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Insignificant Acute Toxicity of TiO_2 Nanoparticles to Willow Trees

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Abstract

Goal, Scope and Background. Manufactured nanoparticles are expected to increase in production in near future. In response, their environmental fate and effects are intensively studied. Phytotoxicity of some types of nanoparticels has been observed for annual species in the seed germination and root elongation test. Yet, no results of toxicity tests with trees have been reported. Woody species, dominant in many ecosystems, may be in particular vulnerable, due to the large porous wood compartment.

Materials and Methods. This study tests the toxicity of TiO_2-nanoparticles on trees with the short-term willow tree transpiration test. TiO2-particles with 25 and 100 nm diameter were suspended in distilled water at concentrations of 0, 1, 10 and 100 mg/L (first test) and 0, 10, 20 and 50 mg/L (second test). Effects on transpiration, growth and water use efficiency of exposed willow cuttings were monitored. The concentration of nanoparticles was measured by spectrophotometry.

Results. None of the measured effect parameters (growth, transpiration and water use efficiency) showed any significant change during the test. Particles were rapidly lost from solution, probably due to sedimentation as a result of aggregation, and also due to adsorption to roots. The loss of nanoparticles from solution was faster for particles with larger diameter and in the presence of trees.

Discussion. Willow trees were not sensitive to short-term exposure to TiO2-nanoparticles. Similar results were obtained for other plant species. Effects of nano-particles were observed for zinc and zinc oxide particles, but these effects were probably due to heavy metal toxicity and not nano-size specific.

Conclusions. In summary, we came to the conclusion that woody species are not in particular vulnerable to nanosized TiO2-particles in the conditions, concentrations and time periods used in this study.

Recommendations and Perspectives. The preliminary results of this study should be confirmed with other types of MNP, other plant species, experiments in soil and experiments combining longer duration and low exposure concentrations, before a final conclusion in this issue can be made.

Keywords: nanoparticles; Salix; titanium dioxide; toxicity; trees; willow

Introduction

The increase in the production and use of manufactured nanoparticles (MNP) have initiated several scientific studies that investigate environmental risks and toxic effects of MNP (Nowack and Bucheli, 2007). A number of papers and reviews deal with "nanotoxicology" (Service 2004, Oberdörster et al. 2005, SCENHIR 2005, Nel et al. 2006, Hansen et al. 2007). Despite the rising interest in environmental fate and effects of nanomaterials, only few published studies have focussed on the effects on higher plants (Nowack and Bucheli 2007, Navarro et al. 2008). Most of these studies were done using the seed germination and the root elongation test (Yang et al. 2006, Zheng et al. 2005, Yang and Watts 2005, Lin and Xing 2007). The tests were subsequently limited to seed plants, and annual agricultural and garden crops were investigated.

On a global scale, trees account for the majority of biomass (Sitte et al. 1991, Trapp 2003) and are dominant constituents of several ecosystem types. Trees are characterized by a large woody compartment, also known as secondary xylem. The xylem is made up of a continuous porous structure of tracheides or tracheae (Sitte et al. 1991). Within the xylem vessels, water and nutrients are drawn upwards by physical forces, mainly to the leaves. The size of these pores, which are developed from dead cells, is in the micrometer range. Nanoparticles have a diameter in the range – as the name indicates – of nanometers and would thus be of a size that allows an accumulation in the xylem structure, eventually blocking the continuity and disturbing or destroying the function of the xylem. This might give woody plants a special vulnerability towards effects from nanomaterials. On the other hand, the vascular system (phloem and xylem) is protected against invasion by external

material (biological and chemical) by the endodermis and the Casparian strip (McFarlane 1995), making a vulnerability to particles in the nanometer size range not very likely.

In consideration of the high importance of trees for the global ecosystems, and given the fact that a special vulnerability to nanomaterial is not completely unlikely, it is surprising that not any toxicity tests have been done so far in order to evaluate the potential hazards of manufactured nanoparticles (MNP) for woody species. In this study, we therefore determined the acute toxic effect of titanium dioxide (TiO_2) MNP to willow trees.

