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Fate and effects assessment of coated nanoparticles

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Summary

This short term research fellowship position is sponsored by the Environmental Chemoinformatics (ECO), Marie Curie Initial Training Network and assesses the fate and effects of coated nanoparticles in the aquatic environment.

In nanotechnology surface modifications such coatings and functionalizing side chains are frequently used to tailor design nanoparticles for a specific application or to preserve their stability in a given solvent. Often these surface modifications provide nanoparticles with new physico-chemical characteristics which have yet to be assessed for their possible ecotoxicological potential.

Beyond focusing on the toxicological implications of selected nanoparticles and surface modifications, this study aims at elucidating the missing link between the physico-chemical characteristics, expressed as the dissolution and aggregation state of nanoparticles in an aqueous environment, and the biological response of nanoparticles using aquatic invertebrates as indicator species.

Introduction

Due to extensive manufacturing and application of nanomaterial products in the last decade the industry is said to be undergoing a "nano revolution" and indeed many industrial branches are solely built upon marketing newly discovered physico-chemical characteristics of nanomaterials. Drug delivery systems in the pharmaceutical industry; stronger, more flexible and antimicrobial textiles; lighter and stronger materials for the automotive and aerospace industry; UV protective day care products; more energy efficient conductor systems and remediation agents for contaminated grounds and waters in the environmental sector are only a few examples where nanomaterials are commonly applied today.

With a future market size of up to USD 3.1 trillion in 2015 and a concurrent demand of 2 million new employees it is not surprising that hardly any other sector benefited from as much public research and development (R&D) investment as nanotechnology in such a short time. And as yet nanotechnology is an integral part of our daily life it is foreseen to emerge to an important industrial sector with a major socio-economic impact in the future (Palmberg *et al.* 2009).

A widely accepted concept defining a nanoparticle (NP) says that at least one dimension has to fall between the 1-100nm range. This small scale and the resulting very high surface to volume ratio are the primary discriminants of new physico-chemical characteristics of a material in the nano range which its corresponding bulk material does not possess.

Once a NP is released into the environment it is important to understand the processes which determine its fate. Environmental factors influence the stability, reactivity, solubility, mobility and

bioavailability of a NP, thereby determining its risk and impact potential. And since it is currently still vastly unknown how to quantify the reactivity of NPs in the environment the issue of dosimetrics, hence assigning quantitative physical or chemical attributes of NPs to a biological response, is a challenging endeavour that is yet to be resolved.

Even though more relevant data are still needed, alarming signals are already given by several studies outlining the toxic effects of a variety of NPs upon aquatic organisms (Baun *et al.* 2008; Handy *et al.* 2008b; Wiench *et al.* 2009). However, the results obtained on ecotoxicity up to now are often inconclusive or contradictious (Menard *et al.* 2011) and the diversity of results may be partly explained by differences in experimental set up. Studies addressing the fate and effects of the same NP in different environmental compartments (e.g. aqueous, soil, sediment) on different species make it merely impossible to directly compared results. Also thus far no consensus has been reached on which NP descriptors to focus on to accurately assess biological responses and the dynamics of NPs in the environment. Confounding factors such ionic strength, pH, dissolved organic matter, salinity or temperature of the solvents essentially determine the stability and bioavailability of a NP and should ideally be monitored throughout experiments.

But next to the lack of monitoring environmental discriminants for the fate of a NP, thus far most "nanotoxicity studies don't even provide coherent information on the physico-chemical characteristics of the tested NPs" themselves (Menard *et al.* 2011). This dramatically highlights the necessity to discriminate relevant physico-chemical characteristics for a routinely description of NPs for better transparency and comparability of results across environmental fate and effect studies.

In nanotechnology there are many procedures that routinely apply NP coatings but also a magnitude of specifically designed NP capping agents can be found today. Capping agents functionalize NPs providing them with new or enhanced physical, optical, chemical and biological properties (Kobayashi and Sakuraba 2008; Liu and Han 2010), and may alter their toxicological implications compared to their non-coated equivalent by determining the NP's bioavailability and uptake by organisms (Fabrega *et al.* 2011).
Even when applying another surface coating to the same core material of a NP may lead to changed characteristics or toxicity. Lee et al. (2010) have shown different toxicities to D. magna when applying different surface coatings to the same quantum dots.

Up to now, however, limited attention was drawn to these surface modifications with regard to their potential in changing a NP's fate in the environment and often no clear distinction is made between a

NP and the same NP but with a capping agent during experiments. Gottschalk and Nowack (2011) criticize that currently used release models lump together different forms of the same NP, such as coated and non-coated NPs, which distorts the prediction of environmental impacts. However, it is currently discussed to what extent surface modifications determine the effects of a NP onto organisms. Studies have shown that different surface modifications of the same NP lead to different toxicological responses and the assumption lies near that the main driver of toxicity of nanomaterials are surface modifications.

Routinely, experiments determining the stability and toxicity of NPs are carried out in ideal laboratory settings using either ultra-pure water or a well-defined reaction medium. However, to address the potential impacts of NPs once they are released into the environment, the heterogeneous nature of fresh water has to be regarded. Ionic strength and organic matter in natural water inevitably affect the stability of NPs and consequently alter the toxicological implications when compared to ideal laboratory settings.

Increasing the ionic strength in a NP suspension leads to destabilization processes, hence aggregation and precipitation of NPs. To the best of our knowledge, the effects of these destabilization processes onto aquatic organisms are largely unknown and more experiments linking the stability of particles in aqueous suspension to a biological response are needed.

Aims

Coatings

This study aims to determine the biological response of the aquatic invertebrate *Daphnia magna* to coated metal based nanoparticles (NPs) with respect to their aggregation and dissolution behaviours. Polyvynilpyrolidone (PVP) coated and uncoated silver (Ag), PVP coated and uncoated iron oxide (Fe3O4) and carbon (C) coated and uncoated copper (Cu) NPs with an average particle size of 25nm were used in this study.

We define the toxicological endpoint as mortality after 48h of the test organism *Daphnia magna*, according to the OECD guideline (no. 202).

We hypothesize that coatings of NPs alter the biological response and that coatings change the aggregation and dissolution behaviour of NPs. Furthermore we hypothesize that surface coatings do not exclusively determine the biological response towards NPs.

Increased Ionic strenght

It is evident that chemical changes of the matrix influences aggregation and dissolution kinetics of a NP suspension but the link to bioavailability and subsequently toxicity of NPs towards aquatic

organisms has received very little attention.
By adding $CaCO_3$, a naturally abundant component of fresh water, the increased ionic strength of the solvent will trigger changes in the aggregation and dissolution dynamics during exposure experiments. We aim at elucidating the relation between $CaCO_3$ induced aggregation and a reduced biological response. We therefore hypothesis that increased aggregation of NPs in standard test medium (STM) spiked with $CaCO_3$ is a primary driver of the reduction of toxicity of D. magna.

