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Toxicity of copper nanoparticles across species

Final Report

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Chapter 1 Introduction

1. Background

Nanotechnology was defined by Professor Norio Taniguchi in 1974 as follows "'Nanotechnology' mainly is mainly consists of the processing of, separation, consolidation, and deformation of materials by one atom or by one molecule". "Nano" means a measure of 10^{-9} in science. According to the suggested definition of EU Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR), Nanomaterial is any form of a material that is composed of discrete functional parts, many of which have one or more dimensions of the order of 100 nm or less (SCENIHR, 2007). In nanoscales, the gravity becomes less important comparing to the surface tension and Van der Waals attraction of particles. Thus, nanomaterials have many unique properties different from bulk counterparts, for instance, the optical and size-dependent properties, antibacterial and antifungi properties etc. With the exponential development of nanotechnology, nanomaterials are widely applied and benefit several aspects of society. Plenty of NPs have been invented and applied in many fields, such as biomaterials, personal care cosmetics, textiles, plastics, animal hygiene, food and energy technologies, drug delivery, medical diagnostics as well as pollutants remediation (EAG, 2009). The global market for nanomaterials is estimated at 11 million tonnes and the potential global value of nanomaterials has been forecast to increase to 2 trillion € by 2015 (REACH, 2008).

2. Nanotoxicology

The awareness of the potential adverse effect of nanoparticles(NPs) in the environment rises sharply with the increasing quantities and diversities of NPs. NPs could enter any compartments in the environment, whereas the potential adverse effect of NPs are largely unknown in terms of health, safety and environment. Many studies illustrated that NMs have potential negative impacts on the exposure environment and living organisms (Bar-Ilan et al., 2009; Allen et al., in press). The toxicity and distribution of NPs are different from bulk particles. The exposure concentration is hard to be determined since NPs is easily aggregated and the stability of NPs is strongly affected by the inherent properties of NPs and their exposure environment. NPs can be easily adsorbed by any particles or organisms due to their highly surface reactivity and surface to volume ratio. They could pass through the skin, lungs and membranes and thus cause damages on various organisms.

Even though the toxicities of NPs have been widely investigated, currently the interactions between NPs and their surroundings as well as the subsequent adverse effects on ecosystems are still partly understood. The fate and toxicity of NPs in the environment depends on many factors. As the toxicity of NPs is widely investigated in terms of inherent properties and exposure surroundings, it is becoming clearer that traditional dose matrices based on mass may be insufficient and even inappropriate to describe the toxicity of NPs (Tinkle, 2008). Many studies illustrated that the toxicity of NPs not only affect by the concentration and solubility of NPs, but also strongly depend on the size, shape of NPs, the composition of chemicals adsorbed onto their surfaces (SCENIHR, 2006; El Badawy et al., 2010; Mukherjee and Weaver, 2010; Choi et al., 2010). Additionally, it is noteworthy that most research on uptake and toxicity of NPs is performed only with limited numbers of (model) species in laboratories. Results achieved in these toxicity tests even can't represent the vulnerable of testing species since the sensitivity of organisms in the different life stages (juveniles and adults) with different sexualities (female and male) could be different towards NPs, not to mention to estimate the risk of NPs on other species and the ecosystem as a whole based on current experimental results. Present research directions face big challenges to evaluate the potential risk of NPs on all the other species in the ecosystem because it is problematic and unrealistic to test diversity of NPs for all taxonomic groups and species under various environmental conditions.

3. Ecological traits and risk assessment of NPs

Ecological traits usually refer to measurable morphological and physiological characteristics and ecological attributes of species, which are comparable across species, such as the life stage, surface area of organisms, target site distribution, food choice, dispersal mode, respiratory mode, reproduction mode (McGill et al., 2006; Webb et al., 2010). The long history of traits in ecology could be traced back to Thienemann (1918), formulating a "law" to describe the traits of invertebrate communities as related to environmental stressors. Even none of research has applied ecological traits in the risk assessment of NPs before, some of them have already indicated that toxicity of NPs could be associated with morphology and physiology of organisms. For instance, Hund-Rinke and Simon (2006) indicate that fast movements of the thoracic appendages of daphnia will increase the likelihood of contact between NPs and the daphnia because the movement could transport NPs to the mouth that subsequently block gills or be adsorbed and take up by the gut. From the perspective of traits approach, the movement of thoracic appendages could be the important ecological traits

which can be used as a potential traits modality to extrapolate toxicity test results of NPs across crustacean and species owning same uptake mode. Another example supporting the application of trait approach in ecotoxicology of NPs can be provided by the adsorption of NPs on the cell wall of algae. NPs interact mainly at the surface of the algae (Van Hoecke et al., 2009). Therefore, surface type, amount of surface area, and inherent mechanisms of membranes to protect the algae from excess of nutrients could be the traits which allow for extrapolation of toxicity patterns of NPs across algae, plants and cells. Furthermore, surface area and filtration ability of gills also may be the traits affecting the toxicity of NPs to fishes. The gill is the primary target of exposure to NPs. NPs caused inhibition of exchange processes across gills, while no histological or biochemical evidence for damage to other organs was observed (Griffitt et al, 2007). It therefore is critical to evaluate the gills morphology or physiological processes to explain the toxicity of NPs to fishes. The traits approach provides a possibility to extrapolate the toxicity across species since traits can be taxon independent.

4. Research objective and report outline

In order to better understand the risk of NPs and protect ecosystem as a whole, the objectives of this work is focus on investigating relation between the biological attributes of species and the toxicity of NPs. It intends to provide theoretic basis and experimental information to assess and predict the toxicity of NPs to untested species.

The preliminary hypothesis of this work is that regressions of toxicity could be developed by including the traits of species, environmental variations and particle properties of NPs with different slopes. This report includes 8 chapters. Chapter 1 gives scientific introduction of this work. Chapter 2-7 will give further details of research questions, including a background and the description of experimental work involved in each work questions. Chapter 8 summarise the activities, output of the whole work period.

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Chapter 2 Smart Nanotoxicity Testing for Biodiversity Conservation

Song, L. & Vijver, M.G. & Peijnenburg, W.J.G.M. & Snoo, G.R., de (2011)

Environmental Science and Technology, 45 (15), pp. 6229-6230.

Risk assessment of nanoparticles (NPs) currently focuses on NP-specific properties and metrics affecting the environmental fate and effects of the particles, with little attention given to extrapolating potentially adverse effects with a view to protecting biodiversity. Given the huge diversity of species in ecosystems, evaluating these effects on the basis of current research directions is problematic. After all, it is unrealistic to either test the diversity of NPs across all taxonomic groups and species under various environmental conditions, or extrapolate the current set of NP toxicity data to other (nontested) species in ecosystems. Indeed, the potential adverse effects of NPs in nature are still largely unknown, and NP risk assessment faces major challenges when it comes to investigating nanotoxicological implications for biodiversity conservation.

We suggest that an approach based on ecological traits could be extremely helpful for interpreting NP toxicity test results and efficiently extrapolating them to living ecosystems. “Ecological traits” usually refer to measurable morphological and physiological characteristics and ecological attributes of species that are comparable within and across species. Examples include the following: organism surface area, target site distribution, dispersal mode, respiratory mode, and reproduction mode, etc.1 Traits are taxa-independent, since the same trait modality can be found across a range of taxa. Baird et al.2 were the first to propose that such traits would help link the potential toxicity of contaminants to wide arrays of organisms and thus allow for better interpretation of the vulnerability of species to toxicants in terms of the ecological functions of the organisms in question. Quantitative, mechanistic links between such traits and the toxicokinetic end points of organisms have been identified experimentally by exposure of organisms to pesticides.3 However, as far as we are aware ecological traits have never been related to risk assessment of NPs. A trait approach is even more meaningful for extrapolating the toxicity profiles of NPs than it is for pesticides and other chemicals. Due to the unique properties of NPs, such as their shape and highly specific reactivity, interactions between NPs and organisms are far more target-oriented and determined by organism morphology and physiology than in the case of “regular” chemicals.

Given the specific interactions between NPs and organisms in ecosystems, a trait-based framework can be established that comprises environmental characteristics, particle properties, and ecological traits (Figure 1).

Normally, external exposure concentrations and internal reaction mechanisms are responsible for the actual damage of toxicants to organisms. On the one hand, environmental characteristics and the particle properties of NPs are the main factors determining the stability (zeta potential), fate, and distribution of NPs in the environment. Subsequently, these parameters influence the effective external exposure concentration of NPs and the potential damage to the species in the different compartments. On the other hand, the internal reaction mechanisms of NPs depend largely on the morphological and physiological characteristics and the life pattern of organisms. By establishing relationships among environmental characteristics, NP particle properties, and ecological traits, the external exposure and internal reaction mechanism of NPs can be systematically described.

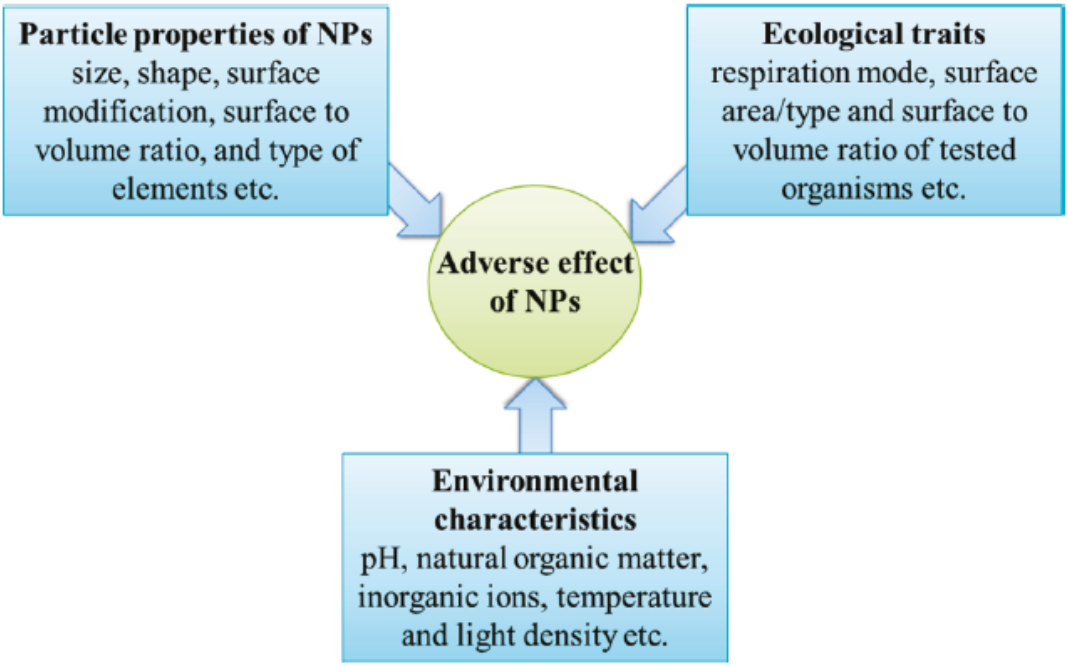

Figure 1. Trait-based framework. Environmental characteristics: determining the fate of NPs; particle properties: including parameters that could be used to describe the properties of NPs; ecological traits: representing any physiological or morphological property and the evolutionary life history of organisms, as long as these are comparable across different species.

Many research studies provide concrete evidence of how to adopt the trait-based framework for assessing nanotoxicity. For instance, it has been found that the interaction between nanoparticles and algae is mainly at the algal surface because of the highly reactive surface of algae and the high surface/volume ratio of both NPs and algae.4 Hence, trait modalities such as surface type, total surface area, and the intrinsic membrane mechanisms that protect algae from excess nutrients could be important factors that affect the potential toxicity of NPs. These trait modalities can be applied across species and therefore permit extrapolation of toxicity patterns of NPs across algae, higher plants, and cells. In addition, it has been demonstrated that NPs cause inhibition of exchange processes across fish gills, while no histological or biochemical evidence for damage to other organs could be observed.5 The gill is therefore the most likely primary target of NP exposure. In the context of the trait approach discussed here, it is critical to evaluate the role of gill morphology and physiological processes in determining the toxicity of NPs to fishes before toxicity data can be extrapolated from one fish species to another.

