Hydra	*Hydra attenuata (1)*	*Hydra attenuata (1)*				*Hydra attenuata (1)*		
Crustaceans	Amphipods	*Hyalella azteca (4)* *Leptocheirus plumulosus (1)*					*Gammarus fossarum (7)*		
	Branchiopods	*Ceriodaphnia dubia (11)* *Daphnia magna (15)* *Daphnia similis (2)* *Thamnocephalus platyurus (1)*	*Daphnia magna (7)* *Daphnia pulex (4)* *Thamnocephalus platyurus (1)*	*Ceriodaphnia affinis (4)* *Daphnia magna (28)* *Thamnocephalus platyurus (10)*	*Ceriodaphnia affinis (4)* *Ceriodaphnia dubia (1)* *Chydorus sphaericus (1)* *Daphnia magna (34)* *Daphnia pulex (4)* *Daphnia similis (2)* *Thamnocephalus platyurus (3)*	*Artemia nauplii (12)* *Chydorus sphaericus (3)* *Daphnia galeata (3)* *Daphnia magna (141)* *Daphnia pulex (3)* *Thamnocephalus platyurus (8)*	*Artemia salina (8)* *Ceriodaphnia affinis (4)* *Ceriodaphnia dubia (3)* *Chydorus sphaericus (1)* *Daphnia magna (78)* *Daphnia similis (17)* *Thamnocephalus platyurus (4)*	*Daphnia magna (33)* *Thamnocephalus platyurus (12)*	*Ceriodaphnia dubia (2)* *Daphnia magna (1)*
	Copepods	*Amphiascus tenuiremis (2)* *Tigriopus japonicus (6)*		*Acartia tonsa (6)*		*Tisbe battagliai (8)*			
	Ostracods			*Heterocypris incongruens (1)*					
Fish		*Danio rerio (2)* *Oncorhynchus mykiss (3)* *Oreochromis niloticus (1)* *Oryzias melastigma (2)*	*Danio rerio (5)*	*Cyprinus carpio (1)* *Danio rerio (6)*	*Danio rerio (9)*	*Danio rerio (30)* *Oncorhynchus mykiss (4)* *Oryzias latipes (2)* *Pimephales promelas (4)*	*Cyprinus carpio (4)* *Danio rerio (10)* *Oncorhynchus mykiss (3)* *Oryzias latipes (6)*		*Danio rerio (4)* *Oryzias latipes (1)*
Insects		*Chironomus dilutus (3)* *Drosophila melanogaster (2)*	*Drosophila melanogaster (1)*	*Folsomia candida (6)*	*Chironomus riparius (6)*	*Aedes aegypti (2)* *Chironomus riparius (7)* *Culex quinquefasciatus (2)* *Drosophila melanogaster (4)* *Folsomia candida (2)*	*Bovicola ovis (1)* *Chironomus riparius (2)* *Hippobosca maculata (1)*	*Allogamus ligonifer (2)*	
Nematodes				*Caenorhabditis elegans (1)*	*Caenorhabditis elegans (7)*	*Caenorhabditis elegans (18)*	*Caenorhabditis elegans (15)*		
Plants		*Cucurbita pepo (1)* *Oryza sativa (3)*	*Lemna gibba (3)* *Oryza sativa (1)*	*Cucurbita pepo (2)* *Fagopyrum esculentum (2)* *Lactuca sativa (1)* *Lepidium sativum (10)* *Lolium perenne (1)* *Raphanus sativus (1)* *Vicia faba (1)*	*Cucumis sativus (3)* *Cucurbita maxima (3)* *Lactuca sativa (3)* *Solanum lycopersicum (3)* *Spinacia oleracea (1)* *Triticum aestivum (3)*	*Allium cepa (2)* *Arabidopsis thaliana (3)* *Cucumis sativus (1)* *Cucurbita pepo (9)* *Hordeum vulgare (10)* *Lactuca sativa (2)* *Lemna gibba (4)* *Linum usitatissimum (6)* *Lolium perenne (7)* *Lycopersicum esculentum (10)* *Nicotiana tabacum (2)* *Zea mays (12)*	*Cucumis sativus (1)* *Lactuca sativa (3)* *Lemna minor (3)* *Lepidium sativum (3)* *Raphanus sativus (1)* *Solanum lycopersicum (1)* *Spinacia oleracea (1)* *Triticum aestivum (5)*	*Lactuca sativa (1)* *Raphanus sativus (1)* *Schoenoplectus tabernaemontani (2)*	*Cucumis sativus (2)* *Lactuca sativa (3)* *Raphanus sativus (1)* *Solanum lycopersicum (1)* *Spinacia oleracea (1)*
Protists		*Tetrahymena thermophila (5)*		*Bodo saltans (1)* *Euglena gracilis (3)* *Tetrahymena thermophila (4)*	*Bodo saltans (2)*	*Tetrahymena thermophila (2)*	*Bodo saltans (2)*	*Tetrahymena thermophila (2)*	
Rotifers							*Brachionus plicatilis (3)*	*Brachionus calyciflorus (2)*	
Echinoderms				*Lytechinus pictus (1)*			*Lytechinus pictus (1)*		
Snails				*Biomphalaria alexandrina (3)*		*Physa acuta (3)* *Potamopyrgus antipodarum (3)*	*Haliotis diversicolor supertexta (4)*	*Potamopyrgus antipodarum (2)*	
Worms		*Arenicola marina (1)* *Eisenia veneta (6)* *Lumbriculus variegatus (3)*	*Eisenia fetida (4)* *Eisenia veneta (4)* *Lumbricus rubellus (5)* *Lumbriculus variegatus (2)*	*Eisenia fetida (6)*		*Eisenia andrei (4)* *Eisenia fetida (4)* *Lumbricus terrestris (13)*	*Arenicola marina (1)* *Eisenia andrei (7)* *Eisenia fetida (10)*		
Yeasts						*Saccharomyces cerevisiae (1)*	*Saccharomyces cerevisiae (1)*		*Saccharomyces cerevisiae (2)*

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PUBLICATION III

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Extracellular conversion of silver ions into silver nanoparticles by protozoan *Tetrahymena thermophila*†

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Katre Juganson,[ab] Monika Mortimer,*[a] Angela Ivask,[a] Kaja Kasemets[a] and Anne Kahru[a]

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In the current study, cell-free exudates of the ciliated protozoan *Tetrahymena thermophila* were shown to progressively convert silver nitrate to silver nanoparticles (Ag NPs) under illumination at ambient temperature. The formation of Ag NPs in the reaction mixture was evidenced by gradual colour changes, appearance of a specific absorbance peak (420–450 nm) and visualization using scanning electron microscopy coupled to an energy-dispersive X-ray spectrometer. After 2 h of incubation the mean hydrodynamic size of the Ag NPs was 70 nm. Seven days of incubation resulted in larger agglomerates and a significant decrease in silver toxicity to *T. thermophila*, accompanied by about 100-fold reduction in the silver ion concentration. Protein analysis indicated an extensive extracellular protein binding by the Ag NPs formed in the protozoan exudates. As protozoa are important components in wastewater treatment, their ability to sequester silver ions into a less bioavailable and less toxic form of silver (*e.g.* NPs) may be one of the adaption mechanisms of ciliate survival in contaminated environments.

Environmental impact

The use of silver nanoparticles (Ag NPs) in various consumer products has remarkably increased during the last few decades. Thus, it is more likely that such materials are released into the environment and the silver ion (Ag^+) concentrations will increase due to dissolution of Ag NPs. It is therefore imperative to assess their potential adverse effects on different trophic levels. The current work describes the possible adaptation mechanisms of the fresh-water ciliated protozoan *Tetrahymena thermophila* to elevated silver concentrations in the environment. It was shown that the protozoan exudates reduced the concentration of Ag^+ by forming Ag NPs, which were less toxic to *T. thermophila* compared to Ag^+.

Introduction

The rapid development of nanotechnology over the past few decades has resulted in an increasing presence of engineered nanoparticles (NPs) in consumer commodities. According to the Woodrow Wilson Database in March 2011 there were more than 1300 consumer products on the market incorporating NPs, and 313 of them containing silver (Ag).[1] Mainly due to their antimicrobial properties, Ag NPs are used in various biomedical applications, food preservation, water and air purification, household products, cosmetics, clothing, and goods for children.[2–4] These antimicrobial properties are shown to rely upon the slow dissolution of NPs,[5,6] as Ag ions are considered one of the most toxic forms of heavy metals to microorganisms as well as other forms of life.[7]

Despite the high toxicity of Ag compounds, a number of organisms have been identified to be capable of forming Ag NPs *in vivo*.[8] Specifically, bacteria, fungi, algae and other organisms that are capable of synthesizing Ag NPs either intra- or extracellularly have already been successfully applied for the green synthesis of nanosilver.[9]

The main driving force for NP biosynthesis by the microorganisms is presumably derived from the need for detoxification of Ag ions into insoluble, less or nontoxic nanoclusters.[10,11] Although the current focus on biosynthesis of Ag NPs has shifted from empirical observations towards mechanistic studies, the exact mechanisms explaining the formation of these NPs remain to be elucidated.[12] For example, it has been shown that peptides containing arginine, cysteine, lysine and methionine were capable of reducing silver ions and could further promote the growth of Ag nanocrystals.[13] Parikh *et al.*[14] reported the formation of Ag NPs by cell-free supernatant of bacteria *Morganella sp.* and suggested that the NPs were formed as a result of silver-specific proteins secreted by the bacteria.

[a]*Laboratory of Environmental Toxicology, National Institute of Chemical Physics and Biophysics, Akadeemia tee 23, Tallinn 12618, Estonia. E-mail: monika.mortimer@kbfi.ee; Fax: +372 639 8382; Tel: +372 639 8382*

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

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Nitrate reductase has been proposed as one of the key enzymes in reducing Ag ions to metallic silver and the activity of this enzyme was shown to increase in certain bacteria, fungi and plants in the presence of $AgNO_3$.[12] Kumar *et al.*[15] provided further evidence on the role of this enzyme in Ag ion reduction, demonstrating that Ag NPs could be synthesized *in vitro* using purified nitrate reductase from fungi *Fusarium oxysporum* in the presence of phytochelatin, 4-hydroxyquinoline and NADPH.

Until now the formation of Ag NPs as a possible route to Ag ion detoxification has been studied mainly in fungi and bacteria.[11] However, the increasing use of Ag NPs in consumer products is expected to result in elevated Ag levels in the environment[16] and thus the capability of other biological species to cope with the toxic amounts of Ag NPs should also be studied. After bacteria, ciliates constitute the second most relevant and abundant community actively participating in the wastewater treatment process.[17,18] The unicellular protozoa of genus *Tetrahymena* are freshwater ciliates that have been used as model organisms for environmental research for decades.[19] However, no information on the interactions between protozoa and Ag ions and their possible conversion to NPs by ciliate protozoa in the environment is available. Moreover, according to a recent review by Kahru and Dubourguier,[20] toxicity data for Ag NPs towards ciliates are practically non-existent in the literature. Kvitek *et al.*[21] showed that a 1 h LC50 value for Ag NPs towards ciliate *Paramecium caudatum* was 39 mg L^{-1} and that the surfactant/polymer modification could increase the toxicity of the Ag NPs against these organisms. According to Shi *et al.*[22] Ag NPs were toxic to *T. pyriformis* (IC50 = 1.5 mg L^{-1}); however, they demonstrated that light irradiation decreased the toxicity.

In this study, we show that the soluble extracellular fraction (SEF) of *Tetrahymena thermophila* can affect the toxicity of $AgNO_3$ by reducing Ag ions and promoting the formation of Ag NPs. The formed Ag NPs were characterized by UV-Vis spectroscopy, dynamic light scattering (DLS) and scanning electron microscopy (SEM). Specifically, we show that the protein fraction of SEF facilitates the formation of the Ag NPs.

Experimental

Materials

$AgNO_3$ was purchased from J.T. Baker and silver NPs (nominal particle size < 100 nm; further designated as "Sigma-Ag NPs") from Sigma-Aldrich. All chemicals used in the study were of analytical grade. The stock suspensions/solutions of Sigma-Ag NPs (54 g L^{-1}) and $AgNO_3$ (54 g Ag L^{-1}) and their further dilutions were prepared in deionized water (MilliQ, Millipore). The stock suspension containing Sigma-Ag NPs was ultrasonicated in Branson Ultrasonic Bath 1510 for 30 min. Both stock suspensions/solutions were stored at room temperature in the dark.

Characterisation of commercial silver nanoparticles

The hydrodynamic diameter and zeta potential of Sigma-Ag NPs (100 mg L^{-1}) in MilliQ water were measured using a Zetasizer Nano ZS (Malvern Instruments). Dissolution of Sigma-Ag NPs in MilliQ water was measured at 205 mg L^{-1} using an Ag ion selective electrode (Van London-pHoenix Company). The fraction of dissolved Ag was calculated based on a calibration curve constructed using aqueous solutions of $AgNO_3$ with different concentrations (10.8 μg–1.08 g Ag^+ L^{-1}). SEM images of Sigma-Ag NPs have been published in our previous article by Ivask *et al.*[23]

Preparation of soluble extracellular fraction of *Tetrahymena thermophila*

Protozoan culture (*T. thermophila* strain BIII) was cultivated essentially as described by Mortimer *et al.*[24] The cells were harvested during the exponential growth phase (5×10^5 cells mL^{-1}) by centrifugation at 300*g* for 5 min at 4 °C and washed twice with MilliQ water. The number of cells was quantified by counting in a haemocytometer (Neubauer Improved, bright line; Germany) after immobilisation in 5% formalin.

Harvested and washed *T. thermophila* was incubated in MilliQ water on an orbital shaker at 100 rpm, 30 °C for 24 h. The cells were pelleted by centrifugation at 300*g* for 5 min at 4 °C and the supernatant was further purified from the cellular debris and membranous fraction by ultracentrifugation at 390 000*g* for 1 h at 20 °C. The obtained supernatant was designated as "soluble extracellular fraction" (SEF).

Characterisation of silver nanoparticles formed from silver nitrate in the soluble extracellular fraction of *Tetrahymena thermophila*

$AgNO_3$ (108 mg Ag L^{-1}) was added to SEF and incubated in 50 mL polypropylene centrifugation tubes (Falcon) on a shaker (150 rpm; Certomat MO II, B. Braun) at 25 °C with illumination from below using Philips TL-D 38 W aquarelle fluorescent tubes. The samples were collected after 0, 2, 24 h and 7 days of incubation. The resulting mixtures were further designated as "SEF-Ag".

Colour changes in SEF-Ag were monitored both visually and using a UV-Vis spectrophotometer. The absorbance spectra induced by the surface plasmon resonance of Ag NPs in SEF were recorded between 300 and 700 nm (Multiskan Spectrum, Thermo Scientific).

The hydrodynamic diameter of formed Ag NPs was measured using a Zetasizer Nano ZS. The particles formed after 2 and 24 h of incubation were visualized using SEM (Zeiss EVO MA 15). Silver ion concentrations in the samples were quantified using an Ag ion selective electrode (see above).

To analyse the protein composition of SEF before and after incubation with $AgNO_3$, SEF and SEF-Ag samples were concentrated 20-fold *via* freeze-drying. For sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), the samples were denatured with SDS-sample buffer for 5 min at 100 °C. The proteins were separated on 14% acrylamide gel using constant amperage (35 mA). The proteins were visualized with silver staining and a quantitative analysis of proteins was performed using TotalLabQuant software (Totallab, UK).