Time effect

Due to the highly dynamic nature of the reactivity of a NP suspension, physico-chemical characterisitcs of a NP suspension are likely to change during the period of ecotoxicological tests. By comparing the phyisco-chemical characteristics and the biological response to freshly prepared and old NP suspensions we aim at investigating the possibility of ruling out repeated handling based inaccuracies by re-using old nanopartilce suspensions.
We hypothesize that there is a difference in biological response and physico-chemical characteristics of fresh and old NP suspensions.

Strategy Summary

- Comparing the biological responses of coated and uncoated NPs:
    - Establishment of dose response relationships of Daphnia magna towards coated and uncoated iron oxide (Fe_3O_4), copper (Cu) and silver (Ag) NPs.
- Comparing physico-chemical characterisitics of coated and uncoated NPs:
    - Collecting data on zetapotential, particle size distribution and ion release of Fe_3O_4, Cu and Ag NPs
- Assessment of the effects of increased ionic strength in standard test medium towards dose response relationships.

Methodology

In a first phase of the study the biological response towards coated and non coated metal NPs was assessed. The aquatic invertebrate Daphnia magna was chosen as indicator species due to its ease in culturing and its importance as a primary consumer in ecological fresh water systems. In a controlled lab based approach, following the OECD guideline 202, a concentration range of NPs dissolved in D. magna standard test medium (STR) with a steady ionic strength of 11mM, the mortality of test animals after 48h was recorded as basis for the development of a dose response relationship and the determination of respective LD50 values.

In the second phase of this study, physico-chemical parameters were measured to determine the NPs behaviour in D. magna standard test medium after 48h. Using dynamic light scattering techniques and measuring the electrophoretic mobility of NPs, the average size distribution and the zetapotential of the particle dispersions were measured to address the aggregation state of the particle dispersions. Additionally, using graphite furnace atomic absorption spectroscopy (GFAAS), the amount of metal ions going into solution from the NPs were measured in order to address the dissolution of the NPs in suspension.

Materials and Methods

Nanopowders

Copper and carbon coated copper and PVP coated iron oxide NPs were purchased from **Nanostructured & Amorphous Materials, Inc.** 16840 Clay Road, Suite 113, Houston, TX 77084, USA. Iron oxide, Silver and PVP coated silver NPs were purchased from **SkySprings Nanomaterials, Inc.** 2935 Westhollow Drive, Houston, TX 77082, USA.

	Cu*	C-Cu*	Ag	PVP-Ag	Fe3O4	PVP-Fe3O4*
Purity	99,8% (metal basis)	99,8% (metal basis, O<10%)	99,95 (trace metal basis)	99,95 (trace metal basis)	98+% (Trace metal basis)	98+%
Average Particle Size	25nm	25nm	20-30 nm	20-30 nm	20-30 nm	20-30 nm
Specific Surface Area	30-50 m^2 / g	30-50 m^2 / g	~ 20m^2 / g	~ 20m^2 / g	~ 40-60 m^2 / g	> 40 m^2 / g
Morphology	Spherical	Spherical	Spherical	Spherical	Spherical	Spherical
True Density	8,94 g /cm^3	8,94 g /cm^3	10,5 g / cm^3	10,5 g / cm^3	4,5-5,1 g / cm^3	
Coating		Carbon		~0,2 wt% Poly vinyl pyrolidone		~1 wt% Poly vinyl pyrolidone

*=NanoAmor

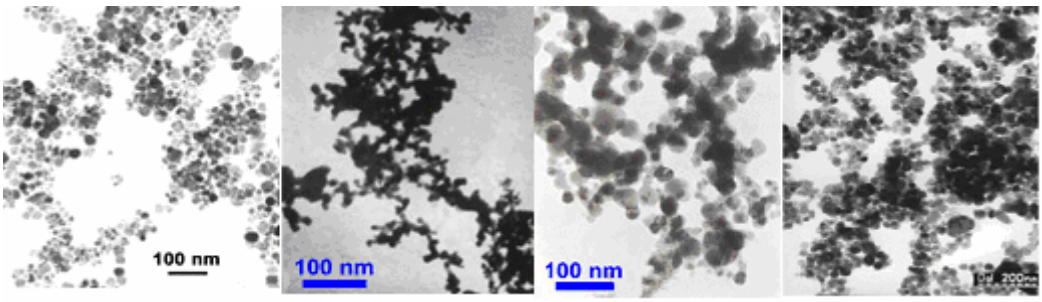


Figure 1: Purchased nanopowders from left to right: Fe3O4 (SkySprings Nano), Cu (NanoAmor), Cu-C (NanoAmor), Fe3O4 – PVP (NanoAmor)

Two cetyltrimethylammonium bromide (STAB) copper NPs (Cu-STAB1 & Cu-STAB2) were synthesized by the Zelinsky Institute of Organic Chemistry, Russian Academy of Sciences in Moscow, Russia. They were synthesized from CuSO4 and determined to be have a size distribution of around 40-45nm (Cu-STAB1) and somewhat lower than 30nm (Cu-STAB2).

Sample Preparation

All exposure experiments and physico-chemical characterization were conducted in standard test medium (STM). STM was composed of CaCl2 (dehydrate) 294mg/L (=2,65mM); MgSO4 (heptahydrate) 123,25mg/L (=0,5mM); NaHCO3 64,75mg/L (=0,77mM); KCl 5,75mg/L (=0,077mM); The ingredients were dissolved in demineralized water and the solution was aerate for 1h. The total ionic strength of the STM solution was I=11mM.

All NP powders were kept refrigerated at 4°C. NP stock suspensions were prepared by weighing about 2mg of nano powder into 250 mL STM in a volumetric flask. The stock suspensions were sonicated in a sonication bath for 10min and all final nominal concentrations used, were prepared by respective dilutions with STM. The NP suspensions were not filtered in order to resemble more realistic environmental exposure scenarios.

To quantify the Ag ions going into solution from a Ag NP dispersion, the desired nominal concentrations of the dispersions were prepared as described above, following a centrifugation step in order to sediment the particles at the bottom. The supernatant, carrying the dissolved ions, was collected and by adding and 40µL aqua regia (68% HNO3 1 : 4 HCL 73%), the ions were kept in solution until analysis. Quantifying the total amount of Ag in a AgNP dispersion, the centrifugational step was omitted and 80µL of auqa regia was added to digest the particles and keep the ions in solution.

The same procedure was carried out for the CuNP, but instead of aqua regia only 20µL and 40µL of 68% HNO3 was used for the quantification of Cu ions and total Cu.

All experiments were a carried out treating coated particles in the same fashion as uncoated particles.

During all exposure experiments negative controls were done using test medium without particles. To check for possible contaminants during the ion release and total concentration determination experiments, blanks using demineralised water were done as well. CuNO3 and AgNO3 solutions served as positive controls.

