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Hazard Evaluation of Metal-Based Nanoparticles and Lanthanides with Freshwater Microcrustaceans

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TTÜ PRESS

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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.

Marge Muna

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Metalliliste nanoosakeste ja lantaniidide kahjulikkuse hindamine magevee pisivähkidega

MARGE MUNA

TTÜ KIRJASTUS

Contents

List of Publications

The list of author's papers, on the basis of which the thesis has been prepared (referred to by their Roman numerals in the text):

I Heinlaan, M., **Muna, M.**, Knöbel, M., Kistler, D., Odzak, N., Kühnel, D., Müller, J., Gupta, G. S., Kumar, A., Shanker, R., & Sigg, L. (2016). Natural water as the test medium for Ag and CuO nanoparticle hazard evaluation: an interlaboratory case study. *Environmental pollution, 216,* 689–699. https://doi.org/10.1016/j.envpol.2016.06.033

II **Muna, M.**, Blinova, I., Kahru, A., Vinković Vrček, I., Pem, B., Orupõld, K., & Heinlaan, M. (2019). Combined effects of test media and dietary algae on the toxicity of CuO and ZnO nanoparticles to freshwater microcrustaceans *Daphnia magna* and *Heterocypris incongruens*: food for thought. *Nanomaterials, 9,* 23. http://doi.org/10.3390/nano9010023

III **Muna, M.**, Heinlaan, M., Blinova, I., Vija, H., & Kahru, A. (2017). Evaluation of the effect of test medium on total Cu body burden of nano CuO-exposed *Daphnia magna*: A TXRF spectroscopy study. *Environmental Pollution, 231,* 1488–1496. https://doi.org/10.1016/j.envpol.2017.07.083

IV 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. http://doi.org/10.1016/j.aquatox.2017.06.002

V Blinova, I., Lukjanova, A., **Muna, M.**, Vija, H., & Kahru, A. (2018). Evaluation of the potential hazard of lanthanides to freshwater microcrustaceans. *Science of The Total Environment, 642,* 1100–1107. https://doi.org/10.1016/j.scitotenv.2018.06.155

VI Blinova, I., Vija, H., Lukjanova, A., **Muna, M.**, Syvertsen-Wiig, G., & Kahru, A. (2018). Assessment of the hazard of nine (doped) lanthanides-based ceramic oxides to four aquatic species. *Science of The Total Environment, 612,* 1171–1176. http://doi.org/10.1016/j.scitotenv.2017.08.274

Author's Contribution to the Publications

I The author conducted the toxicity testing with *D. magna* in three test media and the metal bioavailability tests with metal-specific sensor bacteria in NICPB. She also contributed to the data analysis and the preparation of the manuscript.

II The author designed the study, conducted the toxicity experiments with *D. magna* and *H. incongruens* and performed the DLS-ELS characterisation of the nanoparticles. Data interpretation, writing and editing of the manuscript were also primarily done by the author.

III The author planned and conducted the experiments exposing *D. magna* to CuO nanoparticles and $CuSO_4$ and performing the TXRF copper body burden analysis along with characterisation of the nanoparticles. Data interpretation and statistical analysis of the results was done by the author and she was responsible for the writing of the manuscript.

IV The author assisted with the toxicity testing, post-exposure feeding experiments and microscopy imaging of *D. magna*. She conducted the statistical analysis of the results and prepared the graphs and part of the manuscript.

V The author participated in planning of the study and in the toxicity testing of the lanthanides with *D. magna*, *H. incongruens,* and *T. platyurus*. The microscopic analysis of the algae agglomeration in the test media was also performed by the author. In addition, she contributed to the manuscript preparation.

VI The author conducted part of the toxicity tests exposing crustaceans (*H. incongruens* and *T. platyurus*) and duckweeds (*L. minor*) to lanthanide (doped) particles. The microscopy imaging of the accumulation of the particles in the crustaceans was also performed by the author. She also participated in the data analysis and in the preparation of the manuscript.

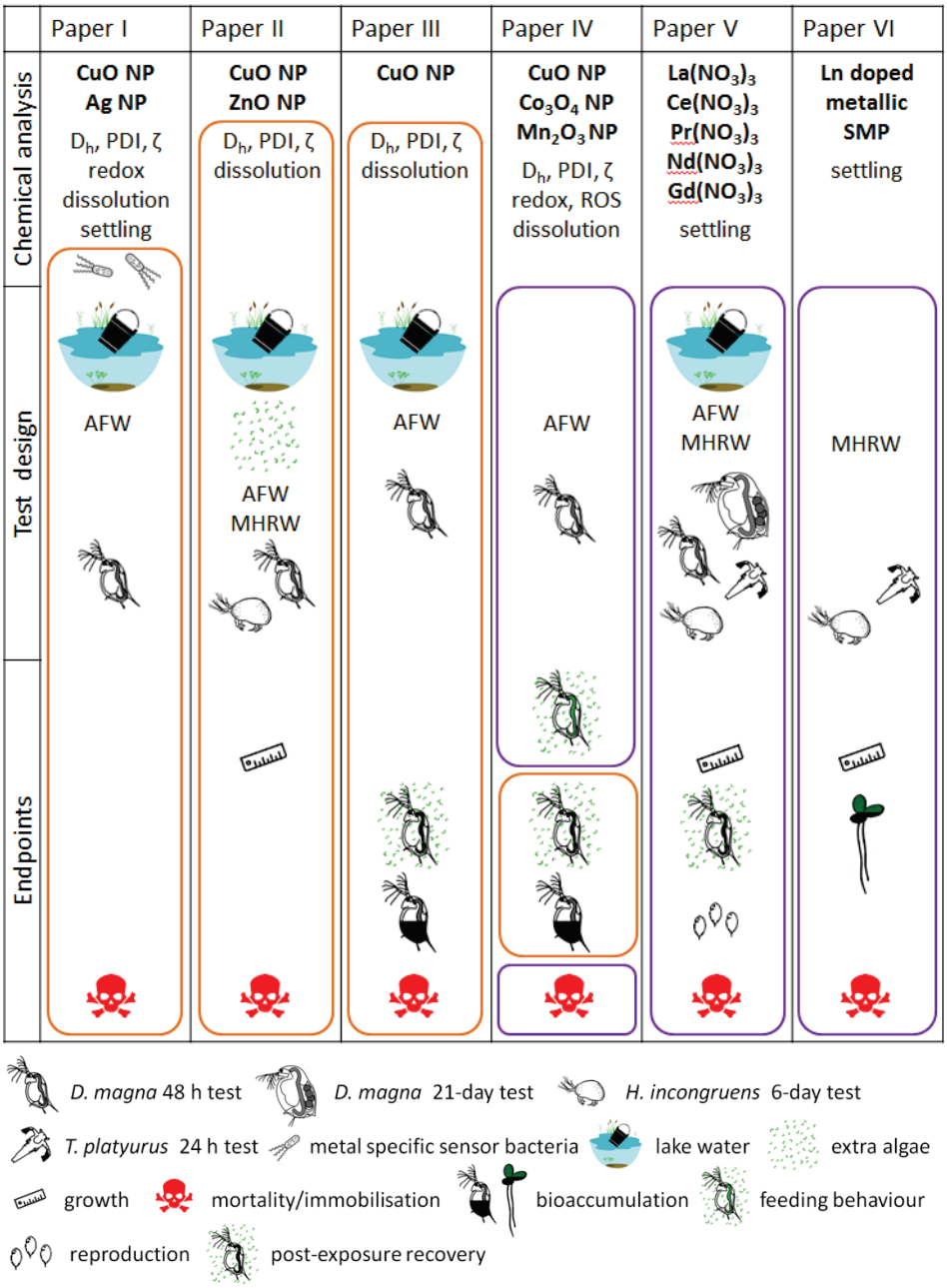

Figure 1. *Scheme of the experimental setup of the publications and author's contribution to the experiments (orange frames – the author was responsible for the experiment; purple frames – the author participated in conducting the experiment). NP – nanoparticles; SMP – submicron particles; D_h – hydrodynamic diameter; PDI – polydispersity index; ζ – zeta potential; ROS – reactive oxygen species; AFW – OECD 202 artificial freshwater; MHRW – US EPA medium hard reconstituted water.*

Other Publications in Peer-Reviewed Journals

Romero-Freire, A., Joonas, E., **Muna, M.**, Cossu-Leguille, C., Vignati, D. A. L., & Giamberini, L. (2019). Assessment of the toxic effects of mixtures of three lanthanides (Ce, Gd, Lu) to aquatic biota. *Science of The Total Environment*, 661, 276–284. http://doi.org/10.1016/j.scitotenv.2019.01.155

Blinova, I., **Muna, M.**, Lukjanova, A., & Kahru A. (2018). Evaluation of the potential hazard of manufactured metal-based nanomaterials to health of aquatic ecosystems: state of the art. *Journal of International Scientific Publications: Ecology & Safety, 12,* 174−182. Retrieved from https://www.scientific-publications.net/en/article/1001659/.

Introduction

An exponential increase in the world's population in the context of a fixed amount of natural resources such as fossil fuels, freshwater, and arable land, makes it difficult to meet the society's expectations towards on-going progress. Different types of (novel) substances with beneficial properties and "green" technologies are developed to fulfil these expectations. On the other hand, application of innovative chemicals and materials can also bring about new types of hazards to the environment by creating emerging contaminants. Determining the potential harmful effects of emerging contaminants to allow safe and sustainable use of these substances is one of the primary aims of environmental toxicology.

Engineered nanomaterials and lanthanides are considered to be emerging contaminants for which environmental standards have not yet been established. The regulatory directives for nanomaterial safety are currently being developed and massive increase is expected in lanthanides' mining and use all over the world. Thus, information on their biological effects is urgently needed for relevant environmental risk assessment.

Standardised toxicity test procedures are used to classify the potential hazard of new chemicals in internationally agreed test conditions. It is known that test design can also have a great impact on the toxicity of chemical compounds, especially on the substances like manufactured metal-based nanoparticles and lanthanides that tend to aggregate, settle, dissolve, precipitate, and/or adsorb to the test vessels before and during the toxicity test.

To determine the potential artificial effects that standard test conditions can impose on nanoparticle and lanthanide toxicity, both standard and modified toxicity test conditions were used in the thesis. Also, additional sublethal toxicity endpoints were included in the test design to increase the environmental relevance of the study. Planktic (*Daphnia magna*, *Thamnocephalus platyurus*) and benthic (*Heterocypris incongruens*) microcrustaceans, as one of the most vulnerable links in the aquatic food chain, were used as test organisms.

Two new approaches for evaluation of possible adverse effects of metal-based nanoparticles to crustaceans were applied in the thesis. These methods enable measuring i) bioaccumulation of metal-based nanoparticles in single microcrustacean specimen and ii) changes in the feeding activity of *D. magna*. The thesis also demonstrated the methodological problems with the standardised acute toxicity test format in toxicity testing of lanthanide salts in both artificial and natural freshwaters for the first time.

The thesis has been published as 6 peer-reviewed scientific articles. The results have also been presented at the following international science conferences: EcoBalt (Tartu, Estonia, 2016; paper **III**), SETAC (Rome, Italy, 2018; paper **V**), Canadian Ecotoxicity Workshop (Vancouver, Canada, 2018; paper **V**).

Abbreviations

AAS	atomic absorption spectroscopy
AFW	artificial freshwater
D_h	hydrodynamic diameter
DLS	dynamic light scattering
DOC	dissolved organic carbon
DOM	dissolved organic matter
dwt	dry weight
EC_{10}	median effective concentration of the test substance that induces an adverse effect in 10% of the test organisms after a specified exposure time
EC_{50}	median effective concentration of the test substance that induces an adverse effect in 50% of the test organisms after a specified exposure time
ECHA	European Chemical Agency
ELS	electrophoretic light scattering
EU	European Union
FP	framework programme
immobilisation	being unresponsive to gentle prodding
ISO	International Organisation for Standardization
LC_{50}	median lethal concentration of the test substance that induces mortality in 50% of the test organisms after a specified exposure time
Ln	lanthanide(s)
NNV	NanoValid, nanosafety project under EU 7th framework programme (Grant Agreement no. 263147)
NP	nanoparticle(s)
N_{tot}	total nitrogen
OECD	Organisation for Economic Co-operation and Development
OECD TG	OECD test guideline
PDI	polydispersity index
P_{tot}	total phosphorus
PVP	polyvinylpyrrolidone
REACH	European Union regulation on Registration, Evaluation, Authorisation and Restriction of Chemicals
REE	rare earth elements
ROS	reactive oxygen species
SD	standard deviation
SMP	submicron particle(s)
TXRF	Total Reflection X-ray Fluorescence
US EPA	United States Environmental Protection Agency

1 Literature review

1.1 Emerging contaminants: state of the art

Emerging contaminants are characterised by the lack of relevant environmental fate and (eco)toxicological data resulting in the absence of regulatory criteria or norms for the protection of human health or the environment (Sauve & Desrosiers, 2014). Potential chemicals of emerging concern can be divided into three categories: i) true emerging contaminants, that were not previously known or that have appeared only recently in the scientific literature, ii) contaminants of emerging interest which have been in the environment for a while but for which concerns have been raised much more recently, and iii) emerging issues about legacy contaminants (Sauve & Desrosiers, 2014).

Engineered nanoparticles (NP) and lanthanides (Ln), both accompanying the advancements in modern technologies, belong to the true emerging contaminants category along with many pesticides, pharmaceuticals, fragrances, plasticisers, hormones, flame retardants, perfluoroalkyl compounds, chlorinated paraffins, siloxanes, algal toxins, radionuclides, etc. (Sauve & Desrosiers, 2014). The true emerging contaminants can be further divided into i) organic substances, ii) inorganic compounds (trace metals such as Ln), and iii) particulate contaminants (nanoparticles, microplastics etc.) (Geissen et al., 2015).

Search in Thomson Reuters Web of Science database (Table 1) shows that NP have been much more extensively studied in terms of environmental toxicology compared to Ln. The search results on NP used in this study vary considerably: Ag, Cu, and Zn based NP toxicity to daphnids (common freshwater test organisms) has been researched much more compared to Co and Mn based NP toxicity.

Table 1. Environmental hazard research on nanoparticles and lanthanides: bibliometric data search results in Thomson Reuters Web of Science database (9.1.2018) omitting the publications that are part of this thesis.

	No. of results	**Search phrases in Title**		
Ecotoxicity of NP		1.	2.	
	286	nano*	ecotoxic* OR "environmental* toxic*" OR "environmental* hazard*"	
NP effects on daphnids		1.	2.	3.
general	314	nano*	*daphni* OR "D. magna" OR cladocera* OR zooplankton OR microcrustacea*	-
bioaccumulation	27			*accumulation OR "body burden"
Ag NP toxicity	69			Ag OR silver
Cu NP toxicity	28			CuO OR copper
Zn NP toxicity	34			ZnO OR zinc
Co NP toxicity	1			Co_3O_4 OR cobalt
Mn NP toxicity	1			Mn_2O_3 OR manganese

Table 1 continued.

Ecotoxicity of Ln		1.	2.
	7	lanthanide* OR "rare earth" OR lanthanum OR cerium OR praseodymium OR neodymium OR samarium OR europium OR gadolinium OR terbium OR dysprosium OR holmium OR erbium OR thulium OR ytterbium OR lutetium	ecotoxic* OR "environmental* toxic*" OR "environmental* hazard*"
Ln effects on daphnids	6		*daphni* OR "D. magna" OR cladocera* OR zooplankton OR microcrustacea*

NP – nanoparticles; Ln – lanthanides

1.1.1 Metal-based nanoparticles

Nanoparticles (NP) are defined as particles with one or more dimensions in the range of 1–100 nm (European Commission, 2013). NP occur naturally as both organic (proteins, viruses, etc.) and inorganic (iron oxyhydroxides, aluminosilicates, metals, etc.) compounds and are products of weathering, volcanic eruptions, wildfires, etc. (Lead & Wilkinson, 2006; Schaming & Remita, 2015). People have non-intentionally produced NP since gaining control of fire and have utilised metal-based NP for up to 16,000 years, mainly as a colorant in the glass and ceramic industries (Schaming & Remita, 2015). Deliberate engineering of NP started in the 1980s with the discovery of the quantum size effect (Heiligtag & Niederberger, 2013) and fullerenes (Kroto et al., 1985). The new chemical, optical, electric, and magnetic properties appearing in materials at nano scale have brought about a paradigm shift in industrial research sometimes referred to as nano-revolution (Alivisatos, 2004; Wonglimpiyarat, 2005).

Engineered NP can be carbon-based (e.g. fullerenes, carbon nanotubes, graphene), organic (e.g. polymers, dendrimers, liposomes), inorganic (e.g. metal, metal oxide, and ceramic NP), or composites (combination of two or more types of NP) (Sudha et al., 2018). This thesis focuses on the biological effects of metal-based NP and more precisely, on ecotoxicologically more studied Ag, CuO and ZnO NP and less studied Mn_2O_3 and Co_3O_4 NP (Table 1).

Metal-based NP are valued for their optical, electronic, and catalytic properties (Alivisatos, 2004) and can be applied in a wide variety of fields from the energy sector to cosmetics (Keller et al., 2013). In addition, Ag NP provide antimicrobial protection in variety of consumer products such as textiles, medical products, domestic appliances, paints, cosmetics, and plastics (Hansen et al., 2016; McGillicuddy et al., 2017; Vance et al., 2015). ZnO NP are common in sun lotions since they strongly absorb ultraviolet rays and also make the emulsion transparent (Cross et al., 2007). CuO NP are most often used as inexpensive alternatives to the rare and expensive noble-metal catalysts suitable for many chemical processes (Gawande et al., 2016; Keller et al., 2013) including the removal of environmental contaminants (Khalaj et al., 2018). Mn_2O_3 and Co_3O_4 NP are both promising lithium ion battery anode materials (Shi at al., 2018; Zhang et al., 2017).

Metal-based NP can reach aquatic ecosystems through several pathways, for example from waste water treatment plants and with surface runoff of biosolids or fertilisers, but also by leaching from coatings and paints, or from cosmetic products,

such as sunscreen worn by bathers (Adeleye et al., 2016; Garner et al., 2017; Keller et al., 2013). Cu based nanoparticles are also widely present in micronised copper wood preservatives (Gottschalk et al., 2015) but the leaching of Cu in NP form from wood is still debatable (Parks et al., 2018). Co and Mn based NP could become water pollutants due to the insufficient recycling of electronic waste such as spent lithium ion batteries (Kang et al., 2013; Zeng et al., 2015). Despite the low estimated global production volumes (< 500 t/year; Keller et al. (2013)), Ag NP have already been detected in the natural waterbodies in concentrations up to 2.5 ng/L (Peters et al., 2018).

NP can have a very different fate (e.g. homo- and heteroaggregation, dissolution) in the waterbodies, depending on the characteristics of the NP and the chemical composition of the water (Garner & Keller, 2014; Garner et al., 2017). The abundance of organic matter and water hardness are of major importance (Bian et al., 2011; Conway et al., 2015; Gao et al., 2009). Ag and CuO NP are predicted to dissolve over days to weeks, while ZnO dissolves over hours to days, the aggregation of all NP being slower in freshwater compared to sea water (Garner & Keller, 2014). The fate of the NP is difficult to study due to the lack of analytical methods to detect and quantify nanoparticles in complex environmental matrices and the fact that natural (metal-based) NP are present in all ecosystems (Holden et al., 2016; Kahru & Dubourguier, 2010). A similar methodological problem occurs in laboratory toxicity testing of NP where the dose of the NP affecting the test organism is difficult to determine and the adverse effects of NP exerted as particles and as ions shed from the particles, are difficult to differentiate (Holden et al., 2016; McGillicuddy et al., 2017). NP-specific guidelines have been just recently published in EU (https://echa.europa.eu/-/reach-guidance-for-nanomaterials-published; 18.01.2019), covering some aspects of ensuring reliable NP toxicity testing.

Regardless of the methodological challenges, it can be generalised that the main toxicity mechanism of metal-based nanoparticles is the shedding of metal ions (Bondarenko et al., 2013; Heinlaan et al., 2008). In fact, nanoecotoxicology gained widespread attention after it appeared that many metal-based NP are (partly) soluble and impose higher toxicity to aquatic biota than the respective (insoluble) bulk particles. NP are considered to be more toxic the more they dissolve, as the ionic form of a metal can be much more toxic than the NP (Garner & Keller, 2014; Notter et al., 2014). However, this does not mean that particle-induced toxicity of NP is irrelevant. For example, ZnO and Mn_3O_4 NP toxicity can be higher than the toxicity of the respective soluble salts (Aruoja et al., 2015; Notter et al., 2014) suggesting particle-dependent toxic effects in addition to ion-induced toxicity. Particle-specific toxicity causes high localised metal concentrations and physical effects after ingestion by or adsorption on the organism (Dabrunz et al., 2011; Golobič et al., 2012), induction of oxidative stress (Zhao et al., 2016) and membrane damage (Otero-González et al., 2013) along with entrapment of unicellular organisms (Aruoja et al., 2015).

Invertebrates, especially filter-feeding crustaceans, are technically suitable, ecologically relevant, and well-studied (Figure 2) test organisms ideal for NP ecotoxicity assessment (Baun et al., 2015). Ag NP are considered hazardous (defined as $L(E)C_{50}$ < 1 mg metal/L by European Commission (2008)) to the crustaceans while ZnO and CuO NP are borderline non-hazardous ($L(E)C_{50}$ 1–10 mg metal/L; Juganson et al., 2015) if the toxicity values were based on nominal metal concentrations. At the same time, modelling results show that ZnO and CuO NP can already reach localised toxic concentrations in the waterbodies in both NP and ionic form despite the current low

release rate (Garner et al., 2017). The toxicity of Mn and Co oxide NP has been substantially less studied compared to Cu, Zn, or Ag based NP (see Table 1). Both Co and Mn oxide NP were shown to induce harm to *D. magna* in a chronic toxicity test (Bozich et al., 2017).

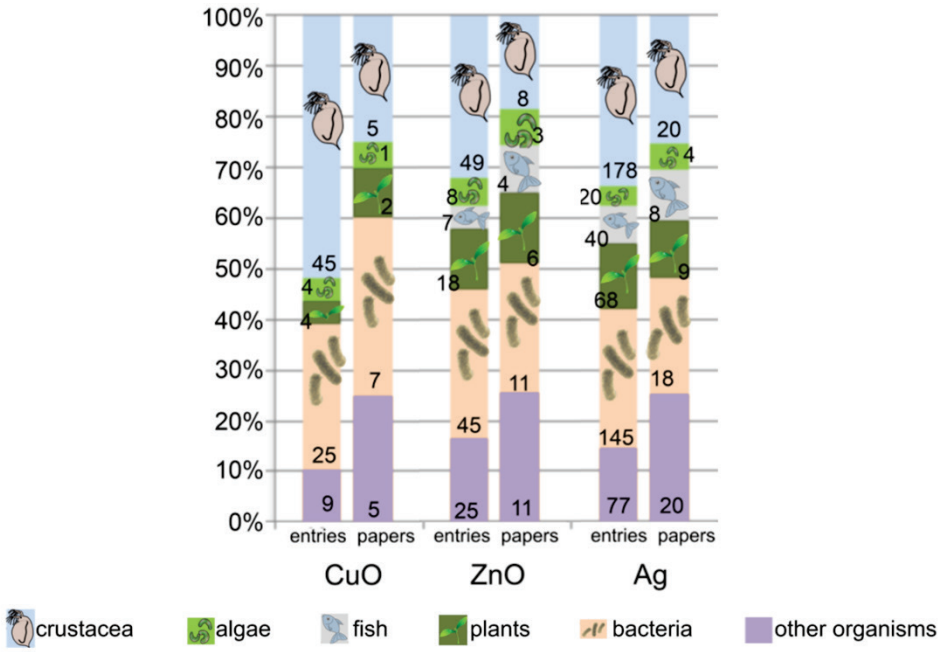

Figure 2. Types of test organisms used for ecotoxicological evaluation of nanoparticles. The left column represents the number of entries and right column represents the number of publications. Modified from Juganson et al. (2015).

Despite extensive research conducted on NP ecotoxicity over the past 15 years, data gaps remain. Methodologically challenging topics such as the effects of environmentally realistic test conditions (exposure concentration, test medium) toxicokinetics, modelling NP behaviour, the effect of aging on toxicity, etc. need further research (Blinova et al., 2018; OECD, 2014). Concerning toxicity tests using daphnids (OECD, 2004, 2012), there is a lack of data on behavioural observations, mechanical effects of the NP, and the potential effects of photoactivity or catalytic properties of the NP (ECHA, 2017a). Also, the extrapolation of the toxicity results obtained by using the standard test procedures to natural ecosystems is still complicated (Blinova et al., 2018).

The main rationale for choosing the metal-based NP for this study were the following: ZnO NP are produced in large volumes (> 30,000 metric tons/year (Keller et al., 2013)) and are highly soluble which makes them likely to cause harm to the environment in the near future (Garner et al., 2017); Cu based NP are well studied model NP that also have high potential of being applied in the environment for the removal of contaminants (Khalaj et al., 2018); Ag NP are some of the most toxic NP to aquatic biota (Juganson et al., 2015) while being easily accessible (and disposed of) by consumers (McGillicuddy et al., 2017); finally, the potential ecotoxicological hazards of Mn_2O_3 and Co_3O_4 have not been sufficiently studied (Table 1).

1.1.2 Lanthanides

The lanthanide (Ln) series consists of 15 chemical elements with atomic number 57 (La) to 71 (Lu) that are also referred to as rare earth elements along with scandium and yttrium. Ln usually occur together in the same mineral, the ones with lower atomic number (except radioactive Pm) being present in higher concentrations (Figure 3; Evans, 1990). However, the elements with even atomic numbers are still more common than the neighbouring elements with odd atomic number (in accordance with the Oddo–Harkins rule) (Figure 3). A slight increase in solubility and decrease in basicity are also associated with increasing atomic number (Wells & Wells, 2001).

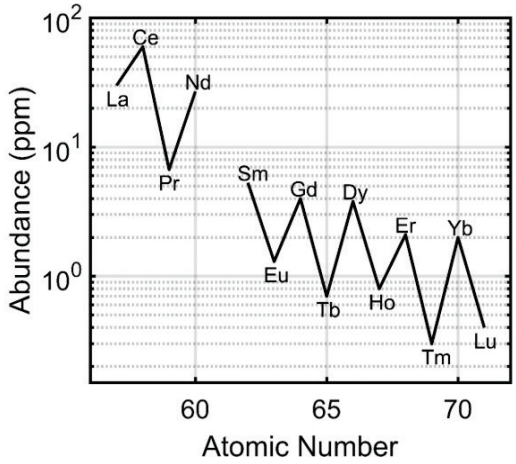

Figure 3. Abundance of the rare earth elements in the Earth's crust. Figure from Kim et al. (2018).

Physical properties of Ln such as magnetism, luminescence, radioactivity, melting point, and optical traits vary greatly between the individual elements (Evans, 1990). These properties also tend to be largely unaffected by the surrounding environment (Cotton, 2006; Kim et al., 2018). Due to the different physical properties, it is desirable to separate Ln into pure elements. On the contrary, chemical properties of Ln are very similar because of the valence electrons of most Ln being positioned in the 4f subshell. All Ln mainly exist in a 3+ oxidation state in the environment. The chemical similarity of Ln makes their separation process into pure elements very complex and is also the reason why Ln belong among *rare* earth elements. Indeed, some Ln are as abundant in the Earth's crust as, for example, zinc and copper (Evans, 1990).

Similarly to NP, lanthanides became industrially important in the 1980s, and over 80% of the cumulative Ln production has taken place since that decade (Graedel et al., 2014). Lanthanides are also considered technology critical elements in many parts of the world as they are technologically largely irreplaceable and a vast majority of Ln are currently produced only in China (Cobelo-García et al., 2015). A recent (2010 to 2015) lowering of lanthanide export quotas by China drove intensive lanthanide exploration activities all over the world and resulted in new large supplies being found in Canada, Greenland, Kenya, Tanzania, Malawi, South Africa (Paulick & Machacek, 2017), and Japan (Kato et al., 2011).

The above-mentioned physical traits of Ln can be applied in high-tech industries (optics, lighting, IT, energy, laser, imaging) but also in agriculture, ceramics, and medicine (Bünzli & Eliseeva, 2010; UNCTAD, 2014). Ln are often used for impurity

doping that can alter or improve the properties of materials. Addition of Ln impurities can, for example induce superconductivity in semiconductors (Cheng et al., 2009; Mukae et al., 1977) and microstructure stability in industrial ceramics (Buban et al., 2006), improve the properties of metal alloys (Yu et al., 2004), and sensitivity and selectivity of sensors (Koshizaki & Oyama, 2000). Ln oxides are also produced in nanoparticle form, e.g. CeO_2 NP, widely used as an auto-exhaust catalyst and a fuel additive, having one of the highest global production volumes among NP (10 000 t/year in 2010; Dahle & Arai, 2015; Keller et al., 2013).

There is a number of ways how anthropogenic Ln pollution can reach waterbodies: with fertiliser runoff (Bosco-Santos et al., 2017; Pang et al., 2001), atmospheric deposition (Cidu et al., 2013) in areas of, e.g. shale oil and coal burning or Ln mining (Li et al., 2018; Sabbioni et al., 1982; Wang & Liang, 2015), application of La-modified bentonite clay (Phoslock®) into lakes for algal bloom management (Spears et al., 2013), and hospital wastewater containing Gd-based contrast agent (Bau & Dulski, 1996). In the Netherlands, for example, the catalyst and artificial fertiliser industries are responsible for the principal emission of Ln into surface water (Slooff et al., 1993). In addition, acid mine drainage mobilises Ln that are naturally present in rocks (Stewart et al., 2017).

Ln concentrations in surface waters are generally very low. The filterable (pore size 0.2–1.2 µm) concentration consisting of the dissolved and fine colloidal fractions is usually in the range of ng/L to low µg/L (Goldstein & Jacobsen, 1988; Han & Liu, 2007; Ingri et al., 2000; Kharitonova & Vakh, 2015; Spears et al., 2013) probably due to the insolubility of lanthanide phosphates and oxides which form most of their ores (Evans, 1990). The total concentrations including larger colloids and suspended species can be much higher (up to 289 mg/L) compared to the filterable fraction (Goldstein & Jacobsen, 1988). Ln concentrations in industrially or agriculturally impacted rivers and in rivers receiving Gd-rich hospital effluent, are often still in the range of concentrations seen in unpolluted waterbodies (Bau & Dulski, 1996; Neal, 2007) but elevated (filterable) concentrations up to 78 µg/L have also been recorded (Kharitonova & Vakh, 2015). The highest filterable concentrations of Ln have been measured in acid mine drainage waters (up to 6.3 mg/L; Chudaeva & Chudaev, 2011; Migaszewski et al., 2016; Noller, 1991; Stewart et al., 2017) and after the application of lanthanum modified bentonite clay for lake restoration (up to 15 mg/L; Spears et al., 2013; Stauber & Binet, 2000). CeO_2 NP have been detected in Dutch surface water in concentrations up to 5.2 ng/L (Peters et al., 2018) that still remains well below the local proposed permitted limit values for total Ce in freshwater (22 µg/L; Sneller et al., 2000).

Human health effects of Ln were first addressed in 1960s when nuclear fission product ^{144}Ce was detected in human lung tissue, plants, molluscs, fish and terrestrial animal bones (Hirano & Suzuki, 1996; Liebscher et al., 1961; Nezu et al., 1960). The presence of radioactive (Th, U) and toxic (Al, As, Pb, etc.) metals in Ln ores is still considered the main hazard of Ln mining to the human health and the environment (US EPA, 2012). Nonradioactive isotopes of Ln have been largely neglected as possible environmental contaminants (Rim, 2016) partly due to the history of Ln mining that has drawn little attention to these elements in countries outside of China (US EPA, 2012).

Beneficial biological effects of Ln on agricultural plants (Pang at al., 2001) but also adverse effects on many other organism groups have been reported (Gonzalez et al., 2015; Hanana et al., 2017; Rim, 2016). Due to the application of Ln in medicine and their presence in fertilisers, toxicologists have been focusing on their potential effects

on humans, soil organisms and plants; data on aquatic organisms are more rare (Blaise et al., 2018; Gonzalez et al., 2014). The toxicity mechanisms of Ln are still relatively poorly understood (Gonzalez et al., 2014) but redox disturbances and the production of nitric oxide and reactive oxygen species (ROS) have been shown (Pagano et al., 2016). Accumulation of La in macrophytes, chironomids, and fish tissues, following La-modified bentonite application has also been recorded (Waajen et al., 2017). In general, the toxicity of individual Ln to living organisms is still assumed to be relatively similar due to the similar chemical properties of Ln (Hirano & Suzuki, 1996) while some synergistic and antagonistic effects between different Ln can occur (Romero-Freire et al., 2019).

The major toxicity mechanism in crustaceans could be the disturbance of Ca^{2+} metabolism (Bosco-Santos et al., 2017) especially for heavy Ln (Tb, Dy, Ho, Er, Tm, Yb, and Lu) that have a similar ionic radius to divalent calcium and possibly interfere with Ca^{2+} metabolism (Wells & Wells, 2001). Toxicity data to daphnids vary a lot depending on the applied Ln compounds, test formats and methods. The 48 h acute Ln toxicity based on measured total concentrations ranges from EC_{50} 1.4 to 24 mg Ln/L (Herrmann et al., 2016; Sneller et al., 2000) while EC_{50} based on dissolved concentrations ranges from 0.04 to 1.2 mg Ln/L (Barry & Meehan, 2000; Stauber, 2000). No acute toxicity of Ln has been shown to ostracods at nominal concentrations up to 6.5 mg Ln/L (Gonzalez et al., 2015; Romero-Freire et al., 2019) but Ln-rich river sediment induced mortality of ostracods (Romero-Freire et al., 2018).

Information on the toxicity of Ln is still scarce (Gonzalez et al., 2014). The behaviour and toxicity of Ln in different aquatic environments should be further studied (Herrmann et al., 2016) along with determination of the relevant chemical constants for accurate modelling of Ln speciation in the environment. In addition, data on long-term biological effects of Ln is urgently needed (Waajen et al., 2017).

1.2 Environmental hazard assessment of chemicals to the aquatic ecosystems

In 2018, 1361 newly developed substances were registered in European Economic Area (ECHA, 2018). The risks that can be posed by the (novel) substances to the environment must be evaluated and managed considering that over a quarter of the production volume of industrial chemicals produced in EU (> 300 million t/year) impose environmental hazard (Eurostat, 2017).

The responsibility to carry out chemical safety assessment can lie with different parties. In the United States, the Environmental Protection Agency (EPA) is responsible for determining the safety of chemicals already released to the market. In the European Union, the safety assessment is an obligation of the chemical manufacturer or importer and takes place before the chemicals are allowed to the market. The most important legislation for chemical safety assessment in EU is the REACH regulation (Registration, Evaluation, Authorisation and Restriction of Chemicals; European Commission, 2006) implemented by European Chemical Agency (ECHA).

According to REACH, the manufacturers and importers must demonstrate the safe use of the chemical and communicate the risk management measures to the users. The type and amount of aquatic toxicology data required by REACH depends on the quantity of chemicals imported or manufactured (Table 2). REACH recommends using test guidelines approved by the OECD or EU for ecotoxicity studies but guidelines

published by the US EPA, ISO, ASTM, and various national guidelines are also accepted (ECHA, 2017b). Safe concentrations of the new substances are usually established based on the no-effect concentrations, i.e., the highest concentration considered non-toxic in the laboratory tests. On the basis of the information presented to ECHA, the use of hazardous substances will be restricted or the substances replaced with less dangerous ones.

Table 2. Aquatic ecotoxicity data required by EU regulation REACH from the chemical manufacturers and importers. Requirements for higher tonnage also include all the requirements for lower tonnage. List of test guidelines corresponding to the OECD test guideline numbers is given in Appendix 7. Modified from Williams et al. (2009).

Information requirement	Tonnage band	OECD test guideline
Short term toxicity testing on invertebrates	1	202
Growth inhibition study on aquatic plants	1	201, 221, 238
Short-term toxicity testing on fish	10	203, 229, 236
Activated sludge respiration inhibition testing	10	209
Long-term toxicity testing on invertebrates	100	211
Long-term toxicity testing on fish	100	204, 210, 212, 215, 230, 234
Bioaccumulation in aquatic species	100	305, 315
Long-term toxicity to sediment organisms	1000	218, 219, 225, 233, 235, 239

Aquatic environment is often the main focus of ecotoxicity testing as many chemicals are eventually washed from the watersheds into waterbodies where they can spread more easily than on land. The majority of the OECD test guidelines for ecotoxicity testing use freshwater organisms. Some of the freshwater test formats are also applicable to seawater organisms but there are few standardised marine species protocols available (ECHA, 2017b).

The primary producers in aquatic ecosystems are microalgae and macrophytes while consumers of different level belong to zooplankton, nekton and benthos. Zooplankton and benthic species also include decomposers. The chemical safety assessment should be based on data covering at least three trophic levels such as algae/aquatic plants, invertebrates, and fish (ECHA, 2017b).

Aquatic species also occupy different habitats in the waterbody. The main habitats in freshwater ecosystems are the water column inhabited by pelagic organisms (nekton and plankton), sediments inhabited by benthic organisms (benthos and rooted macrophytes), and water surface inhabited by free-floating plants (Figure 4). Benthic organisms can live either on the sediment surface or burrow in the sediment.

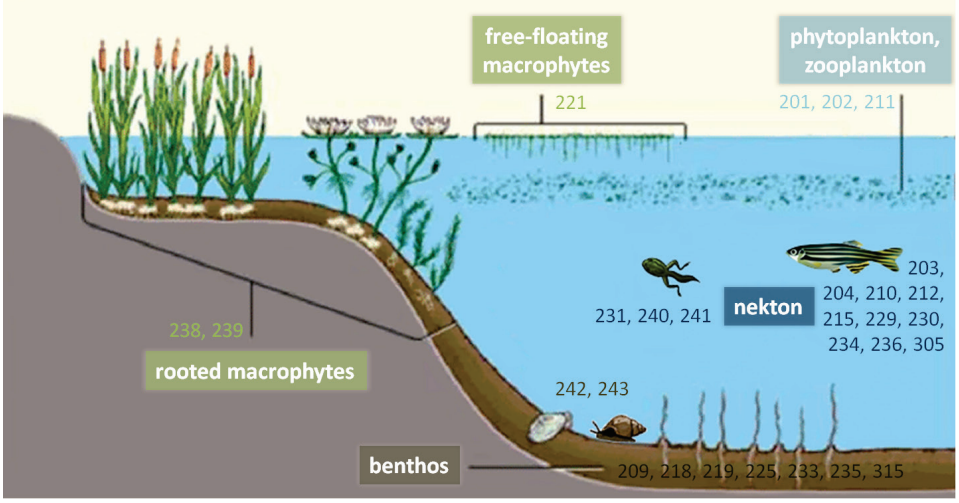

Figure 4. Standardised test protocols for the most common freshwater communities. OECD test guideline numbers are placed next to the relevant organism group. List of test guidelines corresponding to the OECD test guideline numbers is given in Appendix 7. Modified from Encyclopaedia Britannica, Inc. (https://www.britannica.com/science/inland-water-ecosystem).

Most substances affect aquatic organisms upon direct uptake from water rather than from food and thus waterborne exposure is the main focus of REACH (ECHA, 2017b). By the REACH legislation, the environmental toxicity data must always consider the short term toxicity to pelagic water organisms like phyto- and zooplankton or pelagic plants (Table 2). Daphnids (*Daphnia magna, Daphnia pulex, Ceriodaphnia dubia,* and *Ceriodaphnia affinis*) are the preferred pelagic invertebrate taxon in REACH legislation while *Lemna gibba* and *Lemna minor* are suitable for pelagic plant toxicity test (ECHA, 2017b). Acute toxicity tests with pelagic organisms require the test substance to be dissolved and bioavailable throughout the duration of the test in the water medium. Low (< 100 mg/L) and very low (< 1 mg/L) water solubility substances should be studied in long term tests on zooplankton or on fish. These tests also report sublethal endpoints like reproduction and growth.

However, eventually a large part of the pollution entering aquatic ecosystems accumulates in the sediments and may pose a threat to the benthic organisms. Benthic organisms also constitute an important link in the aquatic food chain and play an essential role in the recycling of detritus material. Thus, additional assessment of toxicity to benthic organisms is necessary for substances imported/manufactured in quantities of ≥ 1000 t/year (Table 2) but also for substances imported/manufactured in lower quantities if they are poorly water soluble or with high adsorption potential (ECHA, 2017b). OECD recommends using insect *Chironomus* sp. larvae, oligochaete *Lumbriculus* sp., and rooted macrophyte *Myriophyllum spicatum* for freshwater sediment toxicity tests (ECHA, 2017b).

1.3 Hazard assessment of metal nanoparticles and lanthanides by conventional test formats: limitations and uncertainties

Short, easy to perform, and well standardised toxicity tests using a sensitive test organism are preferred for conventional toxicity testing (Bondarenko et al., 2016). Standardisation allows accessible, comparable, and efficient toxicity evaluation and law-making for different substances. On the other hand, the overarching purpose of the chemical safety assessment is to evaluate the likelihood of the ecosystem being impacted as a result of the exposure to the chemical. Standard test methods required by REACH that are intended for hazard classification and labelling purposes are less suitable for realistic environmental risk assessment of chemicals needed for elaboration of the environmental quality standards (Blinova et al., 2018). These tests neither realistically represent the exposure conditions that occur in nature nor give enough information on the toxicity imposed by emerging contaminants with complex behaviour in the test environment (e.g. metal NP and Ln) (Blinova et al., 2018).

1.3.1 Substance-specific effect of test medium in nanoparticle and lanthanide toxicity testing

One of the key issues in evaluation of the toxicity of low-solubility and readily precipitating and adsorbing substances (e.g. metal NP and Ln) is the ability to determine the concentration of the test substance the test organisms are actually in contact with (Blinova et al., 2018). Metal-based NP can exert toxicity as particulates (physical effects) and in an ionic form depending on the aggregation, dissolution, and metal speciation that are largely determined by the chemical composition of the test medium (Holden et al., 2016). According to REACH, the test medium should be chosen such that exposure of the test substances occurred in a way that resembles the conditions in the environment (ECHA, 2017b). For reproducibility, synthetic mineral freshwater is still most often used as test medium in standard toxicity tests. This is usually a simple mixture of main mineral salts present in natural freshwater and devoid of organic matter and many other components that are present in natural water (e.g. nitrogen and phosphorus).

The absence of these components can alter metal toxicity. For instance, dissolved organic matter (DOM) greatly mitigates metal toxicity to aquatic organisms by binding the metal ions into inactive forms and directly protecting the biomembranes (Wood et al., 2011). A mitigating effect of DOM on toxicity has been recorded for Cu ions (De Schamphelaere & Janssen, 2004; Käkinen et al., 2011) and CuO NP (Blinova, et al. 2010), Ag ions (Blinova et al., 2013; Erickson et al., 1998) and Ag NP (Cupi, et al. 2015), but not for Zn compounds (Blinova et al., 2010) which only show reduction of photocatalytic activity in the presence of DOM (Akhil & Khan, 2017). On the other hand, DOM may increase the stability of NP suspension and NP dissolution which in turn can increase the metal bioavailability (Akhil & Khan, 2017; Grillo et al., 2014). The type of DOM is equally important as mitigating effects are usually mainly delivered by allochthonous (and not by autochthonous) DOM (Wood et al., 2011). Plant nutrients such as phosphates are often missing in synthetic freshwater while it has been shown that the presence of high concentration of phosphorus in eutrophic waters can stabilise CuO NP in the water column (Käkinen et al., 2011). Ln form both colloids (Ingri et al., 2000) and soluble complexes with DOM (Johannesson et al., 2004; J. Tang & Johannesson, 2010) and precipitate in the presence of phosphates (Table 3; Lürling & Tolman, 2010; Tang et al., 2016).

Table 3. Solubility of Ln species in water based on Wells & Wells (2001).

	Insoluble	Sparingly soluble	Soluble
chlorides			$LnCl^{2+}$
nitrates			$LnNO_3^{2+}$
sulfates		$LnSO_4^{+}$	
hydroxides	$Ln(OH)_3$		
carbonates	$Ln_2(CO_3)_3$		
oxalates	$Ln_2(C_2O_4)_3$		
phosphates	$LnPO_4$		
fluorites	LnF_3		

Other components may be more abundant in synthetic freshwater compared to natural water. The high water hardness and absence of DOM in artificial freshwater recommended for *D. magna* toxicity test (OECD, 2004) can be especially favourable for NP aggregation and settling (Chen & Elimelech, 2007; Grillo et al., 2014). High ionic strength also decreases the NP dissolution (Odzak et al., 2014). For these reasons, using an alternative test medium with lower Ca^{2+} content has been recommended for NP toxicity testing (Hund-Rinke et al., 2016). Similarly to metal NP, Ln colloids also destabilise and coagulate in solutions with high ionic strength (Goldstein & Jacobsen, 1988), and this can lead to imposing lower toxicity (Herrmann et al., 2016). Major anions (Cl^-, CO_3^-, SO_4^{2-}) are also often present in high concentrations in microcrustacean standard media (ISO, 2011, 2012a; OECD, 2004) and may affect both NP and Ln toxicity test results. The acute toxicity and sublethal effects of silver ions are reduced in the presence of Cl^- and S^{2-} (Bianchini & Wood, 2008; Levard et al., 2013a; Levard et al., 2013b) while the effect of Cl^- also depends on Cl/Ag ratio and is not mitigating in all the test conditions (Erickson et al., 1998; Levard et al., 2013b; Lin et al., 2015). Chloride-rich media can also increase Ln solubility (Table 3) but soluble Ln sulfates form only at low pH while insoluble carbonates dominate at higher pH (Migaszewski, et al. 2016).

1.3.2 Modifications of standard toxicity testing for increasing the ecological relevance

In addition to the test medium, other test conditions and selection of toxicity endpoints and test organisms can be modified in order to improve the reliability and data quality of laboratory toxicity testing of emerging contaminants (Holden et al., 2016; Petersen et al., 2015). For example, OECD test guidelines include only one zooplankton species *D. magna.* Moreover, some microcrustacean toxicity tests require no feeding at all while others use algal concentrations above the ones seen in nature. There are also only two sublethal endpoints widely used in standardised zooplankton toxicity tests (growth and reproduction) while a variety of such endpoints would help to predict realistic effects of the chemicals on the ecosystem (Holden et al., 2016). Modifying standard aquatic toxicity test methods for hazard assessment is allowed by REACH legislation considering that the results will always be scored less reliable than the results obtained in standard test conditions (ECHA, 2017b). Therefore, the best practice would be to compare the results from the toxicity tests carried out in modified conditions with the ones from the tests carried out in standard conditions and/or with a reference toxicant (Holden et al., 2016; Petersen et al., 2015). The high-quality information acquired that way would make regulatory testing more efficient, promote "green" product design, and allow developing computational tools that would lower

the cost of risk assessment and decrease the need for further animal testing (Holden et al., 2016; Petersen et al., 2015).

The presence or absence of algae is an especially relevant aspect of microcrustacean toxicity test conditions since algal blooms are becoming more and more common in natural waterbodies (Zhang et al., 2017). (Sub)chronic tests usually require feeding the test animals during the toxicity test (ISO, 2012a; OECD, 2012) while acute tests require feeding only prior to the test (OECD, 2004) or no feeding at all (ISO, 2011). The presence of algae in the test medium can change the bioavailability of metals in the test environment but also create an additional route of chemical uptake for the test organism (ECHA, 2017b). Both Ln oxides and metal NP have been shown to form agglomerates with algae (Aruoja et al., 2015; Joonas et al., 2017). Metal accumulation in *D. magna* can increase in the presence of algae (Luo et al., 2018) while toxicity of metal-based NP may decrease in a concentration dependant manner (Stevenson et al., 2017). However, the algal concentrations required in the toxicity tests tend to be unnaturally high. The feeding concentrations required in 21-day *D. magna* reproduction test (OECD, 2012) exceed natural food availability for daphnids up to 400 times (Stevenson et al., 2017) and experimenting with 3–5 times higher algal concentrations has been recommended for NP toxicity studies (Hansen et al., 2017). Thus, the possibility of algae-rich laboratory tests underestimating the toxicity that would possibly occur in the environment should be further studied. In addition to the algal concentration, the type of test medium has been shown to affect the interactions between algae and metals (Lin, et al. 2012) and should be considered.

Including algae in the toxicity test also allows measuring the effects of test chemicals on food uptake by the test organism. Stress-induced effects on feeding behaviour allow rapid prediction of population level effects such as decreased reproduction as a result of the reduced energy intake (Maltby, 1999). *D. magna* feeding activity has been shown to decrease in the presence of toxic metals, the feeding inhibition endpoint being much more sensitive than the acute toxicity (Bitton et al., 1995; Ginatullina et al., 2013).

Another additional sublethal endpoint, especially relevant for safety assessment of low solubility substances such as Ln and NP, is the metal body burden (ECHA, 2017b), i.e. the actual amount of compound associated with the organism after the exposure (Lai et al., 2002). Metal body burden data are a necessary measurement for calculating bioaccumulation, the partitioning of compounds between organisms and their surrounding environment (Lai et al., 2002). An elevated body burden of NP in the prey organism can lead to dietary uptake of NP in the predator via trophic transfer (Holbrook et al., 2008). Measuring the body burden in test organisms also helps to estimate the exposure concentration of low-solubility substances in the test environment (Hansen et al., 2017). OECD test protocols for body burden measurements are available for fish and oligochaetes but use of other organisms is also encouraged. For test species with low individual weight (e.g. *Daphnia*), it may be difficult to provide sufficient biomass to achieve high quality analytical results.

While long-term tests should be preferred for chemical safety assessment of Ln and NP as low solubility substances (ECHA, 2017b), long-term (> 10 days) toxicity tests can be too expensive for initial screening of novel substances. Instead, it has been recommended to slightly elongate the conventional acute toxicity tests for screening purposes of substances such as lanthanum (Stauber & Binet, 2000). Another option to facilitate cost-effective initial screening of emerging contaminants is using subchronic

tests that cover the most vulnerable life stages of the test organisms or whole life cycle tests with animals that have shorter life span. For example, a subchronic test with the ostracod *Heterocypris incongruens* allows measuring sublethal effects after 6 days of exposure instead of up to 100 days necessary in conventional sediment toxicity tests. Crustaceans are also important primary consumers in both pelagic and benthic aquatic communities (Persoone & Janssen, 1994) and tests with sediment organisms are especially relevant for NP and Ln studies, where the studied compounds tend to settle and adsorb in the test environment (ECHA, 2017b; Petersen et al., 2015). Furthermore, including an *H. incongruens* assay into toxicity testing is also valuable for diversifying the crustacean species used as test organisms.

Ecotoxicity testing of emerging contaminants would especially benefit from a wider set of microcrustacean species included in the research as daphnids require relatively high Ca^{2+} concentration in the test medium that can hinder NP and Ln suspension stability (see 1.3.1; Herrmann et al., 2016; Petersen et al., 2015). *D. magna* is also often less sensitive compared to other zooplankton and cladoceran species to e.g. Cu, Cu NP (Sadeq & Beckerman, 2018; Song et al., 2015), and Ln toxicity (Gonzalez et al., 2015; Herrmann et al., 2016). Using a variety of (cladoceran) species with different morphological features has also enabled defining the unique characteristics of the test animal that determine the toxicity of metal NP (Song et al., 2015). Thus, including several microcrustacean species in NP and Ln toxicity testing would serve many purposes and is highly recommended.

Aims of the study

The overall objective of the study was to create new knowledge on the potential environmental hazard of metal-based emerging contaminants (nanoparticles and lanthanides) to the aquatic ecosystem and to propose cost-effective modifications to standard freshwater toxicity test formats to increase the environmental relevance and predictive power of the obtained ecotoxicity data.

Specific aims of the study were:

1) to compare CuO and Ag-PVP NP behaviour and toxicity to *Daphnia magna* in natural *vs* standard freshwater;
2) to explore the combined effects of dietary algae and test media with different nutrient profiles on the toxicity of ZnO and CuO NP to planktic and benthic microcrustaceans;
3) to determine whether the mitigated toxicity of CuO NP in natural water is accompanied by lower Cu accumulation in *Daphnia magna* and to evaluate the suitability of the TXRF method for Cu quantification in individual *Daphnia magna* juveniles;
4) to evaluate the effect of sublethal concentrations of Co_3O_4, Mn_2O_3, and CuO NP on *Daphnia magna* post-exposure feeding behaviour and the following metal body burden;
5) to evaluate the potential hazard of five lanthanides (La, Ce, Pr, Nd, and Gd) to three aquatic microcrustaceans *Daphnia magna*, *Heterocypris incongruens*, and *Thamnocephalus platyurus* using different test conditions;
6) to evaluate the potential hazard of eight multimetal Ln (doped) submicron particles to freshwater microcrustaceans *Hetrocypris incongruens* and *Thamnocephalus platyurus*.

2 Materials and methods

2.1 Tested chemicals

The study focused on ten metals: five transition metals (Mn, Co, Cu, Zn, and Ag) for nanoparticle studies and five metals (La, Ce, Pr, Nd, and Gd) for lanthanide studies (Figure 5).

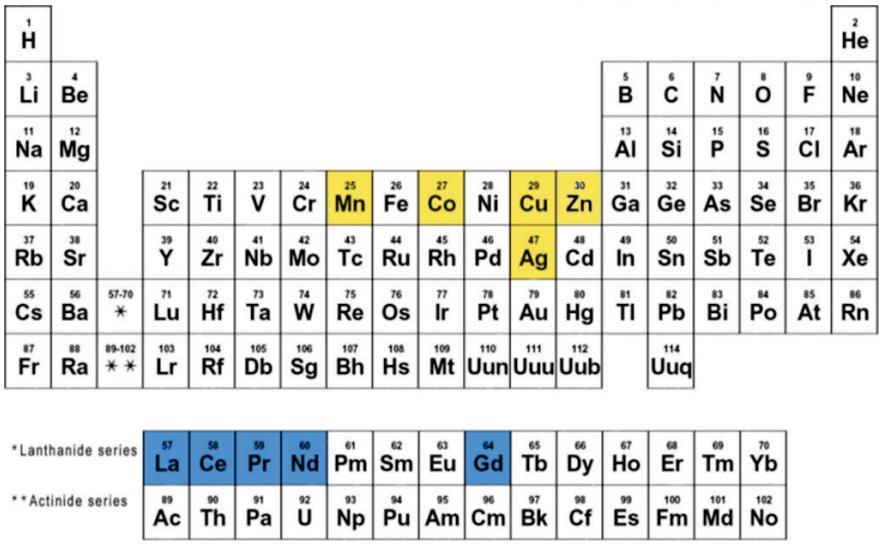

Figure 5. Metals chosen for nanoparticle (yellow) and lanthanide (blue) studies.

2.1.1 Ag, Cu, Zn, Co, and Mn based nanoparticles

The list of nanoparticles used in papers **I–IV** and some of their characteristics are given in Table 4. CuO, ZnO, Ag-PVP, Mn_2O_3, and Co_3O_4 NP were chosen for this study. The NP stock suspensions were sonicated before the experiments to improve the dispersion of the NP. In addition to Mn_2O_3 and Co_3O_4, the toxicity of La, Gd, and Sr doped CoO_3 and MnO_3 submicron particles was evaluated in the lanthanide study (see 2.1.2).

The experiments with NP were accompanied by experiments with the respective soluble metal salts $AgNO_3$ (paper **I**), $ZnSO_4{\cdot}7H_2O$ (paper **II**), $CuSO_4{\cdot}5H_2O$ (papers **I–IV**), $CoCl_2{\cdot}6H_2O$ (paper **IV**), and $MnCl_2{\cdot}4H_2O$ (paper **IV**) as ionic controls. The solubility of Cu and Co salts was > 100 g/L and of Ag, Zn, and Mn salts > 1000 g/L.

Table 4. The studied metal-based nanoparticles.

NP	Primary size (nm)	Form	Origin	Coating/ Stabiliser	Paper
nAg-PVP	21	suspension (40 g Ag/L)	partners in NNV	-/ polyvinyl-pyrrolidone	**I**
CuO	22–25	powder	partners in NNV	-/ -	**I, II, III, IV**
ZnO	10–15	powder	partners in NNV	-/ -	**II**
Co_3O_4	10–30	powder	US Research Nanomaterials	-/ -	**IV**
Mn_2O_3	30	powder	US Research Nanomaterials	-/ -	**IV**

NNV – NanoValid, EU 7th FP nanosafety project (under Grant Agreement no. 263147)

2.1.2 Lanthanide salts and lanthanide (doped) particles

Lanthanide salts were studied in paper **V** and submicron Ln (doped) submicron particles in paper **VI**. $La(NO_3)_3{\cdot}6H_2O$ and $Ce(NO_3)_3{\cdot}6H_2O$ were provided by Treibacher Industrie AG and $Pr(NO_3)_3{\cdot}6H_2O$, $Nd(NO_3)_3{\cdot}6H_2O$, and $Gd(NO_3)_3{\cdot}6H_2O$ by Sigma Aldrich. Solubility of all the salts was > 1000 g/L at 20–25 °C.

The list of submicron Ln (doped) particles and their properties is given in Table 5. The particles were synthesised by Ceramic Powder Technology AS (Norway) for application in solid oxide fuel cell systems or in gas separation membranes. The Ln (doped) particle stock suspensions were sonicated before the experiments to improve the dispersion the particles.

Table 5. The studied lanthanide (doped) particles.

Particles	Primary size (nm)	Dopant
CeO_2	38	none
$Ce_{0.9}Gd_{0.1}O_2$	27	Gd
$Gd_{0.97}CoO_3$	230	Gd
$LaCoO_3$	590	La
$LaFeO_3$	126	La
La_2NiO_4	284	La
$(La_{0.6}Sr_{0.4})_{0.95}CoO_3$	65	La, Sr
$(La_{0.5}Sr_{0.5})_{0.99}MnO_3$	137	La, Sr
$Ce_{0.8}Pr_{0.2}O_2$	23	Pr

2.2 Methods for nanoparticle and lanthanide characterisation

- Dynamic light scattering (DLS) for NP hydrodynamic size measurement (papers **I–IV**);
- electrophoretic light scattering (ELS) for NP zeta potential measurement (papers **I–IV**);

- Total Reflection X-ray Fluorescence (TXRF) spectroscopy for Cu, Mn, and Co quantification in *D. magna* specimen and Ln quantification in *L. minor* and in the water column (papers **III–VI**);
- metal specific sensor bacteria for metal (Cu, Ag) bioavailability analysis (paper **I**);
- ultracentrifugation and the following atomic adsorption spectroscopy (AAS) analysis for measuring NP dissolution assessment and for metal recovery assessment after application of soluble metal salts (papers **I–III**);
- ultrafiltration and the following ICP-MS analysis for NP dissolution assessment (paper **IV**).

2.3 Test media

Test media are listed in Table 6 along with the concentrations of the main components. Standard artificial freshwater (AFW) described in OECD TG 202 (OECD, 2004) and ISO 6341 (ISO, 2012b) was used for acute toxicity testing with *D. magna* (papers **I–V**) while US EPA reconstituted moderately hard water (MHRW) was used for *T. platyurus* and *H. incongruens* tests (ISO, 2011, 2012a; papers **II, V, VI**).

In addition to standard synthetic freshwaters, lake waters were used in parallel to study the effect of more natural conditions on the toxicity. Natural test media used in the experiments was collected from four lakes: oligotrophic Lake Lucerne (paper **I**) and highly eutrophic Lake Greifen (paper **I**) in Switzerland and semi-eutrophic Lake Raku (papers **II, III, V**) and eutrophic Lake Ülemiste (papers **II, III, V**) in Estonia. Lake Ülemiste water was also used in the *D. magna* reproduction test (paper **V**). Use of natural water is allowed by *D. magna* acute and chronic toxicity test guidelines (OECD, 2004, 2012) if the total organic carbon content is below 2 mg/L. This requirement was only met by Lake Lucerne water (Table 6).

Table 6. Main components of the test media.

	***H. incongruens, T. platyurus* MHRW**	***D. magna* OECD AFW**	**Lake Lucerne**	**Lake Greifen**	**Lake Raku**	**Lake Ülemiste**
pH	7.5 (0.2)[1]	7.7 (0.2)	8.3 (0.04)	8.1 (0.07)	8.3 (0.04)	8.2 (0.4)
Hardness (mmol/L)	0.84	2.5	1.1	1.9	1.3 (0.05)	2.0 (0.3)
Ca^{2+} (mg/L)	14	80	38 (1.0)	56 (11)	44 (2.5)	66 (11)
Mg^{2+} (mg/L)	12	12	3.5 (0.2)	15 (0.3)	4.6 (0.1)	7.8 (0.5)
K^{+} (mg/L)	2.1	3.0	<2.5	3.5 (0.07)	1.8 (0.04)	2.7 (0.2)
Na^{+} (mg/L)	26	18	<2.5	18 (2.5)	2.7 (0.02)	6.7 (1.0)
Cl^{-} (mg/L)	1.9	145	2.0 (0.6)	27 (4.4)	3.4 (0.3)	11 (2.1)
SO_4^{2-} (mg/L)	82	48	14 (0.0)	12 (0.7)	22 (0.0)	29 (4.5)
CO_3^{2-} (mg/L)	69	46	n.a.	n.a.	n.a.	n.a.
DOC (mg/L)	0	0	1.0 (0.07)	3.4 (0.07)	5.1 (0.2)	10 (0.5)
P_{tot} (mg/L)	0	0	0.012	0.026	0.035 (0.001)	0.030 (0.012
N_{tot} (mg/L)	0	0	1.4	<0.5	0.62 (0.13)	1.4 (0.4)
Papers	**II, V, VI**	**I–V**	**I**	**I**	**II, III, V**	**II, III, V**

[1] *Mean (standard deviation); n.a. – not analysed.*

As required by the standard test protocols, dietary microalgae were added to the test medium in the *D. magna* reproduction test (OECD, 2012; paper **V**) and in the *H. incongruens* subchronic test (Table 7; papers **II, V, VI**). Dietary algae were also used in modified *D. magna* acute toxicity test formats (paper **II**) and in post-exposure recovery and feeding activity tests (Table 7; papers **III, IV, V**). Two microalgal species *Raphidocelis subcapitata* and *Chlamydomonas reinhardtii* were used.

Table 7. Dietary algae used in the tests.

Type of test	Algal species	Algal concentration (cells/mL)	Feeding	Duration of the test	Paper
D. magna reproduction	*R. subcapitata*	1.5×10^5	daily	21 d	**V**
H. incongruens subchronic	*R. subcapitata*	7.5×10^6 10^8	Once, start of the test	6 d	**II, V** **VI**
D. magna acute	*R. subcapitata*	7.5×10^6	Once, start of the test	48 h	**II**
D. magna post-exposure recovery	*R. subcapitata*	5×10^5	Once, start of the test	24 h	**III**
	C. reinhardtii [1]	6×10^4	Once, start of the test	48 h	**IV**
	R. subcapitata	1.5×10^5	daily	10 d	**V**

[1] These conditions were also used for feeding activity measurements.

2.4 Toxicity testing with microcrustaceans

Toxicity testing was conducted using three different microcrustacean species (Figure 6) and the experiments were based on four OECD and ISO standardised test formats (Table 8). Microcrustaceans are an important link in the aquatic food chain and have been shown to be one of the most vulnerable organism groups to both metal-based nanoparticles and lanthanides (Herrmann et al., 2016; Kahru & Dubourguier, 2010). Planktic (*Daphnia magna*, *Thamnocephalus platyurus*) and benthic (*Heterocypris incongruens*) microcrustaceans were used in this study to compare the traditional pelagic exposure and benthic exposure recommended for low solubility substances (ECHA, 2017b). All the used species have wide distribution and are common in a variety of freshwater bodies including ephemeral waters. They all feed mainly by non-selective filtering. *D. magna* and *H. incongruens* reproduce both sexually and by parthenogenesis while *T. platyurus* reproduces only sexually. All the test species produce dormant eggs that can be hatched by creating favourable laboratory conditions prior to the test. Dormant eggs produced by MicroBioTests Inc. (Belgium) were used in this study.

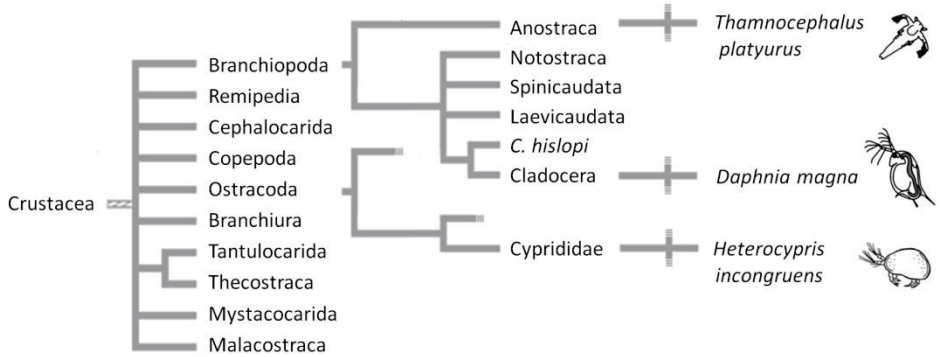

Figure 6. Simplified phylogenetic relationship between the test species based on Tree of Life Web Project (http://tolweb.org/Crustacea).

Table 8. Comparison of test formats for microcrustaceans.

	Daphnia magna	***Daphnia magna***	***Thamnocephalus platyurus***	***Heterocypris incongruens***
Test protocol	OECD 202 (OECD, 2004)	OECD 211 (OECD, 2012)	ISO 14380 (ISO, 2011)	ISO 14371 (ISO, 2012a)
Duration	48 h	21 days	24 h	6 days
Type of test	static acute	semi-static chronic	static acute	static subchronic
Feeding with algae	2 h prior to the test	daily	none	prior to and at the beginning of the test
Medium volume per organism	2 mL	50 mL	0.1 mL	0.4 mL
Temperature	20 °C	20 °C	25 °C	25 °C
Light conditions	dark	16/8 light/dark cycle	dark	dark
Endpoints	immobilisation	mortality, reproduction	mortality	mortality, body length
Papers	**I, II, III, IV**	**IV**	**IV, V**	**IV, V**

The water flea *Daphnia magna* (Figure 7) has been used as a standard aquatic toxicity test species since the 1960s due to its broad distribution, short life cycle, translucent carapace and its suitability for laboratory culturing (Persoone et al., 2009). *D. magna* is by far the most common microcrustacean species used in ecotoxicity testing and is the first invertebrate included in standardised OECD, ISO, and US EPA test protocols. *D. magna* feeds by unselective suspension feeding and occasional grazing at low suspended food concentrations (Horton et al., 1979) ingesting food 0.4–40 µm in diameter (Geller, 1981; Gophen & Geller, 1984). Daphnids are the recommended pelagic invertebrate test organisms by EU REACH legislation (ECHA, 2017b).

Figure 7. Daphnia magna (photo: Aljona Lukjanova).

The fairy shrimp *Thamnocephalus platyurus* (Figure 8) is endemic to the American continents and typically lives in ephemeral waterbodies (Belk, 1977). Fairy shrimps mainly feed by filter-feeding but also by scraping solid surfaces (Belk, 2007). The test protocol for *T. platyurus* (ISO 14380) was standardised relatively recently (2011) and is one of the shortest and simplest tests protocols developed for microcrustaceans.

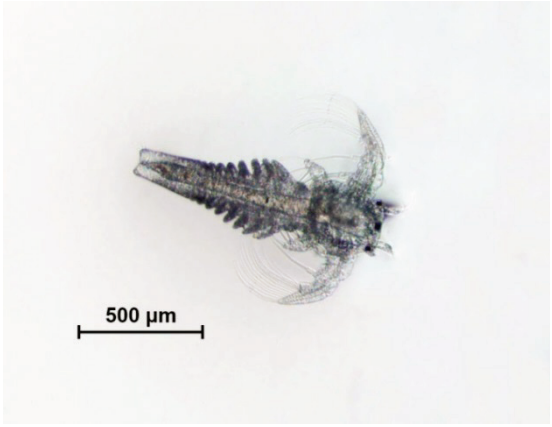

Figure 8. Thamnocephalus platyurus (photo: Marge Muna).

The ostracod *H. incongruens* (Figure 9) lives in the bottom of temperate freshwater ponds and lakes, feeding on bacteria, algae, organic detritus, plant material and other invertebrates (Miličić et al., 2015; Shuhaimi-Othman et al., 2011). The *H. incongruens* test (ISO 14371) is also a relatively recent (2012) addition to benthic invertebrate tests, being a shorter alternative to the conventional tests using chironimids and amphipods. Reference sediment (sand) is provided with the test. As a modification, sand was omitted from part of the *H. incongruens* tests in this study.

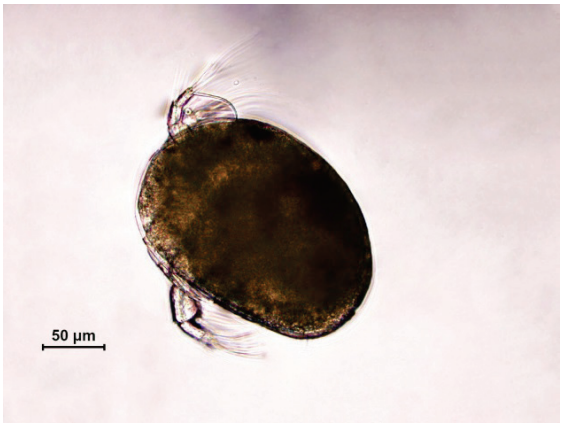

Figure 9. Heterocypris incongruens (photo: Marge Muna).

2.5 Sublethal endpoints

Potential impacts of chemicals on populations and ecosystems can be estimated in laboratory conditions by measuring/recording sublethal endpoints. Sublethal endpoints enable to predict long-term effects on viability and reproduction in environmentally relevant test conditions (Holden et al., 2016). The following standardised sublethal endpoints were measured/recorded in the (sub)chronic tests in this study:

- body length of ostracods (ISO 14371) (papers **II, V, VI**);
- reproduction of *D. magna* (OECD TG 211) (paper **V**).

In addition to standardised sublethal endpoints, unstandardised endpoints were applied. All of the unstandardised endpoints were measured following the *D. magna* acute toxicity assay (OECD, 2004) which is the most widely used test format for chemical safety assessment (see Table 2). Measuring sublethal endpoints such as metal body burden can be especially relevant for low-solubility substances. It is difficult to determine the external concentration to which the test organism is exposed to and thus also the acute toxicity values for these substances (ECHA, 2017b). The following unstandardised sublethal endpoints were measured/recorded subsequent to the *D. magna* acute toxicity test in this study:

- metal body burden in single juvenile daphnids (papers **III, IV**) and in a free-floating macrophyte *Lemna minor* (paper **VI**);
- post-exposure feeding behaviour of juvenile daphnids (paper **III**);
- survival of *D. magna* during post-exposure recovery (papers **II, III, IV**);

2.6 Statistical analysis

In addition to the statistical methods described in the publications (**I–VI**), Pearson correlation was used to calculate the correlation coefficients (r^2) between test medium parameters and characteristics of metal-based NP in this thesis.

3 Results and discussion

3.1 Toxicity evaluation of CuO, ZnO, Ag-PVP, Co_3O_4, and Mn_2O_3 nanoparticles with freshwater microcrustaceans

The aggregation, dissolution, and acute and subchronic toxicity of CuO, ZnO, and Ag-PVP NP were studied in different test conditions (papers **I–III**) along with sublethal endpoints such as feeding behaviour, metal body burden, and recovery after acute exposure to CuO, Co_3O_4, and Mn_2O_3 NP (papers **III–IV**).

3.1.1 Behaviour of CuO, ZnO, and Ag-PVP nanoparticles in different test conditions (papers I–III)

In order to understand the behaviour of CuO, Ag-PVP, and ZnO NP in different test conditions, the aggregation and dissolution of the NP were measured. The aggregation results in standard mineral freshwaters indicated NP aggregation already within the first hour from suspension preparation (Table 9; papers **I–III**) and ζ potential showed (very) low stability of all the suspensions (Table 9; Bhattacharjee, 2016). Analysis of NP fate in the lake waters instead of standard water confirmed the findings of Liu et al. (2018) and Cupi et al. (2015) who showed that despite the (counter)action of several water parameters, organic matter is the principle component to decrease the aggregate size of CuO and ZnO NP (r^2 = 0.52–0.67; papers **I–III**) but not that of Ag-PVP NP possibly due to the presence of stabilising PVP. In contrast to findings of Peng et al. (2017), ZnO NP were found to aggregate more at higher SO_4^{2-} concentrations (r^2 = 0.96; paper **II**). SO_4^{2-} concentration also had a negative correlation with pH which might have been the actual factor impacting ZnO aggregate size (r^2 = 0.87). In general, there was little variation in PVP-stabilised Ag-PVP NP hydrodynamic size between different concentrations and test media compared to ZnO and CuO NP which confirms that the combined effect of the type of the NP and the chemical composition of the test medium will define the stability of the NP suspensions (Garner & Keller, 2014).

Table 9. Hydrodynamic size (D_h), zeta potential (ζ) and polydispersity index (PDI) of CuO, Ag-PVP, and ZnO NP after 0–1 h incubation.

Primary NP size (nm)	Nominal concentration (mg metal/L)	D_h (nm)		ζ (mV)		PDI		Paper
		Standard waters	Lake waters	Standard waters	Lake waters	Standard waters	Lake waters	
CuO	10	573 (A)	197 (R)	-7.3 (A)	-19 (R)	0.22 (A)	0.19 (R)	**II**
22–25		565 (M)	335 (Ü)	-13 (M)	-18 (Ü)	0.24 (M)	0.25 (Ü)	
	100	1830 (A)	2353 (L)	0.41 (A)	-15 (L)	0.64 (A)	0.12 (L)	**I, III**
			2465 (G)		-16 (G)		0.16 (G)	
			980 (R)		-20 (R)		0.26 (Ü)	
			937 (Ü)		-19 (Ü)		0.27 (R)	
Ag-PVP	10	98 (A)	125 (R)	-7.5 (A)	-9.4 (R)	0.24 (A)	0.18 (R)	*
21			106 (Ü)		-6.9 (Ü)		0.22 (Ü)	
	100		120 (L)		-4.4 (L)		0.29 (L)	**I**
			126 (G)		-5.8 (G)		0.36 (G)	
ZnO	10	639 (A)	283 (R)	3.4 (A)	-17 (R)	0.34 (A)	0.56 (R)	**II**
10–15		1225 (M)	177 (Ü)	-5.3 (M)	-16 (Ü)	0.45 (M)	0.38 (Ü)	

**unpublished data; standard waters – OECD 202 artificial freshwater (A) and US EPA moderately hard reconstituted water (M); lake waters – Lake Raku (R), Ülemiste (Ü), Greifen (G), and Lucerne (L).*

Shedding of metal ions is one of the main pathways of adverse effects imposed by metal-based NP (Bondarenko et al., 2013). To understand the effect of test conditions on NP toxicity test results, it is thus crucial to determine the amount of dissolved metals present in the test environment. The data on the bioavailability of the metals is similarly important as not all dissolved metal species are bioavailable (Allen & Hansen, 1996). NP dissolution was measured by quantifying metal ions separated from NP by ultracentrifugation (papers **I–III**). For comparison, metal recovery was also measured after ultracentrifugation of soluble metal salt solutions (papers **I–III**). NP bioavailability was measured by using metal specific sensor bacteria and calculated as a percentage of the bioavailability of the respective soluble salt (paper **I**).

