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## Ecotoxicological assessment of ZnO nanoparticles to *Folsomia candida*

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2013

### **document version**

Publisher's PDF, also known as Version of record

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### **citation for published version (APA)**

Waalewijn-Kool, P. L. (2013). *Ecotoxicological assessment of ZnO nanoparticles to Folsomia candida*. Off-Page, [www.offpage.nl](http://www.offpage.nl).

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Summary and discussion



Although the size-dependent properties make engineered nanoparticles desirable for many applications, the increased use inevitable lead to its release into the environment, for instance via wastewater or sewage sludge. Contamination of the soil environment with engineered nanoparticles may be harmful for soil invertebrates living in there, such as earthworms, isopods and springtails. In the previous five experimental chapters the toxicity of ZnO-NP to the springtail *Folsomia candida* was described for different ZnO-NP (particle size and nanoparticle coating) and different soil properties (soil pH and organic matter content). Full dose-response curves for ZnO-NP were presented for springtail reproduction using a wide range of test concentrations (100-6400 mg Zn/kg d.w.). Also ZnO-NP bioavailability and dissolution were studied by equilibrating spiked soils for one year in the laboratory. A comprehensive ecotoxicological assessment of ZnO-NP was presented and the data provide new and essential information for future risk assessment of ZnO-NP in the soil environment. Here, I would like to discuss methodological issues regarding ecotoxicity testing of metal-based nanoparticles and to reflect on the different toxicity and behaviour of ZnO-NP and ZnCl<sub>2</sub> in soils. Recommendations on ZnO-NP soil toxicity testing will be given and I will highlight some possibilities for further research. The chapter ends with concluding remarks on the main findings of this research.

## 1 Ecotoxicity testing of metal-based nanoparticles in soil

Due to their unique properties, existing analytical methods and test approaches for assessing environmental risk may not be appropriate for metal-based nanoparticles. Nano-ecotoxicology is currently in its infancy and environmental testing of nanoparticles may require developing new test guidelines (Kahru and Dubourguier, 2010; Handy et al., 2008, 2012). However, during investigations of the toxicity of metal-based nanoparticles it is important to take into account the existing knowledge on metal toxicity. At this early stage of nanoparticle research the approaches and tools that were developed for metal toxicity testing in the past can be useful. Additional nanoparticle characterization and adaptations to the test guidelines may be necessary, but only based on good scientific research and careful considerations. This is emphasized in a scientific letter in Appendix II of this thesis (van Gestel et al., 2010. Metal-based nanoparticles in soil: New research themes should not ignore old rules and theories. Comments on the paper by Hu et al., 2010). Methodological issues for soil toxicity testing of ZnO-NP and springtails are specifically addressed here.

Toxicity tests with *F. candida* usually are performed following ISO guideline 11267 (ISO, 1999) or the OECD guideline 232 (OECD, 2009). This method, which focuses on determining the effects on survival and reproduction of the springtails after 28 days of exposure, is suitable without amendments for determining the toxicity of ZnO-NP. The "only" aspects that need consideration are the method of spiking, analytical technique for total Zn analysis and soil type.

Considering the spiking method, two different methods have been tested and evaluated: from a suspension in a soil extract and as dry powder mixed in with dry soil (Chapter 2). The first method is based on dispersing dry powdered ZnO-NP in a soil extract (according to van der Ploeg et al., 2011). Due to association with the dissolved organic carbon and possibly other compounds in the soil extract, a suspension of ZnO-NP is obtained. At high concentrations, however, the suspension was not homogeneous as there still was precipitation. By shaking the suspension and bringing it “in one go” into the soil (in this way also reaching the desired soil moisture content), all ZnO-NP could be transferred into the test soil. This method produced a good recovery and homogeneous distribution of the Zn in the test soils (coefficient of variance (CV) for five subsamples per test concentration < 10%). Also the second method produced a good recovery and homogeneous distribution of Zn in the test soils (CV for five subsamples per test concentration < 10%). This experiment showed that both spiking methods resulted in homogeneous mixing of the ZnO-NP through the soil. The outcome of the toxicity tests with 30 nm ZnO was also reproducible in Lufa 2.2 soil using the dry spiking method with EC50 values of 3493 and 3159 mg Zn/kg (Chapters 2 and 6).