Physico –chemical characterization of nanoparticle suspensions

Atomic absorption spectroscopy:

Using a Perkin Elmer 5100 Graphite Furnace and Flame Atomic Absorption Spectrometer and Zeeman correction, all coated and uncoated CuNP and AgNP dispersions were analyzed for their released ions and total amounts. One concentration of 0,2mg/L for each of the 4 CuNPs was prepared and analyzed for the released ions and total amounts. Triplicates of 1mg/L for each for each of the AgNPs were prepared and analyzed for the released ions and total amounts.

Cu and Ag ion specific electrodes:

Calibration points with CuNO3 or AgNO3 were prepared in MQ water ranging from 0,01-1M. The pH was adjusted to 3 and the ion activities of the standards were measured with the ion specific electrodes. The samples were measured with electrode and the mV reading was extrapolated into active ion concentration. One concentration, varying between 5-30mg/L, for each of the CuNPs and AgNPs was prepared, measured with the electrode and extrapolated into active ion concentration.

Dynamic light scattering:

Using a Malvern Instruments Zetasizer Nano ZS, particle size distribution and zetapotential were determined by measuring the backscatter at 173° at 20°C in disposable folded capillary cells for each particle. Cumulants analysis and Smoluchowski theory were used to determine particle size average and zetapotential. Parameters were adjusted as follows: Viscosity 1,0031cp, refractive index 1,590 and 1,330 for particle and solvent respectively, particle absorption 0,01. 3 measurements with 10 to 100 runs each were performed. Using the software DTS, Ver. 5.00 from Malvern with the general purpose and auto mode for particle size and zeta potential respectively average results were obtained. All NP suspension were prepared in standard test medium and concentrations ranged between 5-30mg/L. Before use and in between measuring replicates, capillaries were rinsed multiple times with milli Q water. Test medium and milli Q water blanks were measured to check for possible contaminants.

Cultures

Due to their wide acceptance, simplicity of cultivation with a fast reproduction cycle and a rapid acute response to toxicants, Daphnia magna (Clone K4) was the test species of choice in this study. Cultures of about 20 adult animals were kept in 0,5L plastic containers and along with changing half of the culture medium and removal of carapaces and dead animals they were fed with algae (species ???) and backing yeast (Dr. Oetker) twice a week. Once every two weeks containers were changed and cleaned to prevent excessive algal growth on the containers. A light/dark rhythm of 16/8 h at 21°C was installed. As a medium Elendt M4 was used, according to OCD 202.

Exposure Tests

The Daphnia magna acute immobilization test was performed as a 48h exposure assay following the “OECD guidelines for testing of chemicals 202”. Using a Pasteur pipette to minimize the transfer of culture water, neonates (age, <24h) were separated from adults and collected in a glass beaker containing 20mL standard test medium as an initial washing step and to allow for acclimatization.

5 animals were transferred to a glass vial with a total volume of 10ml of standard test medium including a specific nominal concentration of nanoarticles. For one concentration range, 7 different

NP concentrations were prepared directly into individual test vials. One concentration range served as the base to calculate the LD50 for the applied NP. As the number of juveniles allowed, up to 5 replicates of the concentration range were encompassed in one experiment, each resulting in an own LD50. Several experiments on different days with different juvenile badges were conducted contributing to the total number of replicates for the same NP. The animals were not fed during the test and the toxicological endpoint was determined as immobility after gently tipping the vial. After 48h the number of dead animals in each vial was collected.

Data treatment

The collected binomial data was analyzed using the open source statistical software package R, version 2.13.0. Using the package "drc" the dose response curves were fit using a 4 parameter logistic function, the model fit was tested and LD50 values were calculated. LD50 values were calculated for all replicates of all NPs and a pair wise t-test of all LD50 values using the Holm adjustment method was performed to compare the data obtained from different particles. To illustrate and compare dose response curves of different NPs all replicates for individual particles were pooled to obtain a single dose response curves. Chi Square tests were run to check the model fit. The data was illustrated in sigmoidal dose response curves of dead daphnids against NP concentrations as well as LD50 values in boxplots showing the interquantile range, median and standard error.

Results and Discussion

1. Coatings of Nanoparticles influence the biological response

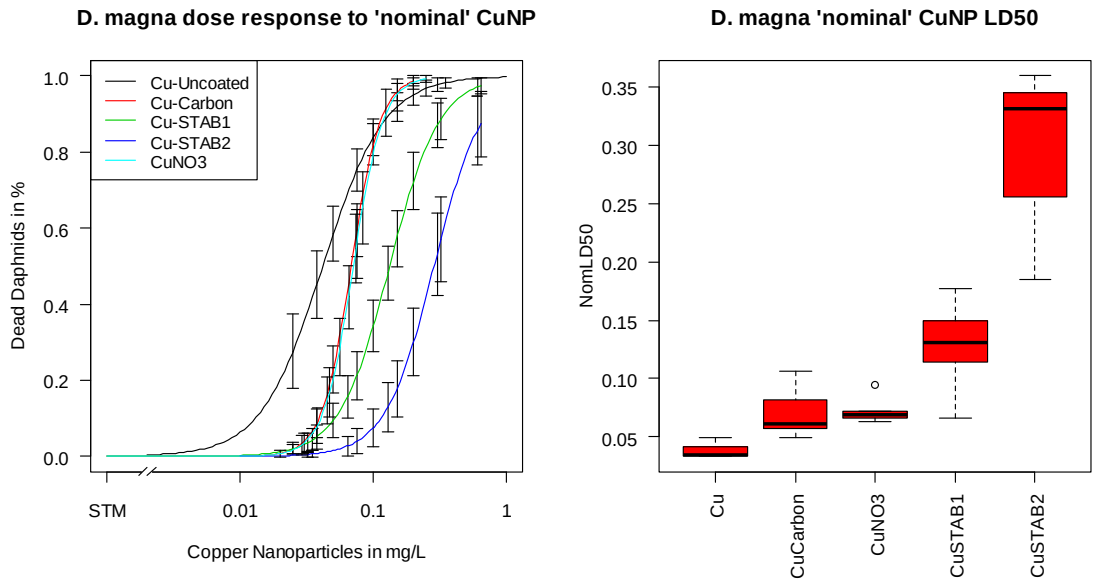


Figure 1: Left: Dose response curves for 4 NPs and a copper reference, using nominal exposure concentrations. Right: Boxplots of LD50 values where the red box is the interquartile range, thick black line is the median and the whiskers are standard error. LD50 values calculated individually for every replicate of each particle or reference.

Table 2: Pairwise t-tests using the Holm adjustment method for LD50 values of 4 copper NPs and a copper reference. LD50 values calculated individually for every replicate (n) of each particle or reference.