1 Material and Methods

1.1 Titanium dioxide nanoparticles

The two types of titanium dioxide (TiO_2) nanoparticles applied were Degussa P25 (20/80% rutile/anatase, BET surface area 47 m^2 g^{-1}) with an average diameter of 25 nm and Hombikat UV100 (100% anatase, BET surface area 288 m^2 g^{-1}) with an average diameter of 100 nm.

The nanoparticles were suspended in deionized water by vigorously shaking for at least 10 min. Stock solutions of 100 mg L^{-1} for each particle size were prepared. Two tests were carried out with P25, with a logarithmic concentration range, namely 0, 1, 10 and 100 mg L^{-1} (TiO_2) in the first, and a refined concentration range, 0, 10, 20 and 50 mg L^{-1} (TiO_2) in the second experiment. For UV100, only one concentration (100 mg L^{-1}) was tested. Six replicates were used for controls and five for each concentration.

1.2 Tree toxicity test

The standard willow tree acute toxicity test was applied to determine acute toxicity of MNP to trees (Trapp et al. 2000). With this test, a series of organic and inorganic compounds has been tested (Larsen et al. 2005, Larsen and Trapp 2006, Trapp et al. 2004, Ucisik et al. 2007, Yu et al. 2005). The toxicity of substrate is monitored by the inhibition of transpiration of willows growing in Erlenmeyer flasks. Other effect parameters are growth (g d^{-1}) and water use efficiency (growth per transpiration, g L^{-1}). The transpiration is normalized relative to the initial transpiration and to the transpiration of unexposed control trees, in order to eliminate variations due to differences in initial size and growth of the trees during the test. The normalized relative transpiration (NRT) is defined as

$$NRT(C,t) = 100 \times \frac{\frac{1}{n} \cdot \sum_{i=1}^{n} T_i(C,t) / T_i(C,0)}{\frac{1}{m} \cdot \sum_{j=1}^{m} T_j(0,t) / T_j(0,0)} \qquad [\%]$$

where C is concentration (mg L^{-1}), t is time period (h), T is absolute transpiration (g h^{-1}), i is replicate 1, 2, …, n and j is control 1, 2, …, m. Controls always have a NRT of 100%. Inhibition of transpiration leads to NRT < 100%. Dead trees have NRT-values at about 10%.

1.3 Determination of the concentration of nanoparticles in solution

The turbidity of solutions was determined and related to the concentration of MNP in solution. The adsorption of solutions was measured at 560 nm against a reference sample of pure deionized water on a UV/VIS spectrophotometer (6405 UV/Vis spectrophotometer, Jenway). Standard curves for both P25 and UV100 were

prepared from 3 replicate measurements in the linear range of adsorption (between 0 and 100 mg TiO_2 L^{-1}). The final concentration of MNP in solution was determined at the end of the experiment (after t = 190 h) in triplicate measurements.

1.4 Microscopical investigations

The adsorption of MNP to roots was observed by microscopical imaging, using a Leica MZ6 light microsope (Leica MZ6) with enlargement factors of 6.3 to 40. At the end of the experiment, bottles were shaken and samples were taken for microscopy.

1.5 Statistical tests

The significance of differences to controls or to other test concentrations was tested using a one-tailed t-test at the significance level α = 5%. The significance was judged using tabulated values (Sachs 1992).

2 Results

2.1 Effect on transpiration

The measured absolute transpiration in the first test (0 to 100 mg TiO_2 L^{-1}) show some variations in time (Figure 1), but the general trend for all doses is practically constant transpiration, with a slight decrease at 10 mg L^{-1}. The results for particles of 25 nm diameter (P25) and 100 nm diameter (UV100) at 100 mg L^{-1} were very similar.

In the second test (Figure 2), all willows show a constant, and overall quite similar, increase of transpiration. Probably, conditions in the second test were more favorable because the trees had larger size and were closer to the light source.

For none of the trees, lethal decay or signs of sickness were observed.