CuO and Ag-PVP NP but also the respective metal salts showed much lower dissolution/recovery in standard waters (≤ 1%) compared to MQ water (7–56%) indicating precipitation of these metals in standard waters (Table 10; papers **I–III**). ZnO NP dissolution (24–27%) and Zn salt recovery (88–102%) were higher and comparable in all the media (paper **II**). For all the NP, metal bioavailability, evaluated by the sensor bacteria, was much higher compared to dissolution suggesting that the metal uptake from NP may be higher than could be expected from the dissolution/recovery data because: I) sensor bacteria were exposed to metal compounds in bacterial growth medium (diluted) which can increase metal dissolution and affect bioavailability (Käkinen et al., 2011); II) bacteria can have different uptake pathways of metals from NP compared to multicellular organisms (von Moos et al., 2014); III) localised metal concentration in the vicinity of the bacterium can be higher than the medium average due to NP sorption to the membrane (Bondarenko et al., 2013; Ivask et al., 2014).

The results confirm that the respective soluble salts should always be included in toxicity testing of metal-based NP (Aruoja et al., 2015; Bondarenko et al., 2016; Handy et al., 2012; Heinlaan et al., 2008; Suppi et al., 2014). Furthermore, metal recovery of soluble salts should always be analysed along with NP dissolution and relevant test concentrations should be used.

Similarly to aggregation, NP dissolution and metal recovery from soluble salts was also measured in natural lake waters. The organic matter in the natural waters increased the dissolution of CuO NP (r^2 = 0.86; papers **I–III**). Interestingly, the dissolution in Swiss lake waters remained even lower than that in the organic free standard water (Table 10). The larger aggregate size in Swiss water compared to standard water (Table 9) probably led to the lower dissolution (Liu et al., 2018) as aggregate size and dissolution were inversely correlated (r^2 = 0.82). Odzak et al. (2015) also showed low solubility of uncoated CuO NP in the studied Swiss lake waters (Greifen and Lucerne). The potentially different profile of dissolved organic matter in the Swiss lakes could also have led to different effects on NP dissolution compared to Estonian lake waters (Raku and Ülemiste) (Gunsolus et al., 2015; Wood et al., 2011). The effect of organic matter might have been further impacted by the fact that Swiss lake waters were filtered through a 0.2 µm pore size filter, while large pore size (0.45 µm) filter was used for Estonian waters. Differently from dissolution, CuO NP bioavailability was higher at higher total nitrogen (r^2 = 0.97) and/or pH (r^2 = 0.83) (the parameters correlated with each other) and at lower Na^+ and Cl^- concentrations (r^2 = 0.89 and 0.80). Cu salt recovery was higher at lower pH (r^2 = 0.49) and/or at higher SO_4 concentration (r^2= 0.59) (the parameters correlated with each other).

Table 10. Dissolution (D) and bioavailability (B) of CuO, Ag-PVP, and ZnO NP and recovery of the respective metal salts.

Nominal concentration (mg metal/L)		MQ		Standard waters		Lake waters		Paper
		D	B	D	B	D	B	
CuO NP	0.005–40		42		18 (A)		32 (L) 25 (G) 35 (19)[1] (Ü)	**I, ***
	10	6.9		0.67 (A) 0.42 (M)		<0.5 (L) 0.6 (G) 0.9 (0.4) (R) 1.2 (0.6) (Ü)		**I–III**
$CuSO_4$	10	84		37 (A) 37 (M)		21 (L) 26 (G) 33 (R) 32 (Ü)		**I–III**
Ag-PVP NP	0.005–40		80		61 (A)		34 (L) 66 (G)	**I, ***
	10	56 (10)		0.26 (0.3) (A)		18 (L) 1.3 (G) 2.7 (1.8) (R) 1.1 (0.9) (Ü)		**I, ***
$AgNO_3$	10	109 (6.7)		0.20 (0.3) (A)		41 (L) 0.8 (G) 4.8 (1.2) (R) 0.8 (0.7) (Ü)		**I, ***
ZnO NP	0.005–40		98 (26)		104 (15) (A)		79 (12) (Ü)	*
	10	27		24 (A) 25 (M)		15 (L) 24 (G) 21 (R) 23 (Ü)		**II, ***
$ZnSO_4$	10	88		102 (A) 97 (M)		46 (L) 67 (G) 90 (R) 91 (Ü)		**II, ***

*Dissolution was measured after 48 h (24 h in MQ) of incubation and is given as a percentage of the nominal concentration. Metal NP bioavailability was measured by using metal specific sensor bacteria after 2 h of incubation and is given as a percentage of soluble salt bioavailability. [1] mean (standard deviation); * unpublished data; MQ – MilliQ ultrapure water; standard waters – OECD 202 artificial freshwater (A) and US EPA moderately hard reconstituted water (M); lake waters – Lake Raku (R), Ülemiste (Ü), Greifen (G), and Lucerne (L).*

Ag-PVP NP dissolution and $AgNO_3$ recovery were decreased by high water hardness (r^2 = 0.52–0.54; paper **I**) the differences between particle behaviour in Lake Greifen and Lake Lucerne being in accordance with results obtained by Odzak et al. (2014). Both dissolution and bioavailability results confirmed high concentration of Ag^+ ions in Ag-PVP NP original stock suspension provided by the manufacturer, the Ag-PVP dissolution being very closely correlated to the metal recovery from $AgNO_3$ (r^2 = 1).

Thus, the dissolution test results of Ag-PVP NP do not represent the true dissolution of the particles.

Both an increase in the dissolution of ZnO NP and a decrease in the free Zn^{2+} concentration in the presence of DOC have been shown (Han et al., 2014; Li et al., 2013). The lake waters used in this study had a moderate effect on the dissolution of Zn compounds (paper **II**). ZnO NP dissolution was slightly higher at higher Mg^{2+} and/or Na^{+} concentrations (r^2 = 0.66 and 0.58; the parameters correlated with each other). Zn recovery from Zn salt solution was higher at high SO_4^{2-} concentrations (r^2 = 0.53). Interestingly, higher ZnO NP dissolution and Zn recovery from $ZnSO_4$ was seen in Lake Greifen compared to Lake Lucerne (Table 10) in this study while the opposite has been shown in the literature for uncoated ZnO NP in these lake waters (Odzak et al., 2015).

NP dissolution was also measured after incubation with algae in different test media (paper **II**). Algae moderately increased the dissolution of CuO and ZnO NP and recovery of Cu from Cu salt solution while Zn recovery from Zn salt solution was not affected (Table 3 of paper **II**). Ag-PVP NP dissolution and Ag recovery from salt exposure were reduced up to 3.9-fold in the presence of algae (unpublished data) especially in natural test media. This indicates possible sorption and uptake of Ag ions by algae similarly to the water plants (Bone et al., 2012).

To conclude, the dissolution and bioavailability of NP was affected by the test medium and addition of algae in a NP-specific manner.

3.1.2 Acute and subchronic toxicity of CuO, ZnO, and Ag-PVP nanoparticles in different test media (papers I–III)

The acute toxicity of CuO, ZnO, and Ag-PVP NP to *D. magna* (OECD, 2004; papers **I–III**) and subchronic toxicity to *H. incongruens* (ISO, 2012a (with sand omitted); paper **II**) were tested in standard media and in lake waters. All the NP induced toxicity to both organisms. According to *D. magna* test results based on nominal metal concentrations, Ag-PVP NP would be classified as (very) hazardous to the aquatic environment (EC_{50} < 1 mg/L; European Commission, 2008) in the standard water similarly to $AgNO_3$ (Table 11) due to the high ion content in the NP stock suspension. CuO and highly soluble ZnO NP along with $ZnSO_4$ would be considered non-hazardous based on the *D. magna* acute test results (EC_{50} > 1 mg/L; European Commission, 2008). However, all EC_{50} values were based on nominal concentrations in this study. As NP tend to settle out of the water column and adsorb to surfaces, the toxicity based on concentrations measured from the water column could classify all the studied NP as hazardous.

Toxicity of all the tested compounds was slightly higher to *H. incongruens* in a 6-day subchronic test compared to *D. magna* acute test except for $CuSO_4$ which was less toxic possibly due to the presence of algae in the *H. incongruens* test. The body length of *H. incongruens* was also affected at sublethal exposure concentrations resulting in growth inhibition after exposure to Cu (up to 23% compared to the control), Zn (up to 53%) (Table S3 in paper **II**), and Ag (up to 36%) compounds (unpublished data). Considerable growth enhancement was seen at some other sublethal exposure concentrations that was especially high after exposure to metal salts: Cu (up to 41%), Zn (up to 17%), and Ag (up to 60%).

Using lake water instead of standard water resulted in drastically different effects on the toxicity of the studied NP to *D. magna* (Table 11). CuO NP toxicity was positively correlated to Cu salt toxicity (r^2 = 0.70) and both salt and CuO NP toxicity was higher at lower DOC concentrations if calculated based on the higher toxicity values obtained in Swiss waters (r^2 = 0.96 for CuO NP and r^2 = 0.84 for Cu salt). In agreement with earlier

studies (Blinova et al., 2010; Yang & Xing, 2009), CuO NP toxicity decrease in lake waters compared to standard mineral test medium (papers **I–III**). However, the toxicity of $CuSO_4$ was not mitigated in Lake Lucerne water despite the fact that the allochthonous DOM with high mitigation potential (Wood et al., 2011) is dominating in both Lake Lucerne and the Estonian lakes (Stücheli et al., 2018; Toming et al., 2013). The different filter pore size used for filtration could have also had an impact as explained in the previous paragraph.

Table 11. The toxicity (48 h EC_{50}) of metal-based NP to D. magna in different test environments. Coloured background illustrates the increase/decrease of toxicity by using lake waters as test media and addition of algae compared to the toxicity in standard freshwater without algae.

	Standard water	Lake water	Standard water + algae	Lake water + algae	Paper
CuO NP	1.6	5.7–75 (L) 5.5–26 (G) 6.3 (R) 28 (Ü)	2.0	 68 (R) >150 (Ü)	I–III
$CuSO_4$	0.053	0.043 (L) 0.12 (G) 0.15 (R) 0.22 (Ü)	0.41	 0.50 (R) 0.65 (Ü)	
Ag-PVP NP	0.00082	0.0017 (L) 0.0029 (G) 0.0023 (R) 0.0033 (Ü)	0.18	 0.93 (R) 0.55 (Ü)	I, *
$AgNO_3$	0.00055	0.00070 (L) 0.0014 (G) 0.0010 (R) 0.0027 (Ü)	0.056	 0.53 (R) 0.50 (Ü)	
ZnO NP	1.9	0.31 (L) 0.81 (G) 0.50 (R) 0.71 (Ü)	1.7	 1.9 (R) 3.1 (Ü)	II, *
$ZnSO_4$	2.3	0.47 (L) 1.3 (G) 0.59 (R) 0.76 (Ü)	1.5	 0.84 (R) 1.3 (Ü)	

< 5 fold increase | *no effect* | *< 20 fold* | *> 20 fold* | *> 100 fold decrease*

**unpublished data; Standard water – OECD 202 artificial freshwater; Lake waters – Lake Raku (R), Ülemiste (Ü), Greifen (G) and Lucerne (L).*

Ag-PVP NP and $AgNO_3$ toxicity were correlated (r^2 = 0.74) as was expected due to the high Ag^+ ion concentration in the Ag-PVP NP stock suspension. Ag-PVP NP toxicity was similar in all the test media (Table 11; paper **I**, unpublished data) which may be because of the stabilising effect of PVP polymers (Blinova et al., 2013). Despite the absence of statistically significant mitigation in natural waters compared to standard waters, there was inverse correlation between DOC concentration and Ag salt and Ag-PVP NP toxicity (r^2 = 0.88 and 0.73) confirming the previous findings (Blinova et al., 2013; Erickson et al., 1998). The steep slope of the dose-response curve and very high

toxicity of both Ag ions and NP possibly increased the variation of the toxicity results (Blinova et al., 2013).

As expected based on the previous studies (Blinova et al., 2010; Yang & Xing, 2009), natural waters did not mitigate Zn compounds' toxicity but on the contrary, increased it (paper **II**). Interestingly, ZnO NP (and $ZnSO_4$) would be classified as hazardous in lake waters but not in standard mineral water probably due to the higher mitigating effect of hardness that was high in standard water (r^2 = 0.80; paper **II**; Akhil & Khan, 2017; Hyne et al., 2005; Li et al., 2013). This indicates that toxicity testing in standard test media could underestimate the potential hazard of Zn compounds to the aquatic ecosystem. ZnO NP toxicity also had a strong positive correlation with pH (r^2 = 0.95) and negative correlation with SO_4^{2-} concentration (the parameters correlated with each other). A similar effect of the water parameters was seen on Zn salt toxicity.

The relatively small absolute variation of NP toxicity between lake waters with different DOC content suggests that the limit concentration of DOC allowed in toxicity tests (2 mg/L; OECD, 2004, 2012) may not be justified. Considering the typical variation of DOC content in nature (2–10 mg/L in rivers and lakes and up to 60 mg/L in swamps according to Thurman (1985)), allowing higher DOC concentrations could increase the environmental relevance of the tests.

3.1.3 Acute and subchronic toxicity of CuO, ZnO, and Ag-PVP nanoparticles in the presence and absence of algae (paper II)

The effect of addition of dietary algae into acute *D. magna* tests was evaluated in addition to the effect a lake waters. The algal concentration was chosen to be similar to that in the *H. incongruens* test (Table 7). Regarding CuO, ZnO, and Ag-PVP NP and $AgNO_3$, the effect of the addition of algae to standard water was different from the effect of using lake water (without algae) instead of standard mineral test medium (Table 11; paper **II**).

CuO and ZnO toxicity did not change in standard water after addition of algae while using lake waters (without algae) instead of standard water either increased or decreased the toxicity (Table 11). The effect of addition of algae on soluble salt toxicity, on the other hand, was similar to the effect of using lake waters (without algae) instead of standard water (Table 11). The mild mitigating effect of algae on $CuSO_4$ toxicity has also been demonstrated before by other authors (Borgmann & Charlton, 1984; De Schamphelaere & Janssen, 2004). The absence of a mitigating effect of algae on CuO and ZnO NP toxicity in standard water may have occurred because of the settling of algae along with NP in this test medium. That may have caused the daphnids to turn to bottom-feeding and increased their exposure to the settled CuO and ZnO NP (paper **II**). Sedimentation of toxicant along with algae has also been shown to induce toxicity in Ln studies (Lürling & Tolman, 2010). The addition of algae drastically decreased the toxicity (up to 220 fold; Table 11) of Ag-PVP NP and $AgNO_3$ similarly to the effect of aquatic plants (Bone et al., 2012). This effect was also very different from that of using lake waters (without algae) instead of standard water where no statistically significant mitigation was seen.

The mitigating effect of the addition of algae on NP toxicity also differed between the standard mineral and natural test media (paper **II**). Much stronger mitigation was seen in lake water with added algae compared to standard water with added algae. The previously described settling of algae in the standard medium was much lower in lake water allowing daphnids to feed in the water column and avoid the settled NP. In addition, Ag-PVP NP dissolution decreased more in lake waters compared to

standard water in the presence of algae (see 3.1.1) which could have contributed to the mitigating effect. The strong mitigating effect of algae on CuO NP toxicity in lake waters compared to standard water was not seen in the subchronic toxicity test with *H. incongruens* possibly due to the inability of the benthic organism to avoid the settled NP (paper **II**).

To conclude, while the chemical water parameters have largely similar effects on metal NP and the respective soluble salt toxicity, the same does not apply for addition of algae into the test medium. Based on our results, using natural freshwater along with addition of algae will significantly increase the environmental relevance of metal-based NP toxicity test results. The effect of environmentally relevant algae concentrations would be a future research perspective. Despite the large variation of CuO and Ag-PVP NP toxicity in different test conditions, CuO NP would still be considered non-hazardous and Ag-PVP NP hazardous to the aquatic ecosystem based on the EC_{50} values calculated from nominal concentrations (and considering the 1 mg/L threshold set by European Commission (2008)). ZnO NP, on the other hand, would be considered hazardous only in lake waters devoid of algae (Table 11).

3.1.4 Exposure of *Daphnia magna* to CuO, Co_3O_4, and Mn_2O_3 nanoparticles: environmentally relevant sublethal endpoints

3.1.4.1 Feeding behaviour (paper IV)

Sublethal toxicity endpoints such as feeding behaviour allow evaluating the long-term consequences of a short term exposure to NP concentrations that do not necessarily induce mortality. Feeding inhibition can, for example, lead to reduced growth and reproduction of test organisms that will eventually affect the viability of the population (Martins et al., 2017). The research on behavioural changes is also one of the priorities of current NP ecotoxicology research (ECHA, 2017a).

D. magna feeding behaviour was monitored after 48 h exposure to sublethal concentrations (≤ 10 mg/L) of Co_3O_4 and Mn_2O_3 NP and low toxicity inducing concentrations (≤ 0.6 mg/L) of CuO NP as a positive control along with respective soluble metal salts. Feeding behaviour in this study was measured by a novel method: quantifying the feeding by-products of *D. magna* fed with microalgae. Measuring feeding by-products instead of using fluorescent dyes or artificial pellets is a more cost-effective method for feeding behaviour analysis.

Exposure to the NP did not have any NP specific effect on the feeding behaviour of *D. magna* (paper **IV**). The effect of Co and Mn compounds on feeding behaviour differed from that of the unexposed (control) *D. magna* only at higher exposure concentrations (≥ 1 mg metal/L). Interestingly, Co salt, CuO NP, and Cu salt, all toxic compounds, but also non-toxic Co_3O_4 NP, temporarily (24 h) increased the post-exposure feeding activity (Figure 2 of paper **IV**). This effect has not been shown before but may lead to distorted results in feeding behaviour tests designed to last for < 24 h (Ginatullina et al., 2013). The non-toxic Mn compounds with high dissolution (35% of the nominal 1 mg Mn/L at 48 h), on the other hand, decreased the feeding activity along with low-toxicity Mn salt (EC_{50} 41 mg Mn/L), indicating the limitations of information delivered by the acute toxicity tests' results.

To conclude, the feeding behaviour study gave important additional information on the effects of metal exposure that were similar for both NP and soluble metal salts.

3.1.4.2 Metal body burden (papers III, IV)

The metal body burden of daphnids induced by ingestion and adsorption of NP aggregates would be transferred into the higher food chain level (e.g. fish) if the daphnids were eaten. The level of metal body burden in microcrustaceans can thus indicate the potential hazard of foodborne metal uptake to fish and eventually to humans.

Metal body burden in single juvenile daphnids was measured in this study using Total Reflection X-Ray Fluorescence (TXRF) spectroscopy, a method that allows accurate metal quantification in very small biological samples but has not been applied on microcrustaceans in ecotoxicity research so far. Daphnids were exposed to CuO (paper **III, IV**), Co_3O_4 (paper **IV**), and Mn_2O_3 (paper **IV**) NP and the respective metal salts for 48 h and allowed to depurate in clean test medium with dietary algae for 24 to 48 h after the exposure.

As anticipated, metal body burden was always higher after the exposure to NP than after the exposure to metal salts at the same metal concentration. *D. magna* can filter aggregated NP (Figure 10) from the test medium (Adam et al., 2014) and adhesion of the aggregates to the exoskeleton was also seen in case of Mn_2O_3 NP exposure (papers **III, IV**). High metal body burden was induced by exposure to both toxic (CuO) and non-toxic (Co_3O_4 and Mn_2O_3) nanoparticles (papers **III, IV**). Non-toxic compounds induced higher metal body burden in *D. magna* compared to sublethal concentrations of the toxic CuO NP at comparable exposure concentrations. This may be explained by the reduced uptake rate induced in *D. magna* exposed to toxic particles (Kamaya et al., 2011) even though no reduction in post-exposure feeding behaviour was seen in this study after exposure to Cu compounds (see 3.1.4.1). The metal uptake from sublethal concentrations of soluble salts was also higher for the metals with lower toxicity (Mn) compared to the toxic Co and Cu salts.

It has also been proposed that low-solubility NP (CuO and Co_3O_4 NP in this study with dissolution < 1% of the nominal 1 mg Co/L and 10 mg Cu/L at 48 h) can reduce the further uptake of NP by inducing a full gut feeling in *D. magna* (Briffa et al., 2018). The feeding behaviour during the exposure to NP was not measured in this study but the post-exposure feeding activity results (see 3.1.4.1) did not confirm the findings. The feeding after the exposure to low-solubility NP increased and after the exposure to high-solubility (35% of the nominal 1 mg Mn/L at 48 h) Mn_2O_3 NP decreased instead. In addition, the metal body burden after exposure to low-solubility Co_3O_4 NP was higher compared to the body burden after exposure to high-solubility Mn_2O_3 NP.

The CuO body burden in *D. magna* was also measured in lake waters. The lake water as test medium mitigated the acute toxicity of CuO NP but increased the total copper body burden in *D. magna* after 48 h exposure compared to the standard water. A similar toxicity mitigating effect of lake water on Cu salt toxicity was observed but no effect on the total body burden compared to standard water was seen.

Daphnids were allowed to depurate in the presence of dietary algae after the exposure. Mn_2O_3 and Co_3O_4 NP body burden remained elevated (> 4-fold compared to the control organism) after 48 h depuration while Cu NP body burden decreased to the control level in most test organisms during 24 h depuration. Mn body burden remained up to 10 times higher compared to Co body burden. The higher dissolution of Mn_2O_3 NP (35% of the nominal 1 mg Mn/L at 48 h) compared to other NP (< 1% of the nominal 1 mg Co/L and 10 mg Cu/L at 48 h) could have caused the higher metal retention after the depuration, for example by different metal compartmentalisation in the body

(Vijver et al., 2004). The pH-driven dissolution of highly soluble NP in the gut of *D. magna* subsequent to particle uptake has been shown to lead to higher uptake of metal in body tissues compared to metals from poorly soluble NP (Briffa et al., 2018). The higher retention of less toxic Mn could have also occurred due to Mn-deficiency of the test organisms as control daphnids reached similar elevated Mn concentration after feeding on algae. Lower post-exposure feeding activity that was recorded for Mn compounds exposed daphnids (see 3.1.4.1) could have also led to higher levels of Mn retained in the gut of daphnids after depuration.

Our study confirmed that metal body burden analysis reveals NP-specific adverse effects and that the low or mitigated acute toxicity of the compound may lead to increased metal body burden. Thus, less toxic NP can also impose a threat of trophic transfer of heavy metals in a NP-contaminated environment and metal body burden should be measured to determine the potential of indirect and long term biological effects. NP with higher solubility induced higher retention of metal during the post-exposure feeding in this study. As the lowest exposure concentrations of metals (0.05–0.1 mg metal/L) used in the body burden tests were still relatively high considering the predicted concentrations in nature (Garner et al., 2017), tests with lower, environmentally more relevant concentrations should be conducted in the future. TXRF proved to be a precise, fast, and cost-effective method for determining Cu, Co, and Mn body burden in *D. magna* specimen.

*Figure 10. Metal accumulation in the gut of D. magna (red arrows) after 48 h exposure to 1 mg metal/L CuO (left), Co_3O_4 (middle), and Mn_2O_3 (right) nanoparticles (papers **III, IV**).*

3.1.4.3 Organism recovery from nanoparticle exposure (papers III, IV)

Along with post-exposure depuration, post-exposure mortality of *D. magna* was recorded to determine the viability/recovery potential of the test organisms that survived the acute exposure. The mortality of organisms transferred to a clean test medium containing dietary algae after acute exposure to a sublethal concentration was often remarkably high. Sublethal concentrations of Cu salt induced higher post-exposure mortality (21%) compared to equitoxic CuO exposure and control (both 12%; Table 3 of paper **III**) in standard test medium indicating that the effect is not NP specific.

On the other hand, post-exposure mortality after 48 h exposure to non-toxic Co_3O_4 and Mn_2O_3 concentrations (paper **IV**) and the following 48 h depuration was also considerable (20–30%; unpublished data) showing that non-toxic NP may also affect

the fitness of the test organism. Moreover, short-term exposure to CuO and $CuSO_4$ in lake waters with toxicity mitigating effect still induced elevated post-exposure mortality (23%; paper **III**). The results demonstrate once more that the mitigating effect of natural freshwaters on Cu toxicity may be short-lived and that even sublethal exposure concentrations can result in irreversible damage to the test organisms. Monitoring of post-exposure mortality is an easy way to obtain additional information on the accuracy of the results obtained in an acute exposure to metals.

3.2 Toxicity evaluation of lanthanides with freshwater biota

Ln may enter the aquatic environment in the form of different compounds. In the current study, the potential hazard of Ln to aquatic ecosystems was evaluated from two different groups of chemicals: soluble Ln nitrates (paper **V**) and low-solubility multimetal submicron lanthanide (doped) particles as an example of a widely used Ln-containing product (paper **VI**). The soluble Ln salts also served as ionic control for Ln (doped) particles. Both salts and particles contained Ln commonly used in different technologies (La, Ce, Pr, Nd, Gd).

3.2.1 Effect of test conditions on the fate of lanthanides (papers V, VI)

The stability of exposure concentrations is vital for chemical toxicity assessment. In toxicity tests of this thesis, total Ln content was measured in the water column of the test vessels and included dissolved Ln, the colloidal fraction, suspended particles, or in some tests, Ln accumulated in algae. Earlier findings have also shown that only a fraction of total Ln concentration in the water column of the test vessel may consist of dissolved Ln species (Gonzalez et al., 2015).

Evaluation of the exposure concentration turned out to be one of the main problems in the acute toxicity testing of Ln. The settling of Ln was concentration and time dependent (paper **V**). At nominal concentrations > 10 mg Ln/L, more than 80% of the applied Ln settled within 48 h while at nominal concentration ≤ 1 mg Ln/L only up to 20% of applied Ln settled in the bioassays as well as in solutions without the test organisms (but containing algae in some cases). As the acute toxicity of Ln was mostly imposed at nominal concentrations > 10 mg Ln/L, the calculation of toxic effect concentration had to be based on the measured concentrations (OECD, 2018).

Settling of Ln also depended on the test medium. In the current study, the experiments with Ln were conducted in two different standard mineral media, OECD AFW and US EPA MHRW, and in two lake waters (Raku and Ülemiste) (Table 6). Ln settled twice as much in MHRW compared to the high-hardness OECD AFW despite the precipitation favouring effect of high water hardness (Goldstein & Jacobsen, 1988; Stewart et al., 2017; paper **V**). The high concentration of Cl^- (145 mg/L) in AFW possibly formed soluble $LnCl_3$ while high SO_4^{2-} (82 mg/L) and CO_3^{2-} (69 mg/L) in MHRW could have precipitated the Ln (Byrne & Kim, 1993; Porvali et al., 2018). Thus, choosing a standard mineral test medium with low concentration of agents such as sulfates and carbonates that form sparingly soluble and insoluble species with Ln (Table 3) can be suggested.

Settling was slightly lower in lake waters (< 15%) compared to AFW at low exposure concentration (1 mg Ln/L; paper **V**) possibly because of the presence of DOM (Ingri et al., 2000) as the lake water with higher DOC content (10 mg/L) had higher total Ln concentration in the water column. The concentration dependent settling of Ln in the lake waters could have occurred due to the increasing ratio of Ln to the strong binding

sites of humic acid along with the increasing Ln exposure concentration (Dupré et al., 1999). Thus, using natural freshwater or adding organic matter to the standard test medium could be one way to stabilise Ln solution as has also been recommended for NP studies (Petersen et al., 2015). The addition of algae, on the other hand, did not affect the total metal concentration in the water column in this study while $LnCl_3$ dissolution can decrease in the presence of algae (Romero-Freire et al., 2019).

Calculation of the toxic values (EC_{50}) based on the measured total Ln concentrations in the water column in the acute exposure was complicated by their 5-fold variability between technical replicates in the presence of test animals (paper **V**). In the long-term tests performed in lake water, the variation was significantly lower (up to 2-fold) possibly due to the low exposure concentrations (≤ 1 mg Ln/L) at which the Ln settling did not exceeded 15% in most cases. The relatively low variation in the chronic test could have also been a result of higher test medium volume per daphnid (50 mL) compared to acute toxicity tests (0.1–2 mL) (paper **V**). Furthermore, high variation was not reported in an acute study conducted in larger vessels (8 mL per daphnid; Barry & Meehan, 2000). Smaller test medium volume is also problematic due to a relatively larger area being in contact with the test vessel walls to which up to 25% of applied Ln can adsorb (Weltje et al., 2002). Thus, using large test vessels and fewer test animals per vessel would decrease the variability of metal concentrations in the water column.

Compared to Ln salts, Ln (doped) particles behaved quite differently (paper **VI**). The settling rate of the particles in US EPA MHRW standard test medium was > 99% from nominal Ln concentration (9.1–81 mg Ln/L) at the end of toxicity tests (at 24 h or 6 days) with only ≤ 0.2 mg Ln/L remaining in the water column at nominal particle exposure concentration of 100 mg/L (Table 2 of paper **VI**). Settling of several other constituent metals (Co, Fe, Mn) was also > 99% of the nominal metal concentration but Ni, Sr and, in case of $(La_{0,6}Sr_{0,4})_{0,95}CoO_3$, Co were present at higher concentrations (3–19% of the nominal metal concentration) in the water column implying higher dissolution of those metals compared to Ln from the particles. Differently from Ln salts, the presence or absence of test organisms did not influence the metal recovery from Ln (doped) particles. Based on the results, Ln leaching from Ln (doped) particles in standard mineral freshwater is very low compared to some other constituent metals at least within a short time span (up to 6 days). Also, all the tests with Ln (doped) particles were carried out in a test medium with high sulfate and carbonate content (MHRW) possibly causing some precipitation of the Ln dissolved from the particles as was shown above.

3.2.2 Acute toxicity and effect of test conditions on lanthanide salts and lanthanide (doped) particles (papers V, VI)

Ln salts' toxicity to *D. magna*, *H. incongruens*, and *T. platyurus* was comparable despite the differences in test protocols (Table 8; paper **V**). The effect concentration values (EC_{50}) based on the measured total Ln concentrations in the water column were 0.2–1.5 mg Ln/L thus being close to the threshold of 1 mg/L set by European Commission (2008) to classify chemicals as "hazardous".

On the other hand, most of the studied Ln (doped) particles (paper **VI**) were not toxic to crustaceans even at nominal concentration of 100 mg/L. Only two types of the particles, La_2NiO_4 and $(La_{0,6}Sr_{0,4})_{0,95}CoO_3$, did impose toxicity to microcrustaceans. The toxic effects of these particles were mainly related to Ni and Co dissolution from the particles (see 3.2.1). Ln concentrations measured in the water column (≤ 0.2 mg/L; paper **VI**) in case of both toxicity-inducing and non-toxic Ln (doped) particle exposure

almost reached the EC_{50} values (0.2–1.5 mg/L mg/L) determined in acute tests with Ln salts (paper **V**). This indicates the presence of the suspended Ln (doped) particles and/or other Ln forms with low bioavailability in the water column. Considering that the toxicity-inducing $(La_{0,6}Sr_{0,4})_{0,95}CoO_3$ particles were nano-sized (65 nm) and relatively sparingly soluble (0.73–2.9% of the nominal 27.5 mg Co/L), similarly to the non-toxic Co_3O_4 NP used in the NP exposure (see 2.1.1), the toxicity of Co oxides should be studied further.

Ln applied as both Ln nitrates and Ln (doped) particles formed complexes and aggregates that were ingested by the test organisms (Figure 11) at all the exposure concentrations (papers **V, VI**). This indicates that the insoluble Ln compounds formed during the exposure were large enough to be filtered by the zooplankton (paper **V**). However, ingestion of neither of the Ln compounds was directly related to the induction of toxic effects seen in the test organisms. Nor was the total metal concentration (see 3.2.1) in the water column reflected in toxicity results indicating different bioavailability of the dissolved and suspended Ln species present in the water column (paper **V**). For example, the higher (DOM-associated) Ln concentration found in the water column of lake waters (see 3.2.1) did not appear bioavailable as the acute toxicity was reduced in lake waters. The change in the concentrations and ratio of major ions in the test media as a result of the high precipitation of Ln at nominal concentrations above 10 mg/L (see 3.2.1) could have also affected the extent of toxic effects recorded in acute tests.

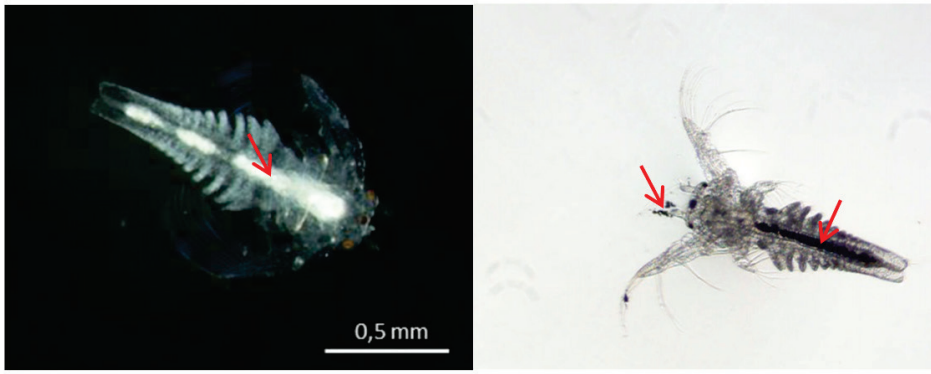

Figure 11. T. platyurus after 24 h exposure to $Pr(NO_3)_3$ at 10 mg Pr/L (left) and to LaSrCoO particles at 6.25 mg particles/L (right). Arrows indicate particle aggregates in the gut and attached to the antennae of the organism.

3.2.3 Sublethal, subchronic, and chronic toxicity of lanthanide salts and lanthanide (doped) particles to *Daphnia magna, Heterocypris incongruens*, and *Lemna minor* (papers V, VI)

Sublethal and long-term effects of Ln were studied by conducting i) a standard subchronic 6-day toxicity test with *H. incongruens* (ISO, 2012) exposed to Ln salts and Ln (doped) particles (papers **V**, **VI**), ii) a 15-day post-exposure recovery tests upon acute exposure of *D. magna* to Ln salts (paper **V**), iii) a standard 21-day reproduction test with *D. magna* (OECD, 2012) exposed to Ln salts (paper **V**), and iv) a bioaccumulation study with the free-floating macrophyte *Lemna minor* exposed to Ln (doped) particles (paper **VI**).

The more variable and slightly higher toxicity seen in subchronic tests with benthic *H. incongruens* compared to the acute toxicity to pelagic organisms was possibly due to the longer exposure period but also the presence of algae in the test (paper **V**). The way feeding of the organism during the exposure influences the contaminant bioavailability depends on the concentrations of both the food and the contaminant and can also increase the metal toxicity at low exposure concentrations (Taylor et al., 1998). The effect of ingested Ln (doped) particles on the feeding behaviour of ostracods cannot be excluded either.

Furthermore, *H. incongruens* tests are conducted in standard mineral freshwater with high sulfate and carbonate concentrations that increased Ln settling. The higher settling rate of Ln salts can also lead to higher exposure of benthic *H. incongruens* to the settled Ln species. Thus, benthic species should be preferential over planktic ones in Ln hazard evaluation. On the other hand, the addition of sand to *H. incongruens* test (as required by the test guideline) had a strong mitigating effect on the toxicity of Ln salts (paper **V**).

In addition, body length was measured in *H. incongruens* tests at sublethal test concentrations. Changes in body length were not seen after exposure to soluble Ln nitrates (paper **V**). On the other hand, ostracod body length was decreased upon exposure to Ln (doped) particles even when no mortality was induced (paper **IV**), indicating food limitation presumably due to algae-particle interactions (Joonas et al., 2017). Formation of crystals was seen in *H. incongruens* exposure to Ln salts (Figure 12) but not in a concentration dependent manner (paper **V**). However, it is not clear whether the crystal formation was induced by the presence of algae.

Thus, the presence of sediment, type of test medium, and food availability should be considered when interpreting the results of the *H. incongruens* test.

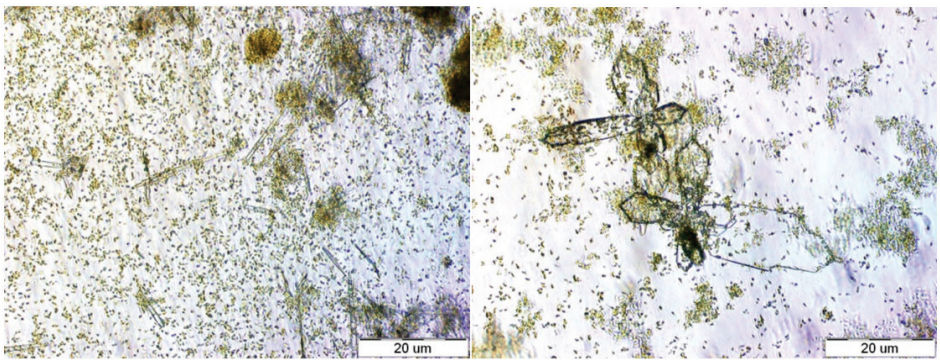

Figure 12. Formation of crystals in H. incongruens test environment during 6-day exposure to $Ce(NO_3)_3$ at 25 mg Ce/L (left) and $Pr(NO_3)_3$ at 50 mg Pr/L (right) in MHRW.

The post-exposure mortality of daphnids was measured after acute exposure and subsequent depuration in clean test medium in the presence of dietary algae (paper **V**). The post-exposure mortality of daphnids previously exposed to sublethal nominal concentration of 3 mg Ln/L, was 100% within just 3 days after transfer to recovery conditions in (paper **V**). This revealed that the duration of the acute test is too short to reveal the acutely toxic effects of Ln. *D. magna* post-exposure mortality subsequent to the acute test was also a more sensitive endpoint in Ln toxicity testing compared to the subchronic *H. incongruens* test where no such mortality was seen after a longer (6-day)

exposure at the same Ln concentration. Interestingly, the post-exposure mortality of daphnids exposed in lake waters was similar to the ones exposed in synthetic freshwater despite the differences in acute toxicity. This confirmed that the medium-induced differences in acute toxicity test are not always relevant for long-term effects (Barry & Meehan, 2000).

The 21 day chronic toxicity (EC_{50}) of individual Ln salts to *D. magna* varied from 0.3 to 0.5 mg Ln/L based on both nominal and measured concentrations and would classify Ln nitrates as hazardous by EU legislation (paper **V**; European Commission, 2008). As mortality was a more sensitive endpoint compared to growth or reproduction in the chronic experiments, the whole life cycle tests may not be necessary for toxicity assessment of Ln to microcrustaceans. Instead, there is a need for additional sublethal endpoints.

The Ln (doped) particles study also included an additional sublethel endpoint – bioaccumulation of metals in aquatic plant *Lemna minor* (paper **VI**). *L. minor* has been previously shown to accumulate La from $LaCl_3$ exposure (Weltje et al., 2002). This study showed no accumulation of Ln into *L. minor* tissue from Ln (doped) particles but did reveal high bioaccumulation of Ni and Co (> 311 mg/ kg dwt; paper **VI**). Thus confirmed our conclusion (see 3.2.2) that the observed toxicity of La_2NiO_4 and $(La_{0,6}Sr_{0,4})_{0,95}CoO_3$ particles was not induced by the constituent Ln. Metal concentration in the tissue is a better metric for Ln toxicity than growth inhibition of *L. minor* as the latter can also be induced by Ln binding phosphorus into biologically unavailable forms (paper **VI**). Metal bioaccumulation results correlated well with the particle toxicity to both *L. minor* and microcrustaceans despite the different test media and formats used. Measuring potential bioaccumulation of Ln in aquatic plants could be a good indicator of Ln bioavailability to a variety of aquatic organisms. The method can be applied in the field, for example upon application of lanthanum-modified bentonite clay (Phoslock®) for lake restoration by phosphorus binding (Waajen et al., 2017).

Conclusions

i) The following new toxicity data on metal-based NP and Ln were obtained as a result of this thesis:
   - Acute toxicity of CuO NP (48 h EC_{50} = 1.6 mg Cu/L) to *D. magna* was mitigated up to 47-fold, Ag-PVP NP toxicity (48 h EC_{50} = 0.0008 mg Ag/L) did not change significantly, and ZnO NP toxicity (48 h EC_{50} = 1.9 mg Zn/L) increased up to 6-fold in natural freshwater. The effect of the presence of algae was strongly medium and compound specific in tests with *D. magna* but not with *H. incongruens*. Co_3O_4 and Mn_2O_3 NP were not toxic to *D. magna* in an acute assay but induced higher metal body burden than toxic CuO NP.
   - Ln would be considered hazardous to aquatic biota based on chronic toxicity test results (21-day EC_{50} = 0.3–0.5 mg Ln/L) of Ln nitrates. Ln bioavailability from lanthanide (doped) submicron particles was low.
   - It was demonstrated that precipitation of metals applied as soluble salts (Ln and Ag nitrates, Cu and Zn sulfates) at high (> 1 mg/L) nominal concentrations in standard and modified test formats is a serious challenge in ecotoxicity evaluation.

ii) To increase the ecological relevance of the laboratory toxicity testing of metal-based NP and Ln, the following recommendations are proposed based on the modified test formats and sublethal endpoints applied in this thesis:
   - Toxicity test results obtained in natural waters in the presence of dietary algae should always be considered in chemical safety assessments along with the results obtained in standard mineral freshwater.
   - Post-exposure sublethal endpoints: feeding behaviour, metal body burden, and recovery assessment are highly applicable for both soluble as well as particulate chemicals, can be easily included to complement standard acute test formats, and yield ecologically more relevant results for environmental hazard evaluation of chemicals.
   - Metal body burden studies are especially suitable for revealing the potential hazard of acutely non-toxic low-solubility substances and should be conducted in natural waters since metal NP bioaccumulation may increase in organics-rich freshwaters. Contribution of individual metals to the toxicity of multimetal particles can also be evaluated by measuring metal concentration in test organism tissue.
   - Total reflection X-ray fluorescence spectroscopy is a suitable method for measuring Cu, Co, and Mn body burden in (single) microcrustaceans.
   - The use of microcrustacean species with different behaviour enables to better understand how the organism's behaviour may influence its exposure to chemicals and consequently, their potential adverse effects.

References

Adam, N., Leroux, F., Knapen, D., Bals, S., & Blust, R. (2014). The uptake of ZnO and CuO nanoparticles in the water-flea *Daphnia magna* under acute exposure scenarios. *Environmental Pollution*, *194*, 130–137. http://doi.org/10.1016/j.envpol.2014.06.037

Adeleye, A. S., Oranu, E. A., Tao, M., & Keller, A. A. (2016). Release and detection of nanosized copper from a commercial antifouling paint. *Water Research*, *102*, 374–382. http://doi.org/10.1016/j.watres.2016.06.056

Akhil, K., & Khan, S. S. (2017). Effect of humic acid on the toxicity of bare and capped ZnO nanoparticles on bacteria, algal and crustacean systems. *Journal of Photochemistry and Photobiology B: Biology*, *167*, 136–149. http://doi.org/10.1016/j.jphotobiol.2016.12.010

Alivisatos, P. (2004). The use of nanocrystals in biological detection. *Nature Biotechnology*, *22*, 47–52. http://doi.org/10.1038/nbt927

Allen, H. E., & Hansen, D. J. (1996). The importance of trace metal speciation to water quality criteria. *Water Environment Research*, *68*, 42–54. http://doi.org/10.1146/annurev.anthro.35.081705.123127

Aruoja, V., Pokhrel, S., Sihtmäe, M., Mortimer, M., Mädler, L., & Kahru, A. (2015). Toxicity of 12 metal-based nanoparticles to algae, bacteria and protozoa. *Environ. Sci.: Nano*, *2*, 630–644. http://doi.org/10.1039/C5EN00057B

Barry, M. J., & Meehan, B. J. (2000). The acute and chronic toxicity of lanthanum to *Daphnia carinata*. *Chemosphere*, *41*, 1669–1674. http://doi.org/10.1016/S0045-6535(00)00091-6

Bau, M., & Dulski, P. (1996). Anthropogenic origin of positive gadolinium anomalies in river waters. *Earth and Planetary Science Letters*, *143*, 245–255. http://doi.org/10.1016/0012-821X(96)00127-6

Baun, A., Hartmann, N. B., Grieger, K., & Kusk, K. O. (2008). Ecotoxicity of engineered nanoparticles to aquatic invertebrates: A brief review and recommendations for future toxicity testing. *Ecotoxicology*, *17*, 387–395. http://doi.org/10.1007/s10646-008-0208-y

Belk, D. (1977). Evolution of egg size strategies in fairy shrimps. *The Southwestern Naturalist*, *22*, 99–105. http://doi.org/10.2307/3670467

Belk, D. (2007). Branchiopoda. In J. T. Carlton (Ed.), *The Light and Smith Manual: Intertidal Invertebrates from Central California to Oregon (4th ed.)* (pp. 414–417). Berkeley, USA: University of California Press.

Bian, S.-W., Mudunkotuwa, I. A., Rupasinghe, T., & Grassian, V. H. (2011). Aggregation and Dissolution of 4 nm ZnO Nanoparticles in Aqueous Enviroments: Influence of pH, Ionic Strength, Size, and Adsorption of Humic Acid. *Langumir*, *27*, 6059–6068. http://doi.org/10.1021/la200570n

Bianchini, A., & Wood, C. M. (2008). Does sulfide or water hardness protect against chronic silver toxicity in *Daphnia magna*? A critical assessment of the acute-to-chronic toxicity ratio for silver. *Ecotoxicology and Environmental Safety*, *71*, 32–40. http://doi.org/10.1016/j.ecoenv.2008.03.006

Bitton, G., Rhodes, K., Koopman, B., & Cornejo, M. (1995). Short-Term Toxicity Assay Based on Daphnid Feeding-Behavior. *Water Environment Research*, *67*, 290–293. http://doi.org/10.1016/S0160-4120(02)00029-6

Blaise, C., Gagné, F., Harwood, M., Quinn, B., & Hanana, H. (2018). Ecotoxicity responses of the freshwater cnidarian *Hydra attenuata* to 11 rare earth elements. *Ecotoxicology and Environmental Safety*, *163*, 486–491. http://doi.org/10.1016/j.ecoenv.2018.07.033

Blinova, I., Ivask, A., Heinlaan, M., Mortimer, M., & Kahru, A. (2010). Ecotoxicity of nanoparticles of CuO and ZnO in natural water. *Environmental Pollution*, *158*, 41–47. http://doi.org/10.1016/j.envpol.2009.08.017

Blinova, I., Muna, M., Lukjanova, A., & Kahru, A. (2018). Evaluation of the potential hazard of manufactured metal-based nanomaterials to health of aquatic ecosystem: state of the art. *Journal of International Scientific Publications: Ecology & Safety*, *12*, 174–182. Retrieved from https://www.scientific-publications.net/en/article/1001659/

Blinova, I., Niskanen, J., Kajankari, P., Kanarbik, L., Käkinen, A., Tenhu, H., … Kahru, A. (2013). Toxicity of two types of silver nanoparticles to aquatic crustaceans *Daphnia magna* and *Thamnocephalus platyurus*. *Environmental Science and Pollution Research International*, *20*, 3456–63. http://doi.org/10.1007/s11356-012-1290-5

Bondarenko, O., Ivask, A., Käkinen, A., Kurvet, I., & Kahru, A. (2013). Particle-Cell Contact Enhances Antibacterial Activity of Silver Nanoparticles. *PLoS ONE*, *8*(5). http://doi.org/10.1080/13683500.2017.1360846

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. http://doi.org/10.1007/s00204-013-1079-4

Bondarenko, O. M., Heinlaan, M., Sihtmäe, M., Ivask, A., Kurvet, I., Joonas, E., … Kahru, A. (2016). Multilaboratory evaluation of 15 bioassays for (eco)toxicity screening and hazard ranking of engineered nanomaterials: FP7 project NANOVALID. *Nanotoxicology*, *5390*, 1–14. http://doi.org/10.1080/17435390.2016.1196251

Bone, A. J., Colman, B. P., Gondikas, A. P., Newton, K. M., Harrold, K. H., Cory, R. M., … Di Giulio, R. T. (2012). Biotic and Abiotic Interactions in Aquatic Microcosms Determine Fate and Toxicity of Ag Nanoparticles: Part 2 – Toxicity and Ag Speciation. *Environmental Science & Technology*, *46*, 6925–6933. http://doi.org/10.1021/es204683m

Borgmann, U., & Charlton, C. C. (1984). Copper complexation and toxicity to *Daphnia* in natural waters. *Journal of Great Lakes Research*, *10*, 393–398. http://doi.org/10.1016/S0380-1330(84)71855-7

Bosco-Santos, A., Luiz-Silva, W., Silva-Filho, E. V. da, Souza, M. D. C. de, Dantas, E. L., & Navarro, M. S. (2017). Fractionation of rare earth and other trace elements in crabs, *Ucides cordatus*, from a subtropical mangrove affected by fertilizer industry. *Journal of Environmental Sciences (China)*, *54*, 69–76. http://doi.org/10.1016/j.jes.2016.05.024

Bozich, J., Hang, M., Hamers, R., & Klaper, R. (2017). Core chemistry influences the toxicity of multicomponent metal oxide nanomaterials, lithium nickel manganese cobalt oxide, and lithium cobalt oxide to *Daphnia magna*. *Environmental Toxicology and Chemistry*, *36*, 2493–2502. http://doi.org/10.1002/etc.3791

Briffa, S. M., Nasser, F., Valsami-Jones, E., & Lynch, I. (2018). Uptake and impacts of polyvinylpyrrolidone (PVP) capped metal oxide nanoparticles on *Daphnia magna*: role of core composition and acquired corona. *Environmental Science: Nano*, *5*, 1745–1756. http://doi.org/10.1039/c8en00063h

Buban, J. P., Matsunaga, K., Chen, J., Shibata, N., Ching, W. Y., Yamamoto, T., & Ikuhara, Y. (2006). Grain boundary strengthening in alumina by rare earth impurities. *Science*, *311*, 212–215. http://doi.org/10.1126/science.1119839

Bünzli, J. C. G., & Eliseeva, S. V. (2010). Lanthanide NIR luminescence for telecommunications, bioanalyses and solar energy conversion. *Journal of Rare Earths*, *28*, 824–842. http://doi.org/10.1016/S1002-0721(09)60208-8

Byrne, R. H., & Kim, K. H. (1993). Rare earth precipitation and coprecipitation behavior: The limiting role of PO_4^{3-} on dissolved rare earth concentrations in seawater. *Geochimica et Cosmochimica Acta*, *57*, 519–526. http://doi.org/10.1016/0016-7037(93)90364-3

Chen, K. L., & Elimelech, M. (2007). Influence of humic acid on the aggregation kinetics of fullerene (C60) nanoparticles in monovalent and divalent electrolyte solutions. *Journal of Colloid and Interface Science*, *309*, 126–134. http://doi.org/10.1016/j.jcis.2007.01.074

Cheng, P., Shen, B., Mu, G., Zhu, X., Han, F., Zeng, B., & Wen, H. H. (2009). High-T_c superconductivity induced by doping rare-earth elements into CaFeAsF. *Epl*, *85*, 0–4. http://doi.org/10.1209/0295-5075/85/67003

Chudaeva, V. A., & Chudaev, O. V. (2011). Accumulation and fractionation of rare earth elements in surface waters of the Russian Far East under the conditions of natural and anthropogenic anomalies. *Geochemistry International*, *49*, 523–549. http://doi.org/10.1134/S0016702911030049

Cidu, R., Vittori Antisari, L., Biddau, R., Buscaroli, A., Carbone, S., Da Pelo, S., … Zannoni, D. (2013). Dynamics of rare earth elements in water-soil systems: The case study of the Pineta San Vitale (Ravenna, Italy). *Geoderma*, *193*, 52–67. http://doi.org/10.1016/j.geoderma.2012.10.009

Cobelo-García, A., Filella, M., Croot, P., Frazzoli, C., Du Laing, G., Ospina-Alvarez, N., … Zimmermann, S. (2015). COST action TD1407: network on technology-critical elements (NOTICE)—from environmental processes to human health threats. *Environmental Science and Pollution Research*, *22*, 15188–15194. http://doi.org/10.1007/s11356-015-5221-0

Conway, J. R., Adeleye, A. S., Gardea-Torresdey, J., & Keller, A. A. (2015). Aggregation, dissolution, and transformation of copper nanoparticles in natural waters. *Environmental Science and Technology*, *49*, 2749–2756. http://doi.org/10.1021/es504918q

Cotton, S. (2006). *Lanthanide and actinide chemistry*. Chippenham, Wiltshire, UK: John Wiley & Sons.

Cross, S. E., Innes, B., Roberts, M. S., Tsuzuki, T., Robertson, T. A., & McCormick, P. (2007). Human skin penetration of sunscreen nanoparticles: In-vitro assessment of a novel micronized zinc oxide formulation. *Skin Pharmacology and Physiology*, *20*, 148–154. http://doi.org/10.1159/000098701

Cupi, D., Hartmann, N. B., & Baun, A. (2015). The influence of natural organic matter and aging on suspension stability in guideline toxicity testing of silver, zinc oxide, and titanium dioxide nanoparticles with *Daphnia magna*. *Environmental Toxicology and Chemistry*, *34*, 497–506. http://doi.org/10.1002/etc.2855

Dabrunz, A., Duester, L., Prasse, C., Seitz, F., Rosenfeldt, R., Schilde, C., … Schulz, R. (2011). Biological surface coating and molting inhibition as mechanisms of TiO_2 nanoparticle toxicity in *Daphnia magna*. *PLoS ONE*, *6*, 1–7. http://doi.org/10.1371/journal.pone.0020112

Dahle, J. T., & Arai, Y. (2015). Environmental geochemistry of cerium: Applications and toxicology of cerium oxide nanoparticles. *International Journal of Environmental Research and Public Health*, *12*, 1253–1278. http://doi.org/10.3390/ijerph120201253

De Schamphelaere, K. A. C., & Janssen, C. R. (2004). Effects of dissolved organic carbon concentration and source, pH, and water hardness on chronic toxicity of copper to *Daphnia magna*. *Environmental Toxicology and Chemistry / SETAC*, *23*, 1115–1122.

Dupré, B., Viers, J., Dandurand, J. L., Polve, M., Bénézeth, P., Vervier, P., & Braun, J. J. (1999). Major and trace elements associated with colloids in organic-rich river waters: Ultrafiltration of natural and spiked solutions. *Chemical Geology*, *160*, 63–80. http://doi.org/10.1016/S0009-2541(99)00060-1

ECHA, (European Chemical Agency). (2017a). Guidance on information requirements and chemical safety assessment. Appendix R7-1 for nanomaterials applicable to Chapter R7b Endpoint specific guidance. European Chemicals Agency. http://doi.org/10.2823/647499

ECHA, (European Chemical Agency). (2017b). Guidance on information requirements and chemical safety assessment. Chapter R.7b: Endpoint specific guidance. European Chemicals Agency. http://doi.org/10.2823/84188

ECHA, (European Chemical Agency). (2018). Registration statistics infograph - ECHA. Retrieved from https://echa.europa.eu/registration-statistics-infograph#

Erickson, R. J., Brooke, L. T., Kahl, M. D., Vende Venter, F., Harting, S. L., Markee, T. P., & Spehar, R. L. (1998). Effects of laboratory test conditions on the toxicity of silver to aquatic organisms. *Environmental Toxicology and Chemistry*, *17*, 572–578.

European Commission. (2006). Regulation (EC) No 1907/2006 of the European Parliament and of the Council of 18 December 2006 concerning the Registration, Evaluation, Authorization, and Restriction of Chemicals (REACH), establishing a European Chemicals Agency, amending Directive 1999/. *Official Journal of the European Communities*. http://doi.org/2004R0726 - v.7 of 05.06.2013

European Commission. (2008). Regulation (EC) No 1272/2008 of the Europaen Parliament and of the Council of 16 December 2008 on classification, labelling and packaging of substances and mixtures, amending and repealing Directives 67/548/EEC and 1999/45/EC, and amending Regulation (EC). *OJ L*, *353*(31.12.2008). Retrieved from http://data.europa.eu/eli/reg/2008/1272/2018-03-01

European Commission. (2013). Communication from the Commission to the European Parliament, the Council and the European Economic and Social Committee. Second Regulatory Review on Nanomaterials. Brussels, 3.10.2012, COM(2012) 572 final. Retrieved from https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=celex%3A52012DC0572

Eurostat. (2017). Chemicals production and consumption statistics - statistics explained. Retrieved from https://ec.europa.eu/eurostat/statistics-explained/index.php/Chemicals_production_and_consumption_statistics#Data_sources_and_availability

Evans, C. A. (1990). *Biochemistry of the lanthanides*. (E. Frieden, Ed.). New York, USA: Springer.

Gao, J., Youn, S., Hovsepyan, A., Llaneza, V. L., Wang, Y., Bitton, G., & Bonzongo, J.-C. J. (2009). Dispersion and Toxicity of Selected Manufactured Nanomaterials in Natural River Water Samples: Effects of Water Chemical Composition. *Environmental Science & Technology*, *43*, 3322–3328. http://doi.org/10.1021/es803315v

Garner, K. L., & Keller, A. A. (2014). Emerging patterns for engineered nanomaterials in the environment: A review of fate and toxicity studies. *Journal of Nanoparticle Research*, *16*, 2503. http://doi.org/10.1007/s11051-014-2503-2

Garner, K. L., Suh, S., & Keller, A. A. (2017). Assessing the Risk of Engineered Nanomaterials in the Environment: development and application of the nanoFate model. *Environ. Sci. Technol.*, *51*, 5541–5551. http://doi.org/10.1021/acs.est.6b05279

Gawande, M. B., Goswami, A., Felpin, F.-X., Asefa, T., Huang, X., Rafael, S., … Varma, R. S. (2016). Cu and Cu-Based Nanoparticles: Synthesis and Applications in Catalysis. *Chemical Reviews*, *116*, 3722–3811. http://doi.org/10.1021/acs.chemrev.5b00482

Geissen, V., Mol, H., Klumpp, E., Umlauf, G., Nadal, M., van der Ploeg, M., … Ritsema, C. J. (2015). Emerging pollutants in the environment: A challenge for water resource management. *International Soil and Water Conservation Research*, *3*, 57–65. http://doi.org/10.1016/j.iswcr.2015.03.002

Geller, W. (1981). The filtration apparatus of cladocera: filter mesh-sizes and their implications on food selectivity, *49*, 316–321.

Ginatullina, E., Chiken, A., Atwell, L., & Kamaya, M. (2013). Engineering of a New Acute Bioassay Design Based on Feeding Behavior of *D. magna* with Fluorescent Microbeads, *Int. J. Emerg. Technol. Adv. Eng. 3*, 671–677.

Goldstein, S. J., & Jacobsen, S. B. (1988). Rare earth elements in river waters. *Earth and Planetary Science Letters*, *89*, 35–47. http://doi.org/10.1016/0012-821X(88)90031-3

Golobič, M., Jemec, A., Drobne, D., Romih, T., Kasemets, K., & Kahru, A. (2012). Upon exposure to Cu nanoparticles, accumulation of copper in the isopod *porcellio scaber* is due to the dissolved Cu ions inside the digestive tract. *Environmental Science and Technology*, *46*, 12112–12119. http://doi.org/10.1021/es3022182

Gonzalez, V., Vignati, D. A. L., Leyval, C., & Giamberini, L. (2014). Environmental fate and ecotoxicity of lanthanides: Are they a uniform group beyond chemistry? *Environment International*, *71*, 148–157. http://doi.org/10.1016/j.envint.2014.06.019

Gonzalez, V., Vignati, D. A. L., Pons, M. N., Montarges-Pelletier, E., Bojic, C., & Giamberini, L. (2015). Lanthanide ecotoxicity: First attempt to measure environmental risk for aquatic organisms. *Environmental Pollution*, *199*, 139–147. http://doi.org/10.1016/j.envpol.2015.01.020

Gophen, M., & Geller, W. (1984). Filter mesh size and food particle uptake by *Daphnia*. *Oecologia*, *64*, 408–412. http://doi.org/10.1007/BF00379140

Graedel, T. E., Gunn, G., & Espinoza, L. T. (2014). Metal resources, use and criticality. *Critical Metals Handbook*. Hoboken, United States: Wiley.

Grillo, R., Rosa, A. H., & Fraceto, L. F. (2014). Engineered nanoparticles and organic matter: A review of the state-of-the-art. *Chemosphere*, *119C*, 608–619. http://doi.org/10.1016/j.chemosphere.2014.07.049

Gunsolus, I. L., Mousavi, M. P. S., Hussein, K., Bühlmann, P., & Haynes, C. L. (2015). Effects of Humic and Fulvic Acids on Silver Nanoparticle Stability, Dissolution, and Toxicity. *Environmental Science & Technology*, *49*, 8078–8086. http://doi.org/10.1021/acs.est.5b01496

Han, G., & Liu, C. Q. (2007). Dissolved rare earth elements in river waters draining karst terrains in Guizhou Province, China. *Aquatic Geochemistry*, *13*, 95–107. http://doi.org/10.1007/s10498-006-9009-1

Han, Y., Kim, D., Hwang, G., Lee, B., Eom, I., Kim, P. J., … Kim, H. (2014). Aggregation and dissolution of ZnO nanoparticles synthesized by different methods: Influence of ionic strength and humic acid. *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, *451*, 7–15. http://doi.org/10.1016/j.colsurfa.2014.03.030

Hanana, H., Turcotte, P., André, C., Gagnon, C., & Gagné, F. (2017). Comparative study of the effects of gadolinium chloride and gadolinium-based magnetic resonance imaging contrast agent on freshwater mussel, *Dreissena plymorpha*. *Chemosphere*, *181*, 197–207. http://doi.org/10.1016/j.chemosphere.2017.04.073

Handy, R. D., Van Den Brink, N., Chappell, M., Mühling, M., Behra, R., Dušinská, M., … Riediker, M. (2012). Practical considerations for conducting ecotoxicity test methods with manufactured nanomaterials: What have we learnt so far? *Ecotoxicology*, *21*, 933–972. http://doi.org/10.1007/s10646-012-0862-y

Hansen, S. F., Heggelund, L. R., Revilla Besora, P., Mackevica, A., Boldrin, A., & Baun, A. (2016). Nanoproducts – What is actually available to European consumers? *Environmental Science: Nano*, *3*, 169–180. http://doi.org/10.1039/c5en00182j

Hansen, S. F., Sørensen, S. N., Skjolding, L. M., Hartmann, N. B., & Baun, A. (2017). Revising REACH guidance on information requirements and chemical safety assessment for engineered nanomaterials for aquatic ecotoxicity endpoints: Recommendations from the EnvNano project. *Environmental Sciences Europe*, *29*, 1–15. http://doi.org/10.1186/s12302-017-0111-3

Heiligtag, F. J., & Niederberger, M. (2013). The fascinating world of nanoparticle research. *Materials Today*, *16*, 262–271. http://doi.org/10.1016/j.mattod.2013.07.004

Heinlaan, M., Ivask, A., Blinova, I., Dubourguier, H.-C., & Kahru, A. (2008). Toxicity of nanosized and bulk ZnO, CuO and TiO_2 to bacteria *Vibrio fischeri* and crustaceans *Daphnia magna* and *Thamnocephalus platyurus*. *Chemosphere*, *71*, 1308–16. http://doi.org/10.1016/j.chemosphere.2007.11.047

Herrmann, H., Nolde, J., Berger, S., & Heise, S. (2016). Aquatic ecotoxicity of lanthanum – A review and an attempt to derive water and sediment quality criteria. *Ecotoxicology and Environmental Safety*, *124*, 213–238. http://doi.org/10.1016/j.ecoenv.2015.09.033

Hirano, S., & Suzuki, K. T. (1996). Exposure, metabolism, and toxicity of rare earths and related compounds. *Environmental Health Perspectives*, *104*, 85–95. http://doi.org/10.1289/ehp.96104s185

Holbrook, R. D., Murphy, K. E., Morrow, J. B., & Cole, K. D. (2008). Trophic transfer of nanoparticles in a simplified invertebrate food web. *Nature Nanotechnology*, *3*, 352–355. http://doi.org/10.1038/nnano.2008.110

Holden, P. A., Gardea-Torresdey, J. L., Klaessig, F., Turco, R. F., Mortimer, M., Hund-Rinke, K., … Nel, A. E. (2016). Considerations of Environmentally Relevant Test Conditions for Improved Evaluation of Ecological Hazards of Engineered Nanomaterials. *Environmental Science and Technology*, *50*, 6124–6145. http://doi.org/10.1021/acs.est.6b00608

Horton, P. A., Rowan, M., Webster, K. E., & Peters, R. H. (1979). Browsing and grazing by cladoceran filter feeders. *Canadian Journal of Zoology*, *57*, 206–212. http://doi.org/10.1139/z79-019

Hund-Rinke, K., Baun, A., Cupi, D., Fernandes, T. F., Handy, R., Kinross, J. H., … Scott-Fordsmand, J. J. (2016). Regulatory ecotoxicity testing of nanomaterials – proposed modifications of OECD test guidelines based on laboratory experience with silver and titanium dioxide nanoparticles. *Nanotoxicology*, *10*, 1442–1447. http://doi.org/10.1080/17435390.2016.1229517

Hyne, R. V, Pablo, F., Julli, M., & Markich, S. J. (2005). Influence of water chemistry on the acute toxicity of copper and zinc to the cladoceran *Ceriodaphnia cf dubia*. *Environmental Toxicology and Chemistry/SETAC*, *24*, 1667–1675. http://doi.org/10.1897/04-497r.1

Ingri, J., Widerlund, A., Land, M., Gustafsson, Ö., Andersson, P., & Öhlander, B. (2000). Temporal variations in the fractionation of the rare earth elements in a Boreal river; the role of colloidal particles. *Chemical Geology*, *166*, 23–45. http://doi.org/10.1016/S0009-2541(99)00178-3

ISO. (2011). Water quality – Determination of the acute toxicity to *Thamnocephalus platyurus* (Crustacea, Anostraca). ISO 14380:2011. Geneva, Switzerland: International Organization for Standardization.

ISO. (2012a). Water quality – Determination of freshwater sediment toxicity to *Heterocypris incongruens* (Crustacea, Ostracoda). ISO 14371:2012. Geneva, Switzerland: International Organization for Standardization.

ISO. (2012b). Water quality – Determination of the inhibition of the mobility of *Daphnia magna* Straus (Cladocera, Crustacea) – Acute toxicity test. ISO 6341:2012. Geneva, Switzerland: International Organization for Standardization.

Ivask, A., Kurvet, I., Kasemets, K., Blinova, I., Aruoja, V., Suppi, S., … Kahru, A. (2014). Size-dependent toxicity of silver nanoparticles to bacteria, yeast, algae, crustaceans and mammalian cells in vitro. *PLoS ONE*, *9*(7). http://doi.org/10.1371/journal.pone.0102108

Johannesson, K. H., Tang, J., Daniels, J. M., Bounds, W. J., & Burdige, D. J. (2004). Rare earth element concentrations and speciation in organic-rich blackwaters of the Great Dismal Swamp, Virginia, USA. *Chemical Geology*, *209*, 271–294. http://doi.org/10.1016/j.chemgeo.2004.06.012

Joonas, E., Aruoja, V., Olli, K., Syvertsen-wiig, G., Vija, H., & Kahru, A. (2017). Potency of (doped) rare earth oxide particles and their constituent metals to inhibit algal growth and induce direct toxic effects. *Science of the Total Environment Journal*, *594*, 478–486. http://doi.org/10.1016/j.scitotenv.2017.03.184

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. http://doi.org/10.3762/bjnano.6.183

Kahru, A., & Dubourguier, H.-C. (2010). From ecotoxicology to nanoecotoxicology. *Toxicology*, *269*, 105–19. http://doi.org/10.1016/j.tox.2009.08.016

Käkinen, A., Bondarenko, O., Ivask, A., & Kahru, A. (2011). The effect of composition of different ecotoxicological test media on free and bioavailable copper from $CuSO_4$ and CuO nanoparticles: comparative evidence from a Cu-selective electrode and a Cu-biosensor. *Sensors (Basel, Switzerland)*, *11*, 10502–21. http://doi.org/10.3390/s111110502

Kamaya, M., Sonamoto, M., Nagashima, K., & Ginatullina, E. N. (2011). A Simple Bioassay Using Fluorescent Microbeads and *Daphnia magna*. *Indian journal of environmental health*, *5*, 1613–1616.

Kang, D. H. P., Chen, M., & Ogunseitan, O. A. (2013). Potential environmental and human health impacts of rechargeable lithium batteries in electronic waste. *Environmental Science and Technology*, *47*, 5495–5503. http://doi.org/10.1021/es400614y

Kato, Y., Fujinaga, K., Nakamura, K., Takaya, Y., Kitamura, K., Ohta, J., … Iwamori, H. (2011). Deep-sea mud in the Pacific Ocean as a potential resource for rare-earth elements. *Nature Geoscience*, *4*, 535–539. http://doi.org/10.1038/ngeo1185

Keller, A. A., McFerran, S., Lazareva, A., & Suh, S. (2013). Global life cycle releases of engineered nanomaterials. *Journal of Nanoparticle Research*, *15*, 1692. http://doi.org/10.1007/s11051-013-1692-4

Khalaj, M., Kamali, M., Khodaparast, Z., & Jahanshahi, A. (2018). Copper-based nanomaterials for environmental decontamination – An overview on technical and toxicological aspects. *Ecotoxicology and Environmental Safety*, *148*, 813–824. http://doi.org/10.1016/j.ecoenv.2017.11.060

Kharitonova, N. A., & Vakh, E. A. (2015). Rare earth elements in surface waters of Priamurye. Features of accumulation and fractionation. *Tomsk State University Journal, 396,* 232–244. http://doi.org/10.17223/15617793/396/40

Kim, P., Anderko, A., Navrotsky, A., & Riman, R. (2018). Trends in Structure and Thermodynamic Properties of Normal Rare Earth Carbonates and Rare Earth Hydroxycarbonates. *Minerals*, *8*, 106. http://doi.org/10.3390/min8030106

Koshizaki, N., & Oyama, T. (2000). Sensing characteristics of ZnO-based NO_x sensor. *Sensors and Actuators B: Chemicals*, *66*, 119–121. https://doi.org/10.1016/S0925-4005(00)00323-3

Kroto, H. W., Heath, J. R., O'Brien, S. C., Curl, R. F., & Smalley, R. E. (1985). C60: Buckminsterfullerene. *Nature*, *318*, 162–163. http://doi.org/10.1038/315279a0

Lai, K. M., Scrimshaw, M. D., & Lester, J. N. (2002). Prediction of the bioaccumulation factors and body burden of natural and synthetic estrogens in aquatic organisms in the river systems. *Science of the Total Environment*, *289*, 159–168. http://doi.org/10.1016/S0048-9697(01)01036-1

Lead, J. R., & Wilkinson, K. J. (2006). Aquatic colloids and nanoparticles: Current knowledge and future trends. *Environmental Chemistry*, *3*(3), 159–171. http://doi.org/10.1071/EN06025

Levard, C., Hotze, E. M., Colman, B. P., Dale, A. L., Truong, L., Yang, X. Y., … Lowry, G. V. (2013a). Sulfidation of silver nanoparticles: Natural antidote to their toxicity. *Environmental Science and Technology*, *47*, 13440–13448. http://doi.org/10.1021/es403527n

Levard, C., Mitra, S., Yang, T., Jew, A. D., Badireddy, A. R., Lowry, G. V, & Gordon E. Brown, J. (2013b). Effect of Chloride on the Dissolution Rate of Silver Nanoparticles and Toxicity to *E. coli*. *Environmental Science & Technology, 47,* 5738–5745. http://doi.org/10.1021/es400396f

Li, D., Li, R., Xue, T., Wang, B., Liu, F., Zhao, B., & Zhao, D. (2018). Characteristic and Geological Implications of Major Elements and Rare Earth Elements of Triassic Chang 7 Oil Shale in Tongchuan City, Southern Ordos Basin (China). *Minerals*, *8*, 157. http://doi.org/10.3390/min8040157

Li, M., Lin, D., & Zhu, L. (2013). Effects of water chemistry on the dissolution of ZnO nanoparticles and their toxicity to *Escherichia coli*. *Environmental Pollution*, *173*, 97–102. http://doi.org/10.1016/j.envpol.2012.10.026

Liebscher, K., Schönfeld, T., & Schaller, A. (1961). Concentration of inhaled cerium-144 in pulmonary lymph nodes of human beings. *Nature*, *192*, 1308. http://doi.org/10.1038/1921308a0

Lin, D., Ji, J., Long, Z., Yang, K., & Wu, F. (2012). The influence of dissolved and surface-bound humic acid on the toxicity of TiO_2 nanoparticles to *Chlorella* sp. *Water Research*, *46*, 4477–4487. http://doi.org/10.1016/j.watres.2012.05.035

Lin, D., Ma, S., Zhou, K., Wu, F., & Yang, K. (2015). The effect of water chemistry on homoaggregations of various nanoparticles: Specific role of Cl^- ions. *Journal of Colloid and Interface Science*, *450*, 272–278. http://doi.org/10.1016/j.jcis.2015.03.027

Liu, Z., Wang, C., Hou, J., Wang, P., Miao, L., Lv, B., & Yang, Y. (2018). Aggregation, sedimentation, and dissolution of CuO and ZnO nanoparticles in five waters. *Environmental Science and Pollution Research*, *25*, 31240–31249. http://doi.org/10.1007/s11356-018-3123-7

Luo, Z., Li, M., Wang, Z., Li, J., Guo, J., Rosenfeldt, R. R., … Yan, C. (2018). Effect of titanium dioxide nanoparticles on the accumulation and distribution of arsenate in *Daphnia magna* in the presence of an algal food. *Environmental Science and Pollution Research, 25,* 20911–20919. http://doi.org/10.1007/s11356-018-2265-y

Luoma, S. N., & Rainbow, P. S. (2005). Why is metal bioaccumulation so variable? Biodynamics as a unifying concept Environmental Science & Technology. *Environ. Sci. Techn.*, *39*, 1921–1931. http://doi.org/10.1021/es048947e

Lürling, M., & Tolman, Y. (2010). Effects of lanthanum and lanthanum-modified clay on growth, survival and reproduction of *Daphnia magna*. *Water Research*, *44*, 309–319. http://doi.org/10.1016/j.watres.2009.09.034

Maltby, L. (1999). Studying Stress: The Importance of Organism-Level Responses. *Ecological Applications*, *9*, 431. http://doi.org/10.2307/2641131

Martins, C., Jesus, F. T., & Nogueira, A. J. A. (2017). The effects of Copper and Zinc on survival, growth and reproduction of the cladoceran *Daphnia longispina*: introducing new data in an "old" issue. *Ecotoxicology*, *26*, 1157–1169. http://doi.org/10.1007/s10646-017-1841-0

McGillicuddy, E., Murray, I., Kavanagh, S., Morrison, L., Fogarty, A., Cormican, M., … Morris, D. (2017). Silver nanoparticles in the environment: Sources, detection and ecotoxicology. *Science of the Total Environment*, *575*, 231–246. http://doi.org/10.1016/j.scitotenv.2016.10.041

Migaszewski, Z. M., Gałuszka, A., & Dołęgowska, S. (2016). Rare earth and trace element signatures for assessing an impact of rock mining and processing on the environment: Wiśniówka case study, south-central Poland. *Environmental Science and Pollution Research*, *23*, 24943–24959. http://doi.org/10.1007/s11356-016-7713-y

Miličić, D. M., Majstorović, A. P., Pavković-Lučić, S. B., & Savić, T. T. (2015). Behaviour and food selection of *Heterocypris incongruens* (Ostracoda). *Crustaceana*, *88*, 1097–1110. http://doi.org/10.1163/15685403-00003479

Mukae, K., Tsuda, K., & Nagasawa, I. (1977). Non-Ohmic Properties Of ZnO-Rare Earth Metal Oxide-Co_3O_4 Ceramics. *Japanese Journal of Applied Physics*, *16*, 1361–1368. http://doi.org/10.1143/JJAP.16.1361

Neal, C. (2007). Rare earth element concentrations in dissolved and acid available participate forms for eastern UK rivers. *Hydrology and Earth System Sciences*, *11*, 313–327. http://doi.org/10.5194/hess-11-313-2007

Nezu, N., Asana, M., & Ouchi, S. (1960). Cerium-144 in food. *Science*, *135*, 102–103. http://doi.org/10.1126/science.135.3498.102

Noller, B. N. (1991). Non-radiological contaminants from uranium mining and milling at Ranger, Jabiru, Northern Territory, Australia. *Environmental Monitoring and Assessment*, *19*, 383–400. http://doi.org/10.1007/BF00401327

Notter, D. A., Mitrano, D. M., & Nowack, B. (2014). Are nanosized or dissolved metals more toxic in the environment? A meta-analysis. *Environmental Toxicology and Chemistry*, *33*, 2733–2739. http://doi.org/10.1002/etc.2732

Odzak, N., Kistler, D., Behra, R., & Sigg, L. (2014). Dissolution of metal and metal oxide nanoparticles in aqueous media. *Environmental Pollution*, *191*, 132–138. http://doi.org/10.1016/j.envpol.2014.04.010

Odzak, N., Kistler, D., Behra, R., & Sigg, L. (2015). Dissolution of metal and metal oxide nanoparticles under natural freshwater conditions. *Environmental Chemistry*, *12*, 138–148. http://doi.org/10.1071/EN14049

OECD. (2004). Test No. 202: *Daphnia* sp. Acute Immobilisation Test, OECD Guidelines for the Testing of Chemicals, Section 2. Paris, France: Organisation for Economic Co-operation and Development. https://doi.org/10.1787/9789264069947-en

OECD. (2012). Test No. 211: *Daphnia magna* Reproduction Test, OECD Guidelines for the Testing of Chemicals, Section 2. Paris, France: Organisation for Economic Co-operation and Development. http://doi.org/https://doi.org/10.1787/9789264185203-en.

