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Aquatic acute species sensitivity distributions of ZnO and CuO nanoparticles

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Abstract

Metal oxide nanoparticles are increasingly being produced and will inevitably end up in the aquatic environment. Up till now, most papers have studied individual nanoparticle effects. However, the implementation of these data into a risk assessment tool, needed to characterize their risk to the aquatic environment, is still largely lacking. Therefore, aquatic species sensitivity distributions (SSDs) were constructed for ZnO and CuO nanoparticles and 5% hazard concentrations (HC₅) were calculated in this study. The effect of individual nanoparticles on these SSDs was estimated by comparison with bulk SSDs. Additionally, the effect of nanoparticle dynamics (aggregation and dissolution) was considered by evaluating the effect of aggregate size on the toxicity, by estimation of the dissolved fraction and comparison with SSDs for ZnCl₂ and CuCl₂ inorganic salt. Bacteria, protozoa, yeast, rotifera, algae, nematoda, crustacea, hexapoda, fish and amphibia species were included in the analysis. The results show that algae (Zn) and crustacea (Zn, Cu) are the most sensitive species when exposed to the chemicals. Similar acute sensitivity distributions were obtained for ZnO nanoparticles (HC₅: 0.06 with 90 % confidence interval: 0.03 - 0.15 mg Zn/l; 43 data points), bulk ZnO (HC₅: 0.06 with CI: 0.03 – 0.20 mg Zn/l; 23 dps) and ZnCl₂ (HC₅: 0.03 with CI: 0.02 – 0.05 mg Zn/l; 261 dps). CuO nanoparticles (HC₅: 0.15 with CI: 0.05 – 0.47 mg Cu/l; 43 dps) are more toxic than the bulk materials (HC₅: 6.19 with CI: 2.15 – 38.11 mg Cu/l; 12 dps) but less toxic than CuCl₂ (HC₅: 0.009 with CI: 0.007 – 0.012 mg Cu/l; 594 dps) to aquatic species. However, the combined dissolution and SSD results indicate that the toxicity of these nanoparticles is mainly caused by dissolved metal ions. Based on the available information, no current risk of these nanoparticles to the aquatic environment is expected.

Keywords: Nano; metal; aquatic toxicity; hazard concentration; dissolution; aggregation

1. Introduction

Nanoparticles are particles with sizes varying between 1 and 100 nm. At this size range, particles may acquire altered properties compared to bulk material such as UV-absorbance, transparency, high strength, antibiotic activity, water and stain repulsion, etc. (Nagarajan, 2008). These specific features are mainly caused by their large surface area-to-volume ratio and varying surface charges (Oberdorster et al., 2005). Particles with such properties have many different applications and economic potential. The exponential increase in the use of nanoparticles in various applications has also raised environmental concerns and the need for the setting of environmental standards. In this work, we will focus on the metal oxides nanoparticles ZnO and CuO. These particles, for which corresponding metal ion toxicity has already been characterised (Wang, 1987), are used in many commercial products (e.g. ZnO nanoparticles in coatings and sunscreens; CuO nanoparticles in catalysts, semiconductors and chemical sensors (Chowdhuri et al., 2004; Ma et al., 2013)). Metal oxide nanoparticles exist in many different sizes, shapes and types (e.g. dispersion, powder, uncoated or diversely coated particles) (Izu et al., 2013; Leite-Silva et al., 2013; Nguyen and Do, 2011). The ever increasing production and application of nanoparticles inevitably results in the release of these materials to aquatic ecosystems via industrial and household waste water (Bystrzejewska-Piotrowska et al., 2009). Once the particles enter the environment, their altered properties, which on the one hand make them attractive for various applications, may on the other hand pose a potential risk

to the environment and especially to aquatic organisms (Baun et al., 2008; Oberdorster et al., 2005).

Several authors have shown a significant toxicity of the metal oxide nanoparticles ZnO and CuO to aquatic organisms. The acute toxicity of ZnO nanoparticles ($(L(E)C_{50}: 0.05 - 1000 \text{ mg/l})$ was already documented for bacteria (Adams et al., 2006; Heinlaan et al., 2008), algae (Aruoja et al., 2009; Franklin et al., 2007), protozoa (Mortimer et al., 2010), nematoda (Ma et al., 2009; Wang et al., 2009), crustacea (Heinlaan et al., 2008; Wiench et al., 2009) and fish (Bai et al., 2010; Zhu et al., 2008). CuO nanoparticles ($(E(L)C_{50}: 0.05 - 569 \text{ mg/l})$ were shown to be toxic to bacteria (Heinlaan et al., 2008), algae (Aruoja et al., 2009), protozoa (Mortimer et al., 2010), crustacea (Heinlaan et al., 2008; Jo et al., 2012; Sovova et al., 2009) and fish (Chen et al., 2011). However, data on the chronic toxicity of metal oxide nanoparticles to aquatic species are more scarce (Adam et al., 2014; Adam et al., 2015; Van Hoecke et al., 2009; Wiench et al., 2009; Zhao et al., 2012).

In all cases, the results show strong variation which is related to the complex behaviour of the nanoparticles including aggregation and dissolution during the course of the exposure. As such, several authors have demonstrated that the observed toxicity of some of these particles is related to the toxicity of the released metal ions rather than the metal oxide nanoparticles (e.g. the toxicity of ZnO nanoparticles to bacteria (Heinlaan et al., 2008), algae (Franklin et al., 2007), yeast (Kasemets et al., 2009), protozoa (Mortimer et al., 2010), nematoda (Ma et al., 2009) and crustacea (Heinlaan et al., 2008)).