For the toxicity analysis the SEF-Ag samples collected after 0, 2, 24 h and 7 days were stored at −18 °C. Prior to the assay, the

samples were thawed and diluted 2-, 4-, 8-, 16- and 32-fold with MilliQ water.

Exposure of *Tetrahymena thermophila* to toxicants

For toxicity analysis 100 μL of harvested and washed *T. thermophila* suspension in MilliQ water was added to 100 μL of the solution/suspension of Ag compounds ($AgNO_3$, SEF-Ag or Sigma-Ag NPs) that were previously diluted in MilliQ water in 96-well polystyrene plates (Falcon). The following nominal concentrations (chosen according to pre-screening results) were used in the toxicity testing: 1.6, 1.9, 3.2, 3.8, 5.1 and 6.4 mg Ag L^{-1} of $AgNO_3$; 1.7, 3.4, 6.8, 13.5, 27 and 54 mg Ag L^{-1} of SEF-Ag; and 50, 100, 150, 200 and 500 mg L^{-1} of Sigma-Ag NPs. Each concentration was tested in three replicates and the final cell density in the test was 5×10^5 cells mL^{-1}. Protozoan suspension in MilliQ water and Ag compounds in MilliQ water were used as non-treated and abiotic controls, respectively. The test plates were incubated on a microplate shaker (300 rpm, Heidolph Titramax 1000) at 25 °C. The pH of *T. thermophila* suspension in MilliQ water did not change significantly upon the addition of Ag compounds and remained unchanged for 24 h (pH = 6.6 ± 0.3). After 2- and 24 h exposure 100 μL of the cell suspension was sampled from each well, and viability of the cells was determined by measuring the ATP content using the luciferin–luciferase method essentially as described by Mortimer *et al.*[24]

Cells were visualized using a light microscope (Olympus CX41) equipped with a DP71 camera.

Data analysis

The ATP concentration in the samples was expressed as a percentage of the non-treated control. The EC50 (effective concentration leading to a 50% loss in cell viability) values with 95% confidence interval were calculated from concentration–effect curves using the REGTOX software for Microsoft Excel™.[25]

Results

Toxicity of silver nitrate to *Tetrahymena thermophila*

The EC50 values of $AgNO_3$ to *T. thermophila* calculated from the ATP concentration data were not significantly different after 2- and 24 h exposure (1.8 and 1.5 mg Ag L^{-1}, respectively, Table 1). However, visualisation of the $AgNO_3$-exposed *T. thermophila* cultures under the light microscope indicated a moderate recovery of the protozoan culture after 24 h exposure compared to the 2 h exposed culture, indicated by the increased share of viable cells. Also, during the visual inspection of the assay wells containing *T. thermophila* culture exposed to the highest concentration of $AgNO_3$ a slight colour change from colourless to maroon was noted. As the toxicity assay was conducted in MilliQ water where no interfering media components were present, it was inferred that the colour change indicated potential changes in the speciation and/or transformation of $AgNO_3$ induced by the protozoan culture. To determine which cellular processes were responsible for the observed changes with $AgNO_3$, we isolated the protozoan exudates and examined their potential role in the transformation of $AgNO_3$.

Table 1 The toxicity[a] (EC50, mg L^{-1}) of silver nitrate and Sigma-Ag NPs to *Tetrahymena thermophila* after 2- and 24 h exposure in MilliQ water

	EC50[b], mg Ag L^{-1}	
Exposure time	$AgNO_3$	Sigma-Ag NPs
2 h	1.8 (1.7–3.6)	286 (233–454)
24 h	1.5 (1.5–1.6)	205 (133–503)

[a] Calculated from dose–response curves using ATP concentration as a viability endpoint. [b] Average values (95% confidence intervals) of at least three independent assays.

Silver nanoparticle formation in the soluble extracellular fraction of *Tetrahymena thermophila*

Similar to the incubation of $AgNO_3$ in the protozoan culture, the addition of $AgNO_3$ to cell-free SEF of *T. thermophila* induced formation of maroon colour (Fig. 1, inset). Remarkably, during the same incubation period (up to 7 days), the colour change was detected only under the illuminated conditions but not in the dark (not shown). The intensity of the colour formed in SEF-Ag under illumination increased over time (Fig. 1, inset). After one week of incubation a black precipitate formed at the bottom of the incubation vessel.

The analysis of the UV-Vis absorbance spectra of the 2- and 24 h incubated SEF-Ag showed a clear surface plasmon resonance peak at 420–450 nm that is characteristic to Ag NPs (Fig. 1). After 7 days of incubation, the plasmon resonance peak of SEF-Ag was significantly broadened and its intensity was low (Fig. 1).

In Fig. 2A the size distribution of the particles formed in SEF-Ag is shown. After 2 h incubation the average hydrodynamic diameter of the formed NPs was 70 nm. In comparison, after

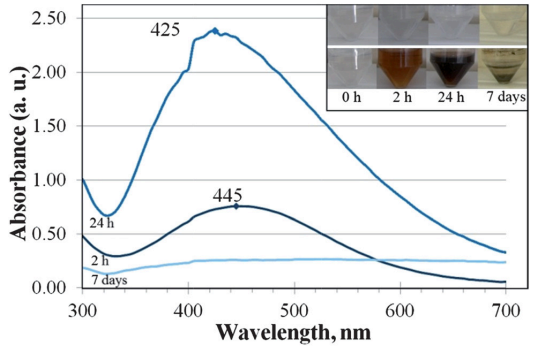

Fig. 1 UV-Vis absorption spectra of silver nanoparticles (NPs) formed after 2 h (middle line), 24 h (upper line) and 7 days (lower line) of incubation of $AgNO_3$ (final concentration 108 mg Ag L^{-1}) with the soluble extracellular fraction (SEF) of *Tetrahymena thermophila*. Inset: photos of SEF of *T. thermophila* (upper panel; no $AgNO_3$ added) and formed Ag NP suspensions in SEF (lower panel) at different incubation times. No specific absorption peak was detected at the beginning of incubation (t = 0 h; data not shown).

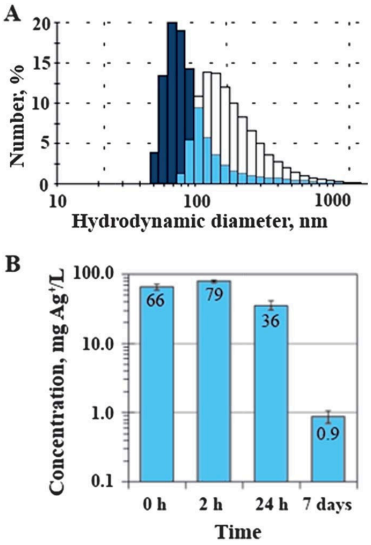

Fig. 2 (A) The size distribution of silver nanoparticles (NPs) formed after 2 h (dark blue bars) and 24 h (white bars) of the reaction of $AgNO_3$ (108 mg Ag L^{-1} added at time zero) with the soluble extracellular fraction (SEF) of *Tetrahymena thermophila*, light blue bars indicate the overlap of the size distributions. (B) The concentration of silver ions in the mixture of 108 mg Ag L^{-1} $AgNO_3$ and SEF of *T. thermophila* after 0, 2 and 24 h and 7 days of reaction; note the logarithmic scale of the *y*-axis.

24 h of incubation the mean particle size increased to 105 nm and after 7 days the particle size exceeded the measurement range of the Zetasizer Nano ZS (up to 10 μm). The formation of particles was accompanied by a decrease of silver ion concentration in SEF-Ag (Fig. 2B).

Interestingly, although 108 mg Ag L^{-1} of $AgNO_3$ was added to SEF, only about 70 mg Ag^+ L^{-1} was detectable by the ion selective electrode at the zero time point (Fig. 2B). Thus, the addition of SEF immediately decreased the concentration of Ag ions in $AgNO_3$ solution, apparently due to the immediate binding of silver ions to proteins and/or changed Ag speciation. The concentration of silver ions decreased even more over time and after 7-day incubation the concentration of silver ions in SEF was about 100 times lower (~0.9 mg Ag L^{-1}) compared to the initial nominal concentration.

The literature suggests that peptides and proteins may be responsible for the biotic formation of Ag NPs from Ag ions. Therefore we studied changes in the protein composition of SEF after its reaction with $AgNO_3$. According to SDS-PAGE, the most prevalent proteins in SEF were within the size range of 22–34 kDa (indicated by a square bracket in Fig. 3). During the reaction of SEF with $AgNO_3$, the amount of those proteins decreased compared to the pure SEF incubated under the same conditions. The intensity of the protein bands of 22–34 kDa size range in the SEF incubated with $AgNO_3$ for 2 h and 24 h decreased by 75% (Fig. 3, lane 4) and 85% (Fig. 3, lane 6), respectively. No proteins within the size range of 22–34 kDa were detected in SEF after 7-day incubation (Fig. 3, lane 8).

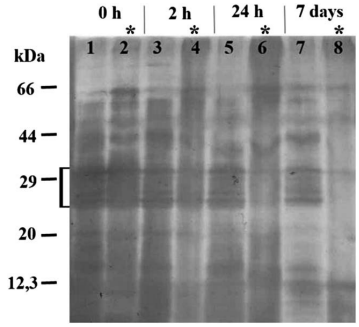

Fig. 3 SDS-PAGE analysis of the soluble extracellular fraction (SEF) of *Tetrahymena thermophila* and SEF incubated with $AgNO_3$ for 2 and 24 h and 7 days. The gel was silver stained; * SEF incubated with $AgNO_3$ (108 mg Ag L^{-1} added at time zero); '[' proteins within the size range of 22–34 kDa.

Comparison of toxicity of SEF-Ag and Sigma-Ag NPs to *Tetrahymena thermophila*

The Ag particles formed in SEF-Ag were further analysed by SEM in the backscattered electron imaging mode. After 24 h of incubation of SEF-Ag, silver NPs were formed (Fig. S1†).

Given that (i) microscopic evaluation revealed a slight recovery of the *T. thermophila* culture upon exposure to $AgNO_3$ for 24 h compared to 2 h exposure and (ii) during that time, Ag NPs were formed, we propose that the observed recovery of the protozoan culture with an increasing exposure period could be associated with the decreasing concentrations of Ag ions *via* Ag NP formation. Indeed, when *T. thermophila* was exposed to SEF-Ag collected after different incubation times, the longer incubated SEF-Ag was less toxic as indicated by the less steep slope of the dose–effect curve and the higher EC50 values (Fig. 4).

To confirm that Ag NPs are less toxic to *T. thermophila* than $AgNO_3$, we chose commercial Ag NPs (herein designated as Sigma-Ag NPs) that were of comparable size with the NPs formed in SEF-Ag. The average hydrodynamic diameter of

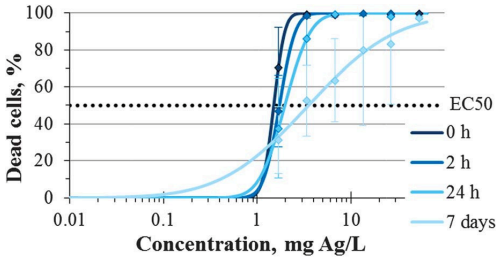

Fig. 4 The effect of $AgNO_3$ pre-incubated in the soluble extracellular fraction (SEF) of *Tetrahymena thermophila* for 0, 2 and 24 h and 7 days on the viability of *T. thermophila*. On the logarithmic *x*-axis are the nominal concentrations of $AgNO_3$ added to SEF at $t = 0$ h. The percentage of dead cells was calculated by measuring the ATP concentration after 2 h of incubation of protozoa with test samples. Data points are the mean values of at least 3 independent experiments, error bars indicate standard deviation.

Table 2 Characterisation of the Sigma-Ag NPs in MilliQ water

Hydrodynamic diameter	147 nm	
Zeta potential	−51 mV	
Polydispersity index	0.48	
Solubility in time[a]		
0 h	0.42 ± 0.01 mg L^{-1}	0.20%
2 h	0.56 ± 0.04 mg L^{-1}	0.3%
24 h	0.86 ± 0.06 mg L^{-1}	0.42%

[a] The concentration of silver ions, measured in the suspension of Sigma-Ag NPs (nominal concentration 205 mg L^{-1} *i.e.*, 24 h EC50 value for *T. thermophila*) in MilliQ water.

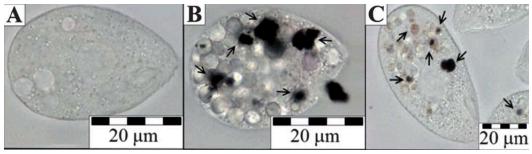

Fig. 5 Images of *Tetrahymena thermophila* under a light microscope. (A) Control cell, (B) after 2 h exposure to Sigma-Ag NPs at 100 mg L^{-1}, and (C) after 24 h exposure to SEF-Ag at 7 mg Ag L^{-1}. Black arrows indicate food vacuoles filled with agglomerates of silver NPs.

Sigma-Ag NPs was 147 nm. On the other hand, the polydispersity index of these commercial NPs was 0.48, indicating that the sample was not monodisperse and contained also larger agglomerates. Also, Ag ion concentration in the suspension of Sigma-Ag NPs in MilliQ water was very low: after 24 h only 0.42% (0.86 mg L^{-1} of 205 mg L^{-1}) of Ag was dissolved (Table 2).

