TEXTE
72/2014

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

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

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).

Index

List of figures

List of tables

Abbreviations

3,4-DCA	3,4 dichloroaniline
3,5-DCP	3,5-dichlorophenol
BAF	Bioaccumulation factor
BET	Brunauer Emmett Teller
BSAF	Biota-sediment accumulation factor
CAS	Chemical abstract service
CL	Confidence limit
C_{mm}	Mean measured exposure concentration
C_{org}	Organic carbon content
DLVO	Derjaguin, Landau, Verwey, Overbeek
DLS	Dynamic light scattering
dw	Dry weight
ECX	Concentration corresponding to X% effect
ELS	Electrophoretic light scattering
FET	Fish embryoacute toxicity test
GLP	Good laboratory praxis
H_2O_2	Hydrogen peroxide
$HClO_4$	Perchloric acid
HCRW	10% higher concentrateted reconstituted water
HF	Hydrogen fluoride
HNO_3	Nitric acid
hpf	Hours post fertilization
ICP-OES	Inductively coupled plasma with optical emission spectrometry
IME	Frauenhofer Institute for Molecular Biology and Applied Ecology
ISO	International Organization for Standardization
JRC	Joint Research Center
LC-MS	Liquid chromatography coupled with mass spectrometry
LCX	Concentration corresponding to X% lethal effects
LL	Laboratory light
LOEC	Lowest observed effect concentration
LOQ	Limit of quantification
Max. WHK	Maximum water holding capacity of soil
MHRW	Moderately hard reconstituted water

Nano-TiO_2	Titanium dioxide nanomaterial
NHM	Natural History Museum, London
NM	Nanomaterial
NOEC	No observed effect concentration
NOM	Natural organic matter
OC	Organic compound
OECD	Organisation for Economic Cooperation and Development
PCA	p-Chloroaniline
PCP	Personal care product
PDI	Poly dispersity index
PP	Primary particle size
Qnano	QualityNano Research Infrastructure
RefeSol 01-A	Natural soil recognized by the German Federal Environment Agency
ROS	Reactive oxygen species
SOP	Standard operating procedure
SSR	Simulated solar radiation
TCC	Triclcocarban
TEM	Transmission electron microscopy
UBA	Federal Environment Agency, Germany
UV	Ultra violet radiation
UVA	Ultra violet A radiation
UVB	Ultra violet B radiation
WPMN	Working Party of Manufactured Nanomaterials
Ww	Wet weight
WWTP	Waste water treatment plant
XRD	X-ray diffraction

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. 2012a, 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)	$\geq$ 50[a]
OECD 236	*Danio rerio*	mortality (96 h)	$\geq$ 100
OECD 209	Activated sludge	respriation rate (3 h)	$\geq$ 1000
OECD 207 OECD 222	*Eisenia fetida*	mortality (14 d) reproduction (56 d)	$\geq$ 1000 $\geq$ 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 Zusammenfassung

2.1 Einleitung

Im letzten Jahrzehnt hat sich die Produktion und die Anwendung von Nanomaterialien vervielfacht: So lag der Weltmarkt für Nanotechnolgie 2009 bei 11.7 Milliarden US $ und 2012 bei 20.7 Milliarden US $ (McWilliams 2012). Ein weiterer Anstieg wird für die nächsten Jahre prognostiziert (48,9 Milliarden US $ in 2017, McWilliams 2012). Die Europäische Kommission empfiehlt folgende Definition für Nanomaterialien: „Ein „Nanomaterial“ ist ein natürliches, bei Prozessen anfallendes oder hergestelltes Material, das Partikel in ungebundenem Zustand, als Aggregat oder als Agglomerat enthält, und bei dem mindestens 50% der Partikel in der Anzahlgrößenverteilung ein oder mehrere Außenmaße im Bereich von 1 nm bis 100 nm haben“ (European-Commission 2011/696/EU). Aufgrund der nanoskaligen Dimension haben Nanomaterialien ein größeres Oberfläche-Volumen Verhältnis als ihre nicht nanoskaligen Gegenstücke. Dies bedingt eine größere Oberfläche für Reaktionen wie z.B. photokatalytische Reaktionen (z.B. Nano-Titandioxid, Wang et al. 2006) bzw. katalytische Reaktionen (z.B. Kohlenstoff-Nanoröhrchen, Lu & Wey 2007). Nanomaterialien werden in einer Vielzahl von Produkten und Anwendungen eingesetzt wie z.B. in Körperpflegeprodukten, Lebensmitteln, Getränken, Farben und Plastik, sowie für Abwasserbehandlung und Grundwasserremediation, als Oberflächenbeschichtungen oder als Katalysatoren (Aitken et al. 2006, Wang et al. 2009, Weir et al. 2012). Während der Produktion und Verwendung von Nanomaterialien können diese bewusst oder unbewusst in die Umwelt gelangen, z.B. wenn sie zur Grundwasserremdiation eingesetzt werden, oder während des Duschens mit Körperpflegeprodukten, die Nanomaterialien enthalten. Im letzten Fall gelangen Nanomaterialien über das Abwasser in Kläranlagen und können somit potentiell in die aquatische Umwelt bzw. über das Ausbringen von Klärschlamm in die terrestrische Umwelt gelangen (Gottschalk et al. 2009). Trotz des exzessiven Anstiegs der Nanomaterialproduktion und dem damit verbundenen Eintrag in die Umwelt werden die spezifischen Eigenschaften von Nanomaterialien bisher noch nicht in der Umweltrisikobeurteilung beachtet. Erklärt werden kann dies zum einen durch das Fehlen von nanospezifischen Verpflichtungen innerhalb von Regulierungen und zum anderen dadurch, dass geprüfte und standardisierte Methoden (z.B. OECD Richtlinien) bis heute noch nicht ausreichend auf ihre Eignung für Nanomaterialien geprüft worden sind.

2006 realisierte die Organisation für Wirtschaftliche Zusammenarbeit und Entwicklung (OECD) die immer größer werdende Lücke zwischen der Verwendung von Nanomaterialien und den Kenntnissen zu ihrem potentiellen Umweltrisiko und gründete die Working Party on Manufactured Nanomaterials (WPMN). Innerhalb dieses Förderprogrammes sammeln die Mitgliedsländer und Organisation der OECD WPMN Informationen zur Sicherheit von ausgewählten synthetischen Nanomaterialien. Diese Informationen umfassen Daten zu mehr als 50 Endpunkten u.a. auch zu ökotoxikologischen Endpunkten. Deutschland – als eins der Mitgliedsländer – ist innerhalb der OECD WPMN dafür verantwortlich, Daten über Umweltverhalten und -verbleib und die Ökotoxikologie von nanoskaligem TiO_2 zu erheben. Diese Daten sollen hauptsächlich über die Anwendung von standardisierten OECD Testrichtlinien ermittelt werden. Bis heute ist unklar, ob mit den jeweiligen Endpunkten, welche in diesen Richtlinien gefordert werden, ausreichend die möglichen Umweltauswirkungen synthetischer Nanomaterialien erfasst werden können. Für Nanomaterialien könnte es z.B. zusätzlich wichtig sein, relevante Expositionsszenarien wie Sonnenlicht (I), Mischungstoxizität (II) und embryonale Entwicklung (III), welche nicht alle in den OECD Richtlinien vorgeschrieben sind, während ihrer Testung zu beachten. Es ist wichtig, diesen Szenarien Beachtung zu schenken, da vorige Studien zeigen, dass manche Nanomaterialien wie TiO_2-NM ein phototoxisches

Potential besitzen, mit anderen Co-Kontaminanten interagieren oder einen Einfluss auf embryonale Entwicklungsstadien haben können (Asharani et al. 2011, Fan et al. 2011, Ma et al. 2012a, Marcone et al. 2012).

Daher wurde in diesem Projekt das ökotoxikologische Gefährdungspotential von zwei verschieden großen TiO_2 Nanomaterialien (Hombikat UV 100 (NM 101), Anatas-Struktur, 7-10 nm und PC 105 (NM 102), Anatas-Struktur 15-25 nm) und einem nicht nanoskaligen TiO_2 Referenzmaterial (Tiona AT 1 (NM 100), Anatas-Struktur, 200-220 nm) für Organismen aus verschiedenen Umweltkompartimenten untersucht. Folgende standardisierte Tests (OECD Richtlinien) wurden dabei angewendet:

- Akuter Daphnien Immobilisationstest, Test Nr. 202 (OECD 2004a)
- Akuter Fischembryo Toxizitätstest, Test Nr. 236 (OECD 2013)
- Belebtschlamm Atmungshemmungstest, Test Nr. 209 (OECD 2010)
- Regenwurm, akuter Toxizitätstest, Test Nr. 207 (OECD 1984)
- Regenwurm, Reproduktionstest, Test Nr. 222 (OECD 2004b)

Die ausgewählten Testrichtlinien decken verschiedene Testorganismen (Daphnien, Fische, Bakterien und Regenwürmer) und Endpunkte ab (Atmung, Mortalität, Mobilität, Reproduktion und embryonale Entwicklung).

Wie weiter oben erklärt, lag der Hauptfokus des Projektes darauf, die TiO_2 Materialien unter relevanten Expositionsszenarien zu testen. Daher wurden (I) der akute Daphnien Immobilisationstest (OECD 2004a) und der Belebtschlamm Respirations Hemmungstest (OECD 2010) unter Bestrahlung mit simuliertem Sonnenlicht durchgeführt. Mischungsexperimente (II) mit TiO_2 und einem organischen Schadstoff (der antimikrobiellen Substanz Triclocarban, TCC) wurden mit dem akuten und chronischen Regenwurmtest (OECD 1984, 2004b) sowie dem Belebtschlamm Respirations Hemmungstest (OECD 2010) durchgeführt. Um einen geeigneten Schadstoff auszuwählen, fand eine ausführliche Literaturstudie statt. In akuten Fischembryo Toxizitätstests (OECD 2013) wurde untersucht, ob die TiO_2 Materialien die embryonale Entwicklung beeinflussen (III).

Ein weiterer Fokus des Projektes war zu überprüfen, ob die verwendeten OECD Richtlinien für die Testung von Nano-TiO_2 geeignet sind. Hierfür wurde ermittelt, ob das vorgeschriebene Testdesign, z.B. die Komposition des Mediums (OECD 2004a) für die Testung von Nano-TiO_2 geeignet ist. Weiterhin wurde untersucht, ob sich durch die Applikation einer TiO_2 Suspension auf Boden reproduzierbar homogene Konzentrationen im Boden herstellen lassen (OECD 1984, 2004b).

Alle TiO_2 Materialien wurden als Beitrag zur Forschung des Förderprogrammes der OECD WPMN bereitgestellt. Chargen der TiO_2 Materialien PC 105 und Tiona AT 1 wurden direkt durch den Hersteller Cristal Global zur Verfügung gestellt. Diese Chargen entsprechen den Nanomaterial Repository NM Serien NM 102 und NM 100 des Joint Research Centre (JRC). Hombikat UV 100 bzw. die NM-Serie NM 101 wurde direkt vom JRC erworben. Um die Lesbarkeit zu erleichtern, werden die TiO_2 Materialien im Folgenden nur noch mit ihren JRC NM Serien Namen NM 100, NM 101 und NM 102 bezeichnet.

2.2 Material und Methoden

2.2.1 TiO_2 Materialien – Charakterisierung

NM 101 (Hombikat UV 100, primäre Partikelgröße (PP): 7-10 nm, 100% Anatas, Sachtleben), NM 102 (PC 105, PP: 15-25 nm, 100% Anatas, Cristal Global) und NM 100 (Tiona AT 1, PP: 200-220 nm, 100% Anatas, Cristal Global).

Die TiO_2 Pulver wurden mittels Transmissionselektronenmikroskopie (TEM), Röntgenbeugung (XRD) und mittels der Bunauer Emmett und Teller Methode (BET, Brunauer et al. 1938) analysiert.

2.2.2 Herstellung und Charakterisierung der Suspensionen

Generell wurden die TiO_2 Materialien als wässrige Suspensionen ins Testmedium eingebracht, indem eine definierte Menge des TiO_2 Materials in deionisiertem Wasser mit einem Ultraschallfinger suspendiert wurde (200 W, 0.2 s Puls und 0.8 s Pause, Sonopuls HD 2200, Bandelin, Berlin, Deutschland). Anschließend wurden ein Teil der Stamm-Suspension oder daraus mit deionisiertem Wasser hergestellte Verdünnungen (Arbeitssuspension) zum Testmedium gegeben. Der hydrodynamische Durchmesser (HD) und das Zeta Potential (ZP) der Partikel in den Stock- und Arbeitssuspensionen wurden mittels dynamischer und elektrophoretischer Lichtstreuung (DLS und ELS) gemessen (Malvern Instruments, Worcestershire, Großbritanien).

2.2.3 Ökotoxizitätstests

Experimente mit Sonnenlicht (I) Akute Daphnien Immobilisationstests (OECD 2004a, 48 h) wurden unter Laborlicht (LL) und unter simuliertem Sonnenlicht (SSL, 280-1000 nm, UVA/UVB Bestrahlungsstärke: 2.5 mW/cm²) in 10-fach verdünntem ISO Medium durchgeführt. In einem zusätzlichen Experiment wurde die Ti-Konzentration im Überstand des ISO Medium gemessen. In diesem Versuch entsprach die nominale TiO_2 Konzentration der EC50 Konzentration (Konzentration bei welcher 50% des untersuchten Effektes im Testsystem auftritt) des jeweiligen TiO_2 Materials. Die Wasserproben (0 h und 48 h) wurden mittels induktiv gekoppeltem Plasma mit optischer Emissionsspektroskopie (ICP-OES) analysiert, nachdem sie mit Mikrowellen unterstütztem Säureaufschluss behandelt wurden. Um zu untersuchen, ob das Testmedium einen Einfluss auf die Ergebnisse der Ökotoxizitätstests der Nanomaterialien hat, wurden NM 101 und NM 102 zusätzlich in unverdünntem ISO Medium (ISO 1996) mit LL und SSR getestet. Zusätzlich zu den Daphnientests wurden auch der Belebtschlamm Atmungshemmungstest (OECD 2010) mit LL (10, 100, 1000 mg/L) und SSL (100 mg/L; 300-800 nm, irradiation UV: 5 mW/cm²) durchgeführt.

Experimente mit Mischungen (II) Mischungsexperimente mit nano-TiO_2 und einem organischen Biozid (Triclocarban, TCC) wurden in Anlehnung an folgende OECD Richtlinien angesetzt: Regenwurm akuter Toxizitätstest (OECD 207, 14 d; mit Exposition gegenüber 1000 mg/kg TiO_2 und TCC Konzentrationen zwischen 42-675 mg/kg), Regenwurm Reproduktionstest (OECD 222, 56 d; mit Exposition gegenüber 400 oder 1000 mg/kg TiO_2 und TCC Konzentrationen zwischen 42-675 mg/kg), sowie Belebtschlamm Atmungshemmungstest (OECD 209, 3 h; mit Exposition gegenüber 100 mg/L TiO_2 und 100 mg/L TCC). Im letzten Test wurden zusätzlich Mischungsexperimente mit nano-TiO_2 und 3,5-Dichlorophenol (mit Exposition gegenüber 100 mg/L TiO_2 und 3,5-DCP, 3.2 mg/L) durchgeführt, da gezeigt werden konnte, dass TCC die Respirationsrate von Mikroorganismen des Belebtschlamms nicht beeinträchtigte. 3,5-Dichlorophenol wurde daher als weitere, toxische Substanz verwendet. TCC wurde in Bodenproben des Regenwurm Reproduktionstests (TCC und NM 101) mittels Flüssigkeits-Chromatographie

gekoppelt mit Massenspektrometrie (LC-MS) analysiert, nachdem die Bodenproben mit Aceton extrahiert wurden. Ti-Konzentrationen wurden mittels ICP-OES in Bodenproben des akuten Regenwurmtests vermessen. Zuvor wurden die Bodenproben mit Hilfe eines Mikrowellen unterstützen Säureaufschlusses behandelt. In jedem Ökotoxizitätstest wurde auch nicht behandeltes Kontrollmedium (TiO_2 und TCC 0 mg/kg) und Medium, welches nur mit den Einzelsubstanzen behandelt wurde, untersucht.

Embryonale Entwicklung (III) Effekte der TiO_2 Materialien auf die embryonale Entwicklung wurden nach der Richtlinie "Akuter Fischembryotoxizitätstest" untersucht (OECD 236, 96 h; Expositionskonzentration von TiO_2: 1, 10, 100 mg/L).

2.3 Ergebnisse und Diskussion

2.3.1 TiO_2 Material Charakterisierung

Die Charakterisierung der TiO_2 Pulver bestätigte die Herstellerangaben zur Partikelgröße, kristallinen Struktur und BET spezifischen Oberfläche. Des Weiteren zeigten die Ergebnisse aus DLS (hydrodynamischer Durchmesser, HD, beschreibt das Agglomerationsverhalten der Partikel) und ELS (ZP, Zeta Potential, beschreibt die Stabilität der Partikel in der Suspension) Messungen, dass mit der verwendeten Ultraschallmethode die Partikel reproduzierbar in den Stamm-Suspensionen (1 g/L) verteilt werden konnten. Demzufolge kann diese Methode als Anweisung für die Herstellung von TiO_2 Stamm-Suspensionen für aquatische Ökotoxizitätstests verwendet werden. Obwohl die Verdünnung der Stamm-Suspensionen in den meisten Fällen zu einem vergleichbaren HD der Partikel führte, waren die absoluten Werte der ZP der Partikel in den Verdünnungen niedriger als die der Partikel in der Stocksuspension. Weitere Untersuchungen sind notwendig, um zu überprüfen, ob die Nutzung von verdünnten Suspensionen in Bezug auf die Beibehaltung von identischer Stabilität und homogene Verteilung der Partikel zulässig ist, da beide Faktoren notwendig sind, um Nanomaterialtoxizität zu untersuchen. Die HD der Partikel in den Stamm-Suspensionen (1 g/L) sind am kleinsten für das größte Material NM 100 (261 nm), gefolgt von NM 101 (512 nm) und NM 102 (625 nm). Wir nehmen an, dass NM 100 Agglomerate schon während der DLS Messung sedimentieren, so dass nur die NM 100 Partikel in der Wasserphase verbleiben und gemessen werden. Die DLS Messung der NM 101 und NM 102 Suspensionen zeigen, dass sich in der Stocksuspension Agglomerate gebildet haben, deren HD viel größer waren als die zugehörigen primären Partikelgrößen.

2.3.2 Ökotoxizitätstests

In dieser Studie wurden nano- und nicht nanoskalige TiO_2 Materialien nach standardisierten OECD Tests und zusätzlich unter Beachtung von relevanten Expositionsszenarien wie simuliertem Sonnenlicht (SSL), Mischungstoxizität, sowie Effekte auf die embryonale Entwicklung getestet, um zu untersuchen, ob diese Expositionsszenarien einen Einfluss auf den Ausgang der Experimente haben. Um festzustellen, ob die Primärpartikelgröße einen Einfluss auf die potentiellen Effekte der TiO_2 Materialien hat bzw. es sich sogar um nanospezifische Effekte handelt, wurden verschieden große TiO_2 Nanomaterialien (NM 101, NM 102) und ein Nicht-Nano Referenz Material (NM 100) getestet. Von weiterem Interesse war, ob die standardisierten Testrichtlinien für die Testung von Nanomaterialien geeignet sind.

Die standardisierten OECD Tests, welche unter Laborlicht oder Dunkelheit (*D. rerio*) stattfanden, erbrachten folgende Ergebnisse: Außer NM 101 (NOEC 18.5 mg/L) im Daphnien Immobilisationstest wurde für alle Materialien NOEC Werte vom mindesten $\geq$ 50 mg/L bestimmt. Tabelle 1 fasst die ermittelten NOEC Werte zusammen:

Tab. 1: NOEC Werte der standardisierten OECD Tests (Laborlicht) mit NM 101, NM 102 und NM 100.

OECD Richtlinie	Organismus	Endpunkt (mg/L)	NOEC (mg/L)
OECD 202	*Daphnia magna*	Mobilität (48 h)	$\geq$ 50[a]
OECD 236	*Danio rerio*	Mortalität (96 h)	$\geq$ 100
OECD 209	Belebtschlamm	Respirations Rate (3 h)	$\geq$ 1000
OECD 207 OECD 222	*Eisenia fetida*	Mortalität(14 d) Reproduktion(56 d)	$\geq$ 1000 $\geq$ 1000

[a] außer für NM 101 (NOEC 18.5 mg/L)

Im Allgemeinen bestätigen Studien, in denen andere TiO_2 Nanomaterialien in ähnlichen Konzentrationen mit Regenwürmern (Heckmann et al. 2011, Hu et al. 2010, McShane et al. 2012, Whitfield Åslund et al. 2011), Fischembryonen (Chen et al. 2011, Zhu et al. 2008) und Belebtschlamm (Zheng et al. 2011) getestet wurden, die von uns gefundenen Ergebnisse. Wie in unserer Studie fanden auch andere Studien, welche Daphnien mit TiO_2 Nanomaterialien exponierten, widersprüchliche Ergebnisse. Manche Materialien beeinträchtigten die Mobilität der Daphnien im mg/L Bereich nicht (Dabrunz et al. 2011, Wiench et al. 2009, Zhu et al. 2010), wohingegen andere in diesem Konzentrationsbereich die Mobilität der Daphnien reduzierten (z.B. EC50 33.7 mg/L, Dalai et al. 2013).

Im Gegensatz zu Studien, welche nach standardisierten OECD Richtlinien durchgeführt wurden, wurden in manchen Studien toxische Effekte von TiO_2 Nanomaterialien beobachtet, wenn andere Endpunkte beobachtet wurden oder die Testdauer verlängert wurde (Chen et al. 2011, Dabrunz et al. 2011, Zhu et al. 2010): Das Schwimmverhalten, beschrieben durch die durchschnittliche und maximale Geschwindigkeit, sowie das Aktivitätsmaß von *D. rerio* Larven wurde von Nano-TiO_2 Konzentrationen zwischen 0,1-1 mg/L nach einer Exposition von 120 h signifikant reduziert (Chen et al. 2011). Zhu et al. und Dabrunz et al. (2010, 2011) zeigten beide, dass eine leicht verlängerte Expositionsdauer zu stärkeren Effekten von nano-TiO_2 auf *D. magna* führte. Die ermittelten EC50 Werte lagen nach 72 h und 96 h Exposition bei 1.62 mg/L (P25, 20% Rutil und 80% Anatas, 21 nm, Zhu et al. 2010) und 0.73 mg/L (A.100, Anatas, 6 nm, Dabrunz et al.). Hingegen lagen die EC50 Werte nach 48 h Exposition bei > 100 mg/L.

In unsere Studie zogen wir keine alternativen Endpunkte in Betracht und verlängerten die Expositionszeit nicht, sondern untersuchten, ob die Beachtung von relevanten Expositionsszenarien wie z.B. Sonnenlicht (I), Mischungstoxizität (II) oder embryonale Entwicklung (III) in standardisierten OECD Tests einen Einfluss auf das Ergebnis der Experimenten mit TiO_2 Materialien hat.

Sonnenlicht (I) Der Daphnien Immobilisationstest demonstrierte, dass SSL die Toxizität aller TiO_2 Materialien im Vergleich zu den Tests unter Laborlicht induzierte bzw. steigerte. Des Weiteren konnte festgestellt werden, dass dieser Effekt für die Nanomaterialien NM 101 und NM 102 (nominal: EC50 0.53 and 1.28 mg/L) stärker ausgeprägt war als für das Nicht-Nano Material NM 100 (nominal: EC50 3.88 mg/L).

Wird der EC50 Wert auf die analytisch gemessenen TiO_2 Konzentrationen bezogen, so liegt der EC50 Wert für z.B. NM 102 (90 µg/L) nah an der modellierten Nano-TiO_2 Konzentration für die aquatische Umwelt (µg/L Bereich, Gottschalk et al. 2009). Demzufolge könnte NM 102 zu Umweltschäden führen, vor allem, wenn beachtet wird, dass die Nano-TiO_2 Produktion in den nächsten Jahren weiter ansteigen wird. Jedoch ist noch unklar, ob die Anwesenheit von natürlichen Komponenten des Oberflächenwassers wie z.B. Humin- und Fulvinsäuren einen Einfluss auf Phototoxizität und ROS Bildung der TiO_2 Materialien hat. Des Weiteren bleibt zu untersuchen, ob die gemessene EC50 Konzentration, welche sich auf die TiO_2 Konzentration in der

obersten Wasserschicht bezieht, ein „worst case scenario“ repräsentiert oder nicht. Um Letzteres zu klären, ist es notwendig zu untersuchen, ob die Partikel in der oberen Wasserphase oder die am Boden sedimentierten Partikel die beobachtete Phototoxizität der TiO_2 Materialien bedingen. Letztendlich vermuten wir, dass die gefundene Phototoxizität der TiO_2 Materialien nicht nur abhängig von einem Faktor, wie z.B. der Photoaktivität (ROS Bildungs Potential) der Partikel ist, sondern zusätzlich von weiteren Faktoren, wie z.B. dem Agglomerationsgrad der Partikel und/oder dem Grad an Partikel/Daphnien Interaktion, abhängt.