OECD. (2014). Ecotoxicology and environmental fate of manufactured nanomaterials: test guidelines. *ENV/JM/MONO(2014)1.* Paris, France: Organisation for Economic Co-operation and Development. Retrieved from http://www.oecd.org/officialdocuments/displaydocument/?cote=ENV/JM/MONO(2014)1&doclanguage=en

OECD. (2018). Guidance document on aqueous-phase aquatic toxicity testing of difficult test chemicals. Series on testing and assessment No. 23 (Second Edition). ENV/JM/MONO(2000)6/REV1. 6.07.2018. Paris, France: Organisation for Economic Co-operation and Development. Retrieved from http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=ENV/JM/MONO(2000)6/REV1&doclanguage=en

Otero-González, L., García-Saucedo, C., Field, J. A., & Sierra-Álvarez, R. (2013). Toxicity of TiO_2, ZrO_2, Fe^0, Fe_2O_3, and Mn_2O_3 nanoparticles to the yeast, *Saccharomyces cerevisiae*. *Chemosphere*, *93*, 1201–1206. http://doi.org/10.1016/j.chemosphere.2013.06.075

Pagano, G., Guida, M., Siciliano, A., Oral, R., Koçba, F., Palumbo, A., … Trifuoggi, M. (2016). Comparative toxicities of selected rare earth elements: Sea urchin embryogenesis and fertilization damage with redox and cytogenetic effects, *147*, 453–460. http://doi.org/10.1016/j.envres.2016.02.031

Pang, X., Li, D., & Peng, A. (2001). Application of rare-earth elements in the agriculture of China and its environmental behavior in soil. *Journal of Soils and Sediments*, *1*, 124–129. http://doi.org/10.1065/iss2001.05.013

Paulick, H., & Machacek, E. (2017). The global rare earth element exploration boom: An analysis of resources outside of China and discussion of development perspectives. *Resources Policy*, *52*, 134–153. http://doi.org/10.1016/j.resourpol.2017.02.002

Peng, Y. H., Tsai, Y. C., Hsiung, C. E., Lin, Y. H., & Shih, Y. hsin. (2017). Influence of water chemistry on the environmental behaviors of commercial ZnO nanoparticles in various water and wastewater samples. *Journal of Hazardous Materials*, *322*, 348–356. http://doi.org/10.1016/j.jhazmat.2016.10.003

Persoone, G., Baudo, R., Cotman, M., Blaise, C., Thompson, K. C., Moreira-Santos, M., … Han, T. (2009). Review on the acute *Daphnia magna* toxicity test – Evaluation of the sensitivity and the precision of assays performed with organisms from laboratory cultures or hatched from dormant eggs. *Knowledge and Management of Aquatic Ecosystems, 393,* 1–29. http://doi.org/10.1051/kmae/2009012

Persoone, G., & Janssen, C. R. (1994). Freshwater invertibrate toxicity tests. In *Handbook of ecotoxicology* (pp. 51–65). Cambridge, UK: Cambridge University Press.

Peters, R. J. B., van Bemmel, G., Milani, N. B. L., den Hertog, G. C. T., Undas, A. K., van der Lee, M., & Bouwmeester, H. (2018). Detection of nanoparticles in Dutch surface waters. *Science of the Total Environment*, *621*, 210–218. http://doi.org/10.1016/j.scitotenv.2017.11.238

Petersen, E. J., Diamond, S. A., Kennedy, A. J., Goss, G. G., Ho, K., Lead, J., … Sayre, P. (2015). Adapting OECD Aquatic Toxicity Tests for Use with Manufactured Nanomaterials: Key Issues and Consensus Recommendations. *Environmental Science and Technology*, *49*, 9532–9547. http://doi.org/10.1021/acs.est.5b00997

Porvali, A., Wilson, B. P., & Lundström, M. (2018). Lanthanide-alkali double sulfate precipitation from strong sulfuric acid NiMH battery waste leachate. *Waste Management*, *71*, 381–389. http://doi.org/10.1016/j.wasman.2017.10.031

Rim, K.-T. (2016). Effects of Rare Earth Elements on the Environment and Human Health: A Literature Review. *Toxicology and Environmental Health Sciences*, *8*, 189–200. http://doi.org/10.1007/s13530-016-0276-y

Romero-Freire, A., Joonas, E., Muna, M., Cossu-Leguille, C., Vignati, D. A. L., & Giamberini, L. (2019). Assessment of the toxic effects of mixtures of three lanthanides (Ce, Gd, Lu) to aquatic biota. *Science of The Total Environment*, *661*, 276–284. http://doi.org/10.1016/j.scitotenv.2019.01.155

Romero-Freire, A., Minguez, L., Pelletier, M., Cayer, A., Caillet, C., Devin, S., … Giamberini, L. (2018). Assessment of baseline ecotoxicity of sediments from a prospective mining area enriched in light rare earth elements. *Science of the Total Environment*, *612*, 831–839. http://doi.org/10.1016/j.scitotenv.2017.08.128

Sabbioni, E., Pietra, R., Gaglione, P., Vocaturo, G., Colombo, F., Zanoni, M., & Rodi, F. (1982). Long-term occupational risk of rare-earth pneumoconiosis. A case report as investigated by neutron activation analysis. *Science of the Total Environment, 26*, 19–32. http://doi.org/10.1016/0048-9697(82)90093-6

Sadeq, S. A., & Beckerman, A. P. (2018). The Chronic Effects of Copper and Cadmium on Life History Traits Across Cladocera Species: A Meta-analysis. *Archives of Environmental Contamination and Toxicology*, 76, 1–16. http://doi.org/10.1007/s00244-018-0555-5

Sauve, S., & Desrosiers, M. (2014). A review of what is an emerging contaminant. *Chemistry Central Journal*, *8*, 15. http://doi.org/10.1186/1752-153X-8-15

Schaming, D., & Remita, H. (2015). Nanotechnology: from the ancient time to nowadays. *Foundations of Chemistry*, *17*, 187–205. http://doi.org/10.1007/s10698-015-9235-y

Shi, Y., Pan, X., Li, B., Zhao, M., & Pang, H. (2018). Co_3O_4 and its composites for high-performance Li-ion batteries. *Chemical Engineering Journal*, *343*, 427–446. http://doi.org/10.1016/j.cej.2018.03.024

Shuhaimi-Othman, M., Yakub, N., Ramle, N. A., & Abas, A. (2011). Toxicity of metals to a freshwater ostracod: *Stenocypris major*. *Journal of Toxicology*, *2011*. http://doi.org/10.1155/2011/136104

Slooff, W., Bont, P. F. H., Van den Hoop, M. A. G. T., Janus, J. A., & Annema, J. A. (1993). *Exploratory report rare earth metals and their compounds. RIVM report no. 710401025.* Retrieved from https://www.rivm.nl/publicaties/exploratory-report-rare-earth-metals-and-their-compounds

Sneller, F. E. C., Kalf, D. F., Weltje, L., & Van Wezel, A. P. (2000). Maximum Permissible Concentrations and Negligible Concentrations for Rare Earth Elements (REEs). RIVM National Institue of Public Health and the Environment, The Netherlands.

Song, L., Vijver, M. G., de Snoo, G. R., & Peijnenburg, W. J. G. M. (2015). Assessing toxicity of copper nanoparticles across five cladoceran species. *Environmental Toxicology and Chemistry*, *34*, 1863–1869. http://doi.org/10.1002/etc.3000

Spears, B. M., Lürling, M., Yasseri, S., Castro-Castellon, A. T., Gibbs, M., Meis, S., … Van Oosterhout, F. (2013). Lake responses following lanthanum-modified bentonite clay (Phoslock®) application: An analysis of water column lanthanum data from 16 case study lakes. *Water Research*, *47*, 5930–5942. http://doi.org/10.1016/j.watres.2013.07.016

Stauber, J. L. (2000). *Toxicity testing of modified clay leachates using freshwater organisms.* Report No: ET/IR267R. CSIRO Land and Water, Australia. Retrieved from https://sepro.com/documents/Phoslock/TechInfo/Eco-Toxicity Report by CSIRO-Australia_Stauber 2000.pdf

Stauber, J. L., & Binet, M. T. (2000). *Canning River Phoslock Testing Final Report*. Report No: ET317R. CSIRO Land and Water and the WA Water and Rivers Commission, Australia. Retrieved from http://www.phoslock.com.au/site/content/scientificreport/Eco-Toxicity_Report_by_CSIRO_Australia_Stauber__Binet_2000_Canning_river_phoslock_field_trial_-_ecotoxicity_testing_final_report.pdf

Stevenson, L. M., Krattenmaker, K. E., Johnson, E., Bowers, A. J., Adeleye, A. S., McCauley, E., & Nisbet, R. M. (2017). Standardized toxicity testing may underestimate ecotoxicity: Environmentally relevant food rations increase the toxicity of silver nanoparticles to *Daphnia*. *Environmental Toxicology and Chemistry*, *36*, 3008–3018. http://doi.org/10.1002/etc.3869

Stewart, B. W., Capo, R. C., Hedin, B. C., & Hedin, R. S. (2017). Rare earth element resources in coal mine drainage and treatment precipitates in the Appalachian Basin, USA. *International Journal of Coal Geology*, *169*, 28–39. http://doi.org/10.1016/j.coal.2016.11.002

Stücheli, P. E., Niggemann, J., & Schubert, C. J. (2018). Comparison of different solid phase extraction sorbents for the qualitative assessment of dissolved organic nitrogen in freshwater samples using FT-ICR-MS. *Journal of Limnology*, *77*, 400–411. http://doi.org/10.4081/jlimnol.2018.1791

Sudha, P. N., Sangeetha, K., Vijayalakshmi, K., & Barhoum, A. (2018). Nanomaterials history, classification, unique properties, production and market. In *Emerging Applications of Nanoparticles and Architectural Nanostructures: Current Prospects and Future Trends* (pp. 341–384). Elsevier Inc. http://doi.org/10.1016/B978-0-323-51254-1.00012-9

Suppi, S., Kasemets, K., Ivask, A., Künnis-Beres, K., Sihtmäe, M., Kurvet, I., … Kahru, A. (2014). A novel method for comparison of biocidal properties of nanomaterials to bacteria, yeasts and algae. *Journal of Hazardous Materials*, *286C*, 75–84. http://doi.org/10.1016/j.jhazmat.2014.12.027

Tang, H., Wang, X., Shuai, W., & Liu, Y. (2016). Immobilization of Rare Earth Elements of the Mine Tailings Using Phosphates and Lime. *Procedia Environmental Sciences*, *31*, 255–263. http://doi.org/10.1016/j.proenv.2016.02.034

Tang, J., & Johannesson, K. H. (2010). Ligand extraction of rare earth elements from aquifer sediments: Implications for rare earth element complexation with organic matter in natural waters. *Geochimica et Cosmochimica Acta*, *74*, 6690–6705. http://doi.org/10.1016/j.gca.2010.08.028

Taylor, G., Baird, D. J., & Soares, A. M. V. M. (1998). Surface binding of contaminants by algae: Consequences for lethal toxicity and feeding to *Daphnia magna* Straus. *Environmental Toxicology and Chemistry*, *17*, 412–419. http://doi.org/10.1002/etc.5620170310

Thurman, E. M. (1985). *Organic geochemistry of natural waters.* Dordrecht, Netherlands: Martinus Nijhof/ Dr. W. Junk Publishers. http://doi.org/10.1007/978-94-009-5095-5

Toming, K., Tuvikene, L., Vilbaste, S., Agasild, H., Viik, M., Kisand, A., … Nõges, T. (2013). Contributions of autochthonous and allochthonous sources to dissolved organic matter in a large, shallow, eutrophic lake with a highly calcareous catchment. *Limnology and Oceanography*, *58*, 1259–1270. http://doi.org/10.4319/lo.2013.58.4.1259

UNCTAD. (2014). *Commodities at a glance: special issue on rare earths*. United Nations Conference on Trade and Development. N°5. Retrieved from https://unctad.org/en/PublicationsLibrary/suc2014d1_en.pdf

US EPA. (2012). *Rare Earth Elements: A Review of Production, Processing, Recycling, and Associated Environmental Issues.* Epa/600/R-12/572. Cincinnati, OH: United States Environmental Protection Agency.

Vance, M. E., Kuiken, T., Vejerano, E. P., McGinnis, S. P., Hochella, M. F., & Hull, D. R. (2015). Nanotechnology in the real world: Redeveloping the nanomaterial consumer products inventory. *Beilstein Journal of Nanotechnology*, *6*, 1769–1780. http://doi.org/10.3762/bjnano.6.181

Vijver, M. G., Van Gestel, C. A. M., Lanno, R. P., Van Straalen, N. M., & Peijnenburg, W. J. G. M. (2004). Internal metal sequestration and its ecotoxicological relevance: A review. *Environmental Science and Technology*, *38*, 4705–4712. http://doi.org/10.1021/es040354g

Von Moos, N., Bowen, P., & Slaveykova, V. I. (2014). Bioavailability of inorganic nanoparticles to planktonic bacteria and aquatic microalgae in freshwater. *Environmental Science: Nano*, *1*, 214–232. http://doi.org/10.1039/c3en00054k

Waajen, G., van Oosterhout, F., & Lürling, M. (2017). Bio-accumulation of lanthanum from lanthanum modified bentonite treatments in lake restoration. *Environmental Pollution*, *230*, 911–918. http://doi.org/10.1016/j.envpol.2017.07.046

Wang, L., & Liang, T. (2015). Geochemical fractions of rare earth elements in soil around a mine tailing in Baotou, China. *Scientific Reports*, *5*, 1–11. http://doi.org/10.1038/srep12483

Wells, W. H., & Wells, V. L. (2001). The lanthanides, rare earth metals. In *Patty's Toxicology* (Vol. 1, pp. 817–840). New York, USA: Wiley. http://doi.org/10.1002/0471125474

Weltje, L., Brouwer, A. H., Verburg, T. G., Wolterbeek, H. T., & de Goeij, J. J. M. (2002). Accumulation and elimination of lanthanum by duckweed (*Lemna minor* L.) as influenced by organism growth and lanthanum sorption to glass. *Environmental Toxicology and Chemistry*, *21*, 1483–1489. http://doi.org/10.1002/etc.5620210721

Williams, E. S., Panko, J., & Paustenbach, D. J. (2009). The European Union's REACH regulation: a review of its history and requirements. *Critical Reviews in Toxicology*, *39*, 553–575. http://doi.org/10.1080/10408440903036056

Wonglimpiyarat, J. (2005). The nano-revolution of Schumpeter's Kondratieff cycle. *Technovation*, *25*, 1349–1354. http://doi.org/10.1016/j.technovation.2004.07.002

Wood, C. M., Al-Reasi, H. A., & Smith, D. S. (2011). The two faces of DOC. *Aquatic Toxicology*, *105*, 3–8. http://doi.org/10.1016/j.aquatox.2011.03.007

Yang, K., & Xing, B. (2009). Sorption of phenanthrene by humic acid-coated nanosized TiO_2 and ZnO. *Environmental Science and Technology*, *43*, 1845–1851. http://doi.org/10.1021/es802880m

Yu, D. Q., Zhao, J., & Wang, L. (2004). Improvement on the microstructure stability, mechanical and wetting properties of Sn-Ag-Cu lead-free solder with the addition of rare earth elements. *Journal of Alloys and Compounds*, *376*, 170–175. http://doi.org/10.1016/j.jallcom.2004.01.012

Zeng, X., Li, J., & Liu, L. (2015). Solving spent lithium-ion battery problems in China: Opportunities and challenges. *Renewable and Sustainable Energy Reviews*, *52*, 1759–1767. http://doi.org/10.1016/j.rser.2015.08.014

Zhang, Y. C., Li, J. T., Wu, Z. G., Huang, L., & Sun, S. G. (2017). Synthesis of hierarchical spindle-like Mn_2O_3 for lithium ion batteries with enhanced lithium storage properties. *Journal of Alloys and Compounds*, *721*, 229–235. http://doi.org/10.1016/j.jallcom.2017.05.305

Zhang, Y., Jeppesen, E., Liu, X., Qin, B., Shi, K., Zhou, Y., … Deng, J. (2017). Global loss of aquatic vegetation in lakes. *Earth-Science Reviews*, *173*, 259–265. http://doi.org/10.1016/j.earscirev.2017.08.013

Zhao, X., Ren, X., Zhu, R., Luo, Z., & Ren, B. (2016). Zinc oxide nanoparticles induce oxidative DNA damage and ROS-triggered mitochondria-mediated apoptosis in zebrafish embryos. *Aquatic Toxicology*, *180*, 56–70. http://doi.org/10.1016/j.aquatox.2016.09.013

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Abstract

Hazard evaluation of metal-based nanoparticles and lanthanides with freshwater microcrustaceans

Along with the economic growth and technological advancements, novel substances are increasingly applied in all the walks of life. Some of these substances may become emerging contaminants with unpredictable effects on human health and the environment due to the lack of respective data or problems with reliable interpretation of the available toxicity data.

Engineered metal-based nanoparticles (NP) and lanthanides (Ln), both industrially relevant since the 1980s, can be considered emerging contaminants. Metal-based NP (with at least one dimension within 1–100 nm) are mainly valued for their novel optical, electronic, and catalytic, but also antimicrobial properties manifesting at nano-scale. Ln are irreplaceable in modern high-tech and green technologies due to their unique physical properties.

As in the aquatic test environment both metal-based NP and Ln tend to aggregate, precipitate, settle and sorb to organisms and vessels, the application of standard toxicity test formats may not provide reliable results that would enable estimating the hazard for the environment. Due to the shortage of high-quality ecotoxicity data on both of these compounds, their safe emission levels into the environment have not yet been established. At the same time, anthropogenic contamination of the substances from both of the groups has already been recorded.

The main aim of this thesis was to improve the understanding of the potential environmental hazards of NP and Ln to the aquatic ecosystem. For that, modified toxicity test formats in parallel to the standard ones using a variety of freshwater microcrustacean test species were applied. On the basis of the obtained results, the author proposes cost-effective modifications of and additions to standard aquatic toxicity test formats to add ecological relevance and predictive power to the obtained ecotoxicity data.

The low stability of NP and Ln suspensions and solutions complicates the toxicity assessment by standard test formats as the bioavailable part of the substance is unknown. Moreover, the tests required to be used for toxicological classification of chemicals are not necessarily suitable for assessing the hazard to the ecosystem. For these reasons, the standardised test formats were modified to better represent the natural environment and the effect of the modifications on the toxicity results was critically analysed. Additional sublethal post-exposure endpoints such as feeding behaviour, metal body burden, and post-exposure recovery potential were used in *D. magna* standard acute test formats in order to gain additional ecologically relevant toxicity information. *Daphnia* feeding behaviour on algae was assessed by quantifying feeding by-products in flow cytometry and metal body burden in single *D. magna* specimen by total reflection X-ray fluorescence spectroscopy (TXRF).

Along with the sublethal effects of CuO, Co_3O_4, and Mn_2O_3 NP, the (combined) effect of test media and dietary algae on CuO, ZnO, and Ag-PVP NP toxicity to crustaceans was examined in this thesis. NP studies were always accompanied by the respective soluble salt as the ionic control. Biological effects of Ln were studied in different test conditions exposing test organisms to La, Ce, Pr, Nd, and Gd nitrates and to multimetal Ln (doped) submicron particles. Since the crustaceans are one of the

most sensitive organism groups to metal-based substances, three species of microcrustaceans, planktic water flea *Daphnia magna* and fairy shrimp *Thamnocephalus platyurus* along with benthic ostracod *Heterocypris incongruens* were used as test organisms.

Natural water as the test medium mitigated CuO NP toxicity, did not change Ag-PVP NP toxicity, and increased ZnO NP toxicity compared to standard mineral test medium. The combined effects of dietary algae and standard vs lake water as test medium were different on NP and the respective metal salt toxicity. Such variation was not seen in tests with *H. incongruens* possibly due to the different behavioural traits of the organisms. Thus, the use of natural water as test medium along with addition of dietary algae to test suspensions may increase the environmental relevance of the NP toxicity test results.

Despite the reduction of CuO NP acute toxicity in natural freshwater, post-exposure Cu body burden in *D. magna* after exposure to CuO NP was higher in natural water than in standard mineral medium. Co_3O_4 and Mn_2O_3 NP did not induce acute toxicity to *D. magna* but did have either increasing or decreasing effect on the post-exposure feeding behaviour of *D. magna* similarly to the respective soluble metal salts. Co_3O_4 and Mn_2O_3 NP also induced higher body burden in *D. magna* compared to toxic CuO or soluble Co and Mn salts. The metal body burden induced by Co_3O_4 and Mn_2O_3 exposure remained over 4-fold elevated compared to the control organisms even after the post-exposure depuration. That could lead to metal transfer to higher food chain levels in the nature. In addition, considerable mortality occurred during the depuration subsequent to Co_3O_4 and Mn_2O_3 exposure. From the sublethal effect studies it was evident that lower or absent acute toxicity does not necessarily indicate lower environmental hazard.

Evaluation of the biological effects of Ln salts was complicated due to the settling of metals in the acute test, resulting in very high variability of the metal concentration in the water column. Ln solutions were more stable in chronic exposure at low nominal concentrations (< 1 mg Ln/L). The results of the chronic experiments and the post-exposure recovery potential after acute exposure showed that Ln may constitute a hazard to the aquatic environment.

In case of Ln (doped) particles, it was demonstrated that the bioavailability of Ln was very low and that other constituent metals (Co and Ni) were responsible for the toxic effects of these particles to the microcrustaceans. The toxicity to microcrustaceans correlated with the accumulation of the toxicity-inducing metals in the floating aquatic plant *Lemna minor*. Therefore, metal concentration in plant tissue could be considered as an indicator of metal bioavailability from low solubility substances to a variety of aquatic organisms.

In summary, the following modifications and additions to standard toxicity tests can be recommended for environmentally relevant toxicity testing of metal-based NP and Ln: i) to carry out toxicity tests in natural water in the presence of dietary algae in addition to the standardised test conditions; ii) to use sublethal endpoints such as feeding behaviour and metal body burden that reveal the potential adverse effects at lower exposure concentrations and of substances of low toxicity; iii) to monitor post-exposure recovery after the acute toxicity test to better predict the long term effects of the toxicants during the initial toxicity screening; iv) to use test organisms with different behavioural traits.

Lühikokkuvõte

Metalliliste nanoosakeste ja lantaniidide kahjulikkuse hindamine magevee pisivähkidega

Seoses majandusliku heaolu kasvu ja tehnoloogia arenguga võetakse kõigis eluvaldkondades kasutusele üha enam uudseid aineid. Osa neist ainetest võivad oma elutsükli jooksul saada saasteaineteks, mille mõju keskkonnale ja elusorganismidele on (veel) raske ennustada, sest vastavat teavet pole piisavalt või pole olemasoleva teabe põhjal võimalik selgeid järeldusi teha.

Ühed sellised uued saasteained on sünteetilised metallinanoosakesed (NO) ja lantaniidid (Ln), mida on tööstuses kasutatud alates 1980. aastatest. NO (vähemalt üks mõõde vahemikus 1–100 nm) hinnatakse nende väiksusega kaasnevate uudsete optiliste, elektrooniliste ja magnetiliste aga ka antimikroobsete omaduste tõttu. Ln on tänu oma unikaalsetele füüsikalistele omadustele asendamatud kõrgtehnoloogilistes ja keskkonnasõbralikes tehnoloogiates.

Kuna nii NO kui ka Ln on kalduvus veekeskkonnas ja katsete käigus agregeeruda, sadeneda, settida ning katseanuma seintele ja katseorganismidele sorbeeruda, ei pruugi toksilisuse hindamine üksnes standardsete meetoditega nende ainete keskkonnaohtu usaldusväärselt ennustada. Lisaks ei sobi standardsed kemikaalide ohutuse klassifitseerimiseks mõeldud toksilisuse testid sageli looduses tekkida võivate ohtude hindamiseks. Vaatamata sellele, et NO ja Ln inimtekkelist reostust on keskkonnas juba mõõdetud, pole olemasoleva keskkonnatoksilisuse teabe ebamäärasus võimaldanud nende saastele piirnorme kehtestada.

Selle töö põhieesmärk oli parandada arusaamist NO ja Ln võimalikust keskkonnaohust veeökosüsteemidele. Selleks võrreldi standardsetes katsetingimustes saadud toksilisuse andmeid looduslähedasemaks kohandatud katsetingimustes saadud andmetega. Töö tulemusena soovitab autor kulutõhusaid muudatusi ja täiendusi, mille sisseviimine standardsetesse toksilisuse katseformaatidesse võimaldaks saadud katseandmete abil NO ja Ln ohte veekeskkonnas paremini ennustada.

Usaldusväärsete katseandmete saamiseks hinnati standardsete toksilisuse näitajate kõrval ka teisi veeökosüsteemi tervise seisukohast olulisi näitajaid nagu *D. magna* eksponeerimisjärgne toitumiskäitumine ja eksponeerimisjärgne taastumise võimekus ning metalli akumulatsioon organismis pärast CuO, Co_3O_4 ja Mn_2O_3 NO eksponeerimist. *D. magna* eksponeerimisjärgse toitumise hindamiseks mõõdeti toiduks kasutatud vetikast pärinevate kõrvalsaaduste hulka ning üksikute *D. magna* isendite metallisisaldus mõõdeti täispeegeldus-röntgenfluorestsents spektromeetriga (TXRF).

Lisaks kohandati standardne toksilisuse hindamise katsekeskkond looduslähedasemaks. Selleks kasutati katsetes standardse vee asemel järvevett ja organisme toideti katse ajal vetikaga. Seejärel analüüsiti muudatuste mõju CuO, ZnO ja Ag-PVP NO toksilisusele. NO katsetesse kaasati alati ka vastava metalli lahustuv sool, et hinnata vabanevate metalliioonide osa NO põhjustatud toksilisuses. Ln bioloogilist mõju uuriti samuti erinevates katsetingimustes eksponeerides pisivähke La, Ce, Pr, Nd ja Gd nitraadile ja mitmest metallist koosnevatele lantaniididega legeeritud (lantaniidi) osakestele. Kuna vähid on üks tundlikumaid organismirühmi metalliliste ainete toksilisusele, kasutati katseorganismidena kolme magevee pisivähi liiki: planktilisi vesikirpu *Daphnia magna* ja paljaskilbilist *Thamnocephalus platyurus* ning põhjaelulist karpvähki *Heterocypris incongruens*.

Järvevee kasutamine standardse mineraalse katsevee asemel vähendas CuO NO toksilisust, ei muutnud Ag-PVP NO toksilisust ning suurendas ZnO NO toksilisust *D. magna* lühiajalises katses. Söödava vetika lisamisel *D. magna* lühiajalises katses kasutatud standardsesse vette ja järvevette tekkis veetüübipõhine ühismõju, mis mõjutas NO ja lahustuva soola toksilisust erinevalt. Erinev mõju ei avaldunud aga *H. incongruens'*i katses tõenäoliselt vähkide erineva toitumiskäitumise tõttu. Seetõttu võib loodusvee ja toiduks sobivate vetikate üheaegne kasutamine NO toksilisuse katsetes looduskeskkonna seisukohast olulisi tulemusi anda.

Vaatamata CuO NO toksilisuse olulisele vähenemisele loodusvees oli *D. magna* Cu sisaldus pärast ekspositsiooni loodusvees oluliselt kõrgem kui pärast ekspositsiooni standardses vees. Co_3O_4 ja Mn_2O_3 NO ei olnud *D. magna*`le akuutselt toksilised, kuid sarnaselt vastavate lahustuvate sooladega suurendasid või vähendasid nad *D. magna* toitumisaktiivsust. Lisaks oli *D. magna* metallisisaldus pärast ekspositsiooni Co_3O_4 ja Mn_2O_3 NO suurem kui pärast ekspositsiooni toksilisele CuO NO või lahustuvatele Co ja Mn sooladele. Co_3O_4 ja Mn_2O_3 NO ekspositsiooni ajal tekkinud metallisisaldus püsis *D. magna*'s kontrollorganismidest üle nelja korra kõrgem ka pärast ekspositsioonijärgset toitumist. See võib viia metallide edasikandumiseni looduslikus toiduahelas. Lisaks suri ekspositsioonijärgse toitumise käigus võrdlemisi suur osa organismidest. Mittestandardsete toksilisuse näitajate kohta saadud tulemused viitasid sellele, et isegi kui NO ei olnud antud tingimustes toksilised või kui toksilisus oli standardsete katsetingimustega võrreldes madalam, ei pruugi see tähendada madalamat keskkonnaohtu.

Ln soolade võimaliku kahjulikkuse hindamine lühiajalistes katsetes oli raskendatud, sest veesamba metallisisaldus kõikus Ln settimise tõttu suures ulatuses. Ln lahuse stabiilsus oli suurem pikaajalises katses madalatel kontsentratsioonidel (< 1 mg Ln/L). Kroonilise katse ja katsejärgse suremuse tulemuste põhjal võib Ln mageveekeskkonnale ohtlikuks liigitada.

Ln biosaadavus lantaniididega legeeritud (lantaniidi)osakestest oli väga madal ja toksilist mõju põhjustasid pisivähkidele teised osakeste koostisesse kuuluvad metallid (Co ja Ni). Toksilisus pisivähkidele korreleerus ujutaime *Lemna minor* kudedesse kogunenud toksilisust põhjustavate metallide sisaldusega. Seega saab metalli sisalduse mõõtmise abil taimekoes hinnata vähelahustuvate saasteainete biosaadavust erinevatele veeorganismidele.

Kokkuvõttes võib soovitada järgnevate muudatuste ja täienduste sisseviimist standardsetesse toksilisuse testidesse looduskeskkonna seisukohast oluliste tulemuste saamiseks metalliliste NO ja Ln toksilisuse hindamisel: i) lisaks standardsetele tingimustele katsed läbi viia ka looduslikus vees, kuhu on katseorganismidele toiduks vetikaid lisatud; ii) hinnata subletaalseid näitajaid nagu toitumisaktiivsus ja bioakumulatsioon, mis annavad keskkonna seisukohast olulist teavet madalamate kontsentratsioonide ja akuutselt vähetoksiliste ainete mõju kohta; iii) jälgida organismide lühiajalise ekspositsiooni järgset taastumist, et ennustada ainete pikaajalist mõju; iv) kasutada erineva eluviisiga katseorganisme.

Appendix 1

Publication I

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Natural water as the test medium for Ag and CuO nanoparticle hazard evaluation: An interlaboratory case study☆

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ABSTRACT

Engineered nanoparticles (NPs) have realistic potential of reaching natural waterbodies and of exerting toxicity to freshwater organisms. The toxicity may be influenced by the composition of natural waters as crucial NP properties are influenced by water constituents. To tackle this issue, a case study was set up in the framework of EU FP7 NanoValid project, performing an interlaboratory hazard evaluation of NPs in natural freshwater. Ag and CuO NPs were selected as model NPs because of their potentially high toxicity in the freshwater. *Daphnia magna* (OECD202) and *Danio rerio* embryo (OECD236) assays were used to evaluate NP toxicity in natural water, sampled from Lake Greifen and Lake Lucerne (Switzerland). Dissolution of the NPs was evaluated by ultrafiltration, ultracentrifugation and metal specific sensor bacteria. Ag NP size was stable in natural water while CuO NPs agglomerated and settled rapidly. Ag NP suspensions contained a large fraction of Ag^{+} ions and CuO NP suspensions had low concentration of Cu^{2+} ions. Ag NPs were very toxic (48 h EC_{50} 1–5.5 μg Ag/L) to *D. magna* as well as to *D. rerio* embryos (96 h EC_{50} 8.8–61 μg Ag/L) in both standard media and natural waters with results in good agreement between laboratories. CuO NP toxicity to *D. magna* differed significantly between the laboratories with 48 h EC_{50} 0.9–11 mg Cu/L in standard media, 5.7–75 mg Cu/L in Lake Greifen and 5.5–26 mg Cu/L in Lake Lucerne. No toxicity of CuO NP to zebrafish embryos was detected up to 100 mg/L independent of the medium used. The results show that Ag and CuO NP toxicity may be higher in natural water than in the standard media due to differences in composition. NP environmental hazard evaluation can and should be carried out in natural water to obtain more realistic estimates on the toxicity.

1. Introduction

Compared to the most produced nanoparticles (NPs) silica and titanium, the production volumes of Ag and CuO NPs are modest. Maximum estimated global production volumes (metric tons/year) are nearly 500 for Ag NPs and about 200 for CuO NP (Cu and CuO), while TiO_2 maximum production is 88 000 (Keller et al., 2013). However, Ag and CuO NP usage in e.g. textiles (Ag), coatings (Ag and CuO), cosmetics (Ag) and pest control (Ag and CuO) entails elevated release potential into waterbodies (Keller et al., 2013; Nowack et al., 2012). Ag and CuO NPs are the most harmful NPs to freshwater biota (Bondarenko et al., 2013a; Juganson et al., 2015) mainly due to the shed Ag- and Cu-ions (Angel et al., 2013; Heinlaan et al., 2008; Jemec et al., 2016; Newton et al., 2013; Notter et al., 2014; Sakamoto et al., 2015). However, it has been suggested that the environmental hazard of ion-releasing metal NPs cannot be directly extrapolated from that of the free metal ion (Blinova et al., 2013). NPs do not dissolve entirely (Dobias and Bernier-Latmani, 2013; Loza et al., 2014) but continue to serve as a source of metal ions. Due to the complexity of natural media as speciation and toxicity determiner

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(Wood et al., 2011), studies in natural waters are suggested (Gunsolus et al., 2015; Park et al., 2014). In freshwater, toxicity mitigation for Ag (Cupi et al., 2015; Gao et al., 2009) and CuO NPs (Blinova et al., 2010; Kramer et al., 2004) is, similarly to Ag- (Karen et al., 1999) and Cu-ions (Park et al., 2009), often ascribed to the presence of organic ligands. Nevertheless, the physico-chemical properties of the NPs themselves are of equal importance in determining the fate and thus subsequent adverse effects of NPs to the aquatic biota (Angel et al., 2013; Blinova et al., 2013).

According to the NanoE-Tox database (on 224 nano-ecotoxicological papers) (Juganson et al., 2015), just 15% of engineered NP toxicity studies have been performed in natural water and in 5% of the studies, natural organic matter has been used as a natural dispersant/coating of the NPs or with an aim to study its influence on NPs' biological effects. This necessitates studies on NP behaviour and toxicity in the natural aquatic environment as under environmentally relevant conditions, acute toxicity of metal formulations may be significantly different compared to the standardized (laboratory) conditions (Handy et al., 2012). Some of the pitfalls in NP toxicity testing include agglomeration processes leading to larger particles, transformation processes such as dissolution with the release of toxic metal ions, interactions of NPs with the medium components and unstable concentrations over experimental time (Handy et al., 2012).

EU FP7 NanoValid project ("Development of reference methods for hazard identification, risk assessment and LCA of engineered nanomaterials"; www.nanovalid.eu) aimed at evaluating the suitability of the existing standard methods and proposing new methods for NP risk assessment. In order to assess NP exposure and hazard under real-life scenarios, several case studies were performed within the project, the current of which dealt with the assessment of NP ecotoxicity under realistic conditions.

Based on the results of an (eco)toxicity profiling battery (consisting of 15 test organisms/cells) (Bondarenko et al., 2016) but also on literature-based nanomaterial hazard evaluation suggestions (Bundschuh et al., 2016; Garner and Keller, 2014; IHCP/2011/I/05/27/OC), Ag and CuO NPs were chosen as model NPs for this inter-laboratory environmental case study using natural freshwater as the test medium in comparison to the standard test media. For that, two different natural waters from two sampling campaigns from Swiss Lake Greifen and Lake Lucerne were used and NP properties in these waters were thoroughly characterized. Aquatic crustacean *D. magna* immobilization assay (OECD202) and zebrafish *D. rerio* embryo assay (OECD236) were chosen for evaluating the hazard of Ag and CuO NPs to freshwater species. Since Ag has been shown to accumulate in sediments and associate with periphyton (Furtado et al., 2015), toxicity testing with the organisms that can ingest particles and also browse sediments for food (*D. magna*) but also that are in direct contact with the sediments upon hatching (*D. rerio*) is highly relevant. The toxicity testing methods, selected for this study, are both standardized at the OECD level for soluble chemicals and, depending on the production volume of the chemical substance (incl. nanomaterials), required for its ecotoxicity evaluation by the European Union chemical regulation REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals). *D. magna* acute immobilization assay has also been specifically proposed for toxicological screening of manufactured nanoparticles (ISO/TR 16197:2014).

The aim of this study was to assess the reference methods for nanomaterial toxicity evaluation, selected in the framework of the NanoValid project, using natural waters as exposure media. Specific aims were i) to evaluate NP transformations and toxicity in natural waters compared to the standard test media and ii) to compare how the results between the participating laboratories deviate. To our knowledge, this is the first international interlaboratory nanoparticle environmental hazard evaluation using natural freshwater as the test medium.

2. Materials and methods

2.1. Participating laboratories

The partners of the EU FP7 project NanoValid, participating in this study, were Eawag (Swiss Federal Institute of Aquatic Science and Technology, Switzerland), NICPB (National Institute of Chemical Physics and Biophysics, Estonia), UFZ (Helmholtz Centre for Environmental Research, Germany) and UoA (Ahmedabad University, India).

2.2. Nanoparticles and sample preparation

The two types of nanoparticles, used in the current study, were provided by EU FP7 NanoValid project partners. Silver nanoparticles (Ag NPs) (Ag NNV 003) were supplied by Colorobia S.p.A. (Firenze, Italy; http://www.colorobbia.com) in the form of aqueous suspension in distilled water, containing polyvinylpyrrolidone (PVP) as the stabilizer (the exact concentration could not be revealed by the supplier). Nominal Ag concentration in the suspension was 40 g/L. The primary particle size of Ag NPs was 21 nm. CuO nanoparticles (CuO NNV-011, provider Intrinsiq Materials, nanoscaled powder with primary particle size of 22–25 nm) were in powder form. The initial (above-mentioned) NP characterization data are from the providers but the same type of Ag NPs have been previously characterized and described in Bondarenko et al., 2016; Böhme et al., 2015, Jemec et al., 2016, Zou et al., 2015 and CuO NPs in Bondarenko et al., 2016 (see SI, Table S1). As ionic controls, analytical grade metal salts $AgNO_3$ and $CuSO_4 \cdot 5H_2O$ were used. CuO NP and all the metal salt working stocks were prepared in ultrapure MilliQ water (>18.2 Ω, Merck Millipore, Germany). For chemical stock and dilution preparation and the tested concentrations, see Table 1.

2.3. Natural water sampling and analysis

Natural water was taken from Lake Greifen, a eutrophic lake and from Lake Lucerne, an oligotrophic lake, both located in the Swiss plateau region of Switzerland (L. Greifen in Maur, N 47° 20.41, E 8° 40.757; L. Lucerne in Kastanienbaum, N 47° 0.104, E 8° 20.024). The sampling was performed twice: in October 2014 and in March 2015. Water was sampled from around 2 m distance from the shore from up to 20 cm depth, using 1 L Teflon (PTFE) bucket on 1.5 m plastic (HDPE) rod. Water temperature and pH were measured on site (pH meter 330i, WTW). A total volume of about 20 L of water was collected from either lake at a time. The water was filtered (0.2 μm cellulose nitrate filters; Sartorius) in the laboratory on the day of sampling and filled into 1 L bottles (polypropylene PP, Nalgene). Major cations and anions (Ca^{2+}, Mg^{2+}, Na^{+}, K^{+}, alkalinity, Cl^{-}, SO_4^{2-}, NO_3^{-}) and ortho-phosphate (o-P) were measured by standard methods (ion chromatography for major ions, titration for alkalinity, colorimetry for o-P) in the analytical laboratory of Eawag. DOC was measured by high-temperature combustion (Shimadzu TOC-L). Trace metals (Cu, Ag, Zn, Fe, Mn) were measured by ICP-MS (Thermo Finnigan Element 2) in acidified filtered water samples. Within two days after sampling, filtered water (3–5 L) was dispatched to the participating laboratories (NICPB, UFZ, UoA) where it was then stored in the dark at 4 °C in the original PP (Nalgene) bottles and used for testing within up to 8 weeks. pH of the natural water was 8.1–8.3, no pH adjustment of the natural waters was performed.

2.4. Hydrodynamic size, zeta potential and sedimentation of Ag and CuO nanoparticles

Dynamic Light Scattering (DLS) and sedimentation analyses were performed by UoA. Hydrodynamic diameters (D_h), zeta potential (ζ) and polydispersity index (pdi) values of Ag and CuO nanoparticles (NPs) were measured by DLS and phase analysis light scattering (PALS), respectively using Zetasizer Nano-ZS (173° angle) equipped with 4.0 mW, 633 nm laser (Model ZEN3600, Malvern instruments Ltd., Malvern, UK). For sample preparation and the analyzed concentration range, see Table 1. The samples were incubated for 1 h at 22 °C in BOD incubator before the analysis. DLS analysis was performed at 22 ± 1 °C.

Sedimentation of Ag and CuO NPs was measured by UV–Vis spectroscopy (BioTek, Synergy HT). For CuO NPs, no peaks in UV–Vis spectroscopy were observed in the range of 200–800 nm. Sedimentation of NPs was also visually observed and captured using a digital camera (Sony Cyber-Shot DSC-HX300V). For that, 1 ml of NP suspension was pipetted into disposable polystyrene cuvettes and kept at room temperature in the dark. The images were captured at 1, 2, 3, 4, 5 and 24 h.

2.5. Nanoparticle dissolution analysis

Nanoparticle dissolution was evaluated using three methods: ultrafiltration, ultracentrifugation and sensor bacteria. These methods have previously been used in the participating laboratories (Ivask et al., 2009, 2014; Odzak et al., 2014) and are applied and compared here in the artificial *vs* natural freshwater.

2.5.1. Ultrafiltration

The NP dissolution analyses using ultrafiltration were performed by Eawag. Ag NP (0.1 mg/L) and CuO NP (1 mg/L) suspensions were prepared in the lake waters (Table 1). Sub-samples for dissolution analysis were taken immediately after preparation of the suspensions (t0) and after 2, 7 and 9 days of incubation. Dissolved fraction of Ag and Cu was separated from NPs by ultrafiltration (UF) using Amicon Ultra Tubes, with a cut-off of 3 kDa and 3 mL volume as described by Odzak et al. (2015). The tubes were centrifuged for 30 min at 1880*g*. The dissolved fraction was diluted to 10 mL and acidified to 0.1 M HNO_3 (Merck, Suprapure). Total (after digestion with HNO_3) and dissolved Ag and Cu were measured by ICP-MS (Thermo Finnigan Element 2). Speciation calculations were carried out using VMinteq version 3.1 (Gustafsson, 2005).

2.5.2. Ultracentrifugation

The NP dissolution analyses using ultracentrifugation were performed by NICPB. Ag NP, CuO NP, $AgNO_3$ and $CuSO_4 \cdot 5H_2O$ samples were prepared (all at 10 mg metal/L, see Table 1) in OECD202 artificial freshwater (AFW) and in the natural waters. The metal salts were included in the analyses for evaluating the recovery of metal ions upon sample preparation. The dissolution analysis was performed as previously explained by Ivask et al. (2014) and Jemec et al. (2016) (the latter on Ag NPs of the same batch). Briefly, incubation was performed at room temperature in polypropylene tubes in the dark and analyzed at t0 h and t48 h. Immediately after preparation and after 48 h of incubation, samples were ultracentrifuged (Beckman L8-M) at 362 769*g* for 30 min (the duration of the whole cycle was 60 min) at room temperature, supernatants were collected for atomic absorption spectroscopy (AAS) analysis. According to calculations, all NPs and complexes with molecular mass above 5 kDa should settle during ultracentrifugation (Tsao et al., 2009). Dissolved Ag and Cu was determined by methods AASL, SpectraAA 220FS, AASET, SpectraAA 220Z in accredited laboratory (EVS-EN ISO/IEC 17025:2005) of Tallinn University of Technology, Estonia.

2.5.3. Nanoparticle dissolution analysis using sensor bacteria

The NP dissolution analyses, using sensor bacteria were performed by NICPB. Metal-specific sensor bacteria are genetically modified to increase bioluminescence in a dose-dependent manner in the presence of intracellular metal ions. The sensor bacteria *Escherichia coli* MC1061 (pSLcueR/pDNPcopAlux) (Ivask et al., 2009) were used for quantifying dissolved (internalized) Ag/Cu ions from Ag and CuO NPs, respectively. The sensor bacteria were maintained at 4 °C on Luria-Bertani (LB) agar medium. Before the test, bacteria were transferred to 3 mL liquid LB medium and cultivated at 30 °C overnight. The next day the culture was diluted 1:50 with fresh LB medium and grown until the exponential phase. LB medium was supplemented with 100 mg/L ampicillin and 10 mg/L tetracycline. After that, cells were centrifuged and washed twice with HMM (Heavy metal MOPS) medium (8.4 g of MOPS, 0.22 g of glycerol-2-phosphate, 3.7 g of KCl, 0.54 g of NH_4Cl, 0.06 g of $MgSO_4$, 0.162 mg of $FeCl_3$ per 1 L of MQ water; LaRossa et al., 1995), supplemented with 0.4% glucose and 0.1% cas-amino acids (acid hydrolysate of casein). Before the test, the washed culture was diluted to $OD_{600} = 0.1$ with the same medium as was used for washing. For initiation of quantification of internalized ions, 100 μL of diluted bacterial culture was added to 100 μL of sample on 96-well white microplate (polystyrene, flat bottom (Greiner Bio-One)). Fluoroscan Ascent luminometer (Thermo Labsystems, Finland) was used to measure bacterial luminescence upon incubation for 2 h at 30 °C in the dark. Bioavailable Ag and Cu ions, dissolved from the NPs, were quantified as described by Bondarenko et al. (2013a) assuming that ions from the respective soluble metal salt were 100% bioavailable to the sensor bacteria.

2.6. Metal analyses in zebrafish embryo exposure media

Metal analyses in zebrafish exposure media were performed by Eawag. Samples for metal analysis were taken at the onset of the experiments and after 96 hpf (hours post-fertilization) for each tested concentration and the negative control. For Ag NP, 0.5 mL sample was mixed with 4.5 mL ultrapure water in a 15 mL PP-plastic centrifuge cone (TPP) and acidified with 40 μL HNO_3 (Merck, Suprapure). For CuO NP, 0.1 mL sample was mixed with 9.9 mL MQ water in a 15 mL PP centrifuge cone and acidified with 80 μL HNO_3 (Merck, Suprapure). Samples were stored at 4 °C until metal analysis by ICP-MS (Thermo Finnigan Element 2).

2.7. Daphnia magna acute toxicity test

The freshwater crustacean *Daphnia magna* 48 h acute immobilization test was conducted according to OECD202 testing guidelines by NICPB and UFZ, with some differences (see Supplementary Information-SI). Briefly, neonate daphnids (<24 h old) were exposed to NPs and the respective metal salts in standard test media and in natural waters. At least 2 independent assays were conducted and 4 technical replicates were performed per concentration. Upon 48 h of incubation at 20 °C in the dark, the immobilization (mortality) of daphnids was recorded by visual observation. The daphnid was considered immobilized if it did not resume swimming within 15 s of gentle agitation. The test was considered valid if the immobilization of control daphnids did not exceed 10%. Toxicity values (EC_{50}) were calculated (see details in SI) based on the immobilization percentage.

Table 1
Chemical stock and dilution preparation for physico-chemical characterization and toxicity testing.

	Working stock				Dilutions for testing			
	partner	medium	conc*. (mg/L)	sonication	storage	medium	conc*. (mg/L)	Test
Ag NP	UoA	MQ[a]	1000	–	prepared fresh	MQ, natural water	25–100	DLS, UV–Vis
Colorobia	Eawag		–		prepared fresh	natural water	0.1	ICP-MS upon ultrafiltration
(MARINA)						OECD 236, natural water	0.00625–0.2 (2014) 0.0125–0.2 (2015)	ZFET
	NICPB	MQ[a]	5000 mg Ag/L	–	dark, rt°, <4 weeks	OECD 202, natural water	10 mg Ag/L	AAS upon ultracentrifugation
						OECD 202, natural water	0.0006–0.1 mg Ag/L	*Daphnia magna*
						HMM[d] medium	0.005–40 mg Ag/L	sensor bacteria
	UFZ	MQ[a]	10	–	prepared fresh	ADaM, natural water	0.001–0.005	*Daphnia magna*
						OECD 236, natural water	0.0025–0.08	ZFET
CuO NP	UoA		1000	–	prepared fresh	MQ, natural water	25–100	DLS, UV–Vis
Intrinsiq	Eawag	MQ[a]	1300	bath[b] 15 min,	prepared fresh	natural water	1	ICP-MS upon ultrafiltration
Materials				60 W, 35 kHz				
						OECD 236, natural water	0.3–1.3 (2014) 0.7–2.6 (2015)	ICP-MS (ZFET conc. recovery)
						OECD 236, natural water	0.3–1.3 (2014) 0.7–2.6 (2015)	ZFET
	NICPB	MQ[a]	5000 mg Cu/L	probe[c] 4 min,	dark, rt°,	OECD 202, natural water	10 mg Cu/L	AAS upon ultracentrifugation
				40 W, 20 kHz	<4 weeks			
						OECD 202, natural water	3.9–200 mg Cu/L (natural water); 0.2–6.25 mg Cu/L	*Daphnia magna*
						HMM[d] medium	0.005–40 mg Cu/L	sensor bacteria
	UFZ	MQ[a]	500	probe[d] 5 min,	prepared fresh	ADaM, natural water	10–100 (2014) 6.25–50 (2015)	*Daphnia magna*
				200 W, 24 kHz				
						OECD 236, natural water	6.25–100	ZFET
$AgNO_3$	NICPB	MQ[a]	100 mg Ag/L	–	prepared fresh	OECD 202, natural water	10 mg Ag/L	AAS upon ultracentrifugation
J.T Baker						OECD 202, natural water	0.0003–0.005 mg Ag/L	*Daphnia magna*
						HMM[d] medium	0.005–20 mg Ag/L	sensor bacteria
$AgNO_3$	UFZ	MQ[a]	100 mg Ag/L	–	prepared fresh	ADaM, natural water	0.001–0.008 (2014) 0.0005–0.008 (2015)	*Daphnia magna*
Merck						OECD 236, natural water	0.025–0.2	ZFET
$CuSO_4{\cdot}5H_2O$	NICPB	MQ[a]	100 mg Cu/L	–	prepared fresh	OECD 202, natural water	10 mg Cu/L	AAS upon ultracentrifugation
Alfa Aesar								
						OECD 202, natural water	0.025–0.5 mg Cu/L	*Daphnia magna*
						HMM[d] medium	0.005–20 mg Cu/L	sensor bacteria
$CuSO_4{\cdot}5H_2O$	UFZ	MQ[a]	100 mg Cu/L	–	prepared fresh	ADaM, natural water	0.05–0.8	*Daphnia magna*
Merck								
						OECD 236, natural water	0.1–1.65	ZFET

UoA – University of Ahmedabad; Eawag – Swiss Federal Institute of Aquatic Science and Technology; NICPB – National Institute of Chemical Physics and Biophysics; UFZ – Helmholtz Center for Environmental Research. NP – nanoparticle; rt° – room temperature; ZFET – Zebrafish embryo test.
*All concentrations in this table are nominal and expressed per compound (unless indicated otherwise) aMQ - MilliQ water (all partners used ultrapure sterile MQ for preparation of working stock suspensions/solutions); bBANDELIN SONOREX, RK 52, BANDELIN electronic, Berlin, Germany; c450 Branson Digital Sonifier (sonicated at continuous mode without temperature adjustment immediately upon suspension preparation); dUP200S, Dr. Hielschler GmbH (sonicated on ice) dHMM medium was supplemented with 0.4% glucose and 0.1% AA.

2.8. Zebrafish embryo acute toxicity test

Zebrafish embryo toxicity tests were performed by Eawag and UFZ according to the OECD236 testing guidelines, with details as described in the SI. Briefly, zebrafish (*Danio rerio*) embryos were obtained from laboratory stock cultures maintained at 26 ± 1 °C at 14:10 h light:dark cycle. Fish were fed daily with flake food and *Artemia spec. ad libitum*. For the egg collection, spawn traps covered with a wire mesh were placed into the fish tanks on the day prior to spawning. After selection of fertilized eggs, the eggs were transferred to either lake water or reconstituted water according to ISO7346-3 in 24-well plates (Cellstar Greiner Bio-One, Frickenhausen, Germany) with one egg per well in 2 mL exposure solution. Exposure experiments with freshly prepared suspensions/solutions were started within 2 h post fertilization (hpf) and every 24 h after onset of the test, zebrafish embryo mortality was monitored up to 96 hpf.

Tests were considered valid if at least 90% of the negative control embryos survived. As lethality endpoints, coagulation, missing formation of somites, no detachment of tail and no heartbeat were used (OECD236). If one or more of these effects were observed, the embryo was considered dead. In total, 20 individuals per concentration were investigated. For EC_{50} calculation, the number of dead embryos was counted in each concentration and expressed in percent mortality. The 50% effect levels were calculated using the software GraphPad Prism 4 (GraphPad Software Inc., La Jolla, CA) with the sigmoidal dose-response (Hill-slope) equation (Eawag) and SigmaPlot12.0 (using 4-parameter Hill model) (UFZ). In Eawag, concentration-response analyses were also based on the geometric mean of measured metal concentrations at the start and at the end of exposure.

3. Results and discussion

3.1. Natural water composition

Selected parameters of the chemical composition of the natural water samples from Lake Greifen and Lake Lucerne (along with those of the standard media) are given in Table 2. The two different samplings were performed in October and March that denote fall and winter seasons in Central Europe. Natural water from the two lakes differed in their composition with respect to major ions (Ca^{2+},

Table 2
Physico-chemical parameters of the studied natural waters, sampled from Lake Greifen and Lake Lucerne on two different dates (indicated under the lake names) and of the used standard media (OECD202 and ADaM for *Daphnia magna* and ISO7346-3 for *Danio rerio* embryos).

	pH	Temp.	DOC	Ag	Cu	o-P	Alkalinity	I	Ca^{2+}	Mg^{2+}	Na^{+}	K^{+}	Cl^{-}	NO_3^{-}	SO_4^{2-}
		°C	mg/L	μg/L	μg/L	μg/L	mM	mM	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L
Lake Greifen															
2.10.2014	8.18	18	3.4	0.005	0.9	2.2	3.71	6	48.6	14.5	16.1	3.4	23.7	1.1	11
26.03.2015	8.08	5.2	3.0	<0.01	1.0	n.a.	4.25	7	63.9	14.9	19.6	3.5	29.9	1.3	12
Lake Lucerne															
2.10.2014	8.29	16.4	1.1	0.006	0.4	<1	2.09	4	37.4	3.3	<2.5	<1.0	1.6	0.4	14
27.03.2015	8.23	8.3	1.0	<0.01	0.5	n.a.	2.12	4	38.9	3.6	<2.5	<2.5	2.5	0.6	14
OECD202	7.8 ± 0.2	20 2	–	–	–	–	n.a.	15.7	80.1	12.1	17.7	3	73.6	–	48
ADaM[a]	7.6 ± 0.2	20 ± 2	–	n.a.	n.a.	n.a	n.a.	n.a	n.a	n.a	n.a	n.a	n.a	n.a	n.a
ISO7346-3	7.4	26 ± 1	–	–	–	–	n.a.	17.2	80.1	12.1	35.4	3	73.6	–	48

Temp. – Temperature *in situ* (natural waters) or during the exposure assays (standard media); DOC – dissolved organic carbon; o-P – ortho-phosphate; I – ionic strength; n.a. not available. For the standard media, calculated data are presented.
[a] ADaM (Aachener Daphnienmedium): the exact composition can not be outlined due to the use of sea salt (333 mg/L) of undefined composition.

Mg^{2+}, Na^{+} and Cl^{-}) and organic carbon (DOC) concentrations. In the waters, sampled in March 2015, the content of Ca^{2+} and Na^{+} was 20–30% higher and the Cl^{-} content about 10-fold higher in Lake Greifen than in Lake Lucerne. The natural Ag (<0.01 μg/L) and Cu (≤1 μg/L) background concentrations in the two samples were very low and did not indicate any specific pollution by these elements. The water composition from these two lakes is representative for lakes and rivers in regions with the geological background of calcium carbonate and with some anthropogenic influence, as characteristic to Central Europe.

3.2. Characteristics of Ag and CuO nanoparticles in natural water

Hydrodynamic size measurements of Ag NPs in MQ water and the natural waters showed that Ag NPs had similar particle sizes at various concentrations in all the media. Neither agglomeration nor sedimentation was observed in lake waters, as shown by low polydispersity indices (pdi) (Table 3). Interactions with natural organic matter may increase the stability of Ag NPs as previously described (Illés and Tombácz, 2006; Mylon et al., 2004; Topuz et al., 2015). DLS data on the same Ag NPs (Ag NNV-003) from Jemec et al. (2016) show that in *D. magna* OECD202 artificial freshwater (AFW), the D_h (at 50 mg/L) was 111 nm and in both ADaM medium (Aachner Daphnienmedium, used here for *D. magna* assay; Klüttgen et al., 1994) and *D. rerio* ISO medium, the D_h (at 0.5 mg/L) was about 135 nm that indicates comparable agglomerate sizes of the Ag NPs in different media. The zeta potential of Ag NPs was also similar in MQ (about −8 mV) and the natural waters (about −7 mV). Despite the low absolute values of the zeta potential, the Ag NP suspensions were also stable as indicated by the lack of (visually observable) precipitates on the bottom of the containers in MQ as in natural water (Fig. S1). This was probably due to the stabilizing (steric) effects of PVP (Topuz et al., 2015; Wang et al., 2005) and organic acids in natural water that may adsorb on the surface of Ag NPs, inducing electrosteric repulsion and increasing the stability of NPs even in the presence low levels of chlorides (Metreveli et al., 2016; Topuz et al., 2015).

Although CuO NPs showed high absolute zeta potential values (Table 3) in MQ (23 ± 0.4 mV at 50 mg/L), L. Greifen and L. Lucerne waters (−15 ± 1.3 mV and −16 ± 0.6 mV, respectively at 50 mg/L), stability (visual observation) of the CuO NPs suspensions was only observed in MQ-water (Fig. S1). The hydrodynamic size of CuO NPs in MQ was about 300 nm and in lake waters 2000–3000 nm and in agreement with the rapid settling of CuO NPs in lake waters after 1 h of incubation (Fig. S1). NICPB's DLS characterization (a separate experiment) of CuO NPs (NNV-011) in OECD202 AFW at 100 mg/L gave D_h values (AVG ± SD; (pdi)) 1497 ± 77 nm; (0.3) and a zeta potential of 4.1 mV (indicative of incipient stability of the suspension). The negative surface charge and agglomeration of CuO NPs in the lake waters was potentially due to combined effects of pH 8.0–8.3 (Sousa and Teixeira, 2013) and ionic strength (Chekli et al., 2015; Conway et al., 2015) of the media.

An increase in dispersion of Ag NPs was observed in Lake Greifen water as higher absorbance of Ag NPs was observed in UV–Vis spectrum compared to MQ and L. Lucerne water (Fig. S2). A change in the colour of Ag NP suspension was observed in L. Greifen water, which may be attributed to the interaction of Ag NPs with organic matter present in the lake water (Fig. S2). CuO NPs formed about 10-fold larger agglomerates in the natural waters compared to MQ water (Table 3). Such sedimentation was not observed for Ag NPs that included PVP as the stabilizing agent in the suspension. On the basis of the NP characterization data, it could be assumed that

Table 3
Characterization of Ag and CuO nanoparticles (upon 1 h of incubation) by Dynamic Light Scattering (Malvern Zetasizer Nano-ZS).

Nanoparticle	D_h[a] (nm)				ζ[b] (mV)			pdi[c]		
	mg NP/L	MQ	Lake Lucerne	Lake Greifen	MQ	Lake Lucerne	Lake Greifen	MQ	Lake Lucerne	Lake Greifen
Ag NP	25	126 ± 11	144 ± 21	132 ± 5,7				0.30 ± 0.04	0.31 ± 0.04	0.38 ± 0.06
	50	113 ± 13	145 ± 20	123 ± 30	−9.0 ± 1.2	−5.2 ± 0.2	−9.9 ± 0.3	0.28 ± 0.03	0.28 ± 0.03	0.36 ± 0.10
	75	121 ± 12	136 ± 31	132 ± 20				0.31 ± 0.05	0.31 ± 0.05	0.31 ± 0.02
	100	132 ± 27	120 ± 13	126 ± 17	−6.8 ± 2.3	−4.4 ± 0.4	−5.8 ± 0.5	0.28 ± 0.04	0.29 ± 0.04	0.36 ± 0.02
CuO NP	25	312 ± 36	2795 ± 1148	3358 ± 1037				0.29 ± 0.1	0.93 ± 0.07	0.64 ± 0.32
	50	292 ± 80	2605 ± 254	2332 ± 634	23 ± 0.4	−16 ± 0.6	−15 ± 1.3	0.33 ± 0.08	0.51 ± 0.14	0.73 ± 0.34
	75	247 ± 33	2295 ± 45	2037 ± 402				0.28 ± 0.07	0.29 ± 0.07	0.68 ± 0.33
	100	266 ± 35	2353 ± 521	2465 ± 282	22 ± 2.5	−15 ± 2.9	−16 ± 0.4	0.33 ± 0.07	0.12 ± 0.01	0.16 ± 0.14

Data are presented as AVG ± SD (n = 3). MQ – MilliQ water.
[a] Hydrodynamic diameter.
[b] Zeta potential.
[c] Polydispersity index.

CuO NPs, entering natural waters, would be eliminated from the water column after a few hours due to rapid sedimentation, referring to higher risk for benthic organisms. In contrast, Ag NPs were stable in lake waters (Fig. S1), posing elevated risk for pelagic organisms.

3.3. Dissolution of Ag and CuO nanoparticles

Dissolved Ag and Cu from Ag and CuO NPs were determined by ultrafiltration and ultracentrifugation at different concentrations (Table 1). It has been shown that both methods are suitable for separating the dissolved fraction from the particulate one (Jemec et al., 2016). Here, the ultrafiltration (UF) method was used with over 9-day incubation, showing a decrease in the total measured metal concentration in time: as an average, 60–70% of recovery of the nominal total concentration in case of Ag NP and 50–60% in case of CuO NP was achieved (Table 4A) possibly due to NP agglomeration, settling and sorption to the lab plastic. In L. Greifen water Ag concentration was stable during the 9-day incubation whereas in L. Lucerne it decreased in time as did CuO NP concentrations in both natural waters. High fractions of dissolved Ag^+ as related to total Ag were measured in both lake waters, whereas only smaller fractions of dissolved Cu^{2+} were observed (Table 4A).

In ultracentrifugation (UC) studies (Table 4B), the NP dissolution was measured upon 24 h incubation in MQ and upon 48 h (duration of the *D. magna* assay) incubation in OECD202 artificial freshwater (AFW) and the natural water. The total metal concentration was not measured. Including $AgNO_3$ and $CuSO_4 \cdot 5H_2O$ in the dissolution analysis for ion recovery control showed that as a result of metal ion speciation in the test media, no more than 1–40% metal ion recovery for $AgNO_3$ and 20–30% for $CuSO_4 \cdot 5H_2O$ (of nominal 10 mg metal/L) was achieved. This may refer to potential underestimation of release of metal ions from Ag and CuO NPs in the test media. On the basis of dissolution analysis, carried out in MQ water, loss of metal ions in UC due to sorption to the plastic is not significant (Table 4B), but this may not apply for NPs. Both NPs but especially Ag NPs yielded much higher dissolution values in MQ than in other media. However, that could be due to the absence of complexing compounds in the medium that may reduce the concentration of metal species in the supernatant of the ultracentrifuged sample. The Ag NP dissolution was comparable to the Ag ion recovery (the former being only higher in L. Lucerne water) whereas the CuO NP dissolution was up to 1%.

In general, UF yielded higher dissolved metal fractions in relation to total Ag and Cu than UC. The main reason for that could be the choice of nominal NP concentration as NP dissolution has been shown to be higher in case of higher solvent/NP ratio (Angel et al., 2013; Jemec et al., 2016; Kasemets et al., 2009). Furthermore, according to Minteq calculations, Ag NP dissolution appeared to be limited by the solubility of AgCl(s) in the respective media (Table 4 and Table S2) as indicated by the calculated concentrations in Table 4. For CuO, pH exerts strong influence on CuO solubility which is also limiting at pH 8.0–8.3 in the natural waters. While the Ag concentrations used in the ultrafiltration experiments were lower

Table 4
Ag and CuO nanoparticle (NP) dissolution in the test media. Dissolution was determined by ICP-MS upon ultrafiltration (panel A), by AAS upon ultracentrifugation (panel B) and by sensor bacteria (panel C). The nominal metal concentrations were 100 μg Ag NPs/L and 1000 μg CuO/L (panel A), 10 mg metal/L (panel B) or in the range of 0.005–40 mg metal/L (panel C). Calculated dissolved Ag at equilibrium with AgCl(s) and Cu at equilibrium with CuO(s) were obtained using VMinteq version 3.1. OECD202 – standard media for *Daphnia magna* assay. For panel C, the NP dissolution (%) is given in relation to the respective soluble metal salt ($AgNO_3$ for Ag and $CuSO_4 \cdot 5H_2O$ for Cu). Limit of detection is the concentration at which the bioluminescence of the bacterial sensor was induced 2-fold.

Panel A Medium	Incubation days	Total Ag (μg/L)	Ag dissolved (μg/L)	Ag dissolved (M)	Total Cu (μg/L)	Cu dissolved (μg/L)	Cu dissolved (M)
Lake Greifen	0	61	27	2.5×10^{-7}	559	5	7.8×10^{-8}
	2	72	25	2.3×10^{-7}	494	14	2.2×10^{-7}
	7	67	22	2.0×10^{-7}	315	21	3.3×10^{-7}
	9	61	17	1.6×10^{-7}	269	19	3.0×10^{-7}
Lake Lucerne	0	79	54	5.0×10^{-7}	542	4	6.3×10^{-8}
	2	76	43	4.0×10^{-7}	475	10	1.6×10^{-7}
	7	54	39	3.6×10^{-7}	371	12	1.9×10^{-7}
	9	44	32	3.0×10^{-7}	363	11	1.7×10^{-7}

Panel B	Incubation days	Ag NP Ag dissolved		$AgNO_3$ Ag "recovered"		Calculated Ag dissolved
		(μg/L)	(M)	(μg/L)	(M)	(M)
MilliQ water	1	4880	4.52×10^{-5}	10,400	9.63×10^{-5}	9.26×10^{-5}
OECD 202	2	50	4.63×10^{-7}	40	3.70×10^{-7}	5.4×10^{-7}
Lake Greifen	2	133	1.23×10^{-6}	79	7.31×10^{-7}	7.6×10^{-7}
Lake Lucerne	2	1810	1.68×10^{-5}	4140	3.83×10^{-5}	5.2×10^{-5}
	Incubation days	CuO NP Cu dissolved		$CuSO_4 \cdot 5H_2O$ Cu "recovered"		Calculated Cu dissolved
		(μg/L)	(M)	(μg/L)	(M)	(M)
MilliQ water	1	800	1.26×10^{-5}	7960	1.25×10^{-4}	4.3×10^{-6}*
OECD 202	2	100	1.57×10^{-6}	3290	3.05×10^{-5}	1.20×10^{-6}
Lake Greifen	2	60	9.45×10^{-7}	2590	2.40×10^{-5}	1.99×10^{-6}
Lake Lucerne	2	<50**	$<8 \times 10^{-7}$	2050	1.90×10^{-5}	8.90×10^{-7}

Panel C	Dissolution (%)		Limit of detection (mg metal/L)			
	Ag NP	CuO NP	Ag NP	CuO NP	$AgNO_3$	$CuSO_4 \cdot 5H_2O$
MilliQ water	80 ± 15	42 ± 10	0.01 ± 0.01	0.33 ± 0.54	0.01 ± 0.01	0.18 ± 0.31
OECD 202	61 ± 5.3	18 ± 0.3	0.02 ± 0.01	0.01 ± 0.01	0.02 ± 0.01	0.01 ± 0.01
Lake Greifen	66 ± 3.1	25 ± 18	0.02 ± 0.01	0.04 ± 0.05	0.01 ± 0.01	0.13 ± 0.17
Lake Lucerne	34 ± 16	32 ± 5.1	0.03 ± 0.0	0.05 ± 0.04	0.01 ± 0.01	0.01 ± 0.0

*Assuming pH 7.0; ** at the detection limit of the method. Sensor bacteria data (Panel C) are presented as AVG ± SD (n = 2).

than the solubility limits, those used in the ultracentrifugation, were exceeding the solubility limit for AgCl(s) (Table 4). The results obtained at lower total Ag concentrations are thus more relevant for the toxicity studies, as shown below. In a previous characterization of the studied Ag NPs (Ag NNV-003) (Jemec et al., 2016), the share of Ag^+-species in the original stock suspensions was quantified as 68% (by UF in Eawag) and 46% (by UC in NICPB) and was thus very high.

Sensor bacteria analysis (performed over a range of concentrations; Table 1), indicated higher concentrations of ions, released from both Ag and CuO NPs (Table 4C), than UF and UC. NP dissolution analysis with sensor bacterial report on the potential bioavailable fraction of the chemical that is able to enter the living cell and induce a measurable response. While comparing the sensor bacteria data with NP chemical characterization data, it must be acknowledged that metal ions, dissolving from NPs, may be of higher concentration around biological receptors than in the surrounding medium (Holden et al., 2014; Käkinen et al., 2011; Leclerc and Wilkinson, 2013). Direct contact between the NP and the bacterial cell induced a 3-fold increase in cellular internalization of Ag ions from Ag NPs compared to the NP-free Ag solution of the same concentration (Bondarenko et al., 2013b). Sensor bacteria are inducible in the sub-toxic concentration range and their limits of detection (LOD) of the sensed metals were very low (in this study about 10 μg metal/L for both Ag and Cu from the respective soluble salts and up to 5 fold higher for Ag and CuO NPs) (Table 4C). The one exception in the current study was a lower LOD (130 μg Cu/L) of copper salt in L. Greifen water. For Cu ions dissolving from CuO NPs, a strong correlation ($R^2 = 0.87$) for copper ion-selective electrode (ISE) and bacterial Cu sensors was observed by Käkinen et al. (2011), indicating that as Cu-ISE, bacterial sensors respond to free copper species. However, in addition the sensor bacteria were shown to access copper fractions that were not detected by the Cu-ISE.

3.4. Toxicity to Daphnia magna – Ag

Among the commonly used environmentally relevant test organisms, the freshwater crustacean *D. magna* has been shown to be the most sensitive organism to Ag and CuO NP and the respective soluble salt toxicity (Bondarenko et al., 2013a). In the current study, Ag NPs showed expectedly high toxicity (1–5.5 μg/L in all media) in 48 h *Daphnia magna* immobilization assay (Fig. 1A and B) and would classify "very toxic" (EC_{50} value < 1 mg/l) to *D. magna* (EC Directive 79/831, 92/93) in all the test media.

48 h EC_{50} values for Ag NPs and Ag^+ were comparably low in both the standard media and the natural waters, indicating low mitigating effects of the studied natural waters. Organic ligands and chloride may complex Ag^+ and thus decrease its toxicity (Blinova et al., 2013; Seitz et al., 2015). This complexation effect does not appear to be efficient at the relatively low DOC and chloride concentrations of these lake waters (Table 2), however at such low EC_{50} concentrations, precipitation of AgCl(s) is not expected to occur, as shown by the calculated solubility limits in Table 4.

Results of the two laboratories (NICPB and UFZ) were in good agreement (Fig. 1A and B). It is assumed that the PVP-mediated high stability of Ag NPs in all the media contributes to the low variation in the results observed between the laboratories. Acute toxicity of $AgNO_3$ (48 h EC_{50} 0.6–2.3 μg Ag/L) was comparable to that of the Ag NPs, probably because of the high fraction (46–68%) of Ag ions in the initial Ag NP stock suspension. This is in contrast to many other studies that have reported significantly higher toxicity of the soluble Ag salt than the NPs (Notter et al., 2014; Pokhrel et al., 2013; Silva et al., 2014; Ulm et al., 2015). In literature, *D. magna* 48 h EC_{50} for $AgNO_3$ in standard test medium is reported 1–1.4 μg Ag/L (comparable to the current study) and for PVP-coated Ag NPs, 15.7–121 μg Ag/L (orders of magnitude higher) (Blinova et al., 2013; Völker et al., 2013). Blinova et al. (2013) showed 3–7-fold lower $AgNO_3$ toxicity in five natural waters (5–35 mg C/L) compared to OECD202 mineral media and a strong correlation between lower $AgNO_3$ toxicity and higher DOC content ($R^2 = 0.88$). At the same time, toxicity of PVP- and casein-coated Ag NPs was lower (up to 4-fold), similar or even higher (1.2-fold for *D. magna* or up to 5-fold for *Thamnocephalus platyurus*) in these natural waters.

In agreement with a number of studies demonstrating Ag NP dissolution-dependent aquatic toxicity (Ivask et al., 2014; Jemec et al., 2016; Lee et al., 2012; Sakamoto et al., 2015), here we show by using the dissolution data from ultrafiltration (Table 4A) which were obtained at lower total Ag concentrations and sensor bacteria (Table 4C), that the Ag^+ ions dissolved from Ag NPs did, as a rule, explain the very high toxicity of the NPs. The dissolved Ag concentrations at *D. magna* Ag NP 48 h EC_{50} concentrations (ranging from the lowest to the highest according to ultrafiltration/sensor bacteria data) would be 0.3–3.6 and 0.3–0.9 μg Ag/L for L. Greifen and L. Lucerne water, respectively. In OECD202 medium, the respective value would be 1.8 μg Ag/L according to the sensor bacteria data (no data for ADaM medium). These values are comparable to the 48 h EC_{50}s for $AgNO_3$ in the corresponding media (Fig. 1A and B). It has however been discussed that the sensor bacteria data cannot be directly used for other organisms (Ivask et al., 2014) but it nevertheless illustrates the higher dissolution/bioavailability potential at the nano-bio interface.