Compared to $AgNO_3$, Sigma-Ag NPs were indeed remarkably less toxic: the EC50 values of the Sigma-Ag NPs were between 205 and 286 mg L^{-1}, while the EC50 values of $AgNO_3$ varied from 1.5 to 1.8 mg Ag L^{-1} (Table 1). This indicates that the formation of Ag NPs from Ag ions could be one of the defence mechanisms of *T. thermophila* against the toxic silver ions. Remarkably, we also observed that Ag NPs could be taken up and stored intracellularly in the food vacuoles of *T. thermophila* (Fig. 5). Visual observations at different time periods revealed that the ingested Ag NPs further agglomerated in the food vacuoles of *T. thermophila.*

Discussion

The increasing use of Ag NPs in industrial and household applications likely leads to the release of Ag NPs to the environment, where the particles may either agglomerate or dissolve to impact the food chain.[16,26] Dissolved silver ions are very toxic to most invertebrate, plant and fish species, and the LC50 values of $AgNO_3$ have been documented to be between 0.1 and 1 mg L^{-1}. According to Ratte[7] the most sensitive freshwater organisms to $AgNO_3$ are the crustaceans ($LC50_{Daphnia\ magna,48\ h}$ = 0.5–35 μg L^{-1}), and the least sensitive the amphipod *Hyalella azteca* (LC50 = 1.6–397.7 mg L^{-1}). According to the current study, the freshwater protozoan *T. thermophila* showed a higher tolerance to silver nitrate (EC50 values 1–2 mg Ag L^{-1}; Table 1) than other freshwater invertebrates. Such results are remarkable considering the fact that the toxicity assays were performed in MilliQ water, where the effects of the medium components on the Ag ion complexation and speciation were eliminated. Nevertheless, our results support the data reported by Shi *et al.*[22] who showed that 1.5 mg Ag L^{-1} of $AgNO_3$ resulted in ~60% growth inhibition of *Tetrahymena pyriformis*. Moreover, *T. thermophila* has been shown to be less sensitive to most toxic substances compared to other aquatic standard test species[27] and it possesses many ABC transporters associated with resistance to multiple drugs and toxins.[28] These features are presumably a result of the adaption to high environmental concentrations of pollutants as protozoa are also present in the wastewater purification process.[29]

Even though in the current study the toxicity testing of $AgNO_3$ to *T. thermophila* was done in MilliQ water, with the aim of minimising any complexation and sedimentation of Ag ions as insoluble salts, the effect of the test organism to the speciation of the toxicant is also an important factor to consider. This was clearly demonstrated as the extensive formation of Ag NPs from Ag ions and further agglomeration of the NPs could occur in the SEF of *T. thermophila* (Fig. 1, inset). As several microorganisms have been shown to be capable of synthesising Ag NPs from Ag ions both intra- and extracellularly,[9] it could be assumed that *T. thermophila* secreted Ag^{+}-reducing compounds into the surrounding environment. Interestingly, under the illuminated conditions the colour changes in the test environment appeared within minutes, whereas no colour change was observed in the dark even after 7 days of incubation. This evidence is in agreement with the data reported by Nam *et al.*[30] and Mokhtari *et al.*[31] who pointed out that the biosynthesis of Ag NPs from Ag ions is promoted by the visible light. Nam *et al.*[30] showed that silver ions were photoreduced in the presence of the carboxylic acid-containing peptides and ambient light. Peptides and proteins could be most likely involved in the Ag NP biosynthesis also in SEF of *T. thermophila* as the protozoan is known to release various acid hydrolases into the extracellular medium.[32] Madinger *et al.*[33] found that under starving conditions *T. thermophila* secreted ~30 different proteins, most of which were proteases. It has been shown that the peptides containing amino acid moieties such as arginine, cysteine, lysine and methionine reduced Ag ions and formed silver NPs of a wide size distribution.[13] The Ag NPs formed in the SEF of *T. thermophila* in the current study were also characterised by high polydispersity in their hydrodynamic size (Fig. 2A) and the broad peaks in the UV-Vis spectra (Fig. 1). It is known that in the biological fluids NPs readily become coated by proteins leading to the formation of protein corona.[34] Such corona has been shown to stabilize the NPs resulting in a more stable aqueous suspension[35] but may also play a role of creating a chemically reducing environment around the silver clusters and promote crystal growth.[12] Our result from SDS-PAGE analysis (Fig. 3) confirmed the involvement of proteins in Ag NP formation as in SEF-Ag the amount of proteins decreased in time. SEF proteins within the size range of 22–34 kDa seemed to show a specifically higher affinity for the Ag NPs at first.

Nevertheless, after 7 days of incubation all of the proteins in SEF-Ag appeared to be bound, as indicated by the lack of the protein bands in SDS-PAGE (Fig. 3). This suggests that under the conditions of the current study, where an excess amount of $AgNO_3$ was used, formation of Ag NPs was limited by the amount of proteins secreted by *T. thermophila*, particularly considering the aggregation and precipitation of Ag particles after 7-day incubation. The role of proteins and peptides in the formation and growth of Ag NPs in SEF was further supported by the UV-Vis analysis of SEF-Ag (Fig. 1). The absorption spectra obtained from the SEF-Ag samples were consistent with the literature, with the absorption maximum of the biosynthesised Ag NPs occurring in the range of 400 to 450 nm.[36,37] As the amplitude of the absorbance peak is proportional to the concentration of Ag NPs,[38] the increase in absorbance observed after 24 h of incubation compared to the first 2 h of incubation indicated the increased number of Ag NPs over time. As it has been discussed previously in a study on the formation of Ag NP-human serum albumin (HSA) corona, the characteristic peak of the surface plasmon resonance of Ag NPs becomes red-shifted upon the binding of dielectric HSA molecules onto the Ag NP.[39] In our study, the characteristic absorbance peak of Ag NPs was identified at 445 nm after 2 h incubation and the peak maximum was shifted towards the shorter wavelength of 425 nm after 24 h incubation of SEF-Ag (Fig. 1). This phenomenon could be explained as follows (schematically illustrated in Fig. 6): during the first hours of the Ag NP formation the number of NPs was low enough to allow sufficient coating of NPs by the proteins excreted by *T. thermophila*. As the concentration of Ag NPs increased over time, as indicated by the higher absorbance peak in the UV-Vis spectrum after 24 h, the free and weakly bound SEF proteins (soft corona) disassembled from the earlier formed Ag NPs to coat the newly formed Ag crystals. During this process the average thickness of the protein corona surrounding the increasing number of Ag NPs decreased (hard corona was formed), which was reflected by the shift of the absorbance peak towards the lower wavelength. At the final stage, when the proteins were entirely consumed by Ag particles, while continuously reducing the excess Ag ions and promoting the NP formation, the uncoated Ag NPs started to aggregate and precipitate. This process was characterised by the broad and less intense absorbance peak obtained after 7 days of incubation that coincided with the visual observations and the DLS measurements (Fig. 2A). The proposed reaction is also supported by the complete disappearance of the protein bands in the 7-day samples of the SDS-PAGE analysis.

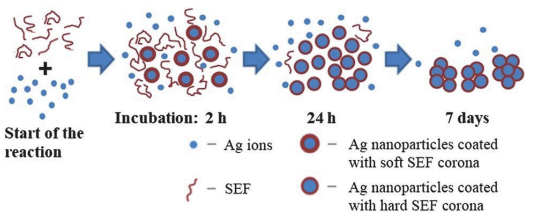

Fig. 6 Schematic illustration of the hypothetical mechanism for the formation of silver nanoparticles assisted by the soluble extracellular fraction (SEF) of *Tetrahymena thermophila*. Soft SEF corona – proteins are weakly bound to nanoparticles; hard SEF corona – proteins are strongly bound to nanoparticles.

The possible toxicity mechanisms of Ag NPs are still under discussion in the scientific community: some have proposed that the toxicity of Ag NPs is solely caused by dissolved Ag ions, others suggest that the particle itself also has a role.[40] Conflicting results can also arise as one particle might have different mechanisms in different organisms as has been demonstrated in the case of CuO NPs.[24,41] Our results support the contribution of Ag ions to Ag NP toxicity in protozoa as longer incubation of SEF-Ag, which resulted in the formation and growth of Ag NPs accompanied by the decrease in Ag ion concentration, leads to the decreased toxicity of SEF-Ag.

It has been proposed in the literature that the biosynthesis of Ag NPs is one of the detoxification routes for Ag ions.[10,11] Although several organisms synthesize Ag NPs intracellularly,[9] *T. thermophila* created an environment that reduced Ag ions to Ag NPs extracellularly. Considering the fact that Ag NPs were less toxic to *T. thermophila* than Ag ions and the toxicity of Ag NPs decreased with the increasing size of the particle, we attribute the formation of Ag NPs catalysed by SEF as one of the primary adaptation mechanisms of *T. thermophila* to toxic metal ions. Further, the formed Ag NPs were taken up and stored intracellularly in the food vacuoles of *T. thermophila*. Indeed, Martín-González *et al.*[42] proposed that biocomplexation could be an important mechanism of resistance of ciliated protozoa to toxic metal ions. The latter mechanism may be one of the adaptational tools for protozoa living in the metal-polluted environments.

Conclusions

The current study demonstrated for the first time that Ag ions were rapidly reduced to Ag NPs by the soluble extracellular fraction (SEF) of the ciliated protozoan *T. thermophila* under illumination at ambient temperature. The proteins of *T. thermophila* SEF were associated with the NP formation and might have also played a role in promoting NP growth. As incubation of silver in SEF reduced its toxicity to *T. thermophila*, formation of Ag NPs may be one of the response mechanisms of the organism to toxic metal ions. The results of the study provide insight into the dynamics of nanomaterials in the aquatic environment by broadening the knowledge on NP formation mediated by cellular exudates, toxicity of NPs and released ions on aquatic species and eventually on other species in the food web.

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Electronic Supplementary Material (ESI) for Environmental Science: Processes & Impacts

Supporting information for: Extracellular conversion of silver ions into silver nanoparticles by protozoan *Tetrahymena thermophila*

Katre Juganson,[a,b] Monika Mortimer[a*], Angela Ivask[a], Kaja Kasemets[a] and Anne Kahru[a]

[a] *Laboratory of Environmental Toxicology, National Institute of Chemical Physics and Biophysics, Akadeemia tee 23, Tallinn, 12618, Estonia*

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

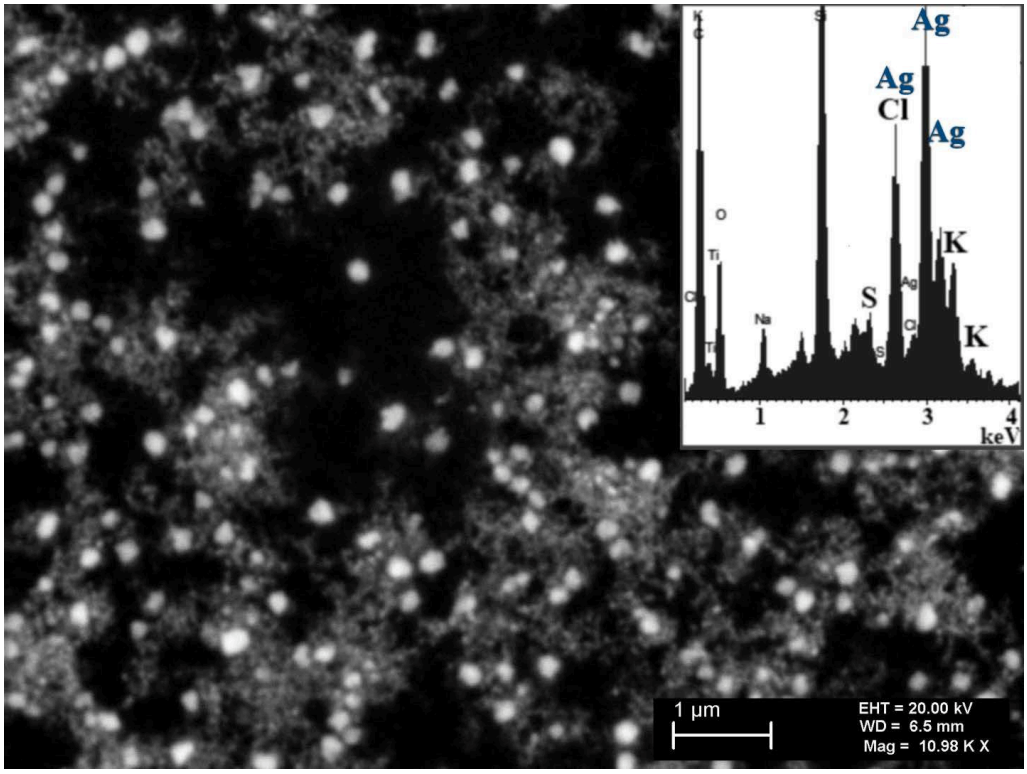

Fig. S1 SEM micrograph (backscattered electron imaging mode) of silver nanoparticles formed after 24-h reaction of $AgNO_3$ (108 mg Ag/L added at time zero) with soluble extracellular fraction (SEF) of *T. thermophila*. The inset represents the spectrum of qualitative energy-dispersive X-ray spectrometry (EDS) analysis of the SEF-incubated $AgNO_3$.

The light spots in the SEM micrograph are Ag NPs and agglomerates. $AgNO_3$ solution with no SEF added resulted in a uniform film (data not shown).

PUBLICATION IV

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Mechanisms of toxic action of silver nanoparticles in the protozoan *Tetrahymena thermophila*: From gene expression to phenotypic events☆

Katre Juganson [a, b, *], Monika Mortimer [a, 1], Angela Ivask [a], Sandra Pucciarelli [c], Cristina Miceli [c], Kaja Orupõld [d], Anne Kahru [a, e]

[a] Laboratory of Environmental Toxicology, National Institute of Chemical Physics and Biophysics, Akadeemia tee 23, 12618 Tallinn, Estonia
[b] Department of Chemistry and Biotechnology, Tallinn University of Technology, Akadeemia tee 15, 12618 Tallinn, Estonia
[c] School of Biosciences and Veterinary Medicine, University of Camerino, Via Gentile III da Varano, 62032 Camerino, Italy
[d] Institute of Agricultural and Environmental Sciences, Estonian University of Life Sciences, Kreutzwaldi 5, 51014 Tartu, Estonia
[e] Estonian Academy of Sciences, Kohtu 6, 10130 Tallinn, Estonia

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ABSTRACT

Silver nanoparticles (AgNPs) are highly toxic to aquatic organisms, however, there is no consensus whether the toxicity is caused solely by released Ag-ions or also by reactive oxygen species (ROS). Here, the effects of protein-coated AgNPs (14.6 nm, Collargol) were studied on viability, oxidative stress and gene expression levels in wild type strains (CU427 and CU428) of ciliate *Tetrahymena thermophila*. Viability-based 24 h EC_{50} values of AgNPs were relatively high and significantly different for the two strains: ~100 mg/L and ~75 mg/L for CU427 and CU428, respectively. Similarly, the expression profiles of oxidative stress (OS) related genes in the two strains were different. However, even though some OS related genes were overexpressed in AgNP-exposed ciliates, intracellular ROS level was not elevated, possibly due to efficient cellular antioxidant defence mechanisms. Compared to OS related genes, metallothionein genes were upregulated at a considerably higher level (36 versus 5000-fold) suggesting that Ag-ion mediated toxicity mechanism prevailed over OS related pathway. Also, comparison between Ag-ions released from AgNPs at EC_{50} concentration and the respective EC_{50} values of $AgNO_3$ indicated that Ag-ions played a major role in the toxicity of AgNPs in *T. thermophila*. The study highlights the importance of combining physiological assays with gene expression analysis in elucidating the mechanisms of action of NPs to reveal subtle cellular responses that may not be detectable in bioassays. In addition, our data filled the gaps on the toxicity of AgNPs for environmentally relevant and abundant organisms. The parallel study of two wild type strains allowed us to draw conclusions on strain to strain variability in susceptibility to AgNPs.

1. Introduction

Silver has been used as an antibacterial agent in the form of silver ions or colloidal solutions for centuries (Fung and Bowen, 1996). Today, silver is widely incorporated into consumer products as nanoparticles: more than 440 nanosilver (AgNP) containing products are on the market (Project on Emerging Nanotechnologies, 2013; http://www.nanotechproject.org/cpi/), large part of these being antimicrobial textiles (Piccinno et al., 2012). According to literature up to 75% of AgNPs may be released from textiles in one washing cycle and, thus, may end up in receiving ecosystems (Mitrano et al., 2016b). Furthermore, in the end of the life cycle, a considerable share of silver-enabled products are disposed of in landfills (Reidy et al., 2013). Even though AgNPs are likely not very mobile in landfills (Mitrano et al., 2016a) they may pose a risk to the environment as silver can be toxic to some fresh water organisms, such as algae and zooplankton, already in

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* Corresponding author. Laboratory of Environmental Toxicology, National Institute of Chemical Physics and Biophysics, Akadeemia tee 23, 12618 Tallinn, Estonia.
E-mail address: katre.juganson@kbfi.ee (K. Juganson).
[1] Present address: Bren School of Environmental Science & Management and Earth Research Institute, University of California, Santa Barbara, California 93106, United States.

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parts per billion (ppb) concentrations (Bondarenko et al., 2013b).