The traits approach provides a third research direction and improves testing systems of NPs by regarding characteristics of species in ecosystems. Moreover, it creates a basis for extrapolating the toxicity of NPs within and across species, because NPs are anticipated to act according to similar toxicity mechanisms and cause similar damage to organisms having the same trait modalities under similar exposure conditions. Another advantage of the trait approach is that it permits distinct treatment of the sensitivities of the same type of organisms to NPs in different life stages and under different sexualities. The drawback of the trait approach lies in the increased complexity of NP risk assessment and the requirement to document species traits during experiments. However, once the system is established and suitable traits are defined, the extrapolation of experimental toxicity data can be easily carried out because initial trait data sets have already been created within the discipline of ecology.3 Consequently, fewer species and less time will be needed for determining the toxicity of NPs experimentally. A major gap in knowledge that presently hampers application of such a framework is the lack of a basis for relating ecological traits and toxicity results, since little experimental data on the individual morphology and physiology of organisms are reported in NP toxicity testing, thus constraining extrapolation of NP toxic effects within and across species. To implement the trait-based framework and protect all species at different life stages under diverse environmental conditions, we suggest that research priority be given to establishing databases that systematically document the trait modalities of individual

experimental species, types and properties of NPs, and exposure conditions, as well as the corresponding toxicity of NPs. Given the rapid growth of nanotechnology and the wide diversity of species in living ecosystems, the trait-based framework deserves priority in the risk assessment of NPs.

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Chapter 3 Species-specific toxicity of copper nanoparticles among mammalian and piscine cell lines.

Lan Song, Mona Connolly, Maria L. Fernández-Cruz, Martina G. Vijver, Marta Fernández, Estefanía Conde, Geert R. de Snoo, Willie J.G.M. Peijnenburg & Jose M. Navas (2013)

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Abstract

The four copper nanoparticles (CuNPs) with the size of 25, 50, 78 and 100 nm and one type of micron-sized particles (MPs) (~500 nm) were exposed to two mammalian (H4IIE and HepG2) and two piscine (PLHC-1 and RTH-149) cell lines to test the species-specific toxicities of CuNPs. The results showed that the morphologies, ion release and size of the particles all played an important role when investigating the toxicity. Furthermore, the authors found that the particle forms of CuNPs in suspensions highly contribute to the toxicity in all exposed cell lines whereas copper ions (Cu2+) only caused significant responses in mammalian cell lines, indicating the species-specific toxicity of CuNPs. This study revealed that the morphologies, ion release rate of NPs as well as the species-specific vulnerabilities of cells should all be considered when explaining and extrapolating toxicity test results among particles and among species.

Keywords: copper nanoparticles, mammalian and fish cell lines, size and morphology of NPs, species-specific cytotoxicity, reactive oxygen species

1. Introduction

Considering the wide application of copper nanoparticles (CuNPs) in a variety of fields, such as in facial spray, as additives in lubricants, in metallic coating and inks, anode materials for lithium ion batteries (Cioffi et al. 2005; Guo et al. 2002; Lei et al. 2008; Liu et al. 2004), CuNPs can enter into diverse environmental compartments and be taken up by organisms through intake of water, food and even from soil by plant species resulting in reduced seedling growth (Lee et al. 2008). Therefore, the potential risks from exposure to CuNPs must be further investigated.

Various studies showed that CuNPs can cause a diversity of toxic effects to biological systems. CuNPs showed a sizeand concentration-dependent toxicity to dorsal root ganglion neurons of rat (Prabhu et al. 2010). Lei et al. (2008) claimed that CuNPs could cause scattered dot hepatocytic necrosis and widespread renal proximal tubule necrosis in the rat. Chen et al.

(2006) showed that only CuNPs can induce toxicological effects and severe pathological injuries to the kidney, liver and spleen of mice when compared with copper at micrometer size. It is already known that the toxicity caused by micro copper is lower than the toxicity of CuNPs and the toxicity caused by copper ions in CuNPs media and the toxicity of copper oxide NPs cannot be simply explained by Cu ions released into the cell medium (Chen et al. 2006; Karlsson et al. 2008). However, little attention has been paid to species-specific NP toxicity and only a limited number of studies have quantified the toxicity contribution of the particle form of NPs and released ions to the total toxicity of particle suspensions (Patra et al. 2007). Therefore, the sensitivity of four different hepatoma cell lines, two mammalian and two piscine in origin, exposed to four sizes of CuNPs and one type of micron-sized copper particles (MPs), was investigated in this study. The aim is to evaluate the species-specific acute toxicity of CuNPs at the cellular level and to evaluate the toxicity contribution of the particle form of CuNPs and ions to the total toxicity of particle suspensions, respectively. Uptake of NPs is not investigated in this study since uptake of NPs cannot always and also is not the only pathway to cause acute toxicity to cells (George et al 2009). For instance, a large amount of uptake may not cause any toxic effect due to the inert characterisation of NPs or the high tolerability of cells (Connor et al. 2005). Cytotoxic effects and reactive oxygen species (ROS) levels were correlated with the physico-chemical properties of the CuNPs and the cell types. Liver being the critical organ for copper storage, homeostasis and excretion of several species and an inherited disorder of copper metabolism can cause Wilson's disease in humans (Tao & Gitlin, 2003). Therefore, hepatocytes were chosen as the cell lines for this research. The preliminary hypotheses of this study are: 1) size or shape of CuNPs can influence their toxicity; 2) the toxicity of CuNPs can be related to the type of the cells that they are exposed to; 3) ion release is not the dominant factor inducing toxicity of CuNPs.

2. Materials and methods

2.1 Chemicals and reagents

CuNPs of 25, 50 and 100 nm sizes were purchased from IoLiTec, Inc., Germany. CuNPs of 78 nm as well as the MPs (nominal size of MPs is 500 nm) were purchased from NanoAmor®, USA (Houston, TX , USA). All particles are uncoated. The resazurin in vitro toxicology assay kit, 6-carboxy-2'7'-dichlorofluorescein diacetate (DCFH-DA) probe and copper (II) nitrate hydrate ($Cu(NO_3)_2$) were purchased from Sigma Aldrich, Madrid, Spain. Ethanol was from Panreac (Barcelona, Spain). Ultraglutamine 1 (200 mM), L-glutamine (200 mM), foetal bovine serum (FBS), penicillin and streptomycin (P/S) (10,000 U/ml/ 10 mg/ml), non-

essential amino acids 100X (NEAA), sodium pyruvate (100 mM), Eagle's Minimum Essential Media (EMEM) for cell culture and Alpha Minimum Essential Media (a-MEM) were purchased from Lonza (Barcelona, Spain). Phenol-red free, serum free Minimum Essential Media (MEM) was sourced from PAN Biotech GmbH, Germany. Analysis grade nitric acid 65% from Scharlau (Barcelona, Spain) purified by sub-boiling distillation in a Milestone Duopur (Milestone srl., Italy) and high purity water (>18 MW/cm) obtained from a Milli- Q Element A10 Century (Millipore Ibérica, Spain) were used for inductively coupled plasma mass spectrometry (ICP-MS) analysis.

2.2 Preparations of CuNPs

Stock copper suspensions (200 mg/ml) of the four types of CuNPs (25, 50, 78 and 100 nm), of the MPs and of Cu(NO3)2 were freshly prepared and dispersed in culture media used for culturing each cell line (Supplementary 1) using sonication for 10 min in an S 40 H Elmasonic water bath sonicator (Elma, Germany). The MPs were included to compare the toxicity of nano/microparticles. Cu(NO3)2 was used as a positive control and the response curves of Cu(NO3)2 were used to calculate the effects of copper ions (Cu2+) present in copper suspensions. Physico-chemical characterisation

2.3 Dynamic light scattering

The size distributions of all particles at 200 mg/ml were measured directly after preparation (0 h) in four types of culture media and after 24 h incubation under relevant culture conditions (see cell cultures section) by dynamic light scattering (DLS) on a Zetasizer Nano-ZS instrument (Malvern, Instruments Ltd., UK). Three independent measurements were taken with each measurement consisting of four measurements. The different types of culture media were also included in measurements to act as background controls as the presence of large proteins and other media components may affect the DLS measurements. Any peaks detected in the same size range as those found in the media were attributed to media components (Supplementary 2). This instrument was also used to ensure that there were no CuNPs but only copper ions in the supernatants of centrifuged media suspensions in the copper ion release experimental setup (see Actual exposure concentrations and copper ion release section). A Zetasizer Nano-ZS instrument was also used to try to measure the zeta potential of nanoparticles in culture medium (200 mg/ml). However due to the high (>9 mS/cm) conductivity of EMEM, medium quality criteria could not be met and measurements were aborted.

2.4 Transmission electron microscopy

Transmission electron microscopy (TEM) analysis was used to characterise the morphology and size distribution of copper suspensions after 24 h incubation. Analysis was only performed in one type of culture medium, EMEM culture medium, because the DLS measurements showed that the different media compositions and temperatures did not influence the hydrodynamic size profiles of the CuNPs. TEM was also used to characterise the primary particle size of the copper suspensions using ethanol as a dispersant. This allowed us to compare profiles and analyse the impact of the media. A JEOL 2100 HT (JEOL Ltd., Japan) TEM was used, operating at an accelerating voltage of 200 kV with integrated energy dispersive X-ray (EDX) spectroscopy (EDX) (Oxford Inca, UK). Stock copper suspensions (200 mg/ml) were deposited onto copper grids and images of CuNPs in ethanol and following 24 h of incubation in EMEM culture medium were collected. Morphology and size distribution of CuNPs were analysed by ImageJ (National Institutes of Health, Bethesda, MD, USA). Size distribution analysis was only performed when individual well-defined NPs could be determined.

2.5 Actual exposure concentrations and copper ion release

The actual exposure concentrations of copper suspensions (including both CuNPs, MPs suspensions and Cu(NO3)2 solution, five concentrations) were prepared freshly and measured using ICP-MS with a quadrupole-based instrument (Thermo X-Series II – Thermo Scientific, Bremen, Germany). Many studies report the release of metal ions as a contributing factor to the toxicity of NPs (Jiang et al. 2009, Wang et al. 2009), therefore the ion release from all copper suspensions (200 mg/ml) in the four different culture media under four culture conditions were quantified using ICP-MS (Supplementary 3). One ml CuNPs suspensions were sampled at time 0, 24 and 48 h after incubation and centrifuged at 13,362 g for 20 min, at 4_C (5415 R series centrifuge, Eppendorf, Germany) to remove CuNPs from suspensions (Fernández-Cruz et al. 2012). The supernatants were analysed using DLS to confirm that all CuNPs were removed. The supernatants were then analysed using ICPMS. Copper ion release (%) was calculated as percentage of the total copper concentration.

2.6 Cell cultures

Four liver cell lines were used in this study, two mammalian in origin; a rat hepatoma (H4IIE) and a human hepatocellular carcinoma (HepG2) and two from fish; the topminnow fish (Poeciliopsis lucida) hepatocellular carcinoma (PLHC-1) and rainbow trout (Oncorhynchus mykiss) hepatoma (RTH-149). All cell lines were obtained from the American Type Culture

Collection (ATTC) (Manassas, VA, USA). According to the cell type, the EMEM culture media was supplemented with necessary components for optimum cell growth (Supplementary 1). H4IIE and HepG2 were cultured at 37_C, PLHC-1 and RTH-149 were cultured at 30 and 20_C, respectively. A 5% CO2 atmosphere was applied to all culture conditions. The media was changed every 48 h and cells were split one to two times per week using 0.5% trypsin/ 0.02% EDTA.