Most studies report individual effect concentrations of metal oxide nanoparticles. Some studies have integrated these data into a review (e.g. Bondarenko et al. (2013); Ivask et al. (2014)). However, the implementation of these data into risk assessment tools, needed to characterize the risk of metal oxide nanoparticles to the aquatic environment, is still largely lacking. Therefore in this study species sensitivity distributions (SSDs) were used as a risk assessment tool. The theory of SSDs is based on the fact that a certain chemical will not cause the same toxicity in all species and this is reflected by the proportion of species that are affected at a certain exposure concentration of the chemical. This way, SSDs represent the variation in sensitivity of species to a chemical. The advantage of SSDs is that the effects of a chemical can be extrapolated from single-species toxicity data (which can show variation in endpoints, exposure conditions, life stages) to effects at ecosystem level (Posthuma et al., 2002). Acute 50% effect/lethal concentration ($L(E)C_{50}$) or chronic no observed effect concentration (NOEC) values are mostly used as toxicity measures in SSDs. Different sorts of distributions have been suggested including log-normal (Wagner and Løkke, 1991), log-triangular (Erickson and Stephan, 1988) and log-logistic (Kooijman, 1987; van Straalen and Denneman, 1989). Species sensitivity distributions are used to calculate the hazard concentration 5% (HC_5), the concentration below which 95 % of the species should be protected. These HC_5 values can be used to derive predicted no effect concentrations (PNEC). Species sensitivity distributions have been constructed for different chemicals including organic compounds (Dom et al., 2012) and metals (Xu et al., 2015). To our knowledge only one study has constructed species sensitivity

distributions for ZnO nanoparticles (Gottschalk et al., 2013), while these distributions are still absent for CuO nanoparticles.

For the evaluation of nanoparticle toxicity, the dynamic behaviour and the effect of exposure conditions and time on their fate and appearance are also important issues to consider. Not only nanoparticle characteristics such as composition, size and shape but also the nanoparticle treatment before testing (e.g. sonication, stirring,...) and environmental test conditions such as ionic strength and pH can influence their aggregation or dissolution state and thus alter toxicity (Jo et al., 2012; Misra et al., 2012; Mohd Omar et al., 2014).

Within this study, aquatic acute SSDs were constructed for ZnO and CuO nanoparticles, as an initiation towards nanoparticle risk assessment. Since not only responses to individual nanoparticles are reflected in these SSDs, the effect of particle dissolution and aggregation on the distribution was estimated as well. Therefore, SSDs of bulk ZnO, CuO and metal salts ZnCl₂, CuCl₂ were also constructed and the effect of individual and aggregated nanoparticle size on the toxicity was statistically evaluated. While some may question the use of species sensitivity distributions for nanoparticles, we believe it is a powerful tool for data integration, which can be used to combine diverse data. Not only variation in endpoints, exposure conditions, life stages of single-species toxicity data are integrated, but also the dynamic behaviour of the nanoparticles is reflected in the SSD.

2. Material and Methods

2.1. Species sensitivity distributions of nanoparticles and nanoparticle specific effects

Acute toxicity data of the metal oxide nanoparticles ZnO and CuO in aquatic organisms were compiled from literature (papers in Web of Science, Science Direct, Ecotox U.S.EPA database, up to July 2014). Species sensitivity distributions (SSDs) were created based on nominal or measured (where available) acute L(E)C₅₀ exposure concentrations. The SSDs were calculated using the SSWD 1.0 program (Duboudin, 2003). This program has been used in different studies (Garnier-Laplace et al., 2008; Smolders et al., 2010). The SSWD software creates cumulative graphs based on species information, (higher-level) taxonomic information and concentration values. These graphs indicate the cumulative weighted probability i.e. the number of test organism that is affected (at a certain exposure concentration) as a percentage of the total number of test organisms present in the data set (representing the total aquatic environment). All the available acute species toxicity data (L(E)C₅₀ with different endpoints and setups) were included. The data were weighted, giving each species the same weight within the distribution (no species was given more importance than any other). By weighing the data, all data points were included in the SSDs and as a result all possible exposure scenarios, including nanoparticle aggregation and dissolution, were included. Three different analyses (log-normal, log-triangular and log-empirical) were made and based on the best-fitted curve (R^2), one analysis was selected. Additionally, 90 % confidence limits (using a bootstrap approach) and HC₅ values were calculated with the software. To test for nanoparticle specific effects, a comparison with the corresponding bulk material (metal oxides with constant physical and chemical properties

regardless of their size) was made by construction of SSDs for bulk ZnO and CuO. The toxicity data used, were obtained in the same way as the nanoparticle data.

2.2. Differences in nanoparticle characteristics, pre-test treatments and test setups influencing nanoparticle stability

Different factors including different test properties: nanoparticle characteristics (types e.g. powder or dispersion, commercial sizes), test pre-treatments (e.g. sonication) and test setup (used media and pH values) were summarised from the papers used to construct the nanoparticle SSDs. These factors, in addition to the chemical composition of the nanoparticles (e.g. ZnO, CuO), influence nanoparticle stability in terms of dissolution and aggregation.

2.3. Effect of nanoparticle dissolution on SSD

The dissolution of the nanoparticle and bulk metal oxides was estimated by calculating the dissolved proportion (total dissolved metal concentration (mg metal/l)/initial total metal concentration (mg metal/l) x 100= dissolved proportion (%)). This dissolved proportion was either derived from the papers directly or calculated when both dissolved and total metal concentration were presented in the papers used for the SSD construction.

The toxicity of the dissolved metal was estimated by making SSDs of the metal salts ZnCl₂ and CuCl₂. Acute toxicity data were obtained from the Ecotox database (U.S.EPA, July 2014). The construction of these SSDs was similar as described above (in section 2.1.) for the nanoparticles.