While working with the ZnO-NP powders and the soils, a personal preference for the dry spiking method was developed, because dry powder was mixed very easily with dry soil. Mixing the dry ZnO-NP directly into the soil allows the transformation and dissolution to occur in the soil, preventing that these processes already can occur in the suspensions. Using suspension spiking, one cannot be certain that the pristine ZnO-NP were tested. But this method is a more realistic scenario, because ZnO-NP will enter natural soils via the sludge resulting from waste water treatment, also in suspensions and not as dry powders. An advantage of suspension spiking is that the dissolution and particle size characterization of ZnO-NP can still be monitored in the soil extracts used for suspension spiking. The scientific debate on spiking nanoparticles to environmental media is ongoing. In the 2<sup>nd</sup> OECD Horizontal Workshop on Ecotoxicity and Environmental Fate (Berlin, 2013), it was suggested that spiking should at least be similar in ecotoxicity and fate tests for the sake of comparison. For soils, a suspension spiking is recommended, but dry spiking may also be justified.

The traditional metal analysis used in my experiments has shown to provide a reliable estimate of Zn concentration of a digested soil sample. Recoveries of Zn in spiked soil samples with ZnO-NP were most times higher than 80% using digestion with concentrated HCl:HNO<sub>3</sub> (1:4) and detection by flame Atomic Absorption Spectroscopy (AAS). Also after one year ageing of ZnO-NP spiked soils the measured of total Zn concentrations were in good agreement with the nominal Zn concentrations. So, the Zn analyses showed that total Zn concentrations can be measured by AAS after digestion of the ZnO-NP spiked soils with aqua regia. It must be realized that the toxicity data is expressed on the basis of total Zn concentrations in soil (as mg Zn/kg) and not on the basis of g ZnO-NP/kg, for example. ZnO-NP powder was weighed on the basis of the amount of Zn needed to reach nominal test concentrations taking into account the molecular

weight of ZnO and Zn. This enables comparison with data obtained from studies with non-nano ZnO and ZnCl<sub>2</sub>, but ignores the nanoparticle form of ZnO-NP in the soil during the 28 day exposure period of the test. The ZnO-NP could have been transformed or dissolved during the test period in the soil. Chemical transformation of test substances in test soils is not a new issue for ecotoxicity testing. Organic compounds may already degrade after 28 days and sparingly soluble metal compounds may partly dissolve. Transformation tests and ageing experiments are therefore proposed to understand the environmental fate in addition to ecotoxicity (McLaughlin et al., 2002).

It is very unlikely that the ZnO-NP remain intact nanoparticles in the soil, but only advanced characterization techniques, such as single particle ICP-MS and FFF could confirm this (Hassellöv et al., 2008; Gimbert et al., 2007). And still there is a great risk that sample preparation may change the properties of the nanoparticles or their aggregates/agglomerates. As a consequence, it will be very difficult to obtain proper insight in nanoparticle characteristics in soils. Another approach for nanoparticle characterization is to look at the Zn composition using electron microscopy techniques. Manzo et al. (2010) studied ZnO-NP at 230 mg/kg soil (nominal concentration) using scanning electron microscopy. After examination of this soil sample, it was not possible to identify any particle, agglomerate or mass portion that could be ascribed to the NP (Manzo et al., 2010). ZnO-NP were also not visible in Lufa 2.2 soil spiked at 6400 mg Zn/kg using SEM (analysis executed by Saskia Kars, Laboratory for Microanalyses at the VU University). Characterization of ZnO-NP in soil was not further investigated and also outside the scope of the ecotoxicological assessment in this thesis. To study ZnO-NP dissolution and bioavailability in soils, its pore water was collected and the dissolved Zn concentrations were measured by flame AAS. Filtration over a 0.45 µm membrane has already been shown to be a suitable method for determination of the dissolved metal fraction in the pore water (Harmsen, 2007; Hobbelen et al., 2004) and it was also applicable for nanoparticle spiked soils. One of the uncertainties with this dissolved Zn fraction is the Zn speciation and composition. Centrifugal ultrafiltration has been applied on the pore water in order to separate nanoparticulate Zn from the dissolved fraction. Unfortunately, no major differences in Zn concentrations were measured before and after ultrafiltration and therefore, this did not reveal potential toxicity of particulate Zn (Chapters 3 and 5).

