COMPARATIVE ECOTOXICITY OF THE NANO Ag, TiO_2 AND ZnO TO AQUATIC SPECIES ASSEMBLAGES

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Abstract. Ecotoxicological testing of engineered nanoparticles (ENPs) began some years ago, but satisfactory knowledge of their environmental effects is still far from acceptable. In this study, the effects of three ENPs, i.e. nano silver (nAg), nano titanium-dioxide (nTiO2) and nano zinc-oxide (nZnO) on surface waters were examined using Species Sensitivity Distributions (SSD) models. Ecotoxicity data (acute EC50/LC50/IC50) from 44 scientific publications were evaluated. The nAg proved to be the most toxic among the three examined ENPs, followed by nZnO and nTiO2 with HC5 values of 0.00015, 0.275, and 3.246 (mg/L), respectively. Risk Quotient (RQ) values of nAg, nTiO2 and nZnO showed irrelevant risk (RQ<0.001) for surface waters in Europe and the USA. However, the risk is expected to increase in the future. No clear relationship was found between toxicity and taxonomical position of the tested species. Some cladoceran species seem to be more sensitive to nAg and nTiO2 than other tested organisms, such as microorganism, plant and animal species. Investigation of the published test results of nAg for *Daphnia magna*, showed that the filtration of the stock solution before testing has statistically significant effect on the toxicity. Our results clearly show that current methods used for ecotoxicological testing of the nano Ag, TiO2 and ZnO are not yet suitable for standardisation.
Keywords: *nano silver, nano titanium-dioxide, nano zinc-oxide, aquatic animal, species sensitivity distribution*

Introduction

The number of nano-scale applications and the amount of nano-products are rapidly growing. Maynard (2006) estimated the production of the engineered nanomaterials to be as much as 58,000 tons in 2011-2020. The market of the nanoproducts is expected to grow to 1 trillion US$ by 2015 (Aitken et al., 2006). As a consequence, investigation of the effects of nanomaterial exposure on aquatic ecosystem is of great importance, since it ultimately receives run-off and wastewater from domestic, industrial and agricultural sources in particular (Battin et al., 2009; Moore, 2006). In spite of the fact that there is an urgent need for standard guide-lines to test ENPs, including nano metals and metal-oxides, there is not any guide-line available yet (Hansen and Baun, 2012).

Common stressors of aquatic ecosystems are in the focus of several scientific programs as e.g. EULAKES (Kováts, 2012). In the last years, the biotic effects of

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several ENPs have been examined and their ecotoxicology testing has begun, but we are still far from satisfactory knowledge of their environmental effects (Nowack and Bucheli, 2007; Peralta-Videa et al., 2011). In aquatic ecotoxicology, most attention was devoted to fullerenes, nano silver (nAg), nano titanium-dioxide ($nTiO_2$) and nano zinc-oxide (nZnO) (Baun et al., 2009). However, our understanding on behaviour and toxicity of these nano particles in aquatic environment is still sparse (Krysanov et al., 2010). Numerous ecotoxicological studies were carried out in aquatic environment with nAg and $nTiO_2$. Fabrega et al. (2011) reviewed effects of silver nanoparticles on algae, invertebrate and fish species. It was pointed out that already as low concentration as some ng/L of nAg can be harmful to various aquatic species. Results of ecotoxicological studies on $nTiO_2$ in aquatic environment were also formerly published. A review of the published papers shows that the results are not conclusive because of the remarkable differences in the products tested, the experimental setups and the lack of standardized bioassays (Clemente et al., 2012). There are certain studies reporting the toxicity of nZnO on algae (Aruoja et al., 2009), crustaceans (Heinlaan et al., 2008), and zebrafish embryo number, larvae survival, hatching rate and malformation (Zhu et al., 2008).

In spite of the fact that several studies proved harmful effects of nano metals and metal-oxides, little is known about their comparative toxicity at assemblage level. That is why effects of nAg, $nTiO_2$ and nZnO on surface aquatic ecosystems were examined in this study using Species Sensitivity Distribution (SSD) models (Posthuma et al., 2002). SSDs are increasingly used in ecological risk assessment (den Besten et al., 2003). The aim of this analysis was to estimate the nAg, $nTiO_2$ and nZnO concentration protective for 95% of the aquatic species (HC_5 values) in order to give reliable estimate for Predicted No-Effect Concentration (PNEC). Besides, some methodological problems related to the applied testing methods and species used in ecotoxicological tests with nano metals and metal-oxides are also discussed.