Die gleichzeitige Exposition von Belebtschlamm gegenüber verschieden großen TiO_2 Materialien und SSL hatte keinen Effekt auf die Respirationsrate. Es kann angenommen werden, dass das gelöste und partikuläre organische Material des Belebschlamms die meiste Strahlung absorbierte, welche für die Induktion der ROS Bildung durch die TiO_2 Materialien verantwortlich gewesen wäre. Dadurch kam es entweder zu keiner ROS Bildung, oder nur zu einer niedrigen ROS Bildung, welche keine negativen Effekte auf die Respirationsrate des Belebtschlamms hatte.

Mischungstoxizität (II) In den Mischungsexperimenten mit Belebtschlamm wurde kein Einfluss der verschieden großen TiO_2 Materialien auf die Toxizität des organischen Schadstoffes Triclocarban und des toxischen Referenzmaterials 3,5-Dichlorophenol (3,5-DCP) auf die Mikroorganismen des Belebtschlamms festgestellt.

Im Gegensatz zu den Belebtschlammtests änderten die verschieden großen TiO_2 Materialien die akute und chronische Toxizität von TCC auf den Regenwurm *E. fetida* in einigen der Tests: Im Allgemeinen wurde die Toxizität von TCC entweder nicht verändert, oder sie war niedriger in der Anwesenheit der TiO_2 Materialien als in den Experimenten, in denen Regenwürmer nur mit TCC exponiert wurden. Dies wird besonders deutlich in den akuten Mischungsexperimenten, in denen die Mortalität von *E. fetida* geringer war, wenn Regenwürmer gegenüber TCC und den beiden größeren TiO_2 Materialien exponiert wurde (NM 102 LC10 nicht berechenbar, oder NM 100 LC10 489 mg/kg TG (Trockengewicht) Boden) als wenn sie nur mit TCC exponiert wurden (LC10 243 mg/kg TG Boden). Chronische Regenwurm Mischungsexperimente der Testsequenz A (welche bei IBACON GmbH durchgeführt wurde) zeigten, dass die Reproduktion in Anwesenheit einer hohen NM 101 Konzentration (400 und 1000 mg/kg; EC50 308 und 384 mg/kg TG Boden) durch TCC weniger beeinträchtigt wurde als wenn die Würmer nur mit TCC exponiert wurden (EC50 243 mg/kg TG Boden). Die Ergebnisse der TCC Analytik der Bodenproben lassen darauf schließen, dass ein Abbau von TCC nicht für die beobachteten niedrigeren Effekte auf die Reproduktion in Anwesenheit von TCC und NM 101 verantwortlich war, da TCC innerhalb des Testzeitraumes von 56 Tagen in Anwesenheit von NM 101 nicht abgebaut wurde.
In der Testsequenz B (welche an der RWTH Aachen durchgeführt wurde) wurde die Reproduktion durch TCC weniger stark beeinträchtigt als es in dem TCC Test der Testsequenz A beobachtet wurde. Um sicherzustellen, dass die Regenwürmer dem Testboden ausgesetzt sind, werden die Testgefäße für 16 h pro Tag beleuchtet. Leichte Unterschiede in der Bestrahlungsintensität haben evtl. die Unterschiede in der TCC Toxizität zwischen den beiden Testsequenzen bedingt. Jedoch wurde in jeder Testsequenz eine Testreihe mit nur TCC angesetzt, so dass ein direkter Vergleich der Mischungsexperimente mit dem jeweiligen TCC Test möglich ist. Die Zugabe einer niedrigen NM 102 und NM 100 Konzentration (400 mg/kg TG Boden, EC50 nicht berechenbar bzw. 1031 mg/kg TG Boden) zu TCC behandeltem Boden führt zu einer geringeren Beeinträchtigung der Reproduktion, während die Zugabe einer höheren Konzentration (1000 mg/kg) zu einer ähnlichen Beeinträchtigung (EC50 692 bzw. 494 mg/kg TG Boden) wie bei Zugabe von TCC als Einzelsubstanz (EC50 956 mg/kg TG Boden). Diese Studie erklärt nicht die Mechanismen, welche zu den beobachteten geringeren Effekten von TCC in Anwesenheit der TiO_2 Materialien geführt hat. Wir nehmen an, dass TCC an die TiO_2 Materialien adsorbierte, welche anschließend entweder nicht durch die

Regenwürmer aufgenommen wurden, oder von welchen TCC im Darm der Würmer nicht remobilisiert und somit nicht aufgenommen wurde. TiO_2 Materialien alleine führten zu keiner Mortalität der Regenwürmer (Testdauer 14 d) und zu keiner Veränderung der Reproduktion (Testdauer 56 d) im Vergleich zu den Kontrollen.

Embyonale Entwicklung (III) Im akuten Fischembryo Toxizitätstest (OECD 236) wurden keine subletalen und letalen Effekte durch die verschieden großen TiO_2 Materialien auf die embryonale Entwicklung von *D. rerio* nach einer Exposition von 96 h (Vorversuch) und 72 h (Hauptversuch) festgestellt.

Im Allgemeinen zeigen die Experimente unserer Studie, dass relevante Expositionsszenarien einen signifikanten Einfluss auf die Ergebnisse von Ökotoxizitätstest haben. Als besonders wichtig für die Umweltrisikobewertung von TiO_2 Materialien stellte sich die Berücksichtigung von Sonnenlicht heraus, das die Toxizität der TiO_2 Materialien auf *D. magna* drastisch erhöhte. Wird die Phototoxizität der TiO_2 Materialien vernachlässigt, wird das Umweltrisiko von TiO_2 Materialien unterschätzt.

Ein weiterer Fokus unserer Studie war die Untersuchung, ob potentielle Effekte der getesteten TiO_2 Materialien von der Partikelgröße bzw. von nanospezifischen Eigenschaften abhängen. Da toxische Effekte der getesteten TiO_2 Materialien nur im Daphnien Immobilisationstest mit SSL beobachtet wurden, können Aussagen darüber nur für dieses Testsystem getroffen werden: SSL induzierte nicht nur die Toxizität der TiO_2 Nanomaterialien NM 101 und NM 102, sondern auch die des Nicht-Nano Referenz Materials NM 100. Dies verdeutlicht, dass die Toxizität nicht nur auf nanospezifischen Eigenschaften beruht sondern auf den Eigenschaften der TiO_2 Materialien selbst (Photoaktivität). Es ist bekannt, dass nicht nanoskalige TiO_2 Materialien auch photoaktiv sind (Almquist & Biswas 2002). Aus den Ergebnissen unserer Studie lässt sich schlussfolgern, dass die Phototoxizität der TiO_2 Materialien zwar von der Partikelgröße abhängt, jedoch nicht nanospezifisch ist. Des Weiteren lässt sich aus den Ergebnissen ableiten, dass es für eine adäquate Umweltrisikoüberprüfung nanoskaliger und nicht nanoskaliger TiO_2 Materialien notwendig ist, zu prüfen, ob die Materialien photoaktiv sind, z.B. indem die Materialien auf Photoaktivität untersucht werden. Bei positivem Befund empfehlen wir, dass Ökotoxizitätstests mit Sonnlicht durchgeführt werden, wenn ein solches Expositionsszenarium relevant für den jeweiligen Test ist.

Zusätzlich haben wir untersucht, ob die standardisierten OECD Richtlinien für TiO_2 Materialien anwendbar sind:

Aufgrund starker Agglomeration der TiO_2 Nanomaterialien war es nicht möglich, eine konstante Expositionskonzentration in den aquatischen Tests zu gewährleisten. Demzufolge nehmen wir an, dass sich innerhalb der Testgefäße rasch ein Konzentrationsgradient ausbildet mit niedrigen Konzentrationen in der oberen Wasserphase und hohen Konzentrationen am Boden des Testgefäßes (Sedimentation). Bedenkt man, dass unbekannt ist, ob die Partikel in der oberen Wasserphase oder die Partikel am Boden des Testgefäßes die beobachteten toxischen Effekte bedingen, stellt sich die Frage, auf welche Konzentration die Effekte bezogen werden sollen. Des Weiteren muss angenommen werden, dass die Probenahme-Methode einen Einfluss auf die gemessenen TiO_2 Konzentrationen hat, z.B. durch Variation der Eintauchtiefe von Messpipetten in der Suspension. Um die Vergleichbarkeit zwischen Studien zu gewährleisten, ist es notwendig, die Probenahme-Methode sowie die Suspensionsherstellung der TiO_2 Materialien zu standardisieren. Für letzteres ist es dringend notwendig, standardisierte Verfahren zu entwickeln.

Wir möchten an dieser Stelle noch einmal die Notwendigkeit betonen, Nanomaterialien auf ihr ROS Bildungspotential zu testen sowie eine Anleitung zu verfassen, die es ermöglicht, den Einfluss von Sonnenlicht in standardisierten OECD Tests zu beachten, um photoaktive Chemikalien und Nanomaterialien unter Sonnenlicht zu testen.

Im Daphnien Immobilisationstest (OECD 2004a) haben wir zusätzlich den Einfluss der Testmediumzusammensetzung auf das Ausmaß der TiO_2 Nanomaterialtoxizität untersucht, indem wir Tests mit den Nanomaterialien NM 101 und NM 102 sowohl in ISO als auch in 10fach verdünntem ISO Medium durchgeführt haben. Hierbei stellten wir fest, dass besonders für NM 102 die Toxizität in verdünntem ISO Medium (EC50 0.5 mg/L) stärker ausgeprägt war als in ISO Medium (EC50 1.1 mg/L). Dies kann mit der DLVO Theorie erklärt werden, welche besagt, dass bei niedriger Ionenstärke (verdünntes ISO Medium) Partikel weniger stark agglomerieren als Partikel in Medium mit einer höheren Ionenstärke (ISO Medium). Aufgrund der niedrigeren Agglomeration ist anzunehmen, dass die Partikel im verdünnten ISO Medium besser bioverfügbar sind und somit stärker mit Daphnien in Wechselwirkung treten als Partikel im ISO Medium. Auf der andren Seite war die Variabilität der beobachteten Effekte in verdünntem ISO Medium ausgeprägter als in ISO Medium. Weil jedoch die beobachtete Toxizität bei Verwendung der beiden Medien keine großen Unterschiede zeigten, empfehlen wir für TiO_2 Materialien weiterhin in ISO Medium zu testen.

Im Fischembryo Toxizitätstest (OECD 2013) bedingt die Agglomeration der TiO_2 Materialien nicht nur eine sich ändernde Expositionskonzentration, sondern birgt auch das Problem, dass es nicht möglich ist, den Test wie in der Richtlinie beschrieben durchzuführen, da keine Vorexposition durchgeführt werden kann. Der Grund hierfür ist, dass Partikel schon während der Auswahlphase der Fischeier agglomerieren würden; es entsteht ein Konzentrationsgradient. Werden die Eier in einem Teil der Vorexposition ins tatsächliche Testmedium überführt, kann sich hierdurch die Konzentration im tatsächlichen Testmedium verändern. Deshalb ist es notwendig, ältere Zell Stadien (8-64; nach der Eiauswahl) in das Testmedium direkt zu überführen, anstatt die Eier zuerst in eine Vorexposition zu überführen.

Die Ergebnisse der TiO_2 Analytik in den Bodenproben des Regenwurmtests zeigen, dass in den Regenwurmtests trotz rascher Agglomerationstendenz die TiO_2 Partikel homogen und reproduzierbar in den Boden eingebracht werden konnten. Dies bestätigt, dass die verwendete Methode der Suspensions-Applikation verwendet werden kann, um TiO_2 Nanomaterialien auf natürliche Böden zu applizieren. Demzufolge sind der akute und chronische Regenwurm OECD Test (OECD 1984, 2004b) für die Testung von TiO_2 Materialien geeignet, sofern Empfehlungen für die Herstellung und Applikation der TiO_2 Nanomaterialien auf Boden in den Richtlinien gegeben werden.

Die Belebtschlamm Atmungshemmung OECD Richtlinie (OECD 2010) ist für die Testung von TiO_2 Nanomaterialien geeignet, obwohl die TiO_2 Materialien in wässriger Suspension verwendet werden, weil in diesen Tests eine konstante Mischung und Belüftung des Testmediums vorgeschrieben ist. Dieses gewährleistet eine konstante Durchmischung der Partikel und des Testmedium und verhindert somit das Absedimentieren agglomerierter Partikel und sichert somit eine konstante Expositionskonzentration.

2.4 Fazit

In unserer Studie bestätigten wir die vom Hersteller angegebenen Partikelgrößen, BET spezifischer Oberflächen und Kristallstruktur der verwendeten TiO_2 Testmaterialien. Wurden standardisierte OECD Tests unter Laborlicht oder Dunkelheit durchgeführt, wurden keine toxischen Effekte auf die Testorganismen festgestellt bis auf NM 101, welches in hohen Konzentrationen, die weit höher lagen als die zu erwartende Umweltkonzentration, einen negativen Effekt auf die Mobilität von *D. magna* hatte. Tests, die unter Beachtung von relevanten Expositionsszenarien wie Sonnenlicht, Mischungstoxizität und embryonaler Entwicklung stattfanden, zeigten, dass insbesondere die Beachtung von Sonnenlicht einen starken Einfluss auf die Toxizität von nano- als auch nichtnanoskaligen TiO_2 Materialien hatte. SSL im Daphnien Immobilisationstest (OECD 2004a) induzierte die Toxizität der TiO_2 Materialien: Effektkonzentrationen lagen im niedrigen mg/L Bereich, wenn sie auf die nominalen Konzentrationen bezogen wurden bzw. im µg/L Bereich, wenn die analytisch verifizierten Konzentrationen zugrunde gelegt werden. Die Mischungsexperimente mit Regenwürmern und Belebtschlamm zeigen, dass in keinem der durchgeführten Tests die Toxizität des organischen Schadstoffes in der Anwesenheit der TiO_2 Materialien erhöht wurde. Offensichtlich wurde die Toxizität des organischen Biozids in der Anwesenheit der TiO_2 Materialien entweder verringert oder nicht verändert. Die akuten Fisch Embryo Toxizitätstests zeigten, dass keines der TiO_2 Materialien die embryonale Entwicklung von *D. rerio* unter den getesteten Bedingungen (in Dunkelheit) beeinflusste.

Die Tests mit Sonnenlicht demonstrieren des Weiteren, dass SSL nicht nur die Toxizität der Nanomaterialien, sondern auch die des Nicht-Nano Materials erhöhte. Dennoch zeigt die Studie, dass die SSL induzierte Toxizität abhängig von der Partikelgröße war, da die EC50 Werte der Nanomaterialien niedriger waren als die der Nichtnanoreferenz. Wir vermuten, dass die festgestellte Phototoxizität nicht nur von einem Faktor wie der Photoaktivität (ROS Bildungspotential) der Partikel abhängt, sondern auch von anderen Faktoren, wie z.B. dem Agglomerationsgrad der Partikel und der Interaktionsfläche zwischen Partikeln und Daphnien.

Es kann zusammengefasst werden, dass der akute Regenwurmtest (OECD 1984), der Regenwurm Reproduktionstest (OECD 2004b) und der Belebtschlamm Respirations Inhibitionstest (OECD 2010) für die Testung von TiO_2 Nanomaterialien geeignet sind, da eine homogene Verteilung der TiO_2 Materialien im Testmedium gewährleistet werden kann. Für die Regenwurmtests konnte bestätigt werden, dass die angewendete Applikationsmethode zu einer homogenen und reproduzierbaren Applikation der TiO_2 Materialien auf Boden führte, während für den Belebtschlammtest eine homogene Verteilung der Partikel im Medium auf Grund ständiger Durchmischung der Partikel mit dem Testmedium angenommen werden kann. Jedoch bedingt in wässrigen Suspensionen die Neigung der Partikel zur Agglomeration und zur anschließenden Sedimentation Probleme für die Testung der TiO_2 Partikel im Daphnien Immobilisationstest (OECD 2004a) und im Fischembryo Toxizitätstest (OECD 2013), da eine konstante TiO_2 Konzentration nicht gewährleistet werden kann. Dieses hat auch zur Folge, dass es nicht möglich ist, die exakte Expositionskonzentration zu bestimmen, und es zeigt die Notwendigkeit, die Wasserprobenahme-Methode zu optimieren, um die Vergleichbarkeit zwischen Studien zu gewährleisten. Wir empfehlen die Entwicklung von modifizierten Anleitungen für aquatische Ökotoxizitätsmessungen, Kriterien für die Stabilität von Partikeln in der Stammlösung und in den Testmedien definiert werden. ISO Medium kann für die Testung von TiO_2 Nanomaterialien im Daphnien Immobilisationstest (OECD 2004a) empfohlen werden.

Diese Studie zeigt die Notwendigkeit, die Phototoxizität von nano- und nicht nanoskaligen TiO_2 Materialien während ihrer Umweltrisikobeurteilung zu beachten, z.B. indem Ökotoxizitätstests unter Bestrahlung mit

Sonnenlicht durchgeführt werden. Wird der Einfluss von Sonnenlicht vernachlässigt, so wird das Umweltrisiko von TiO_2 Materialien unterschätzt. Es sollte verpflichtend sein, das ROS Bildungspotential von Nanomaterialien zu untersuchen, bevor diese in Ökotoxizitätstests untersucht werden.

Zusammenfassend lässt sich sagen, dass es notwendig ist, relevante Expositionsszenarien zu beachten, um das potentielle Umweltrisiko von TiO_2 Materialien genau zu erfassen.

2.5 Ausblick

Eine der Hauptaussagen dieser Studie ist, dass es notwendig ist, Ökotoxizitätstest photoaktiver Materialien unter Sonnenlicht durchzuführen. Dies scheint besonders wichtig für den Fischembryo Toxizitätstest mit TiO_2 Materialien.

Die Daphnien Immobilisationstests haben gezeigt, dass es weiterhin notwendig ist zu untersuchen, ob die beobachtete Toxizität von der TiO_2 Konzentration am Boden der Testgefäße oder im überliegenden Wasser abhängt. Diese Ergebnisse würden Hinweise geben, auf welche Konzentration die Effekte basiert werden sollten. Des Weiteren wäre es interessant, nicht nur in ISO Medium zu testen, sondern zusätzlich in Wasser mit natürlichen organischen Substanzen (z.B. Huminstoffe), um deren möglichen Einfluss auf die Photoxizität der TiO_2 Materialien zu berücksichtigen.

Es bleibt noch zu klären, welche Mechanismen für die geringeren Effekte von TCC in den akuten und chronischen Regenwurmtests in Anwesenheit der TiO_2 Materialien verantwortlich war: dazu sollten Adsorptionsmessungen von TCC an die TiO_2 Materialien durchgeführt werden.

In unserer Studie konnten wir zeigen, dass SSL die Toxiztiät der verschieden großen TiO_2 Materialien unterschiedlich stark induzierte. Dies deutet auf die Notwendigkeit hin, jedes verschieden große TiO_2 Material einzeln zu testen, bis man sich auf eine Vorgehensweise geeinigt hat, wie Nanomaterialien zu kategorisieren sind. Bedenkt man die hohe Diversität an TiO_2 Materialien und die noch größere Diversität von Nanomaterialien im Allgemeinen, empfehlen wir, eine praktikable Methode zur Bestimmung der Photoaktivität von Substanzen/Nanomaterailien zu entwickeln.

An dieser Stelle soll noch einmal betont werden, dass das Nicht-Nano Material (NM 100) unter SSL ebenfalls toxische Effekte auf *D. magna* ausübte. Daher sollten nanoskalige und auch größere TiO_2 Materialien unter SSL in Ökotoxizitätstests getestet werden.

3 Report - 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 are in the size range 1 nm-100 nm' (European-Commission 2011/696/EU). Due to the nanoscale 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 using 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. 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 solar radiation (I), mixture experiments of nanomaterials and other potential contaminants (II) and the testing of embryonic development stages (III). 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. 2012a, Marcone et al. 2012).

Therefore, this project investigated the ecotoxicological risk 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) 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 and embryonic development) were considered.

As explained above the main focuses 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 provided (OECD 1984, 2004b).

Fig. 1 gives an overview of the experiments performed in the present project. Table 1 summarizes the properties of the used TiO_2 materials. All TiO_2 materials were provided as a contribution to the research in the framework of the Sponsorship Programme of the OECD WPMN. Batches of the nanomaterials PC 105 and Tiona AT 1 were directly received by the manufacturer Cristal Global. However, they correspond 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 materials of this report are defined as nanomaterials of the JRC NM-Series.

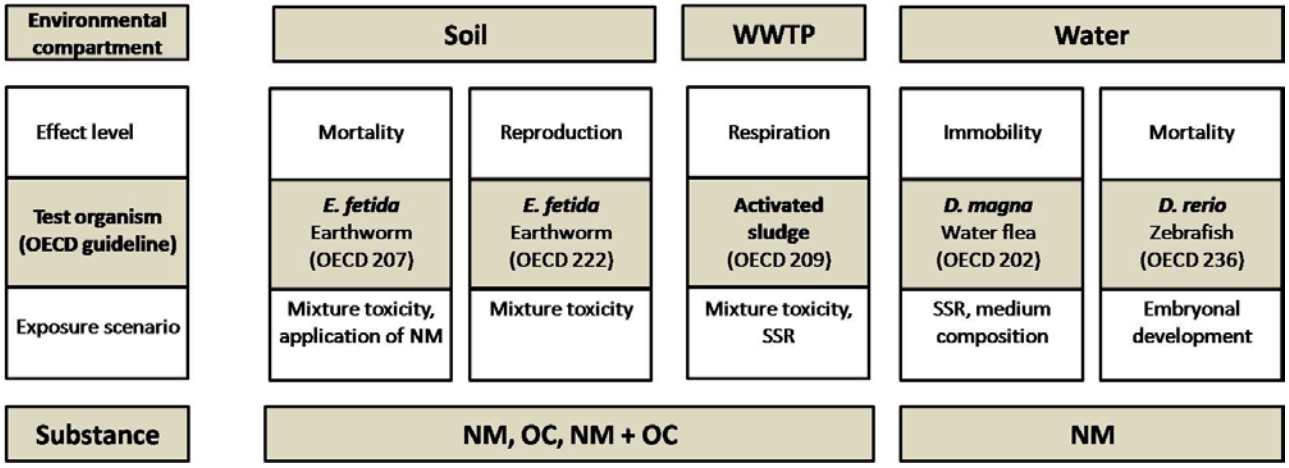

Fig. 1: Overview of the experiments performed in the present study (*NM* nanomaterial, *OC* organic compound, *WWTP* waste water treatment plant, *SSR* simulated solar radiation)

Tab. 1: Properties of the TiO_2 materials used in the project as indicated by the manufacturers.

Property	NM 101 (Hombikat UV 100)	PC 105 (NM 102)	Tiona AT 1 (NM 100)
Manufacturer	Sachtleben	Cristal Global	Cristal Global
Primary particle size (nm)[a]	7-10	15-25	200-220
Crystal structure	anatase	anatase	anatase
BET[a] specific surface area (m^2/g)	320	85	-
Coating	none	none	none

[a] Brunauer Emmett Teller

3.1 Nano titanium dioxide

Nano sized TiO_2 (nano-TiO_2) is one of the most produced nanomaterials (Piccinno et al. 2012). The annual worldwide production of nano-TiO_2 in 2010 was determined to be up to 10.000t/a (Piccinno et al. 2012). Further increase in production is expected for the next decades. However, different estimates exist in the literature: e.g. Robichaud et al. (2009) estimated an exponential increase in production until 2025 resulting in an annual production of 2.5 mil. t/a. In general, a huge diversity of nano-TiO_2 materials exists, differing in the crystalline modification (anatase, rutile and brookite), in size or in the coating used. They are applied especially in PCP as sunscreens and cosmetics or in paints (Aitken et al. 2006). Furthermore, the photo activity of nano-TiO_2 is used in a broad range of products and applications, e.g., for self-cleaning surfaces and for water treatment applications (Pelaez et al. 2012).

Photoactivity of the particles is induced by specific wavelengths of radiation corresponding to the specific band gap energy of the material leading to the formation of an electron-hole pair. Oxygen and water can react with this system yielding in reactive oxygen species (ROS, Fig. 2), capable of degrading organic chemicals. The latter capability may be used in waste water remediation. Beside this beneficial effect, ROS may also induce oxidative stress in organisms (Pan et al. 2009). A study from Ma et al. (2012a) showed that the wavelength range (345-380 nm) corresponding to the band gap energy of the TiO_2 nanoparticle P25 (3.2-3.0 eV) is responsible for the phototoxic effects of P25 towards *Daphnia magna*. These wavelengths are included in solar radiation.

During the lifecycle of nano-TiO_2 it cannot be excluded that it is released into the environment e.g. when TiO_2 containing PCP are washed down the drain it will end up in waste water treatment plants. From here it may enter the aquatic environment via the effluent (estimation: 4 µg/L, Gottschalk et al. 2009) or the terrestrial environment by adsorbing to sewage sludge, which is spread to fields (estimation: increase in sludge treated soil 89 Δµg kg-1 y-1, Gottschalk et al. 2009).