Considering soil type, I recommend to use a natural soil instead of the OECD artificial soil described by the ISO guideline (ISO, 1999). OECD artificial soil can be easily prepared in the laboratory from silica sand, kaolinite clay and sphagnum peat, but it is not always as 'standard' as it intends to be. Substantial differences in soil properties were found for 25 artificial soils prepared according to OECD standardized procedures at various ecotoxicological laboratories. The total organic carbon content, for example, ranged from 1.4 to 6.1% which was responsible for variations in phenanthrene sorption and could also have led to differences in toxicity (Bielská et al., 2012). The use of natural soils provides more realistic exposure situations than artificial soils. And nowadays many soil properties are available for standardized natural soils,

including the Lufa soils (LUFAspeyer, Germany, 2009). Therefore, standard soil Lufa 2.2 ( $\text{pH}_{\text{CaCl}_2} = 5.5$ , ca. 2% OM) was used in my first three experiments (Chapter 2, 3 and 4). In the final two experiments (Chapter 5 and 6) four other natural soils from Europe, ranging in soil pH and organic matter content, were used. In order to evaluate toxicity of ZnO-NP and relate this to the various soil properties,  $\text{pH}_{\text{CaCl}_2}$ , OM content and CEC for these field soils needed to be determined in our laboratory.

## 2 Toxicity of ZnO nanoparticles in relation to particle size

In 28-day toxicity tests using different soils a low hazard of (uncoated) ZnO-NP was found for *F. candida*. EC50 values above 1000 mg Zn/kg were estimated for two types of uncoated ZnO-NP (BASF® Z-COTE, Nanosun ZnO), but also for non-nano ZnO. In order to truly test a particle size effect, the non-nano material (a conventional material with a size above 100 nm) must be exactly the same as the nanoparticle in every aspect, except for the size. This is often impossible to achieve (Handy et al., 2012). The inclusion of the non-nano material in ecotoxicity tests with nanoparticles may reveal insight in potential differences in toxicity between the two. In theory, higher toxicities for smaller sized particles than for larger particles are expected due to the fact that smaller sized particles may have more potential reactive sites for interaction with biotic ligands. In soils, an effect of particle size has not been found on ZnO-NP toxicity to *F. candida*, as the EC50 values for ZnO-NP and non-nano ZnO were not significantly different in any of my experiments (Chapters 2, 3, 4 and 5). In both Lufa 2.2 and Dorset soils the EC50 values based on total Zn and porewater concentrations were in the same range. ZnO-NP toxicity in soil can apparently not be attributed to the size of the ZnO nanoparticles. It is very likely that ZnO nanoparticles in soil undergo various transformations, such as aggregation, sorption and dissolution. There are indications that nanoparticles, due to their high surface reactivity, interact with dissolved organic carbon (Li et al., 2010; Li et al., 2013). This could lead to a decreased surface area and neutralization of a particle size effect. Due to sorption and dissolution behaviour of the particles, size is no longer the most important factor determining ZnO-NP bioavailability and toxicity in soil.

## 3 Toxicity of ZnO nanoparticles in relation to coating

A clear adverse effect on springtail reproduction was discovered for coated ZnO-NP (Chapter 4). In freshly spiked Lufa 2.2 soil the toxicity of triethoxyoctylsilane coated ZnO-NP was significantly higher ( $\text{EC}_{50} = 873$  mg Zn/kg d.w.) than that of uncoated ZnO-NP ( $\text{EC}_{50} = 1964$  mg Zn/kg d.w.). Only after one year equilibration the EC50 value for coated ZnO-NP (1817 mg Zn/kg d.w.) was similar to the one for uncoated ZnO-NP in freshly spiked soil. The properties of a nanoparticle coating are important,