Toxicity and antimicrobial activity of AgNPs has been recently reviewed from the standpoint of AgNP effects to target and non-target organisms, i.e. targeted biocidal effects against pathogenic microbes and biofilm formation *versus* other aquatic organisms (Bondarenko et al., 2013b). Based on the analysis and synthesis of extensive data sets on AgNP toxicity to different types of organisms and in various test conditions it was concluded that the overall trend indicated higher toxicity of AgNPs to mostly eukaryotic non-target than prokaryotic target organisms. This finding highlighted the importance of designing NPs that would be more specific to target species, but also prompted to elucidate the mechanisms of toxic action of AgNPs to non-target species.

Recently we showed that albeit the ecotoxic effects of AgNPs have been studied extensively, there is no consensus concerning the mechanism of action of AgNPs (Juganson et al., 2015). Similar conclusion was reached in several reviews published within the past few years (Duran et al., 2016; Ivask et al., 2014b; McShan et al., 2014). In the current study we used a freshwater ciliate *Tetrahymena thermophila* as a model eukaryotic organism to shed light on the mechanisms of toxic action of AgNPs. *T. thermophila* is an ecologically relevant model organism for nanotoxicology because ciliates play an important role in aquatic food webs being significant grazers of bacteria and a food source for metazooplankton (Sherr and Sherr, 2002). Differently from mostly autotrophic unicellular algae that have previously been used in AgNP toxicity studies (Li et al., 2015; Navarro et al., 2015), the ciliate *T. thermophila* is a heterotroph that internalizes NPs by phagocytosis, thus, is exposed to NPs not only through cell surface but also via phagosome membranes (Kahru et al., 2008; Mortimer et al., 2010). Moreover, its fully sequenced macronuclear genome provides an opportunity for targeted gene expression analysis in response to toxicant exposure (Eisen et al., 2006).

To our knowledge, no study has explored NP effects on different wild type ciliate strains to date, even though it has been acknowledged that attention should be paid to the strain of the test organism used and the differences in wild type phenotypes in toxicity testing (Rogowska-Wrzesinska et al., 2001; Vignet et al., 2013). In this paper we compare the gene transcription and physiological level responses of two wild type strains of *T. thermophila* upon exposure to sub-lethal concentrations of AgNPs. Our approach to connect the effects at gene expression level to the physiological changes aims to shed light on the somewhat controversial literature reports about the AgNP mechanism of toxic action: gene expression studies generally suggest that AgNPs generate oxidative stress (Chae et al., 2009; Niazi et al., 2011; Yeo and Kang, 2008), while bioassay-based studies have reported contradicting results in the induction of different oxidative stress markers such as intracellular reactive oxygen species (ROS) levels, superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX) activities (Gomes et al., 2014; Ulm et al., 2015; Wu and Zhou, 2012). Here we assessed the transcription regulation of metallothionein (MTT), heat shock protein (hsp), SOD, CAT, GPX and glutathione reductase (GSR) genes in parallel with biomarkers often associated with oxidative stress: cell viability, intracellular ROS level, lipid peroxidation, SOD and CAT activities in *T. thermophila* to elucidate the mode of action of AgNPs in this model eukaryotic organism.

2. Experimental

2.1. Chemicals

Casein-coated colloidal silver NPs (AgNPs, primary size 14.6 ± 4.7 nm) were purchased from Laboratories Argenol S. L. (Collargol, batch N° 297) and dispersed in deionized water (MilliQ, Millipore) at 1 g/L. Carboxyl-functionalized polystyrene NPs (PS NPs, primary size 26 nm, 9.97% w/v in deionized water supplemented with 0.05% NaN_3 and ≤0.5% of surfactant) were from Bangs Laboratories (Lot Number 10 048). $AgNO_3$ was purchased from Sigma-Aldrich as a 0.1 M solution. All chemicals used in the study were of analytical grade. Stock suspensions/solutions of Ag-compounds were stored at room temperature in the dark; PS NP stock suspension was stored at +4 °C in the dark. The dilutions of NPs and $AgNO_3$ were prepared in MilliQ water, the pH of the dilutions varied from 6 to 6.5.

2.2. Characterization of nanoparticle dispersions

The hydrodynamic diameters of AgNPs and PS NPs in MilliQ water were measured using a Zetasizer Nano ZS (Malvern Instruments). Dissolution of AgNPs in MilliQ water was quantified as follows: AgNP dispersions at 20, 75 and 100 mg Ag/L were incubated in polystyrene Petri dishes (90 mm) at 25 °C in the dark for 2 and 24 h, NPs were pelleted by ultracentrifugation at 390 000*g*, 20 °C, for 1 h and Ag concentration in the supernatant was quantified by atomic absorption spectroscopy (AAS, Shimadzu AA-6800) equipped with graphite furnace atomizer (GFA-EX7). Detailed physico-chemical characterization of AgNPs has been reported previously (Blinova et al., 2013; Bondarenko et al., 2013a) and is summarized in Table S1.

The potential of NPs and $AgNO_3$ to generate reactive oxygen species (ROS) in abiotic conditions in the dark was determined as described previously (Aruoja et al., 2015), using 2′,7′-dichlorofluorescein-diacetate (DCFH-DA, Life Technologies) and hydroxyl radical-specific 3′-(*p*-hydroxyphenyl) fluorescein (HPF, Life Technologies). Mn_3O_4 NPs (Aruoja et al., 2015) (provided by University of Bremen) and Fenton reaction with 1.1 mg Fe/L of $FeSO_4x7H_2O$ (Reachim, analytical grade) and 15 mg/L H_2O_2 (Sigma-Aldrich) were used as positive controls in general ROS assay (DCFH-DA) and hydroxyl radical generation assay (HPF), respectively.

2.3. Test strains, culturing and viability assay

Standard inbred *T. thermophila* strains CU427 (Chx/Chx [cycloheximide sensitive, mating type VI]) and CU428 (Mpr/Mpr [6-methyl purine sensitive, mating type VII]) of the same background as strain SB210, whose macronuclear genome is sequenced, were used in this study (Bruns and Cassidy-Hanley, 2000). Protozoa were cultured and their viability, based on cellular ATP concentration, was determined as described in Jemec et al. (2016). See the Supplementary material for details.

2.4. Exposure to AgNPs and control chemicals

In bioassays and gene expression studies, *T. thermophila* was exposed to AgNPs at 20 mg Ag/L - a nominal sub-lethal concentration as determined from the dose-response curve (Fig. S1). $AgNO_3$ was used as an ionic silver control, and the exposures were done at a concentration equal to the dissolved silver concentration in the dispersion of AgNPs at 20 mg Ag/L. The respective concentration was determined by AAS analysis after 24-h incubation of AgNPs in MilliQ water as described above, and was 1.5 mg Ag/L. PS NPs served as an insoluble particle control and were used at the exposure concentration of 11 mg/L that was calculated to be an equal number concentration to AgNPs at the concentration of 20 mg/L (see Supplementary material). Positive control for ROS-related damages was Fenton reaction triggered by mixing of 1.1 mg Fe/L of $FeSO_4x7H_2O$ and 15 mg/L H_2O_2 (Aruoja et al., 2015). All the compounds were used at sub-lethal concentrations (Fig. S2).

All exposures were conducted in triplicates in 24-well polystyrene plates (Falcon) at 25 °C in the dark and sampled for analysis at 2 h and 24 h. Protozoa were visualized with light microscope Olympus CX41equipped with DP71 camera to monitor the cells for any changes in the mobility and appearance after the exposures.

2.5. Gene expression quantification and physiological assays

Real-time PCR was used to measure the effects of NPs and control chemicals on the expression levels of selected genes listed in Table S2. Physiological changes were assessed using the following assays: (i) DCFH-DA for intracellular ROS, (ii) thiobarbituric acid reactive substances (TBARS) assay for lipid peroxidation (LPO) (Mortimer et al., 2011), (iii) H_2O_2 degradation for catalase (CAT) activity, and (iv) 19 160 SOD determination Kit (Sigma-Aldrich) for superoxide dismutase (SOD) activity. The assays were performed as described in Supplementary material.

2.6. Data analysis

ATP concentrations in the protozoan samples were expressed as percentages of the untreated control. The concentration-effect curves by the log-normal model were constructed and the EC_{50} values (the effective concentration that induces a response in 50% of the population) with their respective 95% confidence intervals were calculated based on nominal concentrations using REGTOX software for Microsoft Excel™ (Vindimian, 2005). Statistical differences between the untreated control and treated samples were evaluated in R using a one-way analysis of variance (ANOVA) followed by Tukey post-hoc test. Results were considered statistically significant when $p < 0.05$.

3. Results and discussion

3.1. Characterization of AgNPs and selection of control chemicals

One of the key components in toxicity of many metal-containing nanomaterials has been shown to be their dissolution (Bondarenko et al., 2013b; Heinlaan et al., 2008; Kahru and Ivask, 2013). Moreover, as the dissolution strongly depends on the test environment (Kaekinen et al., 2011) the selection of appropriate test medium is of critical importance. Suppi et al. (2015) demonstrated that compared to conventional laboratory assays, often performed in organics-rich growth media, exposure of bacteria, yeasts and unicellular algae to toxic chemicals (especially to metals) in deionized water resulted in 10–1000 times higher toxicity. In the current study, for the purposes of elucidating the mechanism of action of AgNPs to *T. thermophila*, the test system was simplified by conducting the exposures in deionized water to avoid interactions of media components with AgNPs and dissolved Ag-ions. *T. thermophila*, owing to its contractile-vacuole system that is responsible for osmoregulation (Frankel, 2000), is known to survive in deionized water for at least a week (Koppelhus et al., 1994). Additionally, deionized water has proven to be a suitable exposure medium in toxicity assays with *T. thermophila* (Jemec et al., 2016; Juganson et al., 2013). Starved *T. thermophila* are known to undergo physiological, biochemical and molecular changes (Cassidy-Hanley, 2012). However, in the context of the current study these changes were not crucial factors in interpreting the results because the exposures to AgNPs and control chemicals were in the same conditions, i.e., in deionized water, as the cells without added chemicals. Thus, any reported changes in gene expression or physiology of protozoa were assumed to be induced by the chemical exposures.

The ongoing debate (Djurisic et al., 2015; Zhang et al., 2016) over whether engineered nanomaterials (ENMs) have particle-specific adverse effects or the effects are driven by the same mechanisms as for bulk sized materials and/or the ionic constituents of the respective ENMs necessitates the incorporation of proper controls in the toxicity studies. Here, $AgNO_3$ was used as a control for dissolved Ag-ions, expected to be present in AgNP dispersions. Expectedly, 2.1–6.6% of Ag solubilized from AgNPs as a function of dose and time (Fig. S3). These values were in the similar range as the ones reported previously for 4–24-h incubation in deionized water for the same NPs (Bondarenko et al., 2013a; Kaosaar et al., 2016). Polystyrene particles, reportedly incapable of ROS generation (Xia et al., 2006), with an advertised diameter of 26 nm that was in the similar size range of AgNPs selected for the study (Table S1) were used as a control for nanoparticle exposure. According to DLS measurement the hydrodynamic diameter of PS NPs (27.9 ± 0.1 nm) was close to their primary size and the particles were monodisperse in MilliQ water (Fig. S4). Both AgNPs and PS NPs were stable in MilliQ water and did not agglomerate significantly over the time of the experiment.

One of the mechanisms of toxic action proposed for ENMs, including AgNPs, is the production of ROS (Ivask et al., 2014b; McShan et al., 2014; Zhang et al., 2016). Therefore, we first determined the ability of AgNPs to induce ROS in abiotic conditions, i.e. with no test organisms present. AgNPs generated ROS in MilliQ water time- and dose-dependently, as determined by increase in the fluorescence of ROS indicator dye DCFH (Fig. S5A). ROS induction was clearly specific to AgNPs as no increase in the fluorescence of DCFH in the $AgNO_3$ solution or dispersion of PS NPs was observed. Similarly, Ivask et al. (2014c) reported recently that citrate stabilized 10 nm AgNPs at concentrations >10 mg/L and 20 nm AgNPs at concentrations > 50 mg/L generated ROS in abiotic conditions. According to Neal (2008), abiotic generation of ROS is possible through light-dependent detachment of electrons from the surface of ENMs that could initiate a chain of radical reactions resulting in production of ROS. The generation of abiotic ROS by AgNPs in the dark, i.e., in the conditions used in this study, is likely initiated by the oxidative dissolution of AgNPs, a reaction that proposedly produces AgNP-O_2^- adducts and H_2O_2 (Ho et al., 2011). Casein coating of collargol AgNPs likely contributes to generation of ROS by its amine groups and conjugated double bonds. An attempt to assess the potential of AgNPs to generate highly reactive hydroxyl radicals by employing the HPF assay was not successful because HPF fluorescence was quenched in the presence of AgNPs (Fig. S5B). Thus, although successfully used for measurement of abiotic hydroxyl radical production by various different NPs at similar concentrations as used here (Aruoja et al., 2015), the assay was not applicable with AgNPs. The result highlights the importance of considering NP-specific interferences with fluorescence assays. Ag-ions and PS NPs did not produce hydroxyl radicals in the concentration range tested.

3.2. Tetrahymena thermophila viability upon exposure to Ag-compounds

2 and 24 h EC_{50} values of the casein-coated AgNPs for the wild type strains of *T. thermophila* studied in the current work ranged from 72 to 100 mg Ag/L (Table 1). These values were almost two orders of magnitude higher than the EC_{50} values of PVP-coated AgNPs for *T. thermophila* strain BIII (3.2–3.9 mg Ag/L) reported by Jemec et al. (2016). These significantly different values could be explained by higher share of soluble Ag^+-species (approximately 50%) present in PVP-coated AgNP stock. As the toxic effects of AgNPs to aquatic organisms can often be attributed to the release of Ag-ions, *T. thermophila* was exposed to $AgNO_3$. Compared to the tested AgNPs, the EC_{50} values of Ag-ions were about 50-fold lower,

Table 1
The toxicity[a] (2 and 24 h EC_{50}, mg Ag/L) of silver nitrate and AgNPs (Collargol) to two strains of *Tetrahymena thermophila* (CU427 and CU428). The exposure to toxicants was performed in MilliQ water at 25 °C in the dark. Pairwise comparison of the statistical difference of EC_{50} values is presented in Fig. S6.

Exposure time	EC_{50}[b], mg Ag/L			
	AgNPs		$AgNO_3$	
	CU427	CU428	CU427	CU428
2 h	98 (92–103)	72 (67–75)	2.2 (2.1–2.3)	1.8 (1.7–2.0)
24 h	100 (94–114)	79 (76–82)	2.8 (2.5–3.4)	2.6 (2.4–2.8)

[a] Calculated from respective dose-response curves (Fig. S1) using ATP concentration as a viability endpoint.
[b] Average values (95% confidence intervals) based on nominal concentrations of at least three independent assays.

ranging from 1.8 to 2.8 mg Ag/L. Comparison of the dose-response curves (Fig. S1) of both strains for $AgNO_3$ and AgNPs showed that the curves had similar slopes indicating a similar mode of action for these compounds, which is in accordance with previous findings suggesting that Ag-ions are important in the antimicrobial activity of AgNPs (Duran et al., 2016). Indeed, when we re-calculated the EC_{50} values of AgNPs on the basis of the dissolved fraction in NP dispersions, the EC_{50} values were very similar to the respective values of $AgNO_3$ (Fig. S7), supporting the role of Ag-ions in the toxicity of AgNPs.