Once released into the environment nano-TiO_2 might pose a risk to organisms living in the specific compartment (e.g. water, soil, sludge). Several studies have already investigated the effects of nano-TiO_2 on different organisms as algae, daphnids, earthworms or fish (Boyle et al. 2013, Chen et al. 2011, Dabrunz et al. 2011, Hartmann et al. 2010, Hund-Rinke 2010, Ma et al. 2012b, Marcone et al. 2012, McShane et al. 2012). Some studies revealed adverse effects of nano-TiO_2 to e.g. daphnids(Dabrunz et al. 2011, Ma et al. 2012b) , fish (Boyle et al. 2013, Chen et al. 2011) or soil microorganisms (Ge et al. 2011), whereas others did not (McShane et al. 2012, Zhu et al. 2008). However, most of these studies did not consider more complex exposure scenarios as e.g. mixture toxicity of solar radiation during testing.

Therefore, we included the consideration of these kinds of relevant exposure scenarios in our ecotoxicity experiments with different sized TiO_2 materials.

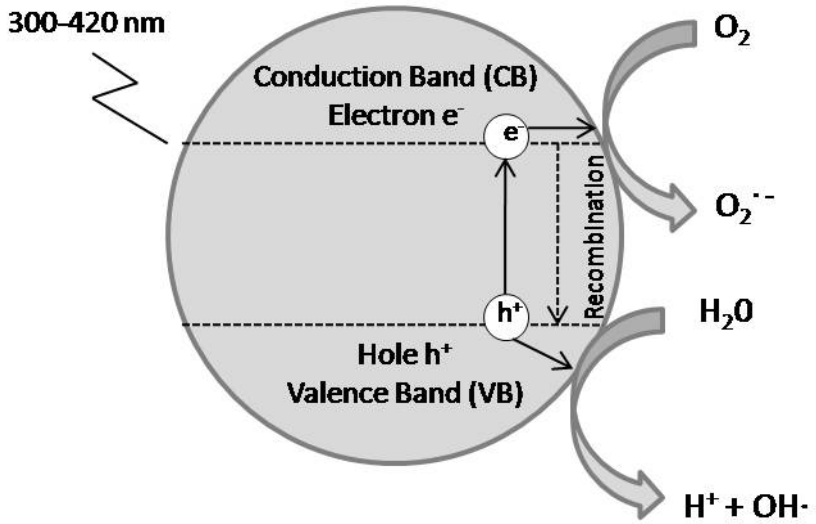

Fig. 2: Formation of reactive oxygen species (ROS, e.g. OH·, $O2^{\cdot-}$) after illumination of nano-TiO_2 with wavelengths between 300-420 nm (modified from Kwon et al. 2008).

4 TiO_2 material characterization

4.1 Material and methods

Dry TiO_2 powder was analyzed by x-ray diffraction (XRD) and by the Brunauer Emmett Teller method. Furthermore, stock suspensions were analyzed by means of dynamic and electrophoretic light scattering (DLS/ELS) and transmission electron microscopy images were taken of particles within diluted stock suspensions.

4.1.1 Chemicals

Following TiO_2 materials were tested: NM 101 (Hombikat UV 100, primary particle size (PP): 7-10 nm, 100% anatase, Sachtleben), PC 105 (NM 102, PP: 15-25 nm, 100% anatase, Cristal Global,) and Tiona AT 1 (NM 100, PP: 200-220 nm, 100% anatase, Cristal Global). 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 TiO_2 material PC 105 and Tiona AT 1 were directly received by the manufacturer Cristal Global. However, they correspond to the batches of the JRC Nanomaterial Repository NM Series NM 100 and NM 102. For the ease of reading all materials of this report are defined as nanomaterials of the JRC NM-Series. Tab. 1 summarizes their properties in regard to primary particle size, crystal structure, BET specific surface area and coating as indicated by the manufacturers.

4.1.2 Transmission electron microscopy (TEM)

NM 101, NM 102 and NM 100 suspensions (2.5 g/L) were prepared as described in the SOP ‘Characterization of a nanomaterial suspension’. After diluting the stock suspensions to 100 mg/L with deionized water, 10 µl of this suspensions were transferred tocopper grids (Plano, Wetzlar, Germany) that were placed on a filter paper. The dry grids were subjected to transmission electron microscopy (TEM; CM 20, Philips, Hamburg, Germany) at the Ernst Ruska-Center for Microscopy and Spectroscopy of the Jülich Research Center, Germany.

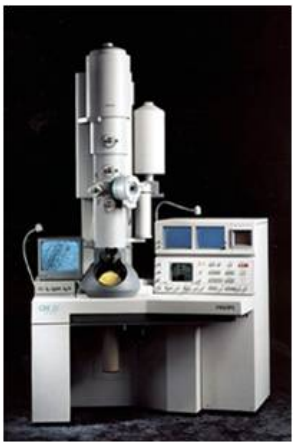

Fig. 3: Transmission electron microscope (CM 20, Philips), Ernst Ruska-Center for Microscopy and Spectroscopy, Jülich Research Center, Germany

4.1.3 X-ray diffraction (XRD)

X-ray diffraction of the TiO_2 powders was carried out on a Enraf Nonius PDS 120 X-ray diffractometer (Bruker, Billerica, MA, U.S.A) equipped with a Co tube at operating conditions 40 kV and 40 mA which emmited monochromatic radiation (K α 1) by using a primary Germanium monochromator and a horizontal slit system (slit size 0.14 mm). A INEL 120 °curved position sensitive detector (PSD; Artenay, France) was used. Rapid data acquisition occurred by simultaneous data collection over 120 ° (2 Θ). The powdered sample was mounted on a zero background holder (sapphire single crystal cut and oriented to give zero background). Measurements were conducted during a stay at the Natural History Museum (NHM, London, Great Britain) which was funded by the Quality Nano (Qnano) Research Infrastructure.

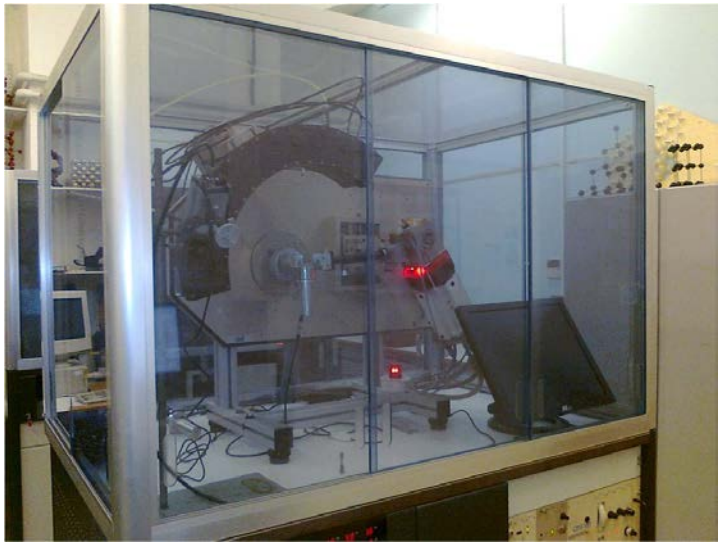

Fig. 4: X-ray powder diffractometer (FR 590 Nonius, Bruker, Billerica, USA), NHM, London, Great Britain

4.1.4 Brunauer Emmett Teller specific surface area (BET)

The BET specific surface area of the TiO_2 powders was measured with a multipoint BET. Analysis was done in the relative pressure range from 0.05 to 0.3, with 5 adsorption points and using equilibrate as analysis mode with samples in liquid nitrogen bath. Prior to BET analysis samples were degassed with N_2 at 100 °C overnight. Further the reference material carbon black (certified surface: 30.6 ± 0.75 m²/g; Micromeritics, Norcross, GA, U.S.A.) was analyzed under the same conditions to validate the method. Measurements were conducted during a stay at the Natural History Museum (NHM, London, Great Britain) which was funded by the Quality Nano (Qnano) Research Infrastructure.

4.1.5 Dynamic and electrophoretic light scattering (DLS/ELS)

Except for the earthworm tests, in which much higher concentrated TiO_2 suspension were used, stock suspensions with a concentration of 1 g/L were prepared in deionized water using ultrasonication (200 W) according to the standard operating procedure (SOP) ‘Preparation of a NM 101, NM 102 and NM 100 Suspension’ (Annex 2-A, 2-B, 2-C). If necessary they were diluted with deionized water to either 100 mg/L or 10 mg/L (working suspension I and II). Suspensions were characterized according to the SOP ‘Characterization of a TiO_2 Suspension’ by means of dynamic light scattering (Annex 3). Furthermore, the zeta potentials of the particles in the suspensions were measured by means of electrophoretic light scattering (ELS). DLS and ELS measurements were carried out with a Zetasizer Nano (Malvern Instruments, Worcestershire, United Kingdom, Fig. 5 B). For the stock and working suspensions I and II of the

nanomaterials NM 101 and NM 102 four to nine independent characterization experiments were performed (Tab. 3). For the reference material NM 100 eight independent characterization experiments were conducted for the stock suspension and two for the working suspension II. The NM 100 working suspension I was not characterized because it was not used for tests.

DLS and ELS measurements of the TiO_2 suspension used in the earthworm tests are described in section 6.2.1.

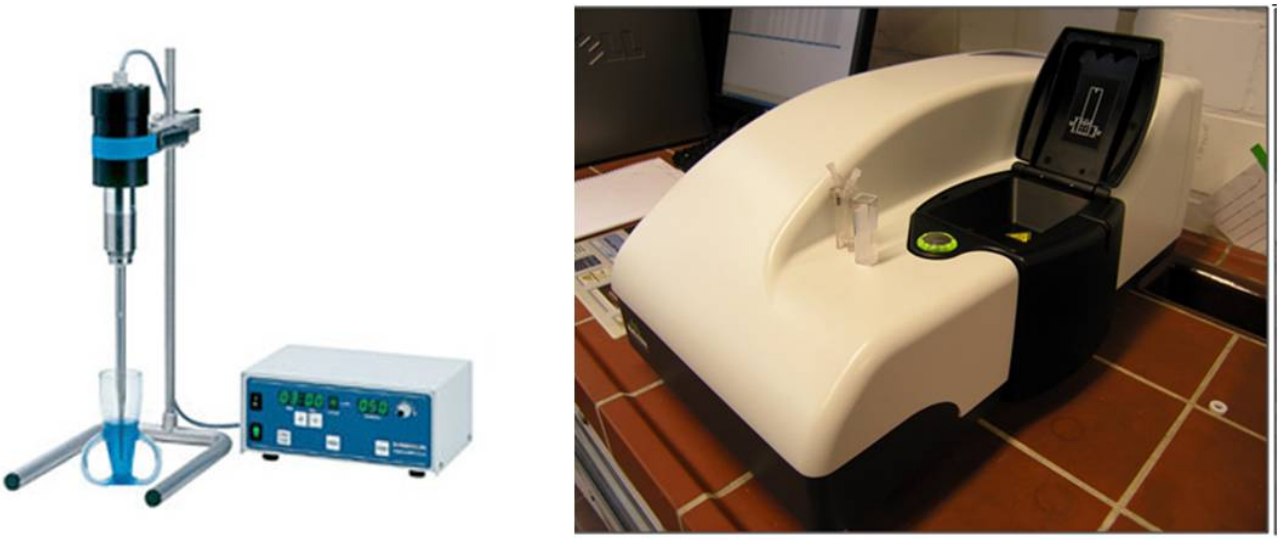

Fig. 5: **Ultrsonic homogenizer (Sonopuls HD 2200, Bandelin, Berlin, Germany; left) and zetasizer nano (**Malvern Instruments, Worcestershire, United Kingdom, right)

4.1.6 Analysis and statistics

Data were statistically analyzed with ToxRat® Professional (version 2.10, ToxRat solutions GmbH). Significant differences between the stock suspensions and the working suspensions were determined using student-t test for homogeneous variances (two sided, *P<0.05).

4.2 Results

4.2.1 TEM

The transmission electron microscopy (TEM) images show that in the tested suspension small (14-240 nm, Fig. 6, B) and large agglomerates of NM 101 (around 1000 nm, Fig. 6 A) were present which consisted of particles with a primary particle size of around 10 nm (Fig. 6, C). These results are in accordance with the primary particle size indicated by the manufacturer for NM 101 (7-10 nm).

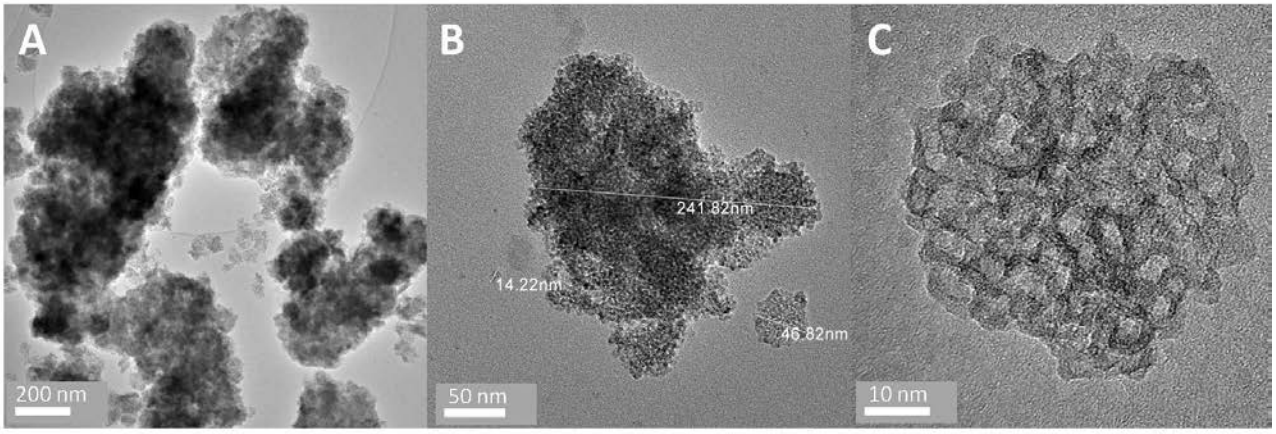

Fig. 6: Transmission electron microscopy images of NM 101 in a 100 mg/L suspension, recorded with a CM 20 (Philips, Hamburg, Germany) are shown. Larger (A) and smaller agglomerates (B,C) consisting of particles with a size of around 10 nm (C) were documented.

NM 102 images of the 100 mg/L suspension show the presence of small (108-350 nm, Fig.7, B) and large agglomerates (900 nm to several 1000 nm, Fig. 7 A) which consisted of particles with a primary particle size of around 20-30 nm (Fig. 7 B, C). These results are comparable with the primary particle of NM 102 indicated by the manufacturer (15-25 nm).

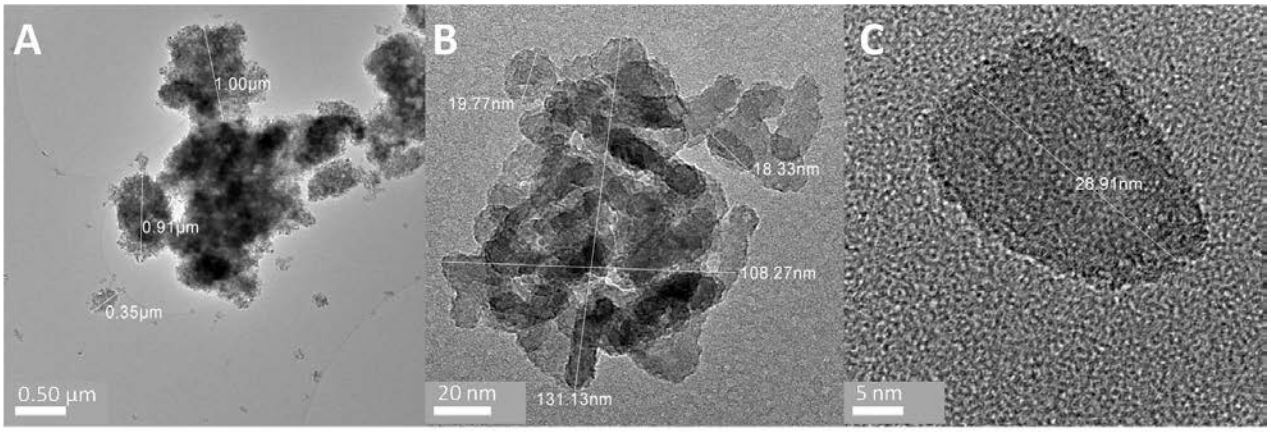

Fig. 7: Transmission electron microscopy images of NM 102 in a 100 mg/L suspension, recorded with a CM 20 (Philips, Hamburg, Germany) are shown. Larger (A) and smaller agglomerates (B,C) consisting of particles with a size of around 18-20 nm (B), as well as single particles (C) were documented.

NM 100 images of the 100 mg/L suspension show the presence of medium sized agglomerates (around 2 µm, Fig. 8, B) and large agglomerates (around 10 µm, Fig. 8 A) which consisted of particles with a primary particle size of around 0.2-1000 nm (Fig. 8 B, C). The particle size indicated by the manufacturer was lower (200-220 nm).

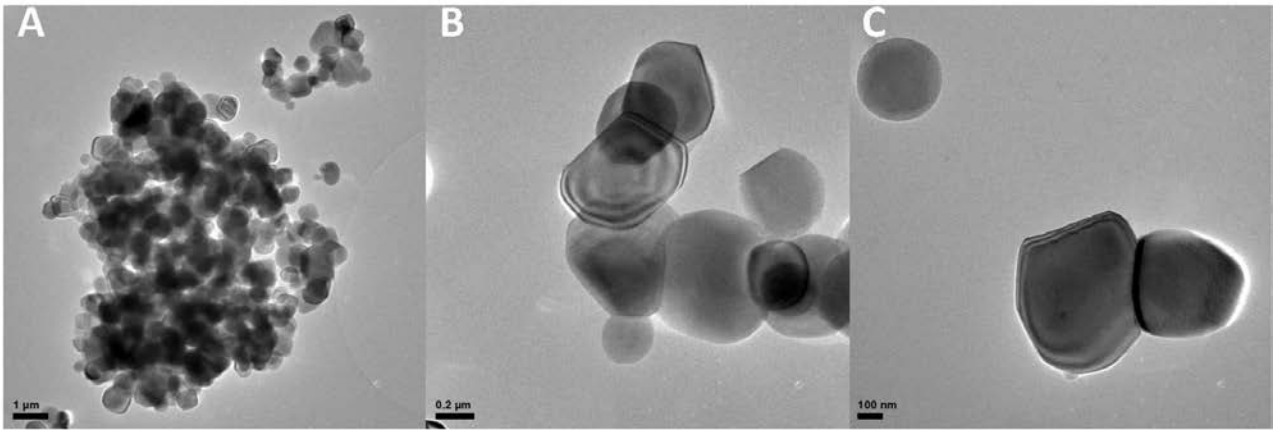

Fig. 8: Transmission electron microscopy images of NM 100 in a 100 mg/L suspension, recorded with a CM 20 (Philips, Hamburg, Germany) are shown. Larger (A) and smaller agglomerates (B,C) consisting of particles with a size of around 0.2-1 µm, as well as single particles (C) were documented.

4.2.2 XRD

Fig. 9 illustrates the determined powder diffractograms of the different sized TiO_2 materials compared to the diffractogram of TiO_2 anatase which was obtained from a database. The diffractograms demonstrate that all TiO_2 materials are of the anatase polymorph.

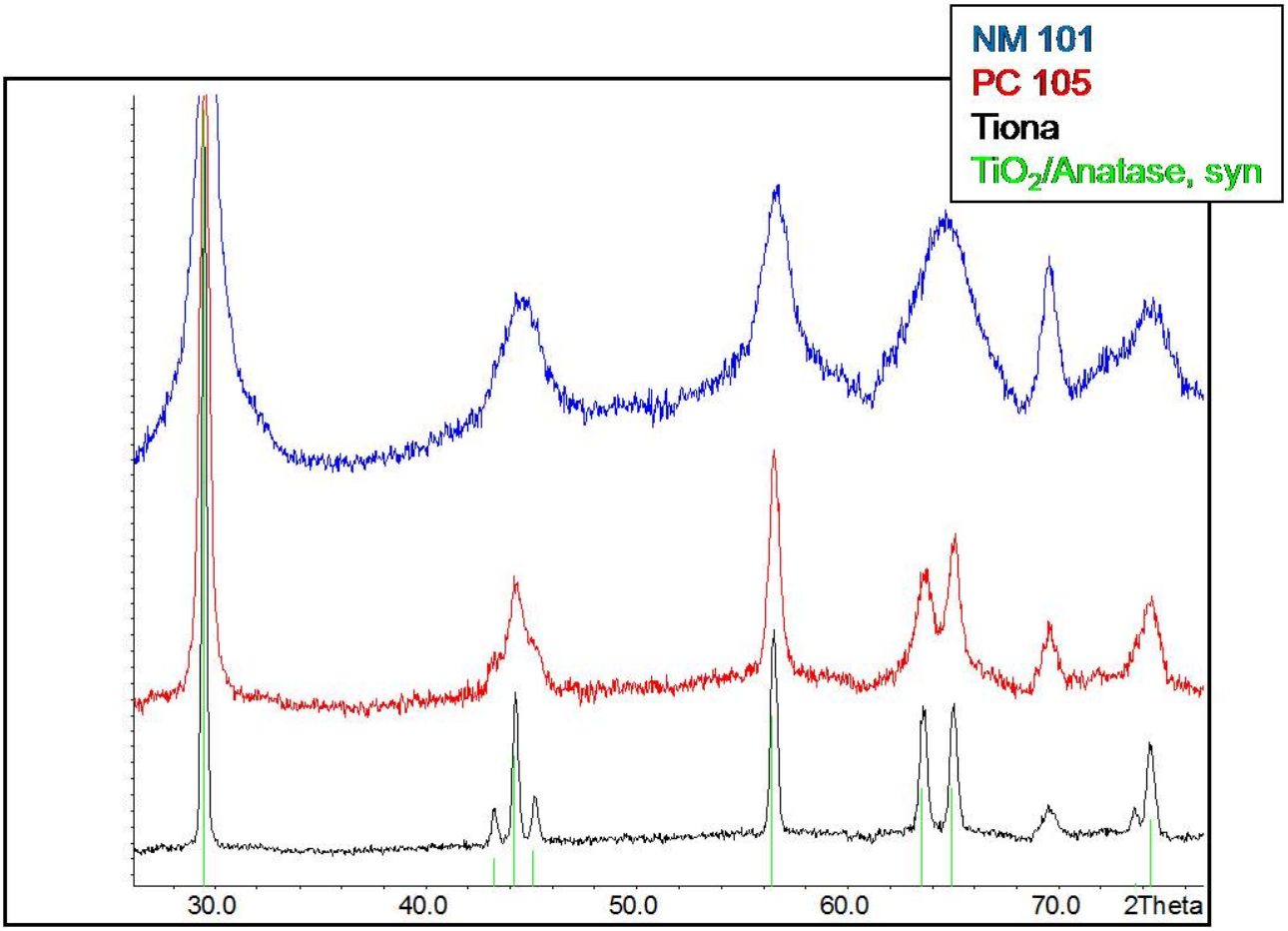

Fig. 9: Powder diffractogram of NM 101 (blue), NM 102 (red) and NM 100 (black). Peak height is not relevant. Diffractorgrams are compared to that of TiO_2 anatase (green) from a database.

4.2.3 BET

Tab. 2 summarizes the measured BET specific surface areas of the different sized TiO_2 materials. These results are in accordance with those indicated by the manufacturers (section 1 Tab. 1). The BET specific surface area of carbon black was the same as the certified value.

Tab. 2: Measured Brunauer-Emmett-Teller specific surface area (m⁴/g) of the different sized TiO_2 materials and the reference material carbon black.

Material	Measured BET specific surface area (m^2/g)
NM 101	280.0
NM 102	77.6
NM 100	9.4
Carbon black[a]	30.7

[a] certified value 30.6 m^2/g

4.2.4 DLS and ELS measurements

Table 3 summarizes the results of the characterization of the NM 101, NM 102 and NM 100 particles in the stock and working suspensions (hydrodynamic diameter, HD/zeta potential, ZP) by means of DLS and ELS. Measurements were conducted directly after their preparation. The HD of the different particles in the stock suspensions can be sorted in following order: NM 102 (625.0 nm) > NM 101 (511.5 nm) > NM 100 (260.9 nm). Dilution of the NM 101 stock suspension to a concentration of 100 mg/L resulted in a significantly higher HD of 1298 nm, whereas dilution to a concentration of 10 mg/L (509.2 nm) did not significantly alter the HD of the particles compared to those in the stock suspension. When NM 102 stock suspensions were diluted to either 100 mg/L (650.9 nm) or 10 mg/L (689.0 nm) no significant change in HD was observed compared to the particles in the stock suspension. The HD of NM 100 particles, after dilution of the stock suspension to 10 mg/L (258.8 nm), was comparable to that of particles in the stock suspension (260.9 nm). Comparable poly dispersity indices (PDI) values, which ranged between 0.31 and 0.39, were observed for the nanomaterials, whereas lower PDI values (0.15-0.19) were monitored for the non-nano reference NM 100. The PDI is a measure for the size distribution within the suspension and is limited to a value of 1, which indicates a broad size distribution. During the measurements attenuators between 3.0 and 6.0 were applied by the instrument.

Zeta potential (ZP) measurement resulted in following values of the NM 100, NM 101 and NM 102 particles in the stock suspensions: -38.2, -31.8 and 12.2 mV. Zeta potentials of NM 101 particles in the working suspensions were significantly lower than that of the particles in the stock suspensions. A significant different zeta potential was also observed for the NM 102 particles in the 10 mg/L working suspension (-9.6 mV) compared to those of the stock suspension. Dilution seems to have had no influence on the zeta potential of the NM 100 particles (-31.4 mV). Illustrations of zeta potentials and HD of all materials are additionally shown in section 3.2.3, Fig. 16, A-C.