3.5. Toxicity to Daphnia magna – Cu

NICPB and UFZ $CuSO_4 \cdot 5H_2O$ toxicity values in standard media differed 4-fold (48 h EC_{50} = 0.05 mg Cu/L and 0.01 mg Cu/L, respectively) (Fig. 1C and D) being however in the same order of magnitude as well as in agreement with literature data (median 48 h EC_{50} 0.024 mg Cu/L; no of data = 8) (review by Bondarenko et al., 2013a). CuO NP toxicity to *D. magna* differed up to 13-fold between partners, being more variable in the natural water. CuO NPs ranged from „very toxic" (EC_{50} = < 1 mg/L) to "harmful" (EC_{50} = 10–100 mg/L) to *D. magna* in standard OECD202 and ADaM test media, respectively. In natural waters, CuO NPs ranged from "toxic" (EC_{50} = 1–10 mg/L) to "harmful" (results by UFZ and NICPB, respectively). Although the used *D. magna* neonates were of different origin (from in-house culture in UFZ *vs* hatched from cysts in NICPB), their acute sensitivity to chemicals has been shown to be similar (Persoone et al., 2009) that was also demonstrated with other chemicals (Ag NPs, Ag- and Cu-salt) in this study. As shown by DLS analyses, CuO NPs agglomerated strongly in the natural media and the suspensions were unstable (Table 3 and Fig. S1). This could potentially lead to more variable experimental conditions in general, explaining the toxicity differences between the two laboratories and high variability of CuO NP toxicity results which did not occur in Cu salt exposures. CuO NP toxicity differences may have also been at least partly due to differences in sample preparation (Table 1) that has been shown to affect the release of Cu ions from the NPs (Jo et al., 2012). Within the study, each partner prepared the NP samples according to their previous personal experience as currently no harmonized method for NP sample preparation for toxicity testing exists (Kühnel and Nickel, 2014). However, despite the variable CuO NP toxicity data, similarly to Ag NPs, if the CuO NP toxicity is expressed on the basis of dissolved Cu using the ultrafiltration (Table 4A) and sensor bacteria data (Table 4C), the toxicity, can be explained by the dissolved Cu ions. The dissolved Cu concentrations at *D. magna* CuO NP 48 h EC_{50} concentrations (ranging from the lowest to the highest according to ultrafiltration/sensor bacteria data) would be 0.08–1.9 and 0.06–1.0 mg Cu/L for L.

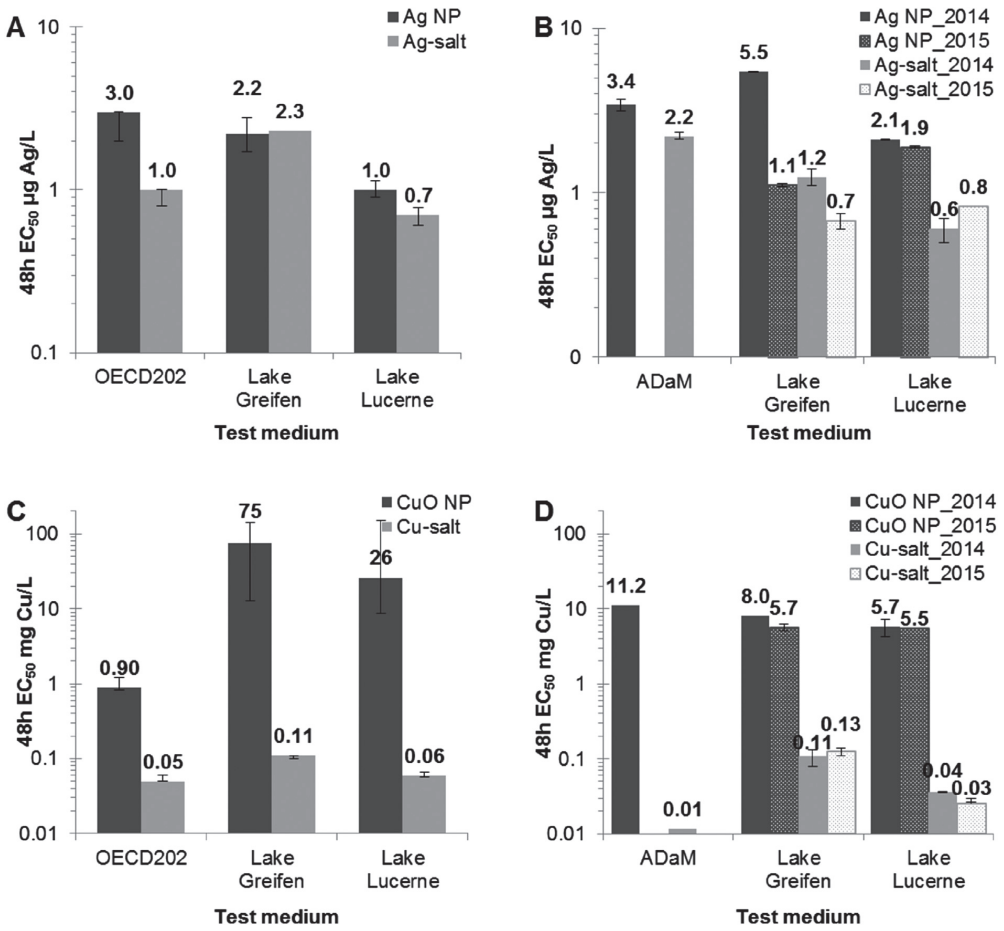

Fig. 1. Acute toxicity of Ag (panels A and B) and CuO (panels C and D) nanoparticles (NP) and the respective salts ($AgNO_3$ and $CuSO_4 \cdot 5H_2O$) to *Daphnia magna*. All the data are presented as 48 h EC_{50} values (AVG) (indicated above the bars) with 95% confidence interval (where available), ($n \geq 2$). NICPB performed the experiments solely in 2014 (panels A and C). UFZ performed the experiments in 2014 and 2015 (panels B and C). All the metal formulations were tested in standard medium (NICPB used OECD202 and UFZ used ADaM medium) and in natural water (from Lake Greifen and Lake Lucerne).

Greifen and L. Lucerne water, respectively. In OECD202 medium, the respective value would be 0.02 mg Cu/L according to the sensor bacteria data (no data for ADaM medium). These values are comparable to the respective 48 h EC_{50}s for $CuSO_4 \cdot 5H_2O$ in the corresponding media (Fig. 1C and D). It can thus be assumed that the released Cu-ions are inducing the CuO NP toxicity.

3.6. Toxicity to zebrafish embryo – Ag

Ag NPs led to clear dose-response relationships of the zebrafish embryo mortality. In general, the variations in effect levels were more obvious when comparing the two sampling dates (performed only by Eawag) than for the standard and natural test media.

The measured total Ag concentrations strongly decreased in time, with 93% ± 16% of the nominal concentration at the start of the test and 21% ± 7% after 96 h (2014), and 75% ± 7% at the start and 57% ± 17% after 96 h (2015), independently of the water tested. These losses may be attributable to sorption to the test vessel or to changes in silver speciation due to the influence of light (Kos et al., 2016; Malysheva et al., 2016; Sekine et al., 2015).

Zebrafish embryo mortality was therefore also expressed as a function of the geometric mean of measured exposure concentrations at the start and the end of the test (Fig. 2A and Table S3). Nominal concentration-based toxicity values for comparative purposes are featured in Fig. 2B. Based on measured concentration data, similar toxicity (96 h EC_{50} = 8.8–11 µg/L) was obtained at all the recorded time points in all the three media in 2014. In 2015, the obtained toxicity values were lower (96 h EC_{50} = 20–35 µg/L) in all the test media. At the concentrations causing medium to high mortality (>50%), sublethal effects, such as delay of development, heart edema and deformation of the axes or head were also observed for about 10–20% of the embryos in all the test media. At exposure concentrations ≥25 µg Ag/L, NP precipitations were observed within the chorion of non-hatched and non-coagulated embryos. Ag NP attachment to fish egg and embryo surfaces as well as malformations in embryo head and heart region have also

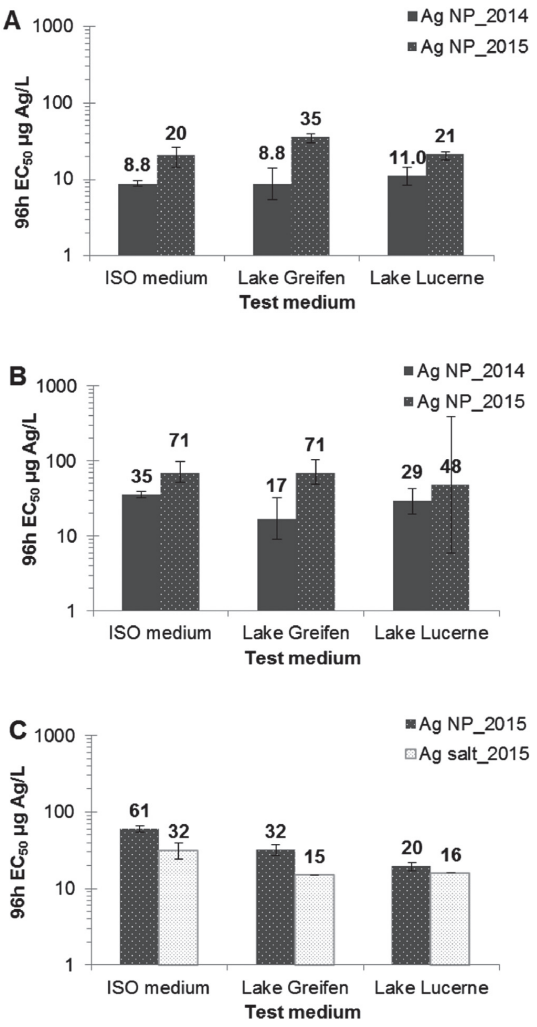

Fig. 2. Acute toxicity of Ag nanoparticles (NP) (panels A, B, C) and $AgNO_3$ (panel C) to *Danio rerio* embryos. All the data are presented as 96 h EC_{50} values (AVG) (indicated above the bars) with 95% confidence interval (where available), (n = 2). Eawag performed the experiments in 2014 and 2015 only on Ag NPs with the toxicity data expressed on the basis of the measured Ag NP concentration (panel A) and nominal Ag NP concentration (panel B). UFZ performed the experiments solely in 2015 on Ag NPs and $AgNO_3$ with all the data expressed on the basis of nominal Ag concentration (panel C). All the metal formulations were tested in standard (ISO) medium and in natural water (from Lake Greifen and Lake Lucerne).

been recorded in other studies (Laban et al., 2010; Osborne et al., 2013; Xin et al., 2015). Attachment of ion-releasing NPs may lead to higher local metal ion concentrations on the organism than in the surrounding medium – a potential scenario which the sensor bacteria results of the current study also refer to (Table 4C).

UFZ performed the zebrafish embryo assays solely in the natural waters sampled in 2015 (Fig. 2C). To evaluate the role of Ag ions in NP toxicity, $AgNO_3$ was tested in parallel to the Ag NPs. In both, the standard test media as well as in natural water, $AgNO_3$ showed higher toxicity than the NPs, which is consistent with Böhme et al. (2015). However, for both the Ag NPs and the ions, the same order of toxicity was observed, with the highest toxicities observed in lake waters compared to the standard medium: 96 h EC_{50} values for Ag NPs were 20 and 32 µg/L for L. Greifen and L. Lucerne, respectively and 61 µg/L for OECD medium (calculated on the basis of the nominal concentration) (Fig. 2C). Often, as for *D. magna*, Ag NP toxicity to *D. rerio* embryos has been linked to the released Ag ions (Böhme et al., 2015; Groh et al., 2015; Wang et al., 2015). In conclusion, a reasonable agreement between the two labs with respect to the EC_{50} for zebrafish embryo mortality was obtained. The toxicity values based on nominal concentrations (Fig. 2B and C) are in the same order of magnitude and follow the same trend, e.g. the highest particle toxicity was observed in L. Lucerne water (2015) by both labs.

3.7. Toxicity to zebrafish embryo – Cu

For CuO NPs, no significant mortality of zebrafish embryos was detected in either of the laboratories at the tested concentrations (Table 1). As in the case of Ag NPs, measured Cu concentrations were much lower than the nominal concentrations, with average recovery of nominal concentrations at the start of the test 44% ± 8% and at the end 15% ± 5% (October 2014) and 65% ± 6% at the start and 39% ± 20% at the end (April 2015), independently of the water tested. The highest observed mortality of 20% at 96 hpf was found at the highest tested concentration of 1.3 mg/L in L. Lucerne water in October 2014, but no mortality was observed in L. Lucerne water in April 2015 at the highest tested concentration of 2.6 mg/L. No mortality was detected in L. Greifen or in OECD water (even at 13 mg/L for the latter, tested once). During early exposures when the embryo was still protected within the chorion, an accumulation of CuO NPs on the chorion was observed at the highest tested concentrations (1.3 and 2.6 mg/L) as did Hua et al. (2014) and Vicario-Parés et al. (2014). After hatching, accumulation of CuO NPs on the body of the embryo, especially around the eyes and the yolk sack was found. At 13 mg/L, CuO NPs in OECD water formed an exoskeleton-like structure around the hatched embryo larvae, although no mortality was recorded. At 96 hpf, slight effects on the hatching success of embryos exposed to the highest tested concentrations (1.3 and 2.6 mg/L) were observed thus the exposure was extended to 120 hpf; however, by 120 hpf, all the embryos had hatched.

At UFZ, CuO NPs exerted no toxicity to zebrafish embryos up to a concentration of 100 mg/L. The Cu^{2+} ions exerted no toxicity in L. Greifen water. In L. Lucerne water, 100% mortality was observed at 0.42 mg Cu/L, whereas at 0.21 mg Cu/L, no mortality occurred. No sublethal effects like deformations or edemas were detected upon CuO NP exposure in none of the waters tested. In literature, adult zebrafish have been shown to be highly sensitive to copper (48 h LC_{50} of 0.25 mg Cu/L for $CuSO_4 \cdot 5H_2O$ and 48 h LC_{50} of 1.56 mg nanocopper/L (Griffitt et al., 2007)). High copper toxicity in standard medium has also been reported for zebrafish embryos (OECD236) in a NP toxicity screening study within the NanoValid project: 96 h EC_{50} of 1.6 mg Cu/L (95% CI 0.84–2.24 mg Cu/L) for CuO NNV-011 (Bondarenko et al., 2016). Hua et al. (2014) also found Cu NPs highly toxic to zebrafish embryos with 120 h LC_{50} for 25 nm Cu NPs 1.03 mg Cu NP/L (95% CI 0.85–1.25) and for $Cu(NO_3)_2$ 0.70 mg Cu/L (95% CI 0.68–0.72).

4. Conclusions

The study was designed as an interlaboratory case study to improve understanding of nanoparticle (on the example of Ag and CuO) effects in natural freshwater compared to the standard test media. We showed that depending on its composition, DOC and

chloride concentrations in particular, natural water may not sufficiently mitigate the toxicity of metal nanoparticles but on the contrary, may enhance it compared to the standard media. Stability of nanoparticles in natural waters is critically dependent on parameters such as ionic strength, DOC and pH. Strong agglomeration of nanoparticles is more challenging with respect to reproducibility of toxicity tests. Nevertheless, using natural waters as exposure media for toxicity testing yields more realistic estimates of nanoparticle biological effects in environmentally relevant conditions.

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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.2016.06.033.

References

Angel, B.M., Batley, G.E., Jarolimek, C.V., Rogers, N.J., 2013. The impact of size on the fate and toxicity of nanoparticulate silver in aquatic systems. Chemosphere 93, 359–365.

Bundschuh, M., Seitz, F., Rosenfeldt, R.R., Schulz, R., 2016. Effects of nanoparticles in fresh waters: risks, mechanisms and interactions. Freshw. Biol. http://dx.doi.org/10.1111/fwb.12701.

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

Blinova, I., Niskanen, J., Kajankari, P., Kanarbik, L., Käkinen, A., Tenhu, H., Penttinen, O.-P., Kahru, A., 2013. Toxicity of two types of silver nanoparticles to aquatic crustaceans *Daphnia magna* and *Thamnocephalus platyurus*. Environ. Sci. Pollut. Res. 20, 3456–3463.

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

Bondarenko, O., Ivask, A., Käkinen, A., Kurvet, I., Kahru, A., 2013b. Particle-cell contact enhances antibacterial activity of silver nanoparticles. PLoS One 8, e64060.

Bondarenko, O., Heinlaan, M., Sihtmäe, M., Ivask, A., Kurvet, I., Jemec, A., Mannerström, M., Heinonen, T., Rekulapelly, R., Singh, S., Pyykkö, I., Drobne, D., Kahru, A., 2016. Multilaboratory evaluation of 15 bioassays for (eco)toxicity screening and hazard ranking of engineered nanomaterials: FP7 project NANOVALID. Early Online 1–14. http://dx.doi.org/10.1080/17435390.2016.1196251.

Böhme, S., Stärk, H.-J., Reemtsma, T., Kühnel, D., 2015. Effect propagation after silver nanoparticle exposure in zebrafish (*Danio rerio*) embryos: a correlation to internal concentration and distribution patterns. Environ. Sci. Nano 2, 553–694.

Chekli, L., Zhao, Y.X., Tijing, L.D., Phuntsho, S., Donner, E., Lombi, E., Gao, B.Y., Shon, H.K., 2015. Aggregation behavior of engineered nanoparticles in natural waters: characterizing aggregate structure using on-line laser light scattering. J. Hazard. Mater. 284, 190–200.

Conway, J.R., Adeleye, A.S., Gardea-Torresdey, J., Keller, A.A., 2015. Aggregation, dissolution, and transformation of copper nanoparticles in natural waters. Environ. Sci. Technol. 49, 2749–2756.

Cupi, D., Hartmann, N.B., Baun, A., 2015. The influence of natural organic matter and aging on suspension stability in guideline toxicity testing of silver, zinc oxide, and titanium dioxide nanoparticles with *Daphnia magna*. Environ. Toxicol. Chem. 34, 497–506.

Dobias, J., Bernier-Latmani, R., 2013. Silver release from silver nanoparticles in natural waters. Environ. Sci. Technol. 47, 4140–4146.

EC Directive 79/831, 7th Amendment, 92/93.

Furtado, L.M., Norman, B.C., Xenopoulus, M.A., Frost, P.C., Metcalfe, C.D., Hintelmann, H., 2015. Environmental fate of silver nanoparticles in Boreal Lake ecosystems. Environ. Sci. Technol. 49, 8441–8450.

Gao, J.I.E., Youn, S., Hovsepyan, A., Llaneza, V.L., Wang, Y., Bitton, G., Bonzongo, J.C.J., 2009. Dispersion and toxicity of selected manufactured nanomaterials in natural river water samples: effects of water chemical composition. Environ. Sci. Technol. 43, 3322–3328.

Garner, K.L., Keller, A.A., 2014. Emerging patterns for engineered nanomaterials in the environment: a review of fate and toxicity studies. J. Nanopart. Res. 16, 1–28.

Griffitt, R.J., Weil, R., Hyndman, K.A., Denslow, N.D., Powers, K., Taylor, D., Barber, D.S., 2007. Exposure to copper nanoparticles causes gill injury and acute lethality in zebrafish (*Danio rerio*). Environ. Sci. Technol. 41, 8178–8186.

Groh, K.J., Dalkvist, T., Piccapietra, F., Behra, R., Suter, M.J.-F., Schirmer, K., 2015. Critical influence of chloride ions on silver ion-mediated acute toxicity of silver nanoparticles to zebrafish embryos. Nanotoxicology 9, 81–91.

Gunsolus, I.L., Mousavi, M.P.S., Hussein, K., Bühlmann, P., Haynes, C.L., 2015. Effects of humic and fulvic acids on silver nanoparticle stability, dissolution and toxicity. Environ. Sci. Technol. 49 (13), 8078–8086.

Gustafsson, J.P., 2005. Visual MINTEQ, 3.0. KTH, Department of Land and Water Resources Engineering, Stockholm.

Handy, R., van den Brink, N., Chappell, M., Mühling, M., Behra, R., Dušinská, M., Simpson, P., Ahtiainen, J., Jha, A., Seiter, J., Bednar, A., Kennedy, A., Fernandes, T., Riediker, M., 2012. Practical considerations for conducting ecotoxicity test methods with manufactured nanomaterials: what have we learnt so far? Ecotoxicology 21, 933–972.

Heinlaan, M., Ivask, A., Blinova, I., Dubourguier, H.-C., Kahru, A., 2008. Toxicity of nanosized and bulk ZnO, CuO and TiO_2 to bacteria *Vibrio fischeri* and crustaceans *Daphnia magna* and *Thamnocephalus platyurus*. Chemosphere 71, 1308–1316.

Holden, P.A., Klaessig, F., Turco, R.F., Priester, J.H., Rico, C.M., Avila-Arias, H., Mortimer, M., Pacpaco, K., Gardea-Torresdey, J.L., 2014. Evaluation of exposure concentrations used in assessing manufactured nanomaterial environmental hazard: are they relevant? Environ. Sci. Technol. 48, 10541–10551.

Hua, J., Vijver, M.G., Ahmad, F., Richardson, M.K., Peijnenburg, W.J.G.M., 2014. Toxicity of different-sized copper nano- and submicron particles and their shed copper ions to zebrafish embryos. Environ. Toxicol. Chem. 33, 1774–1782.

IHCP/2011/l/05/27/OC, 2013. European Commission, Final Report, Examination and Assessment of Consequences for Industry, Consumers, Human Health and the Environment of Possible Options for Changing the REACH Requirements for Nanomaterials.

ISO, 1996. International Standards. Water Quality — Determination of the Acute Lethal Toxicity of Substances to a Freshwater Fish [Brachydanio Rerio Hamilton-Buchanan (Teleostei, Cyprinidae)]. ISO 7346–3: Flow-through method. Available. http://www.iso.org.

ISO/TR 16197, 2014. Technical Report. Nanotechnologies — Compilation and Description of Toxicological Screening Methods for Manufactured Nanomaterials.

Illés, E., Tombácz, E., 2006. The effect of humic acid adsorption on pH-dependent surface charging and aggregation of magnetite nanoparticles. J. Colloid Interface Sci. 295, 115–123.

Ivask, A., Kurvet, I., Kasemets, K., Blinova, I., Aruoja, V., Suppi, S., Vija, H., Käkinen, A., Titma, T., Heinlaan, M., Visnapuu, M., Koller, D., Kisand, V., Kahru, A., 2014. Size-dependent toxicity of silver nanoparticles to bacteria, yeast, algae, crustaceans and mammalian cells *in vitro*. PLoS One 9 (7), e102108. http://dx.doi.org/10.1371/journal.pone.0102108.

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

Jemec, A., Kahru, A., Potthoff, A., Drobne, D., Heinlaan, M., Böhme, S., Geppert, M., Novak, S., Schirmer, K., Rohit, 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. Environ. Int. 87, 20–32.

Jo, H.J., Choi, J.W., Lee, S.H., Hong, S.W., 2012. Acute toxicity of Ag and CuO nanoparticle suspensions against *Daphnia magna*: the importance of their dissolved fraction varying with preparation methods. J. Hazard. Mater. 227, 301–308.

Juganson, K., Ivask, A., Blinova, I., Mortimer, M., Kahru, A., 2015. NanoE-Tox: new and in-depth database concerning ecotoxicity of nanomaterials. Beilstein J. Nanotechnol. 6, 1788–1804.

Karen, D.J., Ownby, D.R., Forsythe, B.L., Bills, T.P., La Point, T.W., Cobb, G.B., Klaine, S.J., 1999. Influence of water quality on silver toxicity to rainbow trout (*Oncorhynchus mykiss*), fathead minnows (*Pimephales promelas*), and water fleas (*Daphnia magna*). Environ. Toxicol. Chem. 18, 63–70.

Kasemets, K., Ivask, A., Dubourguier, H.-C., Kahru, A., 2009. Toxicity of nanoparticles of ZnO, CuO and TiO_2 to yeast *Saccharomyces cerevisiae*. Toxicol. in Vitro 23, 1116–1122.

Keller, A.A., McFerran, S., Lazareva, A., Suh, S., 2013. Global life cycle releases of engineered nanomaterials. J. Nanopart. Res. 15, 1692.

Klüttgen, B., Dülmer, U., Engels, M., Ratte, H.T., 1994. ADaM, an artificial freshwater for the culture of zooplankton. Water Res. 28, 743–746.

Kos, M., Kahru, A., Drobne, D., Singh, S., Kalčíková, G., Kühnel, D., Rohit, R., Gotvajn, A.Ž., Jemec, A., 2016. A case study to optimise and validate the brine shrimp *Artemia franciscana* immobilisation assay with silver nanoparticles: the role of harmonisation. Environ. Pollut. 213, 173–183.

Kramer, K.J.M., Jak, R.G., van Hattum, B., Hooftman, R.N., Zwolsman, J.J.G., 2004.

Copper toxicity in relation to surface water-dissolved organic matter: biological effects to *Daphnia magna*. Environ. Toxicol. Chem. 23, 2971–2980.
Käkinen, A., Bondarenko, O., Ivask, A., Kahru, A., 2011. The Effect of composition of different ecotoxicological test media on free and bioavailable copper from $CuSO_4$ and CuO nanoparticles: comparative evidence from a Cu-selective electrode and a Cu-biosensor. Sensors 11, 10502–10521.
Kühnel, D., Nickel, C., 2014. The OECD expert meeting on ecotoxicology and environmental fate – towards the development of improved OECD guidelines for the testing of nanomaterials. Sci. Total Environ. 472, 347–353.
Laban, G., Nies, L.F., Turco, R.F., Bickham, J.W., Sepúlveda, M.S., 2010. The effects of silver nanoparticles on fathead minnow (*Pimephales promelas*) embryos. Ecotoxicology 19, 185–195.
LaRossa, R.A., Smulski, D.R., Van Dyk, T.K., 1995. Interaction of lead nitrate and cadmium with *Echerichia coli* K-12 and *Salmonella typhimurium* global regulatory mutants. J. Ind. Microbiol. 14, 252–258.
Leclerc, S., Wilkinson, K.J., 2013. Bioaccumulation of nanosilver by *Chlamydomonas reinhardtii* – nanoparticle or the free ion? Environ. Sci. Technol. 48, 358–364.
Lee, Y.J., Kim, J., Oh, J., Bae, S., Lee, S., Hong, I.S., Kim, S.H., 2012. Ion-release kinetics and ecotoxicity effects of silver nanoparticles. Environ. Toxicol. Chem. 31, 155–159.
Loza, K., Diendorf, J., Sengstock, C., Ruiz-Gonzalez, L., Gonzalez-Calbet, J.M., Vallet-Regi, M., Köller, M., Epple, M., 2014. The dissolution and biological effects of silver nanoparticles in biological media. J. Mater. Chem. B 2, 1634–1643.
Malysheva, A., Ivask, A., Hager, C., Brunetti, G., Marzouk, E.R., Lombi, E., Voelcker, N.H., 2016. Sorption of silver nanoparticles to laboratory plastic during (eco)toxicological testing. Nanotoxicology 10, 385–390.
Metreveli, G., Frombold, B., Seitz, F., Grun, A., Philippe, A., Rosenfeldt, R.R., Bundschuh, M., Schulz, R., Manz, W., Schaumann, G.E., 2016. Impact of chemical composition of ecotoxicological test media on the stability and aggregation status of silver nanoparticles. Environ. Sci. Nano 3, 418–433.
Mylon, S.E., Chen, K.L., Elimelech, M., 2004. Influence of natural organic matter and ionic composition on the kinetics and structure of hematite colloid aggregation: implications to iron depletion in estuaries. Langmuir 20, 9000–9006.
Newton, K.M., Puppala, H.L., Kitchens, C.L., Colvin, V.L., Klaine, S.J., 2013. Silver nanoparticle toxicity to *Daphnia magna* is a function of dissolved silver concentration. Environ. Toxicol. Chem. 32, 2356–2364.
Notter, D.A., Mitrano, D.M., Nowack, B., 2014. Are nanosized or dissolved metals more toxic in the environment? A meta-analysis. Environ. Toxicol. Chem. 33, 2733–2739.
Nowack, B., Ranville, J.F., Diamond, S., Gallego-Urrea, J.A., Metcalfe, C., Rose, J., Horne, N., Koelmans, A.A., Klaine, S.J., 2012. Potential scenarios for nanomaterial release and subsequent alteration in the environment. Environ. Toxicol. Chem. 31, 50–59.
Odzak, N., Kistler, D., Behra, R., Sigg, L., 2014. Dissolution of metal and metal oxide nanoparticles in aqueous media. Environ. Pollut. 191, 132–138.
Odzak, N., Kistler, D., Behra, R., Sigg, L., 2015. Dissolution of metal and metal oxide nanoparticles under natural freshwater conditions. Environ. Chem. 12, 138–148.
OECD., 2004. OECD Guidelines for the Testing of Chemicals. Test No 202 *Daphnia sp.* Acute Immobilisation Test OECD, Paris.
OECD., 2013. OECD Guidelines for the Testing of Chemicals. Test No 236 Fish Embryo Acute Toxicity (FET) Test.
Osborne, O.J., Johnston, B.D., Moger, J., Balousha, M., Lead, J.R., Kudoh, T., Tyler, C.R., 2013. Effects of particle size and coating on nanoscale Ag and TiO_2 exposure in zebrafish (*Danio rerio*) embryos. Nanotoxicology 7, 1315–1324.
Park, E.J., Jo, H.J., Jung, J., 2009. Combined effects of pH, hardness and dissolved organic carbon on acute metal toxicity to *Daphnia magna*. J. Ind. Eng. Chem. 15, 82–85.
Park, S., Woodhall, J., Ma, G., Veinot, J.G.C., Cresser, M.S., Boxall, A.B.A., 2014. Regulatory ecotoxicity testing of engineered nanoparticles: are the results relevant to the natural environment? Nanotoxicology 8, 583–592.
Persoone, G., Baudo, R., Cotman, M., Blaise, C., Thompson, K.C., Moreira-Santos, M., Vollat, B., Törökne, A., Han, T., 2009. Review on the acute *Daphnia magna* toxicity test–evaluation of the sensitivity and the precision of assays performed with organisms from laboratory cultures or hatched from dormant eggs. Knowl. Manag. Aquat. Ecosyst. 393, 1.
Pokhrel, L.R., Dubey, B., Scheuerman, P.R., 2013. Impacts of select organic Ligands on the colloidal stability, dissolution dynamics, and toxicity of silver nanoparticles. Environ. Sci. Technol. 47, 12877–12885.
Sakamoto, M., Ha, J.Y., Yoneshima, S., Kataoka, C., Tatsuta, H., Kashiwada, S., 2015. Free silver ion as the main cause of acute and chronic toxicity of silver nanoparticles to cladocerans. Arch. Environ. Contam. Toxicol. 68, 500–509.
Seitz, F., Rosenfeldt, R.R., Storm, K., Metreveli, G., Schaumann, G.E., Schulz, R., Bundschuh, M., 2015. Effects of silver nanoparticle properties, media pH and dissolved organic matter on toxicity to *Daphnia magna*. Ecotoxicol. Environ. Saf. 111, 263–270.
Sekine, R., Khurana, K., Vasilev, K., Lombi, E., Donner, E., 2015. Quantifying the adsorption of ionic silver and functionalized nanoparticles during ecotoxicity testing: test container effects and recommendations. Nanotoxicology 9, 1005–1012.
Silva, T., Pokhrel, L.R., Dubey, B., Tolaymat, T.M., Maier, K.J., Liu, X., 2014. Particle size, surface charge and concentration dependent ecotoxicity of three organo-coated silver nanoparticles: comparison between general linear model-predicted and observed toxicity. Sci. Total Environ. 468, 968–976.
Sousa, V.S., Teixeira, M.R., 2013. Aggregation kinetics and surface charge of CuO nanoparticles: the influence of pH, ionic strength and humic acids. Environ. Chem. 10, 313–322.
Topuz, E., Traber, J., Sigg, L., Talinli, I., 2015. Agglomeration of Ag and TiO_2 nanoparticles in surface and wastewater: role of calcium ions and of organic carbon fractions. Environ. Pollut. 204, 313–323.
Tsao, T.M., Wang, M.K., Huang, P.M., 2009. Automated ultrafiltration device for efficient collection of environmental nanoparticles from aqueous suspensions. Soil Sci. Soc. Am. J. 73, 1808–1816.
Ulm, L., Krivohlavek, A., Jurašin, D., Ljubojević, M., Šinko, G., Crnković, T., Žuntar, I., Šikić, S., Vinković-Vrček, I., 2015. Response of biochemical biomarkers in the aquatic crustacean *Daphnia magna* exposed to silver nanoparticles. Environ. Sci. Pollut. Res. 22, 19990–19999.
Vicario-Parés, U., Castañaga, L., Lacave, J.M., Oron, M., Reip, P., Berhanu, D., Valsami-Jones, E., Cajaraville, M.P., Orbea, A., 2014. Comparative toxicity of metal oxide nanoparticles (CuO, ZnO and TiO_2) to developing zebrafish embryos. J. Nanopart. Res. 16, 2550.
Völker, C., Boedicker, C., Daubenthaler, J., Oetken, M., Oehlmann, J., 2013. Comparative toxicity assessment of nanosilver on Three *Daphnia* Species in acute, chronic and multi-generation experiments. PLoS One 8, e75026.
Wang, H., Qiao, X., Chen, J., Wang, X., Ding, S., 2005. Mechanisms of PVP in the preparation of silver nanoparticles. Mater. Chem. Phys. 94, 449–453.
Wang, Z., Quik, J.T.K., Song, L., Van Den Brandhof, E.-J., Wouterse, M., Peijnenburg, W.J.G.M., 2015. Humic substances alleviate the aquatic toxicity of polyvinylpyrrolidone coated silver nanoparticles to organisms of different trophic levels. Environ. Toxicol. Chem. 34, 1239–1245.
Wood, C.M., Al-Reasi, H.A., Smith, D.S., 2011. The two faces of DOC. Aquat. Toxicol. 105, 3–8.
Xin, Q., Rotchell, J.M., Cheng, J., Yi, J., Zhang, Q., 2015. Silver nanoparticles affect the neural development of zebrafish embryos. J. Appl. Toxicol. 235, 1481–1492.
Zou, J., Hannula, M., Misra, S., Feng, H., Labrador, R.H., Aula, A.S., Hyttinen, J., Pyykkö, I., 2015. Micro CT visualization of silver nanoparticles in the middle and inner ear of rat and transportation pathway after transtympanic injection. J. Nanobiotechnol. 13 (1), 1–9.

Supplementary Information for

Natural water as the test medium for Ag and CuO nanoparticle hazard evaluation: an interlaboratory case study

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Daphnia magna toxicity test

Daphnia magna 48 h acute immobilization test was conducted according to OECD202 (OECD 2004) by NICPB and UFZ, with some modifications. NICPB performed the assay as previously described by Ivask et al. (2014). Neonates used for the toxicity tests were hatched from ephippia (MicroBio Tests, Inc., Mariakerke-Gent, Belgium) that were incubated for up to 96 h at 20°C under continuous illumination of 6000 lux. Prior to initiation of the exposure, the neonates were “pre-fed” with microalgae *Pseudokirchneriella subcapitata* during two hours. Neonates were transferred into the samples *via* clean OECD202 medium. Testing was conducted on 30-well polycarbonate test plates (MicroBio Tests, Inc., Mariakerke-Gent, Belgium) with 5 daphnids per 10 ml sample. OECD 202 artificial freshwater (AFW) (mg per L of DI water: 294 $CaCl_2 \cdot 2H_2O$, 123.25 $MgSO_4 \cdot 7H_2O$, 64.75 $NaHCO_3$, 5.75 KCl; pH 7.8 ± 0.2) was used as the standard test medium in parallel to the natural waters. Toxicity values with 95 % confidence intervals were calculated by log-normal model in MS Excel macro Regtox (Vindimian 2005).

UFZ obtained the neonates for 48 h toxicity tests from in-house daphnid culture where crustaceans were cultivated under controlled conditions of 20 °C at a 16:8 h light:dark cycle. Daphnids were fed three times a week with algae and yeast flakes. Artificial ADaM medium

(Aachener Daphnien Medium), modified after Klüttgen et al. (1994) was used in parallel to the natural waters. ADaM (pH 7.6 ± 0.2) was prepared by dissolving 333 mg/L sea salt ("Tropic Marin", Germany), 270 mg/L $CaCl_2 \cdot 2H_2O$, 55 mg/L $NaHCO_3$, and 1.4 mg/L SeO_2 in deionized water. The tests were conducted in 20 ml test tubes, with 5 neonates per vial exposed in 15 ml of sample as described by Böhme et al. (2015).

Zebrafish embryo toxicity test

Zebrafish embryo toxicity tests were also carried out in two laboratories (Eawag and UFZ) according to the OECD236 guidelines (OECD 2013). In Eawag, the first generation of fish, originally obtained from a local trader (Qualipet (a pet shop), Dietlikon, Switzerland) was used for egg production. Fish were 6–24 months old and kept in single tanks (60 L or 110 L) with a biological filter system and filled with ISO7346-3 (ISO 1996) reconstituted water (294.0 mg/L $CaCl_2 \cdot 2H_2O$, 123.2 mg/L $MgSO_4 \cdot 7H_2O$, 129.48 mg/L $NaHCO_3$, 5.75 mg/L KCl). Nitrate (< 2.5 mg/L), nitrite (≤ 0.025 mg/L) and ammonium (≤ 0.2 mg/L) were checked regularly before and after the weekly exchange of 1/3 of the water. 30–60 fish were kept per 60 or 110 L aquaria, respectively, with random sex distribution and were fed twice a day – once with live *Artemia salina* and once with commercially available flake food (Tropical, Ruhmannsfelden, Germany). The evening before fertilized eggs were needed, glass trays covered with a plastic mesh (3 mm pore size) were placed in the maintenance tanks. To stimulate spawning, green artificial plants and marbles were fixed to the mesh. Eggs were collected from the spawning trays about 1 h after onset of light and pooled into a plastic mesh (0.1 mm pore size) and carefully rinsed several times with reconstituted tank water. After transferring them into a crystallization dish they were incubated at 26°C. Right after egg collection the embryos were randomly distributed to PS-plastic petri dishes (about 40 eggs per dish) loaded with the respective exposure solutions and controls for pre-exposure to ensure even onset of the exposure. From there, fertilized eggs were chosen for the experiments. Exposures were performed within 21 days after sampling of the natural waters (stored at 4°C in PP-plastic bottles (Nalgene)). No pH adjustment of natural waters was performed.

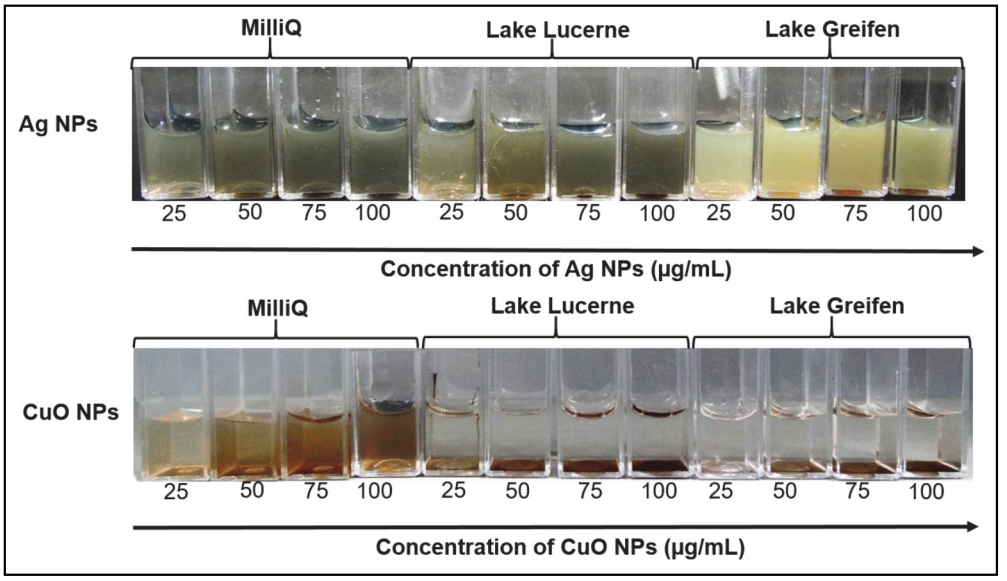

Figure S1. Sedimentation of Ag and CuO nanoparticles in MilliQ and natural water (Lake Lucerne and Lake Greifen) after 1 h of incubation.

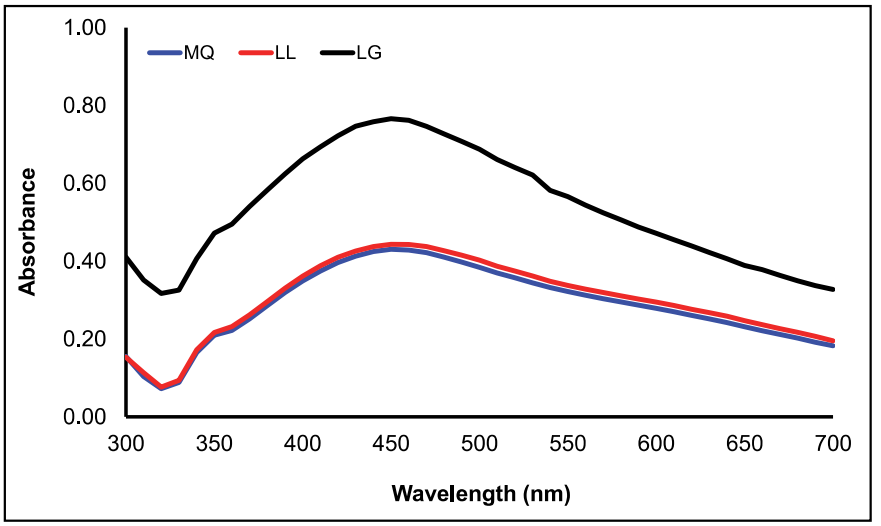

Figure S2. UV-Vis absorbance spectra of Ag nanoparticles (100 mg/L) after 1 h of incubation in MilliQ (MQ) and natural water (LL – Lake Lucerne; LG – Lake Greifen).

Table S1. Physico-chemical characterization of the studied nanoparticles (Ag NNV-003 and CuO NNV-011) in literature (the studies have also been cited in the main text).

Parameter	Medium	Conc. (mg Ag or Cu/L)	Incubation time (h)	Separation	Result	Ref.
Ag NPs NNV-003						
TEM size (nm)	dH2O	50	n.a.	-	20.4 ± 6.8	1
TEM size (nm)	dH2O	n.a.	n.a.	-	21 ± 8	4
D_h (nm)	dH2O	n.a.	n.a.	-	117 ± 24	4
D_h (nm)	dH2O	4-50	0	-	123.8 ± 12.2	1
D_h (nm)	dH2O	100	n.a.	-	132 ± 0.5	2
D_h (nm)	OECD202	50	0	-	111 ± n.a.	1
D_h (nm)	OECD202	100	n.a.	-	111 ± 0.6	2
D_h (nm)	ADaM	0.5	0	-	134 ± n.a.	1
D_h (nm)	ISO7346-3	0.5	0	-	135 ± n.a.	1
pdi	dH2O	100	n.a.	-	0.2	2
pdi	dH2O	4 and 40 (2012)	0	-	0.22	1
pdi	dH2O	4 and 40 (2014)	0	-	0.24	1
pdi	dH2O	40	0	-	0.25	1
pdi	dH2O	50	0	-	0.20	1
pdi	OECD202	50	n.a.	-	0.20	1
pdi	ADaM	0.5	n.a.	-	0.20	1
pdi	ISO7346-3	0.5	n.a.	-	0.19	1
ζ (mV)	dH2O	n.a.	n.a.	-	-20 ± 9	4
ζ (mV)	dH2O	100	n.a.	-	-10.6	2
ζ (mV)	OECD202	100	n.a.	-	-9	2
D (%)	dH2O	40000	n.a.	UF (3 kDa pores, 4000g, 20 min)	68	1
D (%)	dH2O	40000	n.a.	UC (362 769g, 30–60 min)	46	1
D (%)	dH2O	40000	n.a.	C (16 000g, 30 min)	48.3 ± 7.2	3
D (%)	dH2O	10	n.a.	UC (362 769g, 60 min)	49	1
D (%)	dH2O	10	n.a.	UF (3kDa pores, 4000g, 20 min)	51.7	1
D (%)	dH2O	10	24	UC (390 000g, 30–60 min)	49.8	2
D (%)	OECD202	0.1	0–48*	UC (362 769g, 60 min)	25	1
D (%)	OECD202	10	24	UC (390 000g, 30–60 min)	0.5	2
D (%)	ISO7346-3	0.1	n.a.	C (16 000g, 30 min)	44.3 ± 5.3	3
D (%)	ISO7346-3	0.1	0–48*	C (16 000g, 30 min)**	19	1
D (%)	ISO7346-3	10	0–48*	C (16 000g, 30 min)**	1.63	1
CuO NPs NNV-011						

D_h (nm)	dH2O	100	n.a.	-	152 ± 2	2
D_h (nm)	OECD 202	100	n.a.	-	1497 ± 77	2
pdi	dH2O	100	n.a.	-	0.2	2
ζ (mV)	dH2O	100	n.a.	-	45.4	2
ζ (mV)	OECD 202	100	n.a.	-	2	2
D (%)	dH2O	10	24	UC (390 000g, 30–60 min)	9.2	2
D (%)	OECD 202	10	24	UC (390 000g, 30–60 min)	1	2

ADaM – (Aachener Daphnienmedium) *Daphnia magna* toxicity test medium; C – centrifugation; Conc. – concentration; D- dissolution; dH_2O – deionized water; ISO7346-3 – standard zebrafish *Danio rerio* embryo toxicity test medium; n.a. – not available; OECD202 – standard *Daphnia magna* toxicity test medium; UC – ultracentrifugation UF – ultrafiltration.

Separation – sample treatment for particle-ion separation in the nanoparticle dissolution analyses. *incubation period range is given since the result did not vary over this period. **authors found the centrifugation speed insufficient for particle-ion separation.

References: 1 – Jemec et al., 2016; 2 – Bondarenko et al., 2016; 3 – Böhme et al., 2015; 4 – Zou et al., 2015.

Table S2. Calculated dissolved Ag at equilibrium with AgCl(s) and dissolved Cu at equilibrium with CuO(s) using VMinteq 3.1.

Input data	OECD 202	Lake Greifen	Lake Lucerne
Ag(total) (M)	9.26×10^{-5}	9.26×10^{-5}	9.26×10^{-5}
Cu(total) (M)	1.57×10^{-4}	1.57×10^{-4}	1.57×10^{-4}
Cl^- (M)	4.0×10^{-3}	6.6×10^{-4}	4.5×10^{-5}
HCO_3^- (M)	7.0×10^{-4}	3.7×10^{-3}	2.1×10^{-3}
pH	7.8	7.8	8.0
Ionic strength (M)	9×10^{-3}	6×10^{-3}	4×10^{-3}
Calculated dissolved species (M)			
Ag^+	5.52×10^{-8}	3.81×10^{-7}	5.21×10^{-5}
AgCl (aq)	3.62×10^{-7}	3.62×10^{-7}	3.62×10^{-7}
$AgCl_2^-$	1.25×10^{-7}	1.81×10^{-8}	1.33×10^{-10}
$AgCl_3^{-2}$	5.44×10^{-10}	1.14×10^{-11}	6.08×10^{-16}
AgOH (aq)	3.15×10^{-12}	2.18×10^{-11}	4.73×10^{-9}
Total dissolved Ag	5.43×10^{-7}	7.62×10^{-7}	5.26×10^{-5}
Cu^{2+}	1.17×10^{-7}	1.86×10^{-8}	1.02×10^{-8}
$CuOH^+$	1.73×10^{-7}	6.89×10^{-8}	5.28×10^{-8}
$Cu(OH)_2$ (aq)	1.82×10^{-8}	1.82×10^{-8}	1.82×10^{-8}
$Cu(OH)_3^-$	4.95×10^{-11}	1.24×10^{-10}	1.51×10^{-10}
$Cu(OH)_4^{-2}$	3.46×10^{-16}	2.19×10^{-15}	3.01×10^{-15}

$Cu_2(OH)_2^{+2}$	1.16×10^{-9}	1.84×10^{-10}	1.01×10^{-10}
Cu_2OH^{+3}	1.88×10^{-13}	1.19×10^{-14}	4.35×10^{-15}
$Cu_3(OH)_4^{+2}$	1.83×10^{-11}	2.89×10^{-12}	1.59×10^{-12}
$CuCO_3$ (aq)	8.84×10^{-7}	1.72×10^{-6}	7.67×10^{-7}
$Cu(CO_3)_2^{-2}$	6.99×10^{-9}	1.67×10^{-7}	4.56×10^{-8}
$CuHCO_3^{+}$	3.56×10^{-9}	2.76×10^{-9}	9.42×10^{-10}
$CuCl^{+}$	6.14×10^{-10}	1.41×10^{-11}	6.08×10^{-14}
$CuCl_2$ (aq)	5.44×10^{-13}	1.80×10^{-15}	5.73×10^{-20}
Total dissolved Cu	1.20×10^{-6}	2.00×10^{-6}	8.95×10^{-7}

Table S3. Measured total concentrations of Ag and Cu in natural waters (Lake Greifen and Lake Lucerne) and standard ISO test medium at the beginning (0 h) and at the end (96 h) of the zebrafish embryo tests performed at Eawag in 2014 and 2015.

		2014		2015			2014		2015	
	nominal Cu [µg/L]	% of nominal 0h	% of nominal 96h	% of nominal 0h	% of nominal 96h	**nominal Ag [µg/L]**	% of nominal 0h	% of nominal 96h	% of nominal 0h	% of nominal 96h
Lake	2600			58.35	12.56	200	113.56	28.68	80.60	54.35
Greifen	1300	40.85	9.86	56.72	35.93	100	108.02	31.13	82.57	55.13
	1100	35.18	10.12	62.25	45.72	50	101.48	25.36	79.70	68.14
	900	44.66	12.14	63.48	47.67	25	107.36	19.40	79.96	52.45
	700	41.33	14.21	60.13	39.24	12.5	115.04	27.60	84.00	50.71
	500	39.54	17.20			6.25	103.84	28.32		
	300	37.93	26.83							
% (AVG) of nominal		**39.92**	**15.06**	**60.18**	**36.22**		**108.22**	**26.75**	**81.37**	**56.16**
Lake	2600			66.65	25.86	200	94.90	26.51	72.00	69.57
Lucerne	1300	65.33	13.56	71.77	105.55	100	93.99	22.99	66.40	73.22
	1100	43.35	13.19	70.81	42.17	50	91.46	14.44	66.38	63.30
	900	37.66	15.29	70.39	38.02	25	91.00	15.72	75.20	94.81
	700	41.46	17.94	65.87	39.13	12.5	99.84	20.40	60.32	68.13
	500	50.96	20.20			6.25	103.68	28.64		
	300	45.03	23.33							
% (AVG) of nominal		**47.30**	**17.25**	**69.10**	**50.15**		**95.81**	**21.45**	**68.06**	**73.81**
ISO	2600			73.34	17.28	200	70.35	11.95	70.60	30.62
medium	1300	38.98	16.85	56.87	32.02	100	64.04	15.35	72.62	39.79
	1100	42.51	8.15	65.54	26.44	50	73.06	7.94	77.18	37.09
	900	43.04	9.81	74.65	31.59	25	76.20	8.32	76.92	43.15
	700	35.13	9.31	55.58	37.77	12.5	86.16	16.16	84.80	57.05
	500	40.12	12.46			6.25	71.68	20.96		
	300	58.47	19.50							
% (AVG) of nominal		**43.04**	**12.68**	**65.20**	**29.02**		**73.58**	**13.45**	**76.42**	**41.54**
	Total AVG:	***43.42***	***15.00***	***64.83***	***38.46***		***92.54***	***20.55***	***75.28***	***57.17***
	SD:	***7.78***	***5.15***	***6.41***	***21.00***		***15.77***	***7.31***	***7.16***	***16.45***

References

Bondarenko., O., Heinlaan, M., Sihtmäe, M., Ivask, A., Kurvet, I., Jemec, A., Mannerström., M., Heinonen, T., Rekulapelly, R., Singh, S., Pyykkö, I., Drobne, D., Kahru, A., 2016. Multilaboratory evaluation of 15 bioassays for (eco)toxicity screening and hazard ranking of engineered nanomaterials: FP7 project NANOVALID. Early Online: 1–14. DOI: 10.1080/17435390.2016.1196251.

Böhme, S., Stärk, H.-J., Kühnel, D., Reemtsma, T., 2015. Exploring LA-ICP-MS as a quantitative imaging technique to study nanoparticle uptake in *Daphnia magna* and zebrafish (*Danio rerio*) embryos. Analytical and Bioanalytical Chemistry 407, 5477−5485.

ISO 1996. International Standards. Water quality - Determination of the acute lethal toxicity of substances to a freshwater fish *Brachydanio rerio* Hamilton-Buchanan (*Teleostei*, *Cyprinidae*). ISO 7346-3: Flow-through method. Available at [http://www.iso.org].

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.

Jemec, A., Kahru, A., Potthoff, A., Drobne, D., Heinlaan, M., Böhme, S., Geppert, M., Novak, S., Schirmer, K., Rohit, 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.

Klüttgen, B., Dülmer, U., Engels, M., Ratte, H.T., 1994. ADaM, an artificial freshwater for the culture of zooplankton. Water Resources 28, 743-746.

OECD 2004. Guidelines for the Testing of Chemicals. Test No 202 *Daphnia* sp. Acute Immobilisation Test OECD 2004, Paris.

OECD 2013. Guidelines for the Testing of Chemicals. Test No 236 Fish Embryo Acute Toxicity (FET) Test.

Vindimian, E., 2005. MS Excel macro REGTOX_EV7.0.5.xls. http://eric.vindimian.9online.fr/.

Zou, J., Hannula, M., Misra, S., Feng, H., Labrador, R.H., Aula, A.S., Hyttinen, J. and Pyykkö, I., 2015. Micro CT visualization of silver nanoparticles in the middle and inner ear of rat and transportation pathway after transtympanic injection. Journal of Nanobiotechnology 13(1), 1-9.

Appendix 2

Publication II

Muna, M., Blinova, I., Kahru, A., Vinković Vrček, I., Pem, B., Orupõld, K., & Heinlaan, M. (2019). Combined effects of test media and dietary algae on the toxicity of CuO and ZnO nanoparticles to freshwater microcrustaceans *Daphnia magna* and *Heterocypris incongruens*: food for thought. *Nanomaterials, 9,* 23.

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Article

Combined Effects of Test Media and Dietary Algae on the Toxicity of CuO and ZnO Nanoparticles to Freshwater Microcrustaceans *Daphnia magna* and *Heterocypris incongruens*: Food for Thought

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Abstract: The chemical composition of the test medium as well as the presence of algae (microcrustaceans' food) affects the bioavailability and thus the toxicity of metal nanoparticles (NP) to freshwater microcrustaceans. This study evaluated the effect of the addition of algae (*Rapidocelis subcapitata* at 7.5×10^6 cells/mL) on the toxicity of CuO (primary size 22–25 nm) and ZnO NP (10–15 nm) to planktic *Daphnia magna* and benthic *Heterocypris incongruens* in artificial (mineral) and natural freshwater (lake water). The toxicity of ionic controls, $CuSO_4$ and $ZnSO_4$, was evaluated in parallel. When algae were added and the toxicity was tested in mineral medium, 48 h EC_{50} of CuO and ZnO NP to *D. magna* was ~2 mg metal/L and 6-day LC_{50} of *H. incongruens* was 1.1 mg metal/L for CuO and 0.36 mg metal/L for ZnO. The addition of algae to *D. magna* test medium mitigated the toxicity of CuO and ZnO NP 4–11-fold when the test was conducted in natural water but not in the artificial freshwater. The addition of algae mitigated the toxicity of $CuSO_4$ (but not $ZnSO_4$) to *D. magna* at least 3-fold, whatever the test medium. In the 6-day *H. incongruens* tests (all exposures included algae), only up to 2-fold differences in metal NP and salt toxicity between mineral and natural test media were observed. To add environmental relevance to NP hazard assessment for the freshwater ecosystem, toxicity tests could be conducted in natural water and organisms could be fed during the exposure.

Keywords: aquatic toxicology; nanomaterials; water flea; ostracod; zooplankton; feeding; natural waters

1. Introduction

Nanoparticles (NP), defined as particles with at least one dimension in the range of 1–100 nm [1], may pose a hazard to biota when released into the environment. Assessing the environmental hazards of manufactured NP has been a real challenge for the scientific community due to the unique physicochemical properties of NP. Uncertainties regarding the NP behaviour during the toxicity testing and the questionable ecological relevance of respective experimental setups (e.g., unnatural

test conditions and limited number of test species) of the standardised laboratory tests complicate the extrapolation of the laboratory test results to the real ecosystem [2,3]. As safety regulations for nanomaterials (consisting of ≥50% of NP) [1] are still under development, new knowledge on the effects of testing conditions on NP behaviour and toxicity is needed for the correct interpretation of the laboratory test results [4].

Compared to other traditional aquatic test species, microcrustaceans have been shown to be especially sensitive to metal-based NP [5]. However, the considerable variety of the toxicity values between different test species and also crustaceans species can be explained not only by species' sensitivity pattern but partly or even predominantly by the test medium [2,6]. The chemical composition of the test medium and the feeding of aquatic test organisms during the exposure are the main factors which may affect metal bioavailability. ZnO and CuO NP reach the environment mainly due to their use in cosmetics, coatings, paints and pigments [7,8]. Modelling results suggest that ZnO NP pollution in some freshwater bodies may have already reached toxic concentrations while CuO NP pollution can impose localised hazards [9]. In freshwater, CuO and ZnO NP induce toxicity mostly via bioavailable toxic metal ions [5,10–14], causing ionic and osmoregulatory disturbances [13,15]. The dissolved organic matter in natural waters plays an important role in mitigating the toxic effects of not just metal ions [16–18], but also of metallic NP [19–21]. Water hardness is another important NP toxicity mitigator, promoting NP aggregation, decreasing dissolution, and allowing outcompeting of the metal ions at the biotic uptake sites [22–24].

Some standardised (sub)chronic microcrustacean toxicity tests (ISO 14371 [25], OECD 211 [26]) require adding high concentrations of algae in the test medium in order to feed the test organisms. Acute tests such as OECD 202 [27] often do not. As eutrophication and algal blooms are becoming more and more common in freshwater lakes [28], the addition of algae in the test medium helps to mimic environmental conditions. On the other hand, the toxicity results obtained in the presence of algae may not be valid for the periods outside of algal blooms, when algal concentrations may be up to 400 times lower compared to those used in OECD 211 tests [29]. ISO 14371 freshwater sediment toxicity testing with ostracods requires using even more elevated algal concentrations (7.5×10^6 cells/mL) that exceed even the highest possible algal concentrations in nature [30,31].

This study compares the toxicity of two metal-based NP (CuO and ZnO) to planktic and benthic crustaceans. *Daphnia magna* is the most common aquatic invertebrate used for NP toxicity testing [5], while *Heterocypris incongruens* is a relatively novel alternative species to conventional sediment toxicity test organisms [32]. *H. incongruens* is especially relevant for the safety evaluation of NP, which may impose elevated risk to sediment biota by settling quickly in the waterbodies [9,33]. However, data on NP toxicity to benthic organisms are lacking [34,35]. The artificial freshwaters recommended for the standardised *D. magna* and *H. incongruens* toxicity tests have different ionic contents and do not contain dissolved organic matter. In order to add environmental relevance to the experiments, additional tests in natural waters with different organic matter contents were carried out. In addition, algae were added to some of the *D. magna* tests that normally do not require feeding the test organisms. The different combinations of the test media and the presence or absence of dietary algae in the toxicity experiments will give additional information on the environmental relevance of CuO and ZnO NP toxicity results from the laboratory tests. To our best knowledge, no studies have explored the combined effects of dietary algae and test media with different nutrient profiles or compared these effects for metal NP and respective soluble salts.

2. Materials and Methods

2.1. Chemicals

Nanoparticles used in this study were CuO NP (NNV-011; Intrinsiq Materials; powder form) and ZnO NP (NNV-003; Nanogate; powder form) obtained from the EU FP7 NanoValid project ("Developing of reference methods for hazard identification, risk assessment and LCA of engineered

nanomaterials", 2011−2015). $CuSO_4 \cdot 5H_2O$ and $ZnSO_4 \cdot 7H_2O$ (both Alfa Aesar) were used as ionic controls. CuO and ZnO NP stock suspensions (à 20 mL) were prepared at the concentration of 5 g metal/L in MQ water (MilliQ, >18 MΩ cm, Merck Millipore, Darmstadt, Germany) as has been previously described [36]. Stock suspensions were probe-sonicated for 4 min at 20 kHz (40 W) at continuous mode using 450 Ultrasonifier (Branson Ultrasonics Corporation, Danbury, CT, USA) after preparation and used for up to 4 weeks. The primary sizes of the CuO NP and ZnO NP (both uncoated) according to manufacturers' data were 22−25 nm and 10−15 nm, respectively, which was in agreement with the TEM analysis showing 24.5 nm and 13.6 nm particle sizes.

2.2. Test Media

OECD 202 artificial freshwater (AFW) [27] and US EPA moderately hard reconstituted water (MHW) [37] were used as standard exposure media for *Daphnia magna* and *Heterocypris incongruens*, respectively. In addition, water from two Estonian lakes, Lake Ülemiste and Lake Raku, was used (Table 1). Lake Ülemiste is a natural eutrophic lake while Lake Raku is an artificial sandpit lake with a similar phosphorus concentration but lower dissolved organic carbon concentration and hardness. Lake water was collected between September and March and filtered through Millipore nitrocellulose filters (pore size 0.45 µm) and stored in the dark at 4 °C. The chemical analysis of lake waters was performed by an accredited laboratory (Tallinna Vesi Laboratories). The speciation of metal salts in test media was calculated using Visual MINTEQ version 3.1 [38].

Table 1. Experimental setup.

Experiment Type	Test Organism	Test Duration	Algae *R. subcapitata* (Cells/mL)	Test Medium
Acute	*Daphnia magna*	48 h	no	AFW, two lake waters
Acute	*Daphnia magna*	48 h	7.5×10^6	AFW, two lake waters
Subchronic	*Heterocypris incongruens*	6 days	7.5×10^6	MHW, two lake waters

AFW—OECD 202 artificial freshwater; MHW—US EPA moderately hard reconstituted water.

2.3. Physico-Chemical Characterisation of Nanoparticle Suspensions

Dynamic light scattering (DLS) and electrophoretic light scattering (ELS) methodology was employed to measure the hydrodynamic diameter, zeta potential and polydispersity index of NP using ZetaSizer Nano ZS (Malvern Instruments, Malvern, UK) equipped with the 4.0 mW 633 nm laser (Model ZEN3600; 173° angle). NP samples (10 mg metal/L) were incubated for 0 h, 48 h, and 144 h at the toxicity test conditions and vortexed before the measurement.

The dissolution of NP was measured as initially described elsewhere [39]. Briefly, NP suspensions were prepared at 10 mg metal/L and incubated for 48 h (the duration of the *Daphnia magna* acute toxicity test) in OECD 202 AFW, Lake Raku water and Lake Ülemiste water with (7.5×10^6 cells/mL) and without added algae. The samples were then ultracentrifuged at 362,769 × g for 30 min (duration of the whole cycle 60 min). For metal recovery control, salt solutions were always used in parallel. Metal concentrations in the supernatants were measured using graphite furnace atomic absorption spectroscopy (GF-AAS) analysis in the accredited laboratories of the Institute of Medical Research and Occupational Health (Zagreb, Croatia) and Estonian University of Life Sciences (Tartu, Estonia).

2.4. Test Formats of Bioassays

Three different experimental setups in three different test media were used to test the potential toxicity of NP and soluble salts (Table 1). The 48 h *Daphnia magna* acute immobilisation tests were carried out in accordance with the OECD 202 testing guidelines [27]. Briefly, *D. magna* neonates were pre-fed before the test and exposed to NP or metal salts at 21 °C in the dark. After 48 h of exposure, immobilised daphnids were counted. In addition to the standard *D. magna* test format,

a modified one was applied, which included use of natural test media and/or addition of green algae *Raphidocelis subcapitata* at the concentration of 7.5×10^6 cells/mL (the concentration required in the Ostracodtoxkit [30]). Tests were repeated 2 to 8 times with 2 to 4 technical replicates each including 5 daphnids. Based on the immobilisation results, 50% effect concentrations (EC_{50}) were calculated.

Six-day subchronic *Heterocypris incongruens* toxicity testing was performed with a modified version of the OSTRACODTOXKIT F [30] (similar to ISO 14371 guidelines [25]) test procedure. Briefly, ostracod neonates (<24 h) were exposed to the NP or salts in the test media at 25 °C in the dark. Ostracods were pre-fed before the test and food was added to the test media (7.5×10^6 cells of *R. subcapitata*/mL). After 6 days, mortality was recorded. Also, growth inhibition was calculated based on the length measurements under the dissection microscope (Olympus IMT-2, CellB software, Electro Optics, Cambridge, UK) before and after the incubation. As a modification, standard sand was not added to the test to exclude the metal adsorption on sand, which can significantly mitigate metal toxicity [40]. Each test was repeated 2 to 4 times with 2 technical replicates (10 ostracods in each) of all the tested concentrations to calculate EC_{50} values.

2.5. Statistical Analysis

MS Excel macro REGTOX [41] based on non-linear regression was used to calculate EC_{50} values. The "optimal" EC_{50} values were obtained from the log-normal model. Statistically significant differences between EC_{50} values were determined based on the 95% confidence intervals provided by the REGTOX program. The statistically significant differences between metal recovery results were also determined by the absence of overlap between the 95% confidence intervals.

3. Results

3.1. Behaviour of CuO and ZnO Nanoparticles in the Test Media

3.1.1. Stability of Nanoparticle Suspensions

DLS and ELS data (Table S1) showed the low stability of both CuO and ZnO NP suspensions in all the test media (Table 2). Indeed, in lake water, the zeta potential (ζ) measured at the nominal concentration of 10 mg metal/L ranged from −15 to −19 mV, indicating that suspensions were relatively unstable. In both artificial freshwaters, suspensions were also unstable (ζ values ranged from −2 to −3.4 mV) [42].

The hydrodynamic diameters (D_h) of CuO and ZnO NP as well as the polydispersity index (pdi) increased in time in all the test media, indicating intensive aggregation of NP during the exposure (Table S1). After 48 h incubation, the D_h values for CuO and ZnO NP were on average 4 times greater in artificial freshwaters compared to lake waters. Less intensive aggregation of the studied NP in organics-containing natural water probably occurred due to the NP-stabilising effect of dissolved organic matter (DOM) [43,44]. ZnO NP were more stable in Lake Ülemiste water (with higher DOM content) but the stability of CuO NP in both lake waters was similar. This may be explained by the counteraction of DOM concentration and water hardness [23,43,45], with both parameters being higher in Lake Ülemiste water.

Along with aggregation, sedimentation of NP has been shown to increase by high ionic strength and decrease by high organics content [45,46]. Phosphates have also been shown to stabilise CuO NP [47] but at higher concentrations than present in the natural waters of this study. A higher aggregation of NP in artificial waters compared to natural waters indicates the facilitated sedimentation of NP [44] in the former (Table S1). Sedimentation of algae also occurred in CuO and ZnO NP suspensions in mineral test medium but not in organics containing test medium (Figure S1). By contrast, algae in $CuSO_4$ solution settled in all test media (slightly less in AFW) and algae in $ZnSO_4$ solution showed comparable moderate sedimentation in all the test media.

Similarly to the homoaggregation of NP, the heteroagglomeration of NP and algae potentially occurred in artificial test media [19,48,49]. High ionic strength and low DOM concentrations can facilitate homoaggregation as well as heteroagglomeration [50–52]. Compared to dispersed NP, homoaggregated NP themselves enhance algae sedimentation [53], which at the same time is dependent on both algal [54] as well as on NP concentrations [50]. Both these parameters were high in the experimental setup, but the entrapment of algae by CuO NP at lower concentrations (2 mg/L) has also been previously shown [48].

Table 2. Chemical composition of the test media. Hardness values for artificial freshwaters were calculated based on Ca^{2+} and Mg^{2+} concentrations. Water was collected twice from Lake Raku and 4 times from Lake Ülemiste. The mean (SD) of the parameters of lake waters, collected at different times, is given. Conductivity in AFW and MHW was measured; other values were calculated based on the ionic composition.

	D. magna AFW	*H. incongruens* MHW	Lake Raku	Lake Ülemiste
pH	7.8	7.6	8.3 (0.035)	8.2 (0.45)
Conductivity 25 °C (µS/cm)	640 [1]	343 [2]	283 (5.7)	399 (60)
Total organic carbon (mg/L)	0	0	5.1 (0.21)	10 (0.45)
Total hardness (mg-ekv/L)	5	1.7	2.7 (0.10)	3.9 (0.56)
Total phosphorous (mgP/L)	0	0	0.035 (0.00071)	0.030 (0.012)
Total nitrogen (mgN/L)	0	0	0.62 (0.13)	1.4 (0.40)
Cl^- (mg/L)	73	1.9	3.4 (0.28)	11 (2.1)
SO_4^{2-} (mg/L)	48	93	22 (0)	29 (4.5)
Ca^{2+} (mg/L)	80	14	44 (2.5)	66 (11)
Mg^{2+} (mg/L)	12	12	4.6 (0.10)	7.8 (0.50)
Na^+ (mg/L)	18	26	2.7 (0.021)	6.7 (1.0)
Cu^{2+} (µg/L)	0	0	1.0 (0.25)	0.64 (0.19)
Zn^{2+} (µg/L)	0	0	0.66 (0.45)	0.69 (0.36)

MHW—US EPA moderately hard reconstituted water; AFW—OECD 202 artificial freshwater; [1] value from [55]; [2] measured using ZetaSizer Nano ZS (Malvern Instruments, UK).

3.1.2. Dissolution of CuO and ZnO Nanoparticles in the Test Media

The dissolution of NP was evaluated by measuring levels of soluble metal forms released in the NP suspension (10 metal mg/L) after 48 h and 6 days of incubation in the test media (see Section 2.3). The total concentration of Zn and Cu in the supernatants, obtained after the ultracentrifugation of NP suspensions, represents the proportion of dissolved metal species (percentage of nominal concentration) in the test media (Table 3). The soluble forms of metal can be inorganic and organic complexes and free metal ions [56] (Table S2). The concentration of Cu dissolved from CuO NP was ≤2% of the nominal concentration in all the test media (Table 3), indicating a very low dissolution of CuO NP compared to ZnO NP. CuO NP dissolution is usually the highest in water characterised by the lowest pH, DOM and hardness values [47,57], but sometimes the link between these characteristics and dissolution is less straightforward [44]. The presence of algae and incubation duration did not have a significant effect on the CuO NP dissolution or Cu recovery from $CuSO_4$ solutions. The Cu recovery from $CuSO_4$ solutions was only 30−45% in all the test media (Table 3), indicating precipitation/adsorption as was discussed in our earlier work [58]. Visual MINTEQ modelling suggested the precipitation of Cu as tenorite in all the test media (Table S2), which implies that the release of copper ions may be underestimated due to speciation effects and subsequent metal recovery. Accordingly, a common term, "metal recovery", will be used to refer to both metals recovered from NP dissolution experiments as well as from metal recovery control experiments (with metal salts) in the following discussion. Speciation effects were further demonstrated by the parallel analysis of metals in MQ water with the lowest pH value, which

showed a significantly higher dissolution of CuO NP (6.9%) and Cu ion recovery (84%) from $CuSO_4$ than in any of the exposure media (Table 3). The addition of algae (7.5×10^6 cells of *R. subcapitata*/mL) increased the Cu recovery from $CuSO_4$ by 3 to 12% in all test media after 48 h incubation. However, this increase was statistically significant only in lake waters (Table 3).

Table 3. Metal recovery (%) from metal nanoparticles and salt upon ultracentrifugation after 24 h, 48 h or 6 days of incubation of test media at 10 mg metal/L without the test organisms. The addition of algae *Raphidocelis subcapitata* ($7.5 \cdot \times 10^6$ cells/mL) was used in part of the analysed samples. The mean (standard deviation) based on 1-2 experiments is presented.

	MQ	AFW		MHW			Lake Raku			Lake Ülemiste		
Incubation Time	**24 h**	**48 h**		**48 h** [1]		**6 Day** [1]	**48 h**		**6 Day** [1]	**48 h**		**6 Day** [1]
Algae	**No**	**No**	**Yes**	**No**	**Yes**	**Yes**	**No**	**Yes**	**Yes**	**No**	**Yes**	**Yes**
CuO	6.9 (1.6)	0.67 [2] (0.47)	1.5 (0.8)	0.42	1.7	1.8	0.90 [2] (0.42)	1.1 (0.53)	1.0	1.2 [2] (0.58)	1.8 (0.29)	2.0
$CuSO_4$	84 (5.6)	37 (6.2)	47 (4.9)	37	40	26	33 (0.76)	45 (4.1)	31	32 (0.81)	42 (1.3)	37
ZnO	27 (1.9)	24 (6.3)	44 (6.8)	25	55	51	21 (2.7)	57 (37)	83	23 (1.5)	54 (28)	76
$ZnSO_4$	88 (9.1)	102 (14)	94 (5.3)	97	97	81	90 (0.012)	86 (19)	97	91 (2.0)	90 (1.3)	90

MQ—Milli-Q water; AFW—OECD 202 artificial freshwater; MHW—US EPA moderately hard reconstituted water; [1] one experiment was conducted; [2] the values include previously published data [55,58].

The dissolution of ZnO NP was 21−25% depending on the test media, while Zn recovery from Zn salt was 90−102% (Table 3) in all the media, and these values were comparable to Zn recovery in MQ water. ZnO NP dissolution has previously been shown to be high at a variety of ionic strength, pH, and DOM concentration values [57]. The presence of a high concentration of humic acids increases and high medium hardness decreases the ZnO NP dissolution [59]. However, we did not observe similar dissolution behaviour. The addition of algae increased the metal recovery from ZnO NP by 20−36% after 48 h of incubation, but the increase was significant only in AFW (Table 3). According to VisualMINTEQ modelling, the precipitation of Zn as hydrozincite occurred in all the test media (Table S2).

3.2. Toxicity of Cu and Zn Compounds to Daphnia magna and Heterocypris Incongruens

3.2.1. Toxicity of Cu Compounds to *D. magna*

In *D. magna* acute toxicity tests without algae, lake waters significantly mitigated the toxicity of both CuO NP (up to 18-fold) and Cu salt (up to 4-fold) compared to AFW. The toxicity of Cu compounds (especially of CuO NP), was significantly lower in Lake Ülemiste than in Lake Raku water (Table 4). These results are in accordance with earlier published data on toxicity of other types of CuO NP and $CuSO_4$ in natural waters with different DOM concentrations [20]. The direct link between Cu recovery in different media and toxicity was not revealed. This shows that not all dissolved copper species are equally bioavailable to microcrustaceans.

The addition of algae mitigated CuO NP toxicity in lake waters (5 to >10-fold) but not in AFW. Similar to the tests without algae, NP toxicity was lower in Lake Ülemiste water compared to Lake Raku water. The toxicity of Cu salt was mitigated more in AFW (8-fold) compared to lake waters (3-fold) by the addition of alga. Earlier studies have also shown that the addition of algae (*Chlorella*) to mineral test medium decreases Cu toxicity to a lower level than in organics-containing media (without algae) [60].

Table 4. Acute and subchronic toxicity of different metal formulations to *Daphnia magna* and *Heterocypris incongruens* based on nominal concentrations. Data are presented as E(L)C_{50} (95% confidence interval), mg metal/L based on REGTOX "optimal" model. N = 2–8 (*D. magna*) and n = 2–4 (*H. incongruens*).

