TEXTE
72/2014

Environmental hazard of selected TiO_2 nanomaterials under consideration of relevant exposure scenarios

Summary

For our Environment

Umwelt Bundesamt

TEXTE 72/2014

Environmental Research of the
Federal Ministry for the
Environment, Nature Conservation,
Building and Nuclear Safety

Project No. (FKZ) 3710 65 413
Report No. (UBA-FB) 001981/E

Environmental hazard of selected TiO_2 nanomaterials under consideration of relevant exposure scenarios

Summary

by

Anne J. Wyrwoll, Hanna M. Maes, Henner Hollert, Andreas Schaeffer
RWTH-Aachen University, Institute for Environmental Research (Biology V),
Aachen, Germany

Anja Meister-Werner, Ralf Petto
Institute for Biological Analysis and Consulting IBACON GmbH, Rossdorf,
Germany

On behalf of the Federal Environment Agency (Germany)

Imprint

Publisher:
Umweltbundesamt
Wörlitzer Platz 1
06844 Dessau-Roßlau
Tel: +49 340-2103-0
Fax: +49 340-2103-2285
info@umweltbundesamt.de
Internet: www.umweltbundesamt.de

/umweltbundesamt.de
/umweltbundesamt

Study performed by:
RWTH-Aachen University, Institute for Environmental Research (Biology V)
Worringerweg 1
52074 Aachen
Germany

Study completed in:
November 2013

Edited by:
Section IV 2.2 Pharmaceuticals, Washing and Cleaning Agents
Dr. Doris Völker

Publication as pdf:
http://www.umweltbundesamt.de/publikationen/environmental-hazard-of-selected-tio2-nanomaterials

ISSN 1862-4804

Dessau-Roßlau, October 2014

The Project underlying this report was supported with funding from the Federal Ministry for the Environment, Nature Conservation, Building and Nuclear safety under project number FKZ 3710 65 413. The responsibility for the content of this publication lies with the author(s).

1 Summary

1.1 Introduction

In the last decades the production and use of nanomaterials increased extensively. The global market for nanotechnology was 11.7 billion US $ in 2009 and 20.7 billion US $ in 2012 (McWilliams 2012). Further increase is expected for the next years (48.9 billion US $ in 2017, McWilliams 2012). Nanomaterials are defined as 'particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50 % or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm-100 nm' (European-Commission 2011/696/EU). Due to the nano scale dimension they have a higher surface to volume ratio than their bulk counterparts resulting in a decisively larger surface area for reactions as e.g. UV activation (e.g. nano titanium dioxide, Wang et al. 2006) or catalytic reactions (e.g. carbon nanotubes, Lu & Wey 2007). They are used in manifold products and applications as e.g. in personal care products (PCP), in food, beverages, paints and plastics, for waste water treatment, ground water remediation, surface coatings or as catalysts (Aitken et al. 2006, Wang et al. 2009, Weir et al. 2012), to name just a few. During their use and production nanomaterials may intentionally or unintentionally enter the environment e.g. during their use for ground water remediation or while showering with personal care products (PCP) that contain nanomaterials. In the latter case, they are washed down the drain, ending up in waste water treatment plants (WWTP) from which they may enter the aquatic or terrestrial environment via the effluent or by adsorbing to sewage sludge which is spread to fields (Gottschalk et al. 2009).

Despite the high scope of nanomaterial production and subsequent release into the environment, the special characteristics of nanomaterials are often not or not sufficiently considered in environmental risk assessment. This can be explained by a lack of specific obligations for nanomaterials within regulations and by the fact that approved and standardized methods (OECD guidelines) have not been sufficiently analyzed for their applicability for nanomaterial testing yet.

In 2006 the Organization for Economic Cooperation and Development (OECD) recognized the gap between the use and knowledge of the environmental risk of nanomaterials and established the Working Party on Manufactured Nanomaterials (WPMN). In the Sponsorship Programme member states and organizations of the OECD WPMN collected safety information on selected manufactured nanomaterials. This information includes data on more than 50 endpoints regarding also endpoints on ecotoxicology. Germany – as one of the members states to the WPMN – is responsible for the collection of data on environmental fate and ecotoxicology for nanosized titanium dioxide (nano-TiO_2). Data on these endpoints should be primarily collected by utilization of OECD test guidelines. However, it is still unclear, if the parameters considered with these test guidelines are sufficient to describe the potential environmental implications of manufactured nanomaterials. Additional considerations, e.g. the observation of more relevant exposure scenarios which are not covered by performing tests according to the OECD guidelines might be of special importance for manufactured nanomaterials. Relevant exposure scenarios are e.g. the conduction of tests with:

I. solar radiation,

II. mixture experiments of nanomaterials and other potential contaminants,

III. testing of embryonic development stages.

Consideration of these scenarios is important because previous studies show, that some nanomaterials have a phototoxic potential, react with co-contaminants or have an influence on embryonic development stages (Asharani et al. 2011, Fan et al. 2011, Ma et al. 2012, Marcone et al. 2012).