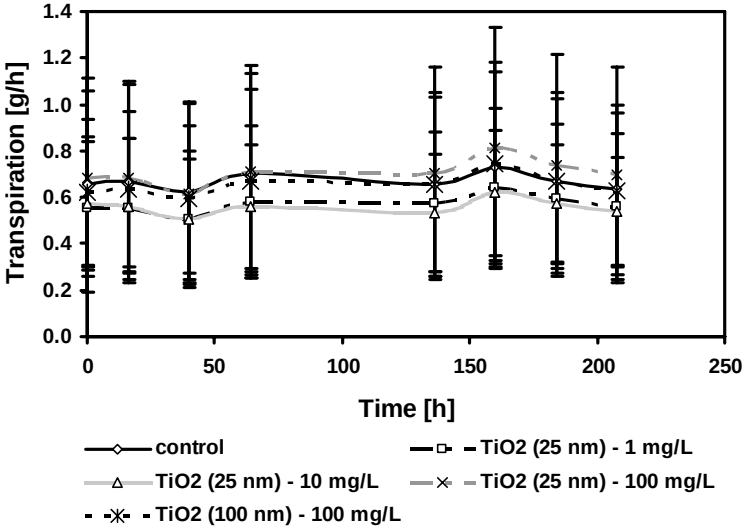

Figure 1. Absolute transpiration (g h^{-1}) of willow trees exposed to solutions with varying nominal concentrations of MNP. Test with logarithmic concentration range. Error bars denote 95% C.I.; n = 6 (controls) or 5 (dosed samples).

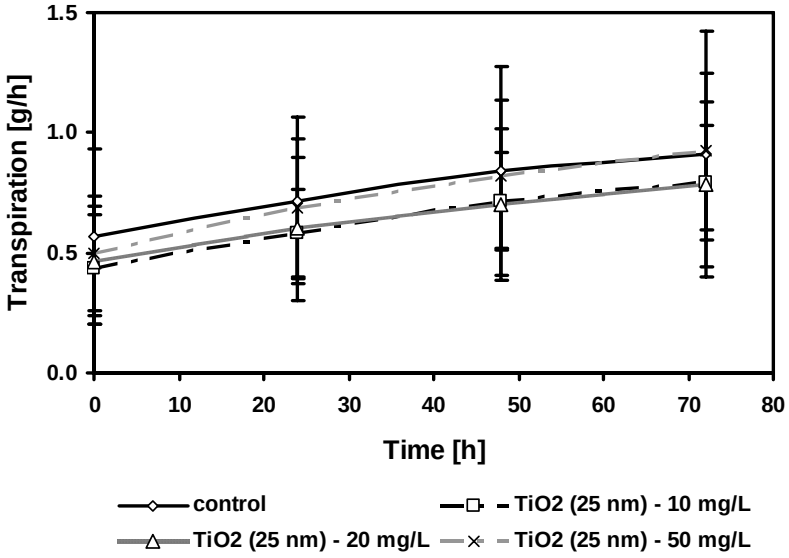

Figure 2. Absolute transpiration (ml h^{-1}) of willow trees exposed to solutions with varying nominal concentrations of MNP. Test with refined concentration range. Error bars denote 95% C.I; n = 6 (controls) or 5 (dosed samples).

2.2 Effect on normalized relative transpiration, growth and water use efficiency

The effect parameters growth (g d^{-1}), water use efficiency (g L^{-1}) and the normalized relative transpiration are shown in Table 1 (logarithmic concentration range) and Table 2 (refined concentration range). In the first test, the NRT (after 64 h) was slightly reduced at all test concentrations, and highest for the trees in the 10 mg L^{-1} solution, but the effect not significant (t-test, α = 5%) for any concentration. The effect was weaker (and also not statistically significant) for the larger particle size (diameter 100 nm). Growth (t = 235 h), too, was at most concentrations slightly reduced, but again this effect was not significant. The water use efficiency (g L^{-1}) (t = 235 h) also did not change significantly at any concentration.

In the second test with concentrations between 0 and 50 mg L^{-1} of diameter 25 nm, the NRT (t = 72 h) of all exposed trees trees was higher than that of controls, but in no case the effect was significantly different. Similarly, neither growth no water use efficiency showed any significant difference to controls. As can be seen, both growth (t = 144 h) and water use efficiency (t = 144 h) were higher in the second test than in the first test, indicating again more favorable conditions.