Methods

Ecotoxicity data (acute EC50/LC50/IC50) were collected from 44 scientific publications (*Table 1*). Papers were carefully selected and only peer reviewed papers were regarded. Collection of the data was closed on 31.01.2013.

Species of the living organisms respond differently to various chemicals. Description of this variation with any statistical distribution model results the SSD curve. The sensitivities of a set of species (ideally from an actual community) are usually described by parametric distribution function (such as log-normal, logistic or log-logistic). The species set can be composed from specific taxa, selected species assemblage or a natural community. The true distribution of toxicity endpoints is unknown, so the SSD is estimated from sample toxicity data and shown as a cumulative distribution function. SSDs are used to calculate concentrations expected to be safe for 95% of the species (PC_{95}, HC_5) which can be used in environmental risk assessments. Although HC_5 can underestimate field data (Smetanová et al. 2014) it is suitable for the comparative analysis used in this study. General description of the SSD methodology is described in detail in Posthuma et al. (2002).

The amount of data used is highly important for the derivation and conclusions based on them. Wheeler et al. (2002) suggested that a minimum of 10 species is required for SSDs used for aquatic risk assessments. Following this suggestions, we calculated HC_5

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from data of 10 or more different species. EFSA recommends (EFSA 2009) the using of EC_x instead of NOEC or LOEC data in relation to all environmental areas. In this study we used EC_{50}/LC_{50}/IC_{50} toxicity data.

The effect of data quality was highlighted by Wheeler et al. (2002). Following their data classification methods, criteria were applied as follows:

- data published in peer reviewed scientific papers were used.
- standard methods were applied
- suitable control was included
- concentrations were measured or no loss would be expected
- if more EC50/LC50/IC50 values were found for a species the geometric average of the data was applied

SSD analyses were made with the ETX 2.0 software (van Vlaardingen et al. 2004).

Risk quotient (RQ) was calculated according to the equation:

$$RQ = PEC / PNEC \qquad \text{(Eq.1)}$$

where PNEC is the Predicted No Observable Effect concentration and PEC is the Predicted Environmental Concentration.

PNEC data are given according to the results of the present SSD analyses. PEC data are compiled from Gottschalk et al. (2009).

The cladoceran, *D. magna* is frequently used in nanoecotoxicological tests. That is why relatively numerous results were found where nAg effect was tested with this species. Results of 39 experiments from 7 studies (Allen et al., 2010, Li et al., 2010, Kim et al., 2011, Zhao and Wang, 2012, Blinova et al., 2012, Jo et al., 2012, Poyton et al., 2012) conducted with *D. magna* and nAg were compiled and further analysed.

As a first step, the methodology (endpoint, duration of the test, filtration, sonication and water type) was specified in the case of all 39 experiments with binary numbers as follows (*Table 1.*). Thereafter, a table was constructed, where toxicity data were registered in decreasing order in the first column and 0 or 1 numbers in the next five ones according to the *Table 1* (e.g. 0, 0, 0, 0, 0 respectively, if LC50 was the endpoint in a 24 hour long test, without filtration and sonication in natural water). This table was analysed with Non-Metric Multidimensional Scaling (NMDS) (SynTax2000 statistical program package, Podani, 2000).

Table 1. *Standardisation of the methodological procedures for NMDS calculations. Any step of the method applied was designated by the number either 0 or 1.*

Endpoint	Duration of the test	Filtration	Sonication	Water
LC50: 0	24 hours: 0	No: 0	No: 0	Natural: 0
EC50: 1	48 hours: 1	Yes: 1	Yes: 1	Artificial: 1

Wilcoxon-Mann-Whitney tests were performed with Free Statistics Software (v1.1.23-r7) (Holliday, 2012) in order to see significant effects of filtration, sonication or water quality on toxicity.