Therefore, this project investigated the ecotoxicological hazard of two different sized TiO_2 nanomaterials (Hombikat UV 100 (NM 101), anatase, 7-10 nm and PC 105 (NM 102), anatase 15-25 nm) and one non-nano sized TiO_2 reference material (Tiona AT 1 (NM 100), anatase, 200-220 nm) to organisms inhabiting different environmental compartments. Following standardized tests (OECD guidelines) were used to investigate the influence of these materials on several test organisms:

- *Daphnia* sp., acute immobilization test, Test No. 202 (OECD 2004a)
- Fish embryo acute toxicity (FET) test, Test No. 236 (OECD 2013)
- Activated sludge, respiration inhibition test, Test No. 209 (OECD 2010)
- Earthworm, acute toxicity test, Test No. 207 (OECD 1984)
- Earthworm, reproduction test, Test No. 222 (OECD 2004b)

Thereby, different organisms and effect levels (respiration, mobility, mortality, reproduction, embryonic development) were considered.

As explained above the main focus of the study were tests under relevant exposure scenarios (I-III). Therefore, *Daphnia* sp. acute immobilization tests (OECD 2004a) and activated sludge tests (OECD 2010) were performed with solar radiation. Mixture experiments with nano-TiO_2 and an organic contaminant (the antimicrobial agent triclocarban, TCC) were conducted with the acute and chronic earthworm (OECD 1984, 2004b) and activated sludge respiration tests (OECD 2010). Prior to the mixture toxicity experiments, a literature study was performed to choose a suitable organic compound for the mixture experiments. Effects of the TiO_2 materials on embryonic development were investigated in the fish embryo acute toxicity test (OECD 2013).

Further focus was set on verifying the applicability of the OECD guidelines for testing nanomaterials. Therefore, we assessed, whether the test design of the OECD guidelines, e.g., the medium composition (OECD 2004a) is applicable for testing TiO_2 nanomaterials. Further, it was examined whether by addition of a TiO_2 suspension to soil a reproducible and homogeneous concentration of the TiO_2 materials in the test soil can be considered (OECD 1984, 2004b).

All TiO_2 materials were provided as a contribution to the research in the framework of the Sponsorship Programme of the Working Party of Manufactured Nanomaterials (WPMN) of the OECD. Batches of the nanomaterials PC 105 and Tiona AT 1 were directly received by the manufacturer Cristal Global, corresponding to the batches of the Joint Research Centre (JRC) Nanomaterial Repository NM Series NM 102 and NM 100. Hombikat UV 100 was directly purchased from the JRC NM-Series as NM 101. For the ease of reading all TiO_2 materials are defined as nanomaterials of the JRC NM-Series.

1.2 Material and methods

1.2.1 TiO_2 materials – characterization

NM 101 (Hombikat UV 100, primary particle size (PP): 7-10 nm, 100% anatase, Sachtleben), NM 102 (PC 105, PP: 15-25 nm, 100% anatase, Cristal Global) and NM 100 (Tiona AT 1, PP: 200-220 nm, 100% anatase, Cristal Global).

Dry particles were characterized by transmission electron microscopy (TEM), X-ray diffraction (XRD) and the Brunauer Emmett and Teller method (BET, Brunauer et al. 1938).

1.2.2 Suspension preparation and application

Generally, TiO_2 materials were applied to the test medium by wet application. This means that TiO_2 suspensions were prepared by mixing a specific amount of the TiO_2 material into deionized water which is thereafter treated with an ultrasonication tip (200 W, 0.2 s pulse and 0.8 s pause, Sonopuls HD 2200, Bandelin, Berlin, Germany). Subsequently an aliquot of the stock suspension or an aliquot of their dilutions (with deionized water, working suspension) are applied to the test medium. The hydrodynamic diameter (HD) and zetapotentials of the particles in the stock and working suspensions were characterized by means of dynamic light (DLS) and electrophoretic light scattering (ELS, Malvern Instruments, Worcestershire, United Kingdom).

1.2.3 Ecotoxicity tests

Tests with solar radiation (I) *Daphnia* sp. acute immobilization tests (OECD 2004a, 48 h) were performed under laboratory light (LL) and simulated solar radiation (SSR, 280-1000 nm, UV irradiation: 2.5 mW/cm²) in 10 fold diluted ISO medium. In an additional experiment the Ti concentration was measured in test vessels containing the EC50 concentration (concentration corresponding to 50% effect in the applied test system) of the different TiO_2 materials. EC50 values were calculated from the acute toxicity tests which were previously performed. TiO_2 analysis was carried out with inductively coupled plasma optical emission spectroscopy (ICP-OES) after microwave assisted acid digestion of the water samples (0 h and 48 h). To investigate whether the test medium has an influence on the outcome of the nanomaterial experiments, NM 101 and NM 102 were not only tested in diluted ISO medium but also in ISO medium (ISO 1996) with LL and SSR. Besides the Daphnia tests, activated sludge respiration inhibition tests (OECD 2010) were also run under LL (10, 100, 1000 mg/L) and SSR (100 mg/L; 300-800 nm, irradiation UV: 5 mW/cm²).