	***D. magna* Acute EC_{50} (48 h)**			***D. magna* acute EC_{50} (48 h) with Algae**			***H. incongruens* Subchronic LC_{50} (6 Days) with Algae**		
	AFW	**Lake Raku**	**Lake Ülemiste**	**AFW**	**Lake Raku**	**Lake Ülemiste**	**MHW**	**Lake Raku**	**Lake Ülemiste**
CuO NP	1.6 * (1.1−3.4)	6.3 (3.9−13)	28 (18−53)	2.0 (1.7−2.2)	68 (57−80)	>150	1.1 (1.1−1.6)	1.9 (1.4−3.3)	2.2 (0.77−4.2)
$CuSO_4$	0.053 * (0.047−0.059)	0.15 (0.089−0.18)	0.22 (0.20−0.25)	0.41 (0.35−0.51)	0.50 (0.35−0.70)	0.65 (0.56−0.76)	0.22 (0.20−0.24)	0.25 (0.23−0.25)	0.44 (0.42−0.49)
ZnO NP	1.9 * (1.7−2.2)	0.50 (0.46−0.58)	0.71 (0.59−0.97)	1.7 (1.6−2.2)	1.9 (1.4−2.6)	3.1 (2.1−4.5)	0.36 (0.30−0.49)	0.51 (0.38−0.62)	0.65 (0.54−0.70)
$ZnSO_4$	2.3 * (1.9−2.9)	0.59 (0.53−0.79)	0.76 (0.66−0.91)	1.5 (1.4−1.7)	0.84 (0.82−0.89)	1.3 (1.3−1.4)	0.36 (0.12−0.46)	0.43 (0.39−0.48)	0.43 (0.40−0.50)

EC_{50}—concentration immobilising 50% of test organisms; LC_{50}—concentration lethal to 50% of test organisms; NP—nanoparticles; AFW—OECD 202 artificial freshwater; MHW—US EPA moderately hard reconstituted water; * The calculation of these values included previously published data [36].

3.2.2. Toxicity of Zn Compounds to *D. magna*

The effect of lake waters on the toxicity of ZnO NP and Zn salt to *D. magna* was quite different from Cu compounds: a 3-4-fold increase in toxicity was observed compared to AFW in acute tests without algae (Table 4). Humic acids can increase ZnO NP toxicity to daphnids while fulvic acids [19] slightly mitigate Zn toxicity [61]. Dissolved organic matter also has much less affinity to Zn ions compared to Cu ions (Table S2). The water hardness, which mitigates Zn toxicity [22], was the highest in AFW, potentially explaining the higher Zn toxicity in natural water with lower hardness. Natural waters also had slightly higher pH which can increase Zn toxicity [61]. Despite the 4-fold difference in Zn recovery upon ultracentrifugation (in the absence of algae), toxicity of ZnO NP and $ZnSO_4$ to *D. magna* was comparable. This may be explainable by the enhanced dissolution of the metal NP upon contact with the living cell [18].

The presence of algae slightly but significantly reduced the toxicity of Zn compounds to *D. magna* in lake waters, but did not change or even increased the toxicity (in case of $ZnSO_4$) in AFW (Table 5). The toxicity of $ZnSO_4$ was significantly higher in Lake Raku water compared to Lake Ülemiste water in the experiments with algae, possibly due to the difference in water hardness [59].

Table 5. The effect of addition of algae on the toxicity of copper (CuO NP and $CuSO_4$) and zinc (ZnO NP and $ZnSO_4$) compounds in 48 h *D. magna* acute immobilisation assay and on the metal recovery (reflects dissolution for NP). Background colour coding is explained below the table and shows statistically significant effects.

		Copper Compounds			Zinc Compounds		
		AFW	Lake Raku	Lake Ülemiste	AFW	Lake Raku	Lake Ülemiste
Change in toxicity [1] (EC_{50} with algae/EC_{50} no algae)	NP	1.3	11	>5	0.9	3.8	4.4
	salt	7.7	3.3	3.0	0.65	1.4	1.7
Change in metal recovery [2] (no algae/with algae)	NP	0.45	0.82	0.67	0.55	0.37	0.43
	salt	0.79	0.73	0.76	1.1	1.0	1.0

increase; no effect; ≤5 fold decrease; ≥5 fold decrease

[1] calculation based on data in Table 4. [2] calculation based on data in Table 3. AFW—OECD202 artificial freshwater; NP—nanoparticles.

3.2.3. Toxicity of Cu and Zn Compounds to *H. incongruens*

Similar to tests with *D. magna*, CuO NP were significantly less toxic than $CuSO_4$ in the 6-day ostracod toxicity tests (Table 4), but there was only one case where toxicity was significantly affected by the test medium. The $CuSO_4$ was up to 2-fold less toxic in Lake Ülemiste water compared to MHW and Lake Raku water, probably due to the highest DOM content being in Lake Ülemiste water (Table 2). Sublethal (mortality <20%) concentrations of $CuSO_4$ enhanced the growth of ostracods (up to 41%) in all test media (Table S3).

As for Cu-compounds, the chemical composition of the test media had very little effect on the toxicity of Zn compounds to *H. incongruens* (Table 4). There were no statistically significant differences between the toxicity of ZnO NP and Zn salt. The toxicity of ZnO NP was significantly lower in Lake Ülemiste water compared to MHW, but no medium effect was observed for Zn salt. Sublethal concentrations of $ZnSO_4$ increased the body length of ostracods by 17% in MHW (Table S3), probably due to the absence of Zn in this water.

Despite being a less common test organism compared to *D. magna*, the data on *H. incongruens* sensitivity to metals obtained in this study were consistent with those available in the literature. The previously published LC_{50} values for $ZnSO_4$ and $CuSO_4$ were in the range from 0.7 to 12 mg Zn/L and from <0.3 to 0.9 mg Cu/L, respectively, despite the fact that reference sediment with possible toxicity mitigating effect was applied [62,63]. Surprisingly, Zn toxicity was the lowest (LC_{50} 12 mg Zn/L) in distilled water as exposure medium [63].

4. Discussion

4.1. Combined Effect of the Media and Feeding on D. magna *Toxicity Test Results*

The effect of the addition of algae on the toxicity of copper and zinc compounds using modified formats of *D. magna* acute immobilisation testing (OECD 202) are summarised in Table 5. The toxicity mitigating effect of the added algae (as seen in both metal NP exposure in lake water and $CuSO_4$ in all the test media) was anticipated, because feeding on organic compounds and the presence of extracellular polymeric substances of some algae have been shown to mitigate metal toxicity [64,65]. In addition, uncontaminated algae may help clear the gut of daphnids of metal NP [66]. The lack of effect and even the increased toxicity of the tested compounds in the presence of algae, as seen for CuO and ZnO NP in artificial freshwater and for Zn salt, was unexpected (Table 5).

The addition of algae could potentially change the toxicant exposure for the test organism. One possible explanation may be the internalisation of metals by algae, leading to foodborne metal exposure [62,66–68] especially in lake Raku water with lower ionic strength [22]. The concurrent sedimentation (or heteroagglomeration) (see 3.1.1) of NP and algae that was observed in AFW could have increased their simultaneous uptake due to daphnids turning to bottom-feeding. Almost no clear correlations were observed for toxicity and changes in the dissolution of either the metal NP or metal salt upon the addition of algae in the test medium (Table 5). As an exception, the absence of a mitigating effect of algae on ZnO NP toxicity in AFW can partly be due to the increased Zn recovery in the presence of algae (Table 5). Altogether, the effects of addition of algae cannot be considered analogous to the addition of dissolved organic matter in the test medium in case of CuO and ZnO NP in AFW.

The use of microalgae for feeding the crustaceans during NP exposure increases the environmental relevance of laboratory testing. The effect of algae concentrations on the interactions between NP and algae is yet to be determined, but high algal concentrations may lead to the heteroagglomeration of algae and NP. According to Stevenson et al. [29], using environmentally relevant algae concentrations in a chronic *Daphnia pulicaria* exposure to nano Ag significantly increased the adverse effects compared to the normal feeding rate recommended by the *D. magna* chronic toxicity test (OECD 211) guidelines.

4.2. Differences between CuO and ZnO Nanoparticle Toxicity to D. magna and H. incongruens

Compared to *D. magna* test with algae, the toxicity of Zn and Cu salts and ZnO NP to ostracod was only slightly higher in artificial freshwaters, but significantly higher (more than 30-fold) for CuO NP in lake waters (Table 4). The differences for Zn and Cu salts, ZnO NP and CuO NP in AFW may be explained by the 3-fold-longer test duration (Table 1) and the different bioavailability of metal species formed in the two different artificial test media (OECD AFW and US EPA MHW, see Table 2). For example, the toxicity of CuO and ZnO NP to *D. magna* has been shown to be higher in MHW compared to OECD AFW despite the higher dissolution in OECD AFW [69].

The negligible effect of the natural water on CuO toxicity to ostracod can be partially explained by the non-permanent nature of changes in metal bioavailability, induced by water parameters such as hardness [70,71] and the presence of humic acids [45]. For instance, the mitigative effects can last long enough to be evident in *D. magna* exposure (48 h) but not in *H. incongruens* exposure (6 days). The behaviour of the test organisms can also influence their exposure to toxicants. The planktic species *D. magna* is mostly exposed to soluble or suspended metal species, whereas benthic *H. incongruens* is more exposed to settled agglomerates of metal compounds. Agglomeration and sedimentation does not necessarily mean that metal compounds are less bioavailable to the test organisms [72]. Ostracods have been shown to be a more vulnerable organism to metal-polluted river sediment compared to water fleas despite the latter ones being more sensitive to metals in an exposure without sediment [73]. As concurrent sedimentation of NP and algae occurred in artificial freshwaters, daphnids may have turned to bottom feeding to access the settled algae. As a result, both daphnids and ostracods could have had similar exposure to the toxicants, feeding on settled NP aggregates along with settled algae.

As algae remained suspended in natural waters, daphnids could feed in the upper layers of the test vessel, avoiding potentially higher metal NP concentrations on the bottom of the test vessel.

The effect of the addition of algae in *D. magna* and *H. incongruens* test medium cannot be directly compared in this study as *H. incongruens* toxicity tests without the addition of algae were not conducted to avoid starvation of the test organisms. Based on the literature, the effect of the addition of algae on toxicity of metals in organics-free test medium seems to be similar for both daphnids and ostracods despite the differences seen in natural waters for CuO as explained in the previous paragraph. Toxicity tests carried out for 48-96 h with adult ostracods *Cypris subglobosa* and *Stenocypris major* in tap water and well water with no addition of algae (and no sediment) resulted in lower LC_{50} for Cu (0.025 to 0.055 mg Cu/L) and slightly to much higher LC_{50} for Zn (1.2 to 85 mg Zn/L) compared to the results obtained in this study, indicating the toxicity mitigating effect of algae on Cu and toxicity enhancing effects on Zn in artificial freshwater [35,74].

5. Conclusions

It is well known that the chemical composition of toxicological test media affects the bioavailability and thus the toxicity of metal nanoparticles (NP) to aquatic test organisms. However, it is poorly understood to what extent the addition of algae—food for microcrustaceans—into the test medium modulates the toxic effect. This aspect must be addressed since, in the (sub)chronic microcrustacean toxicity tests, algae are added as food by default. In this study, the combined effects of artificial versus natural test media and addition of algae (*Raphidocelis subcapitata* at 7.5×10^6 cells/mL) on the toxicity of CuO and ZnO NP to planktic *Daphnia magna* and benthic *Heterocypris incongruens* was evaluated in standardised and modified test formats. Natural freshwater and addition of dietary algae in 48 h *D. magna* exposure were used as modifications.

- Subchronic (6 day) *H. incongruens* LC_{50} for CuO NP was 1.1 mg Cu/L, and 0.22 mg Cu/L for $CuSO_4$, in US EPA mineral water. For both ZnO NP and $ZnSO_4$, the respective 6-day LC_{50} was 0.36 mg Zn/L. For comparison, upon the addition of dietary algae in mineral medium, 48 h EC_{50} of CuO and ZnO NP for *D. magna* was ~2 mg metal/L;
- Compared to standard mineral media, natural freshwater mitigated CuO NP toxicity (4–18-fold) and increased ZnO NP toxicity (3–4-fold) for *D. magna*. For Cu and Zn salts, the toxicity change followed the same pattern with 3–4-fold mitigation and an increase in natural water. In *H. incongruens* tests (all including algae), toxicity was mitigated only up to 2-fold (CuO NP and Cu salt) or remained the same (Zn compounds) in natural water;
- Upon the addition of algae to *D. magna* for 48 h in OECD mineral medium, no toxicity mitigating effect was recorded for CuO NP, possibly due to the sedimentation of algae and NP. $CuSO_4$ 48 h EC_{50}, however, decreased 8-fold. For ZnO NP and $ZnSO_4$, the added algae resulted in comparable or even increased ($ZnSO_4$) toxicity;
- Algae in natural freshwater mitigated both CuO NP (5 to >10 fold) and Cu salt (3-fold) toxicity.5 The toxicity of ZnO was also significantly reduced (4-fold) but Zn salt toxicity remained unchanged.

According to the results of the modified *D. magna* and *H. incongurens* test formats, toxicity data from standardised acute/subchronic exposures may be overestimated for Cu-compounds and underestimated for Zn-compounds for eutrophic freshwaters during algal blooms. Also, our experiments once more demonstrated that the extrapolation of toxicity values obtained using planktic test species to other groups of aquatic microcrustaceans (e.g., benthic) may lead to significant mistakes in the environmental hazard evaluation, especially in the case of metal-based NP.

Supplementary Materials: The following are available online at http://www.mdpi.com/2079-4991/9/1/23/s1, Table S1: Characterisation of CuO and ZnO nanoparticle suspensions at 0 h, 48 h, and 6 days in five different test media. Table S2: Percentage of soluble and solid fraction predicted by Visual MINTEQ simulation results. Tabel S3. Change in *H. incongruens* growth (%) at the end of the 6-day experiment at sublethal concentrations.

Figure S1: Examples of typical sedimentation of algae after 6 days of incubation with CuO and ZnO NP and respective soluble salts at 10 mg metal/L.

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References

1. European Commission. *Communication (COM(2012) 572 Final) from the Commission to the European Parliament, the Council and the European Economic and Social Committee: Second Regulatory Review on Nanomaterials*; European Commission: Brussels, Belgium, 2013.
2. Holden, P.A.; Gardea-Torresdey, J.L.; Klaessig, F.; Turco, R.F.; Mortimer, M.; Hund-Rinke, K.; Cohen Hubal, E.A.; Avery, D.; Barceló, D.; Behra, R.; et al. Considerations of Environmentally Relevant Test Conditions for Improved Evaluation of Ecological Hazards of Engineered Nanomaterials. *Environ. Sci. Technol.* **2016**, *50*, 6124–6145. [CrossRef] [PubMed]
3. Blinova, I.; Vija, H.; Lukjanova, A.; Muna, M.; Syvertsen-wiig, G.; Kahru, A. Assessment of the hazard of nine (doped) lanthanides-based ceramic oxides to four aquatic species. *Sci. Total Environ.* **2018**, *612*, 1171–1176. [CrossRef] [PubMed]
4. Garner, K.L.; Suh, S.; Lenihan, H.S.; Keller, A.A. Species sensitivity distributions for engineered nanomaterials. *Environ. Sci. Technol.* **2015**, *49*, 5753–5759. [CrossRef] [PubMed]
5. Juganson, K.; Ivask, A.; Blinova, I.; Mortimer, M.; Kahru, A. NanoE-Tox: New and in-depth database concerning ecotoxicity of nanomaterials. *Beilstein J. Nanotechnol.* **2015**, *6*, 1788–1804. [CrossRef] [PubMed]
6. Scientific Committee on Emerging and Newly Identified Health Risks. *Risk Assessment of Products of Nanotechnologies*; European Commission: Brussels, Belgium, 2009.
7. Keller, A.A.; McFerran, S.; Lazareva, A.; Suh, S. Global life cycle releases of engineered nanomaterials. *J. Nanopart. Res.* **2013**, *15*, 1692. [CrossRef]
8. European Chemicals Agency. *Literature Study on the Uses and Risks of Nanomaterials as Pigments in the European Union*; European Chemicals Agency: Helsinki, Finland, 2018.
9. Garner, K.L.; Suh, S.; Keller, A.A. Assessing the Risk of Engineered Nanomaterials in the Environment: Development and application of the nanoFate model. *Environ. Sci. Technol.* **2017**, *51*, 5541–5551. [CrossRef] [PubMed]
10. Notter, D.A.; Mitrano, D.M.; Nowack, B. Are nanosized or dissolved metals more toxic in the environment? A meta-analysis. *Environ. Toxicol. Chem.* **2014**, *33*, 2733–2739. [CrossRef] [PubMed]
11. Adam, N.; Leroux, F.; Knapen, D.; Bals, S.; Blust, R. The uptake and elimination of ZnO and CuO nanoparticles in *Daphnia magna* under chronic exposure scenarios. *Water Res.* **2015**, *68*, 249–261. [CrossRef] [PubMed]
12. Adam, N.; Leroux, F.; Knapen, D.; Bals, S.; Blust, R. The uptake of ZnO and CuO nanoparticles in the water-flea *Daphnia magna* under acute exposure scenarios. *Environ. Pollut.* **2014**, *194*, 130–137. [CrossRef]
13. Heinlaan, M.; Ivask, A.; Blinova, I.; Dubourguier, H.-C.; Kahru, A. Toxicity of nanosized and bulk ZnO, CuO and TiO_2 to bacteria *Vibrio fischeri* and crustaceans *Daphnia magna* and *Thamnocephalus platyurus*. *Chemosphere* **2008**, *71*, 1308–1316. [CrossRef]

14. Bondarenko, O.; Juganson, K.; Ivask, A.; Kasemets, K.; Mortimer, M.; Kahru, A. Toxicity of Ag, CuO and ZnO nanoparticles to selected environmentally relevant test organisms and mammalian cells in vitro: A critical review. *Arch. Toxicol.* **2013**, *87*, 1181–1200. [CrossRef] [PubMed]
15. Thurberg, F.P.; Dawson, M.A.; Collier, R.S. Effects of Copper and Cadmium on Osmoregulation and Oxygen-Consumption in 2 Species of Estuarine Crabs. *Mar. Biol.* **1973**, *23*, 171–175. [CrossRef]
16. Erickson, R.J.; Brooke, L.T.; Kahl, M.D.; Vende Venter, F.; Harting, S.L.; Markee, T.P.; Spehar, R.L. Effects of laboratory test conditions on the toxicity of silver to aquatic organisms. *Environ. Toxicol. Chem.* **1998**, *17*, 572–578. [CrossRef]
17. De Schamphelaere, K.A.; Vasconcelos, F.M.; Tack, F.M.G.; Allen, H.E.; Janssen, C.R. Effect of dissolved organic matter source on acute copper toxicity to *Daphnia magna*. *Environ. Toxicol. Chem.* **2004**, *23*, 1248–1255. [CrossRef]
18. Käkinen, A.; Bondarenko, O.; Ivask, A.; Kahru, A. The effect of composition of different ecotoxicological test media on free and bioavailable copper from $CuSO_4$ and CuO nanoparticles: Comparative evidence from a Cu-selective electrode and a Cu-biosensor. *Sensors* **2011**, *11*, 10502–10521. [CrossRef] [PubMed]
19. Akhil, K.; Sudheer Khan, S. Effect of humic acid on the toxicity of bare and capped ZnO nanoparticles on bacteria, algal and crustacean systems. *J. Photochem. Photobiol. B Biol.* **2017**, *167*, 136–149. [CrossRef] [PubMed]
20. Blinova, I.; Ivask, A.; Heinlaan, M.; Mortimer, M.; Kahru, A. Ecotoxicity of nanoparticles of CuO and ZnO in natural water. *Environ. Pollut.* **2010**, *158*, 41–47. [CrossRef]
21. Cupi, D.; Hartmann, N.B.; Baun, A. The influence of natural organic matter and aging on suspension stability in guideline toxicity testing of silver, zinc oxide, and titanium dioxide nanoparticles with *Daphnia magna*. *Environ. Toxicol. Chem.* **2015**, *34*, 497–506. [CrossRef]
22. Barata, C.; Baird, D.J.; Markich, S.J. Influence of genetic and environmental factors on the tolerance of *Daphnia magna* Straus to essential and non-essential metals. *Aquat. Toxicol.* **1998**, *42*, 115–137. [CrossRef]
23. Chen, K.L.; Elimelech, M. Influence of humic acid on the aggregation kinetics of fullerene (C60) nanoparticles in monovalent and divalent electrolyte solutions. *J. Colloid Interface Sci.* **2007**, *309*, 126–134. [CrossRef]
24. Cupi, D.; Hartmann, N.B.; Baun, A. Influence of pH and media composition on suspension stability of silver, zinc oxide, and titanium dioxide nanoparticles and immobilization of *Daphnia magna* under guideline testing conditions. *Ecotoxicol. Environ. Saf.* **2016**, *127*, 144–152. [CrossRef] [PubMed]
25. International Organization for Standardization. *Water Quality—Determination of Freshwater Sediment Toxicity to Heterocypris incongruens (Crustacea, Ostracoda)*; ISO 14371 (E); ISO: Geneva, Switzerland, 2012.
26. Organisation for Economic Co-operation and Development. *Test No. 211: Daphnia magna Reproduction Test, OECD Guidelines for the Testing of Chemicals, Section 2*; OECD Publishing: Paris, France, 2012.
27. Organisation for Economic Co-operation and Development. *Test No. 202: Daphnia sp. Acute Immobilisation Test, OECD Guidelines for the Testing of Chemicals, Section 2*; OECD Publishing: Paris, France, 2004.
28. Zhang, Y.; Jeppesen, E.; Liu, X.; Qin, B.; Shi, K.; Zhou, Y.; Thomaz, S.M.; Deng, J. Global loss of aquatic vegetation in lakes. *Earth-Sci. Rev.* **2017**, *173*, 259–265. [CrossRef]
29. Stevenson, L.M.; Krattenmaker, K.E.; Johnson, E.; Bowers, A.J.; Adeleye, A.S.; McCauley, E.; Nisbet, R.M. Standardized toxicity testing may underestimate ecotoxicity: Environmentally relevant food rations increase the toxicity of silver nanoparticles to *Daphnia*. *Environ. Toxicol. Chem.* **2017**, *36*, 3008–3018. [CrossRef]
30. MicroBioTests Inc. OSTRACODTOXKIT F "Direct Contact" Toxicity Test for Freshwater Sediments. Standard Operational Procedure. Available online: www.microbiotests.be/SOPs/OstracodtoxkitFSOP-A5.pdf (accessed on 24 December 2018).
31. Toxic Cyanobacteria in Water. *A Guide to Their Public Health Consequences, Monitoring, and Management*; Chorus, I., Bartram, J., Eds.; St Edmundsbury Press: Suffolk, UK, 1999; ISBN 0419239308.
32. Belgis, Z.C.; Persoone, G.; Blaise, C. Cyst-based toxicity tests XVI—Sensitivity comparison of the solid phase *Heterocypris incongruens* microbiotest with the *Hyalella azteca* and *Chironomus riparius* contact assays on freshwater sediments from Peninsula Harbour (Ontario, Canada). *Chemosphere* **2003**, *52*, 95–101. [CrossRef]
33. Gondikas, A.P.; Von Der Kammer, F.; Reed, R.B.; Wagner, S.; Ranville, J.F.; Hofmann, T. Release of TiO_2 nanoparticles from sunscreens into surface waters: A one-year survey at the old danube recreational lake. *Environ. Sci. Technol.* **2014**, *48*, 5415–5422. [CrossRef] [PubMed]

34. Coll, C.; Notter, D.; Gottschalk, F.; Sun, T.; Som, C.; Nowack, B. Probabilistic environmental risk assessment of five nanomaterials (nano-TiO_2, nano-Ag, nano-ZnO, CNT, and fullerenes). *Nanotoxicology* **2016**, *10*, 436–444. [CrossRef]
35. Khangarot, B.S.; Das, S. Acute toxicity of metals and reference toxicants to a freshwater ostracod, *Cypris subglobosa* Sowerby, 1840 and correlation to EC_{50} values of other test models. *J. Hazard. Mater.* **2009**, *172*, 641–649. [CrossRef] [PubMed]
36. Bondarenko, O.M.; Heinlaan, M.; Sihtmäe, M.; Ivask, A.; Kurvet, I.; Joonas, E.; Jemec, A.; Mannerström, M.; Heinonen, T.; Rekulapelly, R.; et al. Multilaboratory evaluation of 15 bioassays for (eco)toxicity screening and hazard ranking of engineered nanomaterials: FP7 project NANOVALID. *Nanotoxicology* **2016**, *10*, 1229–1242. [CrossRef] [PubMed]
37. United States Environmental Protection Agency. *Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms*, 4th ed.; United States Environmental Protection Agency: Washington, DC, USA, 2002.
38. Gustafsson, J.P. Visual MINTEQ Version 3.1. Available online: https://vminteq.lwr.kth.se/download/ (accessed on 24 December 2018).
39. Ivask, A.; Kurvet, I.; Kasemets, K.; Blinova, I.; Aruoja, V.; Suppi, S.; Vija, H.; Käkinen, A.; Titma, T.; Heinlaan, M.; et al. Size-dependent toxicity of silver nanoparticles to bacteria, yeast, algae, crustaceans and mammalian cells in vitro. *PLoS ONE* **2014**, *9*, e102108. [CrossRef] [PubMed]
40. Blinova, I.; Lukjanova, A.; Muna, M.; Vija, H.; Kahru, A. Evaluation of the potential hazard of lanthanides to freshwater microcrustaceans. *Sci. Total Environ.* **2018**, *642*, 1100–1107. [CrossRef] [PubMed]
41. Vindimian, E. MSExcel Macro REGTOX_EV7.0.5.xls. Available online: http://www.normalesup.org/~{}vindimian/en_index.html (accessed on 24 December 2018).
42. Bhattacharjee, S. DLS and zeta potential—What they are and what they are not? *J. Control. Release* **2016**, *235*, 337–351. [CrossRef] [PubMed]
43. Grillo, R.; Rosa, A.H.; Fraceto, L.F. Engineered nanoparticles and organic matter: A review of the state-of-the-art. *Chemosphere* **2014**, *119*C, 608–619. [CrossRef] [PubMed]
44. Liu, Z.; Wang, C.; Hou, J.; Wang, P.; Miao, L.; Lv, B.; Yang, Y. Aggregation, sedimentation, and dissolution of CuO and ZnO nanoparticles in five waters. *Environ. Sci. Pollut. Res.* **2018**, *25*, 31240–31249. [CrossRef] [PubMed]
45. Bian, S.-W.; Mudunkotuwa, I.A.; Rupasinghe, T.; Grassian, V.H. Aggregation and Dissolution of 4 nm ZnO Nanoparticles in Aqueous Enviroments: Influence of pH, Ionic Strength, Size, and Adsorption of Umic Acid. *Langumir* **2011**, *27*, 6059–6068. [CrossRef] [PubMed]
46. Han, Y.; Kim, D.; Hwang, G.; Lee, B.; Eom, I.; Kim, P.J.; Tong, M.; Kim, H. Aggregation and dissolution of ZnO nanoparticles synthesized by different methods: Influence of ionic strength and humic acid. *Colloids Surf. A Physicochem. Eng. Asp.* **2014**, *451*, 7–15. [CrossRef]
47. Conway, J.R.; Adeleye, A.S.; Gardea-Torresdey, J.; Keller, A.A. Aggregation, dissolution, and transformation of copper nanoparticles in natural waters. *Environ. Sci. Technol.* **2015**, *49*, 2749–2756. [CrossRef]
48. Aruoja, V.; Pokhrel, S.; Sihtmäe, M.; Mortimer, M.; Mädler, L.; Kahru, A. Toxicity of 12 metal-based nanoparticles to algae, bacteria and protozoa. *Environ. Sci. Nano* **2015**, *2*, 630–644. [CrossRef]
49. Ji, J.; Long, Z.; Lin, D. Toxicity of oxide nanoparticles to the green algae *Chlorella* sp. *Chem. Eng. J.* **2011**, *170*, 525–530. [CrossRef]
50. Ma, S.; Zhou, K.; Yang, K.; Lin, D. Heteroagglomeration of oxide nanoparticles with algal cells: Effects of particle type, ionic strength and pH. *Environ. Sci. Technol.* **2015**, *49*, 932–939. [CrossRef] [PubMed]
51. Quik, J.T.K.; Velzeboer, I.; Wouterse, M.; Koelmans, A.A.; van de Meent, D. Heteroaggregation and sedimentation rates for nanomaterials in natural waters. *Water Res.* **2014**, *48*, 269–279. [CrossRef]
52. Lin, D.; Ji, J.; Long, Z.; Yang, K.; Wu, F. The influence of dissolved and surface-bound humic acid on the toxicity of TiO_2 nanoparticles to *Chlorella* sp. *Water Res.* **2012**, *46*, 4477–4487. [CrossRef] [PubMed]
53. Röhder, L.A.; Brandt, T.; Sigg, L.; Behra, R. Influence of agglomeration of cerium oxide nanoparticles and speciation of cerium(III) on short term effects to the green algae *Chlamydomonas reinhardtii*. *Aquat. Toxicol.* **2014**, *152*, 121–130. [CrossRef] [PubMed]
54. Schlesinger, A.; Eisenstadt, D.; Bar-Gil, A.; Carmely, H.; Einbinder, S.; Gressel, J. Inexpensive non-toxic flocculation of microalgae contradicts theories; overcoming a major hurdle to bulk algal production. *Biotechnol. Adv.* **2012**, *30*, 1023–1030. [CrossRef]

55. Muna, M.; Heinlaan, M.; Blinova, I.; Vija, H.; Kahru, A. Evaluation of the effect of test medium on total Cu body burden of nano CuO-exposed *Daphnia magna*: A TXRF spectroscopy study. *Environ. Pollut.* **2017**, 1–9. [CrossRef]
56. Stiff, M.J. The chemical states of copper in polluted fresh water and a scheme of analysis to differentiate them. *Water Res.* **1971**, *5*, 585–599. [CrossRef]
57. Odzak, N.; Kistler, D.; Behra, R.; Sigg, L. Dissolution of metal and metal oxide nanoparticles under natural freshwater conditions. *Environ. Chem.* **2015**, *12*, 138–148. [CrossRef]
58. Heinlaan, M.; Muna, M.; Knöbel, M.; Kistler, D.; Odzak, N.; Kühnel, D.; Müller, J.; Gupta, G.S.; Kumar, A.; Shanker, R.; et al. Natural water as the test medium for Ag and CuO nanoparticle hazard evaluation: An interlaboratory case study. *Environ. Pollut.* **2016**, *216*, 689–699. [CrossRef]
59. Li, M.; Lin, D.; Zhu, L. Effects of water chemistry on the dissolution of ZnO nanoparticles and their toxicity to *Escherichia coli*. *Environ. Pollut.* **2013**, *173*, 97–102. [CrossRef]
60. Borgmann, U.; Charlton, C.C. Copper complexation and toxicity to *Daphnia* in natural waters. *J. Great Lakes Res.* **1984**, *10*, 393–398. [CrossRef]
61. Hyne, R.V.; Pablo, F.; Julli, M.; Markich, S.J. Influence of water chemistry on the acute toxicity of copper and zinc to the cladoceran *Ceriodaphnia* cf *dubia*. *Environ. Toxicol. Chem.* **2005**, *24*, 1667–1675. [CrossRef]
62. Sevilla, J.B.; Nakajima, F.; Kasuga, I. Comparison of aquatic and dietary exposure of heavy metals Cd, Cu, and Zn to benthic ostracod *Heterocypris incongruens*. *Environ. Toxicol. Chem.* **2014**, *33*, 1624–1630. [CrossRef] [PubMed]
63. Kudłak, B.; Wolska, L.; Namieśnik, J. Determination of EC_{50} toxicity data of selected heavy metals toward *Heterocypris incongruens* and their comparison to "direct-contact" and microbiotests. *Environ. Monit. Assess.* **2011**, *174*, 509–516. [CrossRef] [PubMed]
64. Biesinger, K.E.; Christensen, G.M. Effects of Various Metals on Survival, Growth, Reproduction, and Metabolism of *Daphnia magna*. *J. Fish. Res. Board Can.* **1972**, *29*, 1691–1700. [CrossRef]
65. Xu, H.; Pan, J.; Zhang, H.; Yang, L. Interactions of metal oxide nanoparticles with extracellular polymeric substances (EPS) of algal aggregates in an eutrophic ecosystem. *Ecol. Eng.* **2016**, *94*, 464–470. [CrossRef]
66. Dalai, S.; Iswarya, V.; Bhuvaneshwari, M.; Pakrashi, S.; Chandrasekaran, N.; Mukherjee, A. Different modes of TiO_2 uptake by *Ceriodaphnia dubia*: Relevance to toxicity and bioaccumulation. *Aquat. Toxicol.* **2014**, *152*, 139–146. [CrossRef] [PubMed]
67. Gong, N.; Shao, K.; Li, G.; Sun, Y. Acute and chronic toxicity of nickel oxide nanoparticles to *Daphnia magna*: The influence of algal enrichment. *NanoImpact* **2016**, *3–4*, 104–109. [CrossRef]
68. Wu, F.; Bortvedt, A.; Harper, B.J.; Crandon, L.E.; Harper, S.L. Uptake and toxicity of CuO nanoparticles to *Daphnia magna* varies between indirect dietary and direct waterborne exposures. *Aquat. Toxicol.* **2017**, *190*, 78–86. [CrossRef]
69. Seo, J.; Kim, S.; Choi, S.; Kwon, D.; Yoon, T.-H.; Kim, W.-K.; Park, J.-W.; Jung, J. Effects of Physiochemical Properties of Test Media on Nanoparticle Toxicity to *Daphnia magna* Straus. *Bull. Environ. Contam. Toxicol.* **2014**, *93*, 257–262. [CrossRef]
70. De Schamphelaere, K.A.; Janssen, C.R. Effects of dissolved organic carbon concentration and source, pH, and water hardness on chronic toxicity of copper to *Daphnia magna*. *Environ. Toxicol. Chem.* **2004**, *23*, 1115–1122. [CrossRef]
71. Bianchini, A.; Wood, C.M. Does sulfide or water hardness protect against chronic silver toxicity in *Daphnia magna*? A critical assessment of the acute-to-chronic toxicity ratio for silver. *Ecotoxicol. Environ. Saf.* **2008**, *71*, 32–40. [CrossRef]
72. Zhu, X.; Wang, J.; Zhang, X.; Chang, Y.; Chen, Y. The impact of ZnO nanoparticle aggregates on the embryonic development of zebrafish (*Danio rerio*). *Nanotechnology* **2009**, *20*, 195103. [CrossRef] [PubMed]
73. Havel, J.E.; Talbott, B.L. Life history characteristics of the freshwater ostracod *Cyprinotus incongruens* and their application to toxicity testing. *Ecotoxicology* **1995**, *4*, 206–218. [CrossRef] [PubMed]
74. Shuhaimi-Othman, M.; Yakub, N.; Ramle, N.A.; Abas, A. Toxicity of metals to a freshwater ostracod: *Stenocypris major*. *J. Toxicol.* **2011**, *2011*. [CrossRef] [PubMed]

Supplementary Information

Combined Effects of Test Media and Dietary Algae on the Toxicity of CuO and ZnO Nanoparticles to Freshwater Microcrustaceans *Daphnia magna* and *Heterocypris incongruens*: Food for Thought

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Table S1. Characterisation of CuO and ZnO NP suspensions at 0 h, 48 h, and 6 days in five different test media. Nominal concentration was 10 mg metal/L. The mean (SD) of 3 technical replicates of one experiment is given. NP suspension may be characterised as highly unstable if ζ values are in range of ±0–10 mV; relatively stable for ±10–20 mV; moderately stable for ±20–30 mV, and highly stable for values >±30 mV [40].

		MQ			AFW			MHW			Lake Raku			Lake Ülemiste		
	Time (h)	**0**	**48**	**144**	**0**	**48**	**144**	**0**	**48**	**144**	**0**	**48**	**144**	**0**	**48**	**144**
CuO	D_h (nm)	**207** (14)	**236** (8.9)	**198** (3.9)	**573** (41)	**1826** * (346)	**2142** * (848)	**565** (41)	**1638** * (302)	**2645** * (841)	**197** (10)	**393** (8.9)	**449** (18)	**335** (14)	**440** (20)	**452** (6.5)
	ζ (mV)	**−15** (0.15)	**12** (1.3)	**23** (0.46)	**−7.3** (2.0)	**−4.6** (0.59)	**−3.5** (0.37)	**−13** (0.7)	**−8.4** (1.2)	**−13** (4.0)	**−19** (0.44)	**−18** (0.17)	**−17** (0.36)	**−18** (0.25)	**−17** (0.46)	**−17** (0.55)
	pdi	**0.17** (0.062)	**0.21** (0.015)	**0.24** (0.0040)	**0.22** (0.045)	**0.78** (0.023)	**0.81** (0.12)	**0.24** (0.015)	**0.77** (0.23)	**1** (0.0)	**0.19** (0.010)	**0.28** (0.016)	**0.35** (0.017)	**0.25** (0.016)	**0.26** (0.018)	**0.35** (0.0065)
ZnO	D_h (nm)	**116** (2.5)	**337** (121)	**NS**	**639** (94)	**3855** * (1807)	**3871** * (2556)	**1225** (27)	**2846** * (711)	**3385** * (310)	**283** (50)	**1135** (55)	**1052** * (119)	**177** (4.6)	**609** (27)	**445** (17)
	ζ (mV)	**18** (0.42)	**−10** (1.5)	**NS**	**3.4** (0.40)	**−4.1** (0.14)	**−5.5** (0.52)	**−5.3** (0.29)	**−2.0** (0.11)	**−5.0** (0.30)	**−17** (0.56)	**−15** (0.25)	**−15** (0.49)	**−16** (1.3)	**−16** (0.92)	**−16** (0.21)
	pdi	**0.20** (0.019)	**0.53** (0.14)	**NS**	**0.34** (0.017)	**0.86** (0.25)	**0.84** (0.28)	**0.45** (0.049)	**0.60** (0.14)	**0.30** (0.13)	**0.40** (0.016)	**0.56** (0.11)	**0.46** (0.036)	**0.34** (0.015)	**0.38** (0.023)	**0.37** (0.029)

AFW: OECD202 artificial freshwater; MHRW: US EPA moderately hard reconstituted water; D_h: Hydrodynamic diameter; ζ: Zeta potential; pdi: Polydispersity index; NS: DLS analysis found the sample unsuitable for particle analysis; *: Data did not meet the DLS analysis quality criteria (particle concentration was low etc.).

Table S2. Speciation of metals in different test media predicted by Visual MINTEQ simulation.

	Test medium	pH [1]	Dissolved fraction (%)			Precipitated fraction (%)	
			Inorganic		Bound to DOM	Fraction	Type of mineral
			Free ion	Bound ion			
$CuSO_4$	MQ	4.9	97	2.7	0	0	
	AFW	7.5	0.080	0.12	0	100	Tenorite (CuO)
	MHW	7.7	0.021	0.093	0	100	Tenorite (CuO)
	Raku	7.9	0.0077	0.082	1.0	99	Tenorite (CuO)
	Ülemiste	8.3	0.0016	0.077	2.0	98	Tenorite (CuO), Covellite (CuS)
$ZnSO_4$	MQ	6.4	97	2.6	0	0	Hydrozincite
	AFW	7.4	52	4.5	0	43	Hydrozincite
	MHW	7.5	52	8.3	0	40	Hydrozincite
	Raku	8.1	3.3	1.3	0.071	95	Hydrozincite, Smithsonite, Zn-Al layered double hydroxide
	Ülemiste	8.4	0.47	1.3	0.016	99	Hydrozincite

[1] recorded after 6 days of incubation at 10 mg metal/L at 20 °C; AFW: OECD202 artificial freshwater; MHW: US EPA moderately hard reconstituted water; Ülemiste: water from lake Ülemiste; Raku: water from lake Raku.

Tabel S3. Change in *H. incongruens* growth (%) at the end of the 6-day experiment at sublethal concentrations. Negative values show growth inhibition and positive values growth enhancement in comparison to control organisms. Each value is based on measurements of 5 to 12 organisms from one exposure concentration. The mean (SD) of 2 experiments is given when possible.

	Test concentration (mg metal/L)	MHW	Raku	Ülemiste
CuO NP	0.1	−9.2	−5.6	1.2
	1	−4.2	6.3	−10
$CuSO_4$	0.05		27	
	0.1	41	−23	13
	0.2	4.1		
	0.25			9.8 (7.4)
	0.38			−1.9
ZnO NP	0.1	−9.8 (0.35)		
	0.25		−39	−20
	0.5		−47	−40
$ZnSO_4$	0.01		3.9	−8.6
	0.05		0.7	−17
	0.1	17	−1.7	−21
	0.25		−53	−39
	0.5	−41		

MHW: US EPA moderately hard reconstituted water; Ülemiste: water from lake Ülemiste; Raku: water from lake Raku; NP: nanoparticles.

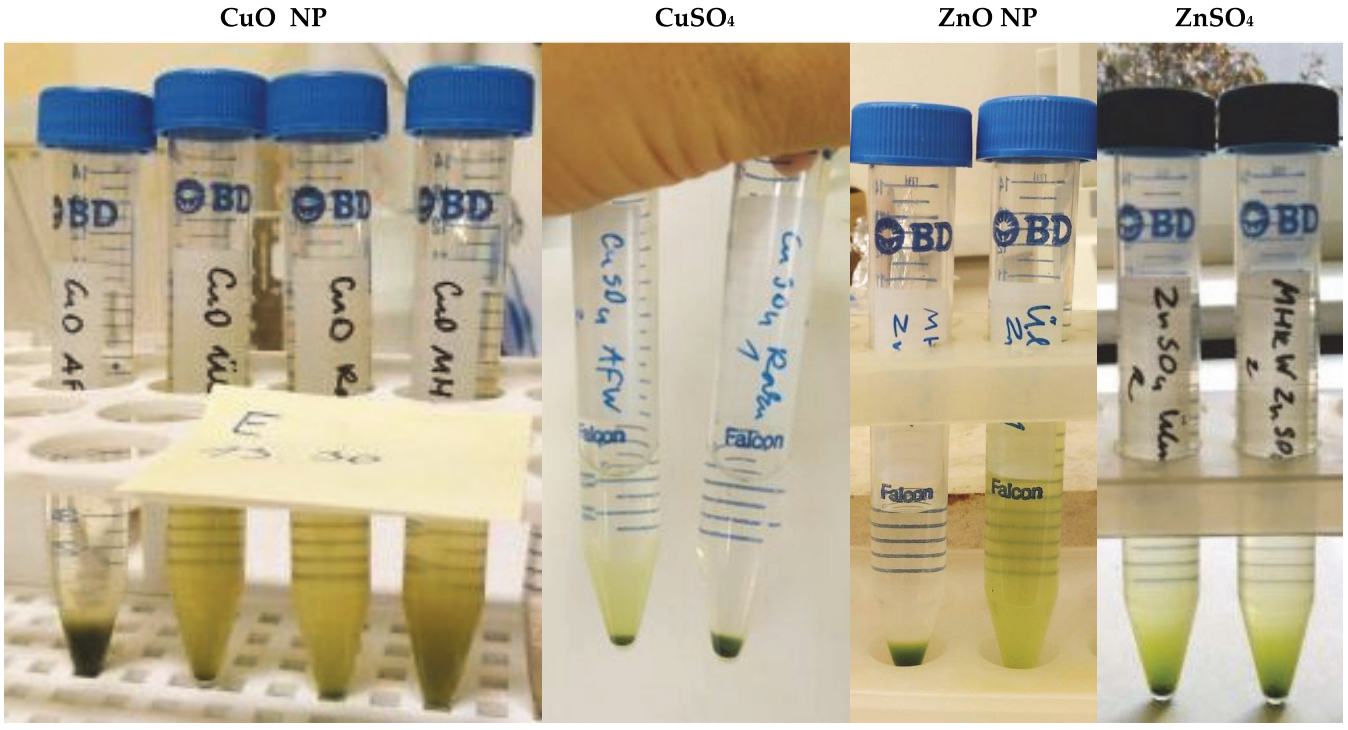

Figure S1. Examples of typical sedimentation of algae after 6 days of incubation with CuO and ZnO NP and respective soluble salts at 10 mg metal/L. MHW: US EPA moderately hard water; AFW: OECD 202 artificial freshwater; Ülemiste: water from lake Ülemiste; Raku: water from lake Raku.

Appendix 3

Publication III

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Evaluation of the effect of test medium on total Cu body burden of nano CuO-exposed *Daphnia magna*: A TXRF spectroscopy study☆

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ABSTRACT

Toxicity of Cu and Cu-based nanoparticles (NPs) to aquatic biota is usually mitigated in natural freshwater compared to organics-free artificial freshwater. The main aim of this study was to evaluate whether mitigated toxicity is accompanied by lower total copper body burden in the freshwater crustacean *Daphnia magna* and whether CuO NPs are more hazardous in this aspect than soluble Cu salts.

Total copper body burden in different media (OECD202 artificial freshwater and two natural freshwaters) was measured by a relatively novel technique - total reflection X-ray fluorescence (TXRF) spectroscopy - which proved suitable for the analysis of individual juvenile daphnids. Mean copper body burden was 2.8–42 times higher in daphnids exposed to CuO NPs (0.05 mg Cu/L and 1 mg Cu/L) than in daphnids exposed to equal or equitoxic concentrations (0.025 mg Cu/L and 0.05 mg Cu/L) of $CuSO_4$. Using natural freshwater instead of artificial one resulted in increased copper burden after exposure to CuO NPs but not after exposure to Cu salt. After 24 h post-exposure depuration in the presence of algae *Raphidocelis subcapitata*, total copper body burden in daphnids exposed to CuO NPs sharply decreased while in daphnids exposed to Cu salt it did not. Despite the CuO NP toxicity mitigating effect of natural freshwater, total copper body burden of aquatic crustaceans in natural waterbodies may be greater than could be predicted based on the results obtained using artificial freshwater as the test medium.

1. Introduction

Excessive copper concentration in surface waters, mainly originating from mining activities, wastewater treatment plants, runoff from urban areas, highways and arable land, is known to impose severe toxicity to aquatic biota (Borgmann and Ralph, 1983; Meyer et al., 2007). The risk of contamination by a novel type of copper compounds, Cu nanoparticles (NPs), that are used as sensors and in catalysis, surfactants, (antifouling) paints, antimicrobials, etc. has increased during the last decade (Adeleye et al., 2016; Bondarenko et al., 2013). Current Cu and CuO NP production volumes are estimated to be low: approximately 200 metric tons worldwide in the year 2010 (Keller et al., 2013). Nevertheless, there can be much higher amounts of Cu NPs on the market as production volume of micronized Cu wood preservatives containing a considerable fraction of Cu NPs possibly already exceeds that of the most widely-used engineered NPs (Civardi et al., 2015; Evans et al., 2008).

Engineered NPs are unbound or agglomerated particles with one or more external dimensions in the size range of 1–100 nm (EC, 2011). CuO NPs have been shown to be much more toxic to filter-feeding water flea *Daphnia magna* than micro CuO. Engineered copper NPs undergo dissolution after reaching waterbodies and copper ions are believed to be the main inducers of copper NP toxicity to *D. magna* (Adam et al., 2015a; Blinova et al., 2010; Heinlaan et al., 2008). However, additional adverse effects of NPs to aquatic organisms, not explainable by dissolution in the environment, have also been shown (Xiao et al., 2015).

To date, the data on direct acute toxicity of CuO NPs to aquatic crustaceans prevail in the scientific literature (Juganson et al., 2015) but information on metal bioaccumulation in crustaceans, exposed to Cu-based NPs, is rare. CuO NPs have been shown to impose greater bioaccumulation risk compared to soluble Cu salts due to ingestion of the NPs by filter feeding organisms and adhesion of NPs to the exoskeleton (Adam et al., 2014; Blinova et al., 2010). This phenomenon may facilitate the transfer of copper and other metal

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nanoparticles to higher levels of the aquatic food chain as cladocerans' (e.g. *D. magna*) bioaccumulation potential is very high (Ferry et al., 2009; Polukhina et al., 1998). Despite the rising importance of considering aspects of bioaccumulation in international legislation, there are still no widespread methods for simple and effective bioaccumulation measurements in small organisms (Schäfer et al., 2015). In particular, NP-specific toxicity testing guidelines currently developed by European Chemical Agency have addressed the need for more experimental data on bioaccumulation potential assessment of NPs (ECHA-17-G-14-EN, 2017). In this study, a method of total reflection x-ray fluorescence (TXRF) spectroscopy was applied to measure total copper concentration in single juvenile *Daphnia magna* specimens. TXRF spectroscopy has been rarely used for elemental analysis of aquatic organisms while it has been shown to enable fast and inexpensive way to measure metal accumulation in individual microzooplankters caught from the wild (Woelfl et al., 2004). Our aim was to test the method on ephippia-born experimentally manipulated juvenile daphnids.

The behaviour of hazardous compounds in water and, as a result, their bioavailability to the test organisms depends on the chemical composition of the test medium. Water chemistry has been shown to impose similar mitigation pattern on soluble Cu salt and CuO NPs (and bulk Cu) indicating (partly) overlapping uptake mechanisms of Cu from these compounds (Blinova et al., 2010; Thit et al., 2015). It may be surmised that composition of the test medium also affects uptake and bioaccumulation of CuO NPs and Cu salt by aquatic organisms. The main parameters influencing bioavailability of copper in the aquatic environment are organic matter, ionic strength, and pH (Conway et al., 2015; Hyne et al., 2005; Meyer et al., 2007). Dissolved organic matter usually has the most significant mitigating effect (Blinova et al., 2010; De Schamphelaere and Janssen, 2004a; Gao et al., 2009; Kramer et al., 2004; Käkinen et al., 2011) while water hardness also contributes (Gao et al., 2009; Park et al., 2009; Yim et al., 2006).

In this study, total copper body burden in *Daphnia magna*, one of the most sensitive freshwater model organisms to CuO NPs (Kahru and Dubourguier, 2010), exposed to nano CuO and Cu salt, was evaluated. Two types of natural water, collected from lakes with different hydrochemical composition (mainly regarding organic matter content and hardness), were used as test media in the total body burden experiments in addition to OECD202 artificial freshwater (AFW; OECD, 2004). The term "total copper body burden" was used in this study to summarize the natural copper background concentration and bioaccumulated copper in the daphnids. The aim of the current study was to evaluate whether mitigated copper toxicity in natural water is accompanied by lower copper accumulation in daphnids and whether the effect is similar for CuO NPs and soluble Cu salt. Additionally, suitability of the TXRF method for Cu measurements in individual *D. magna* juveniles was evaluated.

2. Materials and methods

2.1. Chemicals

Uncoated CuO NPs (NNV-011; Intrinsiq Materials; powder form) from EU FP7 NanoValid project ("Developing of reference methods for hazard identification, risk assessment and LCA of engineered nanomaterials") were used in the study. The primary size of the CuO NPs was 22–25 nm (provided by the manufacturer). $CuSO_4 \cdot 5H_2O$ (Alfa Aesar) was used as the ionic control. CuO NP stock suspension was sonicated as described by Suppi et al. (2015) and used for up to 4 weeks from the preparation. NP and Cu-salt stocks (both 5 g Cu/L in MilliQ water) were stored in the dark at room temperature.

2.2. Physico-chemical characterization of CuO nanoparticle suspensions

Dissolution of CuO NPs was measured by Atomic Absorption Spectroscopy in accredited laboratory of Tallinn University of Technology (Estonia) as previously described by Ivask et al. (2014). Briefly, fresh and 48 h (the duration of the OECD202 acute toxicity test; OECD, 2004) NP suspensions (10 mg Cu/L) prepared in three different test media (see 2.3.) were ultracentrifuged at 362 769 g for 30 min (duration of the whole cycle 60 min). Supernatants were used for AAS analysis.

Malvern Instruments (UK) ZetaSizer Nano ZS (173° angle) equipped with 4.0 mW 633 nm laser (Model ZEN3600) was used for dynamic light scattering (DLS) analysis of the NPs. Hydrodynamic diameter, polydispersity index and zeta potential were measured. Samples were measured at 1 h and 48 h at concentration of 100 mg Cu/L in three different test media (see 2.3.) after shaking.

2.3. Test media

OECD202 artificial freshwater (AFW) (OECD, 2004) was used as a standard exposure medium. Natural water was sampled from two lakes (Lake Raku and Lake Ülemiste, Northern Estonia) that are both part of the freshwater supply system of the city of Tallinn. The lakes represent different hydrochemical types: Lake Ülemiste (944.7 ha) is a natural eutrophic lake while Lake Raku (230.7 ha) is an artificial sandpit lake in the catchment area of Lake Ülemiste. Water samples were filtered through Millipore nitrocellulose filters (pore size 0.45 μm) and stored in the dark at 4 °C. Chemical analysis of the lake water was performed in a certified laboratory (Tallinna Vesi Laboratories). The respective values for AFW were calculated according to the standard protocol. The main chemical parameters of the test media are presented in Table 1. The background total copper concentration in the lakes was low and typical to unpolluted streams in Europe (median value 0.88 μg/L) (http://weppi.gtk.fi/publ/foregsatlas/text/Cu.pdf). The percentage of Cu bound to DOC was calculated using Visual MINTEQ version 3.1. (Nica-Donnan model) (Gustafsson, 2016).

2.4. Daphnia magna acute immobilisation assay

Acute toxicity tests were carried out according to OECD 202 testing guideline (OECD, 2004) in 30-well polycarbonate plates. Daphnids were hatched from ephippia (MicroBioTests Inc., Belgium) in artificial freshwater (OECD202 AFW). Up to 24 h old neonates were fed with cells of *Raphidocelis subcapitata* for 2 h prior to the test and transferred to the test plate *via* clean AFW to minimize carry-over of the algal cells. Immobilized (no mobility recorded within 15 s from gentle agitation) daphnids were counted after 48 h exposure in the dark at 21 °C. EC_{10} (10% immobilisation concentration) and EC_{50} (50% immobilisation concentration) values were calculated using REGTOX Excel Macro program (http://www.normalesup.org/~vindimian/en_index.html). 4–8 independent tests divided into 4 technical replicates each were carried out for CuO NPs and Cu salt in each test media.

2.5. Total Cu body burden experiments

2.5.1. Exposure of daphnids for total Cu body burden measurements

Exposure concentrations for *D. magna* total Cu body burden experiments were selected based on preliminary acute toxicity testing in AFW (see 2.4). Total copper body burden was measured in daphnids hatched from ephippia (MicroBioTests Inc.) and exposed to two concentrations of CuO NPs and $CuSO_4 \cdot 5H_2O$ for 48 h analogously to acute immobilisation test (see 2.4). EC_{10} and EC_{50}

Table 1
Chemical composition of the test media. Calculated values for AFW.

	Lake Raku	Lake Ülemiste	OECD202 AFW
pH	8.23	8.28	7.8 ± 0.2
Conductivity 25 °C (μS/cm)	279	406	640
Dissolved organic carbon (DOC) (mg/L)	4.9	9.7	0
Total phosphorus (mg P/L)	0.034	0.032	0
Total nitrogen (mg N/L)	0.52	1.16	0
Total hardness (mg-eq/L)	2.61	4.11	5
Cl^- (mg/L)	3.2	10	144.5
SO_4^{2-} (mg/L)	22	28	48.0
Ca^{2+} (mg/L)	42.2	70	80.1
Mg^{2+} (mg/L)	4.57	7.5	12.2
Na^+ (mg/L)	2.68	6.17	17.7
K^+ (mg/L)	1.86	2.72	3.0
Cu^{2+} (μg/L)	1.2	0.5	0

AFW — artificial freshwater.

concentrations in AFW were chosen as exposure concentrations for all the three test media. To decrease mortality during the exposure, lower 95% confidence interval (CI) value of EC_{50} was used instead of optimal EC_{50}. Hereinafter, the exposure concentrations will be referred to as EC_{10AFW} and $EC_{50AFW(95\%)}$ as they were determined according to the results obtained in AFW and were expected to have different toxicity in the natural test media. 2–4 independent acute immobilisation tests, divided into 4 technical replicates, were carried out for each test concentration in three test media for total body burden measurements.

In addition, six unexposed adult daphnids from the laboratory culture were analysed for comparison of copper measurements to literature data on wild and cultured daphnids. The culture was maintained in a mixture of Lake Raku and Lake Ülemiste water and fed with algae *Raphidocelis subcapitata*.

2.5.2. Depuration of daphnids after the exposure

To determine whether and how effectively daphnids can depurate from accumulated copper, the daphnids were carefully rinsed in clean exposure medium for 5 min and transferred into the corresponding chemical-free test medium containing algae. Up to 28 actively swimming daphnids from each exposure concentration were incubated at room temperature (21 °C) in the dark (identical to exposure conditions) for 24 h in 50 mL glass vials (1.8 ml medium per daphnid) with $9 \cdot 10^5$ cells of *R. subcapitata* per daphnid.

2.5.3. Total copper body burden analysis

The total copper body burden in *D. magna* was quantified by total reflection X-ray fluorescence (TXRF) analysis performed with Bruker Picofox S2 after 48 h acute copper exposure and the following 24 h depuration. For the analysis, live daphnids were rinsed (see 2.5.2), placed on a glass microscope slide (Delta Lab) and, after removing excess water, frozen at −18 °C. Frozen daphnids were transferred into 2 μL drop of ≥65% HNO_3 on quartz carriers (Bruker). Each daphnid was placed on a separate carrier and analysed individually. The samples on quartz carriers were dried on a hotplate at 50–60 °C followed by adding and drying another drop of HNO_3 and then a mixture of HNO_3 and 2.5 mg/L of Ga (an internal standard). Copper concentration was calculated using Bruker Spectra7 software. 8 to 16 individual daphnids were analysed for each exposure scenario. Additional measurements were performed on daphnids from the laboratory culture for the comparison (see 2.5.1). Element concentration in a daphnid was calculated by subtracting blank (sample carrier with HNO_3 and Ga) Cu concentration from the measured concentration and dividing the resulting element mass by either calculated or measured dry weight of the daphnid (see 2.5.4).

According to the published data (Adam et al., 2015b; De Schamphelaere et al., 2007; Madhav et al., 2017; Polukhina et al., 1998; Quevauviller et al., 1993; Wu et al., 2017; Xiao et al., 2015), Cu concentration of *D. magna* is usually measured using FAAS, GFAAS, ICP-OES, ICP-MS or SR-XRF methods which require digestion of large amounts (>0.5 mg dwt) of sample material (Woelfl et al., 2004) or, in case of SR-XRF, need an access to a synchrotron.

TXRF method, as other X-ray based methods, uses X-rays to radiate the sample and measures the spectrum of the emitted fluorescence which is then used for the interpretation of the elemental composition of the sample. TXRF method is distinctive by the low angle of X-ray beam which enables total reflection of the beam from the sample carrier and thus double excitement of the atoms in the sample: before as well as after the reflection. The resulting magnified signal from the sample and lower levels of background signal from the sample carrier compared to other X-ray methods enable analysis of minute amounts of sample. The sample preparation is fast and simple, as X-rays can penetrate small samples and severe digestion procedures are not needed. Even a benchtop TXRF analyser gives accurate Cu concentration results in a single daphnid after simple wet preparation (Mages et al., 2004, 2001). In addition, the method is low cost and enables relatively fast automated measurements. Disadvantages are the limited number of metals that give a strong signal not overlapping with other elements and limited amount of sample that can be penetrated by X-rays and thus analysed on one sample carrier (liquid samples may need preconcentration). The method also gives no information on speciation of metals e.g. it is not possible to distinguish between dissolved Cu and Cu NPs.

As shown by Heinlaan et al. (2017), the lowest limit of detection (LLD) of Cu in individual *D. magna* neonate was 0.031 ± 0.006 ng (average of LLD values provided by the instrument according to 3-sigma criterion based on 11 individual control daphnids (raised in AFW)); total copper body burden results always given as mean ± SD. Lowest limit of quantification (LLQ) was calculated based on the formulas presented in ICH-Q2B guidelines (1996) by multiplying LLD by 3.3 to acquire the 10-sigma value. Thus the LLQ was estimated to be 0.1 ng. Both LLD and LLQ were below the lowest Cu amount measured in a daphnid sample (0.14 ng). Measurements of a single daphnid spiked with Cu (0.5, 1 and 2 ng) were carried out to reveal possible matrix effects with results within the measurement uncertainty. For quality control and method control, biomass addition experiment (measurements of 1–6 neonates placed on one sample carrier) was conducted and all experiments were accompanied by 1 mg/L (5 ng) Cu sample with constant 85–115% recovery rate. Average Cu amount on the HNO_3 spiked blank sample carrier was 0.048 ± 0.009 ng (7 sample carriers) which was subtracted from all the measured Cu results.

2.5.4. Body length and dry weight measurements

In total, 5–21 juvenile daphnids from each concentration were used to measure daphnid body length (apical spine excluded; Fig. S1) under light microscope (Olympus IMT-2, CellB software) after 48 h exposure and after the 24 h depuration. Additionally, body length measurements of adult daphnids from in-house culture (see 2.5.1) were used to calculate their dry weight. The dry weight was calculated according to the formula weight = 0,0028 · length^{3,6819} (Adam et al., 2015b). The dry weight formula was not suitable for determining the dry weight of juvenile daphnids as it was based on data from adult daphnids only. Thus, dry weight of 220 juvenile daphnids incubated in identical conditions to controls was measured after lyophilisation as described by Heinlaan et al. (2017).

2.5.5. Statistical analysis

As normal distribution of the data could not be shown, Wilcoxon rank sum test, a non-parametric test for independent samples, was used to determine the differences between the exposure groups. Analysis was done using R 3.2.3. Statistical Software (R Foundation for Statistical Computing, Vienna, Austria).

3. Results and discussion

3.1. Characterization of CuO nanoparticles in different test media

Physico-chemical characterization of CuO NPs in three different test media is shown in Table 2. NPs agglomerated more intensively in artificial freshwater than in natural water potentially due to the particle stabilizing effect of dissolved organic matter (Grillo et al., 2014). Zeta (ζ) potential values indicated instability of the CuO NP suspension in AFW and relative stability of NPs in natural waters (Table 2). NP (agglomerate) size range was moderately non-uniform (Table 2) at both time points, similarly in all the test media. However the size of agglomerates (Table 2) in all the test media was within *D. magna* main ingestion size range of 0.4–40 μm (Geller, 1981; Gophen and Geller, 1984). Agglomeration increased in time but interestingly, this had no effect on zeta potential values, possibly due to steric stabilization of the NPs (Grillo et al., 2014). In conclusion, DLS characterization indicate that in the natural water CuO NPs stay suspended in the water column for a longer time than in AFW.

The 48 h dissolution of CuO NPs varied within a small range of 1–1.6% in all the tested media, being slightly higher in natural waters (1.2–1.6%). However, as discussed by Heinlaan et al. (2016), NP dissolution is technically challenging to determine and interpret hence the ionic control should always be used in parallel to the metal-based NP environmental hazard evaluation.

Both solubility and zeta potential results in AFW of the current study were comparable to the results obtained by Bondarenko et al. (2016) on the same batch of CuO NPs.

Table 2
Characterization of CuO nanoparticle suspensions at 0 h and 48 h in three different test media. All the measurements were performed at 100 mg Cu/L.

	OECD202 AFW	Lake Raku	Lake Ülemiste
D_h (nm) 0 h	1830 ± 131	980 ± 94	937 ± 97
D_h (nm) 48 h	2576 ± 427	1855 ± 192	1433 ± 79
pdi 0 h	0.41 ± 0.08	0.27 ± 0.01	0.26 ± 0.03
pdi 48 h	0.38 ± 0.12	0.35 ± 0.06	0.35 ± 0.07
ζ (mV) 0 h	0.64 ± 0.11	−20.13 ± 0.1	−19.23 ± 0.31
ζ (mV) 48 h	0.71 ± 0.1	−19.5 ± 0.1	−18.63 ± 0.31

The mean of 3 measurements ± SD of one experiment is given.
OECD202 AFW – OECD202 artificial freshwater.
D_h – Hydrodynamic diameter.
pdi – Polydispersity index.
ζ – Zeta potential.

3.2. Acute toxicity of the copper compounds

Results of 48 h acute immobilisation test showed that both investigated copper compounds were very toxic to *D. magna*. In AFW, EC_{10} values were 0.051 (95% CI 0.004–0.29) mg Cu/L for CuO NPs and 0.026 (95% CI 0.020–0.036) for $CuSO_4 \cdot 5H_2O$; EC_{50} values were 1.62 (95% CI 1.12–3.40) mg Cu/L for CuO NPs and 0.053 (0.047–0.059) mg Cu/L for copper salt. These results were comparable to previous research data of our laboratory on the 48 h toxicity of a different type of CuO NPs (30 nm) and Cu salt to *D. magna* (CuO EC_{50} = 2.6 mg Cu/L and $CuSO_4$ EC_{50} = 0.07 mg Cu/L; Heinlaan et al., 2008) and as reviewed from different publications by Bondarenko et al. (2013): CuO median L(E) C_{50} = 2.1 mg Cu/L.

Based on the obtained toxicity values, exposure concentrations for total body burden experiments were chosen: $EC_{50AFW95\%}$ values (1 mg Cu/L for CuO NPs and 0.05 mg Cu/L for Cu salt) and EC_{10AFW} values (0.05 mg Cu/L for CuO NPs and 0.025 mg Cu/L for Cu salt).

3.3. Evaluation of the total copper body burden in daphnids

3.3.1. Immobilisation of daphnids during the exposure

Results of neonate immobilisation during the body burden experiments are given in Table 3. The immobilisation after the 48 h exposure to EC_{10AFW} and $EC_{50AFW(95\%)}$ of copper compounds as compared with immobilisation in preliminary acute tests was similar in AFW and lower in natural waters. Toxic effects (immobilisation > 10%) in natural waters was seen only in Lake Raku water upon exposure to $EC_{50AFW(95\%)}$ of Cu salt. These results were in agreement with findings from our laboratory by Blinova et al. (2010) and Heinlaan et al. (2016), who showed that natural water reduced toxicity of both CuO NPs and Cu salt compared to AFW.

CuO NPs are believed to be toxic to freshwater organisms mainly due to dissolved Cu-ions (Heinlaan et al., 2008; Thurberg et al., 1973). Thus, it could be assumed that lower toxicity is a result of exposure to lower concentration of bioavailable Cu-ions. Modelling of $CuSO_4$ speciation in the three test media at 0.025 mg Cu/L exposure showed >99% of Cu to be bound to DOC in both natural waters. The modelling results cannot be directly interpreted for toxicity as it has been shown that the bound copper in natural waters can also impose toxicity (Borgmann and Charlton, 1984). Also, dissolution of NPs measured in this study was not lower in natural waters which might indicate, as some authors have found, that dissolution of Cu based NPs in test medium is less relevant than *in vivo* dissolution in the gut of *D. magna* (Fan et al., 2012; Xiao et al., 2015). The fact that concentration of metal ions may be higher at NP-cell interface than in the surrounding medium is an important consideration (Käkinen et al., 2011; Holden et al., 2014).

There was a remarkable additional immobilisation of daphnids during the post-exposure depuration in the presence of algae (Table 3). This may be explained by compromised viability of neonates as a result of starvation and toxic effect of copper compounds during the 48 h exposure (McWilliam and Baird, 2002). Exposure to soluble copper (as low as 0.01 mg Cu/L) and hardly digestible or Cu-contaminated particles can decrease the filtration rate even in environmentally relevant conditions which can slow down the turnover rate of midgut from the toxic content (Flickinger et al., 1982; Gillis et al., 2005). The higher the exposure concentration of the copper compounds the higher was the immobilisation of daphnids during 24 h depuration (Table 3).

Table 3
Immobilisation (mortality) of daphnids exposed to CuO nanoparticles and $CuSO_4 \cdot 5H_2O$ in the three different test media during the 48 h acute exposure (toxicity test) and during the 24 h post-exposure depuration in the presence of algae.

Sample	Expected Effect*	48 h mortality [%]	24 h depuration mortality [%]
OECD202 AFW			
D. magna control		5.0 (5–5)	12 (0–21)
CuO 0.05 mg Cu/L	EC_{AFW10}	10 (6.5–20)	12 (0–14)
1 mg Cu/L	$EC_{50AFW(95\%)}$	42 (34–52)	38 (21–100)
$CuSO_4$ 0.025 mg Cu/L	EC_{AFW10}	6.9 (5–7.7)	21 (14–40)
0.05 mg Cu/L	$EC_{50AFW(95\%)}$	18 (14–40)	29 (25–40)
Lake Raku water			
D. magna control		1 (0–2.5)	10 (0–17)
CuO 0.05 mg Cu/L	EC_{AFW10}	1.8 (0–6.7)	23 (14–29)
1 mg Cu/L	$EC_{50AFW(95\%)}$	8.3 (5–10)	35 (21–43)
$CuSO_4$ 0.025 mg Cu/L	EC_{AFW10}	0.0 (0–0)	21 (22–23)
0.05 mg Cu/L	$EC_{50AFW(95\%)}$	29 (28–30)	32 (29–46)
Lake Ülemiste water			
D. magna control		0.0 (0–0)	4.3 (0–7.7)
CuO 0.05 mg Cu/L	EC_{AFW10}	1.4 (0–5)	15 (0–21)
1 mg Cu/L	$EC_{50AFW(95\%)}$	1.0 (0–5)	25 (0–54)
$CuSO_4$ 0.025 mg Cu/L	EC_{AFW10}	3.3 (2.5–5)	12 (0–25)
0.05 mg Cu/L	$EC_{50AFW(95\%)}$	1.7 (0–5)	8.0 (0–18)

All the values are presented as mean (min–max).
AFW – Artificial freshwater (OECD202).
*Based on the preliminary toxicity testing.
EC_{AFW10} –10% immobilisation concentration in AFW.
$EC_{50AFW(95\%)}$ – lower 95% confidence interval of the 50% immobilisation concentration in AFW.

3.3.2. Effect of exposure to copper compounds on D. magna body length

As body size partly determines total Cu body burden in an organism, body length was measured before and after post-exposure depuration (Fig. S1). There was no statistically significant difference in the mean body size of control daphnids between the test media despite the presence of different amounts of DOC which has been shown to support the growth of daphnids (McMeans et al., 2015). Nor did the 24 h depuration have a significant effect on the mean body length of control daphnids (mean ± SD values being 863 ± 50 μm after 48 h exposure and 880 ± 65 μm after depuration). In our previous study (Heinlaan et al., 2017), we showed that juvenile daphnids, fed with algae *C. reinhardtii* for 48 h after a 48 h acute immobilisation assay, increased in body length up to 12%. Thus, 24 h may have been too short time for statistically significant growth in body length. As the differences between treatments and time points were inconsistent and small (up to 7% difference in body length), an averaged juvenile daphnid's dry weight (5.9 μg) was used in all body burden calculations.

3.3.3. Reference measurements: copper concentration in unexposed juvenile and adult Daphnia magna

Average copper concentrations in juvenile control daphnids, hatched from ephippia, were 38 ± 17 μg Cu/g after 48 h incubation in AFW, 65 ± 22 μg Cu/g after incubation in Lake Ülemiste water and 87 ± 20 μg Cu/g after incubation in Lake Raku water. The Cu concentration in daphnids increased after the following 24 h depuration: 76 ± 45 μg Cu/g in AFW, 95 ± 45 μg Cu/g in Lake Ülemiste water, and 104 ± 50 μg Cu/g in Lake Raku water. These values are much higher than reported in other studies for unexposed juvenile and adult daphnids originating from laboratory cultures e.g. 12–14 μg/g dwt (De Schamphelaere et al., 2007; De Schamphelaere and Janssen, 2004b) which has also been considered an optimal concentration for daphnids (Bossuyt and Janssen, 2005; all analysed with Flame and Graphite Furnace Atomic Absorption Spectroscopy). Higher Cu concentration (47.8 μg Cu/g dwt) has however been measured in 5 day old culture daphnids with the same method by other authors (Gillis et al., 2005) possibly due to higher Cu concentration in the culture and test medium (tap water, 2–4 μg Cu/L).

Possible explanation for the differences from the concentration in control neonates is the dwt of juvenile daphnids. The mass of <24 h culture neonates was 9.5 μg (Bossuyt and Janssen, 2005) compared to the 5.9 μg of 48 h old juveniles in the current study. In all the mentioned studies dry mass was acquired *via* heating the samples while daphnids in this study were freeze-dried. In addition, the size difference may be due to different origin of the organisms (culture *vs* ephippia).

As to our knowledge, there is no literature on ephippia-born neonates' Cu content and the Cu concentration in the culture medium of the parent animals was unknown. Thus the Cu concentration in adult daphnids from our in-house laboratory culture raised in a mixture of Lake Raku and Lake Ülemiste water was measured for comparison. The results 28.1 ± 12.9 μg Cu/g dwt were significantly lower from ephippia-born juveniles raised in lake waters, comparable to Cu reference of 29.5 μg Cu/g dwt measured from wild *D. magna* biomass (Quevauviller et al., 1993) and higher than the results from laboratory raised daphnids mentioned above. Possibly daphnids, cultured in mineral test media, experience Cu deficiency that might result in low Cu concentration in the neonates (Bossuyt and Janssen, 2005). In these conditions, Cu concentration per neonate may fall below the quantification limit (<0.1 ng Cu) of TXRF protocol used in this study.

3.3.4. Total copper body burden measurements in daphnids exposed to copper compounds

The mean CuO NP accumulation in the current study in different test media was 426–1002 μg Cu/g dwt after exposure to 0.05 mg Cu/L (EC_{10} concentration) and 2028–5503 μg Cu/g dwt after exposure to 1 mg Cu/L ($EC_{50AFW(95\%)}$ concentration) (Fig. 1A). The mean copper body burden measured in the daphnids after exposure to copper salt (0.025 and 0.05 mg Cu/L) in all the test media was 101–160 μg Cu/g dwt (Fig. 1B). Thus, the total copper body burden in daphnids exposed to both concentrations of CuO NPs as well as Cu salt was higher than in the unexposed controls (all p values < 0.005) (Fig. 1A and B) and exposure to CuO NPs resulted in

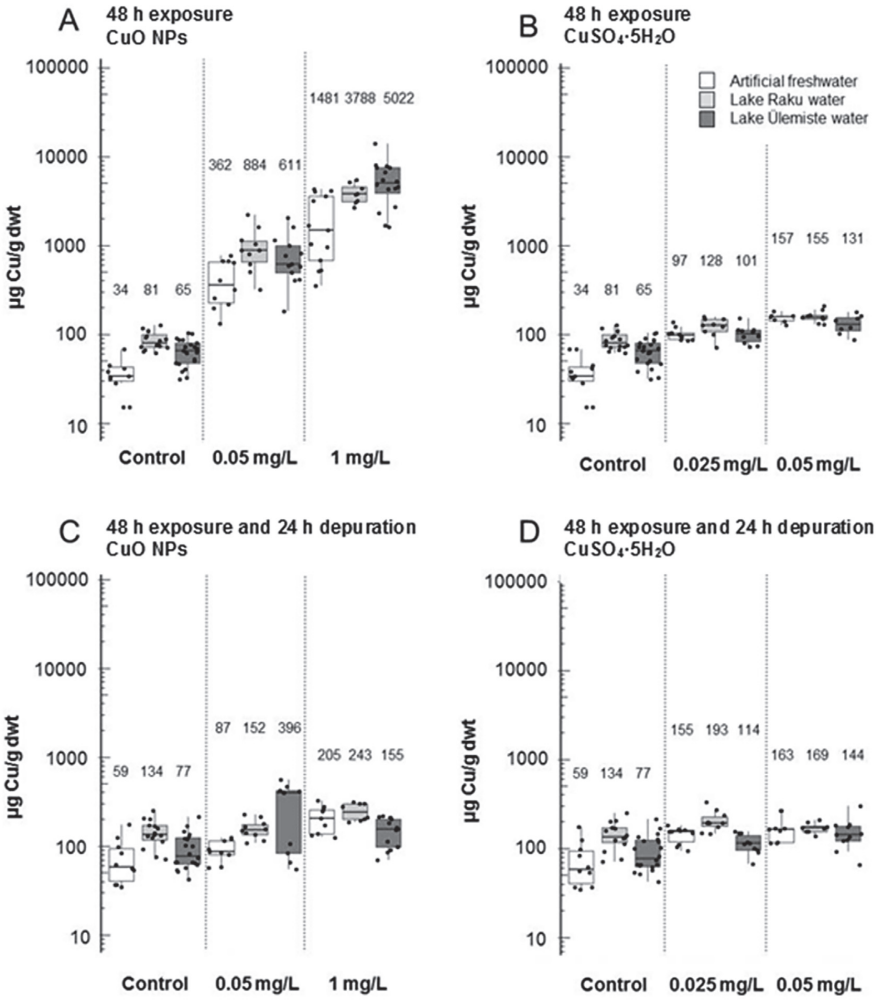

Fig. 1. Total Cu concentration in *Daphnia magna* juveniles after acute exposure (48 h) to CuO nanoparticles (NPs) (A) and $CuSO_4 \cdot 5H_2O$ (B) in three different test media and the following depuration in clean medium in the presence of algae *R. subcapitata* (24 h) (C, D). Each dot represents Cu concentration in one juvenile daphnid. Values between 25th and 75th percentile form the box and the line inside the box show the median. Median body burden values are given above the boxes. Due to logarithmic scale, fences ("whiskers") and outliers are disorted as fences appear longer than the minimum and maximum values within 1.5 times the interquartile range above the upper quartile and below the lower quartile and often include outliers.

much higher mean total Cu body burden than exposure to Cu salt at both equal (2.8–6.2 times; 0.05 mg Cu/L) and equitoxic (4.2–8.1 times; 0.05 mg Cu/L of CuO *vs* 0.025 mg Cu/L of Cu salt and 13–42 times; 1 mg Cu/L of CuO *vs* 0.05 mg Cu/L of Cu salt) concentrations (p always < 0.003) (Fig. 1A and B).