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Results and discussion

The nAg proved to be the most toxic among the three examined nano-metals and metal-oxides, followed by nZnO and $nTiO_2$ (*Table 2.*). HC_5 values based on the minimum concentration values indicate the worst-case situation (0.004 mg/L, 0.82 mg/L, 0.084 mg/L for nAg, $nTiO_2$ and nZnO, respectively). Toxicity calculated with the geometric means of the concentrations is lower (0.013 mg/L, 2.78 mg/L, 0.158 mg/L for nAg, $nTiO_2$ and nZnO, respectively). Differences between results of the two calculations are by factors of 3.25, 3.4 and 1.9 for nAg, $nTiO_2$ and nZnO, respectively. Gottschalk et al. (2013) calculated as low values as 0.00001, 0.062 and 0.001 mg/L for nAg, $nTiO_2$ and nZnO, respectively. This great difference comparing to our results may be a consequence of the model and/or reference data used by Gottschalk et al. (2013). The relative toxicity of the three nano metals examined here is similar to that of found by Kahru and Dubourgier (2010), and Gottschalk et al. (2013) or for toxicity of $nTiO_2$ and nZnO on zebrafish (Danio rerio) (Zhu et al., 2008).

Table 2. *Estimation of the HC5 values with SSD curves for nano-Ag, nano- TiO2 and nano-ZnO. Calculations were made based on minimum and geometric average concentration values. Median (lower; upper) estimate of the HC5 are presented.*

	Ag (mg/L)	TiO_2 (mg/L)	ZnO (mg/L)
Minimum values	0.004 (0.0002; 0.022)	0.82 (0.11; 2.7)	0.084 (0.014; 0.228)
Geometric averages	0.013 (0.001; 0.058)	2.78 (0.56; 7.1)	0.158 (0.028; 0.419)

Shapes of the SSD curves show clear differences in toxicity distribution of the three nano metals (*Figure 1.*). The slope of the nAg curve is smooth comparing to $nTiO_2$. The steepest SSD curve was found in the case of nZnO showing relatively small variation in toxicity according to the species examined.

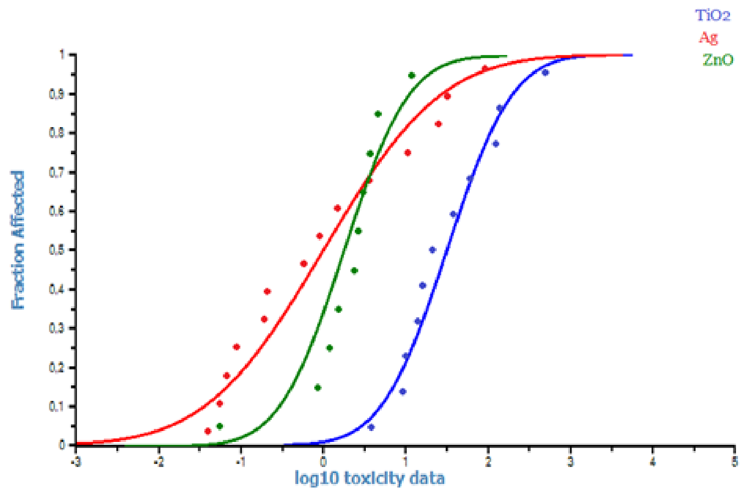

Figure 1. *SSD curves calculated with the geometric means of the EC_{50}/LC_{50}/IC concentrations for the different species*

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The calculated RQ values show irrelevant risk (RQ < 0.001) for both European and US surface waters, respectively (*Table 3*.). The calculated irrelevant risk does not mean that the rapidly growing nano heavy metal exposure will not cause environmental problems in the future. PEC data applied for the calculation originated from the year 2009. However, currently the nano metal and metal-oxide emission is increasing, and this growth will intensify in the next decades (Aitken et al., 2006.). Furthermore, PEC values show high spatio-temporal variation (Gottschalk et al., 2011). Consequently, present RQ estimations must be handled with caution.