because they control the stability and interactions of the particles with components of the soil. Thus, it is appropriate to investigate the influence of surface coatings on fate and toxicity. Microbial mediated nanoparticle coating degradation has been observed and this resulted in nanoparticle aggregation (Kirschling et al., 2011). The dissolved Zn fraction may determine to a large extent the bioavailable fraction of ZnO-NP. In my study, the dissolved Zn fraction was lower for coated ZnO-NP than for uncoated ZnO-NP, suggesting that a coating could prevent the release of Zn. This implicitly implies that toxicity was not related to particle size or to the dissolved Zn fraction, but to the physico-chemical properties of these coated ZnO-NP. From the colour (greyish instead of brown) and deviating structure of the spiked soils, it is understandable that the hydrophobic character of the coated ZnO-NP may have hindered the animals to thrive in the soil. The surface charge of the coated and uncoated ZnO-NP was studied in deionised water by Geert Cornelis (unpublished). He measured a zeta-potential of approx. -30 mV at pH 5 for the coated ZnO-NP (graph not shown in this thesis), which suggests "moderate" stability of these nanoparticles. The zeta-potential for uncoated ZnO-NP was approx. 0 mV at pH 5, so rapid coagulation and flocculation are expected for the uncoated ZnO-NP in solutions with a pH around 5. The higher stability of coated ZnO-NP may lead to higher bioavailability and toxicity than for uncoated ZnO-NP that lose their nanoparticle properties in environmental media. Zeta-potential measurements in soil extracts could be useful to investigate the influence of surface charge and to further examine the influence of a surface coating on ZnO-NP toxicity. General conclusions on the effect of nanoparticle coating cannot be drawn from my tests with only one single type of coating. More studies on different types of coating are needed to unravel the behaviour and toxicity of coated ZnO-NP.

## 4 Toxicity of ZnO nanoparticles in relation to dissolved Zn

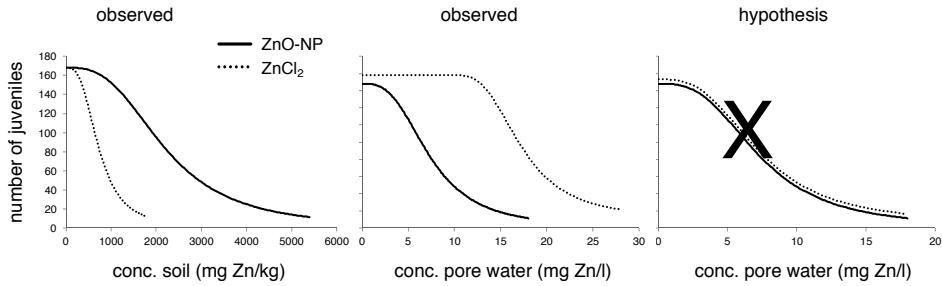
ZnO-NP are soluble particles which means that an effect of the released metal ions can occur. In most aquatic and terrestrial studies, the toxic effects for ZnO-NP were ascribed to the dissolved Zn fraction released from the ZnO-NP (Adams et al., 2006; Aruoja et al., 2009; Blinova et al., 2010; Franklin et al., 2007; Kim et al., 2011). The contribution of the dissolved fraction to ZnO-NP toxicity in soil is unknown.

The toxicity of the soluble metal salt  $\text{ZnCl}_2$  was assessed for comparison with the toxicity of ZnO-NP. If the effects for ZnO-NP were not only nanoparticle effects additional effects of the Zn ions, in theory, could be demonstrated by the soluble metal salt  $\text{ZnCl}_2$ . This was not that simple for soils, because the dissolution and sorption processes of ZnO-NP and  $\text{ZnCl}_2$  were completely different and resulted in another outcome of the toxicity tests than expected. Based on total Zn concentrations much higher toxicity was found for  $\text{ZnCl}_2$  than for ZnO-NP. In Lufa 2.2 ( $\text{pH}_{\text{CaCl}_2} = 5.5$ ) for example the 28-day EC50 was 1964 mg Zn/kg for ZnO-NP and 298 mg Zn/kg for  $\text{ZnCl}_2$  (Chapter 2 and