Paired t-test, Holm adjusted	Cu (n=7)	CuCarbon (n=15)	CuNO3 (n=8)	CuSTAB1 (n=12)
CuCarbon	0.09083			
CuNO3	0.09083	0.88792		
CuSTAB1	**6.0e-07**	**3.0e-05**	**0.00047**	
CuSTAB2 (n=8)	**< 2e-16**	**< 2e-16**	**< 2e-16**	**7.1e-15**

Figure 1 left shows dose response curves for 4 CuNPs with different surface modifications and CuNO3 as copper reference and figure 1 right shows a boxplot for LD50 values of all replicates of each particle. The illustrated curves are based upon nominal concentrations. Model fit tests revealed that all curves fit the applied models (data not shown). C-CuNP show an identical dose response as the CuNO3 reference and the apparent differences in dose response of uncoated CuNPs towards C-CuNP and CuNO3 seen in figure 1 left and right could not be statistically verified by comparing LD50 values, summed up in table 2. Therefore the effects of uncoated CuNP, C-CuNP and CuNO3 appear to be similar. Both particles coated with STAB show significantly reduced toxicities compared to uncoated CuNP, C-CuNP and CuNO3 reference. With the same surface modification but a slightly smaller average particle size, STAB2 shows to be significantly less toxic than STAB1.

Even though NP specific toxicity has been reported for CuNP (Chen *et al.* 2006), our data does not support these findings. The dose response and resulting LD50 values for uncoated CuNP did not reveal significantly different results in comparison to a copper bulk reference, CuNO3. Even though NP specific toxicity is not apparent in this study, the factors affecting NP aggregation and dissolution kinetics are numerous and by choosing a different environmental compartment, these data cannot be directly compared to inhalational or terrestrial studies. A valuable attempt has been undertaken but further effort has to be made to investigate NP specific effects towards D. magna in laboratory toxicity testing, in line with the OECD 202 guideline.

It was previously suggested that a NP's surface modification influences the biological response. Lee et al. (2010)'s finding showed a different response of D. magna to quantum dots equipped with different surface modifications. In our study, the significantly different response to both STAB coated CuNP underlines the general validity of this assumption, using the same indicator species but a different set of particles and surface modifications.

Since other particle characteristics, such as average particle size of 20-30nm, were kept constant, the different responses to uncoated CuNP, C-CuNP and STAB2-CuNP appear to be explained by the

surface modification. However, other particle characteristics cannot be generally disregarded in playing a role towards the toxicity of NP.
A significantly higher LD50 for STAB2-CuNP compared to STAB1-CuNP and an average particle size of 30nm and 40-45nm respectively, indicates that surface modifications are not the exclusive toxicity determining characteristics of NP.

The origin of the toxicity determining characteristics of the particles need to be further addressed by substantial physico-chemical characterization of the particles in the respective test medium. It is assumed that the dissolving copper ions may play a significant role in determining toxicity to *D. magna*.

2. Physico-Chemical Characterization of Coated Copper Nanopoarticles

It is widely discussed that the toxicity inferring characteristics of a metal NP are mainly determined by the release of metal ions into the surroundings. Using atomic absorption spectroscopy to detect and quantify released copper ions after a centrifugation step showed that the extent of released ions differs depending on the coating of the NP.

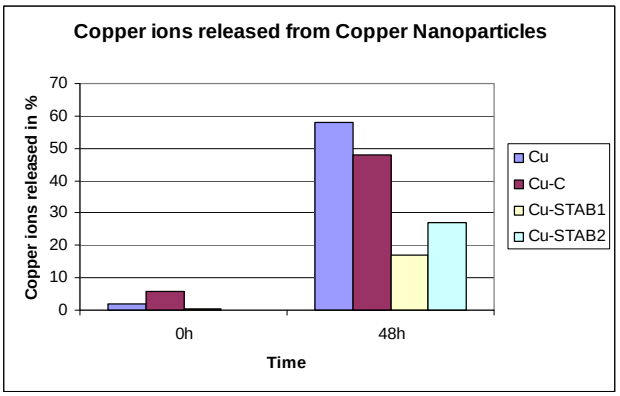


Figure 2: Copper ions released from 4 NPs with different surface modifications were quantified in the supernatant after centrifugation using flame and graphite furnace atomic absorption spectroscopy. Total amounts of copper were also quantified by flame AAS using the same initial concentration as for the ion release measurement but excluding the centrifugation step. The quantified released ions are represented in percentage of the total analytical amounts.

Following our expectations, the uncoated CuNP show the highest release of copper ions closely followed by the C-Cu NP. Both STAB coated CuNP seem to stabilize the CuNP more efficiently in direct comparison as the least amount of ions was released. This finding underlines the general assumption that a metal NP's toxicity may be mainly coming from the released ions, as in our case the least toxic particles released the least amount of ions. However, in this experiment no replicates were performed and experiments with a higher number of replicates are needed to statistically validate these findings.

Not only the metal ions released from a NP, but also the reactivity of those released ions have to be considered of inferring a toxicological response. Therefore, by means of a copper ion selective electrode, the ionic activity of those ions released was measured and the results indicated that the ionic activity does not play an important role.

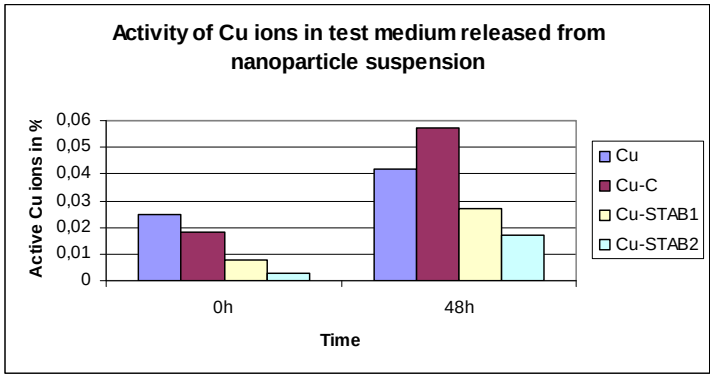


Figure 3: Copper ion activity of 4 different CuNP was measured using a copper selective electrode. By means of a standard regression line the measured potentials of the electrode was transformed into concentrations and are represented as percentage of the nominal concentration of the particle suspension. The activity can be regarded as the quantitative indication for the amounts of free and unbound copper ions in solution.

The test medium, the ions are released into, is providing the basic nutritional requirements for D. magna and therefore most likely scavenges off any free metal ions, as can be seen by the very low percentages in figure 3.

Data on the aggregation behaviour using dynamic light scattering (DLS) and electrophoretic mobility (Zetapotential) techniques are given in figure 4 below. The average size distribution of all CuNP did not confirm the particle size stated by the vendors. However, expectedly, all size distributions were drastically increased which indicates excessive aggregation of the NP due to the ionic strength of the *D. magna* standard test medium (STM). The high average particle size distributions in figure 4 left are in line with the rather low zetapotential in figure 4 right for all particles. The threshold for a stable NP suspension is defined as above +30mV or below -30mV.