Higher accumulation of Cu from CuO NPs compared to Cu salt which has also been shown by other authors (at equitoxic concentrations by Adam et al. (2014)) can be attributed to the uptake by filtration and the resulting accumulation of CuO NPs in the gut. As demonstrated by Lovern et al. (2008) with Au NPs, the gut of unfed adult *D. magna* was saturated with NPs in 12 h from the beginning of the exposure. Accumulation in the gut was also shown in this study (Fig. S1). The similar toxicity induced by far greater concentration of Cu in the body at equitoxic NP and salt concentrations can be explained by the lower bioavailability of Cu from CuO NPs compared to soluble Cu salt.

3.3.5. *The effect of depuration process on total Cu body burden of juvenile daphnids*

Total copper body burden in daphnids exposed to both EC_{10AFW} and $EC_{50AFW(95\%)}$ of CuO NPs concentrations decreased rapidly during depuration (Fig. 1C). Total copper body burden in daphnids exposed to EC_{10} (0.05 mg Cu/L) of CuO NPs decreased to control levels in most daphnids but remained elevated if exposed to $EC_{50AFW(95\%)}$ (1 mg Cu/L) in all the test media ($p < 0.004$) (Fig. 1C). On the contrary, total Cu body burden in daphnids exposed to Cu salt increased or remained the same after depuration (Fig. 1D). Transfer to clean test medium with algae enabled NPs to be defecated and ions excreted but also allowed uptake of copper from algae, used as food during the depuration (Adam et al., 2015b; Khan

et al., 2014; Zhao et al., 2009). The change in the gut content from NP agglomerates to algae was visible by light microscope (Fig. S1). Externally attached copper from CuO NPs or Cu salt has not been shown to have significant effect on total copper concentration as the Cu concentration in the molted carapace has been found to be similar to that in the body (Adam et al., 2015b; Bossuyt and Janssen, 2005; Wu et al., 2017).

3.3.6. The effect of test medium on total Cu body burden

The total Cu body burden after 48 h exposure at both concentrations was higher in natural waters than in AFW for CuO NP ($p < 0.05$) but not for Cu salt (Fig. 1A and B). The higher total copper body burden in daphnids, exposed to CuO NPs in lake waters compared to AFW, could be partly explained by the better stability of CuO NPs in the water phase that facilitated ingestion by daphnids. Smaller hydrodynamic size of NPs in the natural waters may have promoted faster uptake. Skjolding et al. (2014) showed that in case of mercaptoundecanoic acid stabilized 10 nm Au NPs were accumulated faster and depurated slower than 30 nm particles. The difference between zeta potential of the NPs could have also played a role - Feswick et al. (2013) showed increased uptake of negatively charged nanosized quantum dots though no differences in zeta potential were shown in their study. Luoma et al. (2016) showed that humic acid increased the uptake of PVP coated Ag NPs by snail *Lymnaea stagnalis* (non-filtering organism) possibly due to formation of humic acid-NP-biological membrane complexes that retained the particles. In their earlier work (Oliver et al., 2014), no effect of humic acid on Ag NPs bioaccumulation was found. Finally, as only living daphnids were selected for body burden measurements, lower toxicity of Cu compounds in lake waters allowed analysis of specimen with higher total Cu body burden.

Total copper body burden was significantly higher in Lake Raku control and some Cu-exposed groups, compared to other waters both before and after depuration (Fig. 1). This may indicate higher uptake of Cu in Lake Raku water which has the lowest ionic strength of all the tested media (Table 2). Higher concentrations of Ca^{2+} ions in Lake Ülemiste water and in AFW could decrease Cu^{2+} entering the cell (Playle et al., 1992). According to Gillis et al. (2005), *D. magna* exposed to metal-contaminated sediment develops two kinds of Cu reservoirs: one that can be quickly depurated and one that is incorporated into the tissues. Thus, the presence of higher Cu concentrations in daphnids in Lake Raku water also after depuration may indicate higher assimilation of copper into the tissue.

There was no significant difference between copper accumulation between daphnids exposed to CuO in Lake Raku and Lake Ülemiste water despite the differences in toxicity. DLS characterization of CUO NPs (Table 2) showed similar results in both natural waters which could explain the similar accumulation of NPs in both waters. In addition, Cu uptake may have been equally elevated due to lower hardness in Lake Raku water and due to higher DOC concentration in Lake Ülemiste water.

Post-exposure depuration from copper was comparable between different waters for CuO NPs and Cu salt at both concentrations with some exceptions (Fig. 1C and D; paragraph 3.3.5). All the daphnids exposed to EC_{10AFW} (0.05 mg Cu/L) of CuO in AFW and Lake Raku water decreased their total copper body burden to control level during depuration. In Lake Ülemiste water though, part of the specimens, exposed to EC_{10AFW} of CuO, also depurated to control level but other specimens showed no significant decrease in total copper body burden (Fig. 1C).

4. Conclusions

- Exposure of *Daphnia magna* to CuO nanoparticles (NPs) (0.05 and 1 mg Cu/L) resulted in significantly higher total copper body burden than exposure to equitoxic (0.025 and 0.05 mg Cu/L) and equal (0.05 mg Cu/L) concentration of soluble Cu salt ($CuSO_4$).
- Natural water as test medium mitigated acute toxicity of CuO NPs but increased the total copper body burden in *D. magna* after 48 h exposure compared to the artificial freshwater. Similar mitigating effect of natural water on Cu salt toxicity was observed but no effect on the total body burden of Cu from the salt.
- The total copper body burden, induced by CuO NPs exposure, strongly decreased after 24 h post-exposure depuration similarly in all the test media potentially as a result of CuO NP excretion from the gut. The Cu salt induced total body burden did not decrease during the depuration, resulting in all tested body burdens reaching comparable levels (slightly higher than the *D. magna* control) after 24 h post-exposure depuration.
- TXRF method proved suitable method for Cu measurements in individual juvenile daphnids and is well worth considering for routine use in metal accumulation experiments (for daphnis not suffering from Cu deficiency).
- Assumingly, the threat of biotransfer of copper to higher levels of aquatic food chain upon acute exposure to subtoxic CuO NPs is higher than in case of soluble Cu salt.

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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.2017.07.083

References

Adam, N., Leroux, F., Knapen, D., Bals, S., Blust, R., 2014. The uptake of ZnO and CuO nanoparticles in the water-flea *Daphnia magna* under acute exposure scenarios. Environ. Pollut. 194, 130–137. http://dx.doi.org/10.1016/j.envpol.2014.06.037.

Adam, N., Vakurov, A., Knapen, D., Blust, R., 2015a. The chronic toxicity of CuO nanoparticles and copper salt to *Daphnia magna*. J. Hazard. Mater. 283, 416–422. http://dx.doi.org/10.1016/j.jhazmat.2014.09.037.

Adam, N., Leroux, F., Knapen, D., Bals, S., Blust, R., 2015b. The uptake and elimination of ZnO and CuO nanoparticles in *Daphnia magna* under chronic exposure scenarios. Water Res. 68, 249–261. http://dx.doi.org/10.1016/j.watres.2014.10.001.

Adeleye, A.S., Oranu, E.A., Tao, M., Keller, A.A., 2016. Release and detection of nanosized copper from a commercial antifouling paint. Water Res. 102, 374–382. http://dx.doi.org/10.1016/j.watres.2016.06.056.

Blinova, I., Ivask, A., Heinlaan, M., Mortimer, M., Kahru, A., 2010. Ecotoxicity of nanoparticles of CuO and ZnO in natural water. Environ. Pollut. 158, 41–47. http://dx.doi.org/10.1016/j.envpol.2009.08.017.

Bondarenko, O., Heinlaan, M., Sihtmäe, M., Ivask, A., Kurvet, I., Joonas, E., Jemec, A., Mannerström, M., Heinonen, T., Rekulapelly, R., Singh, S., Zou, J., Pyykkö, I., Drobne, D., Kahru, A., 2016. Multilaboratory evaluation of 15 bioassays for (eco) toxicity screening and hazard ranking of engineered nanomaterials: FP7 project NANOVALID. Nanotoxicology 5390, 1–14. http://dx.doi.org/10.1080/17435390.2016.1196251.

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. Arch. Toxicol. 87, 1181–1200. http://dx.doi.org/10.1007/s00204-013-1079-4.

Borgmann, U., Charlton, C.C., 1984. Copper complexation and toxicity to *Daphnia* in natural waters. J. Gt. Lakes. Res. 10, 393–398. http://dx.doi.org/10.1016/S0380-1330(84)71855-7.

Borgmann, U., Ralph, K.M., 1983. Complexation and toxicity of copper and the free metal bioassay technique. Water Res. 17, 1697–1703. http://dx.doi.org/10.1016/0043-1354(83)90030-1.

Bossuyt, B.T.A., Janssen, C.R., 2005. Copper regulation and homeostasis of *Daphnia magna* and *Pseudokirchneriella subcapitata*: influence of acclimation. Environ. Pollut. 136, 135–144. http://dx.doi.org/10.1016/j.envpol.2004.11.024.

Civardi, C., Schwarze, F.W.M.R., Wick, P., 2015. Micronized copper wood preservatives: an efficiency and potential health risk assessment for copper-based nanoparticles. Environ. Pollut. 200, 126–132. http://dx.doi.org/10.1016/j.envpol.2015.02.018.

Conway, J.R., Adeleye, A.S., Gardea-Torresdey, J., Keller, A.A., 2015. Aggregation, dissolution, and transformation of copper nanoparticles in natural waters. Environ. Sci. Technol. 49, 2749–2756. http://dx.doi.org/10.1021/es504918q.

De Schamphelaere, K.A.C., Forrez, I., Dierckens, K., Sorgeloos, P., Janssen, C.R., 2007. Chronic toxicity of dietary copper to *Daphnia magna*. Aquat. Toxicol. 81, 409–418. http://dx.doi.org/10.1016/j.aquatox.2007.01.002.

De Schamphelaere, K.A.C., Janssen, C.R., 2004a. Effects of dissolved organic carbon concentration and source, pH, and water hardness on chronic toxicity of copper to *Daphnia magna*. Environ. Toxicol. Chem. 23, 1115–1122. http://dx.doi.org/10.1897/02-593.

De Schamphelaere, K.A.C., Janssen, C.R., 2004b. Effects of chronic dietary copper exposure on growth and reproduction of *Daphnia magna*. Environ. Toxicol. Chem. 23, 2038. http://dx.doi.org/10.1897/03-411.

EC, 2011. Recommendation 2011/696/EU of the European Commission of 18 October 2011 on the Definition of Nanomaterial. Official Journal of the European Union. L 275/38. European Commission, Brussels, Belgium.

ECHA-17-G-14-EN, 2017. Guidance on Information Requirements and Chemical Safety Assessment. Appendix R7–2 for Nanomaterials Applicable to Chapter R7c Endpoint Specific Guidance Version 2.0. European Chemicals Agency, Helsinki, Finland. http://dx.doi.org/10.2823/647499.

Evans, P., Matsunaga, H., Kiguchi, M., 2008. Large-scale application of nanotechnology for wood protection. Nat. Nano 3, 577. http://dx.doi.org/10.1038/nnano.2008.286.

Fan, W., Shi, Z., Yang, X., Cui, M., Wang, X., Zhang, D., Liu, H., Guo, L., 2012. Bioaccumulation and biomarker responses of cubic and octahedral Cu_2O micro/nanocrystals in *Daphnia magna*. Water Res. 46, 5981–5988. http://dx.doi.org/10.1016/j.watres.2012.08.019.

Ferry, J.L., Craig, P., Hexel, C., Sisco, P., Frey, R., Pennington, P.L., Fulton, M.H., Scott, I.G., Decho, A.W., Kashiwada, S., Murphy, C.J., Shaw, T.J., 2009. Transfer of gold nanoparticles from the water column to the estuarine food web. Nat. Nanotechnol. 4, 441–444. http://dx.doi.org/10.1038/nnano.2009.157.

Feswick, A., Griffitt, R.J., Siebein, K., Barber, D.S., 2013. Uptake, retention and internalization of quantum dots in *Daphnia* is influenced by particle surface functionalization. Aquat. Toxicol. 130, 210–218. http://dx.doi.org/10.1016/j.aquatox.2013.01.002.

Flickinger, A.L., Bruins, R.J.F., Winner, R.W., Skillings, J.H., 1982. Filtration and phototactic behavior as indices of chronic copper stress in *Daphnia magna* Straus. Arch. Environ. Contam. Toxicol. 11, 457–463. http://dx.doi.org/10.1007/BF01056072.

Gao, J., Youn, S., Hovsepyan, A., Llaneza, V.L., Wang, Y., Bitton, G., Bonzongo, J.-C.J., 2009. Dispersion and toxicity of selected manufactured nanomaterials in natural river water samples: effects of water chemical composition. Environ. Sci. Technol. 43, 3322–3328. http://dx.doi.org/10.1021/es803315v.

Geller, W., 1981. The filtration apparatus of *Cladocera*: filter mesh-sizes and their implications on food selectivity. Oecologia 49, 316–321.

Gillis, P.L., Chow-Fraser, P., Ranville, J.F., Ross, P.E., Wood, C.M., 2005. *Daphnia* need to be gut-cleared too: the effect of exposure to and ingestion of metal-contaminated sediment on the gut-clearance patterns of D. magna. Aquat. Toxicol. 71, 143–154. http://dx.doi.org/10.1016/j.aquatox.2004.10.016.

Gophen, M., Geller, W., 1984. Filter mesh size and food particle uptake by *Daphnia*. Oecologia 64, 408–412. http://dx.doi.org/10.1007/BF00379140.

Grillo, R., Rosa, A.H., Fraceto, L.F., 2014. Engineered nanoparticles and organic matter: a review of the state-of-the-art. Chemosphere 119C, 608–619. http://dx.doi.org/10.1016/j.chemosphere.2014.07.049.

Gustafsson, J.P., 2016. Visual MINTEQ Ver. 3.1. Available at:. https://vminteq.lwr.kth.se/download/.

Heinlaan, M., Ivask, A., Blinova, I., Dubourguier, H.-C., Kahru, A., 2008. Toxicity of nanosized and bulk ZnO, CuO and TiO2 to bacteria Vibrio fischeri and crustaceans *Daphnia magna* and Thamnocephalus platyurus. Chemosphere 71, 1308–1316. http://dx.doi.org/10.1016/j.chemosphere.2007.11.047.

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*. Aquat. Toxicol. 189, 123. http://dx.doi.org/10.1016/j.aquatox.2017.06.002.

Heinlaan, M., Muna, M., Knöbel, M., Kistler, D., Odzak, N., Kühnel, D., Müller, J., Gupta, G.S., Kumar, A., Shanker, R., Sigg, L., 2016. Natural water as the test medium for Ag and CuO nanoparticle hazard evaluation: an interlaboratory case study. Environ. Pollut. 216, 689–699. http://dx.doi.org/10.1016/j.envpol.2016.06.033.

Holden, P.A., Klaessig, F., Turco, R.F., Priester, J.H., Rico, C.M., Avila-Arias, H., Mortimer, M., Pacpaco, K., Gardea-Torresdey, J.L., 2014. Evaluation of exposure concentrations used in assessing manufactured nanomaterial environmental hazard: are they relevant? Environ. Sci. Technol. 48, 10541–10551. http://dx.doi.org/10.1021/es502440s.

Hyne, R.V., Pablo, F., Julli, M., Markich, S.J., 2005. Influence of water chemistry on the acute toxicity of copper and zinc to the cladoceran Ceriodaphnia dubia. Environ. Toxicol. Chem. 24, 1667–1675. http://dx.doi.org/10.1897/04-497r.1.

ICH-Q2B, 1996. Validation of analytical procedures: Text and Methodology. International Conference on Harmonization (ICH) of Technical Requirements for the Registration of Pharmaceuticals for Human Use, Geneva.

Ivask, A., Kurvet, I., Kasemets, K., Blinova, I., Aruoja, V., Suppi, S., Vija, H., Käkinen, A., Titma, T., Heinlaan, M., Visnapuu, M., 2014. Size-dependent toxicity of silver nanoparticles to bacteria, yeast, algae, crustaceans and mammalian cells in vitro. PloS one 9, e102108. http://dx.doi.org/10.1371/journal.pone.0102108.

Juganson, K., Ivask, A., Blinova, I., Mortimer, M., Kahru, A., 2015. NanoE-Tox: new and in-depth database concerning ecotoxicity of nanomaterials. Beilstein J. Nanotechnol 6, 1788–1804. http://dx.doi.org/10.3762/bjnano.6.183.

Kahru, A., Dubourguier, H.C., 2010. From ecotoxicology to nanoecotoxicology. Toxicology 269, 105–119. http://dx.doi.org/10.1016/j.tox.2009.08.016.

Keller, A.A., McFerran, S., Lazareva, A., Suh, S., 2013. Global life cycle releases of engineered nanomaterials. J. Nanoparticle Res. 15 http://dx.doi.org/10.1007/s11051-013-1692-4.

Khan, F.R., Kennaway, G.M., Croteau, M.N., Dybowska, A., Smith, B.D., Nogueira, A.J., Rainbow, P.S., Luoma, S.N., Valsami-Jones, E., 2014. *In vivo* retention of ingested Au NPs by *Daphnia magna*: No evidence for trans-epithelial alimentary uptake. Chemosphere 100, 97–104. http://dx.doi.org/10.1016/j.chemosphere.2013.12.051.

Kramer, K.J., Jak, R.G., van Hattum, B., Hooftman, R.N., Zwolsman, J.J., 2004. Copper toxicity in relation to surface water dissolved organic matter: biological effects to Daphnia magna. Environ. Toxicol. Chem. 23, 2971–2980. http://dx.doi.org/10.1897/03-501.1.

Käkinen, A., Bondarenko, O., Ivask, A., Kahru, A., 2011. The effect of composition of different ecotoxicological test media on free and bioavailable copper from $CuSO_4$ and CuO nanoparticles: comparative evidence from a Cu-selective electrode and a Cu-biosensor. Sensors 11, 10502–10521. http://dx.doi.org/10.3390/s111110502.

Lovern, S.B., Owen, H.A., Klaper, R., 2008. Electron microscopy of gold nanoparticle intake in the gut of *Daphnia magna*. Nanotoxicology 2, 43–48. http://dx.doi.org/10.1080/17435390801935960.

Luoma, S.N., Stoiber, T., Croteau, M.N., Römer, I., Merrifeld, R., Lead, J.R., 2016. Effect of cysteine and humic acids on bioavailability of Ag from Ag nanoparticles to a freshwater snail. NanoImpact 2, 61–69. http://dx.doi.org/10.1016/j.impact.2016.06.006.

Madhav, M.R., David, S.E.M., Kumar, R.S., Swathy, J.S., Bhuvaneshwari, M., Mukherjee, A., Chandrasekaran, N., 2017. Toxicity and accumulation of Copper oxide (CuO) nanoparticles in different life stages of *Artemia salina*. Environ. Toxicol. Pharmacol. 52, 227–238. http://dx.doi.org/10.1016/j.etap.2017.03.013.

Mages, M., Woelfl, S., Ovari, M., Tümpling Jr., W.V., Encina, F., 2004. The use of a portable total reflection X-ray fluorescence spectrometer for trace element determination in freshwater microcrustaceans (*Daphnia*). Spectrochim. Acta - Part B At. Spectrosc. 59, 1265–1272. http://dx.doi.org/10.1016/j.sab.2004.05.006.

Mages, M., Woelfl, S., Tümpling, W., 2001. A method for trace element determination of single *Daphnia* specimens using total reflection X-ray fluorescence spectrometry. Spectrochim. Acta - Part B At. Spectrosc. 56, 2209–2217. http://dx.doi.org/10.1016/S0584-8547(01)00325-1.

McMeans, B.C., Koussoroplis, A.M., Arts, M.T., Kainz, M.J., 2015. Terrestrial dissolved organic matter supports growth and reproduction of *Daphnia magna* when algae are limiting. J. Plankton. Res. 37, 1201–1209. http://dx.doi.org/10.1093/plankt/fbv083.

McWilliam, R.A., Baird, D.J., 2002. Postexposure feeding depression: a new toxicity endpoint for use in laboratory studies with *Daphnia magna*. Environ. Toxicol. Chem. 21, 1198–1205. http://dx.doi.org/10.1002/etc.5620210612.

Meyer, J.S., Clearwater, S.J., Doser, T.A., Rogaczewski, M.J., Hansen, J.A., 2007. Effects of Water Chemistry on Bioavailability and Toxicity of Waterborne Cadmium, Copper, Nickel, Lead, and Zinc to Freshwater Organisms. Society of Environmental Toxicology and Chemistry (SETAC) Press, Penscacola (FL), pp. 16–22, 57–59.

OECD, 2004. OECD Guidelines for the Testing of Chemicals. Daphnia Sp. Acute Immobilisation Test (Guideline No. 202). OECD, Paris, France.

Oliver, A.L.S., Croteau, M.N., Stoiber, T.L., Tejamaya, M., Römer, I., Lead, J.R., Luoma, S.N., 2014. Does water chemistry affect the dietary uptake and toxicity of silver nanoparticles by the freshwater snail *Lymnaea stagnalis*? Environ. Pollut. 189, 87–91. http://dx.doi.org/10.1016/j.envpol.2014.02.010.

Park, E.J., Jo, H.J., Jung, J., 2009. Combined effects of pH, hardness and dissolved organic carbon on acute metal toxicity to *Daphnia magna*. J. Ind. Eng. Chem. 15, 82–85. http://dx.doi.org/10.1016/j.jiec.2008.08.007.

Playle, R.C., Gensemer, R.W., Dixon, D.G., 1992. Copper accumulation on gills of fathead minnows: influence of water hardness, complexation and pH of the gill micro-environment. Environ. Toxicol. Chem. 113, 381–391. http://dx.doi.org/10.1002/etc.5620110312.

Polukhina, N.I., Dvurechenskaya, S.Y., Sokolovskaya, I.P., Baryshev, V.B., Anoshin, G.N., Vorotnikov, B.A., 1998. Some toxic microelements in Novosibirsk reservoir's ecosystem (data XRF SR and AAS techniques). Nucl. Instrum. Methods Phys. Res. Sect. A Accel. Spectrom. Detect. Assoc. Equip. 405, 423–427. http://dx.doi.org/10.1016/S0168-9002(97)01046-2.

Quevauviller, P., Vercoutere, K., Muntau, H., Griepink, B., 1993. Certified reference material (CRM 414) for the quality control of trace element analysis in plankton. Fresenius J. Anal. Chem. 345, 12–17. http://dx.doi.org/10.1007/BF00323319.

Schäfer, S., Buchmeier, G., Claus, E., Duester, L., Heininger, P., Körner, A., Mayer, P., Paschke, A., Rauert, C., Reifferscheid, G., Rüdel, H., 2015. Bioaccumulation in aquatic systems: methodological approaches, monitoring and assessment. Env. Sci. Eur. 27, 1–10. http://dx.doi.org/10.1186/s12302-014-0036-z.

Skjolding, L.M., Kern, K., Hjorth, R., Hartmann, N., Overgaard, S., Ma, G., Veinot, J.G.C., Baun, A., 2014. Uptake and depuration of gold nanoparticles in *Daphnia magna*. Ecotoxicology 1172–1183. http://dx.doi.org/10.1007/s10646-014-1259-x.

Suppi, S., Kasemets, K., Ivask, A., Künnis-Beres, K., Sihtmäe, M., Kurvet, I., Aruoja, V., Kahru, A., 2015. A novel method for comparison of biocidal properties of nanomaterials to bacteria, yeasts and algae. J. Hazard. Mater. 286, 75–84. http://

dx.doi.org/10.1016/j.jhazmat.2014.12.027.
Thit, A., Dybowska, A., Køber, C., Kennaway, G., Selck, H., 2015. Influence of copper oxide nanoparticle shape on bioaccumulation, cellular internalization and effects in the estuarine sediment-dwelling polychaete, Nereis diversicolor. Mar. Environ. Res. 111, 89–98. http://dx.doi.org/10.1016/j.marenvres.2015.06.009.
Thurberg, F.P., Dawson, M.A., Collier, R.S., 1973. Effects of copper and cadmium on osmoregulation and oxygen-consumption in 2 species of estuarine crabs. Mar. Biol. 23, 171–175. http://dx.doi.org/10.1007/BF00389481.
Woelfl, S., Mages, M., Mercado, S., Villalobos, L., Ovári, M., Encina, F., 2004. Determination of trace elements in planktonic microcrustaceans using total reflection X-ray fluorescence (TXRF): first results from two Chilean lakes. Anal. Bioanal. Chem. 378, 1088–1094. http://dx.doi.org/10.1007/s00216-003-2273-7.
Wu, F., Bortvedt, A., Harper, B.J., Crandon, L.E., Harper, S.L., 2017. Uptake and toxicity of CuO nanoparticles to Daphnia magna varies between indirect dietary and direct waterborne exposures. Aquat. Toxicol. 190, 78–86.
Xiao, Y., Vijver, M.G., Chen, G., Peijnenburg, W.J.G.M., 2015. Toxicity and accumulation of Cu and ZnO nanoparticles in *Daphnia magna*. Environ. Sci. Technol. 49, 4657–4664. http://dx.doi.org/10.1021/acs.est.5b00538.
Yim, J.H., Kim, K.W., Kim, S.D., 2006. Effect of hardness on acute toxicity of metal mixtures using *Daphnia magna*: prediction of acid mine drainage toxicity. J. Hazard. Mater 138, 16–21. http://dx.doi.org/10.1016/j.jhazmat.2005.11.107.
Zhao, C.M., Fan, W.H., Wang, W.X., 2009. Aqueous and dietary copper uptake and elimination in *Daphnia magna* determined by the (Cu)-C-67 radiotracer. Environ. Toxicol. Chem. 28, 2360–2366. http://dx.doi.org/10.1897/09-069.1.

Supplementary information for

Evaluation of the effect of test medium on total Cu body burden of nano CuO-exposed *Daphnia magna*: a TXRF spectroscopy study

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Figure S1. Light microscope images of *D. magna* after exposure to 1 mg Cu/L CuO nanoparticles (NPs) for 48 h in Lake Ülemiste water (left) and after the following 24 h purification in presence of algae *Raphidocelis subcapitata* (right). Prior to purification, black mass (ingested CuO NPs) can be seen in the gut while after purification, NPs have been replaced by green algae. The body length of daphnids (red lines) was measured excluding the anal spine as recommended in OECD 211 guidelines for *D. magna* reproduction test (OECD, 2004).

References

OECD, 2004. OECD Guidelines for the Testing of Chemicals. *Daphnia sp.* Acute Immobilisation Test (Guideline No. 202). OECD: Paris, France, 2004.

Appendix 4

Publication IV

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.

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Research Paper

Exposure to sublethal concentrations of Co_3O_4 and Mn_2O_3 nanoparticles induced elevated metal body burden in *Daphnia magna*

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ABSTRACT

Despite the significant progress made in ecotoxicological research on nanoparticles (NPs), there is still very limited information available regarding the biological effects of certain types of NPs such as Co_3O_4 and Mn_2O_3. Only a couple of studies provide data on their impact on aquatic organisms whereas, alarmingly, these NPs have been proposed to have high toxicity potential. In addition, more data are needed to determine whether the adverse effects the metal NPs induce on aquatic organisms are rather due to their chemical or particulate nature. To address these open questions, the (sub)lethal effects of Co and Mn NPs in parallel with the respective soluble metal salts on *Daphnia magna* were studied.

The aims of the current study were to i) assess the acute toxicity of Co_3O_4 and Mn_2O_3 NPs (primary size 10–30 nm) to *D. magna*, ii) evaluate whether the acute NP exposure at sublethal concentrations influences *D. magna* post-exposure feeding behaviour and iii) quantify *D. magna* metal body burden after exposure and after the post-exposure feeding to estimate the potential of trophic transfer of metals. Flow cytometry and total reflection X-ray fluorescence spectroscopy were applied for feeding and metal body burden evaluations, respectively. CuO NPs (primary size 22–25 nm) that are very toxic to *D. magna* were included in the study as a positive control. Since the release of metal ions is an important possibility for toxicity of metal NPs, soluble Co-, Mn- and Cu-salts were analysed in parallel.

The solubilisation of Co_3O_4 NPs in the OECD202 assay conditions was 0.1% and Mn_2O_3 NPs 35%. Mn_2O_3 NPs also produced reactive oxygen species in abiotic conditions. However Co_3O_4 and Mn_2O_3 NPs were not acutely toxic to *D. magna* (48 h EC_{50} > 100 mg metal/L) at OECD202 assay conditions. The 48 h EC_{50} values of soluble Co- and Mn-salts were 3.2 mg Co/L and 41 mg Mn/L, respectively. Post-exposure feeding behaviour after 48 h exposure to sublethal concentrations ($\leq$10 mg/L) of Co_3O_4 and Mn_2O_3 NPs differed from that of the unexposed (control) *D. magna* only at the highest exposure concentrations but was comparable to the feeding behaviour of the respective metal salt-exposed organisms. Upon 48 h exposure, dose-dependent increase of *D. magna* total metal body burden in case of both the NPs and the soluble salts was observed. After 48 h post-exposure feeding with algae *C. reinhardtii* (depuration): *D. magna* body burden remained elevated (up to 760-fold compared to the control organism) only in case of the NPs. This may indicate potential for trophic transfer of NPs/heavy metals and thus hazard for freshwater ecosystem.

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1. Introduction

Ecotoxicity of nanoparticles (NPs), particulate (1–100 nm) novel chemicals with unique properties, has been increasingly studied since the mid-2000s. The adverse effects of the most toxic metal NPs (Ag, ZnO, CuO) to freshwater organisms has been shown to be mainly induced by the released metal ions (*i.e.* chemical toxicity) (Kahru and Dubourguier, 2010; Ivask et al., 2014) whereas the toxicity mechanism for water-insoluble NPs (*e.g.* TiO_2, CeO_2) has often been related to mechanical membrane damage (Juganson et al., 2015). In case of aquatic crustaceans and algae, particle-specific effects of the NPs (*i.e.* physical toxicity) such as attachment to outer surfaces (Von Moos et al., 2014), entrapment of algal cells into agglomerates of NPs (Aruoja et al., 2015; Joonas et al., 2017) and impaired mobility (Roberts et al., 2007; Petersen et al., 2015) may interfere with normal feeding behavior of these organisms (Pradhan et al., 2015) but also contribute to trophic transfer of NPs/metals (Chen et al., 2015; Lee et al., 2015) in the freshwater ecosystem. According to a recent literature survey (Juganson et al., 2015) based on 1518 literature entries, crustaceans were the most (33% of the cases) studied organism group in nanoecotoxicology and the water flea *Daphnia magna* was the most (67% of the cases) studied crustacean. *D. magna* was also the organism of choice for the current study since it has suitable characteristics that enable addressing both the chemical and particle-specific toxicity of the NPs. *Daphnia* has been shown to ingest NPs of various composition: carbon nanotubes (Roberts et al., 2007), CuO (Heinlaan et al., 2011), Au (Lee and Ranville, 2012; Skjolding et al., 2014), CeO_2 (Auffan et al., 2013), polystyrene (Nasser and Lynch, 2016), quantum dots (Feswick et al., 2013) that all have different properties and toxic potential which, however, has not been shown to affect their uptake by this non-selective filter-feeder. Whether the daphnid gut epithelium internalizes the ingested NPs (Feswick et al., 2013; Santo et al., 2014) or not (Petersen et al., 2009; Heinlaan et al., 2011; Kwon et al., 2014) is still disputable, potentially due to different properties of the studied NPs and/or methodological approaches. Various sublethal effects in the alimentary tracts of daphnids such as morphological changes and bacterial colonization of the gut (Heinlaan et al., 2011; Kwon et al., 2014) as well as uncomplete NP depuration (Petersen et al., 2009; Zhao and Wang, 2010; Feswick et al., 2013) indicate potential long-term risks regarding nutrient assimilation as well as NP/metal transfer along the food chain (Hou et al., 2013). Nutritional status of *D. magna* has also an important impact on its brood, whose energy reserves it determines (Barata et al., 2005). Thus, the knowledge on sublethal effects of NP exposure is highly sought for and very relevant not only from the organisms' but also from the populations' and ecosystems' perspective.

Co_3O_4 and Mn_2O_3 NPs, which are in the focus of this study, are relevant nanomaterials primarily to electronic applications (Li et al., 2005; Chen et al., 2012) but are also used in food supplements (Project on Emerging Nanotechnologies, 2013). Alarmingly, Co_3O_4 and Mn_2O_3 NPs are less studied for their toxic effects than some other metal NPs such as TiO_2, Ag and ZnO (Juganson et al., 2015). However, Co and Mn NPs have been shown to be potent inducers of oxidative damage in mammalian cells *in vitro* (Papis et al., 2009; Zhang et al., 2012) and in bacteria (Kaweeteerawat et al., 2015). In addition, Co_3O_4 NPs have been shown to induce toxicity for unicellular freshwater algae and also to physically entrap algal cells (Aruoja et al., 2015). Due to the potential of both chemical and particle-specific toxicity that have been described in the literature as well as the scarcity of toxicity data, Co_3O_4 and Mn_2O_3 were chosen as model NPs for the current research.

Since *Daphnia* feeding behaviour is dependent on its nervous system *via* the appendage movement, the related disturbances are associated with neurotoxic chemicals (Agatz et al., 2013). Due to oxidative stress-inducing potential, both Co and Mn formulations could be harmful for *D. magna's* sensitive nervous system (Lilius et al., 1994). As the special focus of the study was on particle-related toxicity of the NPs and since the released metal ions are often associated with the toxicity of the metal NPs, respective soluble metal salts were also included in the study. Using the corresponding dissolved metal salts in NP aquatic hazard assessment is also recommended by the OECD (Petersen et al., 2015). In addition to Co_3O_4 and Mn_2O_3 NPs, CuO NPs (and Cu-salt) were included as positive controls as these are well-known toxicity inducers for *Daphnia* (Bondarenko et al., 2013). Furthermore, copper stress has been shown to reduce the filtration rate of *D. magna* (Flickinger et al., 1982) which is why Cu-formulations were also chosen for post-exposure feeding experiments. Since freshwater crustaceans have been shown to be more sensitive to Co^{2+} (Diamond et al., 1992), Mn^{2+} (WHO, 2004) and Cu^{+} (Bondarenko et al., 2013) than other taxa, data on the biological effects of the respective NPs are needed.

The main aim of this research was to evaluate the fitness of *Daphnia magna* that had been exposed to metal NPs at sublethal concentrations. For that, i) the acute toxicity of Co_3O_4 and Mn_2O_3 NPs to *D. magna* was evaluated to pin-point the sublethal exposure conditions; ii) the feeding behaviour of daphnids upon exposure to sublethal concentrations of these metal oxides and the respective soluble salts was studied and (iii) the effect of the metal NP exposure and the post-exposure feeding on the respective metal body burden in daphnids was assessed. We combined novel techniques: feeding of daphnids on algae was evaluated by flow cytometry and metal body burden in *D. magna* was quantified using X-ray fluorescence spectroscopy. To our knowledge, this is the first study of its kind.

The obtained knowledge is important for environmental risk assessment where the latest recommendations concern advances in ecological relevance of the NP testing (Selck et al., 2016) as well as taking into account the physical effects of the NPs (Petersen et al., 2015) both which the current study aims to address and contribute to. In addition, novel ecotoxicological data are important for QSAR modelling of NPs (Aruoja et al., 2015).

2. Materials and methods

2.1. Nanoparticles and sample preparation

Co_3O_4 (purity 99%, 10–30 nm, specific surface area 50–150 m^2/g, bulk density ~ 6.11 g/cm^3) and Mn_2O_3 (purity 99.2%, 30 nm, spherical, specific surface area 150 m^2/g, bulk density ~ 0.35 g/cm^3) nanoparticles (NPs) were purchased from US Research Nanomaterials (www.us-nano.com). CuO NPs were obtained from Intrinsiq Materials in the framework of EU FP7 NanoValid project (CuO NNV-011, 22–25 nm). All the NPs were uncoated and in powder form with the before-mentioned characteristics provided by the suppliers. NP stock suspensions were prepared at 5 g metal/L of ultrapure water (MilliQ, > 18.2 Ω, Merck Millipore, Germany) in 30 mL glass vials (Fig. S1) and probe sonicated (4 min, 40W) immediately after preparation either by Sonics VibraCell or Branson Digital Sonifier at continuous mode with no temperature adjustment. The pH values (mean ± SD) of the 5 g metal/L NP stock suspensions were 6.2 ± 0.04, 10.6 ± 0.05 and 5.5 ± 0.07 for Co_3O_4, Mn_2O_3 and CuO, respectively. As ionic controls, analytical grade metal salts $CoCl_2{\cdot}6H_2O$, $MnCl_2{\cdot}4H_2O$ (Sigma-Aldrich) and $CuSO_4{\cdot}5H_2O$ (Alfa Aesar) were used. Metal salt stock solutions were prepared at 1 g metal/L and not sonicated. All the stocks were stored in the dark at +4 °C. NP stock suspensions were used for up to 4 weeks after which fresh stocks were prepared. Additional information on the chosen control CuO NPs can be obtained from Bondarenko et al. (2016), Heinlaan et al. (2016) and Käkinen et al. (2016).

2.2. Nanoparticle size and surface charge

Hydrodynamic diameter (D_h), zeta potential (ζ) and polydispersity index (pdi) of the NPs were measured by Dynamic Light Scattering (DLS) with a Malvern Zetasizer Nano ZS (Malvern Instruments Ltd, UK) at 10 mg metal/L (Co_3O_4 and Mn_2O_3 NPs) and 5 mg Cu/L (CuO NPs) at

room temperature. Five parallel measurements were performed for each point with time delay of five seconds. The autocorrelation function accumulated at least ten runs for each parallel measurement. First, electrophoretic mobility was measured and ζ-potential calculated using Henry equation and Smoluchowski approximation. The Stokes-Einstein equation was then used to calculate hydrodynamic diameter of particles from the translational diffusion coefficient (Elimelech et al., 1998; Gregory, 2005).

According to the OECD approach, oxidation-reduction potential (ORP) is one of the physicochemical characteristics that should be measured during in ecotoxicological testing of the NPs as it has been associated with the induction of oxidative stress (Tantra et al., 2011). The ORP is also an indicator of the presence of reducing and oxidative species in the liquid media. pH and ORP were measured in medium before adding the daphnids (0 h) and after 48 h incubation with (biotic conditions) and without daphnids (abiotic conditions) in parallel with the measurements of D_h using Hach Lange HQ40d multiparameter meter with pH probe PHC101 and ORP probe IntelliCAL™ MTC101 (Hach Lange, Switzerland) at room temperature.

2.3. Analysis of nanoparticle dissolution

NP dissolution was analyzed at *Daphnia magna* 48 h immobilization assay conditions immediately upon sample preparation (0 h) and after 48 h incubation with (48 h*) and without (48 h) daphnids. Dissolved fraction was separated from NPs by ultrafiltration using Amicon Ultra-15 Centrifugal Filters (cut-off of 3 kDa, 15 mL). The applied ultrafiltration method has been shown to be suitable for separating the particulate fraction from the dissolved one (Jemec et al., 2016). The respective soluble metal salts that were included in the analyses as protocol controls, yielded expected recovery (data not shown). NPs were analysed at 1 mg metal/L (Co_3O_4 and Mn_2O_3 NPs) and 0.6 mg Cu/L (CuO NPs) and the respective soluble salts at 0.1 mg metal/L ($CoCl_2{\cdot}6H_2O$ and $MnCl_2{\cdot}4H_2O$) and 0.06 mg Cu/L ($CuSO_4{\cdot}5H_2O$). 9.9 mL of NP suspension/metal salt solution and 0.1 mL of 100 µM EDTA (to prevent sorption of metal ions on plastic) was added into the filtration tube and centrifuged at room temperature at 4000*g* for 10 min. The obtained filtrate was acidified to 1.4% (v/v) with ultrapure HNO_3 and stored at +4 °C until the ICP-MS (Agilent 7700) analysis.

2.4. Nanoparticle abiotic reactive oxygen species generation potential

Two different fluorescent probes were used to estimate formation of reactive oxygen species (ROS) by the NPs in OECD202 medium in abiotic conditions (*i.e.*, no daphnids present): 2′,7′-dichlorodihydrofluorescein (H_2DCF, Life Technologies), which reacts with a wide range of reactive oxygen and nitrogen species and the hydroxyl radical (OH·) specific 3′-(*p*-hydroxyphenyl) fluorescein (HPF, Life Technologies). Deacetylation of H_2DCF-DA to H_2DCF and both assays were conducted as described in detail by Aruoja et al. (2015). As a positive control to induce the oxidation of H_2DCF to a fluorescent 2′,7′-dichlorofluorescein (DCF), Mn_3O_4 NPs (Aruoja et al., 2015) were used. Positive control for OH· generation was Fenton reaction with 100 µM $FeSO_4{\cdot}7H_2O$ and 1.5 mM of H_2O_2. Experiments were performed in the dark at room temperature for 24 h. At the end of incubation, fluorescence (excitation at 485 nm and emission at 527 nm) was quantified using microplate fluorometer (Fluoroskan Ascent FL, Thermo Labsystems, Helsinki, Finland).

2.5. Daphnia magna acute immobilization assay

The freshwater crustacean *Daphnia magna* 48 h acute immobilization assay was conducted according to OECD202 testing guidelines with some differences as initially described by Ivask et al. (2014) (OECD, 2004). Neonate daphnids (< 24 h old) used for toxicity testing were hatched from ephippia (MicroBio Tests, Inc., Mariakerke-Gent, Belgium) that were incubated for up to 96 h at 20 °C under continuous illumination of 6000 Lux. Prior to the exposure, the neonates were pre-fed with microalgae *Chlamydomonas reinhardtii* (6.7×10^4 cells/mL) during two hours. Testing was conducted in OECD202 medium – artificial freshwater (AFW) (mg/L of ultrapure water: 294 $CaCl_2{\cdot}2H_2O$, 123.25 $MgSO_4{\cdot}7H_2O$, 64.75 $NaHCO_3$, 5.75 KCl; pH 7.8 ± 0.2) (Fig. S2) in 30-well polycarbonate test plates (MicroBio Tests, Inc., Mariakerke-Gent, Belgium) with 5 daphnids per 10 mL sample in each test well. Neonates were transferred from pre-feeding medium into the samples *via* pure AFW. At least 3 independent assays were conducted and 4 technical replicates were used per concentration. Upon 48 h of incubation at 20 °C in the dark, the immobilization (mortality) of daphnids was recorded by visual observation. The daphnid was considered immobilized if it did not resume swimming within 15 s of gentle agitation. The test was considered valid if the immobilization of control daphnids did not exceed 10%.

2.6. Post-exposure feeding evaluation of Daphnia magna

Unicellular algae *Chlamydomonas reinhardtii* CPCC11 (Canadian Phycological Culture Center; CPCC, Department of Biology, University of Waterloo, Canada) were cultivated in sterile conditions in 4x diluted Tris-acetate-phosphate medium (Harris, 2009) at 20 °C on continuous agitation of 100 rpm. The algal cells were harvested in mid-exponential phase (upon 48 h of cultivation) and washed with OECD202 AFW by centrifugation at 2083*g* for 5 min. The cells were counted in flow cytometer and diluted to concentration of 2×10^5 cells/daphnid (6.7×10^4 cells/mL) at the initiation of the post-exposure feeding (0 h) of *D. magna.* The food loading was chosen on the basis of results from pilot studies so that the algal cell density in control *D. magna* sample would not fall below the incipient limiting level after 24 h of feeding and daphnid feeding rate would not be dependent on the food concentration (McMahon and Rigler, 1965). McMahon and Rigler (1965) and Porter et al. (1983) have shown the incipient limiting levels for adult *D. magna* to be 3×10^5 *C. vulgaris* cells/mL and 10^5 *C. reinhardtii* cells/mL, respectively, but in the present research, juvenile daphnids (< 5 days old by the end of the feeding) were used, thus, according to their feeding activity, the final cell density was adjusted a little lower. The feeding experiments were conducted in 50 mL conical glass flasks, each containing 30 mL of algal suspension in OECD202 AFW. Ten actively swimming previously chemical-exposed or control daphnids that were showing no signs of stress were carefully added to each flask *via* clean OECD202 AFW. Regarding the controls, two kinds of chemical-unexposed *D. magna* were included in the experiment. One group was the so-called “standard *Daphnia* control” (daphnids were not fed during the 48 h OECD202 assay) and the other was the *D. magna* control that was fed 2×10^5 *C. reinhardtii* cells/daphnid already during the 48 h assay (“fed *Daphnia* control”). In addition, each feeding experiment included two “algal controls” (algae only). Flasks were sealed with plastic film and put into dark at 20 °C to rotate at 110 rpm. After 24 and 48 h, mortality of the feeding daphnids was recorded, the sample was re-suspended to ensure representative and homogenuous sampling and 1 mL aliquot was drawn for determining the algal cell numbers using Flow Cytometer (FCM, BD Accuri C6 equipped with an argon-ion excitation laser 488 nm, Accuri Cytometers Inc., Michigan) FL3-A (chlorophyll autofluorescence) gate. BD Accuri C6 Software 264.15 was used for data acquisition and processing. Detailed FCM parameters are provided in the Supplementary information. After both chemical exposure and post-exposure feeding, daphnids were also imaged in visible light with Olympus BX61 (camera Olympus XC30) to measure their body lengths and evaluate their NP association.

2.7. Daphnia magna metal body burden quantification

Total metal body burden in *D. magna* was measured with total reflection X-ray fluorescence (TXRF) spectroscopy (Bruker Picofox S2).

Metal measurements of daphnids were performed after 48 h immobilization assay and after 48 h post-exposure feeding. After the exposure/post-exposure feeding, living daphnids were first transferred into clean OECD202 AFW (5 min) and then frozen at −18 °C for later TXRF analysis. At least 3 individual organisms were analysed from one experiment. For the TXRF analysis, each frozen daphnid was put into 2 µL drop of HNO_3 on an individual quartz carrier (Bruker). The samples were dried at 50°–60 °C, followed by adding another 2 µL drop of HNO_3. After drying the sample for the second time, 2 µL of HNO_3 including 2.5 ppm of Ga (TXRF internal standard) was added to the sample. When samples had been dried and cooled, total metal concentration in the sample was quantified using Bruker Spectra7 software. Element concentration in the daphnid was calculated by dividing element mass by the average dry weight (dwt) of daphnids. Metal concentration of the blank sample carriers (with HNO_3 and Ga) was subtracted from all the body burden results. Dwt of daphnids was determined by weighing 220 lyophilized daphnids (in 3 sets of 110, 110 and 220 in 3 technical replicates each) with Perkin-Elmer AD-2 Autobalance (resolution 0.1 µg). For that, organisms were hatched, prefed and incubated identically to the unexposed controls of the 48 h immobilization assay. For weighing, daphnids were rinsed in MQ water and lyophilized in VirTis SP Scientific BenchTop Freeze Dryer (6KBTEL-85) at −86 °C under 25 µbar vacuum overnight (20 h). The obtained dwt of the juvenile (< 96 h old) unexposed control daphnid was 0.0059 mg and for the ease of comparison and for technical reasons, this value was also applied for metal body burden calculations of chemical-exposed organisms. However, we need to point out that there were some differences in the daphnids' body length (Table S2) that is considered a proxy for their dwt (Adam et al., 2015).

2.8. Data analysis

Data reduction was performed in MS Excel 2010 and R 3.2.3 Statistical Software (R Foundation for Statistical Computing, Vienna, Austria). Toxicity values (ECxs – effective concentrations, leading to X% of daphnid immobilization) with 95% confidence interval were calculated by log-normal model in MS Excel macro Regtox (Vindimian, 2005) based on the daphnid immobilization percentage at the end of the experiment. Regarding the results of post-exposure feeding, the data are presented as the % of the events in the respective gate in the flow cytometry. The data, more than 1.5 interquartile ranges below the first quartile or above the third quartile, were omitted. As normality of the data could not be shown, Wilcoxon rank sum test, a non-parametric test for independent samples, was used to determine the differences between the post-exposure feeding activities of the exposure groups (based on the flow cytometry data from gate 2–*D. magna* feeding byproducts, Fig. S3) and between metal body burden of chemical-exposed and control organisms.

3. Results and discussion

3.1. Nanoparticle characterization

Physicochemical characteristics of the NPs were obtained by Dynamic Light Scattering (DLS) and electrophoretic mobility measurements in OECD202 medium at time 0 h and after 48 h of incubation with (biotic conditions) and without *D. magna* (abiotic conditions) (Table 1). General trend for all the NPs was immediate agglomeration and destabilization due to the presence of monovalent and divalent electrolytes such as $CaCl_2$, $MgSO_4$, $NaHCO_3$ and KCl in the test medium (Fig. S2).

The primary particle size of Co_3O_4 and Mn_2O_3 was about 30 nm (provider data). When introduced to the OECD202 medium (0 h), the D_h of Co_3O_4 NPs increased up to 441 ± 45 nm and of Mn_2O_3 NPs up to 1387 ± 149 nm with ζ-potential values close to zero for both substances (−2.6 ± 1.2 and +0.8 ± 1.3 mV, respectively). After 48 h (and in the presence of *D. magna*), further agglomeration occurred with an increase of the D_h up to 1579 ± 476 nm for Co_3O_4 and 2431 ± 358 nm for Mn_2O_3 NPs. Negative ζ-potential values of both samples were observed. When NPs were released into the medium, their surface charge became progressively negative by passing through the point of zero charge (PZC). pH_{PZC} is equal to 4.7 for Mn_2O_3 (Kosmulski, 2009) (Table S1). It follows logically that at the pH of OECD202 medium (7.8 ± 0.2), *i.e.* above the pH_{PZC}, Mn_2O_3 NP ζ-potential was negative and equal after 48 h to −14.0 ± 1.9 mV and −18.0 ± 1.4 mV in abiotic and biotic conditions, respectively. The same trend was observed for Co_3O_4 for which the ζ-potential value was less negative after 48 h (-5.1 ± 1.7 mV and −7.2 ± 1.2 mV abiotic and biotic conditions, respectively) as the value of pH_{PZC} is close to the OECD202 medium pH (Table S1). The presence of different charged ions in the medium is also expected to have a great influence on the NP surface charge. The main ions present in the *D. magna* test medium are divalent cations such as Mg^{2+} (6%) and Ca^{2+} (22%), but also SO_4^{2-} and HCO_3^- anions up to 8% (Fig. S2). The presence of these ions leads to the NP aggregation in OECD202 medium. Two possible effects are therefore expected: charge screening and specific adsorption, which are both responsible for the final surface charge and playing kinetics roles in the surface charge equilibrium time.

The same trend was observed in the CuO NPs behaviour. The primary particle size of CuO NPs was 22−25 nm (provider data), in OECD202 medium at 0 h, the D_h of CuO NPs increased up to 718 ± 42 nm with a ζ-potential close to zero (+1.6 ± 0.3 mV). At 48 h, further agglomeration of CuO NPs occurred with an increase of the diameter up to 1304 ± 130 nm and 1456 ± 191 nm in the presence of *D. magna* with zeta potential for both samples −18.0 ± 0.7 mV. Indeed, the medium pH was close to the pH_{PZC} (for CuO pH_{PZC} varied from 5 to 9.4) (Kosmulski, 2009) (Table S1) and NP surface charge was expected to be negative below the pH_{PZC}.

When Co_3O_4 and Mn_2O_3 NPs were introduced to the medium, we observed a significant increase of the redox potential. The differences between the redox value of reference medium (277.5 mV) and NP suspensions were 64 and 28 mV for Co_3O_4 and Mn_2O_3 NPs, respectively. After 48 h and in the presence of *D. magna* the ORP showed a tendency of approaching to the value of ORP in the OECD202 medium (Table 1). The presence of CuO NPs was not found to significantly decrease the ORP and at 48 h (with and without *D. magna*), the ORP values were the same as in the medium (Table 1).

3.2. Nanoparticle dissolution

Similarly to the other NP physicochemical characteristics, NP dissolution (Table 1) was also analyzed in both abiotic and biotic conditions to learn whether incubation with daphnids affects the release of ions, which is among the most important toxicity mechanisms for metal ion-releasing NPs (Bondarenko et al., 2013; Notter et al., 2014).

Co_3O_4 NPs showed the lowest dissolution in OECD202 medium (0.1% of the nominal 1 mg Co/L at the 48 h) that was neither changing in time nor in the presence of daphnids. Mn_2O_3 NPs showed the highest dissolution percentage (35% of the nominal 1 mg Mn/L at the 48 h). Dissolution of CuO NPs in OECD202 medium (7% of the nominal 0.06 mg Cu/L at the 48 h) was higher than obtained earlier for the same batch of CuO NPs by Bondarenko et al. (2016) (1% of 10 mg Cu/L dissolved after 24 h of incubation) which could foremost be explainable by the higher solvent/NP ratio (Kasemets et al., 2009; Mwaanga et al., 2014; Heinlaan et al., 2016) used in the current study.

The presence of daphnids decreased the concentration of quantified ions especially for Mn_2O_3 NP suspensions (Table 1). One possible explanation is because the daphnids as constant filter-feeders are removing the NPs from the water column (Skjolding et al., 2014) and potentially retaining them for a prolonged period which is especially likely in the absence of food (Auffan et al., 2013; Yu and Wang, 2014) which is the case in OECD202 acute immobilization assays. The

Table 1
Physicochemical characterization of nanoparticles in OECD 202 test medium.

Sample	Measurement time	D_h [nm]	ζ [mV]	pdi	pH	ORP [mV]	Dissolution [%]
Co_3O_4 NP	0 h	441 ± 45	−2.6 ± 1.2	0.5	6.9	341.0	0.1
	48 h	656 ± 200	−5.1 ± 1.7	0.5	7.2	331.1	0.1
	48 h*	1579 ± 476	−7.2 ± 1.2	0.9	7.2	306.8	0.1 ± 0.0
Mn_2O_3 NP	0 h	1387 ± 149	0.8 ± 1.3	0.8	7.2	305.2	37
	48 h	1726 ± 868	−14.0 ± 1.9	0.8	7.2	299.2	35
	48 h*	2431 ± 358	−18 ± 1.4	0.9	7.2	285.3	17 ± 1.6
CuO NP	0 h	718 ± 42	1.6 ± 0.3	0.3	6.9	267.2	11
	48 h	1304 ± 130	−18 ± 0.7	0.6	7.2	279.4	7
	48 h*	1456 ± 191	−18 ± 0.3	0.8	7.3	279.1	5 ± 0.2
OECD202	n.a	n.a	n.a	n.a	7.6	277.5	n.a

Dynamic Light Scattering (DLS) was used for determining cumulative z-average hydrodynamic diameter (D_h), zeta potential (ζ) and polydispersity index (pdi) of Co_3O_4, Mn_2O_3 and CuO nanoparticles. All DLS measurements were conducted in five technical replicates. The asterisk (*) marks the measurements conducted in biotic conditions. Nanoparticle characterization was performed at 10 mg metal/L (Co_3O_4 and Mn_2O_3) and 5 mg metal/L (CuO). Dissolution was determined at 1 mg metal/L (Co_3O_4 and Mn_2O_3) and 0.6 mg Cu/L (CuO) and is presented as % of nominal metal concentration. The data are presented as means ± SD (where available; n = 1-5). n.a – not available; NP – nanoparticle; ORP – oxidation-reduction potential.

ingested matter (incl. NPs) in the gut is retained within endoperitrophic space but whether it is egested along with the peritrophic membrane, that could indirectly decrease the release of ions from the NPs into the water phase, has not been established (Smirnov, 2014).

3.3. Abiotic oxidative stress generation from nanoparticles

Co_3O_4 and Mn_2O_3 NPs have been shown to induce reactive oxygen species (ROS) formation and the related adverse biological effects (Zhang et al., 2012; Aruoja et al., 2015; Ivask et al., 2015; Kaweeteerawat et al., 2015). In the current study, generation of abiotic ROS was evaluated as by Aruoja et al. (2015) where in-house synthesized NPs were studied. Here, the Co_3O_4 NPs (US Research Nanomaterials) did not induce ROS formation upon 24 h of abiotic incubation at *D. magna* assay conditions (Fig. 1) possibly due to different physicochemical properties of NPs from the Co NPs used in Aruoja et al. (2015). Mn_2O_3 NPs and especially the Mn-salt caused an increase of ROS formation by 15 and 40-fold, respectively, at the highest tested concentration of 100 mg metal/L after 24 h of incubation (Fig. 1). For comparison, the used positive control, Mn_3O_4 NPs (also at 100 mg metal/L), induced ROS increase 70-fold above the background level. Interestingly, the ROS generated by Mn_2O_3 NPs and Mn-salt were of different nature: while Mn_2O_3 NPs at the highest concentration (100 mg Mn/L) produced highly reactive hydroxyl radicals (OH·), these radicals were not detected for Mn-salt (Fig. 1). Despite having shown the highest ROS-induction potential, compared to Co and Cu-formulations, the Mn-formulations (both the NPs and the salt) were of low toxicity to *D. magna* (Table 2).

3.4. Nanoparticle toxicity to Daphnia magna

The 48 h acute toxicity of Co_3O_4, Mn_2O_3 and CuO NPs and the respective soluble metal salts to *D. magna* was assessed according to OECD202 guidelines. The results showed that the chosen types of Co_3O_4 and Mn_2O_3 NPs did not induce acute toxicity to *D. magna* in standard 48 h exposure up to 100 mg metal/L (Table 2). The respective 48 h EC_{50} values for soluble metal salts were 3.2 mg Co/L and 41 mg Mn/L (Table 2). The toxicity of CuO NPs (the positive control) to *D. magna* (48 h EC_{50} = 1 mg Cu/L) was expectedly in agreement with the published data on the same batch of CuO NPs (NNV-011 from NanoValid project) (Bondarenko et al., 2016; Heinlaan et al., 2016) as was the toxicity of Cu-salt (48 h EC_{50} = 0.08 mg Cu/L) (Heinlaan et al., 2008; Heinlaan et al., 2011; Adam et al., 2014).

Also, the toxicity of the soluble Co- and Mn-salts was in line with literature data. Khangarot and Ray, 1989 have shown 48 h EC_{50} of Co^{2+} for *D. magna* to be 1.5 mg Co^{2+}/L. Toxicity of $MnCl_2$ has been

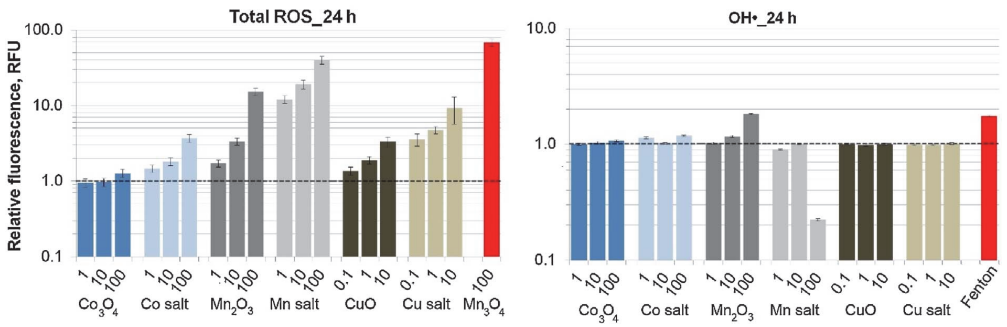

Fig. 1. Abiotic generation of reactive oxygen species (ROS) by Co_3O_4, Mn_2O_3 and CuO nanoparticles and the respective soluble metal salts ($CoCl_2{\cdot}6H_2O$, $MnCl_2{\cdot}4H_2O$, $CuSO_4{\cdot}5H_2O$). The ROS were measured using 2′,7′-dichlorodihydrofluorescein diacetate H_2DCFDA for total ROS and (3′-(*p*-hydroxyphenyl) fluorescein HPF for hydroxyl radicals (OH·). The ROS were quantified after 1 h (1 h) and 24 h of incubation (24 h) in OECD202 artificial freshwater in the dark at room temperature and were normalized by dividing the measured values with background fluorescence of OECD202 artificial freshwater All the sample concentrations (chosen from toxicity testing) are presented as nominal mg metal/L. For total ROS, Mn_3O_4 NPs (from Aruoja et al., 2015) and for OH·, Fenton reaction ($FeSO_4{\cdot}7H_2O$ in H_2O_2) were used as positive controls (red bars). The black dotted line denotes the background fluorescence (1.0) of OECD202 artificial freshwater. Results are presented as means ± SD (n = 3). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Acute (48 h) toxicity values for the studied metal oxide nanoparticles and the respective soluble metal salts for *Daphnia magna*.

	Co_3O_4 NP	$CoCl_2{\cdot}6H_2O$	Mn_2O_3 NP	$MnCl_2{\cdot}4H_2O$	CuO NP	$CuSO_4{\cdot}5H_2O$
EC_{10}	> 100	1.9 (1.6–1.9)	> 100	23 (21.8–23.5)	0.12 (0.08–0.17)	0.04 (0.03–0.04)
EC_{20}	> 100	2.2 (2.0–2.3)	> 100	28 (26.8–28.6)	0.25 (0.19–0.32)	0.05 (0.04–0.05)
EC_{50}	> 100	3.2 (2.9–3.3)	> 100	41 (39.8–41.7)	1.0 (0.89–1.2)	0.08 (0.06–0.08)

All the data (mg metal/L) are presented as average values with 95% confidence interval in the brackets. n ≥ 3. EC – effective concentration; NP – nanoparticle.

shown to vary from 4.0 to 56.1 mg Mn^{2+}/L (Baird et al., 1991; Bowmer et al., 1998). In Teodorovic et al., 2009; *D. magna* was more sensitive to $MnSO_4$ (48 h LC_{50} = 10.28 mg Mn^{2+}/L) than the bacteria (EC_{50} values > 100 mg Mn^{2+}/L).

However, very little data are available about the toxicity of Co_3O_4 and Mn_2O_3 NPs and with an exception of Griffitt et al. (2008), there are no data regarding *Daphnia*. In the current study, the lack of acute toxicity (daphnid immobilization) (Table 2) of the Co_3O_4 NPs could be explained by low dissolution (Table 1) and low ROS-induction potential (Fig. 1). However, despite much more significant dissolution and oxidative potential, the Mn_2O_3 NPs were also non-toxic in the OECD202 test conditions. Based on the studies on mammalian cells *in vitro* and bacteria, Co_3O_4 and Mn_2O_3 (and CuO) NPs have been classified as highly toxic NPs amongst 24 other metal oxide NPs by Zhang et al. (2012) and Kaweeteerawat et al. (2015). Both cited studies propose ROS induction and metal ion release potential as the predictors of metal oxide NP toxicity that could also apply for higher organisms.

The recorded low acute toxicity of the Co_3O_4 NPs in the current study is in line with literature data where no toxicity of Co_3O_4 for particle ingesting protozoa *T. themophila* (Aruoja et al., 2015) up to 100 mg metal/L, despite the generation of abiotic ROS, was shown. Also, in a number of studies, acute toxicity of Co_3O_4 NPs only above 100 mg/L has been shown to occur for mammalian cells *in vitro* (Ivask et al., 2015) and bacteria (Kaweeteerawat et al., 2015; Aruoja et al., 2015; Wang et al., 2016). Griffitt et al. (2008) showed toxicity of Co NPs for crustacean *C. dubia* neonates with the 48 h EC_{50} 1.67 mg Co NPs/L and of Co-salt (tested as Cl_2) 94.66 mg Co^{2+}/L. However, the 95% CI for Co^{2+} (61.94–144.67) was larger than in the present study (2.9–3.3). Aruoja et al. (2015) showed 72 h EC_{50} for *P. subcapitata* to be 1.11 mg Co/L for Co_3O_4 NPs and 0.1 mg Co/L for $CoCl_2{\cdot}6H_2O$. Toxicity of Co NPs, demonstrated in Griffitt et al. (2008) and Aruoja et al. (2015), may be explainable by different physicochemical characteristics of the Co NPs (smaller primary size, higher dissolution), different toxicity endpoints and/or different test organisms.

Compared to cobalt, the biological effects of Mn_2O_3 NPs are even less studied. The few published studies have reported toxicity (expressed as IC_{50}–inhibitory concentration, reducing the cell viability by 50%) to bacteria *E. coli*, yeast *S. cerevisiae* and mammalian cells *in vitro* at 70 (Kaweeteerawat et al., 2015), 170 (Otero-González et al., 2013) and 10.2–13.2 (Zhang et al., 2012) mg Mn_2O_3/L, respectively. In all the cited studies, Mn_2O_3 NPs also induced membrane damage to the test organisms and cells.

Based on the acute toxicity results, sublethal exposure concentrations of Co, Mn and Cu-formulations (Table S2) were chosen for post-exposure feeding and total metal body burden assessment.

3.5. *Daphnia magna* post-exposure feeding

In the literature, negative impact of xenobiotics on *D. magna* feeding activity has been shown in case of exposure of daphnids to various types of chemicals, *e.g.*, metals (Kamaya et al., 2011; Ginatullina et al., 2013), the pesticides imidacloprid (Agatz et al., 2013) and lindane (Hartgers et al., 1999; Furuhagen et al., 2014), the pharmaceutical haloperidol (Furuhagen et al., 2014) and terphenyl coolant (McMahon, 1973).

In the current study, post-exposure feeding of the previously metal NP-exposed *D. magna* was conducted with unicellular algae *Chlamydomonas reinhardtii* and daphnid feeding behaviour was assessed on the basis of the feeding by-products in the respective gate of the flow cytometry data analysis (Fig. S3). Quantification of feeding by-products was used since this value was characteristic only to the algal samples where daphnids were present and missing in „algae only“ control samples. The feeding evaluation by the proportion of *C. reinhardtii* cells or by the proportion of „debris“ (Fig. S3) were neither considered reliable since due to a variety of reasons, the algal cells could have reduced amount of chlorophyll and the experiment was not carried out in sterile conditions. The *C. reinhardtii* cells were used as food for daphnids at density of 6×10^4 cells/mL (2×10^5 cells/daphnid). The algal cell number in algal controls was decreasing about 10% in the first 24 h of *D. magna* feeding experiment and 15% from 24 to 48 h (Fig. S4).

The fed *Daphnia* control was included since *Daphnia* spp. feeding has been shown to be dependent on the body length (Knoechel and Holtby, 1986). Indeed, the fed *Daphnia* control was feeding much more actively (Table S3) at both time-points than the standard *Daphnia* control. This phenomenon could be potentially attributed to the 30% difference in the body length (Lampert, 1987) at both measuring times (Table S2) between the *D. magna* controls. The feeding of the chemical-exposed daphnids was compared to the standard *Daphnia* control (Fig. 2) since the former ones were also not fed during the 48 h assay.

Significantly ($p < 0.05$) higher and lower feeding at the 24 h from that of the standard *Daphnia* control was recorded for Co_3O_4 and Mn_2O_3 (10 mg metal/L) exposed daphnids, respectively (Fig. 2). Similarly to Mn_2O_3 NP, the feeding of Mn-salt (10 mg metal/L) exposed daphnid was also significantly lower from the control organism which indicates that the reduced feeding was not NP-specific but possibly metal-specific. For Co-salt, significantly higher feeding was observed at 1 mg Co/L which was the highest test concentration due to the toxicity of Co^{2+} (Table 2). At the 48 h, the differences only prevailed for Mn-formulations (reduced feeding compared to the control). For Cu-formulations, significantly higher feeding was recorded for 0.06 mg Cu/L for both NP and the salt whereas for Cu-salt, this exposure concentration was the lower limit of 48 h EC_{50} (Table 2) (survived organisms were chosen for the feeding). No differences from the control organisms were seen for Cu at the 48 h of feeding. The obtained post-exposure feeding data indicate that previous exposure to NPs did not affect the *D. magna* feeding on algae significantly more/differently than metal salt

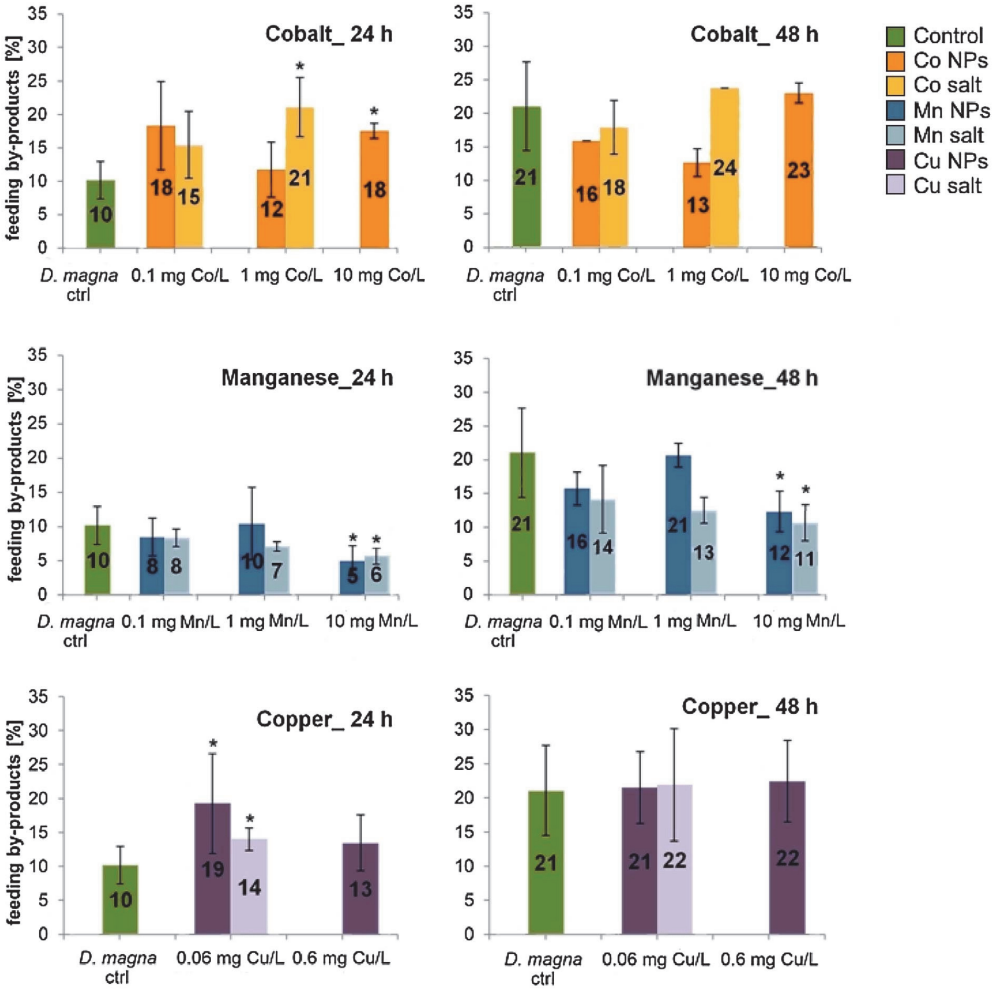

Fig. 2. Post-exposure feeding behaviour of *Daphnia magna*. The 24 and 48 h feeding of daphnids, previously exposed to sublethal concentrations of nanoparticles (Co_3O_4, Mn_2O_3 and CuO) and the respective soluble metal salts ($CoCl_2{\cdot}6H_2O$, $MnCl_2{\cdot}4H_2O$ and $CuSO_4{\cdot}5H_2O$). The data (% of total events) were calculated from the flow cytometer results' gate 2: *D. magna* feeding by-products (Fig. S3). All the *D. magna* metal exposure concentrations (mg metal/L) on the x-axis are nominal. Data are presented as mean ± SD (n = 2–5) with the mean value depicted as the bar. Significant differences from the *D. magna* control daphnids (not previously exposed to chemicals) are indicated by an asterisk (*) ($p < 0.05$).

exposure. However, at this point we are unable to explain why in the case of Co the daphnids were feeding more intensively than the control, but in the case of Mn, less intensively.

Our results are comparable to Nasser and Lynch (2016) where no statistically significant decline in 6 h *D. magna* feeding was recorded during exposure to polystyrene NPs. Similarly, Taylor et al. (1998) found no difference in 24 h of post-exposure feeding between the control and dissolved Cd-exposed organisms (both starved). Fast (upon 2 h of feeding) post-exposure recovery from a chemical (lindane) in *D. magna* was reported by Hartgers et al. (1999). McWilliam and Baird (2002) showed that for Cu, *D. magna* feeding rate in the post-exposure period was concentration-dependent, whereas for other heavy metals (Cd, Zn) this was not the case. In all the cited studies, *Daphnia* were exposed to sublethal chemical concentrations and fed with algae.

3.6. *Daphnia magna* total metal body burden

Common observations for all the tested metal formulations were that *D. magna* total metal body burden after 48 h exposure as well as after the 48 h feeding increased with increasing nominal exposure concentration and was in all cases higher for metal NPs than for the respective salt (Fig. 3).