Mixture toxicity tests (II) Mixture experiments with nano-TiO_2 and an antimicrobial agent (triclocarban, TCC) were conducted according to the acute (OECD 207; with exposure to 1000 mg/kg TiO_2 and concentrations of TCC in the range of 42-675 mg/kg) and chronic earthworm toxicity test (OECD 222; with exposure of 400 or 1000 mg/kg TiO_2 and concentrations of TCC in the range of 42-675 mg/kg) and activated sludge respiration inhibition test (OECD 209; with exposure of 100 mg/L TiO_2 and 100 mg/L TCC). For the latter test additionally mixture experiments were conducted with 3,5-dichlorophenol (3,5-DCP, 3.2 mg/L), because it was shown that TCC did not inhibit the respiration rate of microorganisms of the activated sludge. TCC concentrations were measured by means of liquid chromatography coupled with mass spectrometry (LC-MS) in soil samples of the earthworm chronic mixture toxicity tests after liquid-solid extraction with acetone. TiO_2 concentrations were measured in soil samples of the acute earthworm toxicity test after acid digestion by means of inductively coupled plasma optical emission spectrometry (ICP-OES).

Generally, in all tests untreated control media (TiO_2 0 mg/kg) and media treated with the single substances were additionally tested for ecotoxicological effects.

Embryonic development (III)   Effects of the TiO_2 materials on embryonic development were investigated according to the fish embryo acute toxicity test (OECD 236; exposure concentrations of 1, 10, 100 mg/L).

1.3 Results and discussion

1.3.1 Particle characterization

The characterization of the dry TiO_2 powders used in this study confirmed the sizes, crystalline structure and BET specific surface areas of the particles given by the manufacturer. Furthermore, it was proven that ultrasonication can be used for the preparation of stock suspensions (1 g/L) resulting in reproducible measurements of the hydrodynamic diameter (HD as a parameter to e.g. indicate agglomeration of particles) and zeta potential (ZP, as a parameter to e.g. indicate particle stability) of the particles. Consequently, this method can be used as instruction for preparing TiO_2 stock suspensions for aquatic ecotoxicity tests. Although, dilution of stock suspensions resulted in most cases in comparable HD values of the particles, ZP values were lower in the dilutions than in the stock suspension. Further research is necessary to investigate whether the preparation of diluted suspensions with regard to the maintenance of stability and homogeneous distribution is possible or limited, because both are relevant properties to assess nanomaterial toxicity. HD values of the particles in the stock suspensions (1 g/L) reveal the lowest HD for the largest (non-nano) sized particle NM 100 (261 nm) followed by NM 101 (512 nm) and NM 102 (625 nm). It is assumed that NM 100 agglomerates already sediment during the DLS measurement so that only small NM 100 particles are left in the water phase. On the other hand smaller agglomerates are readily formed in suspensions of NM 101 and NM 102 resulting in HD way above the primary particle sizes.

1.3.2 Ecotoxicity tests

In the present study nano and non-nano scale TiO_2 materials were tested with standard OECD tests and under consideration of relevant exposure scenarios as simulated solar radiation (SSR), mixture toxicity or embryonic development to mainly investigate the influence of these exposure scenarios on the outcome of the tests. Different sized TiO_2 nanomaterials (NM 101, NM 102) and a non-nanomaterial reference (NM 100) were tested to observe whether the potential effects are size dependent or even only relevant for the nano sized materials. Furthermore, it was of interest whether the standardized test guidelines were applicable for TiO_2 nanomaterial testing.

The standard OECD tests which were performed under laboratory light or darkness (*D. rerio*) revealed following results: Except for NM 101 (NOEC 18.5 mg/L) in the *Daphnia* sp. acute immobilization test the determined NOEC values were at least $\geq$ 50 mg/L. Table 1 summarizes the determined NOEC values:

Tab. 1: NOEC values determined in OECD tests (laboratory light) with NM 101, NM 102 and NM 100.