Interestingly, compared to the most vulnerable aquatic organisms – crustaceans, algae and fish – whose median EC_{50} values ranged from less than 1 μg Ag/L to several hundred μg Ag/L upon exposure to Ag-ions and from about 1 μg Ag/L to about 100 mg Ag/L upon exposure to AgNPs (Bondarenko et al., 2013b; Juganson et al., 2015), *T. thermophila* tolerated relatively high concentrations of Ag-compounds. One explanation for that could be the efficient mitigation of silver toxicity by reduction of Ag^+ into less harmful metallic silver by *T. thermophila*'s secreted extracellular substances as we have shown in our previous work (Juganson et al., 2013).

The interspecies differences in silver toxicity indicate the role of cellular physiology in metal and NP toxicity. However, in addition to interspecies differences there can also be variability in toxicities between different strains or organisms of the same species or, as has been demonstrated in AgNP studies, mutant strains of bacteria (Ivask et al., 2010, 2014a), yeast (Kaosaar et al., 2016) and nematode *Caenorhabditis elegans* (Meyer et al., 2010). Here we compared the toxicity of AgNPs and $AgNO_3$ to two wild type strains of *T. thermophila* with almost identical genetic backgrounds (related to the sequenced strain SB210). Interestingly, these two ciliate strains had significantly ($p < 0.05$) different responses to AgNPs with strain CU428 having EC_{50} values about 20–30% lower than the strain CU427 (Table 1 and Fig. S6). On the other hand, the EC_{50} values for $AgNO_3$ were not statistically different in the two strains (Table 1, Fig. S6), and were comparable to previously published $AgNO_3$ EC_{50} values for another *T. thermophila* wild type strain BIII (1.5–2.9 mg Ag/L) (Jemec et al., 2016; Juganson et al., 2013).

3.3. Sub-lethal effects of AgNPs to Tetrahymena thermophila

Although the dissolution corrected EC_{50} values of AgNPs were similar to EC_{50} values of $AgNO_3$ (Fig. S7), indicating the primary role of Ag^+ in NP exerted toxicity, as also reported previously (Burkart et al., 2015; Juganson et al., 2013), it was not unambiguously clear that Ag^+-mediated toxicity was the sole mechanism of action for AgNPs. Namely, AgNPs, differently from $AgNO_3$ and PS NPs, generated ROS in abiotic conditions as described above (Fig. S5A) which was indicative of possible oxidative stress related effects of AgNPs. In order to elucidate the mechanisms of action of AgNPs in *T. thermophila* the expression of selected stress-, oxidative stress-related and metal-binding genes was monitored at sub-lethal concentrations. In total, the expression of ten genes in response to AgNPs, $AgNO_3$ and PS NPs was studied. The selection criteria for the exposure concentrations of AgNPs (20 mg Ag/L) and controls used (1.5 mg Ag/L for $AgNO_3$, 11 mg/L for PS NPs) are described in the Experimental section (2.4).

3.3.1. Expression of general stress related genes

The effect of AgNPs on the induction of genes encoding heat shock proteins (hsp703, 704, 705, belonging to the family of cytosolic Hsp70 proteins (Yu et al., 2012)) was studied to assess the general environmental stress (Barchetta et al., 2008). The involvement of Hsp proteins in Ag-triggered stress response pathways has also been shown in an earlier study where whole genome array of AgNP-exposed nematode *C. elegans* was performed (Roh et al., 2009). Interestingly, although the slopes of the dose-response curves and toxicity of AgNPs and Ag-ions to the two studied protozoan strains (Fig. S1) were similar, the expression profile of the selected Hsp genes in these strains was different (Fig. 1). While in strain CU427 sub-lethal exposure concentration (20 mg Ag/L) of AgNPs up-regulated hsp704 after 2-h and hsp703 expression after 24-h exposure (77- and 6-fold, respectively; Fig. 1A), and similar findings were seen with Ag-ions that induced hsp704 and hsp703 after 2-h exposure (55- and 4-fold, respectively), no changes in expression of these genes were observed in strain CU428 (Fig. 1B). Thus, the mechanism of action of Ag-compounds according to the expression of these two genes is not universal in different strains. As Hsp proteins have important role in many biological processes, e.g. protein folding (Fukuda et al., 2015), up-regulation of Hsp genes should aid the cells to rapidly cope with stress. Indeed, in strain CU427 upregulation of hsp704 and 703 coincided with lower toxicity of Ag-compounds as reported above (Table 1). Interestingly, in strain CU427 also PS NPs induced a slight but significant up-regulation of hsp703 upon 24-h exposure (Fig. 1). This may indicate the potential of AgNPs to trigger NP-specific stress in the cells. Contrarily to hsp 703 and 704, the expression of hsp705 was down-regulated after 2-h exposure to Ag-ions and AgNPs approximately at similar levels in both strains (Fig. 1). A recent study with *T. thermophila* revealed that although hsp705 is heat-inducible, Hsp705 protein could be compensated by other analogous proteins in response to heat-stress (Fukuda et al., 2015). Moreover, hsp705 expression levels in *T. thermophila* are low during starvation (TetraFGD; http://tfgd.ihb.ac.cn/) suggesting that the gene is non-essential; thus, down-regulation of this gene might indicate that the cells need to redirect their resources to cope with Ag-triggered stress in starvation conditions.

3.3.2. Expression of oxidative stress related genes

The other group of genes selected for gene expression analysis was antioxidant defence genes. Most important ROS scavenging enzymes are (i) superoxide dismutase (SOD) that catalyses the dismutation of superoxide ($O_2^{\bullet-}$) into oxygen and hydrogen peroxide, (ii) catalase (CAT) that directly dismutates hydrogen peroxide into water and oxygen, and (iii) glutathione peroxidase (GPX) that uses glutathione to reduce hydrogen peroxide into water and lipid hydroperoxides into their corresponding alcohols (Gill and Tuteja, 2010). In addition, there are several other enzymes involved in antioxidant defence mechanism, e.g. glutathione reductase (GSR) that catalyses the reduction of oxidized glutathione, and some enzymes that based on *Tetrahymena* Genome Database (TGD; http://ciliate.org/index.php/home/welcome) are not present in *T. thermophila*.

Superoxide dismutases. *T. thermophila* features four different SODs – one mitochondrial Mn-SOD (SOD1), and three isoforms of

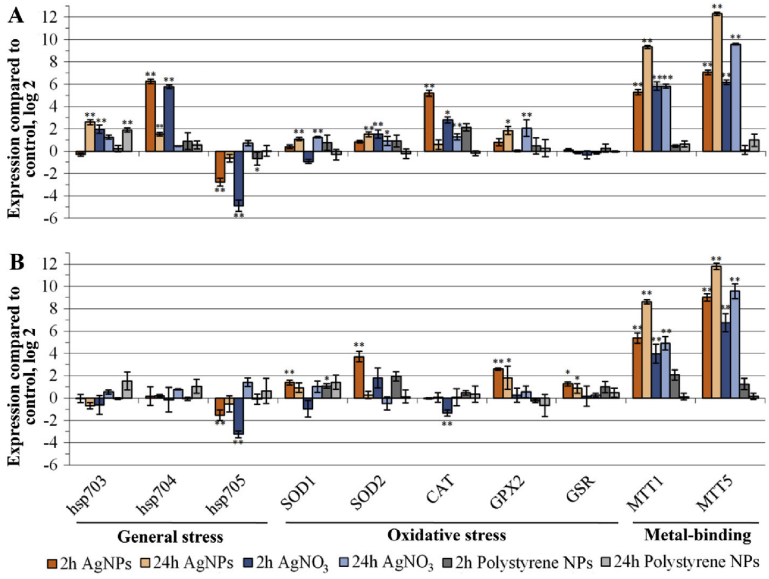

Fig. 1. The alteration of expression of selected genes in two different *Tetrahymena thermophila* strains CU427 (A) and CU428 (B) upon exposure to sub-lethal concentrations of AgNPs, Ag-ions and polystyrene NPs for 2 h and 24 h at 25 °C in the dark. The asterisk (*) marks significant difference ($p < 0.05$) and (**) marks highly significant difference ($p < 0.01$) from the expression levels in the respective strain of protozoa incubated in MilliQ water; note the logarithmic scale of the y-axis. hsp – heat shock protein, SOD – superoxide dismutase, CAT – catalase, GPX – glutathione peroxidase, GSR - thioredoxin and glutathione reductase, MTT – metallothionein.

cytosolic Cu/Zn-SOD. One of the latter isoforms, proposed to have major importance in countering oxidative stress (Ferro et al., 2015), was included in the study (SOD2). Slight but significant 2-fold up-regulation of SOD1 was detected after 24-h exposure to AgNPs in strain CU427 (Fig. 1A), and after 2-h exposure in CU428 (Fig. 1B). In addition, AgNPs induced significant 3-fold up-regulation of SOD2 in CU427 after 24-h exposure (Fig. 1A) and almost 14-fold up-regulation of SOD2 in CU428 after 2-h exposure (Fig. 1B). In strain CU427 Ag-ions elevated SOD1 expression about the same extent as AgNPs after 24-h exposure and SOD2 expression after both, 2- and 24-h exposure. In general, exposure to Ag-compounds resulted in similar expression patterns of both SOD genes in strain CU427. However, SOD responses in strain CU428 appeared to be more AgNP-specific since the exposure to Ag-ions did not alter the expression significantly. Up-regulation of mitochondrial Mn-SOD by AgNPs but not Ag-ions has also been observed for *C. elegans* and the gene has been found important in response to AgNP-induced toxicity (Roh et al., 2009).

Catalase. According to *Tetrahymena* Genome Database (TGD), there is only one CAT gene in *T. thermophila*. In strain CU427, AgNPs elevated CAT expression 36-fold upon 2-h exposure (Fig. 1A), whereas Ag-ions induced up to 7-fold upregulation of CAT after both, 2- and 24-h exposure. Sub-lethal exposures to AgNPs have elevated CAT levels also in other organisms, e.g., the midge *Chironomus riparius*, suggesting excess formation of H_2O_2 by SODs (Nair et al., 2013). Contrarily, no changes in expression levels of CAT were seen in strain CU428 (Fig. 1B), again, indicating a strain-specific response in *T. thermophila*.

Glutathione peroxidase and glutathione reductase. There are several GPX and GSR genes in *T. thermophila*, however, the literature data about effects of different toxicants on the expression of these genes is scarce. Thus, one GPX (designated as GPX2) and one GSR gene were selected based on the expression profiles available in *Tetrahymena* Functional Genomics Database (TetraFGD) and the uniform expression level in starving cells during 24 h. AgNPs induced expression of GPX2 in strain CU427 after 24-h exposure and in CU428 upon both, 2- and 24-h exposure (Fig. 1). Due to somewhat overlapping role of CAT and GPX2, it could be assumed that in strain CU428, reduction of H_2O_2 is primarily catalyzed by GPX2 and thus, induction of CAT gene was not observed as discussed above. Additionally, GPX2 was up-regulated in CU427 but not in CU428 upon 24-h exposure to Ag-ions. Differences in the expression profiles of GPX gene were also observed in AgNP and Ag-ion exposed juvenile rainbow trout livers but, unlike in *T. thermophila*, AgNPs were shown to decrease and Ag-ions increase the GPX expression (Gagne et al., 2012). Interestingly, the expression levels of GSR varied only in AgNP exposed CU428 strain up to 2-fold that was considered significant (Fig. 1) and is in line with GPX2 expression pattern in this strain.

Overall, the gene expression analysis revealed that *T. thermophila* may experience mild oxidative stress upon exposure to AgNPs and Ag-ions. While in strain CU427 exposure to AgNPs and Ag-ions resulted in similar gene expression patterns, the changes in gene expression of strain CU428 appeared to be AgNP-specific. However, these changes could not be attributed to NP size-specific effects as the expression levels of selected genes in protozoa exposed to PS NPs of the same size as AgNPs were not significantly different from those in the control cells (Fig. 1). We also noticed that the two strains differed in the exposure time required for the gene up- or down-regulation to take place: while in strain CU428 the changes in gene expression were generally detected after 2-h exposure, in strain CU427 the differential regulation of selected genes occurred often after 24-h exposure. This may indicate that the metabolism rate in strain CU428 is higher than in strain CU427. Indeed, CU428 was shown to maturate faster upon osmotic shock treatment compared to strain CU427 (Cole and Bruns, 1992).

3.3.3. Expression of metallothionein genes

Metallothioneins (MTT) have diverse roles in the cells, including the scavenging of ROS (Viarengo et al., 2000), and sequestration of toxic metals (Liu and Klaassen, 1996). *T. thermophila* has five different MTTs that fall into two groups – cadmium and copper responsive MTTs (Diaz et al., 2007). Although heavy metal treatments have been shown to result in similar induction patterns of MTT1 and MTT5, and both behave as a multi-stress response proteins, MTT5 responds to the widest range of stressors (Diaz et al., 2007). Here, AgNP exposure caused upregulation of both MTT genes in both *T. thermophila* strains (MTT1 40–650 fold, MTT5 130-5000 fold; Fig. 1). The induction patterns were very similar in both strains. In general, after 2 h the expression levels in AgNP- and Ag-ion exposed cells were similar, while after 24-h treatment the expression of MTT genes was significantly higher in AgNP-exposed cells. These findings suggest that Ag-ions are taken up by *T. thermophila* cells, and AgNPs may act via Trojan horse type mechanism (Park et al., 2010) by releasing additional ions inside the protozoan cell. The hypothesis that MTT levels were elevated due to Ag-ions released from AgNPs was proposed also by Choi et al. (2010) who observed dose-dependent increase in MTT expression levels up to 7.1 fold in zebrafish liver upon treatment with non-lethal dose (120 mg Ag/L) of AgNPs.

3.3.4. Oxidative stress related physiological responses in Tetrahymena thermophila

To associate the findings of the gene expression analysis with the effects at the physiological level we determined the levels of intracellular ROS, lipid peroxidation, and activity of SOD and CAT enzymes in protozoa. In these assays, strains CU427 and CU428 were exposed to the test compounds at sub-lethal concentrations (Fig. S2), in the same conditions as in the gene expression studies. Microscopic observations further confirmed that the cell morphology and mobility in test compound treated cultures were visually comparable to the non-treated control cells (Fig. S8). Visual changes in ciliate cells were observed only in AgNP-exposed samples, where food vacuoles appeared dark after 2-h exposure (Fig. 2B), due to internalised AgNPs, and extracellular AgNP agglomerates consisting of expelled food vacuole contents were evident after 24 h (Fig. 2B, Fig. S8C). The latter finding has been reported also in several previous studies with different ENMs (Aruoja et al., 2015; Blinova et al., 2010; Juganson et al., 2013; Mortimer et al., 2010, 2011, 2014, 2016).

Intracellular generation of reactive oxygen species. Since the AgNPs used in this study generated ROS in abiotic conditions (Fig. S5) and triggered slight oxidative stress responses at gene expression level (Fig. 1), we measured intracellular ROS in AgNP-exposed *T. thermophila* cells. The assay utilizing the ROS indicator dye DCFH-DA did not indicate increased intracellular ROS upon exposure to AgNPs, $AgNO_3$ and PS NPs in neither *T. thermophila* strain (Fig. 3A and B). In strain CU427, 2-h exposure to $AgNO_3$ appeared to decrease ROS levels compared to non-treated control (Fig. 3A), however, we cannot explain this result based on the assays conducted in this study. Fenton reaction, used at sub-lethal level to *T. thermophila* as a positive control in bioassays, elevated ROS levels only in strain CU427, indicating different sensitivities of the two strains. Our observations of similar intracellular ROS levels in AgNP-exposed and unexposed cells are in agreement with some previous studies where ROS generation was monitored with DCFH-DA in live cells/organisms exposed to AgNPs. Namely, no ROS production was detected in green algae *Chlamydomonas reinhardtii* nor cyanobacteria *Synechococcus leopoliensis* (Taylor et al., 2016), and even decrease in the fluorescence intensity of DCF with increasing AgNP dose was seen in *Daphnia magna* (Ulm et al., 2015) and embryonic medaka (Wu and Zhou, 2012). In *T. thermophila*, production of ROS has been demonstrated with the same dye upon exposure to CuO NPs and Cu-ion (Mortimer et al., 2011); however, the effective concentrations studied were much higher (EC_{20}, EC_{50}) than those used in the current study. Hence, although no elevated levels of intracellular ROS were detected in *T. thermophila* upon exposure to AgNPs the changes in the expression of oxidative stress related genes (up to 36-fold upregulation) suggest that gene expression is a more sensitive endpoint in detecting subtle oxidative stress levels in the cells than the bioassay used.