Tab. 3: Hydrodynamic diameters (HD) and zeta potentials (ZP) of the TiO_2 materials in stock and working suspensions (deionized water).

TiO_2 material	Suspension (mg/L)	HD ± SD[a] (nm)	ZP ± SD[a] (mV)	PDI[b] ± SD[a]	Attenuator[c]	N[d]
NM 101	Stock suspension (1000)	511.5 ± 33.7	-31.8 ± 4.2	0.32 ± 0.05	3.7 ± 0.6	9
	Working suspension I (100)	1298 ± 573.7*	-6.4 ± 5.5*	0.33 ± 0.06	4.9 ± 0.4	8
	Working suspension II (10)	509.2 ± 46.1	-14.0 ± 8*	0.36 ± 0.06	5.8 ± 0.5	4
NM 102	Stock suspension (1000)	625.0 ± 43.5	12.2 ± 1.1	0.31 ± 0.06	3.9± 0.4	8
	Working suspension I (100)	650.6 ± 52.5	1.1 ± 12.1	0.35 ± 0.1	5.8 ± 1.0	4
	Working suspension II (10)	689.0 ± 27.1	-9.6 ± 4*	0.39 ± 0.10	6.0 ± 0.0	4
NM 100	Stock suspension (1000)	260.9 ± 9.3	-38.2 ± 10.8	0.19 ± 0.02	3.0 ± 0.0	8
	Working suspension II (10)	258.8 ± 11.7	-31.4 ± 4.0	0.15 ± 0.01	5.0 ± 0.0	2

[a] standard deviation, [b] poly dispersity index, [c] a value of 11 indicates that full power of the laser is used for data collection [d] number of independent experiments, * significant differences to the stock suspension

4.3 Discussion

Except for the TEM measurements of NM 100, the TEM, XRD and BET measurements confirmed that the primary particle size, the polymorph and the BET specific surface area of the different sized TiO_2 materials was in line with the information of the manufacturers. The results give evidence that the different TiO_2 materials only differed in primary particle size and not in their crystalline structure. This confirmation forms the basis of the present study, because one of the main tasks is to observe whether potential TiO_2 toxicity is dependent on size and particularly on nano size as defined by the EC recommendation (EC 2011).

The TEM images were only used to get an idea of the primary particle size of the different particles, no statistical evaluation was performed, e.g. by determining the size of a statistically relevant number of particles. It is known that bulk TiO_2 materials may also contain particles smaller than 100 nm and therefore have a wider particle size distribution than nanomaterials (Weir et al. 2012). This might explain the observed wide primary particle size distribution for NM 100 in our study and makes it difficult to compare our observation to the primary particle size indicated by the manufacturer. The primary particles sizes observed for the nanomaterials did not vary much. Consequently, they can be compared to those indicated by the manufacturers.

In general, the DLS measurements of the stock suspension show that the nanomaterials had a much larger hydrodynamic diameter than their indicated primary particle size, whereas those of the non nano scale NM 100 were in accordance with each other. These results indicate a strong agglomeration behavior of the nanoparticles which might be confirmed by the TEM images showing µm sized NM 101 and NM 102 agglomerates. However, agglomeration of particles might have also occurred as a consequence of drying during the preparation of TEM samples. Micrometer sized agglomerates were also detected via TEM in the NM 100 suspensions, although DLS measurements revealed a HD of around 260 nm. This discrepancy may be explained by assuming that the large NM 100 agglomerates already sedimented during the DLS measurement so that only small NM 100 particles were left in the water phase and were measured. This strong agglomeration behavior is stated in the sedimentation experiment described in section 3.2. NM 101 HD results are comparable to those observed in a study of von der Kammer et al. (2010, 300 nm, -40 mV in deionized water after suspending in a ultrasonic bath, 2x60W, 30 min) and NM 102 HD values are in line

with those observed for NM 102 in the UFOPlan Project No. 3709 65 417 (500 nm, around 18 mV after a sonciation time of 15 min, Kuhlbusch et al. 2012). Except for the NM 101 working suspension I (100 mg/L), the low variability of the HD of the particles in the stock and working suspension confirmed the reproducibility of the ultrasonication method used. It is unclear, why the HD of the NM 101 working suspension I (100 mg/L) was higher and more variable than in the 10 mg/L working suspensions. Perhaps measurement failures were responsible for this discrepancy which is assumed based on the observed high standard deviations of single measurements. A zeta potential of > +30 mV or < -30 mV indicates a stable suspension. Consequently the NM 101 and NM 100 stock suspensions can be defined as stable. The ZP of the nanoparticles in the working suspensions was lower than that observed in the stock suspensions and >-30 mV, indicating that the particles were less stable in the dilutions than in the stock suspensions This was not the case for the non nano reference NM 100 showing also a zeta potential of < -30 mV in the dilution.

4.4 Conclusion

Finally, it can be concluded that the tested TiO_2 materials only differed in primary particle size and not in crystalline structure. Furthermore, the DLS and ELS measurements revealed that the used ultrasonication method was applicable to reach reproducible HD and ZP values of the particles in the stock suspension and that dilution of the stock suspensions to the working suspensions generally had no influence on the HD of the particles, whereas it had an influence on their ZP.

5 *Daphnia* sp., acute immobilization test (OECD 202)

Photoactive nano-TiO_2 are used in a broad range of products and applications, e.g., for self-cleaning surfaces and for water treatment applications (Pelaez et al. 2012). Photoactivity of the particles is induced by specific wavelengths of radiation corresponding to the specific band gap energy of the material leading to the formation of an electron-hole pair (Fig. 2). Oxygen and water can react with this system yielding reactive oxygen species (ROS), capable of degrading organic chemicals. Beside this beneficial effect, ROS may also induce oxidative stress in organisms (Pan, 2009), e.g., those inhabiting TiO_2 polluted water bodies. A study from Ma et al. (2012a) showed that the wavelength range (345-380 nm) corresponding to the band gap energy of the TiO_2 nanoparticle P25 (3.2-3.0 eV) is responsible for the phototoxic effects of P25 towards *Daphnia magna*. These results enlighten the necessity to investigate the toxicity of TiO_2 nanoparticles under more environmental relevant conditions as for example under simulated solar radiation. However, this is not yet considered in environmental risk assessment of TiO_2 nanomaterials.

Therefore, we investigated the influence of environmental realistic levels of simulated solar radiation (SSR) on the acute toxicity of different sized TiO_2 materials (nanomaterials NM 101 and NM 102 and non-nano reference NM 100 with primary particle sizes of 7, 15, and 200 nm (manufacturer information) to *Daphnia magna*. Furthermore, we studied the influence of the ionic strength of the test medium on the outcome of the nanomaterial experiments.

5.1 Material and methods

5.1.1 Chemicals

All three TiO_2 materials NM 101, NM 102 and NM 100 were tested. For details on the materials see section 2.1.1.

5.1.2 Good laboratory praxis (GLP)

The *Daphnia* sp. immobilization tests were performed at the Institute for Environmental Research at RWTH-Aachen University in accordance with Good Laboratory Practice (GLP). Because our institute is a non-GLP testing facility, IBACON and the institute agreed on performing the following, relevant working procedures in accordance to GLP: a) reporting of raw data, b) calibration of pipettes, balances, pH-meters and oxygen measuring instruments (as described in the SOP for the *Daphnia* sp., immobilization Test).

Furthermore, the standard operating procedure (SOP) for the *Daphnia* sp., immobilization test from IBACON was adapted to the testing of TiO_2 materials with SSR. This SOP (‘Investigating the influence of simulated solar radiation on the effect of TiO_2 nanomaterials on the mobility of *Daphnia magna* after exposure for 24 and 48 h‘) is attached in Annex 4-A.

The quality assurance management of IBACON was present during the performance of one of the daphnia immobilization tests (acute toxicity test with NM 101 with SSR and laboratory light (LL)). A study plan (Annex 4-B) was written for the inspected test containing exact instructions regarding e.g. the details of the acute toxicity assay for NM 101 with SSR and LL, the tested concentrations and the test media. It was assessed whether the above-mentioned working procedures were performed in accordance to GLP.

The results of the inspection are summarized in a test report which was written by the quality assurance management of IBACON which can be found in Annex 4-C.

5.1.3 Performance of the *Daphnia* sp., acute immobilization test (OECD 202)

Briefly, acute toxicity tests were performed with < 24 h old neonates of *Daphnia magna* according to the OECD guideline 202 (48 h exposure duration). For each material parallel test series were run with either LL or SSR under a 16 h light/8 h dark regime. Each test series consisted of five or seven treatment groups with different concentrations and one control. Each treatment group consisted of four replicates containing each five neonates. Stock suspensions (1 g/L) were prepared according to the SOPs 'Preparation of a NM 101, NM 102 and NM 100 Suspension' (Annex 2-A, 2-B, 2-C). Working suspensions were prepared by diluting the stock suspension with deionized water to 100 mg/L or 10 mg/L. Suspensions were characterized according to the SOP 'Characterization of a Nanomaterial Suspension' by means of dynamic light scattering (Annex 3). Furthermore, the zeta potentials of the particles in the suspensions were measured with a zetasizer nano (Malvern Instruments, Worcestershire, UK). The desired test concentrations were obtained by diluting either the stock dispersions or the working dispersion with test medium.

As test medium either 10 fold diluted ISO water (medium B) or ISO water (medium A, ISO 1996) was used for the immobilization tests.

10 fold diluted ISO water was used as test medium for the following reason: Römer et al. (2011) found out that the agglomeration of silver (nano-Ag) nanoparticles was lower in diluted ISO water (2, 5, 10 fold) than in undiluted ISO water. They suggested that the increased stability was due to a lower ionic strength in the diluted test medium, leading to a larger diffusive layer thickness that enhances repulsive forces between the particles. This assumption is in accordance with DLVO theory. The DLVO theory (named after Derjaguin and Landau, Verwey and Overbeek) describes the interactions (van der Waals; electrostatic forces) of charged particles in a liquid medium. In the present study, no difference in immobilization was observed between control daphnids exposed to medium B or medium A.

Tests in medium B:

Each test series was performed at least twice (n=8) for each light condition. For the SSR tests with NM 101 and NM 102 following concentrations were tested: 5.00, 1.85, 0.69, 0.25 and 0.08 mg/L, whereas higher concentrations were tested for NM 100: 50.0, 16.7, 5.6, 1.9 and 0.6 mg/L. Except for NM 100 the concentrations used in the LL tests were higher than those used in the SSR tests. Hereby NM 101, which showed toxic effects in a preliminary study under LL; was tested with concentrations up to 100 mg/L (100.0, 60.0, 33.3, 18.5, 10.3 and 5.6 mg/L), whereas NM 102 and NM 100, which did not show toxic effects in the preliminary study, were only tested with concentrations up to 50 mg/L (50.0, 16.7, 5.6, 1.9 and 0.6 mg/L).

Tests in medium A:

To observe whether the ionic strength of the test medium has an influence on the SSR induced toxicity of the nanomaterials NM 101 and NM 102 tests in medium A were performed under the same conditions as tests in medium B, however undiluted ISO medium (medium A) was used instead of 10-fold diluted ISO-medium (medium B) and for NM 101 test suspensions were directly prepared from stock suspensions instead of working suspensions.

For NM 101 three independent SSR experiments with each five concentrations (1.28-50.00 mg/L, dilution factor 2.5) were conducted, whereas for NM 102 three independent experiments were conducted with seven different concentrations (0.21-50 mg/L spacing factor 2.5). Under LL conditions, one independent experiment was conducted for NM 101 with five different NM 101 concentrations (12-100 mg/L, spacing factor 1.7) and for NM 102 two experiments with seven concentrations (0.21-50 mg/L spacing factor 2.5) were performed.

5.1.4 Light sources

Laboratory light (LL)

A normal fluorescent tube was used for testing under laboratory light. As described before, a 16 h light/8 h dark regime was used.

Simulated solar radiation (SSR)

A metal vapor lamp emitting visible radiation comparable to sunlight (280-1000 nm) was used (Bright Sun UV Desert, 70 W, Lucky Reptile, Waldkirch, Germany) as light source for the testing with SSR. The test stand is shown in Fig. 11. The distance between the lamp and the test vessels was 50 cm and a 16 h light/8 h dark regime was used. The manufacturer states that the irradiance of the UVA and UVB radiation of the lamp (3.2 mW/cm² and 50 µW/cm²) is comparable to that of solar radiation at a midsummer day in Germany (4.1 mW/cm² and 120 µW/cm², Sandmann 2001). Consequently, the irradiance of the UVA and UVB radiation as given by the manufacturer is comparable with that of natural sunlight in the troposphere.

The actual spectrum and irradiance of a new (runtime: 0 h) and an already used (runtime: 220 h) metal vapor lamp were recorded with a calibrated spectrometer (AvaSpec, ULS 3648 200-1100 nm, Avantes, Apeldoorn, Netherlands) with a UV compatible glassfiber light conductor (FC-UV200-2, diameter: 200 µm for UV/Vis, Avantes) and a cosinus-corrector (teflon disc, 200-800 nm, diameter: 6.5 mm, height: 1 mm, Avantes) at the Federal Institute for Materials Research and Testing (BAM, Berlin, Germany) together with Anne-Kathrin Barthel. Data was evaluated with AvaSoft 8 (Avantes). Fig. 10 shows the spectrum of the new lamp and Tab. 4 summarizes the results of the measured irradiance of total ultra violet radiation (UV), UVA and UVB radiation. It can be seen that the total UV irradiation was almost 25% lower for the 220 h used lamp than for the new lamp. In detail UVB irradiation was 20% and UVA irradiation was 25% lower in the old than in the new lamp. To ensure that that the UVA irradiance did not vary more than 25% during the experiments, lamps were only used for 220 h.

Tab. 4: Comparison of the irradiance of total UV, UVA and UVB radiation of a new and a 220 h used metal vapor lamp as well as of natural sung light.

UV light (nm)	New lamp (mW/cm²)	220 h used lamp (mW/cm²)	% of new lamp	Natural sun (mW/cm²)
Total UV (280-400 nm)	2.50	1.9	76	5.50[a]
UVA (320-400 nm)	2.36	1.76	75	4.10[b]
UVB (280-320 nm)	0.15	0.12	80	0.12[b]

[a] irradiance at a midsummer day in Neuherberg, Germany (Bundesamt für Strahlenschutz 2012), [b] Irradiance at a mid summer day (04.07.2000, 13:36) in Westerland, Germany (Sandmann 2001).

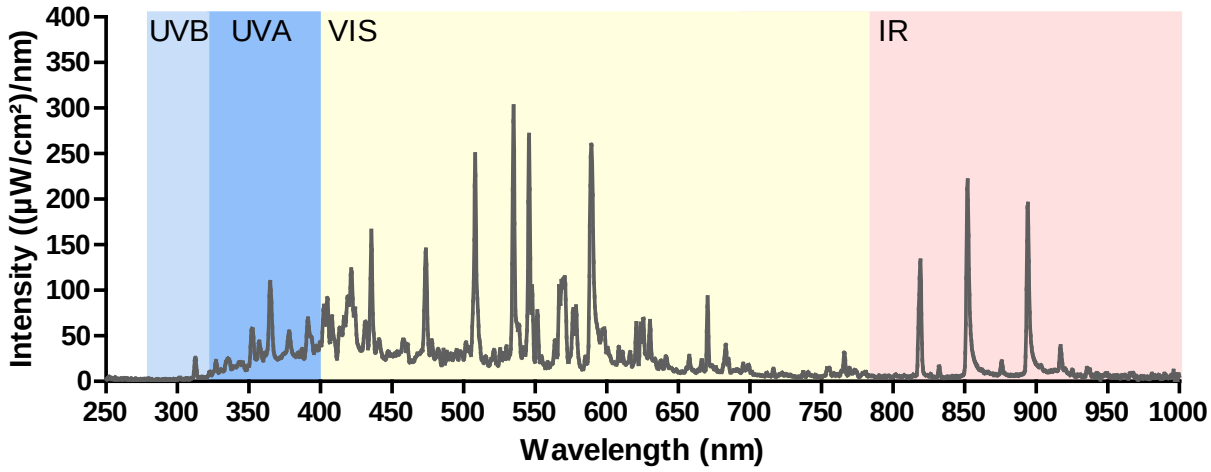

Fig. 10: Spectrum of a new metal vapor lamp which was recorded with a spectrometer at the BAM (Berlin, Germany) together with Anne-Kathrin Barthel. *UVA, UVB* ultraviolet radiation A and B, *VIS* visible light, *IR* infrared

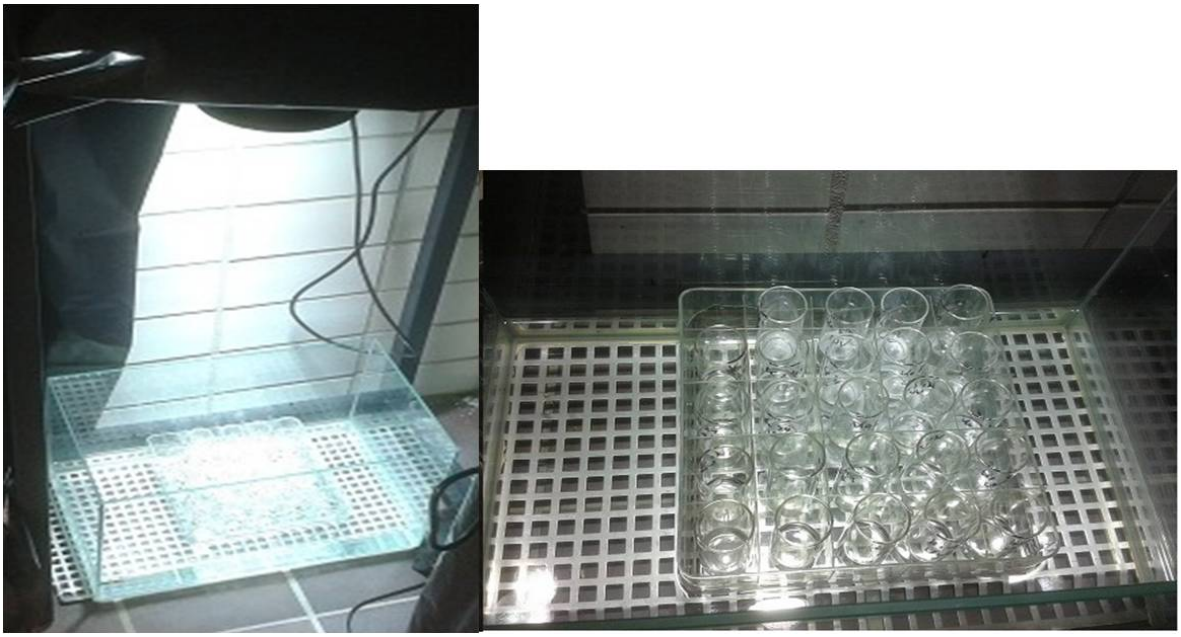

Fig. 11: Overview of the test stand of the *Daphnia* sp., acute immobilization test with simulated solar radiation (left) and close-up of the test vessels (right).

5.1.5 General characterization of particles in the test suspension

The TiO_2 particles in the test suspension (medium B) were characterized in an independent experiment. In this experiment, particles were characterized in the test medium B with regard to their hydrodynamic diameter, zeta potential, concentration and sedimentation behavior. In general these tests were conducted in the same test vessels and under the same test conditions as the ecotoxicity tests with laboratory light in medium B. This includes that daphnia were added to the test medium.

Two different characterization experiments were conducted: In the first experiment the same TiO_2 concentration (1.3 mg/L) was tested for each TiO_2 material. This was done to compare the sedimentation behavior of the different TiO_2 materials to each other (sedimentation experiment). In a second experiment TiO_2 concentrations which corresponded to the nominal EC50 value of each material (NM 101 SSR, NM 101 LL, NM 102 SSR, NM 100 SSR: 1.3, 79.5, 0.5, 3.9 mg/L) were prepared and characterized. Finally, the TiO_2 concentration in these vessels was measured at test initiation and termination (mean t0-t48, Tab. 10). Additionally to these treatment groups controls, which consisted of untreated test medium were conducted.

Fig. 12 gives an overview of the test set up of one treatment group (e.g. NM 101, 1.3 mg/L). At test initiation (0 h) three beakers per treatment group (80 ml) were prepared by diluting three independently prepared stock (1 g/L) or working (100 mg/L) suspensions with test medium B to the specific concentrations. Stock and working suspensions were prepared as explained in section 3.1.3. Immediately after the preparation of the beakers, samples were taken for Ti analysis (each 15 ml) and particle characterization (each approximately 3 ml). Thereafter, the test medium of each beaker was separated to five smaller test vessels containing each 10 ml test medium and five neonates (Fig. 12). From the latter test vessels water samples were collected after 24 h for DLS and ELS measurements and after 48 h for DLS and ELS measurements as well as for Ti analysis. For Ti analysis water samples (each 5 ml) of three smaller test vessels, which were prepared from one beaker, were pooled, resulting in a total of three replicates (each 15 ml) per treatment group (Fig. 12).

For ELS and DLS measurements only one smaller test vessel was sampled per test beaker (approximately 3 ml), resulting in a total of three replicates per treatment group and time point. The test medium was sampled by gently placing the tip of a glass pipette directly under the water column surface. Hydrodynamic diameters and zeta potentials of the particles in the test medium were determined by means of dynamic light scattering (DLS) and electrophoretic light scattering (ELS) according to the SOP 'Characterization of a Nanomaterial Suspension' (Annex 3).

Samples for Ti-analysis were stored in a fridge (8 °C) until they were analyzed at the Institute for Energy and Environmental Technology e.V. (IUTA, Duisburg, Germany). Samples were digested with a mixture of hydrofluoric acid (HF, 40%) and nitric acid (HNO_3, 65%) at 100 bar and 200 °C in a microwave. Thereafter, Ti was analyzed according to (ISO) by means of inductively coupled plasma optical emission spectrometry (ICP-OES).

Ti analysis data was used to determine the mean percentage of the nominal concentration at test initiation and termination for each material. Additionally, the mean measured exposure concentration (C_{mm}) was calculated by forming the mean of the measured concentration at test initiation and termination. Consequently, C_{mm} represents the mean exposure concentration of the specific TiO_2 material in the upper water column during the test duration of 48 h.

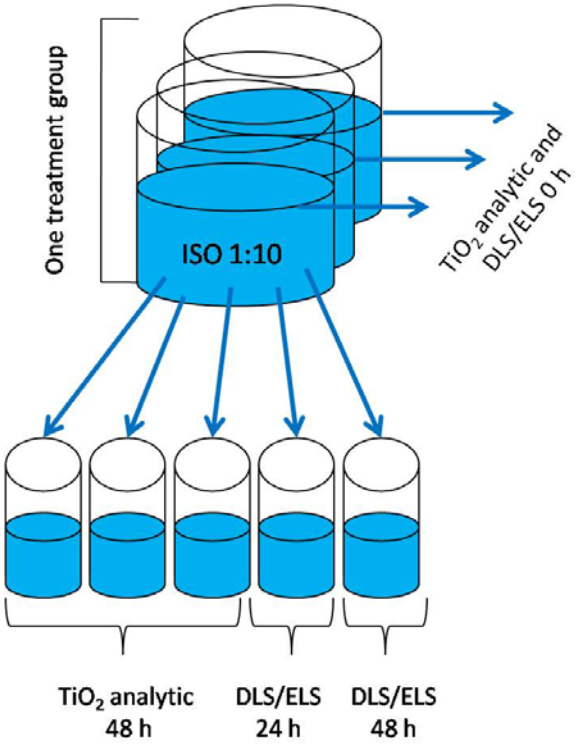

Fig. 12: Test set up of one treatment group (e.g. NM 101 1.3 mg/L). Per treatment group three beakers (80 ml) of test suspension were prepared. The medium of each beaker was separated to five smaller test vessels containing each 10 ml medium and five neonates.

5.1.6 Analysis and statistics

Data were statistically analyzed with ToxRat® Professional (version 2.10, ToxRat solutions GmbH). Concentration response functions were fitted to the data using probit analysis. The median effective concentration (EC50) was calculated from this function. Significant differences to the control (*P<0.05) were determined using Fisher's Exact Binominal Test with Bonferroni Correction to derive the lowest observed effect concentration (LOEC) and no observed effect concentration (NOEC).

Significant differences between the treatment groups of the characterization experiments were determined using student-t test for homogeneous variances (two sided, *P<0.05).

5.2 Results

5.2.1 Immobilization tests with medium B

The measurement of the pH values and of the oxygen content at test initiation and termination in the control media and in the media of the highest treatment group revealed that these values were in line with the validity criteria of the OECD guideline 202. Only tests in which the mortality in the control groups was ≤ 10% were considered for evaluation.

Figure 13 D-F shows the immobility (%) of *Daphnia magna* exposed to NM 101, NM 102 and NM 100 with laboratory light. Under normal laboratory light conditions, no effect of NM 102 and NM 100 on the daphnids was observed (up to 50 mg TiO_2/L). Only for NM 101 a dose response relationship was detected. A lowest observed effect concentration (LOEC, t48) of 33 mg/L and a median effective concentration (EC50, t48) of 79.52 mg/L were calculated (Tab. 5, n=8-16).