OECD guideline	Organism	Endpoint (mg/L)	NOEC (mg/L)
OECD 202	*Daphnia magna*	mobility (48 h)	≥ 50[a]
OECD 236	*Danio rerio*	mortality (96 h)	≥ 100
OECD 209	Activated sludge	respriation rate (3 h)	≥ 1000
OECD 207 OECD 222	*Eisenia fetida*	mortality (14 d) reproduction (56 d)	≥ 1000 ≥ 1000

[a] except for NM 101 (NOEC 18.5 mg/L)

In general, these findings are confirmed by studies which tested other TiO_2 nanomaterials with similar concentrations in tests with earthworms (Heckmann et al. 2011, Hu et al. 2010, McShane et al. 2012, Whitfield Åslund et al. 2011), fish embryos (Chen et al. 2011, Zhu et al. 2008) and activated sludge (Zheng et al. 2011). Like in our study, also other studies in which daphnids were exposed to TiO_2 nanomaterials report controversial results, in some cases no effects of the TiO_2 nanomaterials on the mobility of *D. magna* in the mg/L range were observed (Dabrunz et al. 2011, Wiench et al. 2009, Zhu et al. 2010), whereas in others effects in this concentration range were documented (e.g. EC50 33.7 mg/L, Dalai et al. 2013).

In contrast to the tests which were performed according to standardized OECD test guidelines, some studies revealed toxic effects of TiO_2 nanomaterials when guidelines were slightly modified, e.g., when other end points were observed or the test duration was prolonged (Chen et al. 2011, Dabrunz et al. 2011, Zhu et al. 2010): according to Chen et al. (2011) larval swimming reported as average and maximum velocity and the activity level of the *D. rerio* larvae were significantly affected by nano-TiO_2 concentrations of 0.1-1 mg/L (P25, 25-70 nm) after an exposure period of 120 h. Zhu et al. and Dabrunz et al. (2010, 2011) both demonstrated that a slightly prolonged exposure duration resulted in more pronounced effects of nano-TiO_2 to *D. magna*. EC50 values after 72 h and 96 h exposure accounted to 1.62 mg/L (P25, 20% rutile and 80% anatase, 21 nm, Zhu et al. 2010) and 0.73 mg/L (A.100, anatase, 6 nm, Dabrunz et al.). In contrast, EC50 values after 48 h of exposure were calculated as > 100 mg/L.

In our study, we did not consider alternative endpoints or performed tests with prolonged exposure duration, but we investigated whether relevant exposure scenarios, e.g., solar radiation (I), mixture toxicity (II) or embryonic development (III), in standardized OECD tests will influence the outcome of experiments with TiO_2 materials:

Solar radiation (I) In the *Daphnia* sp. acute immobilization test the toxic effects after exposure of *D. magna* to nano sized (NM 101 and NM 102) as well as non-nano sized (NM 100) TiO_2 materials under simulated sunlight illumination (SSR) were considerably increased. Effects were more pronounced for the nanomaterials NM 102 and NM 101 (nominal: EC50 0.53 and 1.28 mg/L considering nominal concentrations) than for the non-nano reference material (nominal: EC50 3.88 mg/L). Based on measured concentrations, the EC50 of e.g. NM 102 (90 µg/L), is close to the predicted nano-TiO_2 concentration in the aquatic environment (µg/L range, Gottschalk et al. (2009). Therefore, NM 102 may have environmental implications, especially when considering that the production and use of nano-TiO_2 will rise in the future. However, it remains unclear whether the presence of natural components of surface water, e.g., humic and fulvic acids, may influence the ROS formation of TiO_2 materials; furthermore, it has to be further investigated, whether the measured EC50, based on the TiO_2 concentration in the top water layer represents a worst case scenario or not. To clarify the latter it is necessary to investigate whether the particles in the overlaying water phase or those at the bottom of the test vessel caused the observed SSR induced toxic effect of NM 102. We suggest that the observed phototoxicity did not only depend on one factor as e.g. the photoactivity (ROS formation potential) of the particles but also on other factors as e.g. the agglomeration state of the particles and the particle/daphnia interaction area.

Parallel exposure of activated sludge to the different sized TiO_2 materials and SSR did not inhibit its respiration activity. It is reasonable to suggest that the dissolved and particulate natural organic matter of the activated sludge absorb most of the radiation responsible for the ROS formation by the TiO_2 materials resulting in either no ROS formation or in ROS levels too low to induce toxic effects.

Mixture toxicity (II) Mixture experiments with activated sludge revealed that the different sized TiO_2 materials did not alter the toxicity of organic compounds, i.e., the organic compound triclocarban (TCC) and the toxic reference compound 3,5-dichlorophenol (3,5-DCP), for the microbial communities in activated sludge.