Table 1. Nominal initial (C_{init}, mg L^{-1}) and measured final concentration (C_{meas}, mg L^{-1}) of TiO_2 P25 and UV100 particles in solutions, compared to normalized relative transpiration (NRT) after 64 h, growth rate (g d^{-1}) and water use efficiency (WUE, g L^{-1}). In brackets: standard deviation; n = 6 (controls, toxicity tests), 5 (dosed samples, toxicity tests), or 5 (concentration measurements).

Nominal C_{init}	**Final C_{meas}**	**NRT** (%)	**Growth** (g d^{-1})	**WUE** (g L^{-1})
0 (control)	0.009 (0.004) (b)	100 (14.8)	0.15 (0.07)	14 (6)
1	0.3 (0.40) -	96 (14.0) -	0.11 (0.06) -	11(3) -
10	4.3 (1.80) #,*	92 (8.2) -;-	0.11(0.03) -;-	12 (5) -;-
100	73.1 (33.00) #,*	94 (10.4) -;-	0.18 (0.08) -;*	15 (2) -;-
100 (a)	15.1 (7.79) #,*	99 (11.2) -;-	0.12 (0.08)-;-	11(3) -;-

(a) UV 100, diameter 100 nm (b) used as blank for the other concentrations
significantly different from control at α = 5%, one-tailed t-test
* significantly different from next-lowest dose at α = 5%, one-tailed t-test
-;- no significant difference to control ; next lowest dose

Table 2. Nominal initial (C_{init}, mg L^{-1}) and measured final concentration (C_{meas}, mg L^{-1}) of TiO_2 P25 and UV100 particles in solutions, compared to normalized relative transpiration (NRT) after 72 h, growth rate (g d^{-1}) and water use efficiency (WUE, g L^{-1}). In brackets: standard deviation; n = 6 (controls, toxicity tests), 5 (dosed samples, toxicity tests), or 5 (concentration measurements).

Nominal C_{init}	**Final C_{meas}**	**NRT** (%)	**Growth** (g d^{-1})	**WUE** (g L^{-1})
0 (control)	0.0004 (0.0027) (a)	100 (28.1)	0.28 (0.15)	16 (5)
10	6.7 (0.35) #	120 (42.8) -	0.32 (0.11) -	26 (13) -
20	24.5 (17.6) #,*	104 (29.0) -,-	0.26 (0.10) -,-	20 (10) -,-
50	41.4 (7.78) #,*	116 (35.8) -,-	0.37 (0.14) -,-	24 (10) -,-

(a) used as blank for the other concentrations
significantly different from control at α = 5%, one-tailed t-test
* significantly different from next-lowest dose at α = 5%, one-tailed t-test
-;- no significant difference to control ; next lowest dose

Table 3. Nominal initial (C_{init}, mg L^{-1}) and measured final concentration (C_{meas}, mg L^{-1}) of TiO_2 P25 and UV100 particles in solutions without trees, mean of five replicates, t = 165 h; in brackets: standard deviation.

Nominal C_{init}	**Type**	**Initial C_{meas}**	**Final C_{meas}**
100 mg/L	P25	95.1 (0.58)	98.1 (1.89) *
100 mg/L	UV100	78.3 (2.18)	37.8 (9.53) **,#

* significantly different from test with trees at α = 5%, one-tailed t-test
** significantly different from test with trees at α = 1%, one-tailed t-test
# significantly faster than for P 25 at α = 1%, one-tailed t-test

2.3 Measured concentration of particles in solution

Table 1 also shows the nominal initial and the measured final concentration of MNP (t = 235 h) in the growth solutions. As can be seen, the concentration had fallen in all solutions during the first experiment, most likely due to sedimentation of particles. The decrease was 27% for the 100 mg L^{-1} concentration at a diameter of 25 nm, but 85% for the diameter 100 nm. The individual flasks showed a relatively large variation in the measured concentrations.

Table 2 shows the concentrations determined for the second experiment (t = 144 h). At the 10 and 50 mg L^{-1} nominal initial concentration, a decrease of concentration was mesasured, while for 20 mg L^{-1}, an increase was found. However, at this concentration, variations in the measurements were in particular large, as can be seen from a coefficient of variation of 72%.