When daphnids were simultaneously exposed to SSR, a clear dose response relationship was observed for all materials (Fig. 13 A-C). Median effect concentrations (EC50, t48) amounted to 0.53, 1.28, and 3.88 mg/L for NM 102, NM 101, and NM 100, respectively (Tab. 6). The following LOEC (t48) values were determined for NM 102, NM 101, and NM 100: 0.25, 0.69, and 5.56 mg/L (Tab. 6). Fig. 13 A and C show that the immobility of daphnids treated with NM 101 and NM 100 increased with exposure time, whereas immobility of daphnia exposed to NM 102 after 24 h of exposure was almost as high as after 48 h of exposure (Fig. 13 B).

Tab. 5: EC50, LOEC and NOEC values (nominal) derived from the *Daphnia* sp. acute toxicity tests with NM 101, NM 102 and NM 100 performed with laboratory light (LL) in medium B (10fold diluted ISO water).

TiO_2 material (h of exposure)	Light condition	EC50[a] (mg/L)	95%-CL[b] lower/upper (mg/L)	LOEC[c] (mg/L)	NOEC[d] (mg/L)
NM 101 (24 h)	LL	n.c.[e]	n.c.	100	60
NM 101 (48 h)		79.52	62.64/112.71	33.30	18.50
NM 102 (24 h)	LL	n.c.	n.c.	> 50	≥ 50
NM 102 (48 h)		n.c.	n.c.	> 50	≥ 50
NM 100 (24 h)	LL	n.c.	n.c.	> 50	≥ 50
NM 100 (48 h)		n.c.	n.c.	> 50	≥ 50

[a]median effect concentration, [b] 95% confidence limit, [c] lowest observed effect concentration, [d] no observed effect concentration, [e]n.c.: not calculable from the tested concentration series

Tab. 6: EC50, LOEC and NOEC values (nominal) derived from the *Daphnia* sp. acute toxicity tests with NM 101, NM 102 and NM 100 performed with simulated solar radiation (SSR) in medium B (10fold diluted ISO water)

TiO_2 material (h of exposure)	Light condition	EC50[a] (mg/L)	95%-CL[b] lower/upper (mg/L)	LOEC[c] (mg/L)	NOEC[d] (mg/L)
NM 101 (24 h)	SSR	5.85	3.63/13.03	1.85	0.69
NM 101 (48 h)		1.28	0.61/3.66	0.69	0.25
NM 102 (24 h)	SSR	0.99	0.58/1.88	0.69	0.25
NM 102 (48 h)		0.53	0.43/0.65	0.25	0.08
NM 100 (24 h)	SSR	14.08	4.80/100.57	5.56	1.85
NM 100 (48 h)		3.88	0.16/40.73	5.56	1.85

[a]median effect concentration, [b] 95% confidence limit, [c] lowest observed effect concentration, [d] no observed effect concentration

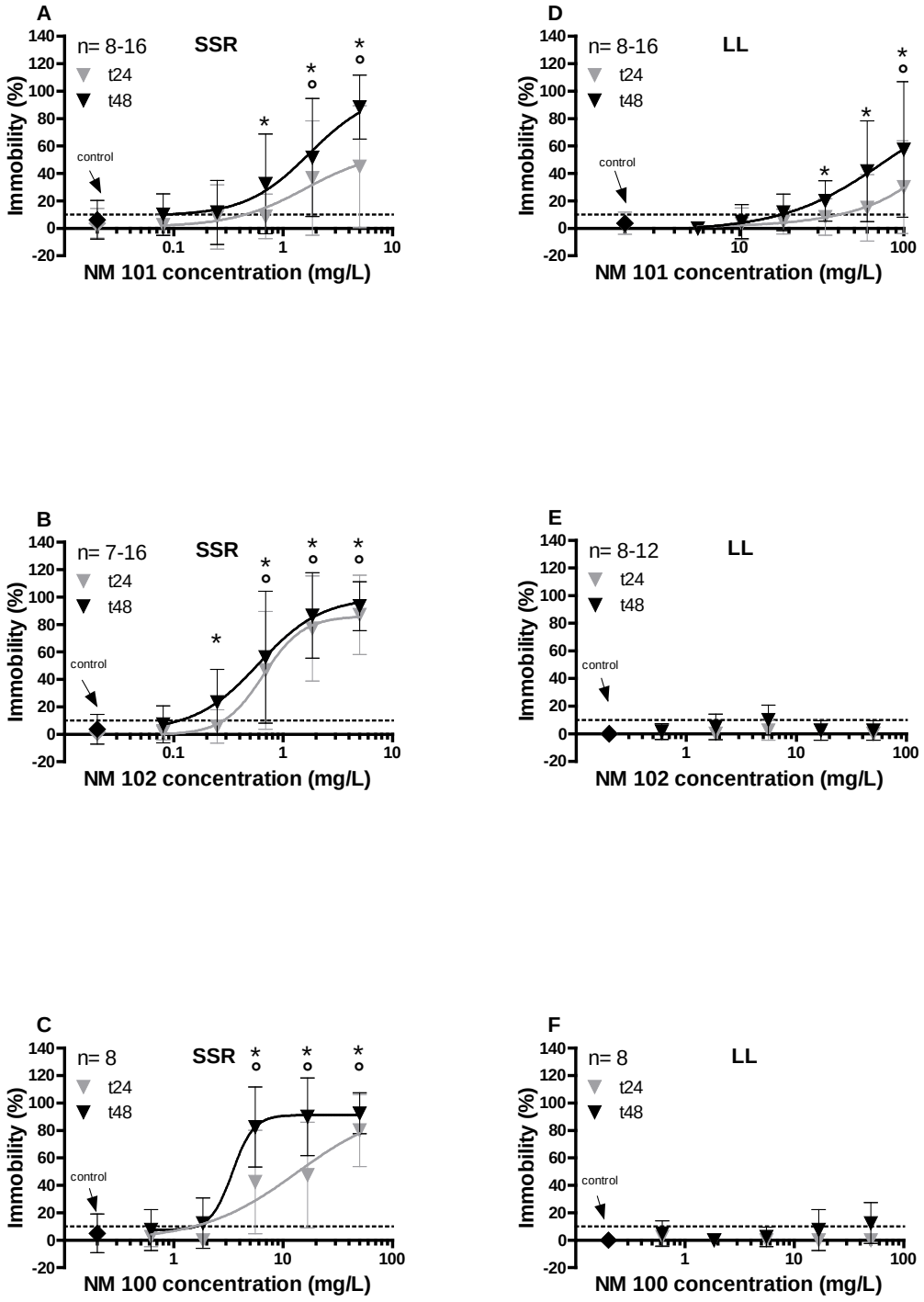

Fig. 13: Immobility (%) of *Daphnia magna* exposed to NM 101, NM 102 and NM 100 with either simulated solar radiation (SSR; A, B, C) or with laboratory light (LL; D, E, F) in medium B. Error bars represent standard deviation derived from the replicates from at least two independently conducted experiments (n=8-16). Circles (24 h of exposure) and asterisks (48 h of exposure) indicate significant differences to the control (*P<0.05).

Table 7 summarizes the results of the characterization of the NM 101, NM 102 and NM 100 stock and working suspensions by means of dynamic light scattering (DLS) which was conducted directly after their preparation. Furthermore, the table contains the results of zeta potential measurements of the previously named materials in the specific suspensions. The hydrodynamic diameters (HD) of the different particles can be sorted in following order: NM 102 (610.9 ± 12.6 nm) > NM 101 (517 ± 37.33 nm) > NM 100 (256.6 ± 7.2 nm). Dilution of the NM 102 stock suspension resulted in comparable hydrodynamic diameters (HD) in the working suspension (705.5 ± 131.0 nm, Table 7) as in the stock suspension. This was not the case for NM 101 (1698.0 ±489.6 nm) which showed a much larger HD in the working than in the stock suspension. Zeta potential measurement of the NM 100, NM 101 and NM 102 particles in the stock suspensions resulted in following values: -43.6, -29.8 and 11.6 mV. Zeta potentials of particles in the working suspension were lower than in the stock suspensions: -3.9 and 2.4 mV for NM 101 and NM 102, respectively. PDI ranged between 0.26 and 0.33 for the nanomaterials and was lower for the non-nano reference (0.17). The attenuator which was applied by the instrument was always lower than five.

Tab. 7: Hydrodynamic diameters (HD) and zeta potentials (ZP) of the TiO_2 materials in stock and working suspensions used for the daphnia tests with medium B.

TiO_2 material	Suspension (mg/L)	HD ± SD[a]	ZP ± SD[a]	PDI[b] ± SD[a]	Atten-uator[c]	N[d]
NM 101	Stock suspension (1000)	517.3 ± 37.3	-29.8 ± 4.4	0.32 ± 0.05	3.8 ± 0.5	4
	Working suspension I (100)	1698.0 ± 489.6	-3.9 ± 3.6	0.33 ± 0.07	5.0 ± 0.5	4
NM 102	Stock suspension (1000)	610.9 ± 12.6	11.6 ± 0.8	0.27 ± 0.02	4 ± 0.0	3
	Working suspension I (100)	705.5 ± 131.0	7.1 ± 8.3	0.26 ± 0.02	5 ± 0.0	3
NM 100	Stock suspension (1000)	256.6 ± 7.2	-43.6 ± 7.6	0.17 ± 0.03	3 ± 0.0	4

[a] standard deviation, [b] poly dispersity index, [c] a value of 11 indicates that full power of the laser is used for data collection, [d] number of independent experiments

5.2.2 Immobilization tests with medium A

Besides the *Daphnia* immobilization tests in medium B, tests were conducted additionally in medium A (ISO water) with the nanomaterials NM 101 and NM 102 under SSR and LL irradiation (Fig. 14, A-D). These additional tests were conducted to elucidate whether the medium composition has an influence on the SSR induced toxicity of the nanomaterials NM 101 and NM 102. Exposure of daphnids to NM 101 and NM 102 with SSR resulted in clear dose response relationships (Fig. 14, A and B). The EC50 (48 h) values were calculated as 2.9 mg/L and 1.1 mg/L and the LOEC (48 h) values as ≤ 1.3 and ≤ 0.2 mg/L (Tab. 8). Fig. 14 B shows that with SSR, NM 102 already had significant effects on the mobility of daphnids after an exposure period of 24 h. This was not observed for NM 101. In general, toxicity of both materials was time dependent, showing higher effects with prolonged exposure duration. When daphnids were exposed simultaneously to the nanomaterials and LL, immobility was not greater than 10% (Fig. 14, C and D). Adsorption of the NM 101 agglomerates (3.1 mg/L) to the carapax and antenna of *D. magna* is documented in Fig. 15 B. This was also observed but not documented for the two other TiO_2 materials.

Tab. 9 summarizes the hydrodynamic diameter (HD) and zeta potentials (ZP) of the nanomaterials in the used stock suspensions. The HD and ZP of NM 101 account to 488.9 nm and -33.4 mV and those of NM 102 to 621 nm and 12 mV. The mean polydispersity indices (PDI) for the measurements are also shown in this table.

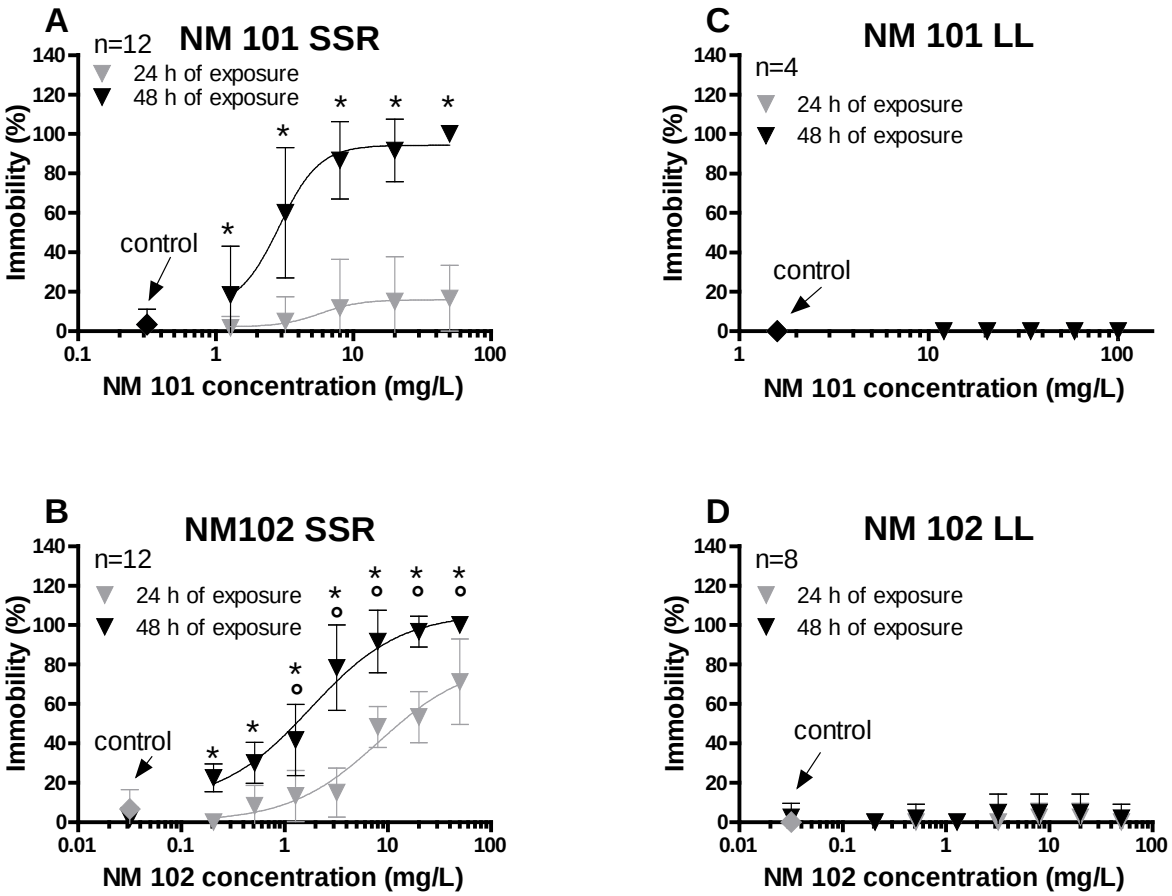

Fig. 14: Immobility (%) of *Daphnia magna* exposed to NM 101 and NM 102 with either simulated solar radiation (SSR) or with laboratory light (LL) in medium A. Error bars represent standard deviation derived from the replicates of one to three independently conducted experiments (n=4-12). Circles and asterisks indicate significant differences to the control at 24 h and 48 h of exposure (*P<0.05).

Tab. 8: EC50, LOEC and NOEC values (nominal) derived from the *Daphnia* sp. acute toxicity tests with NM 101, NM 102 and NM 100 performed with simulated solar radiation (SSR) in medium A (ISO water).

TiO_2 material (h of exposure)	Light condition	EC50[a] (mg/L)	95%-CL[b] upper/lower (mg/L)	LOEC[c] (mg/L)	NOEC[d] (mg/L)
NM 101 (24 h)	SSR[e]	n.c.[e]	n.c.	> 50.0	≥ 50.0
NM 101 (48 h)		2.9	3.5/2.3	≤ 1.3	< 1.3
NM 102 (24 h)	SSR	16.1	24.3/11.5	1.3	0.5
NM 102 (48 h)		1.1	1.4/0.8	≤ 0.2	< 0.2
NM 101 (24 h)	LL[f]	n.c.[g]	n.c.	> 100.0	≥ 100.0

TiO_2 material (h of exposure)	Light condition	EC50[a] (mg/L)	95%-CL[b] upper/lower (mg/L)	LOEC[c] (mg/L)	NOEC[d] (mg/L)
NM 101 (48 h)		n.c.	n.c.	> 100.0	≥ 100.0
NM 102 (24 h)	LL	n.c.	n.c.	> 50.0	≥ 50.0
NM 102 (48 h)		n.c.	n.c.	> 50.0	≥ 50.0

[a]median effect concentration, [b] 95% confidence limit, [c] lowest observed effect concentration, [d] no observed effect concentration, [e]not calculable from the tested concentration range

Tab. 9: Hydrodynamic diameters (HD) and zeta potentials (ZP) of the TiO_2 materials in the stock suspensions (deionized water) used for the daphnia tests with medium A.

TiO_2 suspension (mg/L)	HD ± SD[a] (nm)	ZP ± SD[a] (mV)	PDI[b] ± SD[a]	Attenuator ± SD[a]	N[c]
NM 101 Stock suspension (1000)	488.9 ± 14.7	-33.4 ± 2.1	0.32 ± 0.05	4.0 ± 0.6	3
NM 102 Stock suspension (1000)	621.1 ± 39.3	12.0 ± 08	0.31 ± 0.07	3.7 ± 0.6	3

[a] standard deviation, [b] poly dispersity index, [c] number of independent experiments

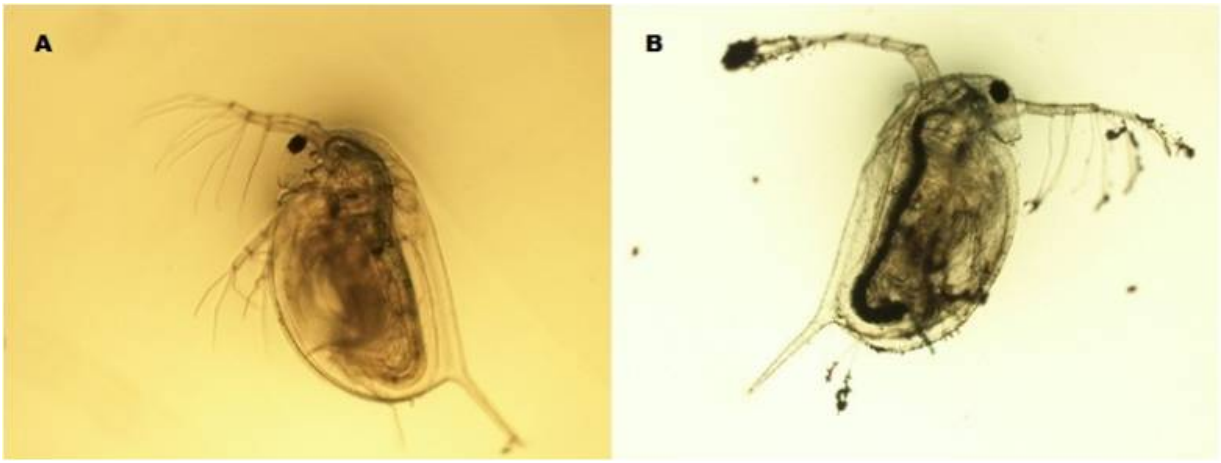

Fig. 15: Adsorption of NM 101 agglomerates (3.1 mg/L) to *Daphnia magna* after an exposure period of 48 h (B). For comparison a control daphnid is shown (A).

5.2.3 General characterization of particles in the stock, working and test suspensions

Dynamic and electrophoretic light scattering - test suspensions

Besides the HD and ZP of the particles in the stock and working suspensions, Fig. 16 depicts the behavior of the different particles in test medium B over a test duration of 48 h. Only the results of valid measurements are shown, which are those of the high concentration NM 101 treatment group (79.5 mg/L) and of both NM 100 treatment groups (1.3 and 3.9 mg/L). However, only the results of the 3.9 mg/L NM 100 treatment group are shown in Fig. 16 B, because both NM 100 treatment groups showed comparable results. No valid results were obtained for the NM 102 treatment groups (0.5 and 1.3 mg/L) and for the low concentration NM 101 treatment group (1.3 mg/L).

For the NM 101 test suspension of 79.5 mg/L it is obvious that the HD of the particles in the test medium at test initiation (753 nm) was larger than in the stock suspension (509 nm) and that the HD was significantly

lower after 24 h (421 nm) and 48 h (289 nm) compared to its HD at test initiation (0 h). Although the zeta potential of the NM 101 particles in the test medium was significantly higher after 48 h (-19.1 mV) than at test initiation (-16.8 mV), the difference was only small (Fig. 16, A). The ZP and HD of the particles in the stock and working suspension did not differ significantly from each other. However the HD and ZP of the particles in the test medium were significantly lower than in the stock suspension. (Fig. 16 B). In general the instrument applied attenuator values of ≥ 8 for the test with NM 102 and for the lower NM 101 concentration, as well as values between 5-7 and 6-8 for the higher NM 101 concentration and the NM 100 tests (data not shown). Attenuator values between 6-9 indicate a good measurement, whereas higher values indicate that the samples were too low concentrated. A value of 11 indicates that full power of the laser is used for data collection.

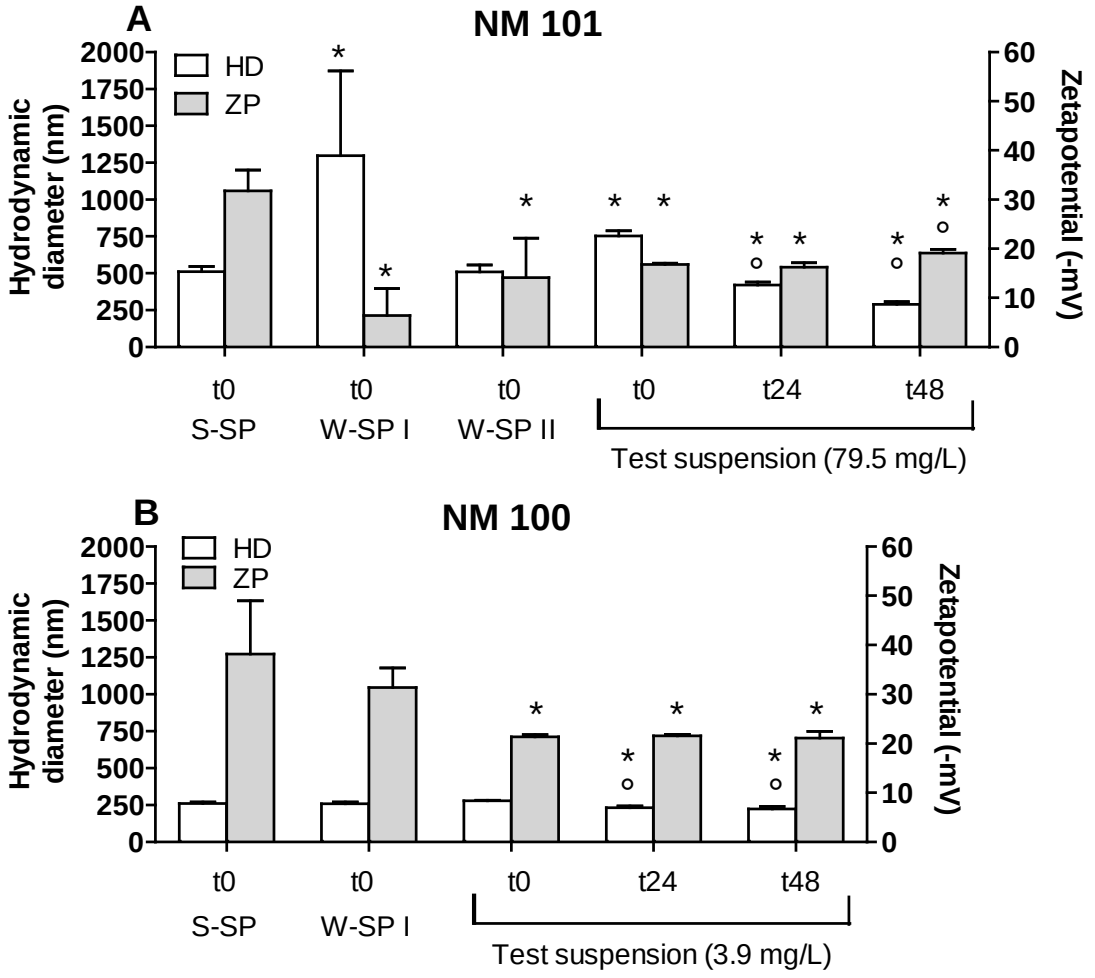

Fig. 16: Hydrodynamic diameters (HD) and zeta potentials (ZP) of NM 101 (A) and NM 100 (B) in the stock (S-SP), working (W-SP I 100 mg/L and W-SP II 10 mg/L) and test suspension at different time points (0, 24, 48 h). Measurements of NM 102 in the test suspension were not valid, (data not shown). Error bars of S-SP and W-SP represent standard deviation derived from a different number of replicates which are summarized in Tab. 3. Three replicates were analyzed for the test suspensions. Asterisks indicate significant differences to the S-SP and circles to the time point t0 of the specific test suspension (*P<0.05).

TiO_2 analysis

To study the sedimentation behavior of the different sized TiO_2 materials in test medium B and to measure the real TiO_2 concentration at the corresponding nominal EC50 concentrations, ICP-OES measurements were carried out in water samples of the overlaying water phase. In the first experiment the real TiO_2 concentration of all materials was analyzed in water samples with one and the same nominal TiO_2 concentration (1.3 mg/L, sedimentation experiment). In a second experiment the real TiO_2 concentration at a TiO_2 concentration corresponding to the EC50 concentration of the specific material was measured (EC50 experiment).