***Table 3**. Calculated RQ values for Europe and the USA. PNEC data are given according to the results of the present SSD analyses. PEC data are compiled from Gottschalk et al. (2009).*

RQ (Europe)	RQ (USA)
0.000005	0.0000007
0.00006	0.000006
0.00006	0.000009

If species' sensitivity according to their taxonomic position is in focus, no clear pattern can be seen (*Table 4*.). As a tendency, Cladoceran species as *Daphnia magna, D. pulex* and *Ceriodaphnia spp*. seem to be more sensitive to nAg and $nTiO_2$ than to nZnO. There are only three species (*Pseudokirchneriella subcapitata, Daphnia magna, Danio rerio*), which were tested for all three nanometals and metal-oxides. The alga *P. subcapitata* was most sensitive to nZnO but not to $nTiO_2$. nAg and $nTiO_2$ exerted high toxicity to the cladoceran *D. magna* but nZnO did not. Both two previous findings are supported by Bondarenko et al. (2013). The zebrafish (*D. rerio*) is not particularly susceptible to any nano metal investigated in this study. No other pattern was observed. Currently, it is not clear if there is really no pattern or the number of available data is modest.

Presently, no official guide-line is available for nano metal or metal-oxide testing. Methods are still under development. Current protocols need to be modified (Handy et al., 2012), at the same time the development / introduction of new methods is necessary. At present, very different methodologies are used in the laboratories. That is why interlaboratory ecotoxicity data for nano metals are comparable with difficulty. In order to study methodological effects on test results, data presented in four papers were analysed. In these cases all other methods were the same except filtration (Jo et al., 2012), sonication (Allen et al., 2010) or water media (artificial or natural) (Blinova et al., 2012). Results show (*Table 5*.) that neither sonication nor water quality (in the case of *D. magna* as well as *T. platyurus*) has any significant effect on the results. However, filtration of the test solution has statistically significant effect on the toxicity. Filtered nAg solution is about two orders of magnitude as toxic as unfiltered one (filtered: 0.01±0.003 mg/L; unfiltered 1.3±0.5 mg/L, average±SD). This is a consequence of the aggregation of ENPs (Buffle et al., 1998).

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Table 4. *Geometric averages of the EC50/LC50/IC50 values for nano-Ag, nano- TiO2 and nano-ZnO of the species involved in the study. Species are ranked according their EC50/LC50/IC50 value. Increasing order.*

nAg		nTiO2		nZnO	
species	EC_{50}/ LC_{50}/ IC_{50}	species	EC_{50}/ LC_{50}/ IC_{50}	species	EC_{50}/ LC_{50}/ IC_{50}
Daphnia pulex	0.04	Daphnia magna	3.8	Pseudokirchneriella subcapitata	0.06
Daphnia magna	0.055	Daphnia pulex	9.2	Tigriopus japonicus	0.85
Ceriodaphnia sp.	0.067	Chlamydomonas reinhardtii	10.0	Elasmopus rapax	1.19
Chlamydomonas reinhardtii	0.09	Ceriodaphnia dubia	13.8	Thamnocephalus platyurus	1.53
Pseudokirchneriella subcapitata	0.19	Chlorella sp.	16.1	Skeletonema costatum	2.36
Thamnocephalus platyurus	0.21	Scenedesmus sp.	21.2	Danio rerio	2.63
Aedes aegypti	0.59	Desmodesmus subspicatus	37.5	Daphnia magna	3.04
Culex quinquefasciatus	0.9	Pseudokirchneriella subcapitata	60.4	Aliivibrio fischeri	3.72
Tetrahymena pyriformis	1.46	Danio rerio	124.5	Thalassiosira pseudonana	4.56
Pimephales promelas	3.61	Haliotis diversicolor supertexta	140.3	Tetrahymena thermophila	11.68
Danio rerio	10.6	Pimephales promelas	500.0		
Paramecium caudatum	25.0				
Aliivibrio fischeri	31.8				
Lemna minor	91.0				

Table 5. *Effects of different preparation methods for nano-Ag toxicity. Filtration: tested solution was filtrated or not, Sonication: tested solution was sonicated or not, Water: standard medium or filtered natural water was applied. p: significance level of the two tailed Wilcoxon-Mann-Whitney test. Data for calculation were applied from papers indicated in last column.*

Effect	Species	p	Author
Filtration	Daphnia magna	0.01	Jo et al. 2012
Sonication	Daphnia magna	0.31	Allen et al. 2010
Water	Daphnia magna	0.91	Blinova et al. 2012
Water	Thamnocephalus platyurus	0.57	Blinova et al. 2012

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According to the results of the NMDS analysis (*Figure 2*), two clear groups could be separated. The difference between the groups originated from the duration of the tests (24 or 48 hours) as commonly practiced. This is a well-known phenomenon (Persoone at al., 2009). No other apparent relationship between toxicity and the applied methodology was detected. This result shows clear evidence to current methods being not suitable for standardisation.