3) and in the Dorset soil at  $\text{pH}_{\text{CaCl}_2} = 5.9$  this was 1481 mg Zn/kg for ZnO-NP and 732 mg Zn/kg for ZnCl<sub>2</sub> (Chapter 5). According to the porewater hypothesis, toxicity could be better explained on the basis of porewater concentration, which is assumed to be the bioavailable fraction (van Gestel, 1997). Negative effects on springtail reproduction could be explained by the porewater concentrations, because clear dose-response curves were estimated on the basis of porewater concentrations. After twelve months ageing for example, when the porewater concentrations peaked at intermediate soil concentrations, the reduction in reproduction was highest (46%) at 1027 mg Zn/kg, which contained the highest porewater concentration (i.e. 67.1 mg Zn/l). However, based on porewater concentrations, the toxicity of ZnO-NP was much higher than that of ZnCl<sub>2</sub>. So, instead of a similar dose-response curve for ZnO-NP and ZnCl<sub>2</sub>, the EC50 values for ZnO-NP were lower than the ones for ZnCl<sub>2</sub> based on the dissolved Zn fraction. Figure 1 illustrates the general pattern I found in all my dose response curves obtained for ZnCl<sub>2</sub> and ZnO-NP based on total soil and porewater concentrations. These graphs and corresponding EC50 values suggest that the effects of ZnO-NP on juvenile production were related to the soluble Zn concentrations in the pore water, but that also other (particle-related?) effects may have occurred. However, direct particle effects of ZnO-NP on the springtail reproduction could not be proven from my experimental data. Ultrafiltration of the porewater samples did not result in a strong evidence for an effect of nanoparticulate Zn in the pore water, as discussed in Chapters 2 and 5. Mathematically, the higher EC50 values for ZnCl<sub>2</sub> could have been a result of the much higher Zn concentrations measured in the pore water from ZnCl<sub>2</sub> spiked soils than from ZnO-NP spiked soils. In Lufa 2.2 the porewater concentrations for the highest soil concentrations were 12.6 and 612 mg Zn/l, and in Dorset soils these were 41.0 and 231 mg Zn/l for ZnO-NP and ZnCl<sub>2</sub>, respectively. But it is more likely that the bioavailability of Zn released from ZnO-NP differed from the soluble salt ZnCl<sub>2</sub> and that other factors in the pore water played a role. In Dorset soil higher calcium concentrations in the soil pore water were measured that may have induced a protective effect in ZnCl<sub>2</sub> spiked soils, leading to higher EC50 values (Chapter 5). The presence of other cations that compete for binding sites with Zn in the soils is a common phenomenon and does explain the lower toxicity of ZnCl<sub>2</sub> based on porewater concentrations in the Dorset soils according to the principles of the terrestrial Biotic Ligand Model (Thakali et al., 2006). Also, ZnO-NP and ZnCl<sub>2</sub> addition differently affected soil pH, with ZnO-NP leading to increased pH levels while ZnCl<sub>2</sub> caused a dose-related pH decrease. Lower EC50 values based on porewater concentrations for ZnO-NP may have resulted from higher pH levels than for ZnCl<sub>2</sub>. According to the free metal ion model, competition with H<sup>+</sup> ions in the pore water can also reduce metal toxicity (Lofts et al., 2004; 2013).

To summarize, a particle size effect of ZnO-NP toxicity was not detected for *F. candida*, but the contribution of a nanoparticle coating and dissolved Zn was responsible for the mild effects of ZnO-NP. In my opinion, the contribution of dissolved Zn to ZnO-NP toxicity in soil cannot be fully unravelled by a simple comparison with the toxicity of ZnCl<sub>2</sub>.



**Figure 1.** General pattern for the effect of ZnO-NP and ZnCl<sub>2</sub> on the reproduction (number of juveniles) of *Folsomia candida* after 28 days exposure observed in this thesis. Total Zn concentrations in the soil (left) and dissolved Zn concentrations in the pore water (middle and right) are provided on the x-axis. See text for explanation.

## 5 Zinc uptake and regulation

It is likely that springtails can be affected following three options: 1) ZnO-NP dissolve in the soil, then the released Zn<sup>2+</sup> moves onto the animal via the ventral tube, is internalized and triggers various negative responses; or 2) ZnO-NP first bind to surface membranes of the animal, then dissolves and zinc ions are taken up; or 3) ZnO-NP are taken up as particles and dissolve in the body, and thus leads to more Zn<sup>2+</sup> inside the gut and/or cells. Zn uptake by *F. candida* exposed to ZnO-NP, non-nano ZnO and ZnCl<sub>2</sub> for 28 days was investigated after finishing the toxicity test described in Chapter 3. Internal Zn concentrations from five animals selected randomly from each exposure concentration were measured using graphite furnace AAS. Unfortunately, the Zn absorption in the blanks was very high and therefore the Zn concentration in the animals was too high. In earlier analysis internal concentrations of 44.5 µg Zn/g d.w. were measured for *F. candida* kept in Lufa 2.2 soil and 126 µg Zn/g d.w. for animals from the breeding culture (Smit et al., 1996). Although the absolute internal Zn concentrations were too high, and for this reason not reported in this thesis, the analyses showed that internal Zn concentrations were regulated for the lower exposure concentrations and increased at higher soil concentrations. They dramatically increased at exposure concentrations above the EC50 values for all three Zn forms. This indicated that the effects on springtail reproduction are related to internal Zn concentrations. Van Gestel and Hensbergen (1997) reported effects on reproduction at internal concentrations of about 97 µg Zn/g d.w. after exposing *F. candida* to ZnCl<sub>2</sub> in artificial soil for 28 days. It remains uncertain whether the effects were induced by particulate Zn, dissolved Zn or Zn<sup>2+</sup> ions. Exposure of earthworms to ZnO-NP and ZnCl<sub>2</sub> resulted in a similar internal distribution of Zn analysed using synchrotron x-ray microspectroscopy (Unrine et al., 2008). Characterization of gut or other tissue could reveal further insight into Zn speciation within the springtails and potential uptake of particulate Zn. A stable