In contrast to the activated sludge respiration tests, the different sized TiO_2 materials changed the acute and chronic toxicity of TCC to the earthworm *E. fetida* in some tests: Generally, the toxicity of TCC was either not altered or toxicity was lower in presence of the TiO_2 materials compared to the exposure of earthworms with TCC alone. This can be seen e.g. in the acute mixture experiments showing a lower mortality of *E. fetida* when they were simultaneously exposed to TCC and to the two larger TiO_2 materials (NM 102 LC10 not calculable, or NM 100 LC10 489 mg/kg dw (dry weight) soil) than when they were exposed to the TCC treatment groups without TiO_2 addition (LC10 243 mg/kg dw soil). Chronic earthworm mixture experiments of the test sequence A (performed at IBACON GmbH) demonstrated that effects of TCC (EC50 243 mg/kg dw soil) on the reproduction of *E. fetida* are less pronounced at high NM 101 concentrations (400 and 1000 mg/kg; EC50 308 and 384 mg/kg dw soil). TCC analysis of soil samples of the latter test confirmed that TCC was not degraded during the test period of 56 days, i.e., lowering the TCC concentration by metaboliszation is not responsible for the observed differences in toxicity in the mixture tests with TCC and NM 101. In test sequence B (performed in the laboratory of RWTH Aachen University) a lower effect of TCC on the reproduction of *E. fetida* was observed compared to test sequence A. To ensure that earthworms are exposed to the test soil, test vessels are illuminated for 16 h. Slight differences in the illumination intensity might have caused the slight variations in TCC toxicity between the two test sequences. However, a TCC (alone) test series and the corresponding mixture toxicity test series with TiO_2 were conducted in each testsequence so that a direct comparison of the mixture and the TCC alone test series is possible. As in the acute toxicity tests the addition of a lower level of NM 102 or NM 100 (400 mg/kg dw soil, EC50 not calculable or 1031 mg/kg dw soil, respectively) to TCC applied soil resulted in less pronounced effects in test sequence B, whereas a higher application level (1000 mg/kg) resulted in comparable effects (EC50 692 or 494 mg/kg dw soil, respectively) than after exposure to TCC without TiO_2 materials (EC50 956 mg/kg dw soil). However, this study does not explain the mechanisms behind the influence of the TiO_2 particles on the chronic toxicity of TCC towards *E. fetida*, except that no degradation of TCC was responsible for the lower effect of TCC in the presence of NM 101. We suggest that TCC adsorbed to the TiO_2 materials which were either not taken up by the earthworms or were taken up but TCC was not remobilized from the particles in their gut, resulting in a lower bioavailability of TCC. It is noteworthy that the survival (test duration 14 d) and reproduction (test duration 56 d) of earthworms exposed to the TiO_2 materials alone were not affected.

Embryonic development (III) In the fish embryo acute toxicity test (OECD 236) no sublethal and lethal effects of the different sized TiO_2 materials on the embryonic development of *D. rerio* were observed within an exposure of 96 h (preliminary study) and 72 h (main experiment).

In general, our experiments in which relevant exposure scenarios during the testing of TiO_2 were considered show that this has an influence on the outcome of ecotoxicity tests. Especially testing simultaneously with solar radiation is very important for the environmental risk assessment of TiO_2 nanomaterials because in our study it was shown that wavelengths of solar radiation induced the toxicity of those to *D. magna*. Neglecting the photoactivity of TiO_2 nanomaterials may lead to an underestimation of the environmental risk of them. .

One further focus of our study was to investigate whether potential effects of the tested TiO_2 materials are dependent on particle size or even more on nano specific characteristics. As the tested TiO_2 materials only exhibited toxic effect in the *Daphnia* sp. acute immobilization test with SSR, statements on this question can be only made for this test system: SSR induced not only the toxicity of the TiO_2 nanomaterials NM 101 and NM 102 but also of the non nano reference NM 100. Consequently, the results of our study indicate that the toxicity is not related to nanomaterial specific characteristics but to TiO_2 materials specific characteristics as e.g. photoactivity. Non-nano scale TiO_2 materials are also known to be photoactive (Almquist & Biswas 2002). Furthermore studies exist, showing that photoactivity among other factors depends on particle size (Allen et al. 2008, Almquist & Biswas 2002, Wang et al. 2006). From our studies, we conclude that TiO_2 toxicity is dependent on particle size but is not limited to nanomaterials. Moreover, for an adequate risk assessment of nano scale and non-nano scale TiO_2 materials we see the necessity to prove whether the materials are photoactive e.g. by performing a screening test for photoactivity. When nanomaterials excert photoactivity we recommend performing ecotoxicity tests with solar radiation when such exposure is relevant for the ecosystem to be tested. This finding may also be relevant for the testing of other nanomaterials.

Besides studying the influence of particle size and specific characteristics of nanomaterials as well as relevant exposure scenarios for the environmental risk assessment we investigated whether the relevant standardized OECD test guidelines are applicable for testing TiO_2 nanomaterials:

Due to strong agglomeration of TiO_2 nanomaterials no constant exposure concentration can be reached. Thus, a concentration gradient develops with low concentrations in the upper overlaying water phase and high concentrations at the test vessel bottom (sedimentation). Considering that it is not known whether the particles in the overlaying water phase or those at the test vessel bottom cause the observed toxic effects the question arises on which concentration the EC50 value should be based. Furthermore, the sampling method for water samples will surely influence the outcome of the determined TiO_2 concentrations. To compare the results of different studies a standardized sampling procedure needs to be established, also with respect on how to prepare suspensions of the TiO_2 materials. Therefore, guidance with respect to define criteria for particle stability is urgently needed.