Despite the decrease in metal body burden after 48 h post-exposure feeding (up to 475 fold for Co_3O_4 and 14-fold for Mn_2O_3 NPs) it remained about 4–760-fold more elevated for Co-NPs and 7–500-fold more elevated for Mn NPs than the background levels of the unexposed control organisms (Fig. 3). This observation may imply long-term adverse effects for the affected organisms themselves but also potential for metal transfer along the food chain. At the same time, residual metal

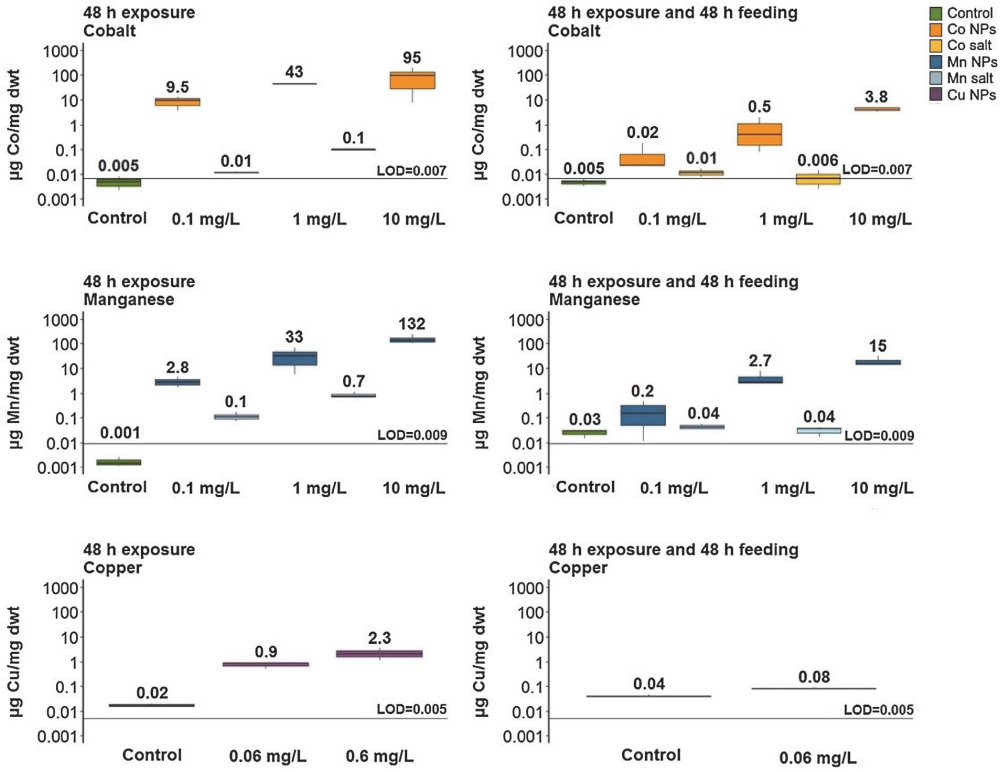

Fig. 3. Total metal body burden of *Daphnia magna* upon exposure to sublethal concentrations of nanoparticles (Co_3O_4, Mn_2O_3 and CuO NPs) and the respective soluble metal salts ($CoCl_2{\cdot}6H_2O$ and $MnCl_2{\cdot}4H_2O$) after 48 h exposure and 48 h post-exposure feeding. All the metal exposure concentrations (on x-axis) are nominal mg metal/L. For the toxicity of the tested compounds, see Table 2 and Table S2. The box contains values between 25th and 75th percentile and the line inside the box shows the median. Fences ("whiskers") indicate minimum and maximum values within 1.5 times the interquartile range above the upper quartile and below the lower quartile. The black line on the figures denotes the median value of limit of detection (LOD) of the respective metal (µg metal/mg *D. magna* dwt) (Table S4). Dwt – dry weight.

body burdens in Co and Mn-salt exposed organisms after 48 h feeding were comparable (1.2–2-fold higher) to the unexposed organisms. CuO NPs induced somewhat lower metal body burdens than Co_3O_4 and Mn_2O_3 NPs at comparable exposure concentrations that after 48 h feeding were similar to the unexposed control.

In the literature, higher metal uptake in CuO NP-exposed daphnids, compared to Cu-salt exposed ones has been observed by Adam et al. (2014). The factors that have been shown to facilitate the depuration of NP-exposed organisms are maternal transfer (Zhao and Wang, 2010), molting (Zhao and Wang, 2010; Adam et al., 2014) and, most importantly, the availability of food (algae) (Zhao and Wang, 2010; Skjolding et al., 2014). In Skjolding et al. (2014) depuration of Au NP-exposed (0.4 mg Au/L) daphnids in the presence of algae has been shown to result in about 3-fold lower residual body burden compared to the depuration, conducted in the absence of food.

Previous studies have suggested that different NPs can be ingested by daphnids and may accumulate inside the organisms or on their surface (Juganson et al., 2015), which may lead to trophic transfer of these NPs. In the current study, light microscope observations after 48 h post-exposure feeding showed dark deposits on the carapace of about 30% (data not shown) of Mn_2O_3 NPs-exposed daphnids (Fig. 4). Such deposits were observed neither in control nor in salt-exposed organisms (Fig. 4). In case of Co_3O_4, potential NP agglomerates were observed in the gut after 48 h exposure and by the end of the 48 h feeding were no longer detectable (Fig. 4). Interestingly, these observations were in agreement with the TXRF results, which showed higher residual metal body burden in *D. magna* after the feeding for Mn_2O_3 than for Co_3O_4 NPs.

4. Conclusions

This study on the biological effects of Co_3O_4 and Mn_2O_3 nanoparticles (NPs) on aquatic crustacean *Daphnia magna* showed that neither of these metal oxides was acutely toxic to *Daphnia magna* in standard OECD202 testing conditions, *i.e.* 48 h EC_{50} > 100 mg metal/L. After having been exposed to sublethal concentrations (≤10 mg/L) of Co_3O_4 and Mn_2O_3 NPs for 48 h, the post-exposure feeding behaviour of *D. magna* on *C. reinhardtii* was largely comparable to non-exposed control and metal-salt-exposed organisms. No particle-related effects of the studied NPs on *D. magna* feeding behaviour were thus recorded. However, we observed a concentration-dependent increase of total metal body burden in both the NP- and soluble metal salt-exposed daphnids. The body burden remained elevated in case of NPs (but not of soluble salts) despite the post-exposure feeding (depuration) of daphnids with algae. This elevated body burden may lead to accumulation of these metals in the food chain.

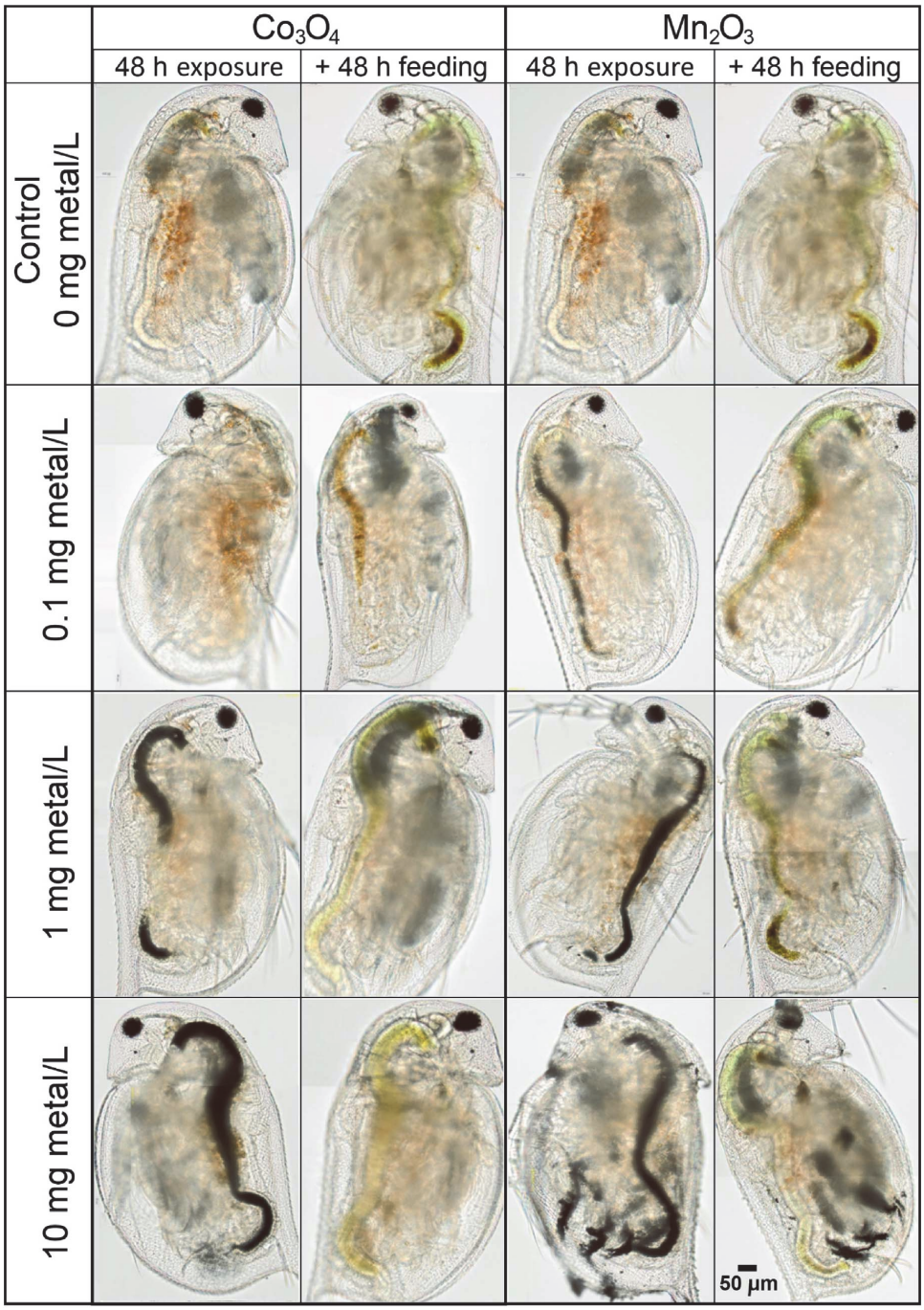

Fig. 4. *Daphnia magna* after 48 h exposure to sublethal concentrations of nanoparticles followed by 48 h post-exposure feeding on algae *Chlamydomonas reinhardtii* (depuration). First row: unexposed control *D. magna* after 48 h exposure and after 48 h post-exposure feeding. *D. magna* after 48 h Co_3O_4 nanoparticle exposure (1st column) and after 48 h post-exposure feeding (2nd column). *D. magna* after 48 h Mn_2O_3 nanoparticle exposure (3rd column) and after 48 h post-exposure feeding (4th column). The nominal exposure concentrations (mg metal/L) are indicated left to the rows.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.aquatox.2017.06.002.

References

Adam, N., Leroux, F., Knapen, D., Bals, S., Blust, R., 2014. The uptake of ZnO and CuO nanoparticles in the water-flea *Daphnia magna* under acute exposure scenarios. Environ. Pollut. 194, 130–137.

Adam, N., Leroux, F., Knapen, D., Bals, S., Blust, R., 2015. The uptake and elimination of ZnO and CuO nanoparticles in *Daphnia magna* under chronic exposure scenarios. Water Res. 68, 249–261.

Agatz, A., Cole, T.A., Preuss, T.G., Zimmer, E., Brown, C.D., 2013. Feeding inhibition explains effects of imidacloprid on the growth, maturation, reproduction, and survival of *Daphnia magna*. Environ. Sci. Technol. 47, 2909–2917.

Aruoja, V., Pokhrel, S., Sihtmäe, M., Mortimer, M., Mädler, L., Kahru, A., 2015. Toxicity of 12 metal based nanoparticles to algae, bacteria and protozoa. Environ. Sci. Nano 2, 630–644.

Auffan, M., Bertin, D., Chaurand, P., Pailles, C., Dominici, C., Rose, J., Bottero, J.-Y., Thiery, A., 2013. Role of molting on the biodistribution of CeO_2 nanoparticles within *Daphnia pulex*. Water Res. 47 (12), 3921–3930.

Baird, D.J., Barber, I., Bradley, M., Soares, A.M.V.M., Calow, P., 1991. A comparative study of genotype sensitivity to acute toxic stress using clones of *Daphnia magna* Straus. Ecotoxicol. Environ. Saf. 21 (3), 257–265.

Barata, C., Varo, I., Navarro, J.C., Arun, S., Porte, C., 2005. Antioxidant enzyme activities and lipid peroxidation in the freshwater cladoceran *Daphnia magna* exposed to redox cycling compounds. Comparative Biochemistry and Physiology Part C. Toxicol. Pharmacol. 140 (2), 175–186.

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. Arch. Toxicol. 87, 1181–1200.

Bondarenko, O., Heinlaan, M., Sihtmäe, M., Ivask, A., Kurvet, I., Jemec, A., Mannerström, M., Heinonen, T., Rekulapelly, R., Singh, S., Pyykkö, I., Drobne, D., Kahru, A., 2016. Multilaboratory evaluation of 15 bioassays for (eco)toxicity screening and hazard ranking of engineered nanomaterials: FP7 project NANOVALID. Early Online 1–14. http://dx.doi.org/10.1080/17435390.2016.1196251.

Bowmer, C.T., Hooftman, R.N., Hanstveit, A.O., Venderbosch, P.W.M., van der Hoeven, N., 1998. The ecotoxicity and the biodegradability of lactic acid, alkyl lactate esters and lactate salts. Chemosphere 37 (7), 1317–1333.

Chen, Z., Jiao, Z., Pan, D., Li, Z., Wu, M., Shek, C.-H., Wu, C.M.L., Lai, J.K.L., 2012. Recent advances in manganese oxide nanocrystals: fabrication, characterization, and microstructure. Chem. Rev. 112, 3833–3855.

Chen, J., Li, H., Han, X., Wei, X., 2015. Transmission and accumulation of Nano-TiO_2 in a 2-step food chain (*Scenedesmus obliquus* to *Daphnia magna*). Bull. Environ. Contam. Toxicol. 95 (2), 145–149.

Diamond, J.M., Winchester, E.L., Mackler, D.G., Rasnake, W.J., Fanelli, J.K., Gruber, D., 1992. Toxicity of cobalt to freshwater indicator species as a function of water hardness. Aquat. Toxicol. 22, 163–180.

Elimelech, M., Jia, X., Gregory, J., Williams, R., 1998. Particle Deposition and Aggregation: Measurement, Modelling and Simulation. Butterworth-Heinemann.

Feswick, A., Griffitt, R.J., Siebein, K., Barber, D.S., 2013. Uptake, retention and internalization of quantum dots in *Daphnia* is influenced by particle functionalization. Aquat. Toxicol. 130–131, 210–218.

Flickinger, A.L., Bruins, R.J.F., Winner, R.W., Skillings, J.H., 1982. Filtration and phototactic behavior as indices of chronic copper stress in *Daphnia magna* Straus. Arch. Environ. Contam. Toxicol. 11, 457–463.

Furuhagen, S., Liewenborg, B., Breitholtz, M., Gorokhova, E., 2014. Feeding activity and xenobiotics modulate oxidative status in *Daphnia magna*: Implications for ecotoxicological testing. Environ. Sci. Technol. 48, 12886–12892.

Ginatullina, E., Chiken, A., Atwell, L., Kamaya, M., 2013. Engineering of a new acute bioassay design based on feeding behaviour of *D. magna* with fluorescent microbeads. Int. J. Emerg. Technol. Adv. Eng. 3 (11), 671–676.

Gregory, J., 2005. Particles in Water: Properties and Processes. CRC Press.

Griffitt, R.J., Luo, J., Gao, J., Bonzongo, J.-C., Barber, D.S., 2008. Effects of particle composition and species on toxicity of metallic nanomaterials in aquatic organisms. Environ. Toxicol. Chem. 27 (9), 1972–1978.

Harris, E.H., 2009. *Chlamydomonas* in the Laboratory, The *Chlamydomonas* Sourcebook. Introduction to *Chlamydomonas* and Its Laboratory Use, 2nd ed. Academic Press, San Diego, pp. 243.

Hartgers, E.M., Heugens, E.H.W., Deneer, J.W., 1999. Effects of lindane on the clearance rate of *Daphnia magna*. Arch. Environ. Contam. Toxicol. 36, 399–404.

Heinlaan, M., Ivask, A., Blinova, I., Dubourguier, H.-C., Kahru, A., 2008. Toxicity of nanosized and bulk ZnO, CuO and TiO_2 to bacteria *Vibrio fischeri* and crustaceans *Daphnia magna* and *Thamnocephalus platyurus*. Chemosphere 71 (7), 1308–1316.

Heinlaan, M., Kahru, A., Kasemets, K., Arbeille, B., Prensier, G., Dubourguier, H.-C., 2011. Changes in the *Daphnia magna* midgut upon ingestion of copper oxide nanoparticles: a transmission electron microscopy study. Water Res. 45 (1), 179–190.

Heinlaan, M., Muna, M., Knöbel, M., Kistler, D., Odzak, N., Kühnel, D., Müller, J., Gupta, G.S., Kumar, A., Shanker, R., Sigg, L., 2016. Natural water as the test medium for Ag and CuO nanoparticle hazard evaluation: an interlaboratory case study. Environ. Pollut. 216, 689–699. http://dx.doi.org/10.1016/j.envpol.2016.06.033.

Hou, W.-C., Westerhoff, P., Posner, J.D., 2013. Biological accumulation of engineered nanomaterials: a review of current knowledge. Environ. Sci. Process. Impacts 15, 103–122.

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.

Ivask, A., Titma, T., Visnapuu, M., Vija, H., Käkinen, A., Sihtmäe, M., Pokhrel, S., Mädler, L., Heinlaan, M., Kisand, V., Shimmo, R., Kahru, A., 2015. Toxicity of 11 metal oxide nanoparticles to three mammalian cell types *in vitro*. Curr. Top. Med. Chem. 15, 1914–1929.

Jemec, A., Kahru, A., Potthoff, A., Drobne, D., Heinlaan, M., Böhme, S., Geppert, M., Novak, S., Schirmer, K., Rohit, 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. Environ. Int. 87, 20–32.

Joonas, E., Aruoja, V., Olli, K., Syvertsen-Wiig, G., Vija, H., Kahru, A., 2017. Potency of (doped) rare earth oxide particles and their constituent metals to inhibit algal growth and induce direct toxic effects. Sci. Total Environ. 593–594, 478–486.

Juganson, K., Ivask, A., Blinova, I., Mortimer, M., Kahru, A., 2015. NanoE-Tox: new and in-depth database concerning ecotoxicity of nanomaterials. Beilstein J. Nanotechnol. 6, 1788–1804.

Käkinen, A., Kahru, A., Nurmsoo, H., Kubo, A.-L., Bondarenko, O.M., 2016. Solubility-driven toxicity of CuO nanoparticles to Caco2 cells and *Escherichia coli*: effect of sonication energy and test environment. Toxicol. in Vitro 36, 172–179.

Kahru, A., Dubourguier, H.-C., 2010. From ecotoxicology to nanoecotoxicology. Toxicology 269 (2–3), 105–119.

Kamaya, M., Sonamoto, M., Nagashima, K., Ginatullina, E.N., 2011. A simple bioassay using fluorescent microbeads and *Daphnia magna*. J. Environ. Eng. 5, 1613–1616.

Kasemets, K., Ivask, A., Dubourguier, H.-C., Kahru, A., 2009. Toxicity of nanoparticles of ZnO, CuO and TiO_2 to yeast *Saccharomyces cerevisiae*. Toxicol. in Vitro 23, 1116–1122.

Kaweeteerawat, C., Ivask, A., Liu, R., Zhang, H., Chang, C.H., Low-Kam, C., Fischer, H., Ji, Z., Pokhrel, S., Cohen, Y., Telesca, D., Zink, J., Mädler, L., Holden, P.A., Nel, A., Godwin, H., 2015. Toxicity of metal oxide nanoparticles in *Escherichia coli* correlates with conduction band and hydration energies. Environ. Sci. Technol. 49, 1105–1112.

Khangarot, B.J., Ray, P.K., 1989. Investigation of correlation between physicochemical properties of metals and their toxicity to the water flea *Daphnia magna* Straus. Ecotoxicol. Environ. Saf. 18 (2), 109–120.

Knoechel, R., Holtby, L.B., 1986. Construction and validation of a body-length-based model for the prediction of cladoceran community filtering rates. Limnol. Oceanogr. 31 (1), 1–16.

Kosmulski, M., 2009. Compilation of PZC and IEP of sparingly soluble metal oxides and hydroxides from literature. Adv. Colloid Interface Sci. 152 (1–2), 14–25.

Kwon, D., Nho, H.W., Yoon, T.H., 2014. X-ray and electron microscopy studies on the biodistribution and biomodification of iron oxide nanoparticles in *Daphnia magna*. Colloids Surf. B: Biointerfaces 122, 384–389.

Lampert, W., 1987. In: In: Peters, R.H., de Bernardi, R. (Eds.), Feeding and Nutrition in *Daphnia*, vol. 45. Memorie dell'Istituto Italiano di Idrobiologia, pp. 143–192.

Lee, B.T., Ranville, J.F., 2012. The effects of hardness on the stability of citrate-stabilized gold nanoparticles and their uptake by *Daphnia magna*. J. Hazard. Mater. 213, 434–439.

Lee, W.-M., Yoon, S.-J., Shin, Y.-J., An, Y.-J., 2015. Trophic transfer of gold nanoparticles from *Euglena gracilis* or *Chlamydomonas reinhardtii* to *Daphnia magna*. Environ. Pollut. 201, 10–16.

Li, W.Y., Xu, L.N., Chen, J., 2005. Co_3O_4 Nanomaterials in lithium-ion batteries and gas sensors. Adv. Funct. Mater. 15, 851–857.

Lilius, H., Isomaa, B., Holström, T., 1994. A comparison of the toxicity of 50 reference chemicals to freshly isolated rainbow trout hepatocytes and *D. magna*. Aquat. Toxicol. 30, 47–60.

McMahon, J.W., Rigler, F.H., 1965. Feeding rate of *Daphnia magna* Straus in different foods labeled with radioactive phosphorous. Limnol. Oceanogr. 10 (1), 105–113.

McMahon, J.W., 1973. Feeding Rate of Daphnia Magna Straus in Different Concentrations of the Terphenyl Coolant – HB-40. Technical Report Vol. 4361 ATOMIC ENERGY OF CANADA LIMITED AECL.

McWilliam, R.A., Baird, D.J., 2002. Postexposure feeding depression: a new toxicity endpoint for use in laboratory studies with *Daphnia magna*. Environ. Toxicol. Chem. 21 (6), 1198–1205.

Mwaanga, P., Carraway, E.R., van den Hurk, P., 2014. The induction of biochemical changes in *Daphnia magna* by CuO and ZnO nanoparticles. Aquat. Toxicol. 150, 201–209.

Nasser, F., Lynch, I., 2016. Secreted protein eco-corona mediates uptake and impacts of polystyrene nanoparticles on *Daphnia magna*. J. Proteomics 137, 45–51.

Notter, D.A., Mitrano, D.M., Nowack, B., 2014. Are nanosized or dissolved metals more toxic in the environment? A meta-analysis. Environ. Toxicol. Chem. 33, 2733–2739.
OECD, 2004. OECD Guideline for Testing of Chemicals. No 202 Daphnia Sp. Acute Immobilisation Test OECD, Paris.
Otero-González, L., Garcia-Saucedo, C., Field, J.A., Sierra-Álvares, R., 2013. Toxicity of TiO_2, ZrO_2, Fe°, Fe_2O_3, and Mn_2O_3 nanoparticles to the yeast *Saccharomyces cerevisiae*. Chemosphere 93, 1201–1206.
Papis, E., Rossi, F., Raspanti, M., Dalle-Donne, I., Colombo, G., Milzani, A., Bernardini, G., Gornati, R., 2009. Engineered cobalt oxide nanoparticles readily enter cells. Toxicol. Lett. 189, 253–259.
Petersen, E.J., Akkanen, J., Kukkonen, J.V.K., Weber, W.J., 2009. Biological uptake and depuration of carbon nanotubes by *Daphnia magna*. Environ. Sci. Technol. 43 (8), 2969–2975.
Petersen, E.J., Diamond, S.A., Kennedy, A.J., Goss, G.G., Ho, K., Lead, J., Hanna, S.K., Hartmann, N.B., Hund-Rinke, K., Mader, B., Manier, N., Pandard, P., Salinas, E.R., Sayre, P., 2015. Adapting OECD aquatic toxicity tests for use with manufactured nanomaterials: key issues and consensus recommendations. Environ. Sci. Technol. 49, 9532–9547.
Porter, K.G., Orcutt Jr, J.D., Gerritsen, J., 1983. Functional response and fitness in a generalist filter feeder, *Daphnia magna* (*Cladocera, Crustacea*). Ecology 64 (4), 735–742.
Pradhan, A., Geraldes, P., Seena, S., Pascoal, C., Cássio, F., 2015. Natural organic matter alters size-dependent effects of nanoCuO on the feeding behaviour of freshwater invertebrate shredders. Sci. Total Environ. 535, 94–101.
Project on Emerging Nanotechnologies, 2013. Consumer Products Inventory. (Retrieved [Sept. 5, 2016], from http://www.nanotechproject.org/cpi.).
Roberts, A.P., Mount, A.S., Seda, B., Souther, J., Qiao, R., Line, S., Ke, P.C., Rao, A.M., Klaine, S.J., 2007. *In vivo* biomodification of lipid-coated carbon nanotubes by *Daphnia magna*. Environ. Sci. Technol. 41, 3025–3029.
Santo, N., Fascio, U., Torres, F., Guazzoni, N., Tremolada, P., Bettinetti, R., Mantecca, P., Bacchetta, R., 2014. Toxic effects and ultrastructural damages to *Daphnia magna* of two differently sized ZnO nanoparticles: does size matter? Water Res. 53, 339–350.
Selck, H., Handy, R.D., Fernandes, T.F., Klaine, S.J., Petersen, E.J., 2016. Nanomaterials in the aquatic environment: a European Union-United States perspective on the status of ecotoxicity testing, research priorities, and challenges ahead. Environ. Toxicol. Chem. 35 (5), 1055–1067.
Skjolding, L.M., Kern, K., Hjorth, R., Hartmann, N., Overgaard, S., Ma, G., Veinot, J.G.C., Baun, A., 2014. Uptake and depuration of gold nanoparticles in *Daphnia magna*. Ecotoxicology 23, 1172–1183.
Physiology of the Cladocera. In: Smirnov, N.N. (Ed.), Elsevier Inc. P. 51(336).
Tantra, R., Cackett, A., Peck, R., Gohil, D., Snowden, J., 2011. Measurement of redox potential in nanoecotoxicological investigations. J. Toxicol. 2012. http://dx.doi.org/10.1155/2012/270651. Article ID 270651, 7 pages.
Taylor, G., Baird, D.J., Soares, M.V.M., 1998. Surface binding of contaminants by algae: consequences for lethal toxicity and feeding to *Daphnia magna* Straus. Environ. Toxicol. Chem. 17 (3), 412–419.
Teodorovic, I., Planojevic, I., Knezevic, P., Radak, S., Nemet, I., 2009. Sensitivity of bacterial *vs.* acute *Daphnia magna* toxicity tests to metals. Cent. Eur. J. Biol. 4 (4), 482–492.
Vindimian, E., 2005. MSExcel Macro REGTOX_EV7.0.5. Xls. http://eric.vindimian.9online.fr/.
Von Moos, N.R., Bowen, P., Slaveykova, V., 2014. Bioavailability of inorganic nanoparticles to planktonic bacteria and aquatic microalgae in freshwater. Environ. Sci. Nano 1 (3), 214–232.
World Health Organization, Geneva, 2004. (Howe, P.D., Malcolm, H.M., Dobson, S.). Manganese and Its Compounds: Environmental Aspects. Concise International Chemical Assessment Document 63.
Wang, D., Lin, Z., Wang, T., Yao, Z., Qin, M., Zheng, S., Lu, W., 2016. Where does the toxicity of metal oxide nanoparticles come from: the nanoparticles, the ions, or a combination of both? J. Hazard. Mater. 308, 328–334.
Yu, Z.-G., Wang, W.-X., 2014. Interaction of functionalized fullerenes and metal accumulation in *Daphnia magna*. Environ. Toxicol. Chem. 33 (5), 1122–1128.
Zhang, H., Ji, Z., Xia, T., Meng, H., Low-Kam, C., Liu, R., Pokhrel, S., Lin, S., Wang, X., Liao, Y.P., Wang, M., 2012. Use of metal oxide nanoparticle band gap to develop a predictive paradigm for oxidative stress and acute pulmonary inflammation. ACS Nano 6, 4349–4368.
Zhao, C.-M., Wang, W.-X., 2010. Biokinetic uptake and efflux of silver nanoparticles in *Daphnia magna*. Environ. Sci. Technol. 44, 7699–7704.

Supplementary information for

Exposure to sublethal concentrations of Co_3O_4 and Mn_2O_3 nanoparticles induced elevated metal body burden in *Daphnia magna*

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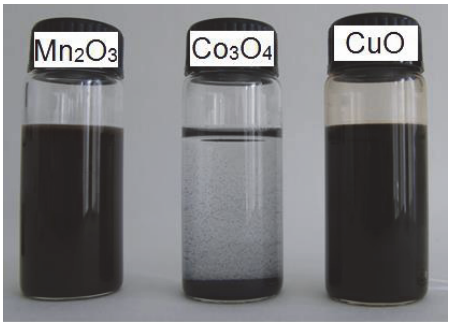

Figure S1. Nanoparticle stock suspensions (all at 5 g metal/L of ultrapure water) imaged immediately upon preparation and probe-sonication (4 min, 40W).

Table S1. Point of zero charge (pH_{PZC}) values for the studied nanoparticles (Kosmulski, 2009).

Nanoparticle	pH_{PZC}
Co_3O_4	7.2–8.9
Mn_2O_3	4.7
CuO	5–9.4

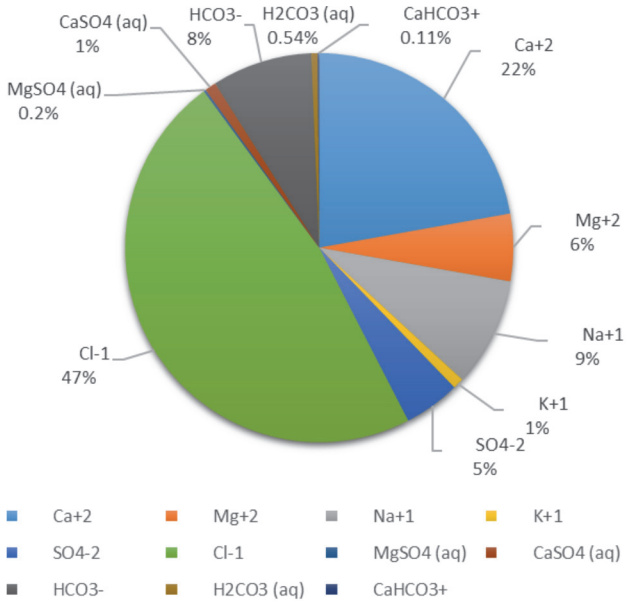

Figure S2. Ion speciation in the OECD202 medium (calculated using MINTEQA2 (Allison Geoscience Consultants Inc. and HydroGeologic Inc.)).

Parameters of flow-cytometry based evaluation of *Daphnia magna* post-exposure feeding

The cells of *Chlamydomonas reinhardtii*, used for post-exposure feeding of *Daphnia magna*, were distinguished from the rest of the events in flow cytometry (FCM) according to the red fluorescence (670 ± 25 nm) channel FL3-A. This channel was used to discriminate between *C. reinhardtii* cells with regular chlorophyll autoflorescence (Fig. S3, gate 3), so-called *D. magna* feeding by-products (recorded only in the algal suspensions that contained daphnids) (Fig. S3, gate 2) and debris (Fig. S3, gate 1). For counting the events in the FCM, 1 ml of well-suspended algal suspension was pipetted into 5 ml polypropylene round-bottom vials just before the analysis and vortexed before the initiation of the measurement. For each sample, data were collected for up to 10,000 events using medium flow rate (35 µl/min). Event size threshold was set to 20,000 on FSC-H (i.e. the events below this limit such as bacteria or microparticles were eliminated from the data).

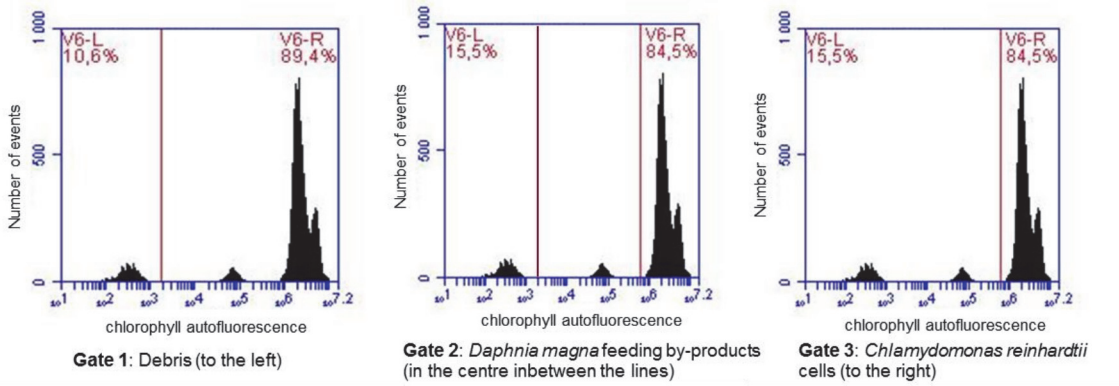

Figure S3. Gating of the events in *Daphnia magna* post-exposure feeding according to the chlorophyll autofluorescence (670 ± 25 nm) of the alga *Chlamydomonas reinhardtii.*

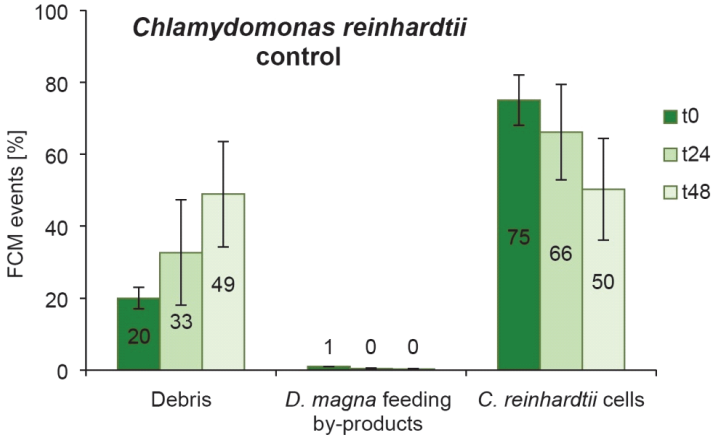

Figure S4. Algal (*Chlamydomonas reinhardtii*) control during *Daphnia magna* post-exposure feeding assay. Counting of the events was performed at the start of the experiment (t0), after 24 (t24) and 48 (t48) h of incubation at *D. magna* feeding conditions. The data are expressed as % of FCM (flow cytometer) events in the gates („Debris", „*D. magna* feeding by-products", „*C. reinhardtii* cells") and presented as AVG ± SD. See the FCM gating strategy in Fig. S3.

Table S2. *Daphnia magna* immobilization (%) and body length (µm) after 48 h chemical exposure and 48 h post-exposure feeding.

	48h immobilization [%]				*Daphnia magna* body length [µm]				Body length increase
	Exposure conc.	exposure		post-exposure feeding		exposure		post-exposure feeding		exposure to feeding
Sample	mg metal/L	AVG	SD	AVG	SD	AVG	SD	AVG	SD	%
D. magna control (unfed)		3	2	5	7	845	35	946	5	12
D. magna control (fed)		0	1	1	2	1168	57	1341	25	15
Co_3O_4 NP	0.1	4	5	5	7	886	36	992	7	12
	1	2	3	11	15	906	n.a	954	33	5
	10	3	3	5	7	905	33	958	8	6
$CoCl_2 \cdot 6H_2O$	0.1	5	5	3	4	870	4	962	20	11
	1	7	5	5	7	878	26	995	16	13
Mn_2O_3 NP	0.1	5	0	8	4	891	n.a	944	29	6
	1	3	0	8	11	901	n.a	917	17	2
	10	3	4	5	7	827	n.a	867	55	5
$MnCl_2 \cdot 4H_2O$	0.1	7	7	13	11	948	n.a	1037	n.a	9
	1	12	4	0	0	893	n.a	1043	n.a	17
	10	7	2	15	7	879	n.a	n.a	n.a	n.a
CuO NP	0.06	7	7	3	4	900	n.a	956	20	6
	0.6	42	18	15	0	865	n.a	936	3	8
$CuSO_4 \cdot 5H_2O$	0.06	17	18	3	4	895	n.a	968	7	8

All the data are averaged from at least 2 (3-5 for controls) independent experiments (4 technical replicates à 5 daphnids each for exposure and 2 à 10 daphnids for post-exposure feeding immobilization).

For body length measurements, each independent experiment included 5 daphnids. Body length data are partially missing due to technical reasons.

conc. – concentration; n.a. - not available; NP - nanoparticle

Table S3. *Daphnia magna* 24 and 48 h post-exposure feeding profiles.

24 h	Exposure conc.	Gate 1 Debris	Gate 2 *D. magna* feeding by-products	Gate 3 *C. reinhardtii* cells
sample	mg metal/L		% of events (AVG ± SD)	
C. reinhardtii	-	33 ± 15	0 ± 0	66 ± 13
D. magna control (unfed)	-	63 ±10	9 ± 2	27 ± 10
D. magna control (fed)	-	55 ±14	35 ± 12	10 ± 4
Co_3O_4 NP	0.1	46 ± 22	18 ± 7	36 ± 15
	1	63 ± 12	12 ± 4	26 ± 8
	10	44 ± 6	18 ± 1	38 ± 7
$CoCl_2•6H_2O$	0.1	49 ± 19	15 ± 5	35 ± 15
	1	48 ± 18	21 ± 4	31 ± 18
Mn_2O_3 NP	0.1	66 ± 9	8 ± 3	25 ± 12
	1	58 ± 2	10 ± 5	32 ± 6
	10	78 ± 15	5 ± 2	23 ± 16
$MnCl_2•4H_2O$	0.1	70 ± 13	8 ± 1	22 ± 12
	1	54 ± 3	7 ± 1	38 ± 4
	10	74 ± 10	6 ± 1	20 ± 9
CuO NP	0.06	52 ± 21	19 ± 7	29 ± 15
	0.6	66 ± 17	13 ± 4	21 ± 14
$CuSO_4•5H_2O$	0.06	48 ± 14	16 ± 4	36 ± 13
48 h				
C. reinhardtii	-	49 ± 15	0 ± 0	50 ± 14
D. magna control (unfed)	-	60 ± 13	19 ± 5	22 ± 12
D. magna control (fed)	-	45 ± 15	52 ± 15	2 ± 1
Co_3O_4 NP	0.1	72 ± n.a.	16 ± n.a.	12 ± n.a.
	1	75 ± 3	13 ± 2	12 ± 1
	10	55 ± 5	23 ± 1	22 ± 4
$CoCl_2•6H_2O$	0.1	71 ± 8	18 ± 4	11 ± 4
	1	65 ± n.a.	24 ± n.a.	11 ± n.a.
Mn_2O_3 NP	0.1	60 ± 19	16 ± 2	24 ± 17
	1	55 ± 13	21 ± 2	24 ± 14
	10	62 ± 18	15 ± 6	23 ± 18
$MnCl_2•4H_2O$	0.1	70 ± 13	14 ± 5	16 ± 13
	1	50 ± 9	13 ± 2	37 ± 7
	10	72 ± 12	11 ± 3	17 ± 12
CuO NP	0.06	61 ± 16	23 ± 7	16 ± 11
	0.6	54 ± 22	22 ± 6	23 ± 18
$CuSO_4•5H_2O$	0.06	48 ± 15	22 ± 8	30 ± 9

The data are presented on the basis of gating of the events (Fig. S3) in flow cytometry. The gating has been performed according to the alga *Chlamydomonas reinhardtii* chlorophyll autofluorescence.

conc. - concentration; n.a. - not available; NP - nanoparticle

Table S4. Total reflection X-ray fluorescence spectroscopy (Bruker Picofox S2) Co, Mn and Cu detection limits for background (measured on empty sample carriers with only HNO_3 and internal standard Ga added) or in *Daphnia magna* after 48 h OECD202 assay (measured in chemical unexposed control organism on sample carrier). The detection limits are given as total metal concentration (ng) in the measured sample and for *D. magna* also as µg metal/mg *D. magna* dwt.

			Detection limit	
Sample	Metal	n	ng	µg metal/ mg dwt
Background	Co	7	0.015 ± 0.001	
Daphnia magna		11	0.038 ± 0.009	0.0064 ± 0.0015
Background	Mn	7	0.019 ± 0.002	
Daphnia magna		11	0.055 ± 0.009	0.0092 ± 0.0015
Background	Cu	7	0.013 ± 0.001	
Daphnia magna		11	0.031 ± 0.006	0.0051 ± 0.0009

n – number of independent measurements for obtaining the average (AVG) value ± standard deviation (SD). Dwt – dry weight.

References

Kosmulski, M. 2009. Compilation of PZC and IEP of sparingly soluble metal oxides and hydroxides from literature. Advances in Colloid and Interface Science 152 (1–2), 14–25. doi:10.1016/j.cis.2009.08.003.

Appendix 5

Publication V

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Evaluation of the potential hazard of lanthanides to freshwater microcrustaceans

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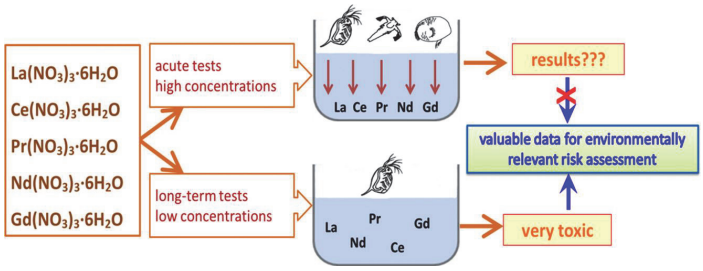
HIGHLIGHTS

- Toxicity of five lanthanides (Ln) to three aquatic microcrustaceans was evaluated.
- Due to low reliability, the acute tests are not suitable for risk assessment of Ln.
- Lanthanides proved very toxic to aquatic crustaceans in the chronic bioassays.
- Chronic toxicity of Ln was high: 21 d LC50 values 0.3–0.5 mg Ln/L.
- Ln could be considered as a uniform group with a similar toxicity mechanism.

GRAPHICAL ABSTRACT



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ABSTRACT

The use of lanthanides in different sectors of industry has significantly increased during the last decades. Although the "anthropogenic" anomalies of lanthanides in the soils, surface and ground waters have already been registered, the ecotoxicological effects of these elements and their fate in the environment are still insufficiently investigated. In this study acute and long-term toxicity of selected lanthanides (La, Ce, Pr, Nd and Gd) nitrates to freshwater crustaceans *Daphnia magna*, *Thamnocephalus platyurus* and *Heterocypris incongruens* were studied and critically evaluated.

The data obtained show that (i) due to the methodical nuances the acute toxicity data of lanthanides are not reliable and have doubtful scientific value even for preliminary toxicity screening and thus should not be used for risk assessment; (ii) toxicity of lanthanides in the 21-day *D. magna* reproduction test was high whereas the mortality of parent daphnids was more sensitive endpoint than reproduction; (iii) the long-term LC50 values for lanthanides varied from 0.3 to 0.5 mg Ln/L and the differences between individual Ln were not statistically significant.

All in all, the results of this study allow us to conclude that the environmental risk assessment of lanthanides should be performed only using long-term toxicity tests. In the environmental risk assessment, lanthanides may be considered as a uniform group of elements with additive mode of action until future investigations will not reveal differences in the ecotoxicity mechanisms of these elements.

Abbreviations: AFW, artificial freshwater; DOC, dissolved organic carbon; E(L)C50, the median effective concentration of the toxicant that induces a designated effect (or death) in 50% of the test organisms after a specified exposure time; Ln, lanthanides (or lanthanoids); LOEC, lowest observed effect concentration; C_{nom}, nominal concentration; REEs, rare earth elements; C_{aq}, measured concentration in the water column of the test vessel.

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0048-9697/

1. Introduction

Lanthanides (Ln) together with scandium and yttrium form a group of 'rare earth elements' (REEs) which also belong among the 'technology-critical elements' since their replacement by other elements in many key technologies is often impossible due to their unique properties (Cobelo-García et al., 2015). The field of REEs application is very wide and the demand for these elements is constantly growing. The use of REEs in many sectors (e.g., agriculture, medicine, electronics, high strength magnets, metal alloys, ceramic pigments, lamps, batteries, biofuel catalysts) has been rising during the last decades (Du and Graedel, 2013; Gonzalez et al., 2014). This process has been accompanied by increasing anthropogenic emissions into the environment via different pathways (Gwenzi et al., 2018), e.g., during mining, from fly ash generated during fossil-fuel combustion and waste incineration, from effluent discharges and leaching from the waste dumps and from fertilizers (Bosco-Santos et al., 2017; Franus et al., 2015). Until now, most REEs are not recycled and end their life-cycle at waste dumps. The use of the lanthanides (Ln) in the agriculture also contributes to the anthropogenic disruption of their natural biogeochemical cycle. As a result, Ln content in the soil and water ecosystems increases (Perelomov and Yoshida, 2008; Tyler, 2004). The "anthropogenic" anomalies of lanthanides in the soils, surface waters, groundwater and even in the tap water have already been registered (Kulaksız and Bau, 2011; Lawrence et al., 2009; Rabiet et al., 2009) indicating that the risks related to the exposure of biota to the elevated concentrations of Ln also rise. Reported Ln concentrations in the surface water usually vary from <1 ng/L to 200 ng/L (Bau and Dulski, 1996; Bau et al., 2006; Neal, 2007; Weltje et al., 2002), however, in the very polluted rivers Ln concentrations may increase up to 10 μg/L (Neal et al., 2005). The increasing contamination by REEs should be a cause for concern as there is uncertainness in REEs' biological effects as well as in their behaviour in the environmental matrices making the prediction of real risks to the ecosystems very difficult. Moreover, it has to be considered that the behaviour of the "anthropogenic" REEs differs from native ones, e.g., REEs supplied as fertilizers are characterised by higher solubility and lower affinity to surfaces of minerals (Kulaksız and Bau, 2013; Tyler, 2004).

Small amounts of Ln exist in all environmental compartments and very small background concentrations of Ln have been reported for the living organisms (Moermond et al., 2001). However, the biological role of Ln is unknown and so far this group of elements is considered as nonessential (Nordberg et al., 2014). Interestingly, both beneficial and adverse biological effects of Ln have been demonstrated in in vitro as well as in in vivo studies (Hua et al., 2017; Rim, 2016). According to some investigations, the pollution by lanthanides may pose the potential hazard for human and animals' health (Pagano et al., 2015; Rim et al., 2013). Specifically, damage to mice liver, kidney and heart were observed after 60 days of intake (via intragastric administration) of a lanthanides' dose of 20 mg/kg body weight (Cheng et al., 2012). On the other hand, application of REEs in the agriculture has improved the crop yield (Pang et al., 2002).

Usually lanthanides are considered as chemically uniform group of elements and, accordingly, it could be expected that behaviour in the ecosystems as well as biological effects of individual Ln are similar. In the nature, Ln occur mainly in the trivalent oxidation state and behave chemically quite similarly, but not entirely (Dahle and Arai, 2015). For example, Chen et al. (1995) experimentally demonstrated that leaching of La, Gd and Y from the soils at the same conditions differs. The comparable toxic effect of Ln on the unicellular algae was reported by Tai et al. (2010) and Joonas et al. (2017). However, Brantley et al. (2001) have found different uptake of individual Ln by the soil bacterium *Arthrobacter* sp. and He et al. (2003) revealed that the addition of different Ln into the rats' food had different effect on animal growth. Thus, in spite of the chemical similarity, the behaviour in the environmental matrices and biological effects of Ln may differ but the significance of the differences is still debatable and the question "should Ln be considered as uniform group of elements in the environmental regulation or each element should be evaluated individually?" is still open. Vast majority of the articles on lanthanides' ecotoxicity deal with La and Ce, and only a few publications report ecotoxicity test results for several Ln performed under identical experimental conditions that allow comparison of toxicity across the lanthanide series (Gonzalez et al., 2014).

Analogously to other metals the bioavailability of Ln to tested species in the different test media may significantly differ due to complexation with inorganic and organic ligands (Pang et al., 2002; Wood, 1990). The previous studies have shown that even minor changes in the chemical composition of the test medium affected the bioavailable fraction of the poorly water-soluble Ln oxides (Blinova et al., 2018; Kurvet et al., 2017; Van Hoecke et al., 2009). To perform adequate evaluation of potential hazard of Ln more data on toxicity of individual lanthanides in different test condition are needed. As Ln is used in fertilizers, the most published studies have been focusing on their potential effects on soil organisms and plants; data on aquatic organisms are more rare (Gonzalez et al., 2014). This study focuses on light Ln which are more abundant in the nature and might thus be a bigger environmental concern, and Gd that is reaching the waterbodies in high concentrations from the hospital wastewaters. Acute toxicity tests were conducted with *Daphnia magna*, *Thamnocephalus platyurus* and *Heterocypris incongruens*, additionally a recovery test and chronic toxicity test were conducted with *D. magna*. The aquatic crustaceans were chosen as model organisms since this group of organisms is very sensitive towards Ln-ions (Herrmann et al., 2016) and toxicity data for a crustacean *Daphnia magna* is widely used for environmental risk assessment.

The main aims of the study are i) to evaluate potential hazard of five lanthanides (La, Ce, Pr, Nd and Gd) to three aquatic microcrustaceans playing very important role in the trophic chain of aquatic ecosystem; ii) to compare toxicity of individual Ln and iii) to evaluate the bioavailability of Ln in the different test media.

2. Methods

2.1. Chemicals

The stock solutions (1000 mg/L) of the lanthanide (III) nitrates - La $(NO_3)_3 \cdot 6H_2O$ (Treibacher Industrie AG, ≥95–100%), Ce$(NO_3)_3 \cdot 6H_2O$ (Treibacher Industrie AG, ≥95–100%), Pr$(NO_3)_3 \cdot$6H2O (Sigma Aldrich, 99.9%), Nd$(NO_3)_3 \cdot 6H_2O$ (Sigma Aldrich, 99.9%), Gd$(NO_3)_3 \cdot 6H_2O$ (Sigma Aldrich, 99.9%) - were prepared in deionized (DI) water.

2.2. Test media

Two types of artificial freshwater (AFW) and natural water from two lakes with different hydrochemical characteristics were used (Table 1). Natural water was collected from Lake Raku (Lake 1) and Lake Ülemiste (Lake 2), filtered through 0.45 μm cellulose nitrate filter and stored at 4 °C in the dark prior to the use in the bioassays.

2.3. Acute bioassays

The toxicity kits for acute toxicity tests with three freshwater crustaceans were purchased from MicroBioTests, Inc. (Mariakerke-Gent, Belgium): Thamnotoxkit F (with *Thamnocephalus platyurus*), Daphtoxkit F magna (with *Daphnia magna*) and Ostracodtoxkit F (with *Heterocypris incongruens*). Test organisms (<24 h old) hatched from the dormant eggs included in the kits were exposed to Ln solutions. Nominal test concentrations of 6.25, 12.5, 25 and 50 mg/L were used in static acute tests. Test conditions are summarized in Table 2.

2.4. Recovery tests (D. magna)

The survivors in *D. magna* acute toxicity test were transferred into clean lake (Lake 2) water and kept at the same temperature and

Table 1
Chemical composition of artificial freshwater and main hydrochemical parameters of lake waters.

Parameter	Lake 1	Lake 2	ISO artificial freshwater[a]	Moderately hard artificial freshwater[b]
pH	8.0 ± 0.2	7.5 ± 0.2	7.4 ± 0.2	7.3 ± 0.2
Ca^{2+} (mg/L)	45.8	75	80.1	13.9
Mg^{2+} (mg/L)	4.71	8	12.1	12.0
K^{+} (mg/L)	1.8	2.98	3.0	2.1
Na^{+} (mg/L)	2.65	8.06	17.7	26.3
Cl^{-} (mg/L)	3.6	14	144.5	1.92
SO_4^{2-} (mg/L)	22	34	48.0	81.5
DOC (mg C/L)	5.2	10.6	0	0
P_{tot} (mg P/L)	0.035	0.016	0	0
N_{tot} (mg N/L)	0.71	1.9	0	0

DOC – dissolved organic carbon; N_{tot} – total nitrogen; P_{tot} – total phosphorus; N_{tot} – total nitrogen.
[a] OECD, 2004.
[b] US EPA, 2005.

illumination conditions as in the reproduction test. The mortality and number of offspring were observed during 15 days. Neonates were fed daily with algae *Raphidocelis subcapitata*.

2.5. Reproduction test (D. magna)

A 21 days semi-static *D. magna* reproduction test was performed according to OECD 211 guidelines (OECD, 1998). Less than 24 h old neonates obtained from laboratory culture were incubated at 21 °C ± 2, with a 16 h/8 h light/dark photoperiod and fed daily with algae *R. subcapitata* ($1.5 \cdot 10^5$ cells/mL). Ten daphnids per every tested concentration (0.01, 0.1, 0.25, 0.5 and 1.0 mg Ln/L) were exposed separately in glass beakers (50 mL test solution). Filtered (0.45 μm) Lake 2 water was used as a test medium. Test solutions were renewed every third day. Mortality of the parent animals and number of offspring per adult female was recorded every day.

2.6. Measurement of Ln concentration in the test media

Total concentrations of lanthanides in the test media (C_{aq}), i.e. sum of all Ln species in the unfiltered water sample, were measured (i) before testing, immediately after preparation of the test solution and (ii) at the end of the test in water from the upper layer of test flask sampled avoiding re-suspension of the settled precipitate. In addition, 20 mL of Ce, Gd and Nd solutions at nominal concentrations (C_{nom}) 1 and 25 mg Ln/L prepared in the different test media (AFWs and lake water) were incubated at room temperature without test organisms to exclude influence of the crustaceans on lanthanides' distribution in the test tubes. After static incubation for 24, 48 and 72 h, metal concentration was measured in the water by sampling from upper layer avoiding re-suspension of the settled precipitate. Total metal concentration in water was quantified by Total Reflection X-ray Fluorescence spectrometer (TRXF) Picofox S2 (Bruker AXS Microanalysis GmbH) as described by Blinova et al. (2018). Detection limit for lanthanides (La, Ce, Gd, Pr) was 0.05 mg/L.

2.7. Statistics

Two to four independent tests with to 2–4 replicates for each tested concentration were conducted. One-way analysis of variance (ANOVA) followed by Students' *t*-test was used to determine statistical significance of the differences between toxic effects of the investigated compounds. The differences were considered significant when $p < 0.05$.

3. Results and discussion

3.1. Uncertainties in evaluation of Ln exposure concentrations in the toxicity test

The quantification of exposure concentrations of tested compound is a requirement for correct interpretation of the toxicity test results. In the current study, the Ln distribution pattern in the test vessel was evaluated by measuring the total Ln concentration, i.e. sum of all Ln forms (dissolved, colloidal and/or accumulated by algae), in the water column (C_{aq}) in bioassays as well as in experiments without crustaceans (see Methods section).

As many salts of trivalent Ln are insoluble (e.g., carbonates, phosphates, hydroxides, oxalates) or sparingly soluble (sulphates) in water, addition of the soluble salt of Ln into the test medium often results in formation of quickly precipitating suspended fraction. The remaining amount of Ln stays in soluble or colloidal form in the water column. The analysis of measured concentrations in the water column (C_{aq}) revealed the similar trends in precipitation for all tested Ln both in bioassays and experiments without organisms depending on (i) nominal concentration, (ii) water composition and (iii) exposure time. However, the variation range of C_{aq} measured in test replicates was significantly larger in the bioassays (i.e. test organisms present) than in the experiments without test organisms. The differences between replicates did not exceed 10% in the solutions incubated without organisms, but measured C_{aq} varied between replicates up to 5-fold in all acute bioassays and up to 2-fold in *D. magna* reproduction test (data not shown). High variability of lanthanide C_{aq} in bioassays may be explained, first of all, by biological factors (crustaceans, algae) modulating Ln speciation in the test vessel through direct

Table 2
Test conditions.

Test species	*Daphnia magna*	*Thamnocephalus platyurus*	*Heterocypris incongruens*
Test medium	ISO AFW	Moderately hard AFW	Moderately hard AFW
Feeding with microalgae *Raphidocelis subcapitata*	2 h prior the test	No	Prior to and during the test
Density of test organisms	5 per 10 mL	10 per 1 mL	10 per 4 mL
Total number of neonates per one tested concentration	20	30	20
Duration	48 h	24 h	6 days
Temperature	20 °C	25 °C	25 °C
Illumination conditions	In dark	In dark	In dark

(biotransformation, biosorption, bioaccumulation) and indirect (modification of the physicochemical properties of the test media) mechanisms (Andres et al., 2000; Francis, 1997; Perelomov and Yoshida, 2008; Schijf and Byrne, 2001).

The precipitation of the Ln increased with increasing nominal concentration (C_{nom}) in all the test formats. For example, in the test without organisms, dynamics of the Ln concentrations in the water column at C_{nom} of 1 and 25 mg Ln/L (corresponding to the exposure levels used in long-term and acute tests) showed that the reduction of C_{aq} was much more remarkable at the high nominal concentrations (25 mg/L) in all three test media used (Fig. 1). In acute bioassays at the high C_{nom} (10–50 mg Ln/L) >80% of applied Ln precipitated during the test whatever the test medium (data not shown). However, at low concentrations (≤1 mg Ln/L) more uniform distribution of the Ln in the test vessels was observed in the lake water. Namely, 80–100% of applied Ln remained in the water column both in the bioassays and solutions without the test organisms (Fig. 1). Our data confirmed that the complexing capacity of organic compounds in lake water depends not only on the chemical composition of the organic matter, but also on the Ln nominal concentrations (Dupré et al., 1999; Tang and Johannesson, 2010). In the AFW, precipitation at 1 mg Ln/L was more intensive than in natural lake water but still significantly lower than at 25 mg/L (Fig. 1). Our data agree with Barry and Meehan (2000) who also showed that La (added as $LaCl_3$) rapidly precipitated in ASTM hard water when C_{nom} exceeded 1 mg/L and no precipitation was observed (~100% stayed in the water column) at concentrations lower than 1 mg/L.

Although the dissolved organic matter (DOC) is of great importance for regulation of Ln speciation in the natural water (Herrmann et al., 2016; Tyler, 2004), significant differences in Ln behaviour were observed also between two different AFWs free from DOC (Table 1). Namely, C_{aq} measured in *D. magna* AFW were nearly 2-fold higher than in *T. platyurus* AFW at the same C_{nom} and time point in experiment without organisms and 2–4-fold higher in the acute bioassays presumably due to different abundance of the main anions in the AFWs (Barry and Meehan, 2000). The higher C_{aq} in experiments without organisms than in *D. magna* acute bioassay may also be explained by effect of organic matter excreted into water by daphnids as exposed neonates were fed with algae prior to testing (i.e. probably colloidal organic complexes were formed). Incubation of Ln solutions with algae ($1.5 \cdot 10^5$ cells/mL) and without algae at C_{nom} 1 mg Ln/L did not lead to statistically significant differences in C_{aq} in all tested media (data not shown).

Fig. 2 illustrates time-dependent changes of Ce in the water column (no test organisms present) as an example for Ln behaviour in test media. One can see that at 1 mg/L Ce precipitation during 3 days was much slower than at nominal concentration 25 mg/L, especially in the lake water. Data pooled from all bioassays showed that after 72 h exposure, the metal concentrations in the upper layer were in average 2–3-fold lower compared to 24 h sample point at high (25 mg Ln/L) concentration in the all used media, however, at low C_{nom} (1 mg Ln/L) these differences did not exceed 30% in AFW and Lake 1 water and were negligible in the Lake 2 water (with the highest DOC concentration). Thus, the higher applied nominal concentration the higher precipitation rate of Ln.

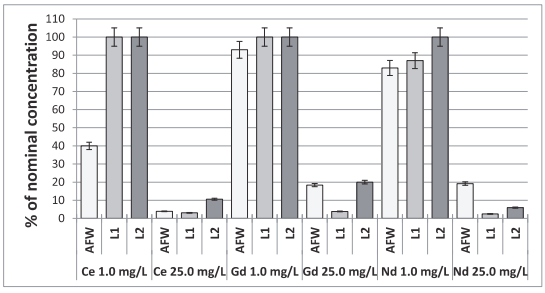

Fig. 1. Share of the metals (% of nominal concentration) remaining in the water column after addition of Ln nitrates into different test media and incubation during 48 h without test organisms. AFW – artificial fresh water used in *D. magna* test; L1 – water from Lake Raku; L2 - water from Lake Ülemiste.

Data pooled from tests with and without animals revealed that in spite of chemical similarity, behaviour of the individual Ln in the same medium and under the same test conditions (test media, C_{nom}, temperature etc.) differed. At C_{nom} exceeding 10 mg/L in case of both types of AFW (Table 2) concentrations of La and Ce in the upper layer were lower than concentrations of Nd, Pr and Gd, but due to very high variability between replicates only for Ce these differences were statistically significant ($p < 0.05$). In the lake water differences between tested elements were not statistically significant at any tested concentrations.

Summing up the above, at low nominal concentrations (<1 mg Ln/L) precipitation of Ln in the investigated three test media was minor and C_{aq} of Ln remained stable for at least 3 days. At the higher C_{nom} precipitation rate of Ln in the same test medium depended both on nominal concentration and exposure time.

3.2. Toxicity of Ln to crustaceans

3.2.1. Calculation of the toxic effects based on nominal concentrations versus concentrations in the water column

According to the guidelines for aquatic ecotoxicity testing of chemicals (OECD, 1998; OECD, 2004) concentration of tested substance in the test solution during the test must be measured. It is recommended that effect concentration should be calculated based on measured concentration in test water (C_{aq}). Though it is considered that calculation of the toxicity values based on the C_{aq} gives more realistic values than calculation based on C_{nom} (Herrmann et al., 2016), in case of Ln, this approach also may yield false results for several reasons, especially in the tests with crustaceans.

As it was shown above, the averaging of measured C_{aq} gives only approximate quantification of exposure of tested organisms to Ln in water as precipitation rate depends both on nominal concentration and exposure time. Furthermore, not all the Ln measured in the water column are bioavailable and at the same C_{aq} Ln bioavailability in different media may significantly vary. For example, acute toxicity of Ln calculated on the basis of C_{nom} to *T. platyurus* and *D. magna* in AFW was similar (Table 3) despite of 2–3-fold higher C_{aq} in *D. magna* medium as compared to *T. platyurus* one (data not shown). At the same time, taking into account that our previous investigations (Blinova, 2004) showed very similar toxicity of heavy metals to *T. platyurus* and *D. magna* given the test formats were similar, it could be assumed that sensitivity of these two species to Ln are probably also comparable. So, in spite of higher total C_{aq} of Ln in daphnia test, the bioavailable fraction of Ln was probably comparable in the both tests, indicating that calculation of the effect concentration on the basis of C_{aq} is actually unjustified. Our study also showed that at comparable C_{aq} in the tests with natural water and AFW the toxic effect of Ln in natural water was always much lower, i.e. Ln species with low bioavailability probably dominated in the natural water. This fact once more confirms weak relationship between measured C_{aq} and observed toxic effect of Ln.

Moreover, crustaceans may ingest precipitated insoluble or partly soluble Ln complexes and toxic effect can either be due to the bioavailable water fraction or ingested particulate fraction. It could be hypothesized that Ln species in the water layer were much more bioavailable than precipitated part, but biological effects of the ingested Ln still have not been investigated enough. On the one hand, in spite of the fact that the gut of *T. platyurus* and *D. magna* was filled with Ln aggregates at all the tested concentrations (Fig. S1), mortality of crustaceans varied from 0 to 100% in acute bioassays, i.e. effect of ingested Ln was low. On the other hand, Stauber and Binet (2000) supposed that not only soluble La, but also particulate lanthanum could be toxic to

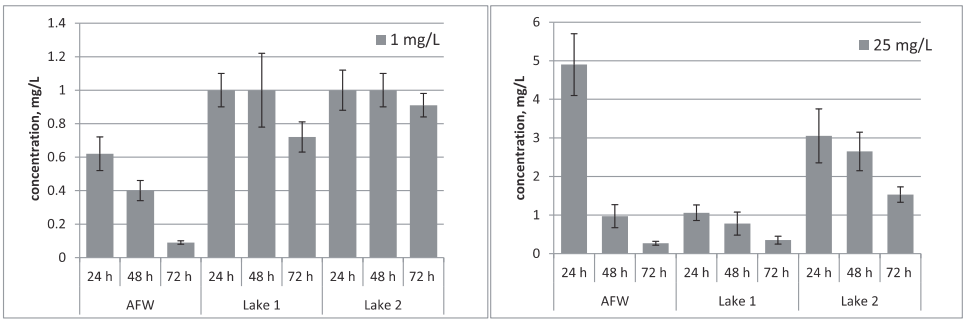

Fig. 2. Dynamics of total Ce concentration in test media after addition of Ce nitrate at nominal concentration 1 mg/L (left) and 25 mg/L (right) and incubation without organisms for 24, 48 and 72 h. AFW – artificial fresh water used in *D. magna* test; Lake 1 – water from Lake Raku; Lake 2 – water from Lake Ülemiste.

Ceriodaphnia dubia. In addition, aggregates of the insoluble Ln compounds may also give indirect effect on the organisms' viability.

In the long-term experiments bioavailable fraction of Ln may enter crustaceans both from water and with algae (food). Moreover, analogously to other metals, algal exudates may modulate Ln speciation (and thus bioavailability) to crustaceans during the test (Bertilsson and Jones Jr., 2002; Vasconcelos and Leal, 2008). According to our knowledge, information on bioavailability of different Ln species and Ln toxicity pathways is limited. Moreover, Ln speciation may also change during experiments as they form labile 'ionic' complexes (Cotton, 2006). Thus, the attempt to tie in concentration of specific Ln species (e.g., Ln ions) and toxic effect will lead to values with very high uncertainty.

Therefore, both nominal concentrations and total Ln concentrations in the water column were used for calculation of the effect concentrations (Table 3).

3.2.2. Acute toxicity

The test formats used for the evaluation of the acute toxicity of Ln to planktonic crustaceans *Thamnocephalus platyurus* and *Daphnia magna* and benthic ostracod *Heterocypris incongruens* slightly differed and are presented in Table 2.

All investigated Ln showed low to moderate acute toxicity to planktonic species *D. magna* and *T. platyurus* when LC50 values were calculated based on C_{nom}. Gd was the most toxic to these two species, however, difference between Gd and other Ln was statistically significant ($p < 0.05$) only in the case of *T. platyurus* test. Toxicity of La and Ce to *H. incongruens* was similar to *D. magna* and *T. platyurus* ones, but Pr, Nd and Gd showed higher toxic effect (LC50 < 10 mg/L). The La toxicity to *H. incongruens* is in agreement with EC50 value (41.5–50.8 mg La/L of C_{nom}) obtained by Khangarot and Das (2009) for ostracod *Cypris subglobosa* in acute test using different from current study format (48 h, well water, ostracods not fed during the test). However, from our point of view, it cannot be concluded that Pr, Nd and Gd are more toxic to ostracods than La and Ce. It should be mentioned that, unlike *D. magna* and *T. platyurus* test results, toxicity of Pr, Nd and Gd to ostracods varied considerably across experiments. Higher variation in Ln bioavailability in *H. incongruens* test compared to the bioassays with planktonic crustaceans at the same C_{nom} may be partly explained by (i) effect of high algae concentration ($7.5 \cdot 10^6$ cells/mL) on Ln bioavailability, (ii) direct contact of benthic ostracods with settled Ln compounds and (iii) consumption of Ln accumulated/adsorbed by algae. All these factors may significantly vary at the same test conditions leading to different Ln availability in test replicates. In particular, it has been shown that at high concentrations Ln may form heteroagglomerates with algae (Joonas et al., 2017; Van Hoecke et al., 2009; Fujiwara et al., 2008) making algae inaccessible for eating by crustaceans, at the same time decreasing bioavailable fraction of the Ln. In the ostracode test, algae *R. subcapitata* were found both as individual cells and as crystal clusters with Ln (Fig. S2). However, we have not found any relationship between formation of heteroagglomerates and C_{nom}, i.e. the ratio between agglomerated and planktonic algae may significantly differ at the same C_{nom} (based on microscope evaluation). This fact also may particularly explain very high variation in the bioavailability of Ln between replicates. The addition of the sand into ostracod test medium significantly decreased the bioavailable Ln fraction (mortality at C_{nom} 50 mg Ln/L did not exceed 30% for all tested Ln) most likely due to Ln affinity to sand particles (Herrmann et al., 2016). Due to the remarkable mitigating effect of sand in AFW, additional tests with lake waters were not conducted.

The relationship between mortality/immobilisation of neonates and Ln concentration in the water column was not revealed for either

Table 3
Acute toxicity (EC50, mg Ln/L) of lanthanides to freshwater microcrustaceans.

Salts	*Thamnocephalus platyurus* 24 h mortality			*Daphnia magna* 48 h immobilisation			*Heterocypris incongruens* 6 days mortality	
	AFW	Lake 1	Lake 2	AFW	Lake 1	Lake 2	AFW	
							Without sand	With sand
L(E)C50 values based on nominal concentrations of tested Ln								
$La(NO_3)_3 \cdot 6H_2O$	34.6 ± 2.3	>50	>50	31.1 ± 9.1	>50	>50	43.1 ± 3.9	>50
$Ce(NO_3)_3 \cdot 6H_2O$	33.0 ± 1.2	>50	>50	26.3 ± 3.5	>50	>50	31.1 ± 6.2	>50
$Pr(NO_3)_3 \cdot 6H_2O$	30.8 ± 0.2	>50	>50	23.8 ± 0.2	>50	>50	<10	>50
$Nd(NO_3)_3 \cdot 6H_2O$	31.8 ± 2.1	>50	>50	20.8 ± 3.8	>50	>50	<10	>50
$Gd(NO_3)_3 \cdot 6H_2O$	18.2 ± 4.8	>50	>50	18.5 ± 0.9	>50	>50	<10	>50
L(E)C50 values (min-max) based on Ln concentration measured in the water by the test end, EC(L)50								
All tested Ln	0.2–1.5	>0.1	>0.2	0.2–1.5	>0.2	>0.2	0.2–1.5	n.a.

n.a. – not analysed.

planktonic or benthic species. As reported above, variation in the total Ln concentrations in water measured at the same C_{nom} in test replicates was very high (up to 5-fold), whereas variation in mortality/immobilisation of crustaceans was much lower. L(E)C50 values calculated based on measured concentrations at the end of the test varied within 0.2–1.5 mg Ln/L (Table 3) for all tested crustaceans. These values are comparable to the ones reported by Barry and Meehan (2000) for La 48 h EC50 of *Daphnia carinata* (1.2 mg measured La/L).

Our study demonstrated very low reliability of toxicity values (LC50) for Ln obtained using acute test format. Too high nominal concentrations and, as a result, very rapid Ln precipitation in the test media and very high variation of measured C_{aq} between replicates complicate interpretation of the observed results. In addition to uncertainties in evaluation of exposure concentrations, too short exposure time is also the limitations of acute test format in the case of Ln. The recovery test performed after acute test of *D. magna* showed that viability of neonates exposed even to concentrations showing no acute toxicity was very low after the test. For example, all neonates exposed to the C_{nom} 3 mg Ln/L in AFW (zero immobilisation in the acute test) died within 3 days in clean (non-spiked) water in spite of food availability (see methods). The same trend was revealed by comparison of mortality in acute and long-term tests, i.e. subtoxic Ln concentrations in acute tests were lethal in chronic test just a few days after the beginning of the experiment. Indeed, immobilisation of daphnids in the lake water was zero at C_{nom} 1 mg Ln/L, whereas in the reproduction test all neonates exposed to this concentration died on the 4th exposure day. Stauber and Binet (2000) also showed that La (added as lanthanum chloride) was not acutely toxic to the *Ceriodaphnia* after short term exposures (48 h) but was toxic after longer exposure times (7 days) at above about 1 mg/L.

Intensive precipitation of Ln modified not only bioavailable concentration of Ln but also chemical composition of test media. Due to formation of insoluble Ln compounds (mainly carbonate complexes in AFW) the concentration ratio of main anions significantly changes. In particular, nitrate concentration in the test medium significantly increases after addition of high concentrations of Ln nitrates. Though nitrate concentrations at LC50 levels (6–11 mg NO_3-N/L) are much lower than reported acute toxicity for *D. magna* (LC50: 323–611 mg NO_3-N/L), they are very close to the LOEC concentration reported for *Ceriodaphnia dubia* (14.1 mg NO_3-N/L) (Scott and Crunkilton, 2000). It could be assumed that modification of chemical composition of test media by spiking it with high concentrations of Ln nitrates may also affect neonates' viability and consequently toxicity test results.

Thus, it may be concluded that results of acute toxicity testing of Ln have doubtful scientific value and due to low ecological relevance cannot be used in the environmental risk assessment.

3.2.3. Chronic toxicity of lanthanides to D. magna

From the ecological point of view, it is not meaningful to perform long-term tests in the synthetic test media as natural dissolved organic matter is very important parameter regulating bioavailability of Ln to crustaceans (Gu et al., 2001; Pourret et al., 2007). Therefore, reproduction test was performed in the natural water (Lake 1) to improve environmental relevance of laboratory test results.

The test results showed that, analogously to our previous results concerning long-term effect of Ag to *D. magna* (Blinova et al., 2013), mortality was more sensitive endpoint than number of offspring per alive adults. For example, at C_{nom} 0.25 mg/L mortality in the test with different Ln varied from 50 to 80%, whereas effect of all tested Ln on fertility was very low (<10%).

The mortality LC(50) values (Table 4) were calculated based on nominal concentrations as C_{aq} was similar to nominal concentration. Namely, at C_{nom} 0.25 and 0.5 mg Ln/L varied from 80 to 110% and from 96 to 105% of C_{nom} at lower exposure concentrations (<0.2 mg/l). The LC50 values (Table 4) are comparable to chronic toxicity of La to *Daphnia carinata* (LC50 0.4 mg/l of C_{nom}) reported by Barry and Meehan (2000) who also showed that mortality was a more sensitive indicator of La toxicity than growth or reproduction. The differences in the LC50 values between investigated Ln were very small and presumably depended on the behaviour of individual Ln in the given (specific) chemical matrix of test media. It is interesting that unlike acute tests results (Table 3), long-term mortality of the daphnids exposed to Gd was lowest as compared with other tested Ln (Table 4), but differences between toxicity values of individual Ln were not statistically significant.

Table 4
Mortality (LC50 based on nominal concentrations) of *Daphnia magna* in the 21 days reproduction test in water from Lake 2.

Salts	LC50, mg Ln/L Mean (min–max)
$La(NO_3)_3 \cdot 6H_2O$	0.46 (0.38–0.53)
$Ce(NO_3)_3 \cdot 6H_2O$	0.3 (0.23–0.36)
$Pr(NO_3)_3 \cdot 6H_2O$	0.29 (0.21–0.38)
$Nd(NO_3)_3 \cdot 6H_2O$	0.31 (0.21–0.44)
$Gd(NO_3)_3 \cdot 6H_2O$	0.49 (0.35–0.52)

pH values in the Ln solutions were not statistically different from the control at tested concentrations (0.01, 0.1, 0.25 and 0.5 mg Ln/L) and varied between 8.2 and 8.8 before test media change (every third day). However, it should be mentioned that at C_{nom} 0.5 mg Ln/L differences were up to 0.5 pH units and nearly 1 unit at concentration 1 mg/L.

The day of the first brood was the same as in the control for all the tested lanthanides at concentration of 0.1 mg/L. However, at concentrations of 0.25 and 0.5 mg Ln/L the first offspring was recorded 1–2 and 2–3 day later than in the control. Thus, exposure to Ln concentrations lower than 0.1 mg/L did not lead to maturation delay. The mortality at nominal concentration of 0.01 mg Ln/L (comparable to the Ln concentrations reported for polluted rivers; Neal et al., 2005) did not exceed mortality in the control in any of the tests.

In the reproduction test, algae (the food of crustaceans) did not form clusters/heteroagglomerates with insoluble Ln compounds and applied Ln concentrations were subtoxic to *P. subcapitata* (Joonas et al., 2017). Thus, adverse effects in the reproduction test cannot be caused by food shortage as it was shown by Lürling and Tolman (2010) for phosphorous rich media. Possible effects of algae on Ln bioavailability were discussed in paragraph 3.2.2.

3.2.4. Lanthanides as a uniform group of elements in the environmental regulation

The behaviour of Ln in the environment, in particular, the mechanism of colloidal particles formation, is very complex and still is incompletely understood (Evans, 1990; Herrmann et al., 2016). The chemical activity of individual Ln slightly differs (Dahle and Arai, 2015). For example, Kulaksız and Bau (2013) showed that in the Rhine River water light Ln were more particle reactive, i.e. more easily bound to colloids and nanoparticles. The chemical properties of Ln change relatively smoothly throughout the group (Evans, 1990). It has been shown that stability of Ln complexes with electronegative ligands, e.g., the chelate-forming oxalates increased along with the gradually decreasing effective ionic radius from La^{3+} to Lu^{3+} (Tyler, 2004). At the same time, the solubility of Ln nitrates in water varies depending on lanthanide atomic number but not monotonically. Namely, the solubility increases between La and Ce, decreases in the interval from Ce to Sm, and then increases between Gd and Lu (Siekierski et al., 1983). In the aquatic environment the distribution of Ln is regulated by diverse processes: precipitation, dissolution, complexation with inorganic and organic ligands (Davranche et al., 2015; Pang et al., 2002). Therefore, even small differences in solubility of Ln nitrates may lead to formation of different species of individual Ln in the same medium, but due to formation of labile complexes these differences are not stable resulting in high variations in the Ln speciation between replicates.