TiO_2 analysis - Sedimentation experiment

Fig. 17 shows the percentage of the nominal TiO_2 concentration (1.3 mg/L) at test initiation (0 h) and termination (48 h) for all TiO_2 materials. In general it is obvious that the measured concentrations at test initiation account to around 20-10% of the nominal concentrations. Furthermore, the nanomaterial concentrations decrease significantly over the test period. This is not the case for the non-nano reference NM 100 for which the concentration seems to be constant during the test duration. The measured test concentration at test initiation can be ordered in the following way, starting with the highest concentration: NM 101 (0.3 mg/L) > NM 102 (0.14 mg/L) > NM 100 (0.10 mg/L). The NM 102 and NM 100 concentration at test initiation were significantly lower compared to that of NM 101.

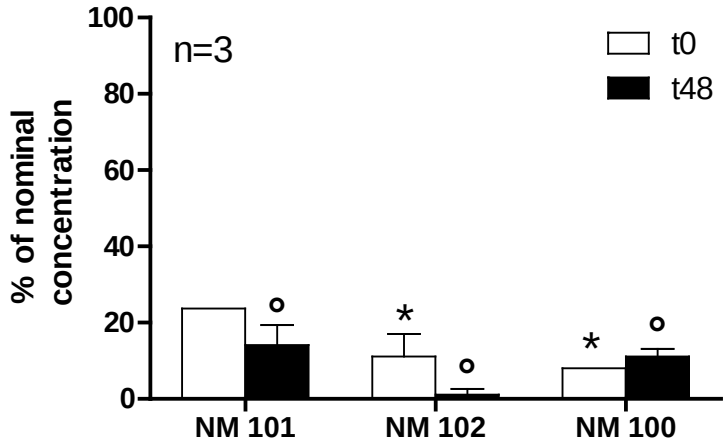

Fig. 17: Sedimentation experiment: The percentage (%) of the nominal TiO_2 concentration (1.3 mg/L), which was measured with ICP-OES at test initiation (0 h) and termination (48) in medium B, is shown for the different TiO_2 materials. Error bars represent standard deviation derived from the replicates of one experiments (n=3). Asterisks indicate significant differences to NM 101 at t0 and circles significant differences between t0 and t48 for each material (*P<0.05).

TiO_2 analysis – EC50 experiment

Tab. 10 summarizes the results of the EC50-experiment. Again it is apparent that for the lower nominal TiO_2 concentrations (0.5-3.9 mg/L) the measured concentrations at test initiation only represent around 25-10% of the nominal concentrations and that further decrease occurs during the testing period for the nanomaterials but not for the non nano-reference. Contrary to the low NM 101 concentration (nominal: 1.3 mg/L), the percentage of the nominal concentration at test initiation accounts to around 80% for the high NM 101 concentration (nominal: 79.5 mg/L). Nevertheless, this treatment group showed the strongest sedimentation

behavior (almost 97%) of all treatment groups. The mean measured exposure concentrations (C_{mm}) can be put in following descending order: NM 101 LL (33.53 mg/L) > NM 100 SSR (0.33 mg/L) > NM 101 SSR (0.24 mg/L) > NM 102 SSR (0.09 mg/L)

Tab. 10: EC50 experiment: Measured TiO_2 concentrations at test initiation (0 h) and termination (48 h) as well as the mean of both values (C_{mm}). Nominal concentrations correspond to the EC50 values of the different materials which were determined in the *Daphnia* sp. acute immobilization tests. Asterisks indicate significant differences to the TiO_2 concentration at test initiation (*P<0.05).

TiO_2 material (Light condition)	TiO_2 nominal (= EC50)	TiO_2 real ± SD[a] (mg/L)			% of nominal ± SD[a]	
		t0	t48	C_{mm}[b] (t0-t48)	t0	t48
NM 101(SSR)	1.3	0.30 ± 0.00	0.18 ± 0.07*	0.24	23.7 ± 0.0	14.1 ± 5.3
NM 101 (LL)	79.5	64.87 ± 3.34	2.18 ± 1.22*	33.53	81.6 ± 4.2	2.7 ± 1.5
NM 102 (SSR)	0.5	0.14 ± 0.03	0.04 ± 0.02*	0.09	26.8 ± 6.6	6.8 ± 3.2
NM 100 (SSR)	3.9	0.31 ± 0.07	0.34 ± 0.36	0.33	8.1 ± 1.7	8.8 ± 9.4

[a] SD standard deviation, [b] C_{mm} mean measured exposure concentration

5.2.4 Summary of the results

Ecotoxicity tests

The results of the present study show that:

- the level of SSR used in this study had no effect on the mobility of control daphnids.
- that toxicity of the different sized TiO_2 materials to *D. magna* was enhanced by environmental realistic levels of SSR (Fig. 13 A-C). Following EC50 values were calculated for the SSR tests: 0.5 mg/L (NM 102), 1.3 mg/L (NM 101) and 3.9 mg/L (NM 100)
- except for NM 101 (smallest NP, EC50 79.5 mg/L) no material showed toxicity under laboratory light exposure only (Fig. 13 D-F).
- EC50 values determined for NM 101 (2.9 mg/L) and NM 102 (1.1 mg/L)in medium A tests with SSR were slightly higher compared to those of tests with medium B and SSR (Fig. 13 A and Fig. 14 A, Tab. 6 and 8). No toxic effect was observed for NM 101 with LL in medium A.

Characterization experiments

- In general, it was not possible to determine the HD of the nanomaterials in the lower concentrated test suspensions (0.5-1.3 mg/L in medium B) whereas it was possible to determine the HD of the particles in the higher concentrated NM 101 test suspension (79.5 mg/L) and that of the particles in the NM 100 test suspension (1.3 and 3.9 mg/L). These results indicate that the HD of NM 101 in the test medium at test initiation was almost 250 nm larger than that in the stock and working suspension II (~500 nm) and decreased during the test period (48 h, 290 nm, Fig. 16, A). In the test suspension the HD of NM 100 (259 nm) was comparable to that in the stock and working suspension (260 nm) as well as to its primary particle size (Fig. 16, C).

- The sedimentation experiment revealed a strong agglomeration behavior of all particles, which is characterized by an immediate sedimentation of the particles from the water phase, resulting, except for the high NM 101 concentration, in very low measured concentrations of 10-20% of the nominal value at test initiation in the upper water column. Furthermore, this agglomeration behavior was more pronounced the larger the primary particle size of the material was: NM 100 >NM 102 >NM 101 (Fig 17). However, this is not reflected by the HD of the particles in the stock suspensions.
- According to the EC50 experiment the measured concentrations were much lower than the nominal values which corresponded to the specific EC50. However, based on the real TiO_2 concentrations NM 102 was still the most toxic material, followed by NM 101 and NM 100 (Tab. 10).

5.3 Discussion

5.3.1 Ecotoxicity tests

In general, the findings of this study prove that except for NM 101 in medium B (10-fold diluted ISO water, i.e. at low ionic strength), no TiO_2 material has an effect on the mobility of *D. magna* when they are simultaneously exposed to LL. Further they reveal, that SSR enhances or induces the toxicity of all materials to *D. magna* and that these effects were slightly more pronounced in the 10-fold diluted ISO-medium (medium B) than in undiluted ISO-medium (medium A).

The laboratory light experiments revealed that except for the smallest TiO_2 material NM 101 in medium B no TiO_2 material had an effect on the mobility of *D. magna*. These results show that in the medium B tests NM 101 particles affected the mobility of the daphnids (48 h-EC50 79.5 mg/L). However, no effect of NM 101 on the daphnids was observed in medium A tests. Thus, the higher ionic strength of the medium counteracts the toxic effect of NM 101, e.g., through a more pronounced agglomeration resulting in a lower availability and toxicity of NM 101 particles for daphnids in medium A than in medium B. Further research is necessary to observe whether NM 101 ROS formation is induced already at laboratory light conditions or if NM 101 itself is toxic to *D. magna*. Generally the results of our study are in line with those of other studies in which daphnids were exposed to TiO_2 nanomaterials with laboratory light. These studies revealed either no toxicity or low toxicity of the tested materials (Dabrunz et al. 2011, Dalai et al. 2013, Wiench et al. 2009).

Preliminary studies in which control daphnids were exposed to SSR confirmed that the artificial sunlight was not harmful (data not shown).The experiments in medium B reveal that SSR enhances or induces the toxicity of nano scale and non-nano scale TiO_2 nanomaterials. In general, our results are consistent with those of Ma et al. (2012b) showing that SSR enhances the toxicity of nano-TiO_2 (P25, 21 nm, 86% anatase, 14% rutile) to *D. magna* (4-5 day old; 48 h-EC50 29.8µg/L) compared to simultaneous exposure with laboratory light (48 h EC50 >500 mg/L). In another study they showed that SSR induced immobility of *D. magna* correlates with the ROS production by P25 and that immobility of *D. magna* was more pronounced the higher the intracellular ROS formation was. The latter finding indicates that probably oxidative stress within the daphnids was responsible for the observed immobility (Ma et al. 2012a). ROS at unphysiological concentrations is known to adversely affect lipids, proteins or DNA in biological tissues (Pan et al. 2009). Regarding these findings we assume that in our study SSR induced ROS production by the nano-scaled as well as by non-nano scaled TiO_2 materials resulted in the observed immobility of daphnids.

Compared to the study of Ma et al. (2012b) we investigated not only one nanomaterial, but two different sized TiO_2 materials as well as one non-nano scale TiO_2 material. Therefore, we were able to examine whether the SSR induced immobility depends on particle size and even more on nano specific characteristics of TiO_2.

As explained above SSR enhanced or induced the toxicity of both, nano scale and non-nano scale TiO_2 materials. However, the effects of the nanomaterials NM 101 and NM 102 on the mobility of *D. magna* were more pronounced than the effect of the non-nanomaterial reference NM 100, resulting in an eight-fold lower EC50 value. A comparison of the LOEC values of the nanomaterials NM 102 and NM 101 (LOEC, 48 h, 0.25 and 0.69 mg/L) to that of the non nano-reference (LOEC, 48 h, 5.56 mg/L) is difficult, because the concentration response curve for NM 100 shows a steep increase between the two tested NM 100 concentrations 1.9 mg/L and 5.6 mg/L, indicating that the present data do not allow a precise LOEC determination of NM 100. However, differences in toxicity get obvious when the effects of the materials at a TiO_2 concentration of 1.9 mg/L are compared with each other: 90% or 60% immobility for NM 102 or NM 101 exposures were monitored, whereas only 10% immobility was detected for exposures to NM 100. Besides the different EC50 values, the latter comparison demonstrates clearly that the SSR enhanced toxicity of the TiO_2 materials is more pronounced for the nano scale than for the non-nano scale TiO_2 materials and that the intermediate sized nanomaterial NM 102 is most toxic.

Several studies investigated the influence of particle size on the photoactivity of nano-TiO_2. Although the optimum particle size varies, many studies observed that in the nano range photoactivity was highest for intermediate anatase TiO_2 particle sizes (Allen et al. 2008, Almquist & Biswas 2002, Grela & Colussi 1996, Wang et al. 1997). Almquist & Biswas (2002) compared the photoactivity of anatase TiO_2 particles with a size range between 5-165 nm with each other and observed an optimum anatase crystal particle size of around 25 nm. They suggest that for particles smaller than 25 nm photoactivity depends more on optical and electrical properties as e.g. light absorption, scattering efficiencies and charge-carrier dynamics, which strongly depend on particle size, whereas for particles greater than 25 nm photoactivity depends more on the surface area available for redox reactions. Based on our assumption that SSR induced toxicity of the different sized TiO_2 materials is related to differences in photoactivity, the observation of Almquist & Biwas may explain our ecotoxicity results, showing the highest SSR induced toxicity for the intermediate sized TiO_2 material NM 102 (PP 15-25 nm). More precisely, we assume that NM 102 shows the highest photoactivity followed by NM 101 and NM 100. It is noteworthy that SSR not only induced/enhanced the toxicity of the nanomaterial but also of the non-nano reference. This finding is in line with the finding of Almquist & Biswas et al. (2002) that bulk TiO_2 (165 nm) is also photoactive. Finally our results show, that the observed SSR induced toxicity is not a nano specific effect but may depend on the photoactivity of the particles which next to the particle size also depends on their crystal structure, optical and electronic properties. Considering that Tong et al.(2013) found out that the phototoxicity of nano-TiO_2 to *Escherichia coli* and *Aeromonas hydrophila* not only depends on the photoactivity of the material but also on the aggregation state as well as the nano-TiO_2/bacteria surface interaction, the observed phototoxicity of NM 101, NM 102 an NM 100 to *D. magna* may not only depend on the photoactivity of the particles but also on other factors as aggregation state of the particles and effective ROS target area. Ma et al.(2012b) suggest that interaction of nano-TiO_2 and *D. magna* is a prerequisite for ROS mediated toxicity of nano-TiO_2 thus emphasizing the importance of ROS target area for the extent of phototoxicity of nano-TiO_2 to *D. magna*.
Finally the findings of this study indicate the importance to a) consider solar radiation in the risk assessment of TiO_2 materials b) to perform a case by case hazard assessment for different TiO_2 materials (unless no valid

concept for a categorization of nanomaterials exists) and additionally c) to not only consider the environmental risk of nano scale but also of non nano scale TiO_2 materials.

In our study, the toxicity of e.g. NM 101 upon SSR exposure was enhanced 60 fold compared to the toxicity observed with LL in medium B based on nominal concentrations. In general, based on the nominal concentrations the SSR induced toxicity of the different sized TiO_2 materials (NM 101 EC50 1.28 mg/L, NM 102 EC50 0.53 mg/L, NM 100 EC50 3.88 mg/L) was lower than the SSR induced toxicity of P25 (4-5 day old; 48 h-EC50 29.8µg/L) as shown in the study of Ma et al (2012b). P25, consisting of a mixture of 20% rutile and 80% anatase, is known to be more photoactive than pure anatase TiO_2. Anatase and rutile crystallites are interwoven with each other forming nanoclusters. This structures allow a fast transfer of electrons between rutile and anatase, thereby rutile acts as an antenna for anatase and thus enhances the photoactivity of P25 compared to pure anatase TiO_2 materials (Hurum et al. 2003). However, based on the mean measured exposure concentration (C_{mm}) of NM 102 which has almost the same primary particle size (15-25 nm) as P25 the SSR induced toxicity to *D. magna* is comparable (EC50 48 h, 90 µg/L). Relating the EC50 to the estimated environmental aquatic concentrations of nano-TiO_2 in the µg/L range (Gottschalk et al. 2009), we conclude that NM 102 may pose a risk to the environment. However, it remains unclear whether the presence of natural components of surface water, e.g., humic and fulvic acids, have an influence on the ROS formation of TiO_2 materials. Humic substances are known to absorb solar radiation. Furthermore, it is also not clear whether the TiO_2 material suspended in the water phase or sedimented at the bottom of the test vessel causes the observed SSR induced toxicity. We observed that the different TiO_2 materials exhibit a strong agglomeration behavior, resulting in a TiO_2 concentration in the upper water phase of only 10-20% of the nominal concentration. The formation of a bottom layer was visually observed for all TiO_2 materials. Thus, during the test period a concentration gradient is rapidly formed within the test suspension, with low concentrations at the top of the test vessel and high concentrations at the bottom of the test vessels. Thus, the EC50 value of NM 102 which is based on the analytically verified concentration (C_{mm}) represents the concentration in the upper water phase and thereby represents a worst case scenario. Due to these uncertainties, in order to assess the environmental risk of TiO_2 materials for *Daphnia magna* further research is necessary to evaluate whether the SSR induced toxicity of the TiO_2 materials is related to the ROS formation potential of the different TiO_2 materials and whether e.g. humic/fulvic acids have an influence on the SSR induced ROS formation and to investigate which part of the TiO_2 in the test vessels is responsible for the SSR induced toxicity of the TiO_2 materials.

In this study, not only the influence of SSR but also the influence of the ionic strength of the medium on the toxicity of the nanomaterials was investigated. The 48 h results of this experiment indicate that SSR induced toxicity of NM 101 (medium A: EC50 2.9 mg/L) and NM 102 (medium A: EC50 1.1 mg/L) was less pronounced in medium A (ISO medium) than in medium B (EC50 1.28 and 0.5 g/L, 10fold diluted ISO medium). Regarding the 95% confidence limits it is obvious that the difference is more pronounced for NM 102 than for NM 101 (Confidence limit (CL) medium A/B (mg/L): 0.8-1.4/0.43-0.65 and 2.3-3.5/0.61-3.66). Differences in SSR induced toxicity between the different media after 24 h were observed for both materials: NM 102 induced earlier toxic effects on the mobility of *D. magna* than NM 101 (EC50 24 h, NM 102: 16.1 mg/L, NM 101 no effect), but for both materials these effects were also less pronounced in medium A than in medium B (EC50 24 h, NM 102 0.99 mg/L and NM 101 5.85 mg/L). Consequently, we suggest that the higher ionic strength of the medium A induced a faster and stronger agglomeration of the nanoparticles. This suggestion is in accordance with the DLVO theory stating that at higher ionic strength surfaces charges of the particles are shielded, thereby reducing the diffusive layer thickness; thus, the particles due to Van-der-Waals interactions have a higher tendency to agglomerate. As suggested above

phototoxicity may not only depend on the photoactivity of the particles but also on the agglomeration state of the particles and the particle/daphnia interaction. A stronger agglomeration results in a stronger sedimentation thereby reducing the particle/daphnia interaction and thus the phototoxicity of the particles in ISO compared to diluted ISO medium.
Due to the lower variability of ecotoxic results if performed in ISO medium compared to the medium at reduced ionic strength (diluted ISO medium) we recommend testing TiO_2 nanomaterials with ISO medium.

5.3.2 Characterization experiments

Additionally to the ecotoxicity tests the behavior of the different particles in the test suspensions was analyzed with regard to their hydrodynamic diameter (HD), zeta potential (ZP) and TiO_2 concentration. Furthermore, the HD and ZP were measured in the stock and working suspension to compare these results with those determined for the particles in the test suspensions. Two experiments were conducted: In the first experiment the sedimentation behavior of the different materials was compared to each other, by measuring the TiO_2 concentrations of the materials at identical nominal TiO_2 concentration (sedimentation experiment). In a second experiment, the measured TiO_2 concentrations, corresponding to the determined EC50 value of the specific materials were measured (EC50 experiment).

To sum up, the most important result of the sedimentation experiment is that all materials at test initiation have a strong tendency to agglomerate and that agglomeration was more pronounced the larger the primary particle size of the material was. Regarding the HD values of the particles in the stock suspension, it is obvious that the sedimentation behavior does not correlate with the HD. This may be related to the fact that DLS measurements of NM 100 probably underestimate the HD due to the fast sedimentation of the agglomerates leaving only the smaller sized particles in the measured water phase. In general, the strong agglomeration behavior of the TiO_2 materials results in very low measured TiO_2 concentrations in the overlaying water phase (10-20% of nominal the nominal value) as consequence of the low zeta potential of the TiO_2 particles in the test medium (around -20 mV, Fig. 16). Dabrunz et al. (2011) and Ma et al. (2012b) also examined the TiO_2 concentration during their test period, but did not observe such strong sedimentation of their tested TiO_2 materials at the beginning of the experiment although the ionic composition of the test medium in their study was comparable to ours, i.e., differences according to the ionic strength of the test medium can be ruled out. Despite of these different observations, the very low variability in the present results and similar results in the sedimentation and EC50 experiment (except for the highly concentrated NM 101 treatment group, 80% of the nominal value) confirm the validity of our results. A possible explanation for the differences in the TiO_2 agglomeration behavior at the beginning of the experiments might be the sampling procedures: we measured TiO_2 concentrations at the end of the test directly in the test vessels, which had a height of 10 cm. To ensure that the already sedimented particles were not resuspended by sampling, the overlaying water phase was collected by introducing the pipette tip on top of the water surface. At test initiation samples were similarly taken but from beakers (diameter: 6.5 cm; height: 9.2 cm) storing 80 ml test medium rather than from the test vessels. In contrast, Dabrunz et al. (2011) and Ma et al. (2012b) sampled from the middle of the water phase, but no information is given concerning the height of their test vessels. As sedimentation probably results in a concentration gradient, the sampling height and the test vessel geometry is expected to have an influence on the outcome on the measured TiO_2 concentration.

Regarding the very low measured TiO_2 concentrations in test medium B, it might be assumed that the application efficiency of TiO_2 to the test vessels was very low in our study. For tests with medium B test suspensions were prepared from working suspensions which were previously diluted from the stock suspension. We guess that this dilution might have resulted in lower measured TiO_2 concentrations in the

working suspensions than expected for the nominal value. Reasons for this might have been a strong sedimentation of the particles in the stock suspension leading to a lower TiO_2 amount which was transferred to the working suspension. However, NM 101 tests in medium A were prepared directly from the stock suspensions. As effects of NM 101 were more pronounced in medium B than in medium A, it is assumed that a dilution failure did not occur during the conduction of tests in medium B. Finally, it is assumed that the measured TiO_2 concentrations do not represent the overall application efficiency for the tests and that the low concentrations at the beginning of the experiment represent the TiO_2 concentration in the upper phase of the test suspension column.

Furthermore, it is assumed that the daphnids are exposed to a TiO_2 concentration gradient within their test vessels, with the highest concentration at the test vessel bottom and the lowest concentration at the water surface. Considering that the measured EC50 concentrations in the present study are based on the TiO_2 concentration in the upper water column they represent a worst case scenario. It is worth noting that it is unclear which part of the applied TiO_2 material (bottom of the vessel or in the water phase) contributes to ROS production and thereby is responsible for the observed effects of the TiO_2 materials under SSR. Based on the measured concentrations NM 102 is the most toxic material, followed by NM 101 and NM 102.

The characterization of the particles in the test suspension by means of DLS and ELS was not possible for all materials and concentrations: the HD of the nanomaterials in the lower concentrated test suspensions could not be measured due a low signal/noise (S/N) ratio, but in the higher concentrated NM 101 test suspension because of a higher S/N. The HD of the particles in the NM 100 treatment group even at low real concentration (real: 0.34 mg/L) was possible since larger particles scatter more light than smaller particles. Fig. 16 demonstrates that only for the non-nano reference material NM 100 the HD in the test suspension did not change compared to that in the stock suspension. This was not the case for NM 101 having a HD almost 200 nm larger than that in the stock suspension.

Regarding the results of this characterization experiment the following recommendations and considerations can be given for the conduction of the *Daphnia* sp. acute immobilization test (OECD 202) with TiO_2 nanomaterials:

- Due to the strong agglomeration behavior of the particles it is recommended to stir the stock and working suspension when they are used to prepare test suspensions.
- DLS measurements are not applicable for low TiO_2 nanomaterial concentrations (nominal: < 1.3 mg/L). Field flow fractionation might be used alternatively, if available.
- From the time of application of the stock suspension to the test medium, the test suspension is a dynamic system driven by sedimentation of the particles resulting in a concentration gradient of the particles. Therefore, different sample collection methods such as the sampling height may influence the outcome of the TiO_2 analysis results. Thus, alternative sampling collection regimes/strategies should be elaborated.
- It is assumed that a TiO_2 concentration gradient is developed in the test vessel with a high concentration at the bottom and a low concentration at the surface of the water column. As it is not known up to now which part is responsible for the extent of the observed toxic effects, this raises the question on which concentration the observed effects should be based.

5.4 Conclusion

The results of the present study show that toxicity of the nano sized as well as the non nano scale TiO_2 materials to *D. magna* was promoted by environmental realistic levels of SSR (Fig. 13 A-C). The effect was more pronounced for the nanomaterials (NM 101 and NM 102) than for the non-nanomaterial reference (NM 100). It is suggested that SSR induced toxicity is a consequence of SSR induced ROS production by the TiO_2 materials and that differences in toxicity between the different TiO_2 materials may be explained by differences in photoactivity. Except for NM 101 (smallest primary particle size), no material showed toxicity under laboratory light exposure (Fig. 13 D-F) indicating either that the material itself is toxic or that ROS production through NM 101 is already induced at wavelengths included in laboratory light. Furthermore, the study showed that the higher ion concentration of medium A may have weakened the SSR induced effect of NM 101 and NM 102 to *D. magna*. A strong sedimentation behavior of the particles was observed at the beginning of the tests resulting in very low TiO_2 concentrations in the upper water column, and in consequence resulting in lower EC50 values with regard to measured concentrations compared to nominal concentrations.

In general, this study emphasizes the need for testing nano-TiO_2 under environmental realistic levels of SSR in ecotoxicity assays because SSR induced ROS production seems to be the main mechanism of TiO_2 toxicity.

5.5 Perspective

Further research is necessary to investigate whether the observed SSR induced toxicity is related to the ROS formation potential of the particles and whether the toxicity is dependent on the induction of oxidative stress within the organisms. Measurements of the activity of antioxidant enzymes such as catalase, gluthathione S-transferase and superoxide dismutase should be performed in exposed daphnids in order to link the observed effects to possible ROS production. Furthermore, it would be necessary to observe whether the documented toxicity is dependent on the TiO_2 at the bottom layer or in the overlaying water concentration. These results would give advice on which concentration the EC50 should be based. Moreover, the mechanisms behind the observed effect of NM 101 under laboratory light should be further examined by measuring the level of ROS in the test medium. In this way, it would be clarified whether ROS production through NM 101 is possibly already induced at laboratory light wavelengths. Testing of NM 100 in medium A with SSR would give further information on the influence of the ionic strength of the medium on the SSR induced effects of TiO_2. Besides testing in artificial water as ISO water it would be interesting to observe the influence of natural organic matter on the phototoxicity of TiO_2 materials.