We again point out the necessity for screening nanomaterials for their ROS formation potential and to develop guidance for including solar radiation in standardized OECD guidelines used for testing photoactive chemicals and nanomaterials.

In the *Daphnia* sp. acute immobilization test (OECD 2004a) we also investigated the influence of medium composition on the extent of the nanomaterial toxicity by testing with ISO medium and 10fold diluted ISO medium. We observed that nanomaterial toxicity, especially for NM 102, was more pronounced in the diluted ISO medium (EC50 0.5 mg/L) than in the ISO medium (EC50 1.1 mg/L). We suggest that in line with the DLVO theory the lower ionic strength in the diluted ISO medium resulted in less agglomeration of the particles in the diluted ISO than in the ISO medium and therefore higher bioavailability/interaction of the particles for/with the exposed daphnids and consequently to a higher toxicity. On the other hand, variability was more pronounced in the diluted ISO medium than in the ISO medium and because differences in toxicity between the two media were not that pronounced we recommend also for nanomaterials to maintain testing in undiluted ISO medium.

In the fish embryo acute toxicity tests (OECD 2013) agglomeration of the TiO_2 materials in aqueous suspensions poses not only the problem of a none-constant exposure concentration but also the problem that it is not possible to perform a pre exposure of the embryos as recommended in the guideline. This is not possible because particles would agglomerate during the egg selection period, so that the concentration in the pre exposure would not be homogeneous. Addition of this inhomogeneous pre-exposure medium to the main test medium would therefore alter the concentration of the main test medium. As a consequence, embryos in older cell stages (8-64) would have to be used and would have to be transferred directly to the main test medium.

In the earthworm tests the tendency of TiO_2 particles to agglomerate did not cause a problem because we were able to apply the particles homogeneously and reproducibly to the soil. This was confirmed by ICP-OES measurements of digested TiO_2 spiked soil samples indicating that the wet application method used in this study can be recommended for the spiking of TiO_2 nanomaterials to natural soils. Thus, the earthworm acute toxicity and earthworm reproduction OECD test guidelines (OECD 1984, 2004b) are applicable for testing TiO_2 nanomaterials as far as recommendations for the preparation and application of nanomaterial suspensions are given in the guidelines.

The guideline for testing TiO_2 nanomaterials in the activated sludge respiration inhibition test (OECD 2010) is appropriate, even though the TiO_2 materials are used in an aqueous suspension, because constant stirring and aeration of the test medium ensures a continuous mixing of the particles with the test medium thereby preventing sedimentation of the particles and ensuring a constant exposure concentration.

1.4 Conclusion

We confirmed that the used TiO_2 test materials were of different particle size, BET specific surface area and of the same crystalline structure in accordance with the information of the providers. Applying standardized OECD tests under laboratory light or darkness we observed no toxic effects to the test organisms except for NM 101 which had a negative effect on the mobility of *D. magna* at concentrations much higher than those expected in the environment. Considering relevant exposure scenarios, e.g., solar radiation, mixture toxicity and embryonic development, during our tests revealed that especially solar radiation has a strong influence on the toxicity of nano as well as non-nano scale TiO_2 materials. SSR in the *Daphnia* sp. acute immobilization test (OECD 2004a) induced toxicity of the TiO_2 material in the low mg/L range when based on nominal concentrations and in the µg/L range when based on analytically measured concentrations. The mixture experiments with earthworms and activated sludge show that in any of the performed tests the toxicity of the organic compound was not enhanced in the presence of the different sized TiO_2 materials. Apparently, toxicity of the organic compounds was either lowered or not altered in their presence. Fish embryo acute toxicity tests demonstrated that neither of the TiO_2 materials altered the embryonic development of *D. rerio* under the conditions tested.

The solar radiation test further indicates that the SSR induced toxicity of the TiO_2 materials was not a nano specific characteristic because SSR induced the toxicity of nano as well as non-nano scale TiO_2 materials. However, SSR induced toxicity was size dependent showing lower EC50 values for the nanomaterials than for the non-nano reference material. We suggest that the observed phototoxicity did not only depend on one factor as e.g. the photoactivity (ROS formation potential) of the particles but also on other factors as e.g. the agglomeration state of the particles and the particle/daphnia interaction area.

It can be concluded that the acute earthworm (OECD 1984), earthworm reproduction (OECD 2004b) and activated sludge respiration inhibition (OECD 2010) tests are applicable for testing TiO_2 materials due to homogeneous distribution of the TiO_2 materials in these test media. For the earthworm tests it was proven that the used wet application method resulted in a homogeneous and reproducible application of the TiO_2 materials to the test soil and in the activated sludge test aeration and mixing ensures the distribution of the particles in the test medium. However, the tendency of the particles to agglomerate and to sediment causes problems for testing TiO_2 nanomaterials in the *Daphnia* sp. acute immobilization (OECD 2004a) and fish embryo acute toxicity (OECD 2013) tests because a TiO_2 concentration gradient quickly develops in the test vessel with low concentrations in the overlaying water phase and high concentration at the test vessel bottom. This problem includes difficulties in determining the exact exposure concentrations and the necessity to standardize the water sampling method. The development of guidance is needed to adapt current aquatic ecotoxicty test guidelines with respect to define criteria for particle stability in stock and test media. ISO medium can be recommended for the *Daphnia* sp. acute immobilization (OECD 2004a) test.