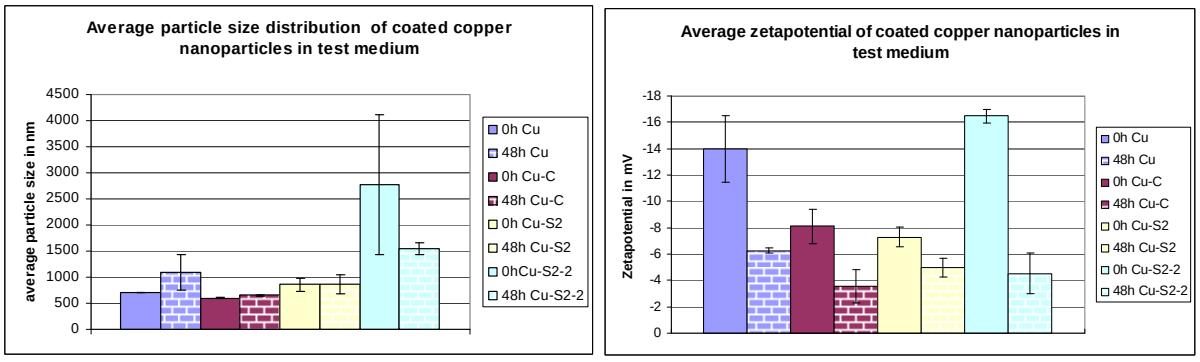
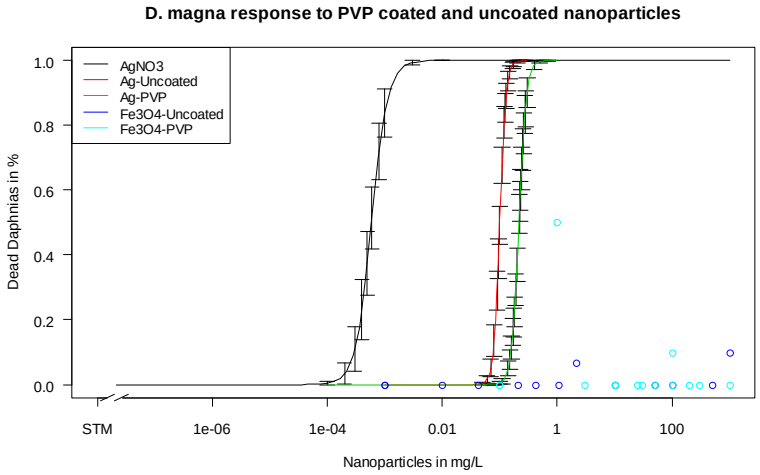


Figure 4: Left: Coated and uncoated CuNP average size distribution in test medium determined by DLS techniques. Right: Average zetapotential of coated and uncoated CuNP in test medium.

Our results suggest that none of the coatings stabilize and prevent the particles from aggregating. However, following Handy et al. (2008a)'s suggestions that "aggregated and unsolved NP would represent more realistic exposure conditions", we omitted filtration of NP suspensions before experiments and measurement, which might exaggerate the size distribution results.

3. A Coated Nanoparticle's Core Material Influences the Biological Response

Iron oxide NPs (Fe3O4NP) have great environmental significance and exposure levels to aquatic organisms may be quite high when applied in heavy metal remediation. However, as can be seen in figure 5, concentrations for Fe3O4NP and PVP-Fe3O4NP resulting in a toxicological response need to be very high.



The D. magna response to these particles is very inconsistent and can be regarded as experimental noise. Thus, both Fe3O4 and PVP-Fe3O4 have proven to be virtually non-toxic.

The highest exposure concentration of these particles was 1g/L and was so high that immediate precipitation of particles was taking place. This raises the questions about the usefulness of Daphnia species as toxicity indicators for these particles when ground feeding organisms may be at higher risk due to higher exposure after precipitation. Further increases in concentrations do not appear useful as toxicological implications upon exposure to higher concentrations will not fall into environmentally relevant concentrations and effects observed will probably not be related to attributes of particles in the nano size range but of physical nature, such as particle unspecific mobility impairment.

With a good dose response model fit (data not shown), AgNP and PVP-AgNP show a very narrow response range, but neither Fe3O4NP nor PVP-Fe3O4NP does. Even though both PVP coated particles share many attributes, such as size, shape and coating, we can still see a clear difference in their biological response. Therefore our results underline the importance of a NP's core material in

environmental assessment and try to appeal for cautiousness when regarding surface modifications as the primary aspect for effect assessment of NPs.

Due to interaction of the surrounding medium and its constituents with the electric double layer on a NP's surface, increased ionic strength of the medium inadvertently increases the hydrodynamic diameter and favors aggregation of NPs (Jiang et al. 2009). Therefore, it is not surprising that our results indicate a substantial degree of NP aggregation in standard test medium, figure 6, with an overall ionic strength of11mM.

As the result above show, a NP's core material does play an important role towards the effects upon organisms. Additionally, illustrated in figure 6 below, the results for the fate determining characteristics of NP in D.magna test medium reveal consensual findings. Controversial to Hoppe et al. (2006), who found *"clear evidence for the protective role of PVP against aggregation"*, the PVP coating in our case does not appear to affect the zetapotential and average particle size of the NP. However, a difference in zetapotential and average particle size between the two core materials, Ag and Fe3O4, is evident. With lower zetapotentials and higher average particle sizes, Fe3O4NP are less stable and tend to aggregate more than AgNP. Relating this increased aggregation states to decreased biological responses lies near, but it is reasonably speculative and requires further research efforts.

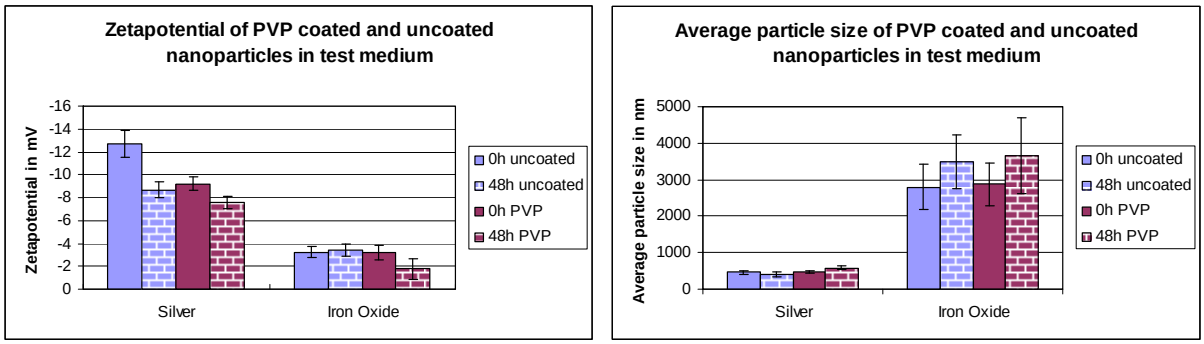


Figure 6: Left: Coated and uncoated AgNP and Fe3O4NP average size distribution in test medium determined by DLS techniques. Right: Average zetapotential of coated and uncoated AgNP and Fe3O4NP in test medium.

Regarding the collected data for Ag ion release, illustrated in figure 7, the assumed enhanced stabilization of the AgNPs by the PVP coating, in terms of dissolution prevention, does not seem to accord striking improvements over the uncoated AgNPs. In contrast, our findings even suggest that slightly more Ag ions are released from the PVP coated AgNPs.