isotope labelling approach to trace the uptake of ZnO-NP may be promising (Fabrega et al., 2011; Lerner et al., 2012). Buffet et al. (2012) showed that Zn from ZnO-NP could be distinguished from natural Zn background in estuarine invertebrates using labelled ZnO-NP. Gene expression profiles could unravel potential differences in detoxification mechanisms of Zn taken up from ZnO-NP and ZnCl<sub>2</sub>. This might also shed light on the possible contribution of particulate Zn to the toxicity of ZnO-NP (Poynton et al., 2011).

## 6 Fate processes of ZnO nanoparticles in soil

A synthetic view of the environmental fate processes of ZnO-NP in soil and the interactions in pore water are illustrated in Figure 2. The dissolution (release of Zn into the pore water) and sorption (binding of Zn to the solid phase) of ZnO-NP in soil is a complex and dynamic process. The hydrolysis of ZnO-NP into Zn hydroxides is a fast process and when Zn<sup>2+</sup> at the particle surface reacts with OH<sup>-</sup> to Zn(OH)<sup>+</sup> (aq), the resulting hydroxide layer may prevent Zn<sup>2+</sup> release (David et al., 2012; Mudunkotuwa et al., 2012). It is already known that slow reactions convert the adsorbed Zn to non-labile Zn in the soil (fixation) (Crout et al., 2006; Voegelin et al., 2002). Ageing processes such as going from bioavailable Zn to non-labile Zn can take months or years (Degryse et al., 2009). Sorption and dissolution depend strongly on pH and the available binding sites on soil components such as organic matter (Chapter 6). Dissolved Zn may form complexes with dissolved organic carbon, which is affected by competing ions such as protons and calcium.

### Sorption

The behaviour of ZnO-NP and ZnCl<sub>2</sub> in soil differs regarding its sorption (and dissolution), and therefore results in different porewater exposures. Sorption constants ( $K_d$ ) for ZnO-NP were higher than for ZnCl<sub>2</sub> with the shape parameter  $n$  higher than 1 for ZnO-NP and lower than 1 for ZnCl<sub>2</sub>. In the lower concentration range, curves can be nearly linear ( $n \rightarrow 1$ ), but at higher metal concentrations the sorption curve levels off, as sorption sites become saturated (Degryse et al., 2009). Therefore,  $K_d$  values can never be evaluated without taking the  $n$  value into account. The  $n$  values found in this research suggested saturation of Zn in the solid phase for ZnCl<sub>2</sub> spiked soils and saturation of Zn in the pore water of ZnO-NP spiked soils. This suggests precipitation or aggregation in the pore water for ZnO-NP, which is rarely observed for metal salts. Further characterization of the NP forms and surface charge in the pore water are needed to unravel the speciation and bioavailability of ZnO-NP.

### Dissolution

Fairly low dissolved Zn concentrations were measured for ZnO-NP aged soils, indicating that during ageing the release of Zn to the pore water is slow. For a nominal concentration of 800 mg Zn/kg, the dissolved Zn fractions increased from 0.253% in