6 Fish embryo acute toxicity (FET) test (OECD 236)

6.1 Material and methods

6.1.1 Chemicals

All three TiO_2 materials NM 101, NM 102 and NM 100 were tested. For details on the materials see section 2.1.1.

6.1.2 Good laboratory praxis (GLP)

The fish embryo toxicity test was performed at the Institute for Environmental Research at RWTH-Aachen University in accordance with Good Laboratory Practice (GLP). Because our institute is a non-GLP testing facility, IBACON and the institute agreed on performing only the following, relevant working procedures in accordance to GLP: a) reporting of raw data, b) calibration of pipettes and balances. During a stay of the quality assurance management of IBACON calibration of pipettes and balances was inspected once.

We did not write a SOP for this test, because except for the preparation of the TiO_2 suspension the test was performed according to the OECD guideline 236. Small deviations from this guideline are summarized in table 11. The preparation of the TiO_2 suspensions is explained in the SOPs 'Preparation of a NM 101, NM 102 and NM 100 suspension' (Annex 2-A, 2-B, 2-C).

6.1.3 Fish embryo acute toxicity test (FET, OECD 236)

Detailed information on the performance of the FET with nanomaterials is given in the OECD guideline 236 fish embryo acute toxicity test.

Briefly, adult fish (*Danio rerio*) were derived from the division Applied Ecology of the Fraunhofer Institute for Molecular Biology and Applied Ecology in Schmallenberg, Germany. They were maintained in dechlorinated tap water at 26°C with a light dark regime of 14:10 hours.

When the light was turned on in the morning, eggs were produced via mass spawning of a group of fish consisting of a gender ratio of 1:2 female and male fish. Eggs were collected in a container which was covered by a mesh so that the adult fish could not eat their own offspring. Directly after spawning fertilized eggs which were within the 8-cell - 64-cell stages, undergoing normal cleavage and showing no injuries of the chorion were selected by using a binocular microscope.

To prevent inadequate exposure by loss of the titanium dioxide material by sedimentation, selected embryos were transferred directly into the test vessels (explanation see tab. 11; six well plastic plate). Five embryos were transferred within 1 ml 10% higher concentrated reconstituted water (HCRW, ISO 1996) to a test medium volume of 9.0 ml. Depending on the treatment group the test medium consisted of 90% HCRW and 10% deionized water or TiO_2 suspension (stock or working suspension) or a mixture of deionized water and TiO_2 suspension. Stock suspensions (1 g/L) were prepared as described in the SOP 'Preparation of a NM 101, NM 102 and NM 100 suspension' (Annex 2-A, 2-B, 2-C) prior to testing and were diluted with deionized water to a concentration of 100 mg/L (working suspension). After test vessels were covered with an air permeable membrane plates were placed in a 26°C tempered incubator under dark conditions. Embryos were exposed to the titanium dioxide materials for 72 h without replacement of the test medium. Sub-lethal and teratogenic effects, which are described in the guideline, were recorded every 24 h.

A preliminary study was performed with NM 101 (1, 10, 100 mg/L) and NM 102 (2.6, 16 and 100 mg/L) independently to the present study under the same test conditions as described in the main study, except that exposure was 96 h. Because it was a preliminary study, raw data were not collected in the style of GLP.

In the main study, all titanium materials were tested simultaneously at three different concentrations (1, 10 and 100 mg/L) with 10 embryos per concentration. Parallel to test material exposures, 40 control embryos were exposed to the test medium consisting of HCRW and deionized water only. Additionally positive controls were conducted. Therefore, 20 embryos were exposed to the reference substance 3,4-dichloroaniline with a concentration of 3.7 mg/L This concentration should induce an embryo mortality of > 30% after an exposure duration of 96 h.

Although the data of the preliminary study was not collected in the style of GLP, for a statistical improvement of data it was decided to consider the results of the 100 mg/L treatment groups for the evaluation of the toxicity of NM 101 and NM 102 to avoid additional animal experiments. It shall be noted that in the German state North Rhine-Westphalia the fish embryo toxicity test is regarded as an animal experiment as soon as embryos are older than 48 hours post fertilization (hpf).

In total 20 embryos were tested for the highest concentration of NM 101 and NM 102 (100 mg/L) in two independent experiments with each 10 eggs. Therefore, according to the guideline the criteria for a limit test, namely the testing of 20 embryos at 100 mg/L are met. NM 100 was only tested once with 10 embryos.

As the preliminary study showed that NM 101 and NM 102 had no effect on the survival and hatching rate of *D. rerio* embryos and larvae it was decided not to perform analytics of the titanium dioxide materials in the main test.

6.1.4 Preparation and characterization of the TiO_2 stock and working suspensions

Stock suspensions (1 g/L) were prepared by suspending the nanomaterials in deionized water using ultrasonication with a microtip (200 W) according to the SOPs ‘Preparation of a NM 101, NM 102 and NM 100 Suspension’ (Annex 2-A, 2-B, 2-C). Working suspensions were prepared by diluting the stock suspension with deionized water to 100 mg/L. Suspensions were characterized according to the SOP ‘Characterization of a Nanomaterial Suspension’ by means of dynamic light scattering (Annex 3-A). Furthermore, the zeta potentials of the particles in the suspensions were measured with a zetasizer (Malvern Instruments, Worcestershire, UK).

6.1.5 Analysis and statistics

As no dose response relationships were observed no EC values were calculated.

6.2 Results

6.2.1 Preliminary study with NM 101 and NM 102

No mortality was observed for NM 101 and NM 102 up to a concentration of 100 mg/L after the exposure period of 96 h. The survival (87.5%; 96.5%) and hatching rate (80%; not documented) of the negative controls and the survival rate (0%; 14%) of the positive controls were as high as required in the OECD guideline. The fertilization rate was 80% and 90%.

6.2.2 Main study with NM 101, NM 102 and NM 100

The fertilization rate of the eggs was 90% and the survival and hatching rate of *D. rerio* control embryos accounted to 97.5% and 95 % after an exposure period of 72 h, so that the validity criteria of the OECD were met. NM 101 and NM 100 did not show any effect on the mortality or hatching rate of *D. rerio* in the same time period. A constant mortality of 20% was observed in the lowest NM 102 concentration (1 mg/L) at each evaluation time. No embryo mortality was observed at 10 and 100 mg/L. 80% mortality was observed for the positive controls after an exposure period of 72 h. This result is in line with the validity criteria given in the OECD guideline (4 mg/L exposure results in a minimum mortality of 30% after an exposure period of 96 h), because mortality after 72 h was already higher than requested in the guideline after an exposure duration of 96 h. Fig. 18 (D, E and F) and Fig. 19 show that the titanium dioxide materials agglomerated and subsequently formed a sediment layer on the bottom of the test vessels. This occurred already a few hours after the test material was applied to the test medium. Furthermore, it was observed that particles tended to adsorb to the chorion of the embryos (no photo shown) and later to the hatched larvae (Fig. 18 A-C). As a consequence the embryos exposed to high concentrations (≥ 10 mg/L) could not be perceived in detail through the chorion so that in these cases sublethal and teratogenic effects were only monitored after the hatching of the larvae which occurred around 48 and 72 hours post fertilization (hpf).

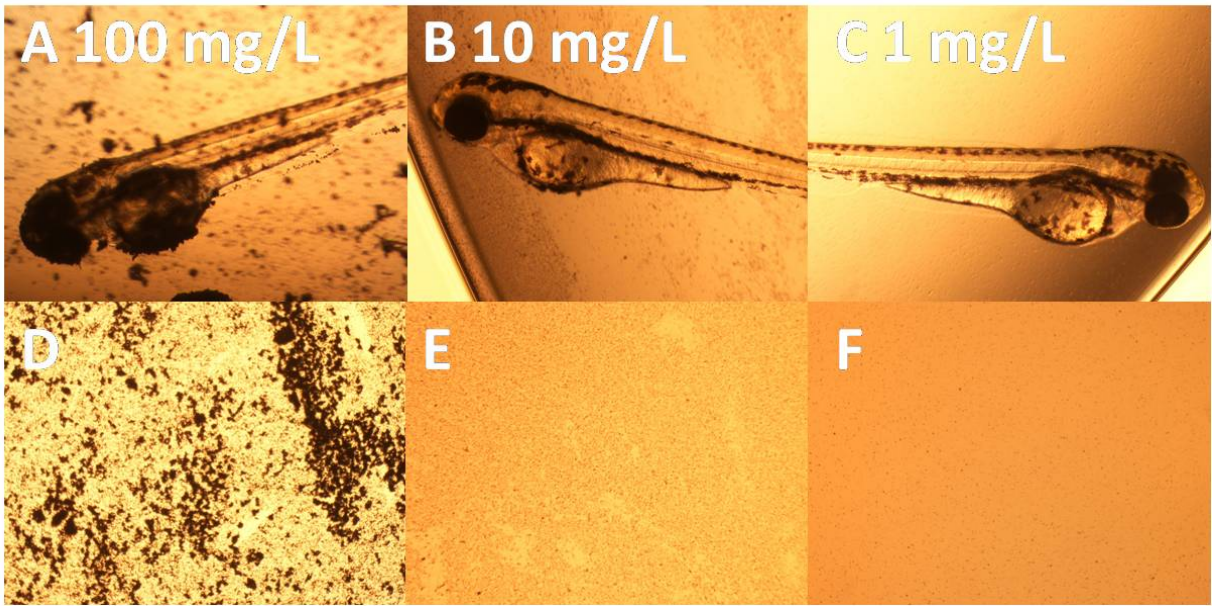

Fig. 18: Documentation of larvae exposed to NM 102 (100-1 mg/L; A-C) for 72 h and documentation of the sediment layer on the bottom of the test vessels (D-F).

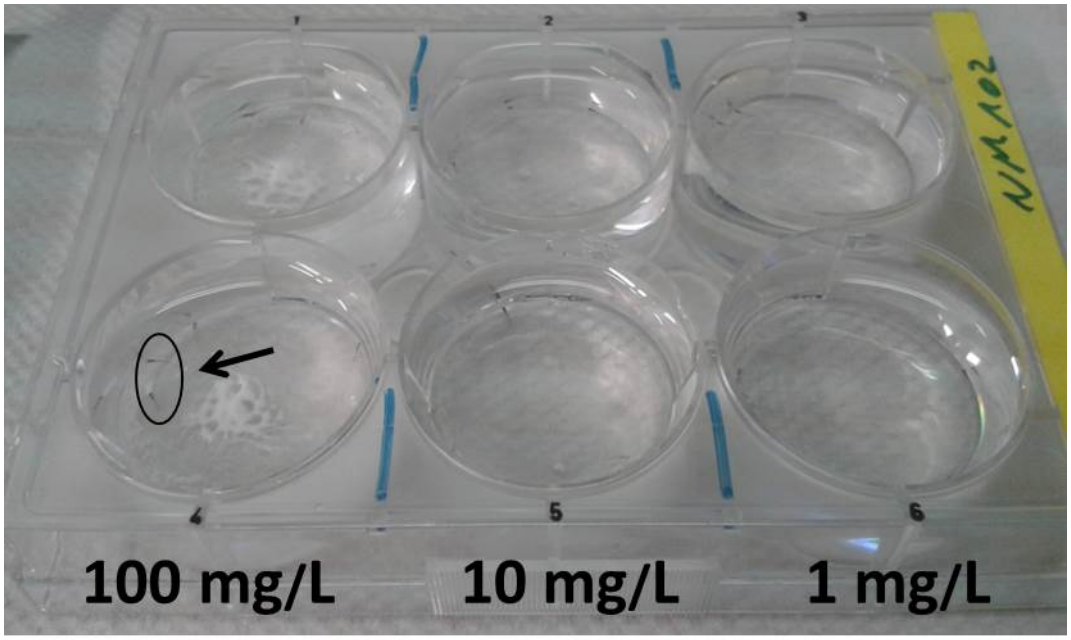

Fig. 19: Test set up of the fish embryo toxicity test with NM 102 after 72 h. Larvae are marked with an arrow.

6.3 Discussion

In general, the fish embryo toxicity test performed in this study deviates in some points from the just approved OECD guideline (approved at 26.07.2013). Tab. 11 summarizes these points and explains the reasons for these deviations.

In general the fish embryo toxicity tests revealed that only NM 102 had a slight effect on embryo survival in the lowest test concentration (1 mg/L) at the end of the exposure period of 72 h. However, this was not the case for higher concentrations (10 and 100 mg/L). Further, no effects were observed at a comparable low NM 102 concentration (2.6 mg/L) in the preliminary study. This demonstrates that the slight effect was not reproducible and does not indicate hormesis, which is characterized by a U-shaped dose response curve (Calabrese & Baldwin 2001).

In conclusion, the results of the fish embryo toxicity test show that all investigated titanium dioxide materials, independent of size, had no effect on embryo survival or the hatching rate of *D. rerio* under the given test conditions. This result complies with studies of Chen et al. (2011) and Zhu et al. (2008) who both did not observe effects on the survival and hatching rate of *D. rerio* after embryos were exposed to P25 (25-70 nm) for 120 h or to an uncoated nano-TiO_2 material (anatase, 230 nm in suspension) as well as an uncoated bulk TiO_2 material (anatase, 1100 nm in suspension) for 96 h.

It might be assumed, that the absence of toxicity of the particles in the present study may be a consequence of missing interaction of the embryos or hatched larvae with the particles. However, Fig. 18 gives evidence that the agglomerated particles adsorbed to the larvae and it was observed that they also adsorbed to the chorion (no photo shown). Nevertheless, this interaction does not verify that the particles were bioavailable for the embryos e.g.it cannot be ruled out that the particles agglomerated to sizes too large to pass through the chorion pores, which have a pore size of 0.5-0.7 µm (Lee et al. 2007). However, some studies indicate that nano-TiO_2 may be bioavailable for larvae. Chen et al. (2011) e.g. observed that larval swimming parameters as average and maximum velocity and the activity level of the *D. rerio* larvae was significantly

affected by nano-TiO_2 concentrations of 0.1-1 mg/L (P25, 25-70 nm) after an exposure period of 120 h. Similar results were observed for juvenile rainbow trout, which were exposed to 1 mg/L nano-TiO_2 (P25, 21 nm, 25% rutile and 75% anatase) for 14 d: The time spent for swimming at high speed was significantly lower compared to control fish. This was not the case for fish exposed to the same concentration of bulk TiO_2 material (134 nm, 25% rutile and 75% anatas; Boyle et al. 2013), indicating a size specific effect. The present study did not focus on behavioral effects of the larvae as these endpoints are not mentioned in the OECD guideline. But it might be necessary based on the results of Boyle et al. (2013) and Chen et al. (2011) to include these endpoints in further studies observing the effects of nano-TiO_2 on the embryonic development of *D. rerio*. For this, it may be necessary to extend the exposure duration to 120 hpf because significant effects on the swimming behavior of *D. rerio* were not recorded before this exposure duration (Chen et al. 2011).

Considering that nano-TiO_2 is known to produce reactive oxygen species (ROS) when it is illuminated with wavelengths corresponding to its band gap energy (3.2 eV, Ma et al. 2012a), it might be assumed that nano-TiO_2 might exhibit toxicity to *D. rerio* embryos under these conditions, especially after the embryos leave their protective chorion. To the best of our knowledge no study exists which investigated the influence of nano-TiO_2 to *D. rerio* embryos in the presence of ultraviolet radiation or solar radiation. However, a study of Ma et al. (2012b) revealed that the toxicity of nano-TiO_2 (P25, 21 nm, 86% anatase and 14% rutile; LC50 2.2 mg/L) to 24 to 48 h old larvae of Japanese medaka (*Oryzias latipes*) was enhanced by two orders of magnitude compared to exposures with laboratory light (LC50, 294 mg/L) after an exposure period of 96 h. Therefore, testing with simulated solar radiation during the conduction of fish embryo toxicity tests with nano-TiO_2 has to be regarded as a necessary exposure scenario. Neglecting this exposure scenario may lead to underestimation of the risk associated with nano-TiO_2 for the embryonic development of *D. rerio*.

Tab. 11: Summary of the deviations between the present study and the instructions given in the OECD guideline 236.

Instruction in the guideline	Deviation from the guideline	Reason for deviation
20 eggs have to be tested for a limit test.	In the main study 10 embryos were tested for the highest concentration (100 mg/L), which was performed in the style of GLP. Further 10 embryos were tested in a preliminary experiment at the same concentration and test conditions. However, the study was not conducted in the style of GLP.	In the German state North Rhine-Westfalia, the FET is considered as an animal experiment when embryos are older than 48 hpf. Therefore, unnecessary animal experiments should be avoided. As no toxic effect was observed for all nanomaterials in the preliminary experiment, only 10 additional eggs were tested in the main experiment for the highest concentration.
If the test item concentration is not ± 20% of nominal concentration a semi-static renewal interval should be applied	Static exposure was used, although it was obvious that more than 20% of the nominal concentration immediately formed a sediment layer on the bottom of the test vessel.	A static exposure was used, because the main focus of this project is to consider relevant exposure scenarios. In nature most probably titanium materials will also agglomerate and form layers on the sediment of lakes and rivers where *D. rerio* embryos are located. Consequently, the static exposure represents a more realistic exposure scenario.

Instruction in the guideline	Deviation from the guideline	Reason for deviation
Pre-exposure of the embryos, when appropriate embryos are selected to ensure the exposure of 8-16 cell stages.	During selection of the eggs, embryos were not pre-exposed to the particles.	This was done, because particles would agglomerate during the 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) were used.
One embryo should be exposed individually.	Five embryos were exposed per well.	Test suspensions were prepared by applying TiO_2 suspensions directly to each test vessel (6 well plate, 10 ml volume per cavity) and not by preparing a large batch of the test suspension which afterwards would be separated to the cavities. This was done to prevent loss of the material during transfer due to sedimentation of the particles in the larger batch. Smaller wells (24 well plates with 2 ml volume per cavity) were not used because then critical small TiO_2 suspension volumes would have been pipetted.

6.4 Conclusion

All materials did not show any effects on the survival or hatching rate of *D. rerio* embryos up to a concentration of 100 mg/L under the conditions tested. A strong sedimentation of the particles was visually observed.

6.5 Outlook

Further studies should investigate the toxicity of different sized titanium dioxide materials in the presence of solar radiation to clarify whether UVA light induced ROS production enhances the toxicity of the materials to the embryo within the chorion and/or to the larvae. Furthermore, as Chen et al. (2011) revealed that P25 had an influence on the behavior of *D. rerio* larvae after an exposure period of 120 h, it would be further interesting to investigate the influence of other, different sized titanium dioxide materials on the behavior of *D. rerio* in order to elucidate if behavior is an appropriate or even necessary additional endpoint to consider.

7 Literature study: Search for an organic co-contaminant with nano-TiO_2 for the mixture experiments (OECD TG 207, 222, 209)

As explained before, it is necessary to include investigations of the mixture toxicity of nano-TiO_2 and a co-contaminant in the risk assessment of nano-TiO_2, because they may occur together with other co-contaminants in specific environmental compartments due to same entry pathways, as e.g. sewage sludge and soil. Nanoparticles have a larger surface area to volume ratio than their bulk counterparts. Therefore, an interaction of organic co-contaminants with nanoparticles is more likely for them than for the bulk counterpart (Hariharan 2006, Haruta 1997).

Thus, not only ecotoxicological effects of nano titanium dioxide (nano-TiO_2) alone but also combinatory effects of nano-TiO_2 in the presence of co-contaminants were considered in this project during the conduction of the earthworm acute and reproduction test (OECD 1984, 2004b) and activated sludge, respiration inhibition test (Fig. 1, OECD 2010).

A literature study was performed to select a representative xenobiotic as a co-contaminant with nano-TiO_2 in the environment. In detail an organic xenobiotic was searched which is likely to occur in soil and sewage sludge along with nano-TiO_2 and for which effect concentrations already are known for organisms inhabiting these environmental compartments. For these reasons xenobiotics, which are present in sewage sludge and manure were screened for their ecotoxicity towards organisms of both environmental compartments, e.g. bacteria of activated sludge (sewage sludge) and worms (soil).

7.1 Key criteria for the organic co-contaminant

Besides the criteria to be of organic origin and to have a toxic potential against organisms inhabiting sewage sludge and soil, further selection criteria were defined during a meeting between the Institute for Environmental Research at RWTH Aachen University and IBACON GmbH in January 2011 (descending importance from 1-9):

1. Organic xenobiotic
2. Application relevance
3. Exposure relevance (occurrence in sewage sludge and/or soil)
4. Known toxicity to bacteria and/or earthworms (*Eisenia fetida*)
5. Chemical analysis methods available and easily accomplishable
6. High persistence (> 14 days)
7. Octanol water partition coefficient (log_{KOW}) 2-5
8. Low photodegradability
9. Possibility to obtain the compound 14C-labeled

The importance of criteria 1, 3 and 4 has already been explained above. As in this project realistic exposure scenarios have to be considered it was important to find a xenobiotic which is not forbidden or restricted in use but will be applied and therefore released into the environment in the following years (see 2. application relevance). Furthermore, it was advantageous if chemical analytical methods of the compound for soil, water, sewage sludge and worm tissue would already be available to simplify analysis of the substance during the ecotoxicity tests (see 5. chemical analysis). Persistence of the chemical during the tests would minimize the formation of metabolites during the test. By this the influence of potential metabolites on the overall toxicity towards the test organisms is reduced and the exposure concentrations are kept more or less constant. Persistence of more than 14 days is aspired because by that time the longest test planned (earth worm acute toxicity test) lasts for 14 days (see 6. persistence > 14 days) (It is worth mentioning here that in the beginning of the project it was not planned to perform the earthworm reproduction test which lasts

56 days.). Log K_{OW} values higher than 5 were not desired due to enhanced adsorption to organic matrices, and thus, reduced bioavailability and chemical extraction efficiency of the xenobiotic. Log K_{OW} values lower than 2 were not favored because of a reduced bioaccumulation potential of the substance and consequently a reduced bioavailability (see 7. log K_{OW} 2-5). If possible, a substance with a low photodegradation potential should be chosen to ensure the stability of the compound during test preparation and testing (see 8. photodegradability). The last criterion was the availability of the 14C-labeled compound. Reason for this was the close collaboration of the present project partners with those of the UFOPlan project No. 3710 65 414 in which the influence on the mobility of nano-TiO_2 and the co-transport of the same environmentally relevant xenobiotic was investigated in soil columns using radioanalytics (Nickel et al. 2013). Hence, the comparison of ecotoxicological data from this project with data of the environmental fate from FKZ 3710 65 414 would be possible (see 9. Availability of the compound as 14C-labeled compound.).

7.2 Reasons for the choice of triclocarban as representative of the organic co-contaminant

During a meeting between IBACON GmbH and the Institute for Environmental Research, RWTH Aachen, the organic substance triclocarban (TCC, point 1 section 5.1) was chosen as the representative co-contaminant of nano-TiO_2 in this project for following reasons:

1. It fulfilled all of our criteria for an organic co-contaminant:
   Besides a high stability in soil (degradation half-life of 108 d, Kwon et al. 2010), a log K_{OW} value of 4.9 (Ying et al. 2007), a potential negative effect on bacteria (antimicrobial substance, point 4, section 5.1), and already known chemical analytical methods (Ying et al. 2007), the main argument for this compound is that it is the only compound in the list of selected candidate co-contaminants which was shown to be detectable in the environment (sewage sludge, 51 mg/kg d.w. sewage sludge, Heidler et al. 2006) at biologically relevant concentrations (LC50 Eisenia fetida 40 mg/kg soil, Snyder et al. 2011). Additionally, the compound is produced at high volumes and has a potential to be distributed to soils through application of sewage sludge onto fields. Heidler et al. (2006) even suggests that three fourths of the used triclocarban in the US is spread to fields (point 3, section 5.1). Further TCC is listed as a priority substance in a review of Clarke and Smith (2011) dealing with emerging pollutants in European sewage sludge. Considering that TiO_2 is an ingredient of sunscreens and TCC of soaps (point 2, section 5.1) they have a similar pathway of release into the environment and therefore a high potential to concomitantly appear in the environment. Besides the above mentioned suitability of TCC, the 14C-labelled isotope is commercially available (point 9, section 5.1), which allows more profound investigations on bioavailability of the compound in soils and sludge, easy biotransformation analyses, and tracking of its interactions with nano-TiO_2 during fate studies.
2. Compared to the other potential co-contaminants TCC and nano-TiO_2 potentially can occur together in soils as well as in sewage treatment plants. All other potential co-contaminants (see Annex 1) would only occur together with nano-TiO_2 in soil after they are applied via manure to fields.
3. None of the other co-contaminants fulfilled all of our criteria (see Annex 1) e.g. the environmental concentrations of all other substances were lower than the ecotoxicological relevant concentrations. Further the 14C labeled substance of all other compounds was not available.