Table 3 shows the measured concentration in the flasks in the absence of trees. The sedimentation of the larger particles, UV100 with 100 nm diameter, is significantly faster than that of partilces with 25 nm diameter, P25. As can be seen, the loss from solution is significantly slower than with trees. This could be due to the adsorption of particles to roots, but also due to the influece of organic substances exuded from roots.

The sedimentation velocity calculated with Stoke's Law (assuming spherical particles and a density of 4 g kg^{-1}) is 13 cm per year at the diameter 25 nm, and 52 cm per year at a diameter of 100 nm. Thus, theoretically, sedimentation may occur only within months or after coagulation of particles to larger agglomerates.

2.4 Agglomeration and adsorption of MNP to roots

Figure 3 shows pictures of willow roots and TiO_2-aggregates from the first experiment. The roots were exposed to aqueous solution of 100 mg L^{-1} P25 TiO_2 (a) and 100 mg L^{-1} UV100 TiO_2 (b). In particular for the larger particle size (d), nanoparticles attached to the roots could be observed. This might have contributed to the rapid decrease of concentration during the experiment. Figures 3 c and d show agglomerates of the particles with diameter 25 nm and 100 nm, respectively. Clearly, the agglomerates are in the micrometer range (for comparison: a willow root has about 500 μm in diameter). Similar observations – flocs of a size up to the micrometer range – have been made by Franklin et al. (2007) with ZnO-nanoparticles. There was no obvious difference in the size of the aggregates for 25 nm particles (P25) and 100 nm particles (UV100), but the amount of aggregate formation and sedimentation seemed to be higher with the larger particles. This is consistent with the measurements of concentration (Table 1).