2.4. Effect of individual and aggregated nanoparticles on SSD

The effects of individual nanoparticles on the SSD were evaluated by considering individual nanoparticle sizes in the analysis. All the available data on nominal individual nanoparticle sizes corresponding to the ZnO and CuO L(E)C₅₀ values, were summarised for the different taxonomic groups. If only size ranges were available, average size values were calculated and used for further analysis. The effects of aggregated nanoparticles on the SSD were evaluated by considering aggregated nanoparticle sizes in the analysis. All the available data on measured average aggregated nanoparticle sizes, corresponding to the ZnO and CuO SSD L(E)C₅₀ values, were summarised. If size ranges were available, average aggregate sizes were calculated and used for further analysis. For all taxonomic groups (for which each taxonomic group contained more than 1 data point), the overall effect of the individual nanoparticle sizes or aggregated nanoparticle sizes on the L(E)C₅₀ values were assessed using linear mixed models (Zuur et al., 2009). Since the data within a taxonomic group are not independent (since the same species reoccur within a taxonomic group), a random intercept and slope for group was added to the models. The effects for each individual taxonomic group (for which more than 2 data points was available) were tested with simple linear models. Significance of the particle size (individual or aggregated) was tested with χ^2 log-likelihood ratio. All analyses (and data visualisations) were performed in the statistical package R (version 2.15.3, (R Development Core Team, 2012)). The lmer function of the lme4 package was used to fit linear mixed models, the lm function of the Stats package for simple linear models. The lrtest function of the lmerTest package was used to calculate the log-likelihood ratio.

3. Results

3.1. Species sensitivity distributions of nanoparticles and nanoparticle specific effects

Acute SSDs were constructed for ZnO nanoparticles (Fig. 1A, 43 data points), bulk ZnO (Fig. 1B, 23 data points), ZnCl₂ (Fig. 1C, 261 data points, described in 3.3) and CuO nanoparticles (Fig. 2A, 43 data points), bulk CuO (Fig. 2B, 12 data points), CuCl₂ (Fig. 2C, 594 data points, described in 3.3). The source data are given in Appendix A. The fittings of the different analyses (log-normal, log-triangular and log-empirical) and corresponding HC₅ values were very similar (Table 1). Log-triangular distributions were visualized, since these resulted in the best fitting curves (R^2). For each graph, the fitting and confidence intervals are visualised. These distributions include both LC₅₀ values (i.e. mortality) and EC₅₀ values (e.g. growth inhibition; immobilisation; luminescence inhibition; fluorescence; hatching rate). Posthuma et al. (2002) indicates that LC₅₀ and EC₅₀ values can be combined for the construction of SSDs since these data have also been combined to assess the toxicity of several chemicals and to derive US water quality criteria. For the ZnO nanoparticles, the mean and median EC₅₀ value (29 data points, i.e. 67 % of the total number of data points) was 74.5 and 6.0 (min: 0.04 – max: 803.5) mg Zn/l, while the mean and median LC₅₀ value (14 data points/33 %) was 63.8 and 2.9 (min: 0.04 – max: 789.0) mg Zn/l. For the CuO nanoparticles, the mean and median EC₅₀ value (26 data points/60 %) was 28.2 and 2.9 (min: 0.04 – max: 129.0) mg Cu/l and the mean and median LC₅₀ value (17 data points/40%) was 49.2 and 3.7 (min: 0.01 – max: 454.6) mg Cu/l. When combining the ZnO and CuO dataset, the most prominent test endpoints were mortality (31 data points/36 %) and immobilization (17 data points/20 %). The acute tests lasted maximally up to 96 hours, with most studies conducted during a period of 24 hours (33 data points/40 %).

ZnO nanoparticles (Fig. 1A) and bulk material (Fig. 1B) show toxicity towards bacteria, algae, protozoa, yeasts, nematoda, crustacea and fish. The individual data of these SSDs were used to calculate average $L(E)C_{50}$ values with minima and maxima for the different exposed taxonomic groups (Fig. 3A and B). These different graphs (SSD and average values) indicate that the freshwater algae are most sensitive to ZnO nanoparticles and bulk material. Their population growth is inhibited by 50 % at concentrations below 0.07 mg Zn/l for the nanoparticles and the bulk fraction. For the other species, there is no clear pattern and the different taxonomic groups cover a wide toxicity range ($L(E)C_{50}$): bacteria (0.04 – 803.5 mg Zn/l for nanoparticles; 1.4 mg Zn/l – 682.1 mg Zn/l for bulk), protozoa (4 – 10 mg Zn/l for nanoparticles and bulk), yeast (97.2 – 105.3 mg Zn/l for nanoparticles; 107.7 – 126.9 mg Zn/l for bulk), nematoda (1.8 – 789.0 mg Zn/l for nanoparticles; 1.8 – 65.1 mg Zn/l for bulk), crustacea (0.2 – 17.7 mg Zn/l for nanoparticles; 0.2 – 7.1 mg Zn/l for bulk), fish (1.4 – 18.5 mg Zn/l for nanoparticles, 1.2 – 2.7 mg Zn/l for bulk), amphibia (8.3 mg Zn/l for nanoparticles). For both ZnO nanoparticles and bulk material a similar HC_5 of 0.06 (with 90 % CI: 0.03 – 0.15 for nanoparticles and 0.03 – 0.20 for bulk) mg Zn/l was calculated based on the log-triangular SSDs (the HC_5 values for the log-normal and log-empirical distributions are given in Table 1).

Species sensitivity distributions for CuO nanoparticles (Fig. 2A) and bulk material (Fig. 2B) are presented and show the acute toxicity in different species of which bacteria, algae, protozoa, yeasts and crustaceans are the most prominent ones. The average $L(E)C_{50}$ values with minima and maxima for the different taxonomic groups are given in Fig. 3C and D. The SSDs and toxicity

ranges indicate that the different taxonomic groups cover a wide toxicity (L(E)C₅₀) range when exposed to the CuO nanoparticles: bacteria (3.7 – 63.1 mg Cu/l), algae (0.7 – 57.0 mg Cu/l), protozoa (97.9 – 129.0 mg Cu/l), yeasts (10.7 – 16.5 mg Cu/l), crustacea (0.01 – 9.8 mg Cu/l) and fish (193.3 mg Cu/l). For Bulk CuO only a limited dataset is available. The toxicity value for algae is 11.5 mg Cu/l, for which only one data point was found and for crustaceans between 75.5 and 139.8 mg Cu/l. The L(E)C₅₀ values of bacteria, yeast and protozoa species when exposed to bulk CuO range from 345.0 to 3044.5 mg Cu/l. Based on the obtained distributions a HC₅ value of 0.15 (90 % CI: 0.05 – 0.47) mg Cu/l was calculated for the CuO nanoparticles, compared to a value of 6.19 (90 % CI: 2.15 – 38.11) mg Cu/l for bulk Cu.