2.7 Cell exposure

Due to different growth rates and morphologies, cells were seeded in 96-well plates (Greiner-Bio one, CellStar, Spain) with different densities (2.5 _ 104 cells/well for H4IIE, 7.5 _ 104 for HepG2 and 5 _ 104 for RTH-149 and PLHC-1) in 100 ml culture media per well under each culture condition, respectively. After 24 h, the culture media was removed and the cells were washed with phosphate buffer saline (PBS). Cells were exposed to all copper suspensions and the Cu(NO3)2 using the nominal concentration range of 12.5–200 mg/ml immediately following 10 min water bath sonication. A cell free 96-well plate with the same generated nominal concentration range was also prepared for each culture media and used to measure copper concentration by ICP-MS to determine the actual exposure concentrations. Toxicity evaluation

2.8 Cytotoxicity

The assay based on the ability of mitochondrial oxidoreductases to reduce the indicator dye resazurin (7-hydroxy-3Hphenoxazin- 3-one-10 oxide) to resorufin has been used (O'Brien et al. 2000). After 24 h exposure under relevant incubation conditions, the medium was removed and the cells were washed once with PBS; 100 ml culture media together with 5 ml of resazurin dye was added to the wells. Fluorescence intensity (532 nm excitation and 595 nm emission) was quantified on a GENios microplate reader (Tecan, Männendorf, Switzerland) after 2 h incubation. Potential interference of all copper suspensions with the fluorescence of the indicator dyes was checked by preparing a plate with corresponding concentration ranges as exposures (12.5– 200 mg/ml) but without cells and quantifying the fluorescence intensity of wells after 2 h. Cellular toxicity (%) was calculated as the decrease in fluorescence intensity and expressed as a percentage of control.

2.9 Oxidative stress

Intracellular ROS production was determined using the fluorescent probe DCFH-DA. The probe was prepared in phenol-red free, serum free MEM media (100 mM) under dark

conditions just prior to carrying out the assay. Culture media was removed from the exposed cells following the exposure timeframe (24 h) and cells were washed with PBS. The DCFH-DA probe was loaded to the wells, dark conditions were maintained and the plates were incubated under culture conditions for 30 min. Following the 30 min period cells were washed twice with PBS to remove any extracellular probe. Cells were then reconstituted with phenol-red free, serum free MEM media. Fluorescence (485 nm excitation and 530 nm emission) was measured on a GENios microplate reader immediately upon reconstitution and then 60 min after incubation under respective culture conditions. Potential interferences of copper suspensions with the fluorescence of the probe were checked by preparing a sample plate of copper suspensions without cells and quantifying the fluorescence intensity of wells after 60 min. Oxidative stress (%) was calculated as the percentage of fluorescence increase over 60 min.

3. Data analysis and statistics

3.1 Statistics

All exposures were performed in triplicate, with the mean ± standard deviation of three independent tests being represented in the final results. The data were analysed by one-way ANOVA followed by Dunnett's post hoc-test (treatment vs. control) in SigmaPlot_ 12.0 (Systat Software Inc., Chicago, IL, USA). The normality and homoscedasticity of all data was checked prior to carrying out statistical analysis. The calculation of the IC50 (concentration causing a 50% of inhibition with respect to the controls) caused by copper suspensions and by Cu(NO3)2 (resazurin assay) was performed by SPSS 16.0 using the function of the Probit regression (IBM SPSS, Armonk, NY, USA). The statistical significances ($p < 0.05$) were compared among different particles and among different cell lines using TTest2 (Matlab, MathWorks, Natick, MA, USA), respectively. The results are listed in Supplementary 6. The dose–response curves of oxidative stress production (exposure concentration range: 12.5–200 mg/ml) were plotted in Figure 6. The increase of ROS production (%) caused by 50 and 80 mg/ml (measured exposure concentration) of each copper suspension was calculated from the fitted curve in order to further compare the different responses among cell lines. These values were then used for pairwise comparisons to detect significant differences in the abilities of the different sized NPs to increase ROS production. Multiple comparisons among groups were carried out using a one-way ANOVA followed by a Holm-Sidak method ($p < 0.05$). This allowed determining any size-dependent

differences in toxicity as well as providing a statistical way for species-dependent sensitivity analysis.

3.2 Toxic contribution of the particle form of CuNPs and Cu2+

Both the Cu2+ and the particle form of CuNPs contribute to the toxicity of copper suspensions in living cell lines. The toxicity of Cu2+ in copper suspensions can be determined by the concentration–response curve of Cu(NO3)2. The actual concentrations of Cu2+ released by CuNPs (200 mg/ml) have been measured by ICP-MS (see Actual exposure concentrations and copper ion release section). When calculating the contribution of Cu2+ and CuNPs to the overall toxicity of the suspensions, it was assumed that the release of copper ions is independent of the concentration of CuNPs. Subsequently, the toxicity of Cu2+ (ECu 2+) in the copper suspensions could be determined according to the concentration–response curve of Cu(NO3)2. Furthermore, it is assumed that there are no interactions between Cu2+ and CuNPs. The total toxicity of copper suspensions was assessed experimentally. Therefore, the toxic effect of the particle form of the CuNPs (ECuNPs) can be estimated using the response addition model (Backhaus et al. 2000): $E_{CuNPs} = 1 - [(1 - E_{total}) / (1 - E_{Cu2+})]$ (1) Where Etotal represents the total cell toxicity caused by the copper suspensions. ECuNPs and ECu 2+ represent the cell toxicity caused by the particle form of CuNPs and Cu2+, respectively. The cellular toxicity (%) caused by copper suspensions was plotted as a function of the total copper concentration, together with the corresponding toxic contribution of the particle form of CuNPs and Cu2+. The IC50 caused by copper suspensions and the IC50 caused by the particle form in each copper suspension were plotted together with the IC50 values caused by Cu(NO3)2. The IC20, IC50 and IC80 caused by the particle form of each suspension were calculated and listed together with the IC20, IC50 and IC80 of copper suspensions for comparison in the Supplementary 5.

4. Results

4.1 Physico-chemical characterisation of CuNPs

Transmission electron microscopy

Figure 1 shows the TEM micrographs of the copper particles indicating the size, shape and distribution status in ethanol and after 24 h incubation in culture media. The results revealed that none of the CuNPs appear in the specified size according to the suppliers. The pristine CuNPs are present as aggregates and therefore it is very difficult to differentiate individual NPs to determine their size from TEM images except for the MPs. CuNPs of 25, 50 and 100

nm are spherical particles but are present in aggregates of irregular shape that appear conjoined.

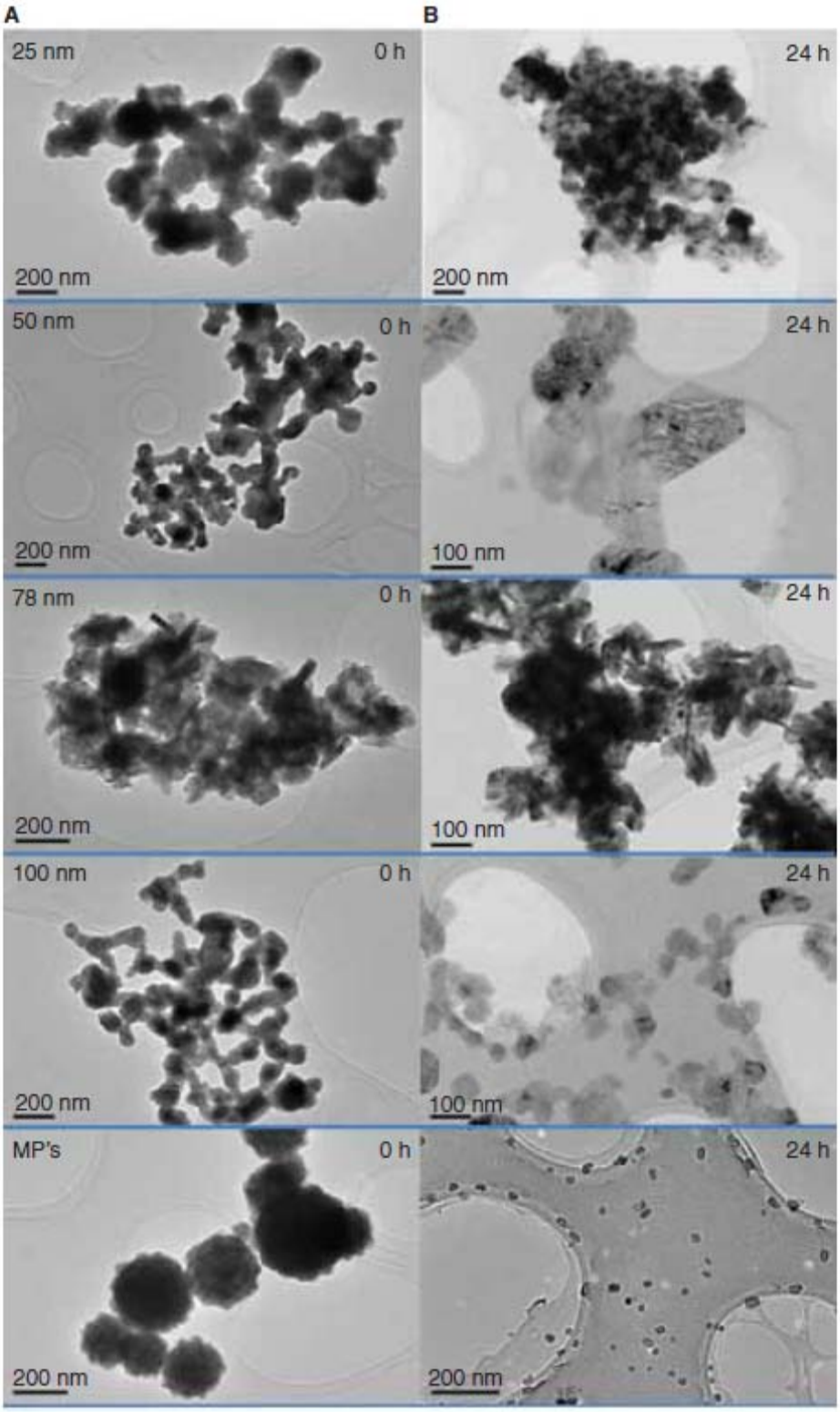

Figure 1. TEM images of copper particles (A) in their pristine form (prepared in ethanol) and (B) following 24 h incubation in EMEM culture medium under culture conditions (37_C/5% CO2). Scale bars indicate size (nm).

The 78 nm CuNPs interestingly appear to have rod-shaped nanostructures visible within aggregates. The MPs aggregates have a defined shape, distinctly spherical with a rough surface in nature. After 24 h incubation under culture conditions, 25, 50 and 100 nm CuNPs retain the aggregate status with a lighter appearance. The form of the 78 nm CuNPs after 24 h incubations was similar as the initial forms. The MPs retain their rough edged appearance with lighter appearances as well but the size decreased by a factor of 10 according to size distribution analysis from TEM images. The mean size at 0 h was 360.2 nm with more than 85% of the particles between 200 and 600 nm. However, after 24 h incubation in culture media, 90% of the MPs were smaller than 60 nm and the mean size decreased to 35 nm (Supplementary 4).

Dynamic light scattering

The hydrodynamic sizes of suspensions before and after 24 h incubation in the four culture media are presented in Figure 2. The results illustrate that all the copper particles are present largely in aggregates with sizes 200–700 nm in diameter depending on the type of particles. Slight differences in sizes were measured in the different culture media, with no apparent trend or temperature/media compositions influencing factor seen. Following 24 h of incubation, the general trend was a decrease in the hydrodynamic diameters of the particles under each culture condition.