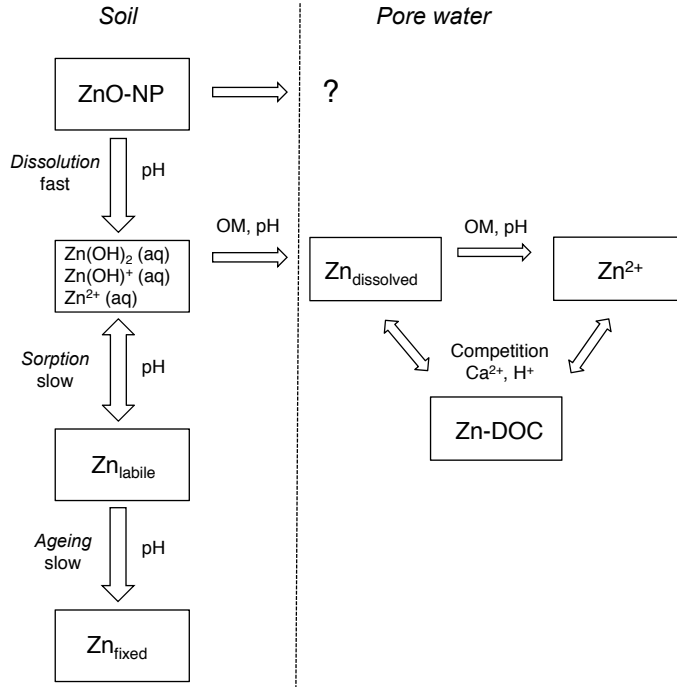


Figure 2. Synthetic view of the environmental fate processes of ZnO-NP in soil (dissolution, sorption, and fixation) and the interactions in the pore water. OM = organic matter; DOC = dissolved organic carbon.

freshly spiked soils to 1.95%, 2.39% and 2.94% after three, six and twelve months ageing. For  $ZnCl_2$  aged soils, the dissolved Zn fractions were much higher and a maximum of 30.4% was calculated at the highest nominal Zn concentration. In  $ZnCl_2$  spiked soils, the dissolved fractions remained the same for each test concentration during the ageing period, which can be explained by the use of closed systems. In experimental field plots with leaching, the water extractable Zn fraction at the highest nominal concentration (3200 mg Zn/kg spiked as  $ZnCl_2$ ) declined from 36.8 to 1.5% of the total Zn concentration in three successive years (Smit et al., 1997). Also in the pH-amended soils (no ageing) low amounts of Zn were present in the pore water, indicating low bioavailability of ZnO-NP in soil. The maximum percentage of dissolved Zn was 0.317%, calculated for the soil with the lowest pH.

The non-linear increase in porewater Zn concentrations for ZnO-NP with total soil concentrations can be explained by pH. The decrease in porewater concentrations at higher total soil concentrations is most likely a cause of higher pH levels. After six months ageing, the soil pH increased from 5.1 for the lowest test concentration to 5.6 for soils spiked with 800 mg Zn/kg (giving the highest dissolved Zn concentration) and to 6.55 for the highest soil concentration (6400 mg Zn/kg). This suggests that

the release of Zn is limited by a solid-phase reaction, reaching a pH-dependent limit around a pH of 5.6. Below this pH level  $H^+$  ions will force the soluble Zn into solution, while at higher pH levels the Zn will adsorb to the solid phases, as was also confirmed by the decrease in Freundlich sorption constants with decreasing pH (Chapter 5). The slight decrease of pH with time, probably due to microbial activity, may explain the continuous release of Zn during one year ageing. As the dissolution of ZnO-NP in soil was strongly influenced by pH it was not possible to establish a reliable dissolution rate for ZnO-NP. In addition, maximum dissolved Zn concentrations were measured for intermediate spiking concentrations so a single average dissolution rate would clearly overestimate the dissolution rate at the highest exposure concentrations. Modelling porewater concentrations from total ZnO-NP concentrations is still a future exercise when more experimental data on ZnO-NP dissolution is available. The voltammetric technique AGNES seems to be a promising tool for actual measurements of the free Zn concentrations in soil solutions (David et al., 2012).

## 7 Toxicity of ZnO nanoparticles in relation to soil properties

The influence of soil properties on ZnO-NP toxicity to *F. candida* was described in Chapter 5 and 6. No effect of ZnO particle size was found on toxicity in pH amended field soils, but the effect on reproduction significantly decreased with increasing soil pH. EC50s of 553, 1481 and 3233 mg Zn/kg were estimated for uncoated ZnO-NP, at pH 4.5, 5.9 and 7.2, respectively. No correlation between EC50s and OM content was found in four different natural soils. Also, in these soils ZnO-NP toxicity was more related to soil pH, as EC50s increased with increasing pH from 1695 to 4446 mg Zn/kg at  $pH_{CaCl_2}$  5.0 to 6.8. These studies showed that ZnO-NP dissolution and toxicity is more dependent on soil pH than on OM content. Dissolved organic carbon in the pore water may protect springtails from adverse effects of dissolved nanoparticles, but further research is needed on this aspect.