3.2. Differences in nanoparticle characteristics, pre-test treatments and test setups influencing nanoparticle stability

The different characteristics are provided in appendix A. Most of the tested nanoparticles was purchased as a powder form (26 data points, i.e. 60 % of the total number of data points for ZnO, 32 data points/74 % for CuO), compared to a small number of purchased dispersions (6 data points/14 % for ZnO, 2 data points/5 % for CuO). In 26 % (ZnO) and 21 % (CuO) of the investigated studies the original nanoparticle form was not mentioned. Most of the particles had average individual sizes between 10 and 70 nm (33 data points/80 % for the ZnO, 22 tests/69 % for CuO). A smaller number of the particles were below 10 nm (3 data points/7 % for the ZnO) or above 70 nm (5 data points/12 % for ZnO, 10 tests/31 % for CuO). Concerning the pre-treatment of the particles, there were large differences in the type of the treatment and the treatments' duration. In 12 % (5 tests) of the ZnO and 19 % (8 data points) of the CuO studies,

the nanoparticles were not treated at all. In most of the studies (34 data points/79 % for CuO, 34 data points/79 % for ZnO) sonication or sonication in combination with shaking, stirring or filtration was applied in order to get the particles equally dispersed in the spiking solution. In addition, also the time that the particles were treated varied among the studies. The sonication time varied between 5 min and 2 hours of continuous sonication. Most of the studies were performed with nanoparticles that were sonicated for 30 min. Concerning the experimental exposure medium, a large difference in test media composition and pH was observed. Beside the OECD (recommended) medium (ISO test medium, M4 or M7 used in 6 data points/14 % for ZnO, 4 data points/9 % for CuO) and moderately hard U.S.EPA medium (3 data points/7 % for ZnO, 10 data points/23 % for CuO), also 13 other test media were used. Most experiments were performed in freshwater media (28 data points/65 % for ZnO and 34 data points/79 % for CuO), while experiments in brackish water (14 data points/33 % for ZnO and 9 data points/21 % for CuO) or seawater (1 data point/2 % for ZnO and none for CuO) were limited. The freshwater, brackish water and seawater data are scattered within the SSDs. So there is no clear indication of overall differences in sensitivity between freshwater and saltwater species. The pH of the used media varied between 5.5 and 8.5, with most pH values ranging between 7 and 7.9 (11 data points/26 % for ZnO and 23 data points/54 % for CuO). It has to be mentioned that in 23 % and 28 % of the studies on CuO and ZnO nanoparticles, the pH was not indicated at all. All the above mentioned factors (type, individual particle size, pre-treatments, medium and pH) are known to influence nanoparticle behaviour and thus their dissociation or aggregate formation during the toxicity tests (Jo et al., 2012; Misra et al., 2012; Mohd Omar et al., 2014).

3.3. Effect of nanoparticle dissolution on SSD

The nanoparticle and bulk metal oxide dissolution concentrations are given in Table 2. Different methods have been used to determine the dissolved fraction. Centrifugation in combination with filtration, dialysis, ultrafiltration and metal ion sensitive bacteria (*Escherichia coli*) or yeasts (*Saccharomyces cerevisiae*) were commonly used techniques in the acute toxicity studies used in this paper. In addition, the time at which these measurements were performed after addition of the nanoparticles to the test medium varied (from 60 min to 10 days in different studies). About 0.6 to 100 % of the ZnO nanoparticles dissolved, similar to bulk ZnO (0.6 – 100 %). In general, a trend of higher dissolution at lower nanoparticle or bulk material exposure concentrations can be observed. However, as indicated in the table, the pH, also plays an important role. Other test media characteristics (including ionic strength, water hardness, temperature) which are not indicated in the table also influence the dissolution. The dissolution of the CuO nanoparticles is between 0.3 and 75.8%. For the highest exposure concentrations (about 100 mg Cu/l), low dissolution proportions were observed (about 2-3 %). The pH and other test media characteristics also influence the dissolution of these nanoparticles. The dissolution of bulk CuO was much lower (0.04 to 0.57%) than the CuO nanoparticle dissolution. Species sensitivity distributions of the metal salts ZnCl₂ (Fig. 1C) and CuCl₂ (Fig. 2C) were constructed. Different species of algae, molluscs, crustaceans, insects, other invertebrates, fish and amphibians were included in the ZnCl₂ SSD analysis. In general, the algae species appeared to be the most sensitive to the metal. A HC₅ value of 0.03 (90 % CI: 0.02 – 0.05) mg Zn/l was calculated for the zinc salt. In the CuCl₂ SSD analysis, algae, molluscs, crustaceans, insects, other

invertebrates and fish were included. Crustaceans were the most sensitive to copper. For the Cu salt a HC₅ value of 0.009 (90 % CI: 0.007 – 0.012) mg Cu/l was calculated.

3.4. Effect of individual and aggregated nanoparticles on SSD

The nominal individual size of ZnO nanoparticles (Fig. 4 left) has no overall effect ($\chi^2=0.61$, $p = 0.436$) on the L(E)C₅₀ value for all taxonomic groups. When analysed individually, there is a positive trend between individual particle size and L(E)C₅₀ for algae ($\chi^2= 3.74$, $p= 0.053$), a negative trend for nematodes ($\chi^2= 2.78$, $p= 0.095$) and no trend for the other groups (all $p > 0.657$). For CuO nanoparticles (Fig. 4 right) the individual size does not have a significant overall influence on the L(E)C₅₀ ($\chi^2=3.24$, $p= 0.072$) values. There is a positive trend for algae ($\chi^2=3.29$, $p= 0.07$), but no trend for the other taxonomic groups (all $p > 0.286$). For both ZnO and CuO nanoparticles none of these trends were significant.