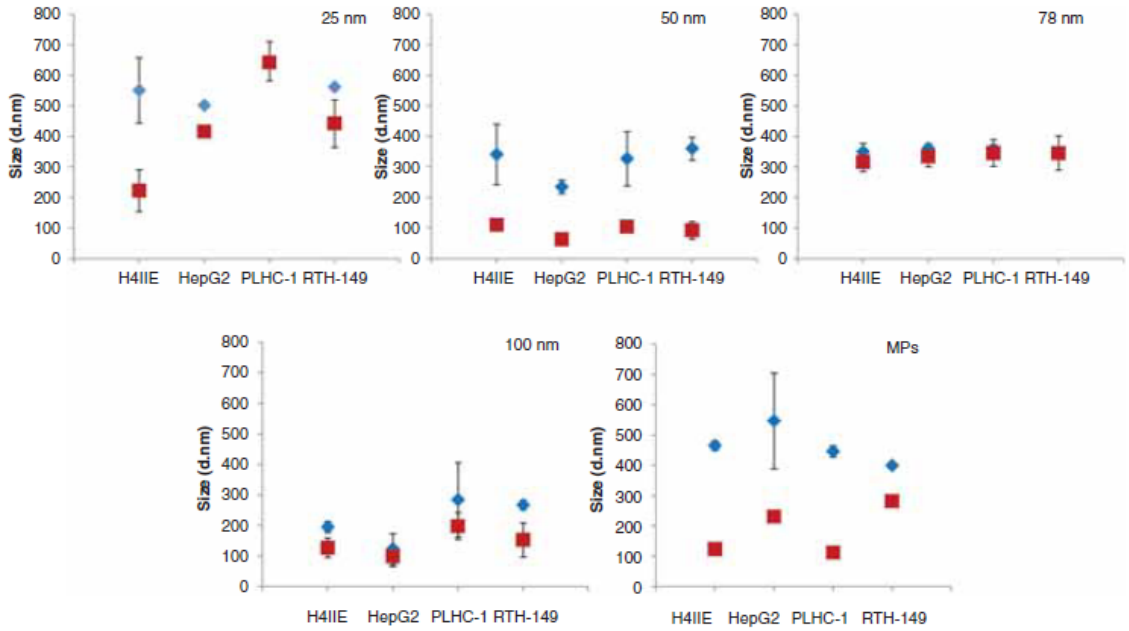

Figure 2. Size distributions of the copper particles measured by DLS directly after preparation (0 h) and after incubation (24 h) in four different culture media, designated H4IIE (37_C),

HepG2 (37_C), PLHC-1 (30_C) and RTH-149 (20_C) according to the which cell line it is used for. Results are expressed as means ± standard deviation.

The 78 nm CuNPs showed higher stability than all other particles under culture conditions, with similar sizes at 0 and 24 h after incubation, which is consistent with the TEM results. The DLS profiles of all the supernatants showed that there are no peaks except the media profiles, indicating no particles present in the supernatants after centrifugation and the measured copper concentration using ICP-MS in the supernatants were solely copper ions.

Actual exposure concentration and ion release

Measured concentrations of copper suspension versus nominal exposure concentrations are presented in Table I. Measured concentrations are the mean of four measurements in different culture media. The measured copper concentrations in experimental media deviated from 23% to 55% from the desired nominal concentrations depending on the CuNPs. The 78 nm CuNPs and the MPs showed the largest deviations, possibly due to the difficulties encountered in their manipulation (partly due to visible adherence of the particles to the plastic). Therefore, all responses in toxicity assays are correlated with the measured concentrations in this study.

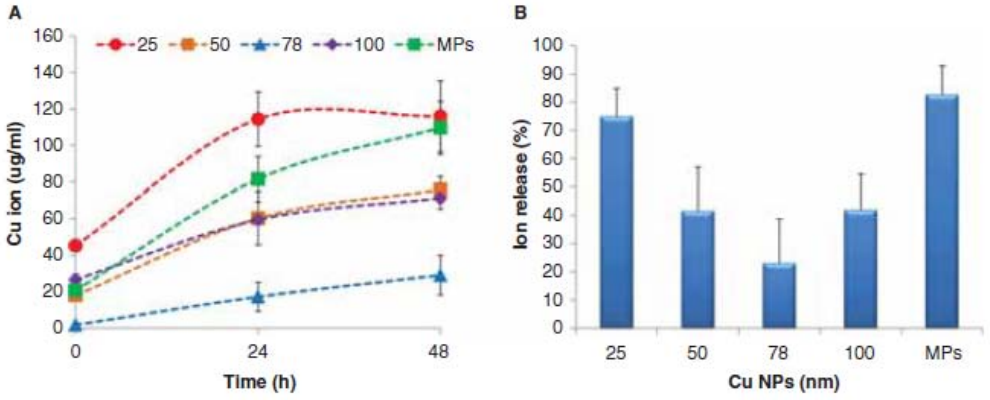

Figure 3. Copper ion release profiles for the copper suspension (a) over time and (b) the ion release after 24 h incubation, expressed as percentage of the total copper suspension concentration. Results are expressed as means ± standard deviation.

The ICP-MS results showed that there were no significant differences in measured ion release rates between the different media. Therefore, the mean of four measurements in different media and standard deviation (SD) are shown in Figure 3A. Ion release profiles were distinct for all CuNPs. The release of copper ions was time-dependent. At 0 h copper ions were present at low levels in particle suspensions of 200 mg/ml, with Cu2+ concentrations ranging from 2 ± 1 to 45 ± 3 mg/ml depending on the types of copper particles. The dissolution rate was very fast in the first 24 h, with the majority of ions being released in the culture media

during this time period. The 78 nm CuNPs showed the lowest rate of dissolution among all suspensions. Figure 3B illustrates the percentage of ion release from CuNPs and the MPs calculated as a percentage of the measured total copper concentration. Only 23 ±15% of 78 nm CuNPs was present as dissolved Cu2+ after 24 h. The 50 and 100 nm CuNPs showed similar percentages of dissolution, approximately 41%. Approximately 70–80% of 25 nm CuNPs and the MPs were dissolved after 24 h. The ion release of all CuNPs slows down after 24 h. Only 3% and 11% of 25 and 78 nm suspensions, respectively, were released as ions during the later 24 h. The percentage of Cu2+ released by 50 and 100 nm CuNPs was very similar after 48 h (approximately 50%). However, about 98% of the MPs were dissolved after 48 h.

4.2 Toxicity evaluation

Cellular toxicity

There was a clear dose–effect relationship after exposure of the different cell lines with the different copper suspensions. Values of the IC50 for each copper suspension and of the IC50 for each particle form in each suspension in different cell lines are plotted in Figure 4. The IC50 of Cu(NO3)2 to each cell line was plotted as a reference in this figure. The corresponding significant statistical analyses between different particles and between different cell lines are shown in Supplementary 6. There is a strong contrast between the sensitivities of cell lines to copper suspensions. In general, the mammalian cell lines are more sensitive than the piscine cell lines, most strikingly in the case of exposure to Cu(NO3)2 and 25 nm CuNPs. The lowest IC50 was seen following exposure to 25 nm in the H4IIE cell line. Exposure to the MPs induced a lower IC50 value in both the mammalian cell lines and the piscine cell lines as compared with the CuNPs. The piscine cell line RTH-149 shows the highest resistance to all copper suspensions and Cu(NO3)2. The lowest IC50 was 74 ± 14 mg/ml when RTH-149 was exposed to the MPs.

Toxic contribution of the particle form of CuNPs and Cu2+

The IC50 of the particle form in each suspension in different cell lines was much lower than the IC50 of the Cu2+ ions, except when H4IIE was exposed to the 78 nm CuNP (Figure 4). The IC50 of the particle form of 78 nm CuNPs is 77 ± 17 mg/ml for the H4IIE cell line, whereas the IC50 of the Cu2+ is 54 ± 9 mg/ml for H4IIE cells; 25 nm CuNPs and the MPs were the most toxic particles to all cell lines. The lowest value of the IC50 4 ± 3 mg/ml was found when H4IIE was exposed to MPs, and the second lowest value of IC50 was 7 ± 1

mg/ml when HepG2 cells were exposed to 25 nm CuNPs. RTH-149 shows the highest resistance to all copper particles.

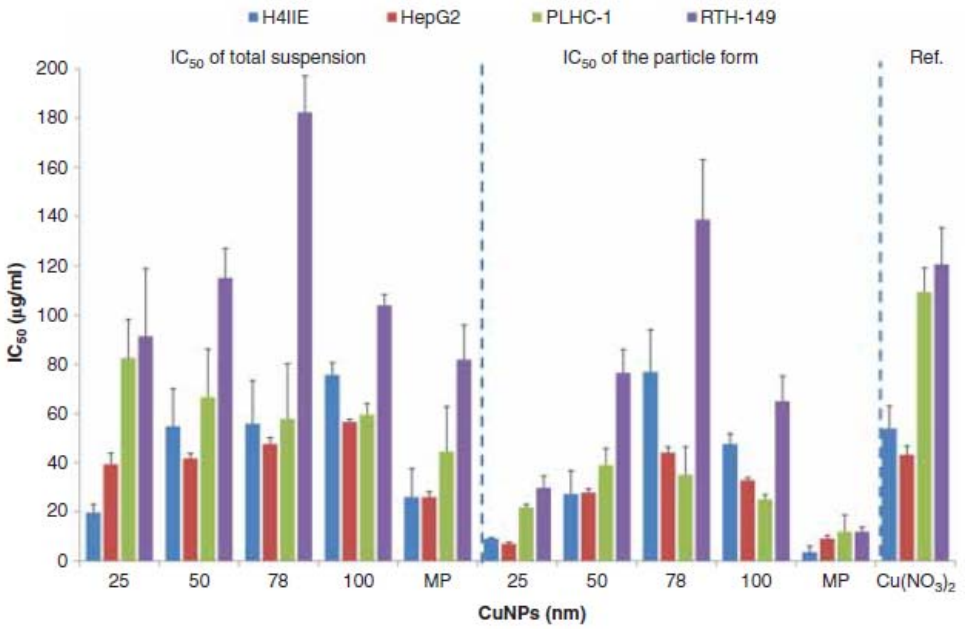

Figure 4. The IC50 caused by each copper suspension and the IC50 caused by the particle form in each copper suspensions to mammalian (H4IIE, HepG2) and piscine cell lines (PLHC-1 and RTH-149). The IC50 of Cu(NO3)2 for four types of cell lines is shown separately. Results are expressed as means ± standard deviation.

The cellular toxicity(%) caused by copper suspensions is plotted against the total copper concentration (measured values) in Figure 5, together with the corresponding toxic contribution of the particle form of CuNPs and Cu2+, respectively. The particle form of CuNPs significantly contributed to the toxicity of the copper suspensions in all four cell lines. However, the toxic contribution of Cu2+ and of the particle form of CuNPs is dependent on the type of CuNPs and cell lines. The IC50 of Cu2+ in Cu(NO3)2 in mammalian cell lines (54 ± 9 mg/ml for H4IIE and 43 ± 4 mg/ml for HepG2) was about half of the value found for piscine cell lines (109 ± 10 mg/ml for PLHC-1 and 120 ± 15 mg/ml for RTH-149). Cu2+ only significantly contributed to the total toxicity in the H4IIE and HepG2 cell lines exposed to 25, 50, 100 nm CuNPs and the MPs as shown in Figure 5. Cu2+ exhibited little toxicity to the total toxicity when H4IIE and HepG2 exposed to 78 nm CuNPs, because of the low levels of Cu2+ in the 78 nm suspensions. The piscine cell lines showed a high resistance to Cu2+ in all cases.

The IC20, IC50 and IC80 of Cu(NO3)2, of the copper suspensions and of the particle form in each copper suspension when exposed to each cell line are given in Supplementary 5. The particle form of CuNPs can cause 20% of cellular toxicity at much lower concentration compared with Cu(NO3)2 in most of cases (Supplementary 5). For instance, the particle form of the 25 nm CuNPs and of the MPs can cause 20% cellular toxicity at 6 ± 2 and 4 ± 0 mg/ml to HepG2 cell lines, respectively. But the IC20 of Cu(NO3) is 18 ± 2 mg/ml to HepG2 cell lines, which is three times higher (less toxic) than the IC20 of the particle form of the 25 nm CuNPs and the MPs.