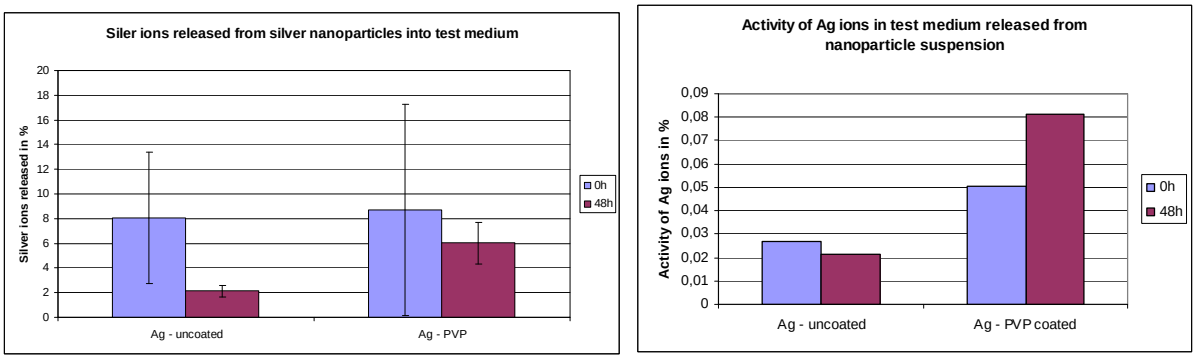


Figure 7: Left: Ag ion release from coated and uncoated AgNP dispersions as determined by AAS. Right: Determination of Ag ion activity in coated and uncoated AgNP dispersions.

With this finding and the extremely low ionic activity of the released Ag ions indicates that silver ion dissolution is unlikely to be a major contributor to AgNP toxicity in *D.magna*, when exposed in test medium. However, our data only consider the NP dynamics in the test medium, ion release and ionic activity may change and still play a significant role *in vivo* upon ingestion of the test organisms, which has to be addressed in future studies.

4. The Effects of Increased Ionic Strength on the Fate and Effects Coated Nanoparticles

Limestone is one of the major contributors towards the calcium input to natural waters, and calcium carbonate (CaCO3) is the most abundant chemical compound in fresh water. Spiking test medium during a toxicological response tests with CaCO3 attempts to resemble natural fluctuations in ionic strength. Increases in ionic strength have been shown to enhance aggregation and precipitation of NP. Presuming toxicological effects are caused by the physico-chemical characteristics of NP in suspension, enhancing particle aggregation suggests a reduction in toxicity as aggregates fall out of the nano range and precipitate. Initial negative control/solvent control tests with concentrations of up to 10mM CaCO3 revealed no toxicity to *D. magna* (data not shown). Therefore, observed changes in effects in toxicity tests spiked with CaCO3 concentrations up to 10mM, can be referred to the induced physico-chemical changes of the NPs due to the increased ionic strength. The carbon coating of the CuNP serves to sterically stabilize the particles to preserve particle dispersion in suspension. It has been reported that sterically stabilized particles are not subject to changes in aggregations kinetics under increased ionic strength.

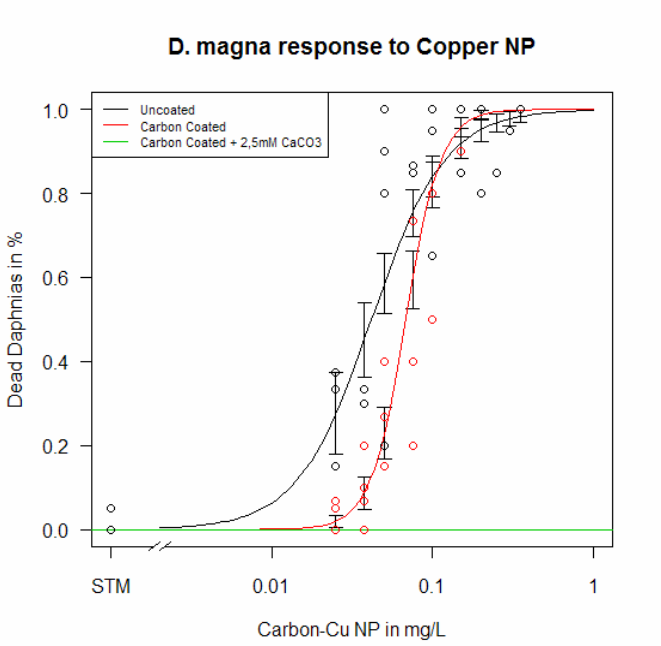


Figure 8: Dose response curve of 48h exposure of carbon coated and non-coated copper NPs to D. magna. Non-coated copper particles are more toxic than carbon coated particles and the addition of 2,5mM CaCO3 drastically reduces the toxic effects of carbon coated copper particles.

The fact that there is virtually no response to the C-CuNP under conditions of increased ionic strength during exposure to *D. magna* (figure 8), the finding of constant aggregation kinetics cannot be validated by our findings. In contrast, the lack of any response during exposure to *D. magna* rather suggests that substantial changes of physico-chemical characteristics can be expected which determine the stability and the effects of the coated particle.

Further characterization of particles using light scattering techniques has to be performed in order to determine the particles stability in test medium with increased ionic strength.

Applying CaCO3 as supplement to toxicity tests in order to increase the ionic strength of the medium and to resemble more environmentally relevant conditions during exposure tests has shown different responses of D. magna and indicate changing physico-chemical charactersitcs in contrast to other sterically stabilized particles stated in the literature. This finding underlines the frequently reported call for a case by case assessment of NPs.

Due to the fact that increased ionic strength interferes with zetapotential and DLS measurements, it is of value knowing the minimum effect inhibition concentration of CaCO3 for the 100% mortality concentration of CuNPs of 0,2mg/L. In figure 9 , a CaCO3 gradient shows that the *D. magna* response to constant 0,2mg/L of C-CuNP and CuNPs changes with increasing CaCO3 concentrations.

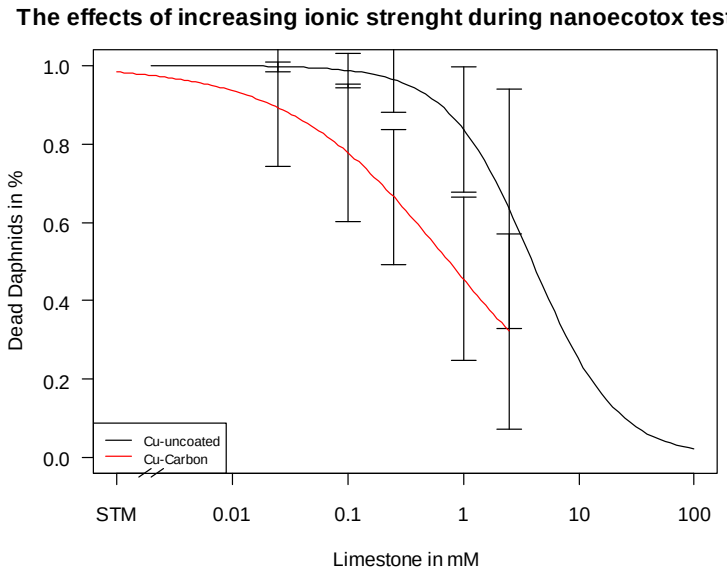


Figure 9: D. magna exposure tests with constant 0,2mg/L C-CuNP and CuNP but increasing concentrations of CaCO3. Toxicitiy mitigating effects with increasing concentrations of CaCO3 can be observed.