Lipid peroxidation (LPO). One of the cellular targets of ROS are lipids. The resulting lipid peroxides decompose rapidly and the main product of this process is malondialdehyde (MDA). Interestingly, even when no intracellular ROS were detected in the protozoa after AgNP exposure (Fig. 3A and B), significant time-dependent increase in LPO was seen after 24-h exposure to AgNPs in strain CU427 (Fig. 3C), but not in strain CU248. (Fig. 3D). $AgNO_3$ induced significant LPO in both strains (Fig. 3C and D), indicating that LPO seen with AgNPs was likely triggered by released Ag-ions. Expectedly, PS NPs did not induce LPO. Our results on LPO upon AgNP exposure are in agreement with previous reports where LPO was demonstrated in AgNP-exposed early-stage embryonic medaka

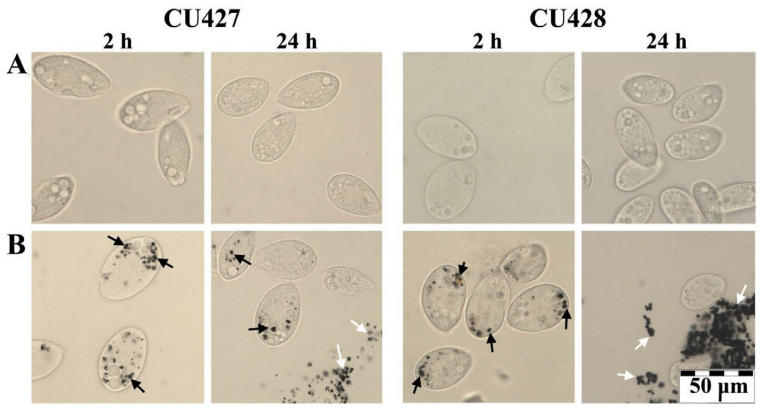

Fig. 2. Bright field images of live *Tetrahymena thermophila* cells (strains CU427 and CU428) after 2- and 24-h incubation in MilliQ water (A) and exposure to nominal sub-lethal concentration of 20 mg Ag/L of AgNPs in MilliQ water (B). Black arrows indicate food vacuoles filled with AgNPs, white arrows indicate extracellular AgNP agglomerates.

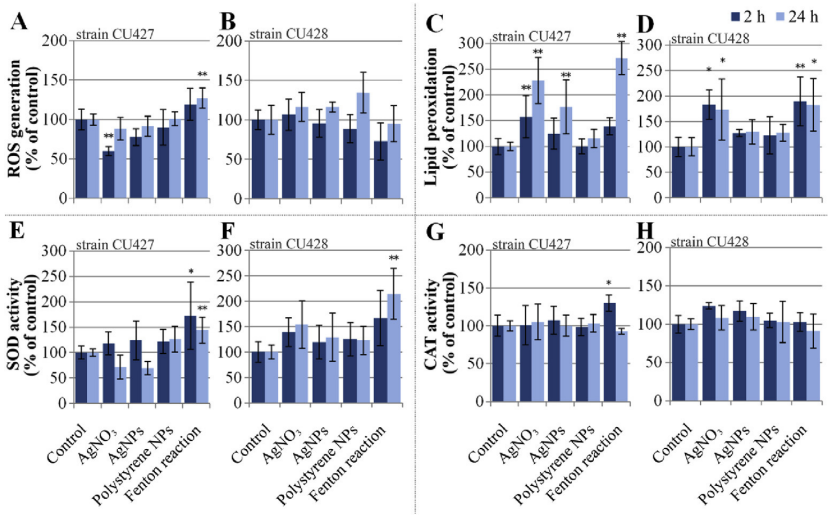

Fig. 3. The potential of sub-lethal concentrations of Ag-compounds, polystyrene NPs, and Fenton reaction to generate ROS (A, B), lipid peroxidation (C, D), and their effect on superoxide dismutase activity (E, F) and on catalase activity (G, H) in *Tetrahymena thermophila* strains CU427 (A, C, E, G) and CU428 (B, D, F, H). The asterisk (*) marks significant difference ($p < 0.05$) and (**) marks highly significant difference ($p < 0.01$) from the respective *T. thermophila* strain incubated in MilliQ water (Control). The bars are the average values of at least 6 replicates and error bars indicate standard deviations.

(Wu and Zhou, 2012), and zebrafish liver (Choi et al., 2010). On the other hand, a few studies have shown that Ag-ions are not as powerful inducers of LPO as AgNPs. Increase in LPO has been observed with AgNPs but not Ag-ions in rainbow trout (Gagne et al., 2012), and although both treatments increased LPO levels in the gills of mussels, LPO levels in AgNP-treated mussels continued increasing in time while no time-dependent changes were detected for Ag-ion-treated mussels (Gomes et al., 2014).

Activity of antioxidant enzymes. Although no excess ROS levels in *T. thermophila* were detected, gene expression study indicated slight to moderate up-regulation of SOD and CAT genes in ciliates exposed to Ag-compounds which may be indicative of increased SOD and CAT enzyme activities. Contrarily, enzymatic assay showed that SOD and CAT activities were not elevated in *T. thermophila* after exposure to AgNPs or $AgNO_3$ (Fig. 3E, F, G, H). This result is in line with the data for AgNP-exposed *D. magna* where no change was observed in SOD levels (Ulm et al., 2015). On the other hand, no increase in SOD and CAT activity does not necessarily prove that the enzymes were not affected by AgNPs as silver might act as SOD and CAT enzyme inhibitor due to its high affinity towards sulfhydryl groups of the protein cysteine residues (Kaekinen et al., 2013). For example, AgNPs have been shown to interact with SOD enzyme (Zhang et al., 2015) and in this study, Ag-ions were inhibitory to catalase (Supplementary material). Thus, the up-regulation of SOD and CAT genes may have been promoted by the necessity to restore the level of active SOD and CAT enzymes in response to inhibition of the activity of these enzymes by silver compounds. We suggest that at sub-lethal concentrations, Ag-compounds likely interacted with SOD and CAT, necessitating overexpression of the respective genes for production of additional enzymes.

4. Conclusions

In this study, we showed that the main cause for AgNP-triggered toxicity in *T. thermophila* wild type strains CU427 and CU428 is NP dissolution. The central role of Ag-ions in AgNP toxicity was confirmed by (i) the similar EC_{50} values of $AgNO_3$ and dissolved-silver based EC_{50} values of AgNPs and similar slopes of dose-effect curves, (ii) up to 5000-fold overexpression of metallothionein genes upon exposure to both AgNPs and $AgNO_3$, and (iii) similar response profiles of AgNPs and $AgNO_3$ in all bioanalytical assays. Collargol AgNPs exerted different effects on the two strains both at physiological and transcriptional level: compared to strain CU427, AgNP EC_{50} values were 20–30% lower and regulation of oxidative stress gene expression was AgNP-specific in strain CU428. Although AgNPs showed the potential to generate ROS in abiotic conditions and some oxidative stress related genes were up-regulated upon exposure to sub-lethal concentrations of Ag-compounds, intracellular ROS levels and SOD and CAT activities were not elevated in protozoa. This suggests that *T. thermophila* has efficient antioxidant defence mechanisms and regulatory pathways to counteract AgNP toxicity. This study highlights the importance of combining physiological assays with gene expression analysis in elucidating the mechanisms of action of NPs. Subtle cellular responses such as oxidative stress related mechanism of AgNPs, undetectable in bioassays, were revealed at the transcriptional level. In addition, the difference in the magnitude of gene upregulation indicated that Ag-ion mediated toxicity mechanism of AgNPs prevailed over oxidative stress related pathway.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.envpol.2017.03.013.

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Supplementary material for

Mechanisms of toxic action of silver nanoparticles in the protozoan *Tetrahymena thermophila*: from gene expression to phenotypic events

Katre Juganson,*[a, b] Monika Mortimer,‡[a] Angela Ivask,[a] Sandra Pucciarelli,[c] Cristina Miceli,[c] Kaja Orupõld[d] and Anne Kahru[a, e]

[a] Laboratory of Environmental Toxicology, National Institute of Chemical Physics and Biophysics, Akadeemia tee 23, 12618 Tallinn, Estonia
[b] Department of Chemistry and Biotechnology, Tallinn University of Technology, Akadeemia tee 15, 12618 Tallinn, Estonia
[c] School of Biosciences and Veterinary Medicine, University of Camerino, Via Gentile III da Varano, 62032 Camerino, Italy
[d] Institute of Agricultural and Environmental Sciences, Estonian University of Life Sciences, Kreutzwaldi 5, 51014 Tartu, Estonia
[e] Estonian Academy of Sciences, Kohtu 6, 10130 Tallinn, Estonia
* Corresponding author: katre.juganson@kbfi.ee
‡ Present address: Bren School of Environmental Science & Management and Earth Research Institute, University of California, Santa Barbara, California 93106, United States

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Methods

Calculation of polystyrene NP mass-based concentration

Polystyrene particles were assumed to be ideal spheres with even densities.

$$C_{PS} = n_{Ag} * m_{PS} = \frac{20\ ^{mg}/_{L}}{m_{Ag}} * V_{PS} * \rho_{PS} = \frac{20\ ^{mg}/_{L}}{V_{Ag} * \rho_{Ag}} * \frac{4}{3} * \pi * {r_{PS}}^3 * \rho_{PS} = \frac{20\ ^{mg}/_{L}}{\frac{4}{3} * \pi * {r_{Ag}}^3 * \rho_{Ag}} * \frac{4}{3} * \pi * {r_{PS}}^3 * \rho_{PS} =$$

$$= \frac{20\ ^{mg}/_{L}}{{r_{Ag}}^3 * \rho_{Ag}} * {r_{PS}}^3 * \rho_{PS} = \frac{20 * 13^3 * 1.06}{7.3^3 * 10.49}\ ^{mg}/_{L} \approx 11\ ^{mg}/_{L}$$

C_{PS} – polystyrene NP mass-based concentration, mg/L
m_{PS} – mass of a polystyrene NP, mg
n_{Ag} –number of AgNPs/L
ρ_{Ag} – density of silver (10.49 g/cm^3)
ρ_{PS} – density of polystyrene (1.06 g/cm^3)
r_{Ag} – radius of an AgNP (7.3 nm)
r_{PS} – radius of a polystyrene NP (13 nm)
V_{Ag} – volume of an AgNP, cm^3
V_{PS} – volume of a polystyrene NP, cm^3

In vitro enzyme activity assays

The enzyme inhibitory effects of NPs and $AgNO_3$ were assessed by measuring the activity of purchased pure superoxide dismutase (SOD) and catalase (CAT) after incubation with the test chemicals. SOD activity was measured using 19160 SOD determination kit (Sigma-Aldrich). 10 µL of SOD (2 U/mL, Sigma) was mixed with an equal volume of test dispersion/solution at a range of concentrations and pipetted into the wells of a transparent 96-well polystyrene microplate (Falcon). 200 µL of water-soluble tetrazolium salt (WST-1, 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) working solution and 20 µL of Enzyme Working Solution or Dilution Buffer were added to samples or blanks, respectively. The plate was incubated at 37 °C with agitation (Titramax 1000 Incubator) for 20 min, followed by the absorbance measurement at λ=450 nm using a multi-mode microplate reader (SpectraMax Paradigm). Inhibition curve was constructed with SOD over a concentration range of 0.1...2 U/mL. SOD activities in the samples were calculated following the manufacturer's protocol.

CAT activity was assessed using the method described by Iwase et al. (Iwase et al., 2013) Briefly, 100 µL of 1% Triton X-100 (Fluka) was pipetted to 5-mL round bottom polypropylene tubes (Greiner Bio-One). 50 µL of CAT (0.5 mg/L, ~2000 U/mg, Sigma) in 50 mM potassium phosphate buffer (PPB, pH 7) was mixed with 50 µL of NPs or $AgNO_3$ at a range of concentrations and added to Triton X-100. For the calibration curve, 50 µL of CAT at the concentration range of 0.125...1 mg/L in 50 mM PPB was mixed with 50 µL of MilliQ water and added to Triton X-100. Subsequently, 100 µL of 30% H_2O_2 was added to the

mixtures and the tubes were incubated at room temperature for 30 min. CAT activity was quantified by taking an image of the tubes and measuring the height of foam produced by released O_2 by counting the pixels in the image.

Tetrahymena thermophila cultivation and viability measurements

100 µl of *T. thermophila* (strains CU427 and CU428) stock culture was pipetted into 10 mL of SSP medium consisting of 2% proteose peptone (Fluka), 0.1% yeast extract (Lab M) and 0.2% glucose, supplemented with antibiotics streptomycin sulphate (Sigma-Aldrich) and penicillin G (Fluka), each at 250 µg/mL, and 1.25 µg/mL of fungicide amphotericin B (Sigma-Aldrich), and incubated in a Petri dish at 30 °C for 18-24 h. The cells were harvested at the exponential growth phase by centrifugation at 300 *g* for 5 min at 4 °C and washed twice with MilliQ water. For experiments with NPs and $AgNO_3$, *T. thermophila* was suspended in MilliQ water at 10^6 cells/mL. For toxicity assays, 100 µL of *T. thermophila* culture was added to 100 µL of test dispersion/solution. After 2- and 24-h exposure in 96-well polystyrene plates at 25 °C in the dark, the toxicity of NPs and $AgNO_3$ was evaluated by measuring the cellular ATP content using the luciferin-luciferase assay (Sigma-Aldrich). ATP was extracted from the cells essentially as in Kahru et al. (Kahru et al., 1982) with modifications as described previously (Jemec et al., 2016; Mortimer et al., 2010).

Gene expression studies with *Tetrahymena thermophila*

For gene expression studies, *T. thermophila* cells were exposed to AgNPs, $AgNO_3$ and PS NPs in above-mentioned concentrations and conditions in Petri dishes with 4 compartments, each treatment in two replicates. After 2- and 24-h exposure, total RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. RNA concentration was measured at λ=260 nm and the purity of RNA was in the range of 1.8-2.2 ($\lambda_{260\ nm}/\ \lambda_{280\ nm}$). The samples were further digested with DNase I (Fermentas) and reverse transcribed into cDNA using M-MuLV Reverse Transcriptase (Thermo Scientific) following the procedure suggested by the provider. For real-time PCR, Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific) was used and the reaction mix was prepared according to the manufacturer's protocol. The primers used to amplify selected genes are listed in Table S2. Real-time PCR was performed in a 96-well thermal cycler (Bio-Rad) using the following cycling program: 10 min at 95 °C, followed by 40 cycles of 15 sec at 95 °C and 60 sec at 55 °C. Two replicate reactions were performed for each primer pair. The relative ratio of each gene was calculated according to Yu et al. (Yu et al., 2012) by using *T. thermophila* 17srRNA gene (GenBank ID M10932) as a reference.