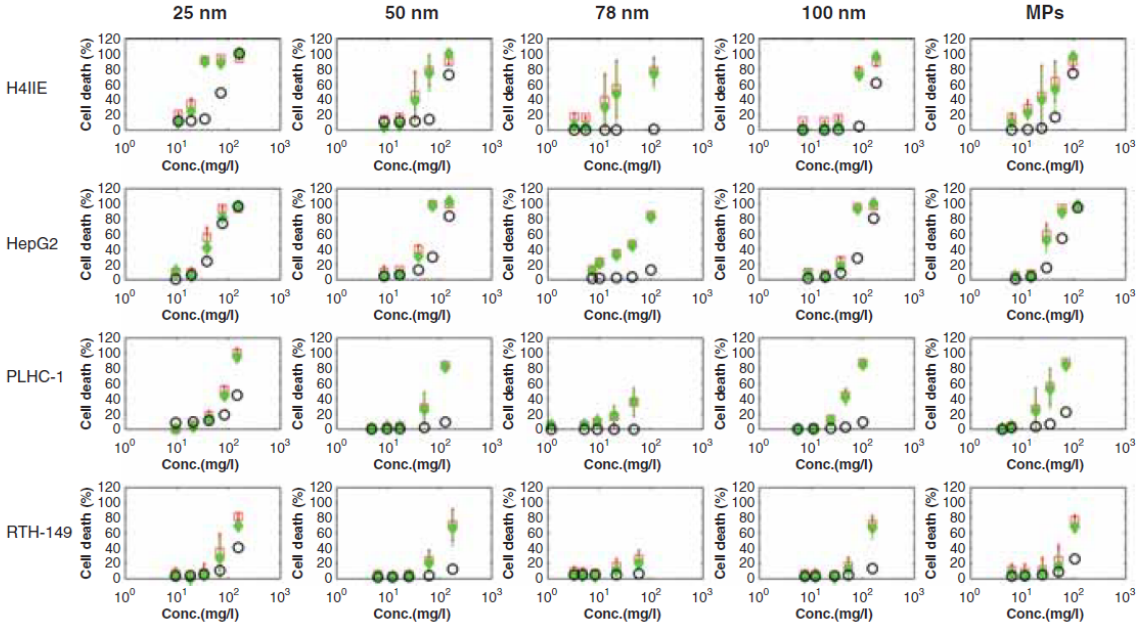

Figure 5. The total cellular toxicity (%) caused by copper suspensions (red, square), and the cellular toxicity (%) caused by particle forms of each copper suspension (CuNPs) (green, solid diamond) and Cu^{2+} (blue, circle) plotted against the total copper concentrations, respectively. Results are expressed as means ± standard deviation.

ROS levels

The ability of copper suspensions and Cu(NO3)2 to elicit the generation of intracellular ROS was also investigated. Curves representing the response of each cell line to all copper particles and Cu(NO3)2 are compared in Figure 6. The ability of CuNPs to increase intracellular ROS levels is concentration and cell type dependent. The corresponding responses to two different concentrations (50 and 80 mg/ml) are extrapolated from the fitted curves to compare the different responses of different cell lines (Supplementary 7). Although no differences in ROS generation were detected among cell lines after exposure to low concentrations, at the highest concentrations the mammalian cell lines (H4IIE and HepG2) exhibited a higher sensitivity to CuNPs than the piscine cell lines used in this study. Mammalian cell line H4IIE responses are

significantly different ($p < 0.01$) from both piscine responses upon exposure to the 25 nm CuNPs and Cu(NO3)2. The 25 nm CuNPs elicit the highest increase in ROS levels in H4IIE compared with all other suspensions, even above those elicited by Cu(NO3)2. However, there is no significant difference of ROS response among cell line when exposed to other CuNPs. Furthermore, the difference of ROS generation among CuNPs in same cell lines is not apparent. The results showed that only the ROS response of 25 nm in H4IIE is significantly different ($p < 0.05$) from the response to the 100 nm CuNPs for the 50 mg/ml dose. And only the 78 nm CuNPs elicits an increased response significantly different ($p < 0.05$) from the 25 nm NP and the MPs in the PLHC-1 cell line for the 80 mg/ml.

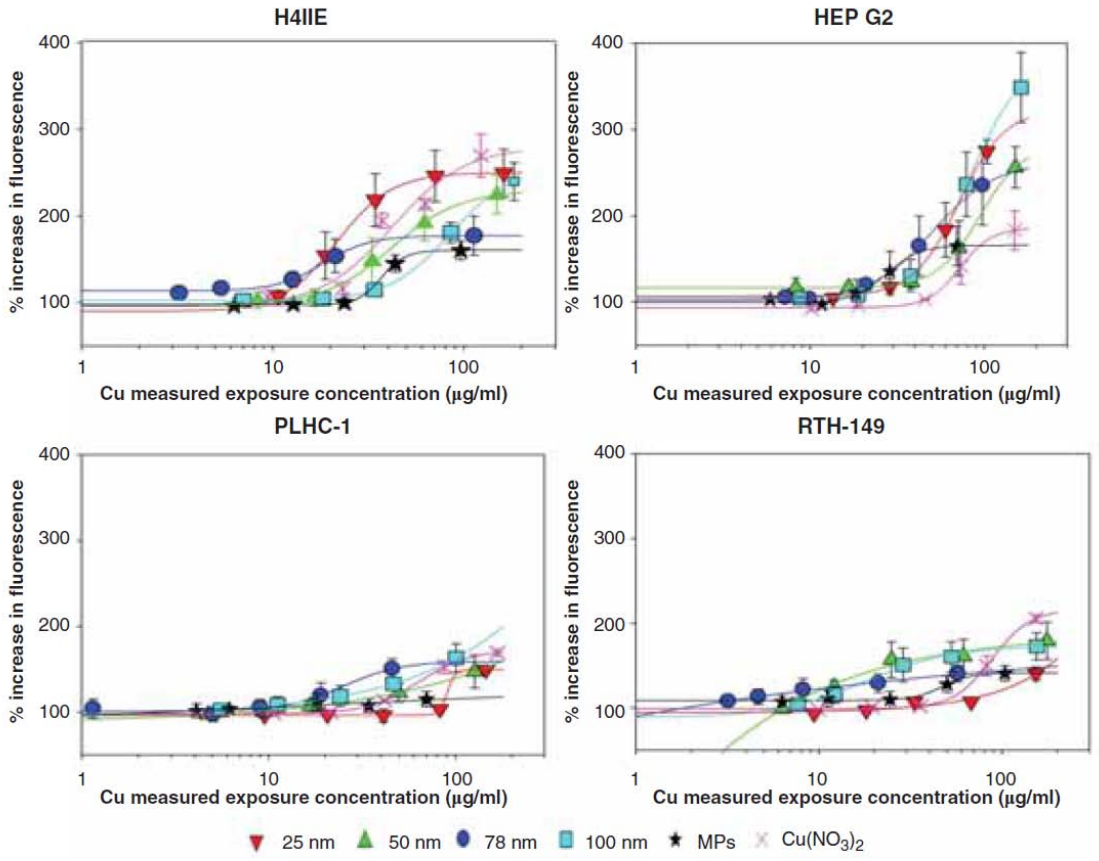

Figure 6 Intracellular ROS levels in the four cell lines following 24 h exposure to the different copper suspensions and Cu(NO3)2. ROS levels are quantified by measuring the % of increase in fluorescence with respect to the control (100%). Results are expressed as means ± standard error of the mean. Standard curves are presented using a four parameter logistic function to fit the data to a sigmoidal curve.

5. Discussion

5.1 Behaviour of NPs in culture media

It was found that all tested CuNPs aggregated immediately in the culture media. All CuNPs and the MPs underwent dissolution releasing copper ions under exposure conditions, however to different extents depending on the type of CuNPs. The increase in dissolution rates over time is consistent with the fact that the hydrodynamic sizes of CuNPs in culture media decreased after 24 h, as shown by the DLS and TEM measurements. This suggests that the decrease in size of the CuNPs is due to dissolution. The net surface area may be a dominating factor affecting ion release of CuNPs. The smaller the NPs, the larger the net surface area. The large dissolution rate of 25 nm CuNPs could be explained by the largest net surface area. The rough surface of MPs causes fast decomposition of MPs. Therefore, the size of MPs decreases sharply as the net surface area and ion release rate of MPs increases dramatically. Much lower Cu2+ ion levels and constant morphologies were detected before and after 24 h incubations of 78 nm CuNPs which may be due to the 78 nm CuNPs being rod-shaped according to the TEM observation. The net surface area of rod-shaped particles is much smaller compared with the same amount of spherical CuNPs according to mathematic calculation. Therefore, the rod shape of 78 nm CuNPs has much lower dissolution compared with other CuNPs.

Although (Liu and Hurt 2010) reported that the ion release rates of silver NPs can increase with temperature in the range 0_37_C, no clear relationship was found between the rate of ion release, temperature (20, 30 and 37_C) and the original size of the CuNPs in this study (Figure 2). The ionic strengths of the culture media for all cell lines are expected to be quite similar (Supplementary 1). It is also difficult to conclude if the ion strength of the culture media has clear impact on Cu2+ ion release from Figure 2. Further studies are needed to investigate the effect of temperature and different compositions of culture mediums on aggregation size and the rate of ion release of CuNPs.

5.2 Physical properties and toxicities of CuNPs suspensions

The toxicity of CuNPs suspensions (except MPs) in mammalian cell lines increased with a decrease of the nominal size of the particles (Figure 4, left part). The toxicity of CuNPs showed the same trend in RTH-149 when the nominal particle size of the particle is less than 100 nm. If we only consider the IC50 caused by the particle form of 25, 50, 78 nm CuNPs

suspensions, then the toxicity of CuNPs increased with the decrease of nominal particle size in H4IIE, HepG2 and RTH-149 cell lines (Figure 4, middle part). Therefore, the original size could be one of parameters which affect the toxicity of CuNPs suspensions. However, the particles were present as big aggregates in the culture media and all particles went through dissolution processes. Whereas also the shape of the particles was different (78 nm CuNPs is rod, the rest of CuNPs is spherical). Furthermore, there is no significant difference of toxicities between different copper suspensions in PLHC-1 cell lines (Supplementary 6). No correlation could be established between the initial size of aggregates or the aggregates after dissolution and the toxicity of CuNPs suspensions. Therefore, the size of particle matters, but size is not the only dominant factor regarding the toxicity of CuNPs.

Both the particle form of CuNPs and Cu2+ are responsible for the adverse effects observed for the copper suspensions (Figure 5). However, the particle form of CuNPs was the dominant source of toxicity of copper suspensions in all cases. Cu2+ only contributed significantly when mammalian cell lines exposed to 25, 50, 100 nm CuNPs and the MPs. The IC50(particle) found for the particle form of 25 nm CuNPs and the MPs was extremely low due to the large surface area of the particles before (25 nm) and especially after dissolution. The high surface reactivity of CuNPs can cause oxidative stress via the Fenton reaction or induce mitochondrial depolarisation of cells (Karlsson et al. 2009). The high ion concentrations also caused severe damage to all cells. Therefore, the total toxicities were quite high in both cases. All copper suspensions and Cu(NO3)2 can generate a high level of ROS following 24 h exposure, indicating that ROS may be the potential mechanism causing cell toxicity in both cases. In addition, all copper suspensions generated higher ROS levels than Cu(NO3)2 in HepG2 and some CuNPs also produced higher ROS levels than Cu(NO3)2 in the other cell lines. These results indicate that the particle form of CuNPs also plays an important role in ROS production to cells in all suspensions.

Morphology of CuNPs is one of the important properties affecting their toxicity. Compared with spherical CuNPs, the rod particle form of CuNPs (78 nm) expressed quite low toxicity to H4IIE and HepG2 cell lines. Rod-shaped particles have a smaller surface area as compared with the same quantity of spherical-shaped particles according to the mathematic calculation. Therefore, the lower toxicity of the rod particle form of 78 nm CuNPs can be explained by the low reactivity of the surface area of the rod-shaped particles. In addition, there is evidence that spherical NPs are easier to be taken up by H4IIE cells than rod-shaped NPs (Arnida et al.

2010). Singh et al. (2007) reported that the spherical shape of CuNPs facilitates physical interaction with cells, allowing them to be more efficiently attached to the surface of cells and be taken up more efficiently through endocytosis, specifically pinocytosis processes.

5.3 Species-specific toxicity of CuNPs suspensions

The uptake and toxicities of CuNPs were associated with the properties of the cells. The RTH-149 cell lines have higher resistance to toxicity of copper suspensions compared with mammalian cell lines and PLHC cell lines in general. The ROS generation in piscine cell lines was lower than the ROS generation in mammalian cell lines. Particularly, the IC50 of 25 nm CuNPs suspensions and of Cu(NO3)2 in mammalian cell lines was two times lower than the IC50 of piscine cell lines, indicating the higher vulnerability of mammalian cell lines. The mammalian cells were susceptible to both the particle form of CuNPs and Cu2+ in the suspensions, but piscine cells seem to be only vulnerable to the particle form of CuNPs (Figure 5). The particle form of CuNPs can induce severe damage to all cell lines, revealing the low resistance of all cell lines to CuNPs in particle forms. The different toxicities of copper ions to all tested cell lines were due to variations in biological properties and the responses of the four cell lines. Copper ions can be metabolised in hepatoma cells and be transferred in metallothionein by reduced glutathione (Chen et al. 2006). The overloading of copper ions leads to cellular toxicity. Cu2+ contributes significantly when exposed to mammalian cells but little effect was found on piscine cells indicating that piscine cells have greater abilities to metabolise and transport copper ions than mammalian cells. Therefore, species-specific cell features significantly affect the toxicity profile of copper suspensions.