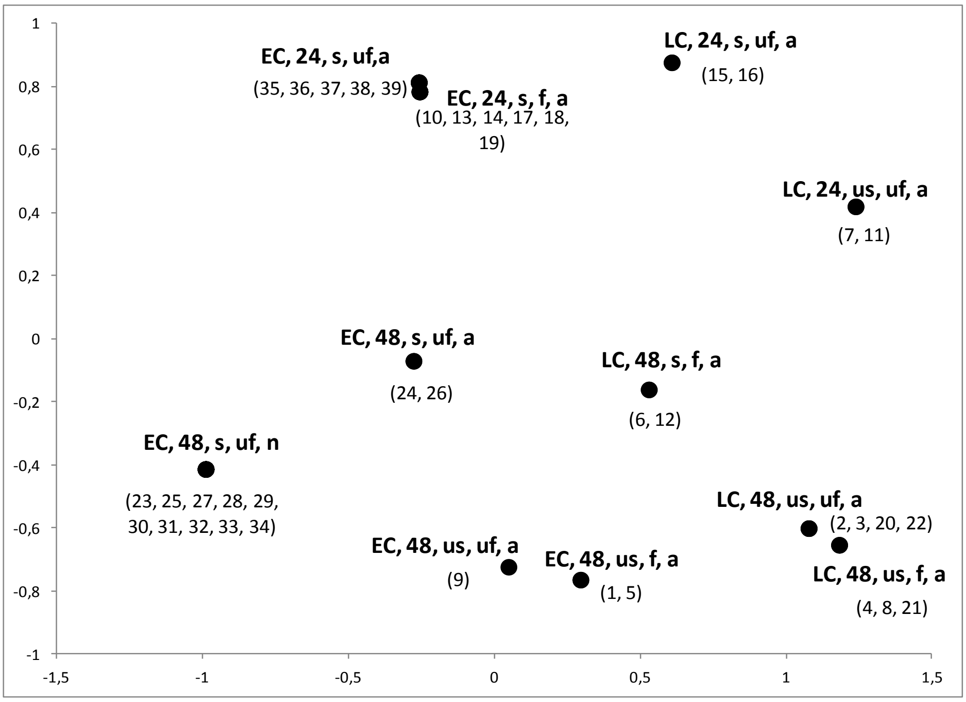

Figure 2. *Result of the NMDS analysis. Bold: EC – effective concentration, LC – lethal concentration, 24- test duration was 24 hours, 48 - test duration was 24 hours, s – sonicated, us – unsonicated, f – filtrated, uf – unfiltrated, n – natural water, a – artificial water. Normal letter: rank order of the toxicity data. Number 1 is the highest, 39 the lowest toxicity data.*

Conclusions

The relative toxicity of nAg, nZnO and $nTiO_2$ analysed with SSD models showed decreasing effect, respectively. This finding confirms previous results. An estimation of the RQ does not indicate any danger of the nano metals for aquatic communities at the moment, but an increase of the RQ is expected in the future with the increasing quantities of exposure. Filtration of the test solution before applying to the test vessels seems to be an important factor in ecotoxicological experiments with nano metals and metalloids. However, currently no guide-lines are available for the assessment of toxic effects caused by these materials. There is an urgent need for widely applicable standard test methods in aquatic nanoecotoxicology.

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Appendix

Appendix 1. *Analyses were based on the publications as follows:*

Allen, H.J., Impellitteri, C.A., Macke, D.A., Heckman, J.L., Poyton, H.C., Lazorchak, J.M., Govindaswamy, S., Roose, D.L., Nadagouda, M.N. (2010): Effects from filtration, capping agents, and presence/absence of food on the toxicity of silver nanoparticles to Daphnia magna. - Environmental Toxicology and Chemistry 29: 2742-2750.

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