For a more controlled laboratory based attempt to evaluate the fate and effects of NP in medium, in order to resemble natural fresh waters during exposure tests, these results provide a basis for the establishment of more standardize protocols.

5. Nominal versus Analytical Nanoparticle Concentrations

Nominal concentrations, the concentrations the researcher assumes to apply, and analytical concentrations, the factual concentration the researcher applies, often vary in practice for multiple reasons. Applying minute amounts of nanopowders during the preparation of nanosuspension probably accounts for the highest degree of diverging nominal and analytical concentrations due to weighing and dilution inaccuracies in our case. But also introducing quantitative inaccuracies by disregarding the weight of a surface modification might result in an overestimation of the core material quantities. Figure 10 below shows the ratios of analytical over nominal concentrations in percent and illustrates how drastically these concentrations may vary for NPs with different coatings and different core materials.

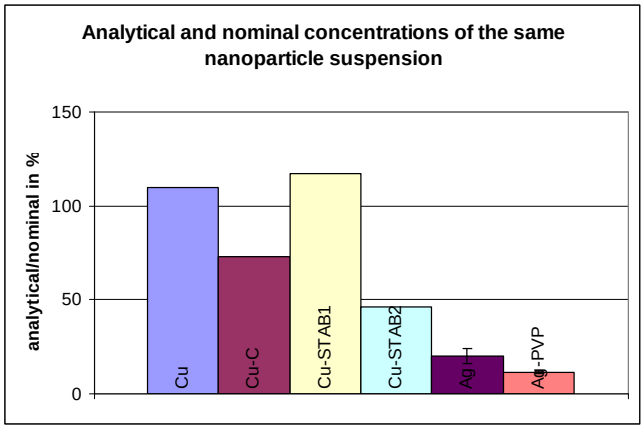


Figure 10: Comparison of analytical NP concentrations in percentage of nominal concentrations for 4 Cu NPs and 2 Ag NPs in test medium after 48. No replicates were preformed for the 4 CuNPs and a total of 3 replicates were performed for the 2 AgNPs.

Predetermining a dosage correction factor for individual NPs appears as a handy solution to dose response experiments, to clarify the actual exposure concentrations. This is of relevance as dose response results may change significantly. Figure 11 below illustrates LD50s for the 4 CuNPs and exemplifies the dilemma of working with nominal and analytically corrected concentrations during dose response experiments and the subsequent diverging interpretation of those results.

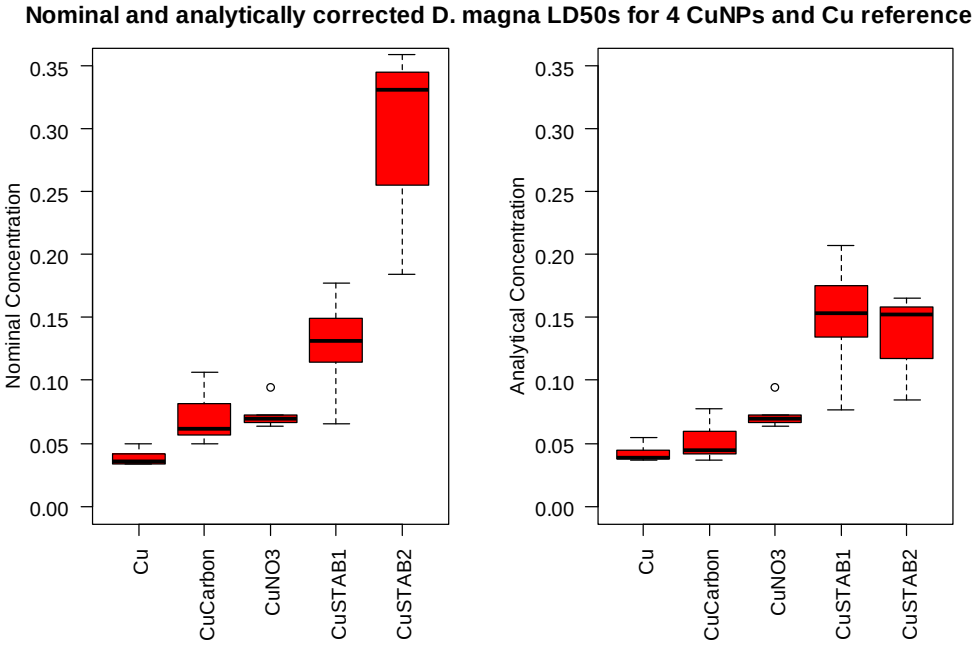


Figure 11: AAS was performed on all copper particles after 0h and 48h for two different concentrations. Those results were used to correct the exposure concentrations during biological response tests. All concentration are given in mg/L.

Table 3: Pairwise comparison using t-test with pooled SD of analytical data. P-value adjusted method: holm.

Pairwise t-test	Nominal				Analytically corrected			
Holm adjusted	Cu (n=7)	CuCarbon	CuNO3	CuSTAB1	Cu	CuCarbon	CuNO3	CuSTAB1
CuCarbon (n=15)	0.09083				0.38			
CuNO3 (n=8)	0.09083	0.88792			0.05	0.11		
Cu-STAB1 (n=12)	**6.0e-07**	**3.0e-05**	**0.00047**		**8.0e-13**	**1.6e-14**	**1.7e-09**	
CuSTAB2 (n=8)	**< 2e-16**	**< 2e-16**	**< 2e-16**	**7.1e-15**	**6.1e-10**	**9.7e-11**	**1.5e-06**	0.30

The results in figure 11 an table 3 show that analytically corrected data can drastically change the outcome of an experiment. These findings underline the necessity to find ways, allowing for high throughput strategies for exposure tests with known analytical concentrations.

More replicates are necessary to clarify if the determined analytical concentrations always differ in the same way towards the nominal concentrations.

6. Time Effect on Nanoparticle Suspensions

In order to assure good quality and consistency of data it is important to keep the dosage of NP during exposure tests constant. One potential error source for inconsistencies lies within the preparation of the NP suspensions. Weighing milligram amounts of powder into a specific volume is often the only option as nanopowders are often not at hand in vast amounts. Thus, preparation methods are often prone to result in fluctuations of actual analytical concentrations, as discussed in the previous section. Therefore preparing and re-using suspensions for daphnia exposure tests may propose a good way in order to keep the analytical concentration fluctuations constant by repeating experiments with the same suspension.