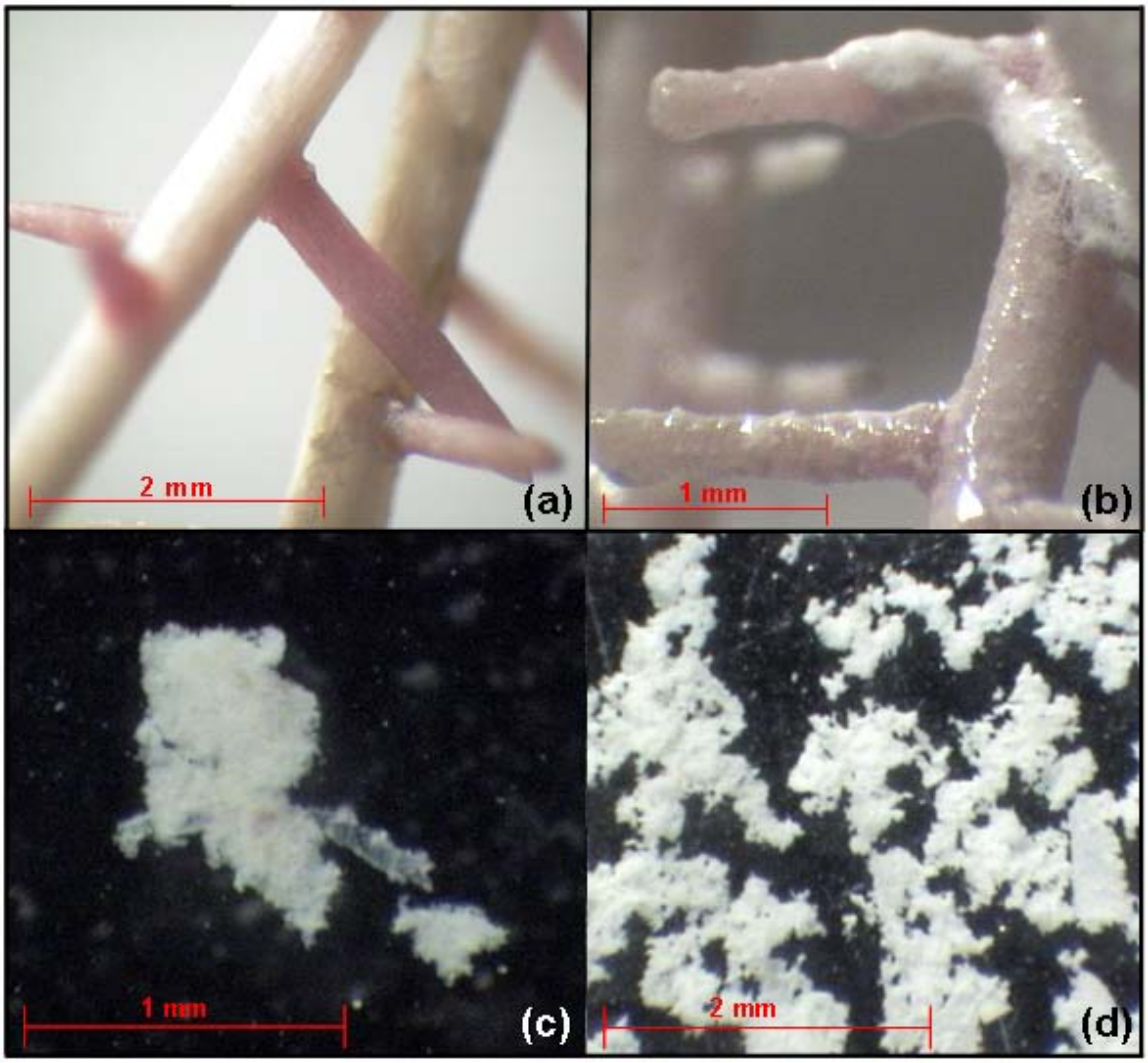

Figure 3. Microscope image of the willow roots and precipitated TiO_2: (a) roots (100 mg L^{-1} P25 TiO_2), (b) roots (100 mg L^{-1} UV100 TiO_2), (c) sedimented TiO_2 (100 mg L^{-1} P25 TiO_2), and (d) sedimented TiO_2 (100 mg L^{-1} UV100 TiO_2).

3 Discussion

3.1 Toxicity of TiO_2 nanoparticles to willow trees

The performed experiments did not show any significant toxic effects of aqueous TiO_2 (size 25 nm and size 100 nm) to willow cuttings. A small, insignificant effect of TiO_2 with particle size of 25 nm could be observed, which was not linearly related to the applied dose. The inability to establish a concentration-response relationship may be linked to aggregation of TiO_2 particles.

3.2 Comparison to other findings

For annual plants, result from the seed germination and the root elongation test are available (Yang et al. 2006, Zheng et al. 2005, Yang and Watts 2005, Lin and Xing 2007, Hond et al. 2005, 2005b).

The study of Zheng et al. (2005) showed that soaking in high-strength TiO_2-nanoparticles-solution (0.25 to 4 ‰) had positive effects on germination of aged spinach seeds and on the growth of seedlings. Best results were found at 2.5 ‰ (2500 mg L^{-1}) nano-TiO_2. Similarly, Yang et al. (2006) could show that nano-TiO2 significantly promoted growth of spinach and accelerated nitrogen assimilation. Hong et al. (2005) also showed that photosensity of chloroplasts was reduced by TiO_2. Due to these positive effects, an application of nanoparticles for seed coating was suggested.

The first report of negative effects of nanoparticles on plants at relatively low dosage was by Yang and Watts (2005). These authors investigated the phytotoxicity of uncoated and phenanthrene-coated nanoscale alumina and concluded that uncoated

alumina particles in 2 mg L^{-1} concentration inhibited the root elongation of corn, cucumber, soybean, cabbage and carrot. It was commented that the toxic effect might not be nanospecific but as well be due to dissolution of aluminium (Murashov 2006).

Lin and Xing (2007) tested the phytotoxicity of five types of nanoparticles, namely multi-wall carbon nanotubes (MWCNT), metallic nanoparticles made from aluminum and zinc, and metal oxides (Al_2O_3 and ZnO) with six different plant species, namely radish, rape, ryegrass, lettuce, corn and cucumber. The germination rate was only affected by Zn and ZnO-particles, and only in rye grass and corn, respectively. In the root elongation test, all plants were affected, and all except corn severely, when suspended in 2000 mg/l nanoparticles. The 50% inhibitory concentrations (IC50) of Zn and ZnO-nanoparticles with a diameter of 20 and 35 nm were estimated to be near 50 mg L^{-1} for radish and near 20 mg L^{-1} for rape and ryegrass.