The sizes and the shapes of different cells are different. The average diameter of HepG2 ranges from 1.5 to 3.5 mm and they normally grow in clusters because of the irregular cytoplasmic expansions and microvilli on the plasma membranes connecting them (Bouma et al. 1989). PLHC-1 cell lines have round shapes and these cells appear loosely organised and form monolayers of 4–8 mm thick in culture (Hightower & Renfro, 1988). H4IIE cells have the fastest growth rate compared with the rest of the cell lines and RTH-149 is the only one with fabric shapes, multiple nuclei and connecting filaments. All these factors can also affect the exposure, uptake and toxicity of CuNPs and Cu2+. Also the culture conditions of the cell lines are different, and the metabolism inside the cells may proceed slower at lower temperatures, all of which may affect the uptake of CuNPs and their level of toxicity. For instance, the higher resistance of the piscine cell lines and in particular of the RTH-149 cell

line to the toxicity of copper suspensions compared with the other cell lines used could be related with the lower temperature of culture of the piscine cell lines, and in particular of RTH-149 cells. The properties of cells and the exposure conditions should therefore also be taken into account when discussing the toxicity of the CuNPs.

6. Conclusions

By investigating the behaviour and toxicity of CuNPs using four different cell lines, this study revealed that the toxicity of CuNPs cannot be simply linked with a single physicochemical property of CuNPs. The decrease in particle size can be linked to the toxicity of CuNPs, but the morphologies of CuNPs and the species-specific vulnerabilities of cells also play important roles in evaluating the toxicity profile of CuNPs. The particle form of CuNPs highly contributes to the toxicity in all copper suspensions whereas copper ions only caused significant impacts on mammalian cell lines. More research should be carried out to investigate the mechanisms of uptake and toxicity of NPs in different species regarding the specific properties of cell lines, the contribution of particle and ion form of NPs as well as the exposure conditions. It is clear that the extrapolation of toxicities among species and different test concentrations needs to be handled carefully.

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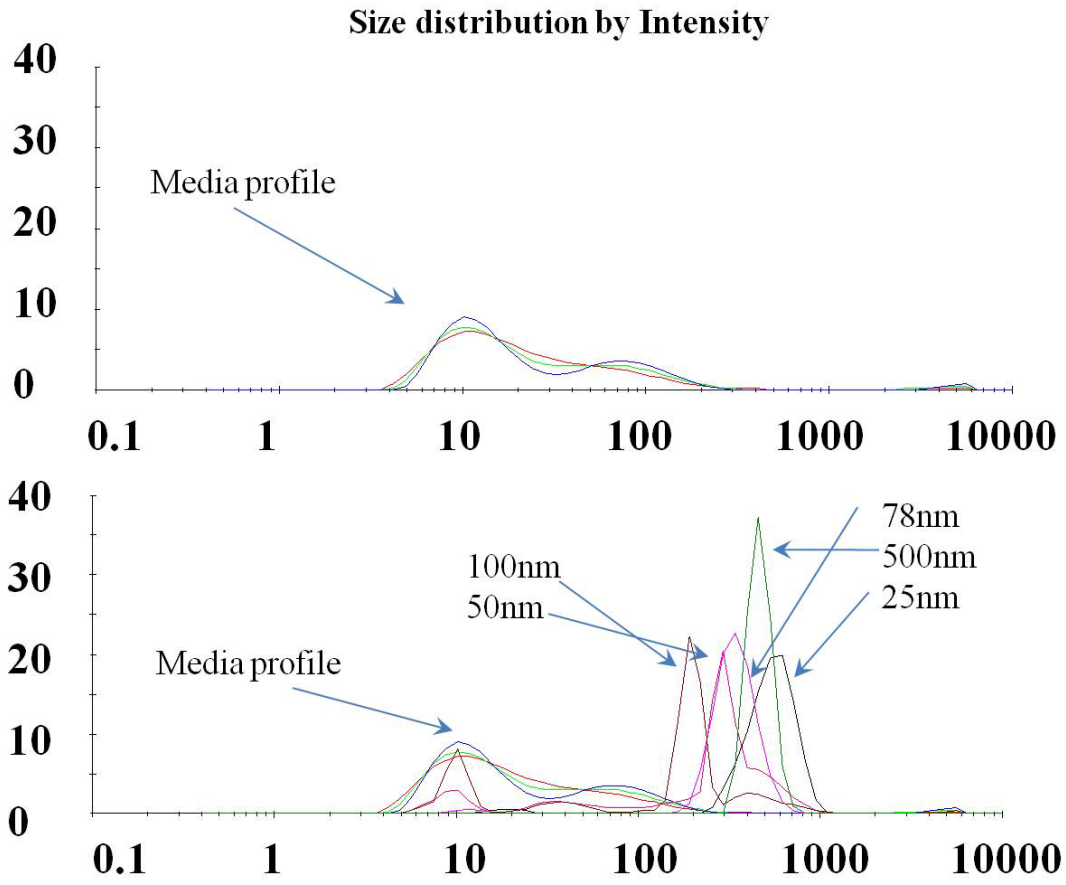
Supplementary Information

Supplementary 1. Supplements of cell culture media

H4IIE and HepG2 cells were maintained at 37ºC and 5% CO_2. EMEM culture medium for H4IIE cells was supplemented with 10% FBS, 1% L-glutamine (200 Mm), 1% of penicillin/streptomycin (10 000 u/mL and 10 mg/mL for penicillin and streptomycin respectively and 1% 100XNEAA. For culturing HepG2, the 1% L-glutamine was replaced by 1 % ultraglutamine (200 mM). PLHC-1 cells were cultured at 30 ºC in a 5% CO_2 atmosphere in α-MEM culture medium supplemented with 5 % FBS, 1 % L-glutamine, and 1 % of penicillin/streptomycin, at the same initial concentrations as indicated before. RTH-149 was cultured at 20 ºC under 5% CO_2 atmosphere in EMEM supplemented with 10 % FBS, 1 % L-glutamine, 1 % penicillin/streptomycin, at the same initial concentrations as indicated before, and 1 % sodium pyruvate (100 mM).

Supplementary 2. An example of subtracting particle profiles from media profiles in the DLS graphs. The culture media profile of H4IIE cell lines has been measured first, which was shown in the upper part of this figure. Then the profiles of CuNPs in H4IIE culture media were measured. The peaks with similar sizes and intensity as H4IIE media profiles were neglected. Only the peaks with higher intensity were taken into consideration to determine the size distribution of CuNPs.

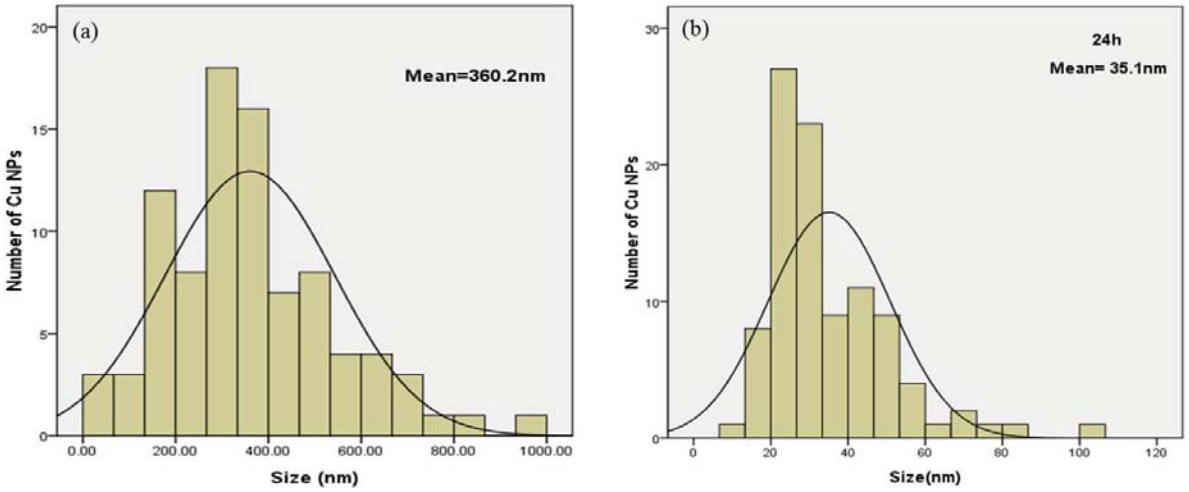


Supplementary 3. ICP-MS measurement

A certified multi-element solution (As, Ba, Be, Bi, Ce, Co, In, Li, Ni, Pb and U - Analytika Ltd, Czech Republic) was used to establish and optimize the operating instrument conditions. Copper quantification was carried out using external calibration and internal standardization (Ga) to minimize the impact of signal instability during the analysis. Calibration standard solutions of copper and internal standard solutions of Ga were prepared daily with subsequent dilutions of the 1000 mg/L Cu in 2 % HNO_3 (v/v) and 1000 mg/L Ga in 2 % HNO_3 (v/v) stock standard solutions (Alfa Aesar, Madrid, Spain), respectively.

Limits of detection (LOD) and limits of quantification (LOQ) were calculated as being respectively three times and ten times the standard deviation of the blank, considering as such each one of the different media studied (H4IIE, HepG2, PLHC-1 and RTH-149) subject to the same treatment as samples. In all cases, the LOD ranged from 0.16 to 0.27 μg/L and the LOQ from 0.53 to 0.90 μg/L. The instrumental response was linear over the calibration range from 0.1 to 100 μg/L, with a relative standard deviation (RSD) < 2 %. For copper concentration measurements, the media from each well was transferred into a polypropylene flask and the well washed twice with nitric acid 2 % (v/v). The rinses were added to the corresponding sample media and made up to a final volume of 10 mL with nitric acid 2% (v/v). Just before ICP-MS analysis, samples were ultrasonicated for five minutes.

Supplementary 4. Size distribution of (a) the pristine micro particles (MPs) prepared in ethanol and (b) 24 h after incubation under culture conditions.