## 8 Hazard assessment

Bioavailability and toxicity of metals and metal oxides may change over time because of changes in soil conditions affecting metal availability. Ageing decreased the toxicity of ZnO-NP and  $ZnCl_2$  already after three months, shown by significantly higher EC50 values for both Zn forms (Chapter 4). Longer equilibration times would provide information on the further development of the dissolved Zn fractions in the soil, but in all probability this would not change the hazard assessment considering the low toxicity for springtails in one-year aged soils. After one year ageing, negative effects on springtail reproduction were no longer found for uncoated ZnO-NP at a measured

concentration of 5855 mg Zn/kg. The EC50 for ZnCl<sub>2</sub> increased to 707 mg Zn/kg after one year ageing. In this study, ZnO-NP, non-nano ZnO and ZnCl<sub>2</sub> spiked soils were aged in closed systems in the laboratory. Percolating the test soils with water after spiking (leaching) would have increased the environmental realism of the toxicity tests (Smit and van Gestel, 1998). Leaching has been demonstrated to be effective for metal salts, but the applicability of leaching for nano-sized metals has not been proven and was not performed in this study. The effect of leaching of ZnO-NP spiked soils could be investigated as a next step in long-term soil studies.

Toxicity data could be used to translate EC50 values into a Predicted No Effect Concentration (PNEC) for hazard assessment in soils. The PNEC can be defined as the maximum concentration which is tolerated by an organism without producing any adverse effects. PNEC values are estimated by division of the lowest value for the toxicity with the relevant assessment factor (REACH guidance, 2008). The size of the assessment factor depends on the type of data that are available i.e. short-term or long-term toxicity tests, the number of trophic levels tested and the general uncertainties in predicting ecosystem effects from laboratory data (Crane et al., 2008). For deriving a PNEC for the terrestrial environment a leaching-ageing correction factor is usually applied to make toxicity field-relevant. The term leaching-ageing factor (L/A factor) refers to the combined effect of leaching (due to changing ionic strength) and ageing (due to long-term reactions) on Zn bioavailability and toxicity in soil (Smolders et al., 2009). This L/A factor can be quantified as the ratio between the EC50 values before and after equilibration (van Gestel et al., 2012). In this research in which only ageing was studied for one year without leaching, a factor of 2.36 for ZnCl<sub>2</sub> and 2.98 for uncoated ZnO-NP can be derived from the EC50 values estimated at T=0 and T=12. In the literature, an L/A factor of 3 is proposed for Zn based on CEC, background Zn and pH of different soils tested (Smolders et al., 2009). So, multiplying EC50 values derived from short-term soil toxicity tests with a factor 3 for leaching/ageing effects seems to be safe enough for coated and uncoated ZnO-NP.

## In conclusion

Scientific research and ecotoxicological testing of ENPs must be performed for environmental risk assessment and legislative purposes. Potential adverse effects on our environment cannot be excluded at this stage of nanotechnology development. With the rapidly increasing nanotechnology we must not wait for uncontrolled high exposures in the soil environment that may affect the organisms living there. The behaviour of ZnO-NP in soils suggests that the environmental risk assessment could be similar to non-nano ZnO, as the ZnO-NP lose their pristine properties in natural soils. Nanoparticle coatings should get more attention in future environmental research. ZnO-NP dissolution in soils is a slow process and ZnO-NP are less toxic than

the soluble metal salt  $\text{ZnCl}_2$ . Based on total Zn concentrations in the soil, the hazard assessment of  $\text{ZnCl}_2$  using a L/A factor of 3 would still be safe for ZnO-NP. Based on porewater concentrations Zn toxicity may be reduced by a protective effect of DOC and protons. Soil pH and organic matter content are important soil properties to take into account, but soil pH is the most important soil property affecting ZnO-NP dissolution and toxicity. Although it is possible that ZnO-NP may create toxic effects by releasing the toxic  $\text{Zn}^{2+}$  ions, there are currently no conclusive data or scenarios that indicate that these effects will become a major problem in natural soils or that they cannot be evaluated using the current risk assessment of Zn.