However, toxicity experiments using the freshwater alga *Pseudokirchnerilella subcapitata* yielded a IC50 of 0.06 mg L^{-1} for nanoparticulate ZnO, bulk ZnO and dissolved $ZnCl_2$, attributable solely to dissolved zinc (Franklin et al. 2007). The authors warned that care must be taken in toxicity testing when the effects may be related to simple solubility.

In summary, both positive and negative significant effects have been observed after exposure of plants to various kinds of nanoparticles, while in our study the observed effect was quite small. Since roots are the first tissue in contact with MNP in solution, toxic symptoms can more be expected in roots than in the shoots. This may be the reason for the lower toxic thresholds, but also the positive effects, of some nanomaterial in the root elongation test (Lin and Xing 2007).

TiO_2 nanoparticles may produce reactive oxygen species upon interaction with the organisms or with ultraviolet radiation (Kus et al. 2006). Accordingly, damaging effects of TiO_2 nanoparticles have been observed on algae, daphnids (Hund-Rinke and Simon 2006) and bacteria (Adams et al. 2006) to be enhanced by sunlight or UV illumination. Even though the suspensions in the willow tree test were not shielded from light, severe damage from photoinduced production of reactive oxygen species was not observed.

3.3 Limitations of the approach

There are several limitations of the approach which have to be kept in mind when relying on the results. First of all, in this study we did not attempt and had no means to determine whether nanoparticles enter the plants and the xylem. Uptake into wood may be a very slow process and not become apparent during the short experimental period. The willow tree toxicity test is a short-term test, which can of course only identify acute toxic effects. A slow accumulation of persistent nanomaterials in the large compartment wood may continue over a long time, and long-term effects at far lower doses can not be excluded. It also needs to be considered that transpiration is regulated from leaves and may continue even though roots are already damaged to some extend.

A shortcoming of the approach may also be the mixing of the test solutions from a stock solution. The high concentration in the stock solution may enhance agglomeration, which in turn lowers the concentration of "free" nanoparticles (Franklin 2007). In cases where effects are related to size and surface, agglomerates are less effective.

3.4 Transferability of results to other conditions

Other MNP. Manufactured particles vary in many properties, for example diameter, surface area, surface charge, active groups, composition and others. Experiments with TiO_2-particles can thus not be transferred to other types of nanomaterial.

Soil. Trees generally do not root in distilled water, but in soil. Nanoparticles in soil might behave different from those in distilled water. Sorption to soil colloids, and reduced mobility is likely. On the other hand, the soil solution contains a relevant fraction of dissolved organic cabon DOC (Schachtschabel et al. 1984). DOC may lead to agglomeration as well as to stabilization of MNP (Franklin et al. 2007). DOC may increase bioavailability of lipophilic compounds by acceleration of diffusive transport (Mayer et al. 2007). On the other hand, adsorption to soil organic matter is likely to reduce the concentration in solution. Thus, a more pronounced effect in soil is not expected.

Other woody species. In earlier experiments with toxic organic chemicals, differences between tree species in uptake, metabolism and phytotoxicity were not very pronounced (Larsen et al. 2004, Yu et al. 2004). Therefore, it is unlikely that the majority of other tree species is more sensitive to an exposure with/to MNP than the basket willows tested in this stuy. However, this does not exclude the possibility that some species might be far more vulnerable than others.

4 Conclusions

The acute toxic effects of manufactured TiO_2-nanoparticles were low. The effects did not follow a clear dose-effect relationship, probably due to the formation of aggregates and subsequent sedimentation. The aggregates were similar in size for both diameters, but more aggregates were formed with the larger particles, and in the presence of tree roots.

Even with increased production of nanomaterials, we do not expect concentrations above 100 mg L^{-1} of manufactured nanoparticles in the soil solution of forest ecosystems in the future. Thus, we may conclude from our results that the acute toxicity of TiO_2-MNP to trees due to physical effects is low.

The preliminary results of this study should be confirmed with other types of MNP, other plant species, experiments in soil and experiments combining longer duration and low exposure concentrations, before a final conclusion in this issue can be made.

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