The present study shows the necessity of considering the phototoxicity of nano and non-nano scale TiO_2 materials in their environmental risk assessment, e.g., by conducting ecotoxicity tests with simultaneous irradiation by sunlight. Neglecting the influence of sunlight results in a clear underestimation of the environmental risk associated with TiO_2 materials. It should be mandatory to test the ROS formation potential also for other nanomaterials before conducting ecotoxicity tests.

Summing up, realistic exposure scenarios are necessary to properly assess the potential environmental risks of TiO_2 materials.

1.5 Outlook

One of the main outcomes of our study is the requirement to perform more ecotoxicity tests in the presence of simulated solar radiation.

At least fish embryo acute toxicity tests with the tested TiO_2 materials should be repeated in the presence of solar radiation.

Regarding the *D. magna* tests further research is necessary to observe whether the documented toxicity is dependent on the TiO_2 concentration at the bottom layer or on the overlaying water concentration. These results would give advice on which concentration the EC50 should be based. Furthermore, it would be interesting to test not only in clear ISO water but in water containing natural organic matter (NOM) to investigate the influence of NOM on the phototoxicity of TiO_2 materials.

The mechanisms responsible for the lowered acute and chronic earthworm toxicity of the organic compound in the presence of the TiO_2 materials has to be further evaluated e.g. by investigating whether TCC adsorbs to the TiO_2 materials.

In our study it was shown that the SSR induced toxicity of the different sized TiO_2 materials was, although not a nanospecific effect, particle size dependent. This indicates the necessity to test each TiO_2 material differing in size unless a considerable approach to categorize nanomaterials was agreed on. Considering the high diversity of TiO_2 materials and the much higher diversity of nanomaterials in general, it is recommended to establish a screening tool for photoactive substances.

It should be emphasized that the non nano reference (NM 100) also exhibited toxic effects to *D. magna* when illuminated with SSR. Thus, phototoxicity is not limited to nanosized TiO_2 materials and more non nano scale TiO_2 materials should be tested under SSR in ecotoxicity tests.

2 Literatur

Aitken RJ, Chaudhry MQ, Boxall ABA, Hull M (2006): Manufacture and use of nanomaterials: current status in the UK and global trends. Occup Med-Oxford 56, 300-306

Allen NS, Edge M, Verran J, Stratton J, Maltby J, Bygott C (2008): Photocatalytic titania based surfaces: environmental benefits. Polymer degradation and stability 93, 1632-1646

Almquist CB, Biswas P (2002): Role of synthesis method and particle size of nanostructured TiO_2 on its photoactivity. Journal of Catalysis 212, 145-156

Asharani PV, Yi LW, Gong ZY, Valiyaveettil S (2011): Comparison of the toxicity of silver, gold and platinum nanoparticles in developing zebrafish embryos. Nanotoxicology 5, 43-54

Brunauer S, Emmett PH, Teller E (1938): Adsorption of gases in multimolecular layers. Journal of the American Chemical Society 60, 309-319

Chen TH, Lin CY, Tseng MC (2011): Behavioral effects of titanium dioxide nanoparticles on larval zebrafish (*Danio rerio*). Marine Pollution Bulletin 63, 303-308

Dabrunz A, Duester L, Prasse C, Seitz F, Rosenfeldt R, Schilde C, Schaumann GE, Schulz R (2011): Biological Surface Coating and Molting Inhibition as Mechanisms of TiO_2 Nanoparticle Toxicity in *Daphnia magna*. Plos One 6

Dalai S, Pakrashi S, Chandrasekaran N, Mukherjee A (2013): Acute Toxicity of TiO_2 Nanoparticles to *Ceriodaphnia dubia* under Visible Light and Dark Conditions in a Freshwater System. PloS one 8, e62970

European-Commission (2011/696/EU): Commission Recommendation on the definition of nanomaterials. Official Journal of the EU L 275/38. 20.10.2011

Fan WH, Cui MM, Liu H, Wang CA, Shi ZW, Tan C, Yang XP (2011): Nano-TiO_2 enhances the toxicity of copper in natural water to *Daphnia magna*. Environmental Pollution 159, 729-734

Gottschalk F, Sonderer T, Scholz RW, Nowack B (2009): Modeled Environmental Concentrations of Engineered Nanomaterials (TiO_2, ZnO, Ag, CNT, Fullerenes) for Different Regions. Environmental Science & Technology 43, 9216-9222