Assays for intracellular ROS generation and SOD and CAT activity

T. thermophila samples were subjected to intracellular ROS analysis immediately after exposure. For lipid peroxidation analysis, samples were frozen and kept at -20 °C until analysis, and for catalase (CAT) and superoxide dismutase (SOD) activity analysis, the samples were frozen with liquid nitrogen in order to disrupt the cells.

Generation of ROS in the cells was measured by adding 10 µL of 1.03 mM DCFH-DA to 100 µL of protozoan suspension pipetted in the wells of a black 96-well polypropylene microplate (Greiner Bio-One) and incubated for 1 h at 25 °C in the dark. The fluorescence was quantified using the Fluoroskan Ascent FL microplate reader (excitation 485 nm, emission 527 nm) and normalized to cellular ATP content (biomass of viable cells).

CAT activity in the samples was determined as described in Li and Schellhorn (Li and Schellhorn, 2007). Briefly, thawed protozoan samples from different exposure scenarios (see above) were diluted 10-fold in 50 mM potassium phosphate buffer (pH 7). 200 µL of sample was transferred into transparent 96-well UV-Star microplate (655801, Greiner Bio-One). 50 µL of 0.2% H_2O_2 was added to the sample and the decrease in absorption was measured at λ=240 nm every 15 sec for 5 min using a spectrophotometer (Thermo Scientific Multiskan Spectrum). Calibration curve was constructed with CAT (Sigma) over the concentration range of 0.5-2 U/mL.

For SOD activity measurements the samples were thawed and diluted 5-fold in the Dilution Buffer from 19160 SOD determination kit (Sigma-Aldrich). 20 µL of the diluted sample was transferred into a transparent 96-well polystyrene microplate (Falcon) and SOD activity was determined following the manufacturer's protocol as described above for the pure SOD activity (*In vitro* enzyme activity assays).

Results

Effect of Ag-compounds and polystyrene NPs on SOD and CAT activity *in vitro*

Prior to using the SOD assay for assessment of the effect of Ag-compounds on purified SOD activity, the effect of Ag on the performance of the assay was tested first. AgNPs and $AgNO_3$ at concentrations used in *T. thermophila* study (1.5 mg Ag/L for Ag ions and 20 mg Ag/L for AgNPs) interfered with the assay performance by inhibiting an assay component - xanthine oxidase (Fig. S9). When Ag concentrations that did not interfere with the assay performance (0.3 mg Ag/L for Ag-ions and 4 mg Ag/L for AgNPs) were tested, no significant inhibitory effect by Ag-compounds on SOD enzyme was found. On the other hand, Ag-compounds did not appear to interfere with the SOD assay in the cell-based tests, i.e., where intracellular SOD activity was measured in *T. thermophila* after exposure to sub-lethal concentrations of Ag-compounds. This could be explained by possible binding of Ag to cellular compounds that reduced the concentration of free Ag ions inhibitory to xanthine oxidase (data not shown).

The conventional method for CAT activity assessment (Li and Schellhorn, 2007) where H_2O_2 is added and the change in optical density at 240 nm is measured was not applicable because AgNPs absorbed light at this wavelength and H_2O_2 bleached the AgNP suspension (data not shown). Instead, a foam based method (Iwase et al., 2013) was applied (Fig. S10). However, this method could not be used for determining the effect of AgNPs on CAT activity either: no dose dependent inhibition of CAT activity by AgNPs was detected, both sub-lethal

concentration of 20 mg Ag/L and close to 100% lethal concentration of 100 mg Ag/L appeared to equally inhibit CAT activity by 69%. This may have been an experimental artefact caused by H_2O_2 interactions with the protein coating of Collargol AgNPs. Ag-ions, on the other hand, inhibited CAT dose-dependently and 2 mg Ag/L inhibited approximately 50% of the activity.

Table S1. Physico-chemical parameters of silver nanoparticles (Collargol, AgNPs)

Parameter		Reference
Primary size (diameter)	14.6 ± 4.7 nm	(Bondarenko et al., 2013)
Coating	casein (30% of total mass)	(Bondarenko et al., 2013)
Shape according to TEM	spherical	(Blinova et al., 2013)
Hydrodynamic diameter in MilliQ water	44 nm (pdi = 0.2)	(Bondarenko et al., 2013)
Zeta potential in MilliQ water	-42.7 mV	(Blinova et al., 2013)
Solubility in MilliQ water	7.6% at 10 mg/L in 4 h	(Bondarenko et al., 2013)
	6.9% at 5 mg/L in 24 h	(Kaosaar et al., 2016)

TEM – transmission electron microscopy
pdi – polydispersity index
MilliQ water – ultrapure water

Table S2. List of primers used in this study.

Gene	Primer	Sequence
HSP70a paralog SSA4 (TTHERM_01080440)	hsp703	FW: 5' TCTAAAAGCTAAGTCCACGAAGTT 3' RV: 5' AACCAGTTAGAATTGCAGCCTATA 3'
HSP70 (TTHERM_00105110)	hsp704	FW: 5' CAAGAACCAAGTCCACGAAGTC 3' RV: 5' GAACCAGAACCAGTCAAGATAGC 3'
HSP70a paralog SSA5 (TTHERM_00558440)	hsp705	FW: 5' TCTCAAAGCCAGTCAAGAATGC 3' RV: 5' GCCATAAGCAATAGCAGCAGC 3'
Metallothionein (TTHERM_00241640)	MTT1	FW: 5' GCGGATGTTGCTGCGTAAGTAA 3' RV: 5' GGGATCAAAGCAGCAGGGTTTA 3'
Metallothionein (TTHERM_00660230)	MTT5	FW: 5' GTCGGTTCAGGAGAAGGATGC 3' RV: 5' CCTCCAGGGCAGCATTCTTTAG 3'
Superoxide dismutase (TTHERM_00357070)	SOD1	FW: 5' GGGTGGTCATATTAACCACGCT 3' RV: 5' GGATAGCAGCAGTACGACCG 3'
Cu/Zn SOD (TTHERM_00059350)	SOD2	FW: 5' CCCTGCTCCAGGATATGATG 3' RV: 5' GTGAGGACCAACAGATTCTGTT 3'
Catalase (TTHERM_01146030)	CAT	FW: 5' GAGGTACTCCCGATGGTTAC 3' RV: 5' GGGCATCAGCTTCAGCAGC 3'
Glutathione peroxidase (TTHERM_00895660)	GPX2	FW: 5' CGTCTCATTGAAGAACTTTAACAA 3' RV: 5' CAGCCCAAGGTTCTTATTCACC 3'
Thioredoxin and glutathione reductase (TTHERM_00047660)	GSR	FW: 5' CCGTGGTACTGTACCTCACC 3' RV: 5' CCATAGTCTCTACACCGATATTC 3'
Ribosomal protein	17S	FW: 5' GAATTGACGGAACAGCACACC 3' RV: 5' TCACTCCACCAACTAAGAACGGC 3'

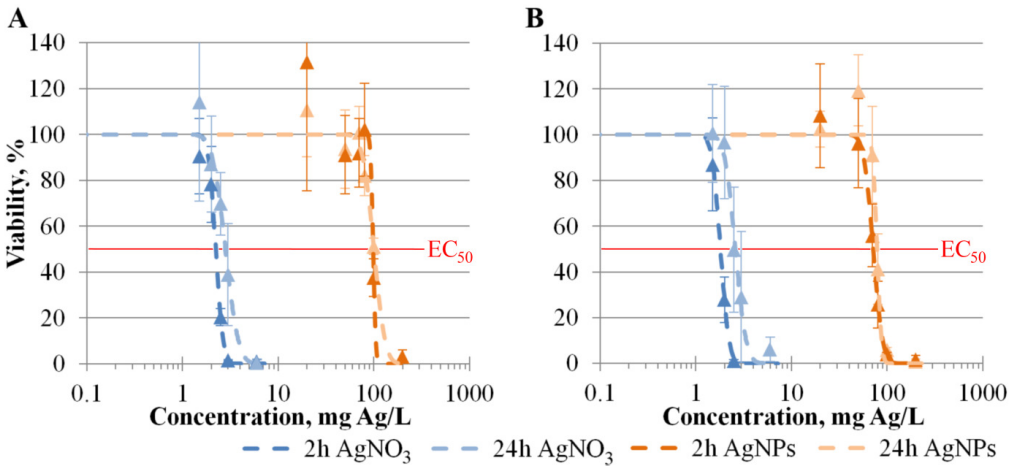

Fig. S1 Viability of *Tetrahymena thermophila* strains CU427 (A) and CU428 (B) upon exposure to Ag-compounds for 2 and 24 hours at 25 °C in the dark: a concentration-effect analysis. Data points are the average values of at least 3 replicates and error bars indicate standard deviations. ATP concentration was used as a viability endpoint. Concentration-effect curves were generated using REGTOX software for Microsoft Excel™.

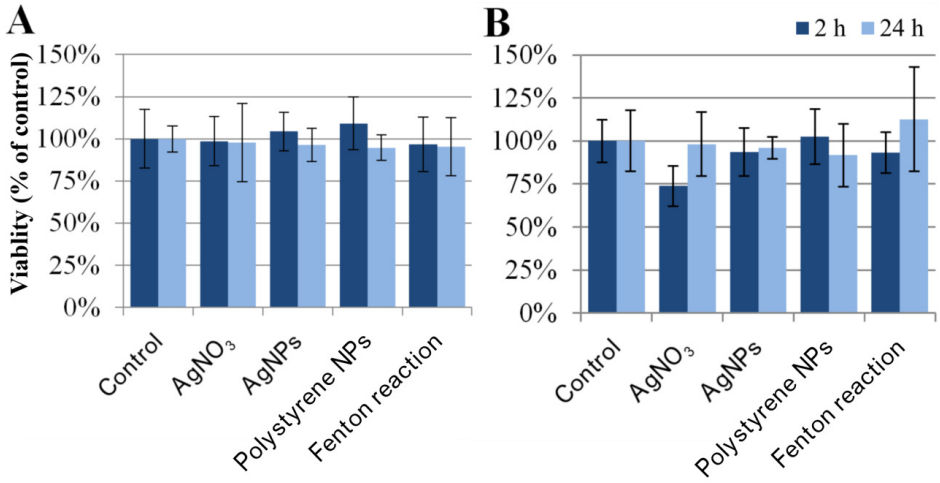

Fig. S2 Viability of *Tetrahymena thermophila* strains CU427 (A) and CU428 (B) upon 2- and 24-h exposure at 25 °C in the dark to 1.5 mg Ag/L of $AgNO_3$, 20 mg Ag/L of AgNPs, 11 mg/L of polystyrene NPs and a mixture of 1.1 mg Fe/L of $FeSO_4x7H_2O$ and 15 mg/L H_2O_2 (a Fenton reaction). These sub-lethal exposure concentrations were used for gene expression study, ROS assay, lipid peroxidation test and for the measurement of the activity of antioxidant enzymes. The bars are the average values of at least 6 replicates and error bars indicate standard deviations. Protozoan viability was measured using an ATP assay (Jemec et al., 2016). The concentrations are nominal.

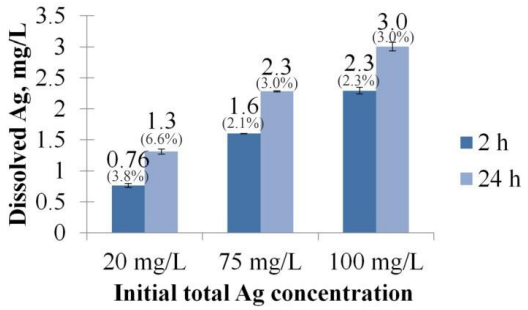

Fig. S3 Dissolution of different nominal concentrations of AgNPs after incubation for 2 and 24 h in MilliQ water at 25 °C in the dark. The bars are the average values of 2 to 4 replicate measurements and error bars indicate standard deviations. The values on top of the bars (with the fraction of initial Ag in parentheses) are Ag concentrations quantified by AAS as described in the methods.

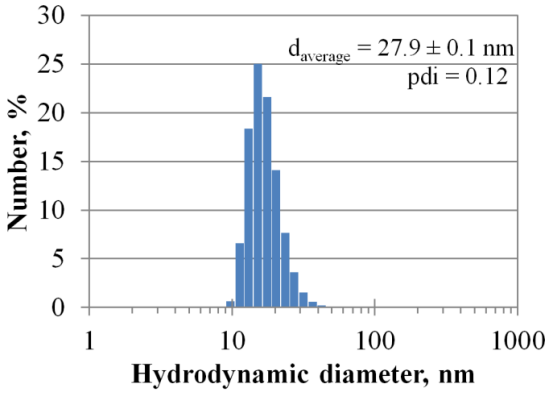

Fig. S4 Hydrodynamic size distribution of polystyrene NPs. $d_{average}$ – average hydrodynamic diameter of 3 replicate measurements ± standard deviation, pdi – polydispersity index.

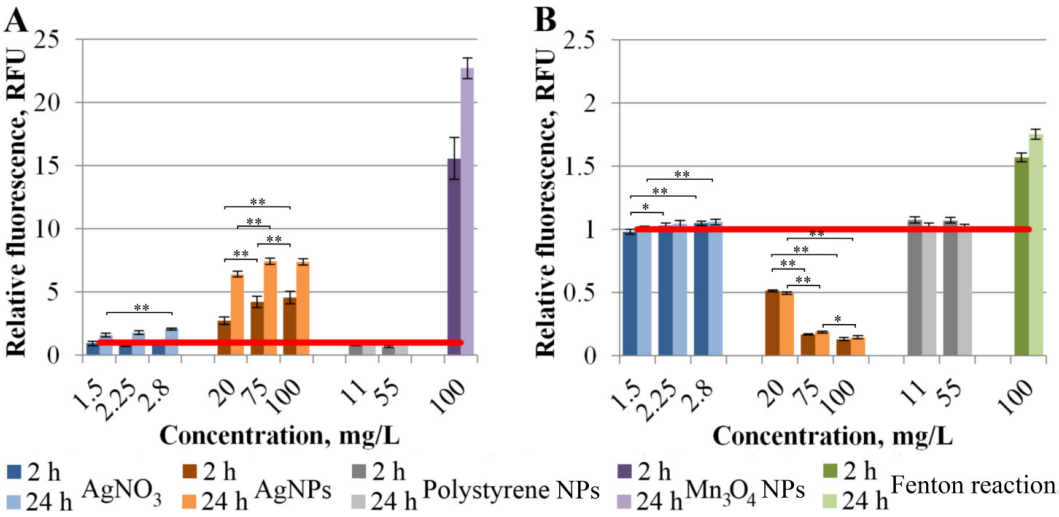

Fig. S5 Production of reactive oxygen species (ROS) by Ag-compounds and polystyrene NPs in abiotic conditions (in the absence of protozoa) at 25 °C in the dark as quantified by DCFH-DA (A) and HPF (B) fluorescence. All the exposure concentrations are nominal and sub-lethal. The concentrations of Ag-compounds are presented as mg Ag/L. The bars are average values of 3 replicates and error bars indicate standard deviations. Red lines indicate background fluorescence (= 1.0) of MilliQ water in the respective assays. Panel B demonstrates that HPF fluorescence was quenched in the presence of AgNPs, i.e., indicates that the assay could not be used for quantifying abiotic ROS in AgNP dispersions. Fenton reaction was induced with 1.1 mg Fe/L of $FeSO_4x7H_2O$ and 15 mg/L H_2O_2. In pairwise comparison, the asterisk (*) marks significant difference ($p < 0.05$) and (**) marks highly significant difference ($p < 0.01$).