Examples are given in the literature where analytical concentrations of every exposure concentration were determined and compared to nominal concentrations and it proved to be important for the interpretation of the biological response. However in the era of high throughput data collection and rather time consuming and expensive methodologies to determine total concentrations of one specific element, performing analysis for the total concentration determination is in no way feasible for experiments performed on a daily basis. Therefore, we are trying to prove that the biological response will not change for a NP suspension that was stored and re-used after a given amount of time.

The ageing effects towards the toxicity of carbon coated CuNP and non-coated CuNP onto *D. magna* was assessed by testing a freshly prepared NP suspension (0d old) and re-assessing the same suspension after 52 days of ageing (figure 12). It is known that coatings provide enhanced stability for NP (Alejandro-Arellano *et al.* 2000) and that “carbon coated nanomaterials, especially metals, are of

great significance due to their stability to oxidation and degradation" (Li and Liu 2009). Thus we expected to observe only a minor increase in toxicity of carbon coated CuNP over time. Yet to this day the dissolution rates for non-coated particles are somewhat ambiguous and no clear cut conclusion has been drawn on the cause of modality (Elzey and Grassian 2010).

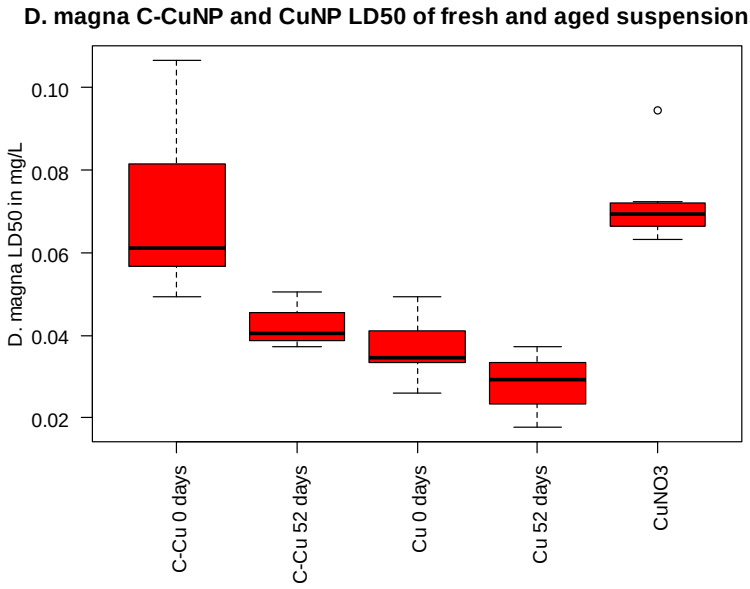


Figure 12: Boxplot comparison of the LD50 values of a fresh, 0 days old, C-CuNP and CuNP suspensions to the same suspensions after an ageing period of 52 days.

Regarding the LD50 values in figure 12, only minor differences in effect concentration range can be observed between fresh and old particles respectively. However, the slightly lower LD50 values for both of the 52 days old suspensions indicates that aged suspensions have slightly greater toxicological potential.

Table 4: Pairwise comparison using t-test with pooled SD of analytical data. P-value adjusted method: holm.

Paired t-test, Holm adjusted	C-Cu 0 days (n=15)	Cu 0 days (n=8)
C-Cu 52 days (n=3)	**0.01659**	
Cu 52 days (n=3)		**1.00000**

The direct comparison produced somewhat ambiguous, as can be seen at the t-test results in table 4. The increased toxicity of the 52 days old C-CuNP suspension appears to be significant in comparison to the 0 days old C-CuNP suspension. Vice versa, the toxicity of the 52 days old CuNP suspension appears to be insignificantly higher than the toxicity of a 0 days old CuNP suspension.

However, the reduced number of replicates during the tests does not allow for statistically representative outcomes and tests should be repeated with larger numbers of replicates.

Nevertheless, even though NP suspensions are highly dynamic systems, the results indicate that the changes of those dynamic processes over time are likely to be less important for the assessment of biological effects upon D. magna. This carries great potential for the establishment of more accurate methodologies towards the effect assessment of NP suspensions.

Activities during the ECO-ITN Fellowship

Training during 1st quarter

August - October 2011

- Training in and taking over the maintenance of invertebrate aquacultures.
- Initial training in conducting ecotoxicological dose response tests using aquatic invertebrates.
- Initial training in methods for physico-chemical characterization (zeta potential, nanotracking analysis) of NPs at RIVM.
- Participating in the two weeks ECO-ITN Summer School 2011
    - Theoretical and practical lessons on REACH, integrated testing strategies, exposure modeling and fate and effect assessment of NPs.
- Preparation of the general outline of the research plan for the 12 months fellowship.
- Preparation of the first toxicity tests on aquatic invertebrates (D. magna).

November - January 2011/2012

- Collecting first NP dose response data.
- Integrating and wrting on the first results section.
- The initial training on the cultivation of aquatic invertebrates of the family Daphniidae and introduction to dose response tests provided the means for a routinely ecotoxicological response testing.
- The introduction to a Malvern Zetasizer at the RIVM, provided the means to determine the surface charge of NPs as well as with the Dynamic Light Scattering mode to determine the hydro dynamic diameter of a NP suspension. These methods represent fundamentally important tools for the physico-chemical characterization of NPs.

February - April 2012

- Brief introduction to NP wet synthesis and instrumentation for physico-chemical characterization (UV-Vis, electrochemical cells, ICP-MS) at the Department of Chemistry at the Leiden University.
- Participating in the one week ECO-ITN Winter School 2012

    - Theoretical and practical lessons on intellectual property patenting, CADASTER projects, exposure modeling and fate and effect assessment of NPs using in-vitro assays and physico-chemical characterization.
- Attempting to establish toxicologically relevant concentrations for appropriate dose response tests of iron oxide and PVP coated iron oxide NPs.
- Performing toxicity tests on aquatic invertebrates (D. magna) using carbon coated and uncoated copper NPs.
- Excluding toxicological effects of CaCO3 as supplement to increase the ionic strength in standard test medium for dose response tests using *Daphnia magna*.
- Performing toxicity test of copper NPs with increased ionic strength.
- Integrating and writing on 2nd results section.

May – July 2012

- Writing on a paper draft.
- Arrange agreements on collaborations on the synthesis and evaluation of coated NPs.
- Conduct experiments on toxicity response, aggregation and dissolution kinetics in daphnia standard test medium supplemented with CaCO3 and humic acids using:
    - Coated and non-coated copper particles
    - PVP coated and non-coated silver and iron oxide particles
- Planning and conducting experiments on the toxicity, aggregation and dissolution kinetics of gold NPs functionlized with straight chain aliphatic carbon and different functional groups.

July – August 2012

- Writing the ECO-ITN final report
- Write project proposals on the fate and effects assessment of functionalized gold NPs and aged NP suspensions.
- Re-writing the ECO-ITN finalreport into paper form through Willie Peijnenburg, ECO-ITN will be informed.

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