In the current study, C_{aq} measured at low C_{nom} (<1 mg/L) did not reveal any statistically significant differences between individual Ln exposed in the same test medium. Thus, the small differences in toxicity

values obtained from long-term reproduction test (Table 4) may be mostly explained by differences in the speciation of individual Ln and not by different toxicity of individual Ln. The toxicity investigation of the La, Ce, Pr, Nd and Gd to protozoa *Tetrahymena thermophila* and bacteria *Vibrio fischeri* also revealed that toxic effect of these Ln was very similar under the same test conditions (Kurvet et al., 2017). Toxicity investigation of Ln to *Hyalella azteca* also showed similar LC50 values for all Ln (Borgmann et al., 2005). Based on the foregoing data it could be assumed that toxicity mechanisms of Ln are similar. Thus, in the environmental assessment, Ln may be considered as a uniform group with additive mode of toxicity until future investigations will not reveal differences in the ecotoxicity mechanisms of Ln.

4. Conclusions

We assert that due to the methodical nuances the acute toxicity data of lanthanides are not reliable and have doubtful scientific value even for preliminary toxicity screening and thus should not be used for risk assessment of Ln.

In 21 days reproduction test, lanthanides were very toxic to crustaceans showing potential hazard for aquatic ecosystems. However, as even minimal effect concentrations obtained from reproduction test were still two to three orders higher than concentration reported for surface waters, the further investigations using environmentally relevant concentrations are needed for realistic assessment of environmental hazard of Ln.

Results from long-term *D. magna* test allow it to conclude that Ln should be considered as a uniform group of elements with similar mode of action in the environmental risk assessment until the future investigations will not reveal differences in the ecotoxicity mechanisms of Ln.

To make it possible to compare Ln toxicity between different experiments, the toxicity values calculated from nominal concentrations should always be presented along with calculations based on measured total Ln concentration in the water or Ln speciation (if any). This could be also useful from environmental risk assessors' point of view as it is unlikely that measurements of Ln species could be included into routine aquatic monitoring (Khan et al., 2017).

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Appendix A. Supplementary data

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References

Andres, Y., Thouand, G., Boualam, M., Mergeay, M., 2000. Factors influencing the biosorption of gadolinium by micro-organisms and its mobilisation from sand. Appl. Microbiol. Biotechnol. 54, 262–267.

Barry, M., Meehan, B.J., 2000. The acute and chronic toxicity of lanthanum to *Daphnia carinata*. Chemosphere 41 (10), 1669–1674.

Bau, M., Dulski, P., 1996. Anthropogenic origin of positive gadolinium anomalies in river waters. Earth. Planet. Sci. Lett. 143, 245–255.

Bau, M., Knappe, A., Dulski, P., 2006. Anthropogenic gadolinium as a micropollutant in river waters in Pennsylvania and in Lake Erie, northeastern United States. Chem. Erde-Geochem. 66 (2), 143–152.

Bertilsson, S., Jones Jr., J.B., 2002. Supply of dissolved organic matter to aquatic ecosystems: autochthonous sources. In: Findlay, S., Sinsabaugh, R.L. (Eds.), Aquatic Ecosystems: Interactivity of Dissolved Organic Matter. Academic Press, pp. 3–24.

Blinova, I., 2004. Use of freshwater algae and duckweeds for phytotoxicity testing. Environ. Toxicol. 19 (4), 425–428.

Blinova, I., Niskanen, J., Kajankari, P., Kanarbik, L., Käkinen, A., Tenhu, H., Penttinen, O.-P., Kahru, A., 2013. Toxicity of two types of silver nanoparticles to aquatic crustaceans *Daphnia magna* and *Thamnocephalus platyurus*. Environ. Sci. Pollut. Res. 20 (5), 3456–3463.

Blinova, I., Vija, H., Lukjanova, A., Muna, M., Syvertsen-Wiig, G., Kahru, A., 2018. Assessment of the hazard of nine (doped) lanthanides-based ceramic oxides to four aquatic species. Sci. Total Environ. 612, 1171–1176.

Borgmann, U., Couillard, Y., Doyle, P., Dixon, D.G., 2005. Toxicity of sixty-three metals and metalloids to *Hyalella azteca* at two levels of water hardness. Environ. Toxicol. Chem. 24 (3), 641–652.

Bosco-Santos, A., Luiz-Silva, W., da Silva-Filho, E.V., de Souza, M.D.C., Dantas, E.L., Navarro, M.S., 2017. Fractionation of rare earth and other trace elements in crabs, *Ucides cordatus*, from a subtropical mangrove affected by fertilizer industry. J. Environ. Sci. 54, 69–76.

Brantley, S.L., Liermann, L., Bau, M., Wu, S., 2001. Uptake of trace elements and rare earth elements from hornblende by a soil bacterium. Geomicrobiol J. 18, 37–61.

Chen, Z.X., Wang, X.R., Tian, L.Q., Dai, L.M., 1995. Simulation study in the effects of acid precipitation on the environmental chemical behaviour and plant utility of REEs. Acta Sci. Circumst. 15 (1), 32–38.

Cheng, J., Li, N., Cai, J., Cheng, Z., Hu, R., Zhang, Q., Wan, F., Sun, Q., Gui, S., Sang, X., Wang, L., Hong, F., 2012. Organ histopathological changes and its function damage in mice following longterm exposure to lanthanides chloride. Biol. Trace Elem. Res. 145, 361–368.

Cobelo-García, A., Filella, M., Croot, P., Frazzoli, C., Du Laing, G., Ospina-Alvarez, N., Rauch, S., Salaun, P., Schäfer, J., Zimmermann, S., 2015. COST action TD1407: network on technology-critical elements (NOTICE)—from environmental processes to human health threats. Environ. Sci. Pollut. Res. 22, 15188–15194.

Cotton, S.A., 2006. Lanthanide and actinide chemistry. Inorganic Chemistry: A Textbook Series, 2nd ed. John Wiley and Sons, Ltd., UK.

Dahle, J.T., Arai, Y., 2015. Environmental geochemistry of cerium: applications and toxicology of cerium oxide nanoparticles. Int. J. Environ. Res. Public Health 12 (2): 1253–1278. https://doi.org/10.3390/ijerph120201253.

Davranche, M., Gruau, G., Dia, A., Marsac, R., Pédrot, M., Pourret, O., 2015. Biogeochemical factors affecting rare earth elements distribution in shallow wetland groundwater. Aquat. Geochem. 21, 197–215.

Du, X., Graedel, T.E., 2013. Uncovering the end uses of the rare earth elements. Sci. Total Environ. 461, 781–784.

Dupré, B., Viers, J., Dandurand, J.L., Polve, M., Benezeth, P., Vervier, P., Braun, J.J., 1999. Major and trace elements associated with colloids in organic-rich river waters: ultrafiltration of natural and spiked solutions. Chem. Geol. 160, 63–80.

Evans, C.H., 1990. Biochemistry of the Lanthanides. Plenum Press, New York.

Francis, A.J., 1997. Biotechnology of radioactive wastes: a general overview. In: Ronneau, C., Bitchaeva, O. (Eds.), Biotechnology of Waste Management and Site Restoration. Kluwer Academic, The Netherlands, pp. 19–28.

Franus, W., Wiatros-Motyka, M.M., Wdowin, M., 2015. Coal fly ash as a resource for rare earth elements. Environ. Sci. Pollut. Res. 22 (12):9464–9474. https://doi.org/10.1007/s11356-015-4111-9.

Fujiwara, K., To, Y.M., Kawakami, H., Aoki, M., Tuzuki, M., 2008. Evaluation of metal toxicity in *Chlorella kessleri* from the perspective of the periodic table. Bull. Chem. Soc. Jpn. 81:478–488. https://doi.org/10.1246/bcsj.81.478.

Gonzalez, V., Vignati, D.A., Leyval, C., Giamberini, L., 2014. Environmental fate and ecotoxicity of lanthanides: are they a uniform group beyond chemistry? Environ. Int. 71, 148–157.

Gu, Z.M., Wang, X.R., Gu, X.Y., Cheng, J., Wang, L.S., Dai, L.M., Cao, M., 2001. Determination of stability constants for rare earth elements and fulvic acids extracted from different soils. Talanta 53, 1163–1170.

Gwenzi, W., Mangori, L., Danha, C., Chaukura, N., Dunjana, N., Sanganyado, E., 2018. Sources, behaviour, and environmental and human health risks of high technology rare earth elements as emerging contaminants. Sci. Total Environ. 636, 299–313.

He, M.L., Wang, Y.Z., Xu, Z.R., Chen, M.L., Rambeck, W.A., 2003. Effect of dietary rare earth elements on growth performance and blood parameters of rats. J. Anim. Physiol. Anim. Nutr. 87, 229–235.

Herrmann, H., Nolde, J., Berger, S., Heise, S., 2016. Aquatic ecotoxicity of lanthanum - a review and an attempt to derive water and sediment quality criteria. Ecotoxicol. Environ. Saf. 124:213–238. https://doi.org/10.1016/j.ecoenv.2015.09.033.

Hua, D., Wang, J., Yu, D., Liu, J., 2017. Lanthanum exerts acute toxicity and histopathological changes in gill and liver tissue of rare minnow (*Gobiocypris rarus*). Ecotoxicology 26, 1207–1215.

Joonas, E., Aruoja, V., Olli, K., Syvertsen-Wiig, G., Vija, H., Kahru, A., 2017. Potency of (doped) rare earth oxide particles and their constituent metals to inhibit algal growth and induce direct toxic effects. Sci. Total Environ. 593, 478–486.

Khan, A.M., Bakar, N.K.A., Bakar, A.F.A., Ashraf, M.A., 2017. Chemical speciation and bioavailability of rare earth elements (REEs) in the ecosystem: a review. Environ. Sci. Pollut. Res. 24, 22764–22789.

Khangarot, B.S., Das, S., 2009. Acute toxicity of metals and reference toxicants to a freshwater ostracod, *Cypris subglobosa* Sowerby, 1840 and correlation to EC50 values of other test models. J. Hazard. Mater. 172 (2–3), 641–649.

Kulaksız, S., Bau, M., 2011. Anthropogenic gadolinium as a microcontaminant in tap water used as drinking water in urban areas and megacities. Appl. Geochem. 26, 1877–1885.

Kulaksız, S., Bau, M., 2013. Anthropogenic dissolved and colloid/nanoparticle-bound samarium, lanthanum and gadolinium in the Rhine River and the impending destruction of the natural rare earth element distribution in rivers. Earth Planet. Sci. Lett. 362, 43–50.

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 (7), 754.
Lawrence, M.G., Ort, C., Keller, J., 2009. Detection of anthropogenic gadolinium in treated wastewater in South East Queensland, Australia. Water Res. 43 (14), 3534–3540.
Lürling, M., Tolman, Y., 2010. Effects of lanthanum and lanthanum-modified clay on growth, survival and reproduction of *Daphnia magna*. Water Res. 44 (1):309–319. https://doi.org/10.1016/j.watres.2009.09.034.
Moermond, C.T.A., Tijink, J., van Wezel, A.P., Koelmans, A.A., 2001. Distribution, speciation, and bioavailability of lanthanides in the Rhine-Meuse estuary, The Netherlands. Environ. Toxicol. Chem. 20 (9):1916–1926. https://doi.org/10.1002/etc.5620200909.
Neal, C., 2007. Rare earth element concentrations in dissolved and acid available particulate forms for eastern UK rivers. Hydrol. Earth Syst. Sci. 11 (1), 313–327.
Neal, C., Whitehead, P.G., Jeffery, H., Neal, M., 2005. The water quality of the River Carnon, west Cornwall, November 1992 to March 1994: the impacts of Wheal Jane discharges. Sci. Total Environ. 338 (1), 23–39.
Nordberg, G., Fowler, B., Nordberg, M., 2014. Handbook on the Toxicology of Metals. 4th ed. Academic Press.
OECD, 1998. OECD Guideline for Testing of Chemicals: *Daphnia magna* Reproduction Test (211). OECD, Paris, France.
OECD, 2004. OECD Guideline for Testing of Chemicals: *Daphnia sp.* Acute Immobilisation Test (202). OECD, Paris, France.
Pagano, G., Guida, M., Tommasi, F., Oral, R., 2015. Health effects and toxicity mechanisms of rare earth elements–knowledge gaps and research prospects. Ecotoxicol. Environ. Saf. 115:40–48. https://doi.org/10.1016/j.ecoenv.2015.01.030.
Pang, X., Li, D., Peng, A., 2002. Application of rare-earth elements in the agriculture of China and its environmental behavior in soil. Environ. Sci. Pollut. Res. 9 (2): 143–148. https://doi.org/10.1007/BF02987462.
Perelomov, L.V., Yoshida, S., 2008. Effect of microorganisms on the sorption of lanthanides by quartz and goethite at the different pH values. Water Air Soil Pollut. 194 (1–4), 217–225.
Pourret, O., Davranche, M., Gruau, G., Dia, A., 2007. Organic complexation of rare earth elements in natural waters: evaluating model calculations from ultrafiltration data. Geochim. Cosmochim. Acta 71 (11):2718–2735. https://doi.org/10.1016/j.gca.2007.04.001.
Rabiet, M., Brissaud, F., Seidel, J.L., Pistre, S., Elbaz-Poulichet, F., 2009. Positive gadolinium anomalies in wastewater treatment plant effluents and aquatic environment in the Herault watershed (South France). Chemosphere 75, 1057–1064.
Rim, K.T., 2016. Effects of rare earth elements on the environment and human health: a literature review. Toxicol. Environ. Heal. Sci. 8 (3):189–200. https://doi.org/10.1007/s13530-016-0276-y.
Rim, K.T., Koo, K.H., Park, J.S., 2013. Toxicological evaluations of rare earths and their health impacts to workers: a literature review. Saf. Health Work 4, 12–26.
Schijf, J., Byrne, R.H., 2001. Stability constants for mono- and dioxalato-complexes of Y and the REE, potentially important species in groundwaters and surface freshwaters. Geochim. Cosmochim. Acta 65, 1037–1046.
Scott, G., Crunkilton, R.L., 2000. Acute and chronic toxicity of nitrate to fathead minnows (*Pimephales promelas*), *Ceriodaphnia dubia* and *Daphnia magna*. Environ. Toxicol. Chem. 19, 2918–2922.
Siekierski, S., Mioduski, T., Salomon, M., 1983. Solubility Data Series Volume 13. Scandium, Yttrium, Lanthanum and Lanthanide Nitrates. Pergamon Press, Oxford.
Stauber, J.L., Binet, M.T., 2000. Canning River Phoslock Field Trial – Ecotoxicity Testing Final Report. Report No: ET317R. CSIRO Centre for advanced analytical chemistry energy technology, Australia.
Tai, P., Zhao, Q., Su, D., Li, P., Stagnitti, F., 2010. Biological toxicity of lanthanide elements on algae. Chemosphere 80, 1031–1035.
Tang, J., Johannesson, K.H., 2010. Ligand extraction of rare earth elements from aquifer sediments: implications for rare earth element complexation with organic matter in natural waters. Geochim. Cosmochim. Acta 74, 6690–6705.
Tyler, G., 2004. Rare earth elements in soil and plant systems — a review. Plant Soil 267, 191–206.
US EPA, 2005. Standard methods for examination of water and wastewater. http://www.standardmethods.org/store.
Van Hoecke, K., Quik, J.T.K., Mankiewicz-Boczek, J., De Schamphelaere, K.A.C., Elsaesser, A., Van der Meeren, P., Barnes, C., McKerr, G., Howard, C.V., Van De Meent, D., Rydzynski, K., Dawson, K.A., Salvati, A., Lesniak, A., Lynch, I., Silversmit, G., De Samber, B., Vincze, L., Janssen, C.R., 2009. Fate and effects of CeO_2 nanoparticles in aquatic ecotoxicity tests. Environ. Sci. Technol. 43:4537–4546. https://doi.org/10.1021/es9002444.
Vasconcelos, M.T., Leal, M.F., 2008. Exudates of different marine algae promote growth and mediate trace metal binding in *Phaeodactylum tricornutum*. Mar. Environ. Res. 66 (5):499–507. https://doi.org/10.1016/j.marenvres.2008.07.002.
Weltje, L., Heidenreich, H., Zhu, W., Wolterbeek, H.T., Korhammer, S., de Goeij, J.J., Markert, B., 2002. Lanthanide concentrations in freshwater plants and molluscs, related to those in surface water, pore water and sediment: a case study in the Netherlands. Sci. Total Environ. 286, 191–214.
Wood, S.A., 1990. The aqueous geochemistry of the rare-earth elements and yttrium: 1. Review of available low-temperature data for inorganic complexes and the inorganic REE speciation of natural waters. Chem. Geol. 82 (1–2):159–186. https://doi.org/10.1016/0009-2541(90)90080-Q.

Supplementary Information

Evaluation of the potential hazard of lanthanides to freshwater microcrustaceans

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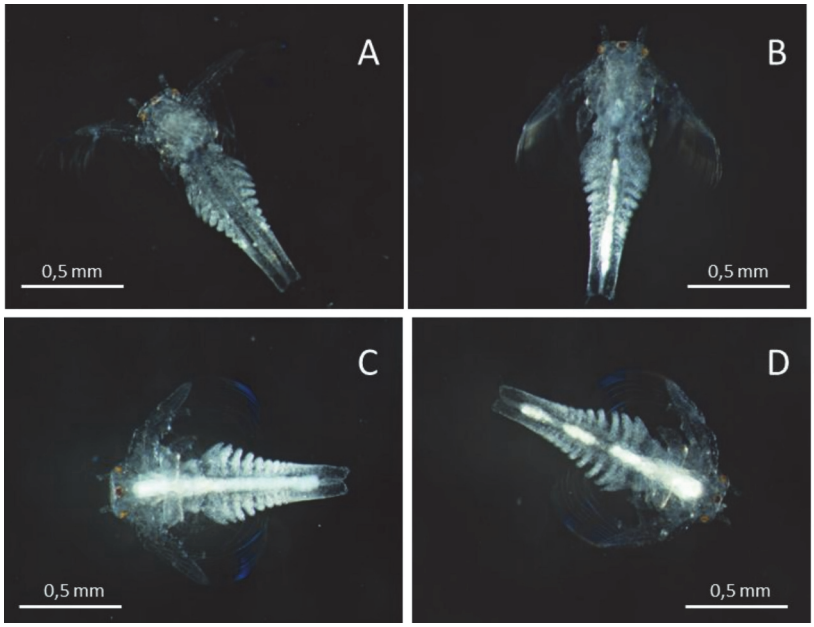


Fig. S1. Accumulation of the insoluble Ln compounds in the gut of *Thamnocephalus platyurus* during 24 h acute test: A – control; B - at nominal concentration 25 mg Ce /L; C- at nominal concentration 20 mg La/L; D - at nominal concentration 10 mg Pr/L.

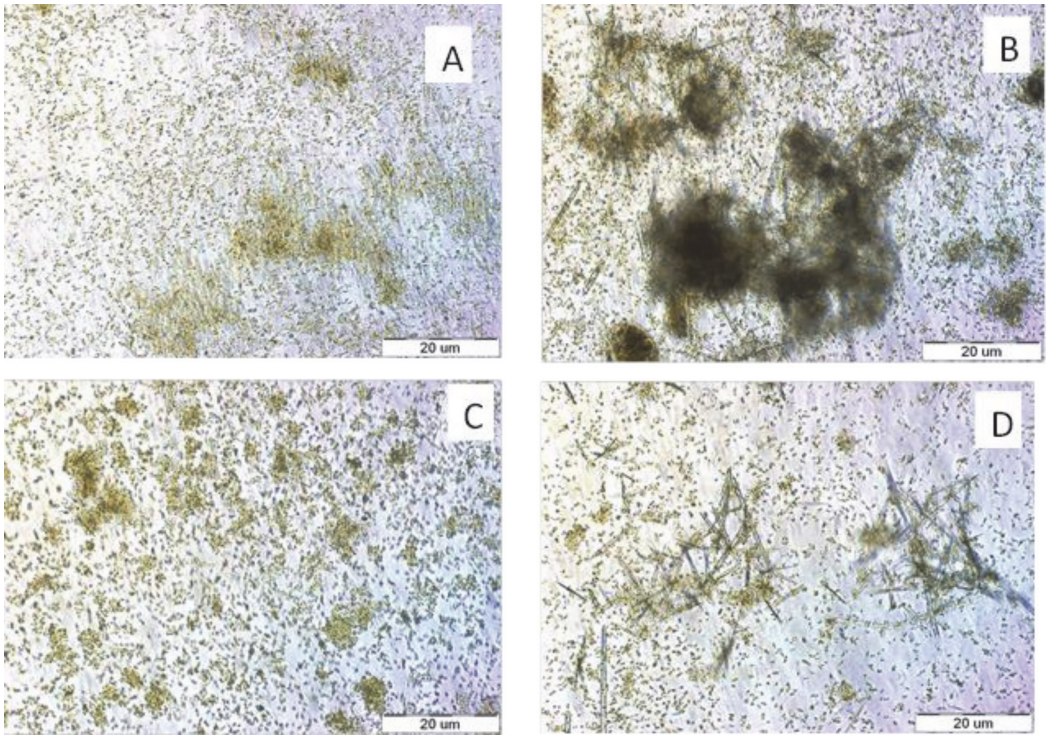
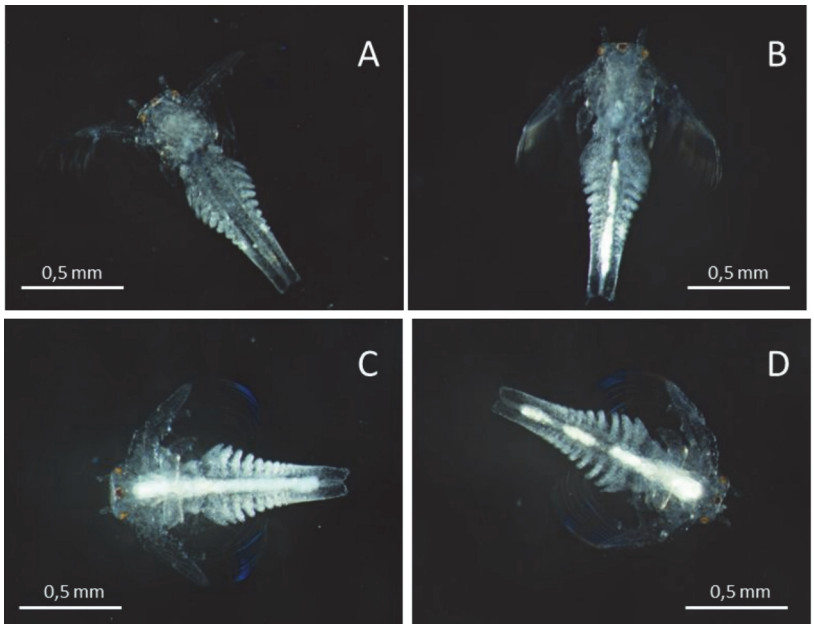


Fig. S2. Algae *Raphidocelis subcapitata* were found both as individual planktonic cells and as crystal clusters with Ln in ostracod *Heterocypris incongruens* test. Examples of algae agglomeration in control (A) and at nominal concentration 25 mg Ln/L of Ce (B), Nd (C) and Pr (D).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Appendix 6

Publication VI

Blinova, I., Vija, H., Lukjanova, A., **Muna, M.**, Syvertsen-Wiig, G., & Kahru, A. (2018). Assessment of the hazard of nine (doped) lanthanides-based ceramic oxides to four aquatic species. *Science of The Total Environment, 612,* 1171–1176.

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Assessment of the hazard of nine (doped) lanthanides-based ceramic oxides to four aquatic species

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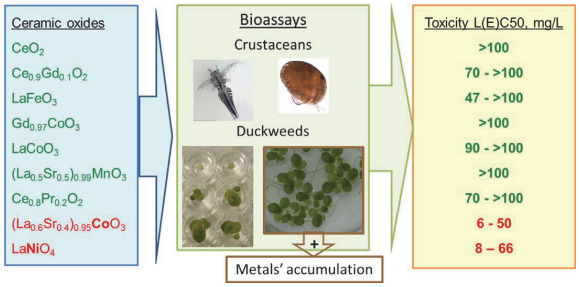
HIGHLIGHTS

- Toxicity of (doped) lanthanide-based ceramic oxides to crustaceans and duckweeds was studied
- Toxicity of (doped) lanthanide-based ceramic oxides was mostly due to bioavailable fraction of Ni and Co
- Ceramic oxides not containing Ni or Co had very low toxicity to crustaceans and duckweeds (EC50: 50 -> 100 mg/L)
- The test design may significantly affect bioavailability of tested compound to test species.
- Accumulation of metals in the duckweed *Lemna minor* is a good indicator of potential hazard of poorly soluble metal oxides.

GRAPHICAL ABSTRACT



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ABSTRACT

The risk of environmental pollution with rare earth oxides rises in line with increasing application of these compounds in different sectors. However, data on potential environmental hazard of lanthanides is scarce and concerns mostly Ce and Gd. In this work, the aquatic toxicity of eight doped lanthanide-based ceramic oxides ($Ce_{0.9}Gd_{0.1}O_2$, $LaFeO_3$, $Gd_{0.97}CoO_3$, $LaCoO_3$, $(La_{0.5}Sr_{0.5})_{0.99}MnO_3$, $Ce_{0.8}Pr_{0.2}O_2$, $(La_{0.6}Sr_{0.4})_{0.95}CoO_3$, $LaNiO_4$) and one non-doped oxide (CeO_2) with primary size from 23 to 590 nm were evaluated in four short-term laboratory assays with freshwater crustaceans and duckweeds. Results showed no acute toxicity (EC50 > 100 mg/L) or very low acute toxicity for most studied oxides. Observed toxicity was probably due to bioavailable fraction of dopant metals (Ni and Co) but in the case of aquatic plants, decrease of nutrient availability (complexing of phosphorus by lanthanides) was also presumed. Studied oxides/metals accumulated in the aquatic plant tissue and in the gut of crustaceans and thus may be further transferred via the aquatic food chain. Accumulation of metals in the duckweed *Lemna minor* may be recommended as a cost-effective screening bioassay for assessment of potential hazard of poorly soluble oxides to aquatic ecosystems.

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0048-9697/

1. Introduction

The worldwide production of rare earth oxides (REO) is rapidly increasing as these materials are used in different technologies (catalysis, electronics, fuel cells, etc.) (Campbell, 2014; Graedel et al., 2015). The widespread application of REO increases the hazard of environmental contamination at different stages of their life cycle: production-transportation-use-waste deposition. However, the current knowledge on the ecotoxicity of rare earth elements (REEs) (i.e., lanthanides and yttrium) and their behaviour in the water and soil ecosystems is insufficient for evaluation of risks related to elevated concentrations of these metals (Cobelo-García et al., 2015). In respect of environmental hazard, the most studied REEs are cerium and lanthanum, while information on other REEs is scarce and inconsistent (Herrmann et al., 2016; Babula et al., 2008). The search in ISI WoS made on Feb 02, 2017 showed that there were 99 papers on (ecotoxic* AND cerium), 61 on (ecotoxic* AND lanthan*) and 19 on (ecotoxic* AND gadolinium) and just 2 papers on (ecotoxic* AND praseod*). For comparison, there were 879 papers on (ecotoxic* AND titan*). Although remarkable amount of ecotoxicological information on widely used REOs such as nano-CeO_2 already exists (Juganson et al., 2015; Montini et al., 2016), there is still no consensus on its environmental hazard (Dahle and Arai, 2015). The data on potential environmental hazard of other types of REOs is practically absent. The composition and physicochemical properties of the metal oxides can be tuned via doping that allows widening the field of applications of these metal oxides (Pokhrel et al., 2013; Xu et al., 2017). As a result, a diverse range of lanthanide-based ceramic metal oxides enter the market while their environmental hazard evaluation required for risk assessment is very costly. The "read-across" approach may significantly reduce costs of hazard evaluation of lanthanide-based ceramic oxides, but application of the grouping concept requires generation of relevant ecotoxicity data for a representative set of chemicals belonging to this group.

In the current work, the aquatic toxicity of eight doped lanthanide-based ceramic oxide powders designed for application in solid oxide fuel cell systems or in gas separation membranes (CerPoTech, 2007) was evaluated using short-term laboratory assays with crustaceans and duckweeds. Non-doped CeO_2 was included in the study to compare our test results with previously available data. The applied test species represent different aquatic trophic levels and are widely used to screen the potential hazard of pollutants to aquatic ecosystems. To receive more relevant information for hazard assessment we applied different exposure scenarios and quantified tentative bioavailability and bioaccumulation of metals in the plant tissue. REOs are poorly water-soluble substances and therefore their hazard evaluation needs special methods (OECD 23, 2000). There are two main approaches for hazard evaluation of the poorly soluble substances: (i) toxicity evaluation of water available fraction (leachates) of the solid particles (OECD 23, 2000) and/or (ii) exposure of the test organisms to oxides' suspension, e.g., prepared as recommended for nanoparticles (NPs) (OECD, 2014; Käkinen et al., 2016). From our point of view, the latter protocol is more environmentally relevant for oxides with primary size at nano- or micro scale as these compounds may enter the organism as ions and via ingestion of pure particles or particles attached to the food.

2. Methods

2.1. Chemicals

The (doped) lanthanides-based ceramic oxides were synthesised using spray pyrolysis technique (Messing et al., 1993) by CerPoTech AS, Norway (CerPoTech, 2007). The list of oxides is presented in Table 1 and described in more detail in Joonas et al. (2017). The specific surface area (Brunauer–Emmett–Teller method) and primary size of oxide particles were provided by the producer. For the preparation of the stock suspensions (100 mg/L) oxides were suspended in the corresponding test medium, sonicated using 450 Ultrasonifier (Branson Ultrasonics Corporation) probe for 2 min at 20 kHz 40 W and used for preparation of the test series dilutions on the same day.

Table 1
The (doped) lanthanide-based ceramic oxides used in the experiments.

	Specific surface area, m^2/g	Primary particle size, nm
CeO_2	22.0	38
$Ce_{0,9}Gd_{0,1}O_2$	31.1	27
$Gd_{0,97}CoO_3$	3.4	230
$LaCoO_3$	1.4	590
$LaFeO_3$	7.2	126
La_2NiO_4	3.0	284
$(La_{0,6}Sr_{0,4})_{0,95}CoO_3$	15.0	65
$(La_{0,5}Sr_{0,5})_{0,99}MnO$	7.0	137
$Ce_{0,8}Pr_{0,2}O_2$	36.1	23

2.2. Quantification of the metal oxides in the test environment and plant tissue

Total concentrations of the metals in the oxides suspensions were measured before and after testing. Before testing, concentration was measured after shaking of the suspensions. At the end of the test, samples from the upper layer of exposure medium (at the concentrations 100 and 10 mg/L) were collected avoiding re-suspension of settled oxides. Total metal concentrations in the test medium and in plant tissue were quantified using the total reflection X-ray fluorescence spectrometer (TRXF) Picofox S2 (Bruker AXS Microanalysis GmbH). For quantification of metal concentrations in duckweed's tissue ~10 mg of dry biomass was dissolved in 200 μl of 65% HNO_3 and digested at 60 °C. After that the samples were dried in the airflow at 60 °C and 1 ml of water was added to the dry pellet, vortexed, centrifuged and pellet dried in SpeedVac overnight. Then 200 μl of concentrated HNO_3 including 1 ppm Ga (internal standard) was added to the dry pellet and samples were incubated in ultrasonication bath for 1 h before analysis for metals with TRFX. Concentration of metals was quantified with Spectra software (Bruker AXS Microanalysis GmbH). Detection limit for lanthanides (La, Ce, Gd, Pr) was 0.05 mg/L and for other measured metals — 0.005 mg/L.

2.3. Toxicity tests

2.3.1. Bioassays with crustaceans

The larvae for the *Thamnocephalus platyurus* (a fairy shrimp) acute toxicity assay were obtained after hatching of the cysts at 25 °C for 24 h under continuous illumination. Larvae (<24 h old) were incubated for 24 h in suspensions of (doped) REOs at 25 °C in the dark. Before and during the test larvae were not fed. Four independent tests with 3 replicates (10 larvae per 1 ml of exposure medium) for each concentration of the chemical were conducted.

Six-day acute toxicity test with the benthic ostracod *Heterocypris incongruens* was performed according to OSTRACODTOXKIT F test procedure (Ostracodtoxkit, 1995). Briefly, ostracods hatched from the cysts (<24 h old) were incubated with suspensions of (doped) rare earth oxides for 6 days at 25 °C in the dark. Ostracods were provided with food (algae *Raphidocelis subcapitata*) immediately after hatching from the cysts as well as once at the start of the test. The concentration of algae at the beginning of the test was about 10^8 cells/ml. The toxicity endpoint was mortality and growth inhibition of ostracods. The length of ostracods was measured under the dissection microscope before and after incubation. The test was performed in two test formats: with and without the standard sand (included in the Toxkit). Each test was repeated twice with 2 replicates of each tested concentration of the chemical (10 larvae per 4 ml of exposure medium).

Cysts of *T. platyurus* and OSTRACODTOXKIT F were purchased from MicroBio-Tests, Inc. (Mariakerke-Gent, Belgium). In both tests with

crustaceans the moderately hard synthetic freshwater (mg/L: $NaHCO_3$–96, $CaSO_4$*$2H_2O$–60, $MgSO_4$*$7H_2O$–123, KCl–4) was used as a test medium. Exposure concentrations of REOs for ostracod bioassay were selected on the basis of *T. platyurus* test results.

2.3.2. Bioassays with duckweeds

In the *Lemna minor* (common duckweed) growth inhibition test (OECD 221, 2006) plants from laboratory culture were incubated with suspensions of (doped) rare earth oxides in glass vessels containing 100 ml test medium for 7 days. Growth inhibition of *Lemna* was determined by number of fronds and dry biomass of the plant. Each test was repeated three times and each concentration of the chemical was analysed in triplicate.

In the 3 days growth inhibition assay of *Spirodela polyrhiza* (giant-duckweed), inhibition of the growth of germinated "turions" (resting fronds) was measured. The areas of the first fronds were measured before and after the exposure of the turions in plastic multiwell plates (one plant per 1 ml of suspension). For details, see test operation procedure (Spirodela Duckweed Toxkit, 2013).

Both duckweeds were incubated under continuous illumination (7000 lx) at 25 °C. The Swedish Standard (SIS) growth medium for L. *minor* and Steinberg medium for *S. polyrhiza* were used in the experiments (OECD 221, 2006). SIS medium contains less nitrate and phosphate ions and has lower conductivity (320 μS/cm) than Steinberg medium (940 μS/cm).

Accumulation of metals in L. *minor* tissue was measured at the end of the test after washing the whole plant in deionized water and then drying it at 90 °C. It should be mentioned that in spite of careful washing small amount of suspended oxides remained attached to the plant surface.

In all bioassays used in the current study the highest nominal exposure concentration of REOs was 100 mg/L. The upper concentration limit used in the toxicity assays (100 mg/L) was chosen based on the hazard ranking criteria for aquatic environment (EC Regulation No 1272/2008, 2008), according to which the substances with L(E)C50 > 100 mg/L are considered "not classified" (i.e., not harmful). The selection of the range of exposure concentrations was based on the preliminary tests.

2.3.3. Data analysis

The EC50 values were calculated using the REGTOX software for Microsoft Excel (Vindimian, 2001). The statistical significance of the differences between mean values from different tests was evaluated using one-way analysis of variance (ANOVA) followed by the Student's *t*-test, $p < 0.05$ was considered to be statistically significant.

3. Results and discussion

3.1. Test media characterisation

The behaviour of metal oxide particles in the aquatic ecosystems is determined by following processes: sedimentation, dissolution, absorption on the sediments or organic ligands and uptake by organisms. Thus, the key parameters which may significantly affect bioavailability of the tested compounds in bioassays are chemical composition of the test media, presence/absence of sediment and/or organic ligands (Käkinen et al., 2011).

In the current study, the same test medium was used in both crustacean assays. The main differences between bioassays with crustaceans *T. platyurus* and *H. incongruens* were test duration and feeding or not feeding the test organisms with microalgae. In addition, the ostracod test was performed in two formats: with and without addition of sand into the test wells. Thus, presence of algae and sand were the main factors which may affect speciation and distribution of metals in the ostracod bioassay. To evaluate the effect of test design on the metals concentration in the water column the chemical analysis of upper layer sampled from the test- wells was made i) after 24 h settling without test organisms present, ii) at the end of 24 h *T. platyurus* test (no algae added), iii) at the end of 6 days ostracod test (with algae added; test conducted with and without addition of sand into the test wells). In Table 2 total metal concentrations measured at highest exposure concentration (100 mg compound/L) in the upper layer of the water column pooled from three different experiments (without test organisms, *T. platyurus test* and ostracod test without sand) are presented. For more details on sampling, see Table 2. We use term 'total metal concentration' as water samples from tests tubes may contain both soluble and insoluble forms (suspended oxide particles) of metals.

The chemical analysis showed that the share of metals that remained in the water column in tests with crustaceans was very small (Table 2). Content of most of the metals in the water column was < 1% of nominal oxide concentration, and only in the case of Ni, Co and Sr, i.e. metals used for doping of studied REOs (Table 1), the concentrations in the water column were up to 10% (Ni, Co) or close to 20% (Sr) reflecting the different metals leaching from REOs.

Although there were differences in the exposure scenarios (i.e., duration of the assay, presence or absence of food/algae or test organisms) the concentration of the metals in the water column varied only slightly in different tests with crustaceans (Table 2). The highest metal concentrations in the water column were observed for REOs incubated without test organisms present but in general the concentrations did not vary remarkably (Table 2). However, in the ostracod test with sand (these data are not included in Table 2) concentration of metals in the water column were 3–10-fold lower than without sand in the test wells.

In duckweed tests, metal concentration at the end of the test (day 7) was not measured as preliminary experiments showed that concentrations of practically all REOs' metals in the test medium at the end of the test before harvesting of the duckweeds were under detection limit.

The pH during the test slightly increased, on average from 6.8 to 7.4 in the crustacean tests and from 5.8 to 7.1 in the duckweed tests. In all test media most of the suspended REO particles rapidly settled.

3.2. Toxicity of REOs

Chemical analysis showed that in all bioassays the metal concentrations in the oxide suspensions at the beginning of tests did not differ from nominal concentration by >5% (data not shown), therefore, nominal concentrations of oxides were used for the calculation of EC50 values (Table 3). The highest exposure concentration of oxides in all bioassays was 100 mg/L as recommended by OECD202 guideline (OECD, 2004).

Toxicity testing showed that three oxides - CeO_2, $Gd_{0,97}CoO_3$ and $(La_{0,5}Sr_{0,5})_{0,99}MnO_3$ were not toxic at 100 mg/L for crustaceans and duckweeds. Only two oxides - La_2NiO_4 and $(La_{0,6}Sr_{0,4})_{0,95}CoO_3$ - proved toxic to all four test species (Table 3). Our results on CeO_2 particles are in agreement with Van Hoecke et al. (2009) who also did not observe acute toxicity of 14, 20, and 29 nm CeO_2 to *T. platyurus* and *D. magna* even at 1000 mg/L.

The results of the bioassays showed that toxicity of the tested REOs to crustaceans and duckweeds did not depend on size or specific surface area of the oxide particles as it has also been shown for microalgae (Joonas et al., 2017).

In Table 4 acute EC50 values for Ni, Co, La, Fe, Mn and Sr obtained from the literature for *Daphnia magna* and *Lemna minor* are presented. Our previous studies have demonstrated that acute toxicity values for different metal-based nanoparticles and heavy metals to *D. magna* and *T. platyurus* were very similar (Blinova et al., 2010; Blinova et al., 2013; Uudeküll et al., 2017). We also observed comparable toxicity of the (doped) rare earth oxides studied in this work towards *T. platyurus* and *D. magna* (preliminary tests, data not shown). Therefore, data for *D. magna* will be used for further comparison with

Table 2
Total metal concentration (mg Me/L) in water samples collected from the water column at the end of the tests with crustaceans.

Compound (nominal exposure concentration)	**Ce**	**Gd**	Co	**La**	Fe	Ni	Sr	Mn	**Pr**
CeO_2	0.13–0.17[a]								
(100 mg/L)	(81.4)[b]								
Ce$_{0.9}$**Gd**$_{0.1}O_2$	0.06–0.09	0.05–0.09							
(100 mg/L)	(72.5)	(9.05)							
Gd$_{0.97}CoO_3$		< 0.05–0.08	0.04–0.1						
(100 mg/L)		(58.1)	(23.1)						
LaCoO_3			0.12–0.18	0.14–0.16					
(100 mg/L)			(24.0)	(56.5)					
LaFeO_3				<0.05–0.07	0.05–0.07				
(100 mg/L)				(57.2)	(23.0)				
La$_2NiO_4$				0.18–0.22		1.8–2.2			
(100 mg/L)				(53.1)		(22.4)			
(**La**$_{0.6}$**Sr**$_{0.4}$)$_{0.95}CoO_3$			0.2–0.8	0.10–0.12			2.4–2.8		
(100 mg/L)			(27.5)	(35.2)			(14.8)		
(**La**$_{0.5}$**Sr**$_{0.5}$)$_{0.99}MnO_3$				< 0.05–0.08			0.71–1.15	< 0.05–0.1	
(100 mg/L)				(31.8)			(20.1)	(25.7)	
Ce$_{0.8}$**Pr**$_{0.2}O_2$	<0.05–0.06								< 0.05–0.16
(100 mg/L)	(65.1)								(16.4)

Rare earth elements are in bold.
[a] Min–max values pooled from three different experiments. Water was collected avoiding re-suspension of settled oxides after: i) 24 h incubation of oxide suspensions in crustacean test medium (no organisms present), ii) 24 h incubation in the test with crustaceans *Thamnocephalus platyurus* (not fed with algae) and iii) after 6 days incubation with ostracods *Heterocypris incongruens* (fed with algae, no sand added to the test wells).
[b] Nominal exposure metal concentration is presented in the brackets.

observed results since the available information on toxicity of metals to *T. platyurus* is limited.

3.2.1. Toxicity to crustaceans *Thamnocephalus platyurus* and *Heterocypris incongruens*

Only two of the tested oxides - La_2NiO_4 and $(La_{0.6}Sr_{0.4})_{0.95}CoO_3$- showed relatively high toxicity to crustaceans (Table 3). Most likely Ni and Co were responsible for the toxic effect of these oxides as the concentration of Ni and Co in the water column at the end of exposure (Table 2) was comparable to the reported toxicity values for these elements to *D. magna* (Table 4). In particular, total concentration of Ni in the test water at 10 mg/L nominal La_2NiO_4 exposure concentration was 0.6–0.9 mg Ni/L that exceeded minimum reported EC50 concentration for *D. magna* (Table 4). High toxicity of $(La_{0.6}Sr_{0.4})_{0.95}CoO_3$ particles to *H. incongruens* in the test without sand addition may also be related to high concentration of Co in the water column: 0.6 mg Co/L at 10 mg/L nominal exposure concentration. The concentrations of lanthanides (Ce, Gd, La, Pr) in the water column were very low <0.2 mg/L (Table 2) even at highest exposure concentration (100 mg/L), that is much lower than respective acute EC50 values reported for *D. magna* (Table 4). Concentration of Sr was also considerably lower than LC50 values from the literature (Table 4). Thus, it could be concluded that in case of La_2NiO_4 and $(La_{0.6}Sr_{0.4})_{0.95}CoO_3$ main toxicity was due to bioavailable fraction of dopant metals - Ni and Co. This conclusion is also supported by the results of ostracod tests where addition of the sand to the test wells decreased the concentration of Co in the water column by 2–3 fold and that of Ni by 10-fold (data not shown). The decrease of Ni and Co concentrations in the water column was accompanied by the toxicity reduction of REOs to *H. incongruens* (Table 3) indicating that sand was binding toxic metals. Khangarot and Das (2009) showed that acute toxicity values for Ni^{2+} (EC50–86.8 mg/L) and Co^{2+} (EC50–25.5 mg/L) to a freshwater ostracod *Cypris subglobosa* (belonging to same family as *Heterocypris incongruens*) was significantly higher than to *D. magna* (Table 4), i.e. *D. magna* is more sensitive than ostracods to these metals. However, in our study, mortality of *H. incongruens* exposed to REOs containing Ni and Co (without sand) was only slightly higher than of toxicity of these oxides to *T. platyurus* (Table 3). This disagreement may be due to the different test designs: Khangarot and Das (2009) exposed ostracods to toxicants for 48 h without addition of food but in test format used in the current study exposure time was 3-fold longer (6 days) and ostracods were fed with algae during the test. Longer exposure to the toxicants as well as additional exposure route of metals via ingested with food may increase availability of Ni and Co to ostracods.

In the ostracod assay we also measured the body length of test organisms. In both test formats, with and without sand, there was a

Table 3
Toxicity of (doped) lanthanide-based ceramic oxides to crustaceans and duckweeds (L(E)C50[a], mg/L).

	Crustaceans			Duckweeds	
Test	*Heterocypris incongruens* 6 days mortality (organisms fed with algae)		*Thamnocephalus platyurus* 24 h mortality (organisms not fed)	*Lemna minor* 7 days growth inhibition	*Spirodela polyrhiza* 3 days growth inhibition
Test medium	Moderately hard synthetic freshwater			Swedish Standard medium (OECD 221)	Steinberg medium (OECD 221)
Test conditions	with sand	without sand	–	–	–
CeO_2	>100 (0)[b]	>100 (6.7)	>100 (3.3)	>100 (−3.3)	>100 (−7.0)
Ce$_{0.9}$**Gd**$_{0.1}O_2$	>100 (0)	>100 (3.1)	70 ± 14.7	>100 (16.2)	>100 (4.9)
Gd$_{0.97}CoO_3$	>100 (0)	>100 (20)	>100 (6.7)	>100 (22.0)	>100 (1.2)
LaCoO_3	>100 (5.4)	>100 (10.0)	>100 (11.7)	>100 (46.0)	90 ± 16.6
LaFeO_3	>100 (0)	>100 (0)	>100 (3.3)	47.1 ± 9.7	>100 (15.4)
La_2NiO_4	30 ± 9.8	8.5 ± 5.7	13 ± 0.3	29.1 ± 4.8	66.7 ± 6.9
(**La**$_{0.6}$**Sr**$_{0.4}$)$_{0.95}CoO_3$	51.5 ± 10.8	6.2 ± 2.8	33.3 ± 8.7	7.53 ± 1.90	6.38 ± 1.10
(**La**$_{0.5}$**Sr**$_{0.5}$)$_{0.99}MnO_3$	>100 (0)	>100 (0)	>100 (2.2)	>100 (14.9)	>100 (−10.6)
Ce$_{0.8}$**Pr**$_{0.2}O_2$	>100 (0)	>100 (0)	>100 (0)	69.2 ± 3.9	>100 (13.8)

Rare earth elements are in bold.
[a] LC50 values were calculated based on nominal exposure concentrations of oxides.
[b] In the brackets the highest adverse effect (mortality or inhibition, %) recorded at the highest exposure concentration (100 mg/L) is presented.

Table 4
Acute toxicity of selected metals collected from the literature (EC50, mg/L).

Test/metal	Ni	Co	La	Fe	Mn	Sr
Daphnia magna	0.2–8.0[a,b]	0.7–4.4[a,b]	1.6–2.8[a,c]	12.9–17.3[d]	9.3–60[a,b]	94–125[a,b]
Lemna minor	0.2–0.7[a]	0.2–16.0[a]	1.2–2.8[a]	–	31.0[a]	94–162[a]

[a] US Environmental Protection Agency, 2000.
[b] Okamoto et al., 2015.
[c] Petersen et al., 1974.
[d] Birge et al., 1985.

good correlation ($r^2 = 0.69$) between the average inhibition of ostracods' body length and their mortality. However, the length of ostracods in the test without sand was 10–25% smaller ($p < 0.05$) than the length of the organisms exposed to the same oxides concentration with sand present, even if there was no mortality. This indicates that body length is more sensitive parameter than mortality. Smaller size of exposed test organisms may be partly explained by indirect effect of oxides. Namely, Aruoja et al. (2015) studied the effects of metal oxides on growth of algae *Pseudokirchneriella subcapitata* and showed that metal oxides tend to agglomerate and entrap algae. This process can lead to the formation of the clumps of particle agglomerates with algae rendering them too large for the ingestion by crustaceans. Analogous hypothesis was suggested by Van Hoecke et al. (2009) who reported decrease of the size of daphnids in the reproduction test due to formation of the clumps of CeO_2 NPs aggregates and algae.

It should be also mentioned that in the case of non-selective filter-feeder organisms such as crustaceans, toxicity testing integrates the toxic hazard of both dissolved and insoluble fraction of oxides. However, in spite of the fact that at the end of all tests the gut of *T. platyurus* was fully filled with oxides (see Supplemental Fig. 1) in most cases acute toxic effects were not observed. Heinlaan et al. (2017) also showed that marginally soluble Co_3O_4 nanoparticles were not toxic to *D. magna* (48 h EC50 > 100 mg metal/L) although accumulated in the gut. This confirms once more that dissolved metals are mostly responsible for toxic effect of metal oxides (Kahru and Ivask, 2013). However, oxides accumulated in the gut may be transferred to the upper food chain levels.

3.2.2. Duckweeds Lemna minor and Spirodela polyrhiza

The study revealed very similar phytotoxicity ($r^2 = 0.72$) of investigated oxides to floating aquatic plants *Lemna minor* and *Spirodela polyrhiza*. Although it has been reported that *L.minor* has similar or lower sensitivity to heavy metals than *T.platyurus* (Blinova, 2004), in the current study some of the tested oxides such as $LaFeO_3$ and $Ce_{0.8}Pr_{0.2}O_2$ were not toxic to crustaceans but inhibited growth of L. *minor* (Table 3). This effect may be partly explained by complexation of lanthanides (La, Ce and Pr) with phosphate in the duckweed growth medium decreasing the availability of phosphate (a nutrient) needed for plant growth. This assumption is confirmed by the fact that growth inhibition after $LaFeO_3$ and $Ce_{0.8}Pr_{0.2}O_2$ exposure was observed in L. *minor* but not in *S. polyrhiza* test which was performed in the test medium that contained nearly 3-fold higer phosphate concentration than medium used in L. *minor* test (see Methods). The indirect negative effect of the same REOs was also shown in the growth inhibition test with microalgae (Joonas et al., 2017).

Analogously to crustaceans, bioavailable Ni and Co mainly accounted for observed toxicity of La_2NiO_4 and $(La_{0.6}Sr_{0.4})_{0.95}CoO_3$ to duckweeds. The accumulation of these metals in plant tissue supported this conclusion. Indeed, concentration of Ni in the tissue of L. *minor* exposed to La_2NiO_4 at 40% growth inhibition concentration was 390 mg/kg dry weight that is comparable to value (311 mg/kg at EC50) reported by Drost et al. (2007) for Ni salt. In the case of $(La_{0.6}Sr_{0.4})_{0.95}CoO_3$, concentration of Co in the tissue of L. *minor* was 1355 mg/kg at 60% inhibition concentration which is also comparable with data reported by other researchers for Co salt (Begović et al., 2016).

The concentrations of all metals from investigated oxides were measured in duckweeds' tissue after the end of the test. Comparison of the metal concentrations in L. *minor* tissue and toxicity to tested species (Table 3) showed that toxic effect was always accompanied by elevated concentration of one or more metals in the plant tissue. For example, Co concentration in plants exposed to 10 mg/L of $(La_{0.6}Sr_{0.4})_{0.95}CoO_3$ (EC50 = 7.5 mg/L, Table 3) was 1355 mg/kg dry weight, whereas in L. *minor* exposed to 100 mg/L of non-toxic $LaCoO_3$ or $Gd_{0.97}CoO_3$ cobalt content was correspondingly only 20.1 and 15.7 mgCo/kg DW, i.e. accumulation of the toxic metals in the tissue was in agreement with toxicity test results (Table 3).

As different factors, for example, accumulation in the duckweeds or metabolites egested into water by plants may change solubility of settled oxides and, as a result, also change the concentrations of the soluble metals in the test medium over time, the metal accumulation in the plant tissue may be a relevant indicator on the bioavailability of the metals from poorly water-soluble REOs in the test vessel.

4. Conclusions

- Evaluation of the potential hazard of nine (doped) lanthanides-based ceramic oxides to four aquatic test species revealed that in short-term tests:
  - Most oxides - CeO_2, $Gd_{0.97}CoO_3$, $(La_{0.5}Sr_{0.5})_{0.99}MnO_3$, $Ce_{0.9}Gd_{0.1}O_2$, $LaFeO_3$, $LaCoO_3$ and $Ce_{0.8}Pr_{0.2}O_2$ - were not toxic (EC50 > 100 mg/L) or slightly toxic (EC50: 50–100 mg/L) to tested species;
  - La_2NiO_4 and $(La_{0.6}Sr_{0.4})_{0.95}CoO_3$ were toxic to all tested species, probably due to bioavailable fraction of Ni and Co, but additional adverse effect of other factors (dissolved lanthanides or ingested oxide particles) cannot be excluded;
  - Even not acutely toxic, the accumulation the metals/metal oxides in the aquatic plants and in the gut of crustaceans may lead to transfer of metals from studied ceramic oxides to the upper levels of the aquatic food chain;
  - Accumulation of metals in the duckweed L. *minor* may be recommended as cost-effective screening bioassay for assessment of potential long-term hazard of poorly soluble oxides to aquatic ecosystems.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2017.08.274.

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References

Aruoja, V., Pokhrel, S., Sihtmäe, M., Mortimer, M., Mädler, L., Kahru, A., 2015. Toxicity of 12 metal based nanoparticles to algae, bacteria and protozoa. Environ. Sci. Nano. 2: 630–644. http://dx.doi.org/10.1039/c5en00057b.

Babula, P., Adam, V., Opatrilova, R., Zehnalek, J., Havel, L., Kizek, R., 2008. Uncommon heavy metals, metalloids and their plant toxicity: a review. Environ. Chem. Lett. 6: 189–213. http://dx.doi.org/10.1007/s10311-008-0159-9.

Begović, L., Mlinarić, S., Dunić, J. Antunović, Katanić, Z., Lončarić, Z., Lepeduš, H., Cesar, V., 2016. Response of *Lemna minor* L. to short-term cobalt exposure: the effect on photosynthetic electron transport chain and induction of oxidative damage. Aquat. Toxicol. 175:117–126. http://dx.doi.org/10.1016/j.aquatox.2016.03.009.

Birge, W.J., Black, J.A., Westerman, A.G., Short, T.M., Taylor, S.B., Bruser, D.M., Wallingford, E.D., 1985. Recommendations on Numerical Values for Regulating Iron and Chloride Concentrations for the Purpose of Protecting Warm Water Species of Aquatic Life in the Commonwealth of Kentucky. Memorandum of Agreement No. 5429. Kentucky Natural Resources and Environmental Protection Cabinet, Lexington, KY.

Blinova, I., 2004. Use of freshwater algae and duckweeds for phytotoxicity testing. Environ. Toxicol. 4:425–428. http://dx.doi.org/10.1002/tox.20042.

Blinova, I., Ivask, A., Heinlaan, M., Mortimer, M., Kahru, A., 2010. Ecotoxicity of nanoparticles of CuO and ZnO in natural water. Environ. Pollut. 158:41–47. http://dx.doi.org/10.1016/j.envpol.2009.08.017.

Blinova, I., Niskanen, J., Kajankari, P., Kanarbik, L., Käkinen, A., Tenhu, H., Penttinen, O.P., Kahru, A., 2013. Toxicity of two types of silver nanoparticles to aquatic crustaceans *Daphnia magna* and *Thamnocephalus platyurus*. Environ. Sci. Pollut. R. 20: 3456–3463. http://dx.doi.org/10.1007/s11356-012-1290-5.

Campbell, G.A., 2014. Rare earth metals: a strategic concern. Miner. Econ. 27, 21–31.

CerPoTech, 2007. Applications. Ceramic Powder Technology AS, Heimdal, Norway Available at:. http://www.cerpotech.com/applications (Cited 2016 October 14).

Cobelo-García, A., Filella, M., Croot, P., Frazzoli, C., Du Laing, G., Ospina-Alvarez, N., Rauch, S., Salaun, P., Schäfer, J., Zimmermann, S., 2015. COST action TD1407: network on technology-critical elements (NOTICE)—from environmental processes to human health threats. Environ. Sci. Pollut. Res. 22, 15188–15194.

Dahle, J.T., Arai, Y., 2015. Environmental geochemistry of cerium: applications and toxicology of cerium oxide nanoparticles. Int. J. Environ. Res. Public Health 12, 1253–1278.

Drost, W., Matzke, M., Backhaus, T., 2007. Heavy metal toxicity to *Lemna minor*: studies on the time dependence of growth inhibition and the recovery after exposure. Chemosphere 67:36–43. http://dx.doi.org/10.1016/j.chemosphere.2006.10.018.

EC Regulation No 1272/2008, 2008. Official J. Of the European Union. European Commission, Brussels, Belgium.

Graedel, T.E., Harper, E.M., Nassar, N.T., Nuss, P., Reck, B.K., 2015. Criticality of metals and metalloids. PNAS 112:4257–4262. http://dx.doi.org/10.1073/pnas.1500415112.

Heinlaan, M., Muna, M., Juganson, K., Oriekhova, O., Stolle, 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*. Aquat. Toxicol. 189, 123–133.

Herrmann, H., Nolde, J., Berger, S., Heise, S., 2016. Aquatic ecotoxicity of lanthanum – a review and an attempt to derive water and sediment quality criteria. Ecotox. Environ. Saf. 124:213–238. http://dx.doi.org/10.1016/j.ecoenv.2015.09.033.

Joonas, E., Aruoja, V., Olli, K., Syvertsen-Wiig, G., Vija, H., Kahru, A., 2017. Potency of (doped) rare earth oxide particles and their constituent metals to inhibit algal growth and induce direct toxic effects. Sci. Total Environ. 593–594, 478–486.

Juganson, K., Ivask, A., Blinova, I., Mortimer, M., Kahru, A., 2015. NanoE-Tox: new and in-depth database concerning ecotoxicity of nanomaterials. Beilstein J. Nanotechnol. 6: 1788–1804. http://dx.doi.org/10.3762/bjnano.6.183.

Kahru, A., Ivask, A., 2013. Mapping the dawn of nanoecotoxicological research. Acc. Chem. Res. 46:823–833. http://dx.doi.org/10.1021/ar3000212.

Käkinen, A., Bondarenko, O., Ivask, A., Kahru, A., 2011. The effect of composition of different ecotoxicological test media on free and bioavailable copper from $CuSO_4$ and CuO nanoparticles: comparative evidence from a Cu-selective electrode and a Cu-biosensor. Sensors 11:10502–10521. http://dx.doi.org/10.3390/s111110502.

Käkinen, A., Kahru, A., Nurmsoo, H., Kubo, A.L., Bondarenko, O.M., 2016. Solubility-driven toxicity of CuO nanoparticles to Caco2 cells and *Escherichia coli*: effect of sonication energy and test environment. Toxicol. In Vitro 36:172–179. http://dx.doi.org/10.1016/j.tiv.2016.08.004.

Khangarot, B.S., Das, S., 2009. Acute toxicity of metals and reference toxicants to a freshwater ostracod, *Cypris subglobosa* Sowerby, 1840 and correlation to EC50 values of other test models. J. Hazard Mater. 172:641–649. http://dx.doi.org/10.1016/j.jhazmat.2009.07.038.

Messing, G.L., Zhang, S.C., Jayanthi, G.V., 1993. Ceramic powder synthesis by spray pyrolysis. J. Am. Ceram. Soc. 76:2707–2726. http://dx.doi.org/10.1111/j.1151-2916.1993.tb04007.x.

Montini, T., Melchionna, M., Monai, M., Fornasiero, P., 2016. Fundamentals and catalytic applications of CeO_2-based materials. Chem. Rev. 116, 5987–6041.

OECD, 2014. Ecotoxicology and Environmental Fate of Manufactured Nanomaterials: Test Guidelines. Series on the Safety of Manufactured Nanomaterials 40 (Paris, France).

OECD 221, 2006. Organisation for economic cooperation and development (OECD) guidelines 221 for the testing of chemicals. Lemna sp. Growth Inhibition Test (Paris, France).

OECD 23, 2000. Organisation for Economic Cooperation and Development (OECD) Guideline 23 for the Testing of Chemicals, Aquatic Toxicity Testing of Difficult Substances and Mixtures (Paris, France).

Okamoto, A., Yamamuroa, M., Tatarazako, N., 2015. Acute toxicity of 50 metals to *Daphnia magna*. J. App. Toxicol. 35:824–830. http://dx.doi.org/10.1002/jat.3078.

Ostracodtoxkit, F., 1995. "Direct contact" Toxicity Test for Freshwater Sediments. MicroBioTests Inc, Mariakerke, Belgium. Available at: http://www.microbiotests.be/SOPs/Ostracodtoxkit%20F%20SOP%20-%20A5.pdf. (Cited 2016 September 9).

Petersen, S.A., Sanville, W.D., Stay, F.S., Powers, C.F., 1974. Nutrient Inactivation as a Lake Restoration Procedure: Laboratory Investigations. Report EPA-660/3–74-032. p. 118 (Corvallis, Oregon).

Pokhrel, S., Nel, A.E., Mädler, L., 2013. Custom-designed nanomaterial libraries for testing metal oxide toxicity. Acc. Chem. Res. 46 (632–41). http://dx.doi.org/10.1021/ar300032q.

Spirodela Duckweed Toxkit, 2013. Growth Inhibition Microbiotest with Spirodela polyrhiza. MicroBioTests Inc, Mariakerke, Belgium. http://www.microbiotests.be/SOPs/Spirodela%20Duckweed%20Toxkit%20SOP%20-%20A5.pdf. (Cited 2016 September 15).

US Environmental Protection Agency, 2000. Ecotox Knowledgebase. EPA ECOTOX Database, Washington, DC Available at:. http://cfpub.epa.gov/ecotox/.

Uudeküll, P., Kozlova, J., Mändar, H., Link, J., Sihtmäe, M., Käosaar, S., Blinova, I., Kasemets, K., Kahru, A., Stern, R., Tätte, T., Kukli, K., Tamm, A., 2017. Atomic layer deposition of titanium oxide films on As-synthesized magnetic Ni particles: magnetic and safety properties. J. Magn. Magn. Mater. 429, 299–304.

Van Hoecke, K., Quik, J.T.K., Mankiewicz-Boczek, J., De Schamphelaere, K.A.C., Elsaesser, A., Van der Meeren, P., Barnes, C., McKerr, G., Howard, C.V., Van De Meent, D., Rydzynski, K., Dawson, K.A., Salvati, A., Lesniak, A., Lynch, I., Silversmit, G., De Samber, B., Vincze, L., Janssen, C.R., 2009. Fate and effects of CeO_2 nanoparticles in aquatic ecotoxicity tests. Environ. Sci. Technol. 43:4537–4546. http://dx.doi.org/10.1021/es9002444.

Vindimian, E., 2001. MSExcel macro REGTOX EV7.0.6.xls. Available at:. http://www.normalesup.org/~vindimian/en_index.html.

Xu, B., Zhang, Q.T., Yuan, S.S., Liu, S.X., Zhang, M., Ohno, T., 2017. Synthesis and photocatalytic performance of yttrium-doped CeO_2 with a hollow sphere structure. Catal. Today 281:135–143. http://dx.doi.org/10.1016/j.cattod.2016.06.049.

Supplemental data

Assessment of the hazard of nine (doped) lanthanides-based ceramic oxides to four aquatic species

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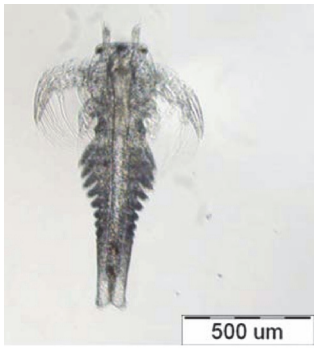
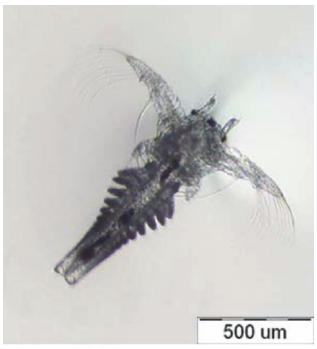
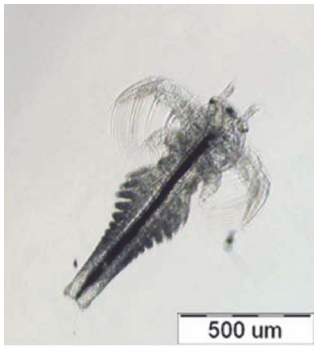
Fig. 1. Accumulation of the ceramic oxides in the gut of *Thamnocephalus platyurus* after 24 h exposure



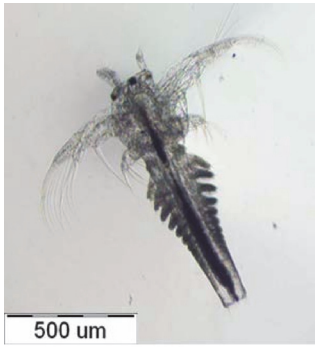
Control



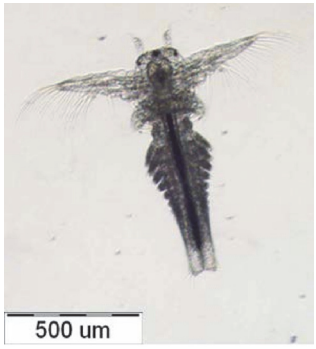
$Ce_{0,9}Gd_{0,1}O_2$ 100 ppm



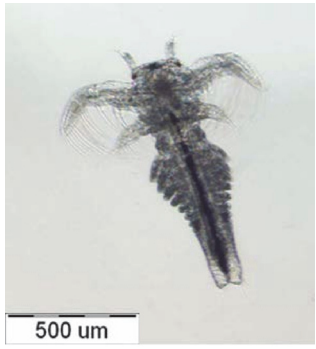
CeO_2 100 ppm



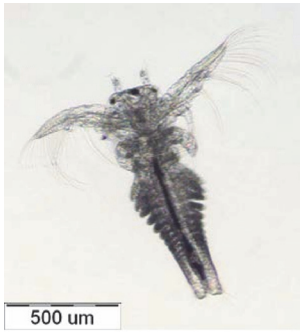
$Ce_{0,8}Pr_{0,2}O_2$ 100 ppm



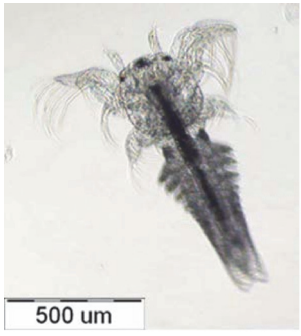
$Gd_{0,97}CoO_3$ 100 ppm



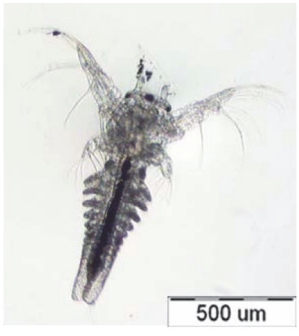
$LaCoO_3$ 100 ppm



$LaFeO_3$ 100 ppm



La_2NiO_4 3.125 ppm



$(La_{0,6}Sr_{0,4})_{0,95}CoO_3$ 6.25 ppm

Appendix 7

OECD Test Guidelines for the hazard assessment to aquatic biota (Source: http://www.oecd.org/env/ehs/testing/oecdguidelinesforthetestingofchemicals.htm, 20.02.2019)

OECD test guideline number

201 Freshwater Alga and Cyanobacteria, Growth Inhibition Test
202 *Daphnia* sp. Acute Immobilisation Test
203 Fish, Acute Toxicity Test
204 Fish, Prolonged Toxicity Test: 14-Day Study
209 Activated Sludge, Respiration Inhibition Test (Carbon and Ammonium Oxidation)
210 Fish, Early-life Stage Toxicity Test
211 *Daphnia magna* Reproduction Test
212 Fish, Short-term Toxicity Test on Embryo and Sac-Fry Stages
215 Fish, Juvenile Growth Test
218 Sediment-Water Chironomid Toxicity Using Spiked Sediment
219 Sediment-Water Chironomid Toxicity Using Spiked Water
221 *Lemna* sp. Growth Inhibition Test
225 Sediment-Water *Lumbriculus* Toxicity Test Using Spiked Sediment
229 Fish Short Term Reproduction Assay
230 21-day Fish Assay
231 Amphibian Metamorphosis Assay
233 Sediment-Water Chironomid Life-Cycle Toxicity Test Using Spiked Water or Spiked Sediment
234 Fish Sexual Development Test
235 *Chironomus* sp., Acute Immobilisation Test
236 Fish Embryo Acute Toxicity (FET) Test
238 Sediment-Free *Myriophyllum Spicatum* Toxicity Test
239 Water-Sediment *Myriophyllum Spicatum* Toxicity Test
240 Medaka Extended One Generation Reproduction Test (MEOGRT)
241 The Larval Amphibian Growth and Development Assay (LAGDA)
242 *Potamopyrgus antipodarum* Reproduction Test
243 *Lymnaea stagnalis* Reproduction Test
305 Bioaccumulation in Fish: Aqueous and Dietary Exposure
315 Bioaccumulation in Sediment-dwelling Benthic Oligochaetes

Curriculum vitae

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Additional training

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2. Muna, M., Blinova, I., Kahru, A., Vinković Vrček, I., Pem, B., Orupõld, K., & Heinlaan, M. (2019). Combined effects of test media and dietary algae on the toxicity of CuO and ZnO nanoparticles to freshwater microcrustaceans *Daphnia magna* and *Heterocypris incongruens*: food for thought. *Nanomaterials, 9,* 23. http://doi.org/10.3390/nano9010023
3. Blinova, I., Vija, H., Lukjanova, A., Muna, M., Syvertsen-Wiig, G., & Kahru, A. (2018). Assessment of the hazard of nine (doped) lanthanides-based ceramic oxides to four aquatic species. *Science of The Total Environment, 612,* 1171–1176. http://doi.org/10.1016/j.scitotenv.2017.08.274
4. Blinova, I., Muna, M., Lukjanova, A., & Kahru A. (2018). Evaluation of the potential hazard of manufactured metal-based nanomaterials to health of aquatic ecosystems: state of the art. *Journal of International Scientific Publications: Ecology & Safety, 12,* 174−182. Retrieved from https://www.scientific-publications.net/en/article/1001659/.
5. Blinova, I., Lukjanova, A., Muna, M., Vija, H., & Kahru, A. (2018). Evaluation of the potential hazard of lanthanides to freshwater microcrustaceans. *Science of The Total Environment, 642,* 1100–1107. https://doi.org/10.1016/j.scitotenv.2018.06.155
6. Muna, M., Heinlaan, M., Blinova, I., Vija, H., & Kahru, A. (2017). Evaluation of the effect of test medium on total Cu body burden of nano CuO-exposed *Daphnia magna*: A TXRF spectroscopy study. *Environmental Pollution, 231,* 1488–1496. https://doi.org/10.1016/j.envpol.2017.07.083
7. 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. http://doi.org/10.1016/j.aquatox.2017.06.002
8. Heinlaan, M., Muna, M., Knöbel, M., Kistler, D., Odzak, N., Kühnel, D., Müller, J., Gupta, G. S., Kumar, A., Shanker, R., & Sigg, L. (2016). Natural water as the test medium for Ag and CuO nanoparticle hazard evaluation: an interlaboratory case study. *Environmental pollution, 216,* 689–699. https://doi.org/10.1016/j.envpol.2016.06.033

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Hariduskäik

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Teenistuskäik

2015– …	Keemilise ja Bioloogilise Füüsika Instituut, Keskkonnatoksikoloogia labor, nooremteadur
2014–2015	Keemilise ja Bioloogilise Füüsika Instituut, Keskkonnatoksikoloogia labor, insener
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Täiendkoolitused

aprill 2018	COST NOTICE TD 1407 töötuba "Technology Critical Elements in Ecosystem and Human Health" ja koolitus "Technology Critical Elements: Toxicity Testing and Assessment of Risks", Tallinn, Eesti
aprill–juuli 2017	COST NOTICE TD 1407 lühiajaline teadusmissioon Lorraine'i Ülikoolis Metzis Prantsusmaal teemal "Ecotoxicological Impact of REE Discharge in Water and Sediments"
jaanuar 2017	COST NOTICE TD 1407 töötuba "Environmental Concentrations, Cycling and Modeling of Technology Critical Elements" ja kursus "Environmental Analytical Chemistry of Technology Critical Elements (TCEs)", Rehovot, Iisrael
sept–dets 2015	Ljubljana Ülikool (Sloveenia), Biotehnoloogia teaduskond, Bionanoteam, doktorandi semester välismaal
aprill 2016	EUROTOXi toksikoloogia aluskursus "Principles of Toxicology", Zagreb, Horvaatia

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Keelteoskus

eesti keel – emakeel
inglise keel – kõrgtase
vene keel – algtase
rootsi keel – algtase

Publikatsioonid eelretsenseeritud ajakirjades

1. Romero-Freire, A., Joonas, E., Muna, M., Cossu-Leguille, C., Vignati, D. A. L., & Giamberini, L. (2019). Assessment of the toxic effects of mixtures of three lanthanides (Ce, Gd, Lu) to aquatic biota. *Science of The Total Environment*, 661, 276–284. http://doi.org/10.1016/j.scitotenv.2019.01.155
2. Muna, M., Blinova, I., Kahru, A., Vinković Vrček, I., Pem, B., Orupõld, K., & Heinlaan, M. (2019). Combined effects of test media and dietary algae on the toxicity of CuO and ZnO nanoparticles to freshwater microcrustaceans *Daphnia magna* and *Heterocypris incongruens*: food for thought. *Nanomaterials, 9,* 23. http://doi.org/10.3390/nano9010023
3. Blinova, I., Vija, H., Lukjanova, A., Muna, M., Syvertsen-Wiig, G., & Kahru, A. (2018). Assessment of the hazard of nine (doped) lanthanides-based ceramic oxides to four aquatic species. *Science of The Total Environment, 612,* 1171–1176. http://doi.org/10.1016/j.scitotenv.2017.08.274
4. Blinova, I., Muna, M., Lukjanova, A., & Kahru A. (2018). Evaluation of the potential hazard of manufactured metal-based nanomaterials to health of aquatic ecosystems: state of the art. *Journal of International Scientific Publications: Ecology & Safety, 12,* 174−182. Retrieved from https://www.scientific-publications.net/en/article/1001659/.
5. Blinova, I., Lukjanova, A., Muna, M., Vija, H., & Kahru, A. (2018). Evaluation of the potential hazard of lanthanides to freshwater microcrustaceans. *Science of The Total Environment, 642,* 1100–1107. https://doi.org/10.1016/j.scitotenv.2018.06.155
6. Muna, M., Heinlaan, M., Blinova, I., Vija, H., & Kahru, A. (2017). Evaluation of the effect of test medium on total Cu body burden of nano CuO-exposed *Daphnia magna*: A TXRF spectroscopy study. *Environmental Pollution, 231,* 1488–1496. https://doi.org/10.1016/j.envpol.2017.07.083
7. 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. http://doi.org/10.1016/j.aquatox.2017.06.002
8. Heinlaan, M., Muna, M., Knöbel, M., Kistler, D., Odzak, N., Kühnel, D., Müller, J., Gupta, G. S., Kumar, A., Shanker, R., & Sigg, L. (2016). Natural water as the test medium for Ag and CuO nanoparticle hazard evaluation: an interlaboratory case study. *Environmental pollution, 216,* 689–699. https://doi.org/10.1016/j.envpol.2016.06.033