There is no overall effect of aggregated particle size on the L(E)C₅₀ values for the aggregated ZnO nanoparticles for all taxonomic groups combined ($\chi^2= 0.28$, $p= 0.596$; Fig. 5 left). When tested individually, the toxicity to fish ($\chi^2= 8.42$, $p= 0.004$) and nematodes ($\chi^2= 8.23$, $p= 0.004$) significantly decreases when exposed to larger aggregates. For the other groups there is no effect (all $p > 0.328$). There is a trend that increasing aggregated particle size for CuO increases L(E)C₅₀ ($\chi^2= 2.76$, $p= 0.097$; Fig. 5 right). This trend is highly influenced by the significant trend for crustaceans ($\chi^2= 12.99$, $p < 0.001$) when looking at the individual taxonomic groups, while there is no relationship for the other groups ($p > 0.819$).

4. Discussion

In this study, species sensitivity distributions were constructed and the effect of individual nanoparticles and nanoparticle dissolution and aggregation on these distributions was evaluated. ZnO nanoparticles, bulk ZnO and ZnCl₂ were the most toxic to algae and crustacea, which was also shown by Bondarenko et al. (2013). Algae and crustacea have also been shown to be one of the most sensitive organisms when exposed to different chemicals (e.g. to organic compounds (Dom et al., 2012)). The SSD of ZnO nanoparticles is very similar to the bulk ZnO and ZnCl₂ distribution, with comparable acute hazard concentrations 5 % and overlapping 90 % confidence intervals for nanoparticles (0.06 mg Zn/l with 90 % CI 0.03 - 0.15 mg Zn/l; Fig. 1A), bulk (0.06 mg Zn/l with 90 % CI 0.03 – 0.20 mg Zn/l; Fig. 1B) and salt (0.03 mg Zn/l with 90 % CI 0.02 – 0.05 mg Zn/l; Fig. 1C). Gottschalk et al. (2013) found a lower median HC₅ value of 0.01 mg/l for ZnO nanoparticles. However, these lower values may be explained by extrapolations (i.e. acute to chronic and EC₅₀ to NOEC) performed on the data prior to input into the SSD model. The similarity in HC₅ values between nanoparticles, bulk and metal salt in our study suggests similar toxicity effects of zinc, zinc oxide nanoparticles and zinc oxide bulk material, which were reported by different authors (Aruoja et al., 2009; Bondarenko et al., 2013; Franklin et al., 2007; Heinlaan et al., 2008). In most studies, high nanoparticle solubilities were observed (up to 100 %; Table 2). The dissolution was dependent on the nanoparticle concentration, with the lowest dissolution percentage at the highest ZnO exposure concentrations. The high dissolution of the ZnO nanoparticles and the comparable toxicity with zinc salts indicates that the acute toxicity of ZnO nanoparticles is due to the toxic zinc ions. Several studies already indicated that the toxicity of ZnO nanoparticles is due to the zinc ions dissolved from the

particles (Aruoja et al., 2009; Franklin et al., 2007; Mortimer et al., 2010). However, the hypothesis that the toxicity of ZnO nanoparticles is exclusively caused by dissolved ions is still somewhat debatable since in some studies differences in toxic responses (between nanoparticles and metal salts) were observed. As such, gene expression patterns in *D. magna* exposed to zinc ions and ZnO nanoparticles (2.2 and 9.0 mg/l) have been shown to be different (Poynton et al., 2011). Furthermore, Zhu et al. (2009b) have observed higher zebra fish embryo toxicity of ZnO nanoparticles, compared to free zinc ions. After 96 hours of exposure to 10 mg/l ZnO nanoparticles (causing effects on embryo development) at pH 8, only 1.01 ± 0.43 mg/l occurred as dissolved Zn^{2+} . When exposing zebra fish embryos to this dissolved fraction (1 mg Zn^{2+} /l), no toxicity was observed. However, these effects were usually observed at high exposure concentrations (> 10 mg/l) which usually also is associated with the formation of aggregates.

Other factors that have the potential to cause toxicity are the physical characteristics of the individual nanoparticles or their aggregates. It is likely that these nanoparticles or aggregates have a different mode of action than metal ions (Poynton et al., 2011). The size of the individual ZnO nanoparticles had no significant effect on the L(E)C_{50} values for the different taxonomic groups (Fig. 4 left). This independence between the ZnO nanoparticle size and its toxicity has been shown for different species. For example, Wiench et al. (2009) have shown that the particle size had no effect on the acute toxicity in *D. magna*. Similarly, Adams et al. (2006) indicated that the antibacterial activity of nanoparticles to *Bacillus subtilis* and *Escherichia coli* was not affected by differentiating advertised nanoparticle size (67 nm, 820 nm, 44 μm). The absence of an observed effect of individual nanoparticle size on toxicity may on the one hand be

because most nanoparticle preparations are not monodisperse but include various nanoparticle sizes and on the other hand because the ZnO nanoparticles mostly dissolve (with high to full dissolution at low exposure concentrations) (Adam et al., 2014) and/or aggregate (with high aggregation at high exposure concentrations > 10 mg/l) (Adams et al., 2006; Ma et al., 2011; Wang et al., 2009; Zhu et al., 2009a). Our results show no general trend between the size of the ZnO aggregates and the L(E)C₅₀ values (Fig. 5 left). Only for fish and nematodes, smaller nanoparticle aggregates cause a significantly higher toxicity. However, due to the limited data set, these results have to be treated with caution. The effect of ZnO nanoparticle aggregate size on toxicity was also shown by Zhang et al. (2007). This study also indicated that small aggregates (230 nm) were more toxic to bacteria (*E. coli*) than larger aggregates (2417 nm). The authors contributed the higher toxicity of the smaller aggregates to their larger surface area-to-volume ratio. However, it is also possible that the dissolution of smaller aggregates is faster compared to larger aggregates as a result of the larger surface area-to-volume ratio. Overall, the results of the SSDs comparison clearly indicate that the zinc ions dissolved from the ZnO nanoparticles are the main cause of the observed toxicity. However, at high exposure concentrations (> 10 mg/l (Ma et al., 2011; Wang et al., 2009; Zhu et al., 2009a)), in some species and exposure scenarios, the aggregated nanoparticles may also contribute to the observed effect.