Heckmann L-H, Hovgaard MB, Sutherland DS, Autrup H, Besenbacher F, Scott-Fordsmand JJ (2011): Limit-test toxicity screening of selected inorganic nanoparticles to the earthworm *Eisenia fetida*. Ecotoxicology 20, 226-233

Hu C, Li M, Cui Y, Li D, Chen J, Yang L (2010): Toxicological effects of TiO_2 and ZnO nanoparticles in soil on earthworm *Eisenia fetida*. Soil Biology and Biochemistry 42, 586-591

ISO (1996): Water quality -- Determination of the acute lethal toxicity of substances to a freshwater fish [*Brachydanio rerio* Hamilton-Buchanan (Teleostei, Cyprinidae)] -- Part 1: Static method ISO 7346-1:1996. ISO

Lu CY, Wey MY (2007): The performance of CNT as catalyst support on CO oxidation at low temperature. Fuel 86, 1153-1161

Ma H, Brennan A, Diamond SA (2012): Photocatalytic reactive oxygen species production and phototoxicity of titanium dioxide nanoparticles are dependent on the solar ultraviolet radiation spectrum. Environmental Toxicology and Chemistry 31, 2099-2107

Marcone GPS, Oliveira AC, Almeida G, Umbuzeiro GA, Jardim WF (2012): Ecotoxicity of TiO_2 to *Daphnia similis* under irradiation. Journal of Hazardous Materials 211, 436-442

McShane H, Sarrazin M, Whalen JK, Hendershot WH, Sunahara GI (2012): Reproductive and behavioral responses of earthworms exposed to nano-sized titanium dioxide in soil. Environmental Toxicology and Chemistry 31, 184-193

McWilliams A (2012): Nanotechnology: A Realistic Market Assessment. Acessed 15.01.2014. BCC Research, http://www.reportlinker.com/p096617-summary/Nanotechnology-A-Realistic-Market-Assessment.html

OECD (1984): Earthworm, Acute Toxicity Tests, Test Guideline No. 207. Guidelines for the testing of chemicals, OECD, Paris

OECD (2004a): *Daphnia* sp., Acute Immobilisation Test , Test Guideline No. 202. Guidelines for the testing of chemicals, OECD, Paris

OECD (2004b): Earthworm Reproduction Test (*Eisenia fetida*/*Eisenia andrei*) , Test Guideline No. 222. Guidelines for the testing of chemicals, OECD, Paris

OECD (2010): Activated Sludge, Respiration Inhibition Test (Carbon and Ammonium Oxidation), Test Guideline No. 209. Guidelines for the testing of chemicals, OECD, Paris

OECD (2013): Fish Embryo Acute Toxicity (FET) Test, Test Guideline No. 236. Guidelines for the testing of chemicals, OECD, Paris

Wang N, Zhao CL, Shi ZX, Shao YW, Li HW, Gao N (2009): Co-incorporation of MMT and MCM-41 nanomaterials used as fillers in PP composite. Mater Sci Eng B-Adv 157, 44-47

Wang XH, Li JG, Kamiyama H, Moriyoshi Y, Ishigaki T (2006): Wavelength-sensitive photocatalytic degradation of methyl orange in aqueous suspension over iron(III)-doped TiO_2 nanopowders under UV and visible light irradiation. J Phys Chem B 110, 6804-6809

Weir A, Westerhoff P, Fabricius L, Hristovski K, von Goetz N (2012): Titanium Dioxide Nanoparticles in Food and Personal Care Products. Environmental Science & Technology 46, 2242-2250

Whitfield Åslund ML, McShane H, Simpson MJ, Simpson AJ, Whalen JK, Hendershot WH, Sunahara GI (2011): Earthworm sublethal responses to titanium dioxide nanomaterial in soil detected by 1H NMR metabolomics. Environmental science & technology 46, 1111-1118

Wiench K, Wohlleben W, Hisgen V, Radke K, Salinas E, Zok S, Landsiedel R (2009): Acute and chronic effects of nano-and non-nano-scale TiO_2 and ZnO particles on mobility and reproduction of the freshwater invertebrate *Daphnia magna*. Chemosphere 76, 1356-1365

Zheng X, Chen Y, Wu R (2011): Long-Term Effects of Titanium Dioxide Nanoparticles on Nitrogen and Phosphorus Removal from Wastewater and Bacterial Community Shift in Activated Sludge. Environmental Science & Technology, null-null

Zhu X, Chang Y, Chen Y (2010): Toxicity and bioaccumulation of TiO_2 nanoparticle aggregates in *Daphnia magna*. Chemosphere 78, 209-215

Zhu XS, Zhu L, Duan ZH, Qi RQ, Li Y, Lang YP (2008): Comparative toxicity of several metal oxide nanoparticle aqueous suspensions to Zebrafish (*Danio rerio*) early developmental stage. J Environ Sci Heal A 43, 278-284