A: $AgNO_3$

	CU427 2h	CU428 2h	CU427 24h	CU428 24h
CU427 2h				
CU428 2h	ns			
CU427 24h	*	**		
CU428 24h	ns	**	ns	

* $p < 0.05$ ** $p < 0.01$

B: AgNPs

	CU427 2h	CU428 2h	CU427 24h	CU428 24h
CU427 2h				
CU428 2h	***			
CU427 24h	ns	***		
CU428 24h	***	ns	***	

*** $p < 0.001$ ns - not significant

Fig. S6 Pairwise comparison of 2 h and 24 h EC_{50} values for $AgNO_3$ (A) and AgNPs (B) for *Tetrahymena thermophila* strains CU427 and CU428. EC_{50} values are presented in Table 1 in the main paper.

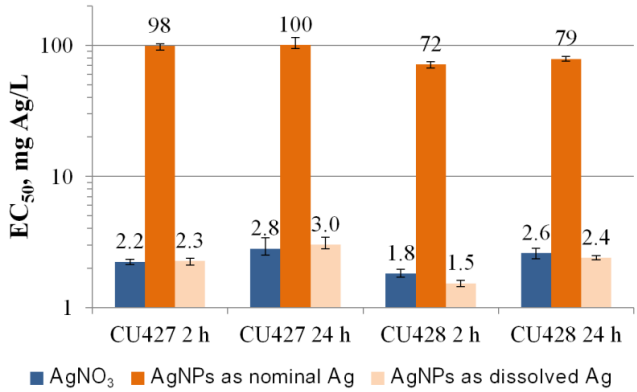

Fig. S7 Toxicity of AgNP and $AgNO_3$ to *Tetrahymena thermophila* strains CU427 and CU428: 2 h and 24 h EC_{50} values based on nominal *versus* dissolved silver concentrations. EC_{50} values based on nominal concentrations are from Table 1; EC_{50} values based on dissolved Ag ions are calculated using data presented in Figure 1. Data represent the average of 3 replicates and the error bars indicate 95% confidence intervals.

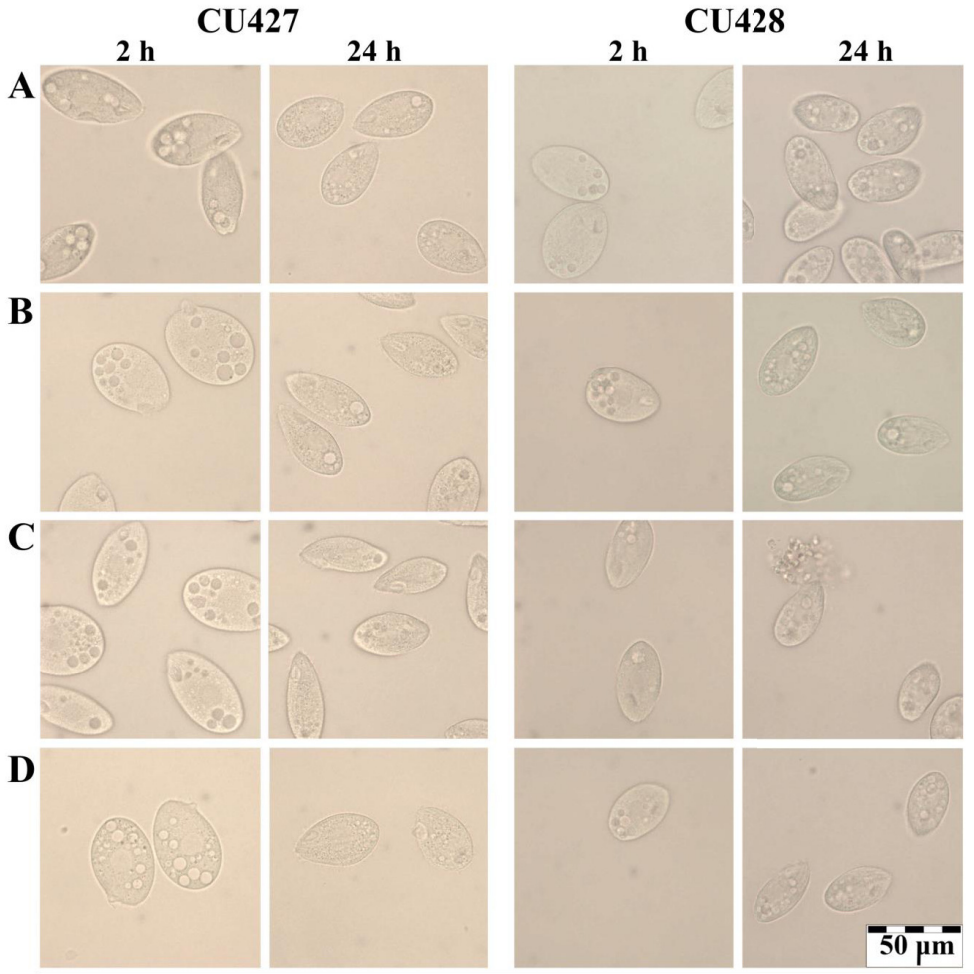

Fig. S8 Bright field images of live *Tetrahymena thermophila* cells (strains CU427 and CU428) after 2- and 24-h incubation in MilliQ water (A) and exposure to 1.5 mg Ag/L of $AgNO_3$ (B), 11 mg/L of polystyrene NPs (C), and mixture of 1.1 mg Fe/L of $FeSO_4x7H_2O$ and 15 mg/L H_2O_2 (a Fenton reaction), (D) in MilliQ water. All the exposure concentrations are sub-lethal and nominal.

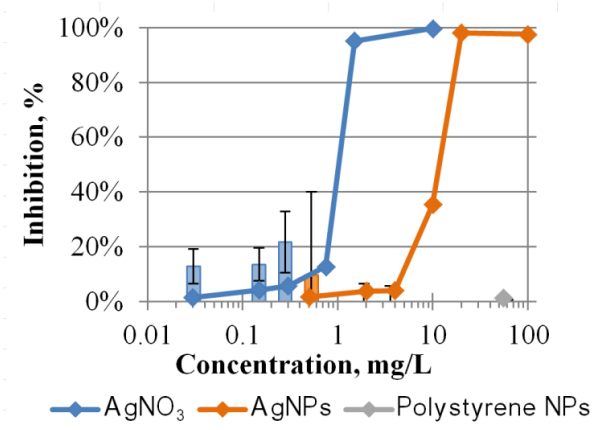

Fig. S9 Effect of Ag-compounds and polystyrene NPs on SOD assay performance, i.e., inhibition of the assay component – xanthine oxidase (lines) and on enzymatic activity of SOD standard (full bars). The concentrations of Ag-compounds are nominal and presented as mg Ag/L.

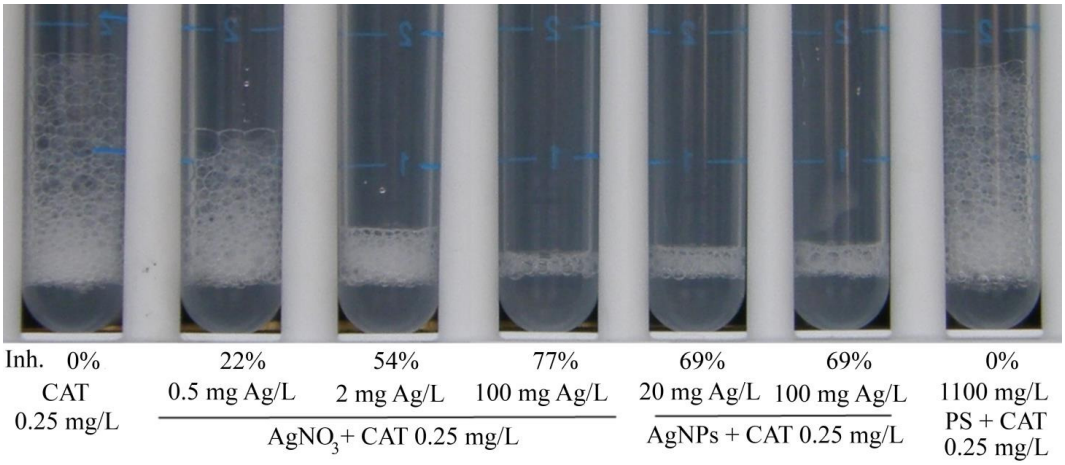

Fig. S10 Effect of Ag-compounds and polystyrene NPs on catalase (CAT) activity in the foam test. Concentrations are nominal. Inh. – inhibition percentage compared to enzymatic activity of the same concentration of CAT standard in 50 mM potassium phosphate buffer (pH 7). PS – polystyrene NPs. No dose dependent CAT inhibition by AgNPs was established using the assay which led us to conclude that the 69% inhibition was an experimental artefact caused by H_2O_2 interactions with the protein coating of Collargol AgNPs.

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CURRICULUM VITAE

Katre Juganson

Date and place of birth: 09.06.1988, Tallinn, Estonia

E-mail address: katre.juganson@eesti.ee

Education

2012-2018	Tallinn University of Technology, PhD student of Chemistry and Gene Technology
2010-2012	Tallinn University of Technology, Master of Science in Natural Sciences (*cum laude*)
2007-2010	Tallinn University of Technology, Bachelor of Science in Natural Sciences (*cum laude*)
2004-2007	Tallinn Secondary School of Science (silver medal)

Language competence/skills

Estonian, English (fluent)
Russian (average)
Finnish (basic)

Special courses

Sept. 26-30, 2016	Sweden, Karolinska Institutet, course "Safety assessment in drug discovery and development"
May 4-5, 2016	Estonia, NICPB, training school "Assessing the dose of nanomaterials in toxicological studies: Advanced approaches utilizing experimentation and modelling"
Nov. 4-9, 2013	Italy, University of Camerino, training school "Genomics and Evolutionary Biology"
May 6-10, 2013	Portugal, University of Aveiro, course "Implications of Nanomaterials: A hands on course on Synthesis, Characterization, and Ecotoxicology, 5th Edition"
Apr. 22-26, 2013	Sweden, Karolinska Institutet, course "Nanotoxicology - potential risks of engineered nanomaterials to human health and the environment"
Nov. 14-18, 2011	Turkey, course "EUROTOX Advanced Toxicology course"
Oct. 10-11, 2011	Estonian University of Life Sciences, short course on methods in transmission, scanning and immunoelectron microscopy
Jun. 27-Jul. 1, 2011	Serbia, University of Belgrade, course "EUROTOX Basic Toxicology course"

Professional Employment

2010-… National Institute of Chemical Physics and Biophysics, Laboratory of Environmental Toxicology, junior researcher (2015-…), engineer (2012-2015), MSc student (2010-2012)

2009-2010 Tallinn University of Technology, Chair of Semiconductor Materials Technology, BSc student

2008–2009 Tallinn University of Technology, Chair of Molecular Diagnostics, BSc student

Honours & Awards

2017 Conference award for the best contribution at NanoImpact conference (Ascona, Switzerland), oral presentation

2016 Finalist and nominee of the three-minute lecture competition of the Estonian Academy of Sciences

2016 nanoTOX2016 Travel award for participation in the 8th International Nanotoxicology Congress

2013 COST action BM1102 Ciliates as model systems to study genome evolution, mechanisms of non-Mendelian inheritance, and their roles in environmental adaptation Short-Term Scientific Mission Grant for research at the University of Camerino, Italy (2 months)

2013 Katre Juganson and Urmas Joost; Tartu University and Tallinn University of Technology doctoral school "Functional Materials and Technologies"; Grant for interdisciplinary research project “Photocatalytic properties of nano-TiO_2 thin films: Effects on biomolecules and living cells”

2012 EUROTOX 2012 fellowship for participation in the 48th EUROTOX Congress

ELULOOKIRJELDUS

Katre Juganson

Sünniaeg ja -koht: 09.06.1988, Tallinn, Eesti

E-posti aadress: katre.juganson@eesti.ee

Hariduskäik

2012-2018	Tallinna Tehnikaülikool, keemia- ja geenitehnoloogia doktorant
2010-2012	Tallinna Tehnikaülikool, loodusteaduste magister (*cum laude*)
2007-2010	Tallinna Tehnikaülikool, loodusteaduste bakalaureus (*cum laude*)
2004-2007	Tallinna Reaalkool (hõbemedal)

Keelteoskus

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

Täiendusõpe

26.-30. sept. 2016	Rootsi, Karolinska Institutet, kursus: "*Safety assessment in drug discovery and development*"
4.-5. mai 2016	Eesti, KBFI, kursus: "*Assessing the dose of nanomaterials in toxicological studies: Advanced approaches utilizing experimentation and modelling*"
4.-9. nov. 2013	Itaalia, Camerino ülikool, kursus "*Genomics and Evolutionary Biology*"
6.-10. mai 2013	Portugal, Aveiro ülikool, kursus "*Implications of Nanomaterials: A hands on course on Synthesis, Characterization, and Ecotoxicology, 5th Edition*"
22.-26. apr. 2013	Rootsi, Karolinska Institutet, kursus "*Nanotoxicology - potential risks of engineered nanomaterials to human health and the environment*"
14.-18. nov. 2011	Türgi, kursus "*EUROTOX Advanced Toxicology course*"
10.-11. okt. 2011	Eesti Maaülikool, transmissioon-, skaneeriva ja immuunoelektronmikroskoopia kursus
27. juuni-1. juuli 2011	Serbia, Belgradi ülikool, kursus "*EUROTOX Basic Toxicology course*"

Teenistuskäik

2010-… Keemilise ja Bioloogilise Füüsika Instituut, keskkonnatoksikoloogia laboratoorium, nooremteadur (2015-…), insener (2012-2015), magistrant (2010-2012)

2009-2010 Tallinna Tehnikaülikool, pooljuhtmaterjalide tehnoloogia õppetool, bakalaureus

2008–2009 Tallinna Tehnikaülikool, molekulaardiagnostika õppetool, bakalaureus

Teaduspreemiad ja -tunnustused

2017 Konverentsiauhind parima suulise ettekande eest rahvusvahelisel konverentsil NanoImpact (Ascona, Šveits)

2016 Eesti Teaduste Akadeemia kolme minuti pikkuste loengute konkursi finalist ja laureaat

2016 nanoTOX2016 stipendium 8. rahvusvahelisel nanotoksikoloogia konverentsil osalemiseks

2013 COST tegevuse BM1102 *Ciliates as model systems to study genome evolution, mechanisms of non-Mendelian inheritance, and their roles in environmental adaptation* lühiajalise teadusmissiooni stipendium teadustööks Itaalias, Camerino Ülikoolis (2 kuud)

2013 Katre Juganson ja Urmas Joost; Tartu Ülikooli ja Tallinna Tehnikaülikooli doktorikooli "Funktsionaalsed Materjalid ja Tehnoloogiad" toetus interdistsiplinaarseks uurimisprojektiks “TiO_2 nanoosakeste ning nanokilede fotokatalüütiliste omaduste uurimine bioloogiliste molekulide ning elusrakkude näitel”

2012 EUROTOX 2012 stipendium 48. EUROTOX'i konverentsil osalemiseks