When applying assessment factors of 10 (assessment factors for acute-to-chronic extrapolation; European Commission (2003)) and 5 (maximal assessment factor to derive predicted no effect concentrations based on SSDs; European Commission (2003)) to the calculated HC₅ values of ZnO nanoparticles (HC₅: 0.06 mg Zn/l), a predicted no effect concentration (PNEC) of 1.2 µg Zn/l

can be calculated. Based upon information currently available, the theoretically predicted average environmental concentration (PEC) of ZnO nanoparticles is 0.07 (with 85 % confidence intervals: 0.04 – 0.23) $\mu\text{g Zn/l}$ in European surface water (Sun et al., 2014). Since the predicted PEC value is lower than the calculated PNEC value, no current risk of these nanoparticles to the aquatic environment is expected, on the basis of the information available. However, it should be emphasized that these assumptions are based on predicted PEC values and not actual measurements. The average measured baseline concentration for zinc in European surface waters is 2.68 (with min: 0.09 – max: 310) $\mu\text{g Zn/l}$ (Merag, 2007). Considering the toxicity of ZnO nanoparticles was shown to be caused by dissolved zinc and the amount of total zinc added by ZnO nanoparticles is low compared to the average measured baseline concentration, effects caused by these added nanoparticles in European surface waters are expected to be low.

The CuO nanoparticles and copper salt were the most toxic to crustacea, which was also shown by Bondarenko et al. (2013). In contrast to the ZnO case, the SSD for CuO nanoparticles differs from the distributions obtained for bulk CuO and CuCl_2 . The HC_5 value for CuO nanoparticles (0.15 mg Cu/l with 90 % CI 0.05 – 0.47 mg Cu/l; Fig. 2A) is more than 40 times lower than for bulk CuO (6.19 mg Cu/l with 90 % CI 2.15 – 38.11 mg Cu/l; Fig. 2B), indicating a higher toxicity of the nanoparticles. This higher CuO nanoparticle toxicity was observed in different studies (Aruoja et al., 2009; Mortimer et al., 2010). On the other hand, dissolved copper (HC_5 : 0.009 mg Cu/l with 90 % CI 0.007 – 0.012; Fig 2C) is more toxic than CuO nanoparticles. However, there is a high probability that the toxicity of all three (nanoparticles, bulk, salts) is simply due to the ionic copper. When considering the dissolution values (Table 2), a relationship can be seen

between the dissolution and the toxicity. In general, bulk CuO shows low dissolution (on average 0.27 % of the total copper occurred as dissolved, with minimum 0.04 % – maximum 0.57 % dissolution measured after 2 hours to 48 hours of exposure) and is the least toxic. On average, 17.9 % of the CuO nanoparticles dissolved (with minimum 0.3 % – maximum 75.8 % measured after 60 minutes to 10 days of exposure) with intermediate toxicity, while Cu salts completely dissolved at the tested concentrations and this is reflected in the high toxicity caused by the free Cu^{2+} ions. Based on these average dissolution percentages, new HC_5 values, only referring to the dissolved fraction of the nanoparticles and bulk, can be calculated. For CuO nanoparticles and bulk CuO this dissolved HC_5 value would be 0.027 (0.009 – 0.084) mg Cu/l and 0.017 (0.006 – 0.103) mg Cu/l, values that compare quite well with that obtained for CuCl_2 (HC_5 : 0.009 mg Cu/l with 90 % CI 0.007 – 0.012 mg Cu/l). As such, overlapping confidence intervals between the dissolved fraction of the nanoparticles, bulk and salt indicates that the toxicity is largely attributable to the toxic copper ions. This was also suggested by several authors (Jo et al., 2012; Kasemets et al., 2009).

Other factors that may play a role in the nanoparticle toxicity are the individual and aggregated nanoparticles. The effect of individual nanoparticle size was tested using the available acute toxicity data. However, the results showed no significant effect of nanoparticle size on the L(E)C_{50} values for any taxonomic group (Fig. 4 right). The absence of an effect of individual nanoparticle size to aquatic species may be due to the heterogeneous nanoparticle preparations and because upon entering the aquatic environment, most CuO nanoparticles form larger aggregates (Gomes et al., 2012; Sousa and Teixeira, 2013). A significant trend of increased toxicity associated with smaller aggregates was only observed for crustaceans (Fig. 5 right).

However, also in this case, it is likely that the smaller aggregates dissolve more and faster. Due to the limited data set, these results have to be treated with caution. In addition, Jo et al. (2012) indicated that aggregation limits the acute nanoparticle toxicity to *D. magna*. This was observed since CuO nanoparticles exposed after filtration (0.05 – 0.45 µm filtered) caused higher acute toxicity to daphnids than exposed in unfiltered medium (where nanoparticles are still present).

When applying assessment factors of 10 (acute-to-chronic extrapolation) and 5 (predicted no effect concentrations based on SSDs), a PNEC value of 3 µg Cu/l can be calculated based on the obtained HC₅ (0.15 mg Cu/l) for CuO nanoparticles. Currently there are no production volume or predicted environmental concentration values available for CuO nanoparticles. However, these nanoparticles are currently manufactured in lower volumes than ZnO nanoparticles (Bondarenko et al., 2013). As a result, no current risk of these nanoparticles to the aquatic environment is expected, on the basis of the information available. The average measured baseline copper concentration in European surface waters is 0.88 (with min: 0.08 – max: 14.6) µg Cu/l (Merag, 2007). The effects caused by CuO nanoparticles in European surface waters are expected to be low since the toxicity was shown to be caused by the dissolved fraction and the amount of copper added from the CuO nanoparticles is expected to be low compared to the average measured baseline copper concentration.