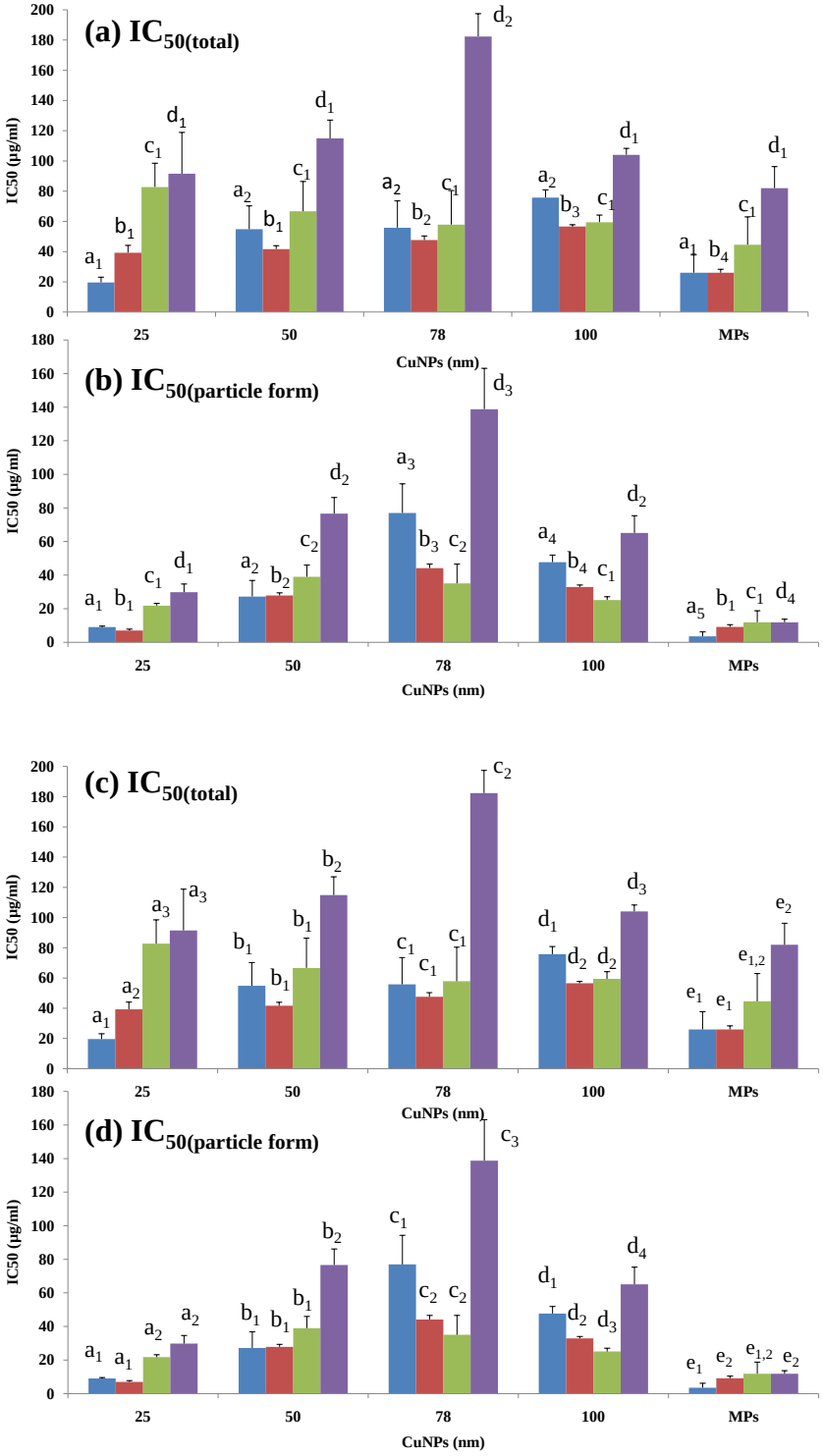


Supplementary 5. $IC_{20,}$ IC_{50} and IC_{80} of each copper suspension after exposure to the four different cell lines after 24 hours and the $IC_{20,}$ IC_{50} and IC_{80} of particle form in each copper suspensions(CuNPs), expressed as mean ± standard deviation (µg Cu /mL of medium suspension).

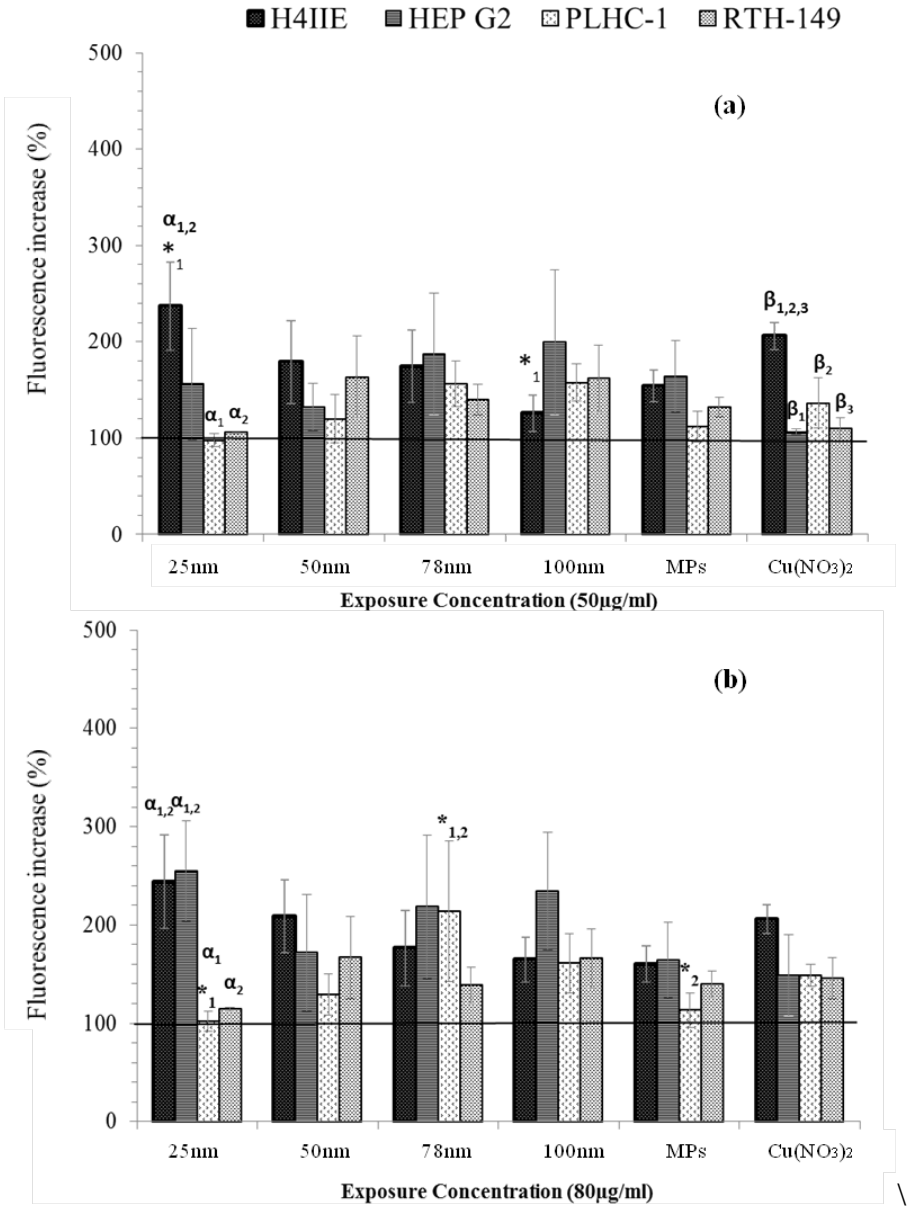
		H4IIE			HepG2			PLHC-1			RTH-149		
	ICx (µg/ml)	**IC_{20}**	**IC_{50}**	**IC_{80}**	**IC_{20}**	**IC_{50}**	**IC_{80}**	**IC_{20}**	**IC_{50}**	**IC_{80}**	**IC_{20}**	**IC_{50}**	**IC_{80}**
IC_x of copper suspension	**25nm**	10±0*	20±3	70±4	17±4	39±5	68±4	53±13	83±16	124±2	53±18	91±27	144±15
	50nm	20±13	55±15	95±14	22±3	42±2	72±1	45±19	67±20	117±9	63±13	115±12	201±71
	78nm	15±3	56±18	112±55	11±5	48±3	88±5	29±17	58±22	87±28	69±32	182±74	260±13
	100nm	29±0	76±5	136±24	28±2	57±1	81±0	32±5	59±5	88±4	67±30	104±4	166±22
	MPs	6±5	26±12	71±24	15±2	26±12	53±4	38±5	45±18	81±12	35±18	74±14	112±10
	$Cu(NO_3)_2$	18±7	54±9	90±14	18±2	43±4	71±4	79±14	109±10	180±9	68±36	120±15	179±16
ICx of Particle form in copper suspensions	**25nm**	3±0	9±1	15±1	6±2	7±1	17±1	14±3	22±1	29±0	17±7	30±2	43±3
	50nm	16±7	27±10	39±12	19±3	28±2	37±1	23±11	39±7	62±4	49±14	77±10	143±57
	78nm	33±14	77±17	98±43	12±4	44±2	76±5	17±9	35±12	53±14	64±34	139±24	213±15
	100nm	34±6	48±4	62±4	22±2	33±1	44±1	15±2	25±2	36±2	32±2	65±10	98±3
	MPs	1±0	4±3	6±5	4±0	9±1	12±1	6±0	12±7	28±3	6±2	12±2	17±1

*Interpolation from Fig. 6.

Supplementary 6. Significant differences (P<0.05) of $IC_{50\ (total)}/IC_{50\ (particle)}$ between different particles (a, b) in the same cell lines and between different cell lines exposed to same particles (c, d), $IC_{50\ (total)}/IC_{50\ (particle)}$ were plotted as mean ± standard deviation.



Supplementary 7. Intracellular ROS in hepatocellular cell lines following exposure to a concentration extrapolated from the fitted curves: (a) 50 µg/ml and (b) 80 µg/ml of different types of CuNPs, the MPs and copper nitrate. ROS levels are quantified by measuring the percentage increase in fluorescence with respect to the control (100%). Results are expressed as the mean of three independent assays ± standard error of the mean. Significant differences between CuNPs exposures are represented by (*) (P<0.05) and same numbers designate relationship, while significant differences between cell line responses are represented by (α, β) (P<0.01) and same numbers designate relationship.



Chapter 4 Toxicity of copper nanoparticles across cladoceran species

Song, L.; Vijver, M.G.; Peijnenburg, W.J.G.M.; De Snoo, G.R.
In preparation.

1. Introduction

Nanoparticles (NPs) could eventually enter in natural system due to the widely application nowadays. Currently, potential adverse effect of NPs has been widely studied and it already known that toxicity of NPs is largely associated with properties of NPs and environmental characterization (Auffan et al., 2009, Fabrega et al., 2009). Puzyn et al. (2011) developed model to predict the toxicity of metal oxides NPs to Escherichia coli, which may used to evaluate untested NPs. However, it is still lack information to interpret toxicity of NPs among different species and toxicity of NPs to untested species is largely unknown. It is necessary to investigate the potential detrimental effects of NPs in ecosystems and protect more species in ecosystem for biodiversity conservation (Behra and Krug, 2008, Song et al., 2011).

Song et al (2011) proposed that ecological traits could be the potential parameters help to interpret and extrapolate toxicity of NPs across species because of the unique properties of NPs, such as their shape and highly specific reactivity, interactions between NPs and organisms are target-oriented and which may strongly associated with morphology and physiology of organisms. In order to explore suitable ecological traits to interpret adverse effect NPs, five cladoceran speices were exposed to four sizes of CuNPs and one type of micron sized copper particles (MPs), to investigate if biological attribute of cladocerans can be used to predict the toxicit of CuNPs across species. Cladocerans can only select food according particle sizes. Particles filtered by thoracic appendages are transported to mouth without any selective mechanisms (Hund-Rinke and Simon, 2006). NPs could significantly interfere with the morphology and physiology of cladocerans during filtration due to the small size, high surface reactivity of NPs. Therefore, it is imperative to understand the interaction between cladoceran species and NPs and the subsequently toxicity effect. Our aim is to provide, the first time, possible biological traits of species which can be used to predict toxicity of NPs. The preliminary hypotheses of this study are:

1. LC_{50} of CuNPs increases with the increasing of body length, body volume and suface areas of spcecies.
2. Solubilisation of Cu NPs is not expected to explain the toxicity of Cu NPs.

This chapter is in preparation. Outputs will be delivered as a peer reviewed scientific paper.

Reference

Auffan M, Rose J, Bottero J, Lowry GV, Jolivet J & Wiesner MR.2009. Towards a definition of inorganic nanoparticles from an environmental, health and safety perspective. Nature Nanotechnology, 4: 634 – 641.

Behra R & Krug H.2008. Nanoecotoxicology: Nanoparticles at large. Nature Nanotechnology, 3:253 – 254

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Puzyn T, Rasulev B, Gajewicz A, Hu X, Dasari TP, Michalkova A, Hwang H, Toropov A, Leszczynska D& Leszczynski J. 2011. Using nano-QSAR to predict the cytotoxicity of metal oxide nanoparticles. Nature Nanotechnology, 6:175–178

Song, L.; Vijver, M.G.; Peijnenburg, W.J.G.M.; De Snoo, G.R. 2011. Smart Nanotoxicity Testing for Biodiversity Conservation. Environmental Science & Technology, 45: 6229–6230

Chapter 5 DOC effect on toxicity of copper nanoparticles to cladoceran species

Song, L.; Vijver, M.G.; Peijnenburg, W.J.G.M.; De Snoo, G.R.
In preparation.