5. Conclusions

Species sensitivity distributions were constructed for ZnO and CuO nanoparticles. Additionally, the effects of individual nanoparticles, particle dissolution and aggregation on these

distributions were estimated on the basis of the existing data sets. ZnO nanoparticles, bulk ZnO and ZnCl₂ showed similar distributions and hazard concentrations. Overall, CuO nanoparticles were more toxic than their bulk counterparts but less toxic than the corresponding CuCl₂ salt. When taking into account the effect of the dissolved fraction in the species sensitivity distributions, the toxicity of ZnO and CuO nanoparticles appears to be largely caused by the free metal ions. Based on the available information, there is currently no risk of ZnO and CuO nanoparticles to the aquatic environment. However, future work should focus on measuring environmental nanoparticle concentrations to accurately determine PEC values. Given the complexity and dynamics of metal oxide nanoparticle exposure there is also a need for more focus on chronic and realistic exposure scenarios.

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Tables

Table 1: HC₅ values (hazard concentrations 5%) with 90 % confidence intervals (mg metal/L) for the log-triangular, log-normal and log-empirical distribution and goodness of fit of these distributions (R^2) when exposed to ZnO nano, bulk, Zn salt, CuO nano, CuO bulk, Cu salt. Since the log-empirical distribution was not fitted, no R^2 values are available here.

		log-triangular	log-normal	log-empirical			log-triangular	log-normal	log-empirical
ZnO nano	HC ₅	0.06	0.05	0.05	CuO nano	HC ₅	0.15	0.27	0.16
	90 % CI	0.03 - 0.15	0.02 - 0.13	0.04 - 0.14		90 % CI	0.05 - 0.47	0.11 - 0.79	0.05 - 0.44
	R^2	0.9763	0.9668	N/A		R^2	0.9806	0.9325	N/A
ZnO bulk	HC ₅	0.06	0.04	0.05	CuO bulk	HC ₅	6.19	12.94	11.54
	90 % CI	0.03 - 0.20	0.02 - 0.14	0.04 - 0.19		90 % CI	2.15 - 38.11	3.58 - 71.28	11.54 - 79.81
	R^2	0.9740	0.9671	N/A		R^2	0.9585	0.8443	N/A
Zn salt	HC ₅	0.03	0.03	0.03	Cu salt	HC ₅	0.009	0.007	0.010
	90 % CI	0.02 - 0.05	0.02 - 0.05	0.02 - 0.03		90 % CI	0.007 - 0.012	0.005 - 0.009	0.008 - 0.015
	R^2	0.9869	0.9806	N/A		R^2	0.9848	0.9846	N/A

Table 2: The dissolved proportion, calculated based on the dissolved metal concentration and total metal concentration. The pH, time (between addition of nanoparticles or bulk to the medium and measurement of the dissolved metal concentration) and method used to measure the dissolved fraction is indicated.

Metal oxide	Initial metal concentration (mg metal/l)	Dissolved metal concentration (mg metal/l)	Dissolved proportion (%)	Time	pH	Method of analysis	Reference
ZnO nano	< 1	< 1	69-97	2 h	6.5	<i>Escherichia coli</i> MC1061 centrifuged (19,000 g for 20 min)	(Heinlaan et al., 2008)
	0.04		79.6	-	-	and filtered (0.2 µm membrane filters)	(Dasari et al., 2013)
	2.2	0.4	18.2	24 h	8.1	Ultrafiltration (10000K, 2nm filter)	(Poynton et al., 2011)
	4.7	3.9	83.0	6 h	6.5	<i>Escherichia coli</i> MC1061	(Mortimer et al., 2010)
	9	0.4	4.4	24 h	8.1	Ultrafiltration (10000K, 2nm filter)	(Poynton et al., 2011)
	32.7	5.8	17.7	96 h	7	Centrifuged (13,000 g for 20 min) and filtered (0.2 µm polytetrafluoroethylene filter), ICP-MS	(Wang et al., 2009)
	97.4	77	79.1	8 h	-	<i>Escherichia coli</i> MC1061	(Kasemets et al., 2009)
	80.3	16	19.9	72h	7.6	Dialysis (1000 Da)	(Franklin et al., 2007)
	100.4	6.8	6.8	-	-	filtered with 0.2 µm membrane, ICP-AES	(Baek and An, 2011)
	4017.3	25.6	0.6	70h	8	vacuum filtrated through 0.02 µm membrane	(Manzo et al., 2013)
			100	2 h	-	<i>Escherichia coli</i> MC1061	(Ivask et al., 2010)
	< 1	< 1	69-97	2 h	6.5	<i>Escherichia coli</i> MC1061	(Heinlaan et al., 2008)
	4.4	3.4	77.3	6 h	6.5	<i>Escherichia coli</i> MC1061	(Mortimer et al., 2010)
			100	2 h		<i>Escherichia coli</i> MC1061	(Ivask et al., 2010)
ZnO bulk	32.7	5.6	17.1	96 h	7	centrifuged at 13,000 g for 20 min and filtered (0.2 µm polytetrafluoroethylene filter), ICP-MS	(Wang et al., 2009)
	80.3	16	19.9	72 h	7.6	Dialysis (1000 Da)	(Franklin et al., 2007)
	108	69	63.9	8 h	-	<i>Escherichia coli</i> MC1061	(Kasemets et al., 2009)
	4017.3	23.1	0.6	70 h	8	vacuum filtrated through 0.02 µm membrane	(Manzo et al., 2013)
CuO nano	0.71		25	-	-	<i>Escherichia coli</i> MC1061 and <i>Saccharomyces cerevisiae</i> BMA64-1A	(Aruoja et al., 2009)
	0.1		2.7-10.6	60 m	6.8-8.5	centrifuged (12,000 rpm for 10 min), ICP-OES	(Fan et al., 2012)
	0.13		75.8	-	-	centrifuged (19,000 g for 20 min) and filtered (0.2 µm membrane filters)	(Dasari et al., 2013)
	19.1	0.6	3.1	10 d	5.8	centrifuged (75,600 g for 60 min) and filtered (0.2, 0.05 µm, 0.025 µm), flame-AAS	(Pradhan et al., 2012)
	56.86	1.87	3.3	10 d	5.8	centrifuged (75,600 g for 60 min) and filtered (0.2, 0.05 µm, 0.025 µm), flame-AAS	(Pradhan et al., 2012)
	3.2	0.01	0.3	48 h	-	<i>Escherichia coli</i> MC1061	(Heinlaan et al., 2011)
	16.6	3.8	22.9	8 h	5.5-5.6	<i>Saccharomyces cerevisiae</i> BMA64-1A	(Kasemets et al., 2009)
	16.6	5.8	34.9	24 h	5.5-5.6	<i>Saccharomyces cerevisiae</i> BMA64-1A	(Kasemets et al., 2009)
	99.9	2.7	2.7		-	filtered with 0.2 µm membrane, ICP-AES	(Baek and An, 2011)
	128	2.7	2.1	6 h	6.5	<i>Escherichia coli</i> MC1061	(Mortimer et al., 2010)

CuO bulk	0.01 - 1000	<2.5	-	2 h	6.5	<i>Escherichia coli</i> MC1061	(Heinlaan et al., 2008)
		0.01–200	20	2 h	-	<i>Escherichia coli</i> MC1061	(Ivask et al., 2010)
	11.55		0.18	-	-	<i>Escherichia coli</i> MC1061 and <i>Saccharomyces cerevisiae</i> BMA64-1A	(Aruoja et al., 2009)
	139.8	0.05	0.04	48 h	-	<i>Escherichia coli</i> MC1061	(Heinlaan et al., 2011)
		0.2–10,000	0.4	2 h	-	<i>Escherichia coli</i> MC1061	(Ivask et al., 2010)
	1036	3.2	0.31	8 h	5.5-5.6	<i>Saccharomyces cerevisiae</i> BMA64-1A	(Kasemets et al., 2009)
	1036	5.9	0.57	24 h	5.5-5.6	<i>Saccharomyces cerevisiae</i> BMA64-1A	(Kasemets et al., 2009)
	1705	1.9	0.11	6 h	6.5	<i>Escherichia coli</i> MC1061	(Mortimer et al., 2010)
	0.01 - 1000	<1.5	-	2 h	6.5	<i>Escherichia coli</i> MC1061	(Heinlaan et al., 2008)

Figures

Fig. 1. Aquatic (log-triangular) species sensitivity distribution (based on nominal or measured acute $L(E)C_{50}$) of ZnO nanoparticles (a; LC_{50} values in red; EC_{50} values in black), bulk ZnO (b; LC_{50} values in red; EC_{50} values in black) and $ZnCl_2$ (c; LC_{50} and EC_{50} values in black), indicating the cumulative weighted probability (% , Y-axis) at a certain exposure concentration (mg Zn/l, X-axis). The slope (R^2) and hazard concentration 5% (with 90 % confidence intervals) are indicated. Data from (Adams et al., 2006; Aruoja et al., 2009; Baek and An, 2011; Dasari et al., 2013; Franklin et al., 2007; Heinlaan et al., 2008; Hu et al., 2009; Ivask et al., 2010; Kasemets et al., 2009; Li et al., 2013; Li

et al., 2011; Ma et al., 2009; Ma et al., 2011; Manzo et al., 2013; Mortimer et al., 2010; Nations et al., 2011; Poynton et al., 2011; Wang et al., 2009; Wiench et al., 2009; Xiong et al., 2011; Yu et al., 2011; Zhao et al., 2012; Zhu et al., 2009a; Zhu et al., 2009b; Zhu et al., 2008) were used for the construction of the SSD. The other invertebrates (for ZnCl_2) represent species of sea urchin and protozoa.

Fig. 2. Aquatic (log-triangular) species sensitivity distribution (based on nominal or measured acute $L(E)C_{50}$) of CuO nanoparticles (a; LC_{50} values in red; EC_{50} values in black), bulk CuO (b; LC_{50} values in red; EC_{50} values in black) and CuCl_2 (c; LC_{50} and EC_{50} values in black), indicating the cumulative weighted probability (% , Y-axis) at a certain

exposure concentration (mg Cu/l, X-axis). The slope (R^2) and hazard concentration 5% (with 90 % confidence intervals) are indicated. Data from (Aruoja et al., 2009; Baek and An, 2011; Chen et al., 2011; Dasari et al., 2013; Fan et al., 2012; Heinlaan et al., 2008; Heinlaan et al., 2011; Hu et al., 2009; Ivask et al., 2010; Jo et al., 2012; Kasemets et al., 2009; Manusadzianas et al., 2012; Mortimer et al., 2010; Pradhan et al., 2012; Zhao et al., 2012) were used for the construction of the SSD. The other invertebrates (for CuCl_2) represent species of sea urchin and protozoa.

Fig. 3. Average L(E)C₅₀ values with minima and maxima for the different taxonomic groups when exposed to ZnO nanoparticles (a), ZnO bulk (b), CuO nanoparticles (c), CuO bulk (d). For each taxonomic group, the number of available data points (p) and the percentage (X) of the total number of data points is indicated.

Fig. 4. Effect of ZnO (left) and CuO (right) individual nominal nanoparticle size on the toxicity. The $L(E)C_{50}$ concentrations (mg metal/l) are indicated in function of the individual nanoparticle size (nm).

Fig. 5. Effect of ZnO (left) and CuO (right) aggregated measured nanoparticle size on the toxicity. The $L(E)C_{50}$ concentrations (mg metal/l) are indicated in function of the aggregated nanoparticle size (nm).