1. Introduction

Copper nanoparticles (CuNPs) have been widely applied and could eventually enter in natural system. Song et al. (2011) studied the toxicity of different nominal size of CuNPs to five cladoceran speices to investigate the relation between biological attribute of cladocerans and toxicity of CuNPs across species. It already known that toxicity of NPs is largely associated with properties of NPs, biological attributes of organisms and environmental characterization (Song et al, 2013, Auffan et al., 2009, Fabrega et al., 2009). It is also important to investigate the effect of environmental parameters on the fate and toxicity of NPs to apply the experimental results more close to real field.

Organic matter widely present in the aquatic environment. CuNPs has high reactive surface and therefore can be easily bound with organic matter. Subsequently, organic substance can affect the fate and toxicity of CuNPs in aquatic systems. It is more realistic to consider the effect of organic substance when evaluate the potential adverse effect of CuNPs in aquatic environment.

In this study, our objective is to determine the toxicity of coated and uncoated 25nm CuNPs on three species of cladocerans to study the effect of coating and DOC on fate and toxicity of CuNPs in aquatic environment. The preliminary hypotheses of this study are:

1. DOC can bind with CuNPs and ions, and the binding effect will affect the fate of CuNPs in aquatic environment.
2. The interaction between DOC and CuNPs can reduce their toxicities to cladocerans.

This chapter is in preparation. Outputs will be delivered as a peer reviewed scientific paper.

Reference

Auffan M, Rose J, Bottero J, Lowry GV, Jolivet J & Wiesner MR. 2009. Towards a definition of inorganic nanoparticles from an environmental, health and safety perspective. *Nature Nanotechnology,* 4: 634 – 641.

Fabrega J, Fawcett SR, Renshaw JC,Lead JR. 2009. Silver Nanoparticle Impact on Bacterial Growth: Effect of pH, Concentration, and Organic Matter. Environmental Science & Technology, 43: 7285-7290.

Song, L.; Vijver, M.G.; Peijnenburg, W.J.G.M.; De Snoo, G.R. 2011. Smart Nanotoxicity Testing for Biodiversity Conservation. Environmental Science & Technology, 45: 6229–6230

Chapter 6 Toxicity of copper nanoparticles across Lemnaceae species

Song, L.; Vijver, M.G.; Peijnenburg, W.J.G.M.; De Snoo, G.R.
In preparation.

1. Introduction

Since Copper nanoparticles (CuNPs) have been widely used in electronics, biocide, ceramics, films, polymers, metallic inks, paints, textiles and coatings due to their optical, electrical, and catalytic properties, CuNPs can potentially end up in diversity of environment. However, the potential adverse effects of CuNPs on ecosystem and human health are still largely unknown. Plants provide a potential pathway for the transport and bioaccumulate NPs into food chain since NPs with a diameter less than the pore diameter of cell wall could easily pass through the cell wall, uptake, translocation and specific localization by plants (E. Corredor, et al., 2009;Nair et al., 2010.). Lee et al., 2009 investigated the effect and bioaccumulation of CuNPs on the growth of Phaseolus radiatus (mung bean) and Triticum aestivum (wheat) seedling. CuNPs were uptake by both of plants cells, but Phaseolus radiatus was more sensitive than T. aestivum to CuNPs. Copper ion released from CuNPs only had negligible effects in this study. Shah and Belozerova 2009 showed that CuNPs can significantly increase the shoot/root ratio of lettuce at a concentration of 0.066% (w of CuNPs/w of soil) after 15 days. However, Stampoulis et al., 2009 reported that CuNPs with a nominal size of 50nm can reduce the length of emerging roots by 77% of the control. Different flora responds differently to NPs and hence, it is necessary to evaluate the potential adverse effect of NPs to different plants species to provide systematic information for the risk evaluation of NPs.
Lemnaceaes are aquatic plants and they are widely spread in many aquatic environments. They can reproduce with quite high rate and they are a valuable food source for livestock, poultry and fish (Vermaat et al., 1998). Perreault et al., 2013 reported that toxicity of copper based NPs to lemna gibba were mainly driven by copper ions and NPs can be accumulated inside of plants and bioaccumulated to higher trophic level. However, other study showed that copper oxide NPs is inhibitorier to Landoltia punctata than the soluble copper ion (Shi et al., 2011). It is still unclear if CuNPs have the same effect on other Lemnaceae species. Therefore, the object of this study is to evaluate the toxicity of copper nanoparticle on three different lemnaceae species to compare the toxicity across species.

This chapter is in preparation. Outputs will be delivered as a peer reviewed scientific paper.

Reference

Corredor E, Testillan PS, Coronado MJ, González-Melendi P6, Fernández-Pacheco R, Marquina C, Ibarr MR, de la Fuente JM, Rubiales D, Pérezde-Luque A and Risueño MC. 2009. Nanoparticle penetration and transport in living pumpkin plants: in situ subcellular identification, BMC Plant Biology, 9: 45.

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Nair R, Varghese SH, Nair BG, Maekaw T , Yoshida Y, Kumar D.2010. Nanoparticulate material delivery to plants. Plant Science 179: 154–163.

Perreault F, Samadani M. Dewez D. 2013. Effect of soluble copper released from copper oxide nanoparticles solubilisation on growth and photosynthetic processes of Lemna gibba L. Nanotoxicology. DOI: 10.3109/17435390.2013.789936

Shah V, Belozerova I. 2009. Influence of metal nanoparticles on the soil microbial community and germination of lettuce seeds, Water Air Soil Pollution. 197: 143–148.

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Stampoulis D, Sinha SK, White JC. 2009. Assay-dependent phytotoxicity of nanoparticles to plants, Environmental Science and Technology. 43: 9473–9479.

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Chapter 7 Establishing common principles for copper nanoparticles toxicity across divergent fish species.

"Quality Nano" exchange

1. Introduction

"Quality Nano" funded our work in Exeter University for 20 working days, which allows us to access highly advanced equipment needed for evaluation the toxicity of nanoparticles.

The aim of this chapter is focus on:

1. It will provide preliminary data about the relations between morphological and physiological properties of fish species and corresponding uptake and toxicity of CuNPs.

2. The results will give more systematical information and a cleaner view for extrapolating the toxicity of nanoparticles in different fish species and subsequently predict and protect for toxicity of hitherto untested fish species in ecosystems.

Outputs will be delivered as a peer reviewed scientific paper, which will be included in the PhD thesis of Lan Song with the working title: "Establishing common principles for copper nanoparticles toxicity across divergent fish species", funded by Qnano and Environmental ChemOinformatics (ECO) Marie Curie Network sponsored by the European Union.

"Quality Nano" is the European Union-funded infrastructure for Quality in nanomaterials safety testing. This four year project integrates 28 top European analytical & experimental facilities in nanotechnology, medicine and natural sciences with the goal of developing and implementing best practice and quality in all aspects of nanosafety assessment.

More information is available at: http://www.qualitynano.eu/

Chapter 8 Output of this work

In this chapter, all the main activities during August 2010 till July 2013 are summarized. The main achievement and output are listed.

Main activity

Aug.2010 - Jul.2011	● Literature review: get familiar with the research field. ● Made working plan for the whole PhD. ● Prepared experiment facilities and did range finding test for the toxicity of CuNPs to cladocerans. ● Wrote the first article: Smart Nanotoxicity Testing for Biodiversity Conservation. ● Prepared and wrote the proposal for the secondment in INIA, Mardrid, Spain. ● Teaching assistant, Leiden University.
Aug.2011 - Jul.2012	● Secondment in INIA during Oct. till Nov. 2011, Madrid, Spain. Carried out experiment to test toxicity of CuNPs to cell lines. ● Prepared and revised the draft: Species specific toxicity of copper nanoparticles among mammalian and piscine cell lines. ● Teaching assistant, Leiden University. ● Supervised Bachelor students, Leo Smit, (Bachelor thesis) ● Experiment: toxicity of five different CuNPs to five different cladoceran species.
Aug.2012 - Jul.2013	● Published the article: Species specific toxicity of copper nanoparticles among mammalian and piscine cell lines. ● Experiment: effect of dissolve organic carbon (DOC) on toxicity of CuNPs to different cladoceran species. ● Experiment: toxicity of five different CuNPs to five different cladoceran species. ● Prepared draft: 1. Toxicity of copper nanoparticles across cladoceran species. 2. DOC effect on toxicity of CuNPs to cladoceran species. 3. Toxicity of copper nanoparticles across Lemnaceae species ● Supervised Bachelor students, Jorn Adriaan Bom (Bachelor thesis) ● Volunteered the SETAC Europe 23rd Annual Meeting. Glasgow, UK ● Applied and funded by “Quanlity Nano”, the European Commission under FP7 Capacities Programme.

Honors and Awards

2013 Funded by “Quanlity Nano” for 20 working days to work in Exeter University.

2010 Marie Curie ITN network fellow, Environmental ChemOinformatic (ECO)

Publication

Song, L.; Vijver, M.G.; Peijnenburg, W.J.G.M.; De Snoo, G.R. Smart Nanotoxicity Testing for Biodiversity Conservation. Environ. Sci. Technol., 2011, 45 (15), pp 6229–6230

Song, L.; Connolly, M.; Fernández-Cruz M.L.; Vijver M.G.; Fernández, M.; Conde, E.; de Snoo, GR.; Peijnenburg, W.J.G.M.; Navas, J.M. Species specific toxicity of copper nanoparticles among mammalian and piscine cell lines. Nanotoxicology, early online. doi:10.3109/17435390.2013.790997.

Song, L.; Vijver, M.G.; Peijnenburg, W.J.G.M.; De Snoo, G.R. Toxicity of copper nanoparticles across cladoceran species. In preparation (Chapter 4).

Song, L.; Vijver, M.G.; Peijnenburg, W.J.G.M.; De Snoo, G.R. DOC effect on toxicity of copper nanoparticles to cladoceran species. In preparation(Chapter 5).

Song, L.; Vijver, M.G.; Peijnenburg, W.J.G.M.; De Snoo, G.R. Toxicity of copper nanoparticles across Lemnaceae species. In preparation (Chapter 6).

Conference

18. - 22. October 2010: Attended the Marie Curie Initial Training Network "Environmental ChemOinformatics (ECO)" was holding the first Autumn School at the Helmholtz Zentrum München (HMGU).

21. - 25. February 2011: Attended the Marie Curie Initial Training Network "Environmental ChemOinformatics (ECO)" was holding the Winter School 2011 at the Hochschule Fresenius (HSF) in Idstein, Germany.

Feb. 2011: Give Presentation named “Smart nanotoxicity testing for biodiversity conservation” in Institute of Environmental Science.

Mar. 2011: Give Presentation named “Smart nanotoxicity testing for biodiversity conservation” in National Institute for Public health and the Environment (RIVM), The Netherlands."

19. - 30.September 2011: Attended the Marie Curie Initial Training Network "Environmental ChemOinformatics (ECO)" will hold the ECO Winter School 2012 at Leiden University.

Feb. 2012: Oral presentation: Traits could be helpful to predict toxicity of Nano copper among cladoceran species. Netherlands Annual Ecology Meeting (NAEM). Lunteren, The Netherlands.

27. February - 02.March 2012: Attended the Marie Curie Initial Training Network "Environmental ChemOinformatics (ECO)" will hold the ECO Winter School 2012 at INIA, Madrid, Spain.

Sep. 2012: Oral presentation: Toxicity of Nano copper among cladoceran species, Nanotoxicology, Beijing, China.

11. - 15. June 2012: Attended the Marie Curie Initial Training Network "Environmental ChemOinformatics (ECO)" will hold the ECO Summer School 2012 in Verona (UNIMIB).

25.February - 01.March 2013: Attended the Marie Curie Initial Training Network "Environmental ChemOinformatics (ECO)" will hold the ECO Winter School 2013 at Linnaeus University in Kalmar, Sweden.

12-16 May 2013: Attended and volunteered the SETAC Europe 23rd Annual Meeting. Building a better future: Responsible innovation and environmental protection. Glasgow, Scotland, United Kingdom