7.2.1 Triclocarban

Triclocarban (TCC, or 3, 4, 4'-trichlorocarbanilide, CAS 101-20-2) is used as an antibacterial agent in household products as bar and liquid soaps and in body washes (0.1-0.3%, Clarke & Smith 2011). Since July 2013 its use in European personal care products (PCP) is restricted to a maximum content concentration of 1.5% ((EG) 2009). However, this threshold is above the typical applied concentration of TCC in PCP (0.1-0.3%, Clarke & Smith 2011). Combined with the fact that TCC is a 'high production volume' substance it is assumed that the restriction will not have an influence on the release of TCC into the environment. Recently, TCC was found in the influent of waste water treatment plants (median concentration of 4.2 ppb, Heidler & Halden 2009) sewage sludge (51 mg/kg d.w., Heidler et al. 2006), and the effluent (median concentration of 0.23 ppb, Heidler & Halden 2009) of waste water treatment plants and subsequently in biosolid treated soils (Cha & Cupples 2009). The fact that TCC was detected in 100 % of 25 wastewater treatment plants observed in the U.S. (Heidler & Halden 2009) strengthens its categorization as a high production volume compound. In sediments high concentrations of TCC were monitored e.g. Miller et al. (2008) detected 25 mg/kg in a sediment core of a New York, USA estuary. *Lumbriculus variegates* may remobilize TCC from sediments. These oligochaetes have a high TCC bioaccumulation potential (Biota-sediment accumulation factor (BSAF) 1.6 g organic carbon/g lipid, Higgins et al. 2009) and serve as food for predators. Therefore, TCC may enter the food chain. Coupled with a considerable persistence of TCC in soil (half-life of 108 d in aerobic soil, Ying et al. 2007) and a high acute toxicity towards soil organisms (LC50 40 mg/kg, Eisenia fetida, Snyder et al. 2011) TCC poses a high risk to the environment. Furthermore TCC has an endocrine disrupting potential, e.g. its presence enhances the activity of estrogen and testosterone in reporter gene assays in recombinant endocrine and testosterone responsive cells (Ahn et al. 2008). Furthermore, metabolism studies in activated sludge showed that TCC decomposes to p-chloroaniline (PCA) and 3, 4-dichloroaniline (3,4-DCA, Gledhill 1975). 3, 4-DCA occurs as an intermediate during the production of dyestuff, pigments, pharmaceuticals and herbicides (Janicke & Hilge 1980, Wegman & De Korte 1981) and is a metabolite during the biodegradation of herbicides in the environment (Crossland 1990, Miller et al. 1980, Saxena & Bartha 1983, Viswanathan et al. 1978). p-Chloroaniline is an aromatic amine used as a starting material in the dye, textile and rubber industries (Beard and Hoe, 1981). In some pharmaceutical preparations it has been detected as a degradation product (Ciarlone et al., 1976). Both substances are known to elicit toxic effects in several organisms (Ensenbach & Nagel 1997, Froehner et al. 2000, Kühn & Pattard 1990).

8 Earthworm tests (OECD 207, 222)

8.1 Material and methods

8.1.1 Chemicals

For a detailed description of the used TiO_2 materials see section 2.1.1. Triclocarban was purchased from Sigma Aldrich (3,4,4,-trichlorocarbanilide, CAS 101-20-2, chemical purity 99%, Sigma Aldrich, Steinheim, Germany).

8.1.2 Soil

RefeSol 01-A (Fraunhofer Institute for Molecular Biology and Applied Ecology (IME), Schmallenberg, Germany), a natural, slightly loamy, middle acidic, very slightly humic soil was used as test soil. The soil was air dried, sieved through a 2 mm sieve and stored at room temperature until use. It has a maximum water holding capacity (max. WHK) of 29.8% and an organic carbon content of 0.93% (C_{org}). At test initiation and termination pH values and dry weight of the prepared soil were determined. pH values were measured in a soil/$CaCl_2$ (0.01 M) suspension (2:5 (w/v)) after a mixing period of 2 h. Dry weight was measured after a drying period of 15 h at 105 °C.

8.1.3 Organism (*Eisenia fetida*)

Adult earthworms (*Eisenia fetida*) that had a clitellum and weighed between 250-600 mg wet weight (ww) were used for the earthworm tests. Worms were derived from a culture maintained at IBACON GmbH (Rossdorf, Germany). Worms were acclimated in uncontaminated test soil under test conditions (20 °C and a light dark rhythm of 16:8 hours) for 24 h before they were transferred to the test soils. Dung which was used for feeding in the chronic earthworm tests was obtained from an organic cow farm, dried and ground to a fine powder.

8.1.4 Preparation and characterization of the TiO_2 suspensions

Suspensions used for the earthworm tests were higher concentrated than those used for the other ecotoxicity tests. Therefore, they were additionally characterized by means of DLS and ELS according to the SOP ‘Characterization of a nanomaterial suspension’. Characterization of the particles was performed in an additional experiment rather than characterizing the suspensions used in the test: A specific amount of TiO_2 material (e.g. 1000 mg for the acute and 1000 mg or 400 mg for the chronic toxicity tests) was weighed into a 200 ml beaker. Thereafter, the material was suspended with as much deionized water as necessary to reach 55% of the maximum water holding capacity of the test soil (e.g. 164 ml deionized water per kg dry weight (dw) RefeSol 01-A), according to the SOP ‘Application of a nanomaterial suspension to soil’. When more than one kg soil was prepared at a time the suspension application occurred in several batches (e.g. 2x164 ml per 2 kg dw soil). This was done to prevent the usage of too high concentrated suspensions. The procedure of application of suspensions to the soil is described in section 6.1.5.

8.1.5 Earthworm, acute toxicity test (OECD 207)

Four test series were performed to evaluate whether the TiO_2 materials have an influence on the acute toxicity of TCC. Each test series consisted of controls (untreated soil, n=4) and five TCC treatment groups (TCC concentrations: 42; 84; 168; 338; 675 mg/kg dry weight (dw); n=4). In the test series I only TCC was

tested. In the other three test series (II-IV) mixtures of TCC and one of the TiO_2 materials NM 101 (II), NM 102 (III) and NM 100 (IV) (1000 mg/kg) were tested with 5 different concentrations of TCC in a co-exposure with one static concentration of one of the TiO_2 materials. Furthermore an additional treatment group with the TiO_2 material in the corresponding concentration was tested (1000 mg/kg dw; n=4).. Earthworm acute toxicity tests were performed in accordance to the OECD guideline 207 (OECD 1984).

One day before the test a specific volume of TCC acetone solutions or only acetone (controls) were applied to soil aliquots (10% of the total soil amount). Soil aliquots were left in a fume hood over night for evaporation of the acetone. The next day the TiO_2 materials were applied to the remaining soil as described in the SOP 'Application of a TiO_2 suspension to soil' (Annex 5). After an amount of freshly prepared test soil corresponding to 500 g dw was weighed into the test beakers ten acclimatized worms were placed in each test vessel. The test vessels were closed loosely with a lid and exposed at 20 °C under continuous light (400-800 lux). After seven and 14 days living worms were counted. At test termination (14 d) worms were additionally washed and weighed. Thereafter, living worms were left on moist papers to clear their guts for 24 h. The number of living worms and the worm weights without gut content were determined after this period.

The biomass change in comparison to the control which was based on the worm weights with gut content (W_{with}) was calculated according to equation 1:

$$W_{with} = ((W_{t0}-W_{t14})/(W_{t0C}-W_{t14C}))*100 \quad \text{Equation 1}$$

The biomass change in comparison to the control which was based on the worm weights without gut content ($W_{without}$) was calculated according to equation 2:

$$W_{without} = ((W_{t0}-W_{t15})/(W_{t0C}-W_{t15C}))*100 \quad \text{Equation 2}$$

W_{tx} mean weight per worm of one treatment group after 0, 14 and 15 days of exposure

W_{txC} mean weight per worm of the control group after 0, 14 and 15 days of exposure

8.1.6 Earthworm, reproduction test (OECD, 222)

Earthworm reproduction tests were performed according to the OECD guideline 222 (OECD 2004b). The mixture experiments were done in two sequences. In test sequence A TCC and NM 101 were tested in the laboratories of IBACON GmbH and in test sequence B TCC, NM 102 and NM 100 were tested in the laboratories of the RWTH-Aachen. The test conditions were the same and test organisms were taken from the same culture.

To test whether the TiO_2 NM 101 has an influence on the chronic toxicity of TCC towards *E. fetida* three test series were performed. In the following the test set up is described: Each test series consisted of controls (untreated soil, n=8) and five TCC treatment groups (TCC concentrations: 42; 84; 168; 338; 675 mg/kg dry weight (dw); n=4). In the first test series only TCC was tested. In the other two test series a mixture of TCC and one of two concentrations (400 or 1000 mg/kg dw) of the TiO_2 material (NM 101) were tested. Besides the five TCC treatment groups the latter test series included additionally treatment groups in which only the TiO_2 material itself in the corresponding concentration (400 or 1000 mg/kg dw; n=4) was applied to the soil.

After an amount of freshly prepared test soil corresponding to 500 g dw was weighed into the test beakers ten acclimatized worms were placed in each test vessel. The test vessels were closed with a perforated plastic lid and were exposed at 20 °C under a light dark rhythm of 16:8 h (400-800 lux). After 28 days living adult

worms were counted, washed and weighed. Thereafter, test vessels were exposed for further 28 d. Subsequently the number of juvenile worms was counted at test termination.

Earthworm reproduction tests were performed in accordance to the OECD guideline 222. For detailed description of the test preparation see section 6.1.5.

In the test sequence B an additional TCC test series was run. Additionally, four mixture test series with TCC and NM 100 as well as TCC and NM 102 were run as explained above for NM 101. The used TCC and TiO_2 concentrations were the same as in the tests described above.

8.1.7 Chemical analysis

Triclocarban analysis

Sampling Soil samples of the earthworm reproduction test were collected at test initiation and test termination from the controls and from one of the lower (84 mg/kg) as well as the highest (675 mg/kg) TCC treatment groups of the TCC test and the mixture tests with TCC and NM 101 (test sequence A).

Extraction Three soil replicates (5 g wet weight (ww)) per time point and treatment group were extracted two times with acetone (1:4 w/v; p.a. AppliChem, Darmstadt, Germany; recovery rate was 85%).

LC-MSMS TCC was analyzed in the soil extracts with liquid chromatography mass spectrometry (LC-MS) after the addition of an internal standard (13C-TCC, Cambridge Isotope Laboratories, Andover, Massachusetts, USA) to the diluted soil extracts. Measurements were carried out with LC-MS on an Eclipse XDB C-18 column (Agilent Technologies, Santa Clara, USA). A gradient was run with the mobile phase: acetonitrile (B) and water (A) which were both applied with 5 mM NH_4AC (ammonium acetate; Tab. 12). The retention time of TCC was 9.1 min.

The recovery rate of the method was determined to be 85%. The sample concentrations were corrected for recovery rate.

Tab. 12: Gradient for LC-MS analysis. Only the polar phase (A) is given.

Total Time (min)	Flow rate (↔l/min)	A (%)
0.00	500	50
2.00	500	50
2.10	500	10
7.00	500	10
7.10	500	50
11.00	500	50

TiO_2 analysis

Ti concentrations in soil and worm samples of the earthworm acute toxicity tests were analyzed by means of inductively coupled plasma optical emission spectrometry (ICP-OES) after digestion of the samples with different mixture of acids (for a detailed description see below).

Sampling Soil aliquots of the earthworm acute toxicity tests were sampled from each three replicates of the controls (0 mg TiO_2 material/kg; n=3), the highest TCC treatment groups of each test series (0 mg TiO_2 material/kg; n=3), the mixture treatment groups with the highest TCC concentration of each test series (675 mg TCC/kg and 1000 mg TiO_2 material/kg, n=3) the TiO_2 controls (1000 mg TiO_2 material/kg; n=3) and of each test series at the end of the test (14 d). Samples were stored in a fridge at -20 °C until analysis. Worms of these controls and treatment groups, which already had purged their guts for 24 h, were also frozen at -20 °C until analysis. Two worms per replicate were digested. In total, the worms of three replicates per treatment group were analyzed, resulting in six independently digested worms per treatment group.

Digestion Aliquots of the soil samples were finely ground with a bowl mill (mortar material: agate). Thereafter, 0.1 mg of the fine soil powder was digested by using a sequence of different acids and temperatures. Details are given in Tab. 13. Digestion was carried out in PTFE tubes which were placed in a heating block (HotBlock^{TM} Pro, Environmental Express, Charleston, South Carolina, USA). For each material one TiO_2 control (10 mg) was run containing only pure TiO_2 powder. Additionally, a blank control was conducted. After digestion, samples were filled up with Milli-Q water to 50 ml.

Worms were dried over night at 60 °C in separate test tubes. For NM 101 and NM 102 TiO_2 controls were prepared containing 500 µg and 670 µg TiO_2 powder. Worms were digested with a mixture of nitric acid (HNO_3) and hydrogen peroxide for 60 h at room temperature. Then samples were heated at 60 °C for 3 h and thereafter at100 °C until the samples turned clear and yellow (next morning). Subsequently samples were made up to a volume of 10 ml with Milli-Q water.

ICP-OES Measurements were carried out with an ICP-OES (iCAP 6000 Duo, Thermo Scientific, Waltham, MA, USA). Standards were diluted from a Ti-standard stock solution (10.00 mg/L in water with 2.4% F-, SPEX CertiPrep, Metuchen, New Jersey, USA) with Milli-Q water containing 4% HNO_3 to 0.05-100 ppm Ti. Additionally to the standards and the samples rock samples with known Ti concentrations were measured. By multiplying the measured Ti concentrations with a Ti-TiO_2 conversion factor (1.668), TiO_2 concentrations were calculated.

Tab. 13: Sequences of soil digestion for Ti-analysis.

Sequence	Acid	Temperature	Duration (h)	Lid
1	1 ml HNO_3[a]	60°C	~ 1 h to evaporate the water	open
2	-	95°C	4 h	open
3	4 ml HF[b], 1 ml $HClO_4$[c]	100 °C	14 h	closed
4	-	100 °C	8 h	open
5	-	150 °C	2 h	open
6	-	150 °C	60 h	closed
7	-	150 °C	4 h	open
8	0.5 ml H_2O_2[d,e] 2 ml HNO_3 4-5 ml deionized water	60°C	1 h	open
9	-	70 °C	5 h	closed

[a] nitric acid [b] hydrogen fluoride [c] hydrochloric acid [d] hydrogen peroxide [e] for the TiO_2 controls and the blanks 1 ml H_2O_2 was added instead of 0.5 ml

8.1.8 Analysis and statistics

Data were statistically analyzed with ToxRat® Professional (version 2.10, ToxRat solutions GmbH). Concentration response functions were fitted to the data using probit analysis. The concentrations causing 10 and 50% mortality (LC10 and 50) were calculated from this function. Significant differences to the control (*P<0.05) were determined using Fisher's Exact Binominal Test with Bonferroni Correction to derive the lowest observed effect concentration (LOEC) and no observed effect concentration (NOEC).

Significant differences between the treatment groups and the controls as well as between the treatment groups and the corresponding TCC treatment group were determined using student-t test for homogeneous variances or one way ANOVA (*P <0,05).

8.2 Results

8.2.1 Characterization of the TiO_2 suspensions

The hydrodynamic diameters (HD) of the particles were measured in the different TiO_2 suspensions. In the two NM 101 suspensions (400 mg/160 ml and 1000 mg/160 ml) intended for application in the earthworm reproduction test, HDs were comparable to each other (540 nm and 528 nm). Because the concentration in the higher concentrated suspension (1000 mg/160 ml) was too high, no zeta potential was measurable. However, that of the particles in the lower concentrated suspension (400 mg/160 ml) accounted to -41 mV (Tab. 14), indicating a stable suspension.

Tab. 14 Hydrodynamic diameters (HD) and zeta potentials (ZP) of the particles in the NM 101, NM 102 and NM 100 suspension used for spiking soils of the earthworm tests.

TiO_2 material	Concentration (mg/160 ml)	HD ± SD[a] (nm)	ZP ± SD (mV)	n[b]
NM 101	400	540 ± 11	-33 ± 8	3
	1000	528 ± 69	-[c]	5
NM 102	400	873 ± 191	9 ± 1	10
	1000	1062 ± 20	-[c]	2
NM 100	400	241 ± 4	-5.5 ± 1	4
	1000	206 ± 9	-[c]	3

[a] Standard deviation [b] number of repetitions [c] Measurement was not possible due to too high concentrations

The HD of the particles in the NM 102 suspensions were 873 nm and 1062 nm (400 and 1000 mg/160 ml) and the zeta potential of the particles in the lower concentrated suspension was 9 mV (Tab. 14). Because the concentration in the higher concentrated suspension was too high, no zeta potential was measurable.

For NM 100 only the lower concentrated suspension was measured. A HD of 241 nm and a zeta potential of -5.5 mV were determined (Tab. 14).

8.2.2 Earthworm, acute toxicity test (OECD 207)

No mortality greater than 10% was observed for control worms therefore the validity of all tests was confirmed.

Mortality Fig 20 A, shows the mortality of the earthworms exposed either to TCC (I), TCC and NM 101 (II), TCC and NM 102 (III) or TCC and NM 100 (IV) for seven days. No mortality higher than 10% was determined in all test series after this exposure period.

Compared to the mortality after 7 d of exposure, mortality was higher after 14 d of exposure. Negative concentration response relationships were determined between the TCC concentration and the survival of the earthworms for the test series I and II (TCC and TCC + NM 101; Fig. 20, B). In general, the survival was more affected in the test series I and II (TCC and TCC + NM 101) than in the test series III and IV (TCC+ NM 102 or NM 100). E.g. mortality in the highest treatment groups can be put in following descending order: test series II (40%) > test series I (33%) > test series III (7%) and test series IV (7%). This trend is reflected by the concentrations causing 10% mortality (LC10) and the no observed effect concentrations (NOEC; Tab. 15): test series II (243 mg/kg; 169 mg/kg) < test series I (262 mg/kg; 338 mg/kg) < test series IV (489 mg/kg; ≥ 675 mg/kg) < test series III (not calculable; ≥ 675 mg/kg). Significant differences to the controls (0%) were identified for the highest treatment group of the test series I (TCC; 33%) and in the test series II (TCC + NM 101) for the treatment groups containing a TCC concentration equal to or higher than 338 mg/kg of the test series II (TCC+ NM 101; 17% and 40%). Significant differences between the mortality of the treatment groups of the TCC test series (I) and the corresponding treatment groups of the other test series were only determined for the treatment group with 338 mg/kg TCC in the test series III (TCC + NM 102). Mortality was around 40% lower than in the corresponding treatment group of the TCC test series. It is worth noting that generally the mortality after 14 days of exposure varied a lot (standard deviation (SD) up to 23%; Fig. 20, B).

The comparison of Fig. 20 B and C shows that during the gut purging phase of 24 h, further earthworms died, resulting in a maximum mortality of 70%, 50%, 20% and 13% in the highest treatment groups of the test series II, I, IV and III. Significant differences to the controls (0%) were observed for the test series I, II and IV for all treatment groups with TCC concentrations ≥ 338 mg/kg. Lethal concentrations causing 50% mortality (LC50) and NOEC values can be put in following order (Tab. 16): test series II (444 mg/kg, 169 mg/kg) < test series I (652 mg/kg; 84 mg/kg) < test series IV (not calculable; 169 mg/kg) and test series III (not calculable, ≥ 675 mg/kg). The mortality in the treatment groups with TCC concentrations ≥ 338 mg/kg in the test series III and in the treatment groups with a TCC concentration of 675 mg/kg in the test series IV was significantly lower than in the corresponding treatment groups of the TCC test series (Fig. 20, C). Exposure of TiO_2 materials alone (1000mg/kg) induced no toxicity upon 7 and14 days and after purging of the gut content for additional 24 hours.

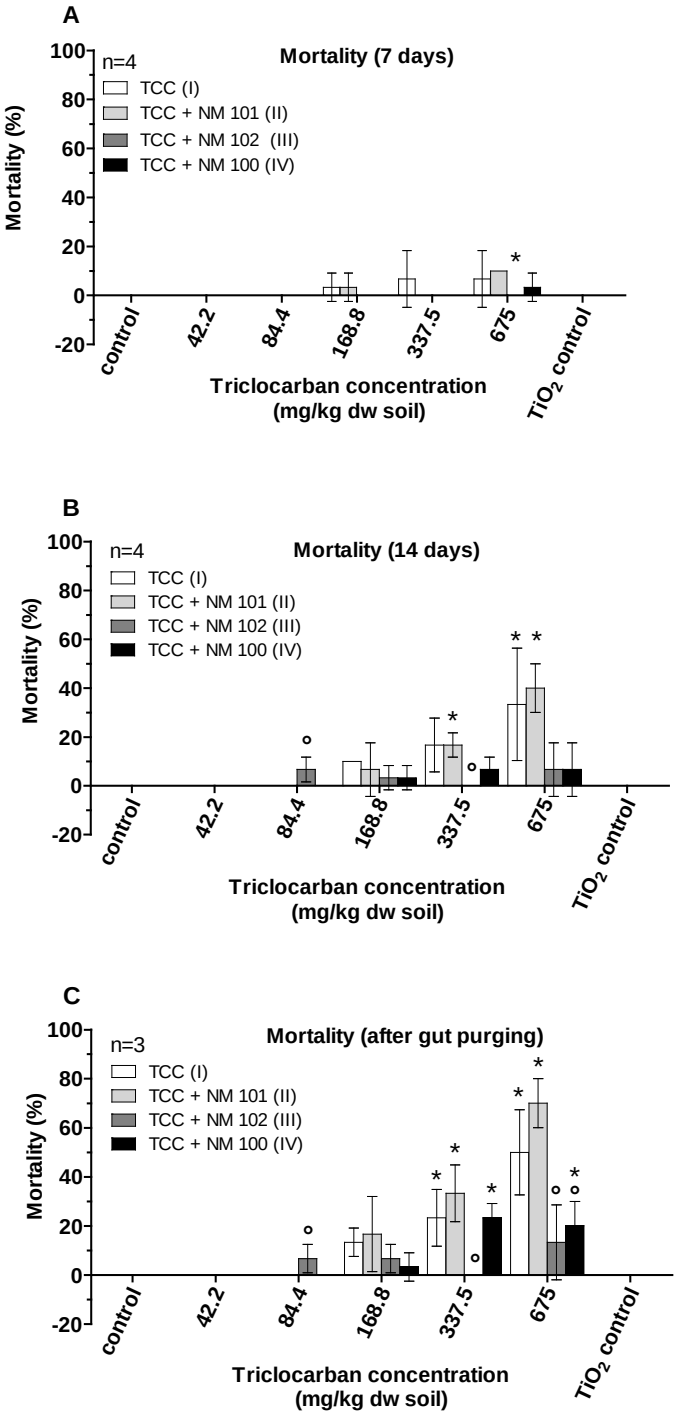

Fig. 20: Mortality (%) of *Eisenia fetida* exposed to triclocarban (TCC, white bars), TiO_2 materials (TiO_2 control, 1000 mg/kg dw soil) NM 101, NM 102 and NM 100 and mixtures of both at differing TCC concentrations (grey, dark grey and black bars) for 7 and 14 days as well as after the gut purging phase of 24 h. Error bars represent standard deviation derived from the replicates of one independently conducted experiment (n=3-4). Circles and asterisks indicate significant differences to the control respectively to the corresponding TCC treatment group.

Tab. 15 TCC concentrations causing 10% and 50% mortality (LC10 and LC50) and no observed effect concentrations (NOEC) of the earthworm, acute toxicity tests with triclocarban (TCC) and combinations of TCC and the TiO_2 materials after an exposure period of 14 d.

Test series	LC10 (mg TCC/kg dw soil)	LC50 (mg TCC/kg dw soil)	NOEC (mg TCC/kg dw soil)
TCC (I)	262	1177	338
TCC + NM 101 (II)	243	967	169
TCC + NM 102 (III)	n.c.	n.c.	≥ 675
TCC + NM 100 (IV)	489	n.c.	≥ 675

n.c. not calculable

Tab. 16 TCC concentrations causing 10% and 50% mortality (LC10 and LC50) and no observed effect concentrations (NOEC) of the earthworm, acute toxicity tests with triclocarban (TCC) and combinations of TCC and the TiO_2 materials after the gut purging phase of 24 h.

Test series	LC10 (mg TCC/kg dw soil)	LC50 (mg TCC/kg dw soil)	NOEC (mg TCC/kg dw soil)
TCC (I)	161	652	84
TCC + NM 101 (II)	160	444	169
TCC + NM 102 (III)	678	n.c.	≥ 675
TCC + NM 100 (IV)	280	n.c.	169

n.c. not calculable

Biomass changes Biomass changes were calculated by first subtracting the worm biomass at test termination from that at test initiation. Thereafter the percentage of this difference from the initial biomass is calculated. Biomass changes of worms were determined with two different methods. For the first method biomass was determined by weighing worms with their gut content (referred to as biomass with gut content), for the second method worms were weighed after they had purged their guts for 24 h. The latter weight corresponds to the biomass without gut content (referred to as biomass without gut content). In the following the biomass results with gut content will be explained first: For all test series a concentration response relationship was observed between the TCC concentration and the change of the biomass with gut content of *E. fetida* (Fig. 21, A-D). Biomass with gut content was around 15% (test series I and III) and 20% (test series II and IV) lower than in the corresponding controls. Significant differences compared to the control were observed for the treatment groups with concentrations ≥ 169 mg/kg in the test series I with TCC alone, whereas this was already the case for the treatment groups with concentrations ≥ 84 mg/kg in the other test series (II-IV). Regarding Fig. 21 A-D it is obvious that for all test series except for the test series II, the concentration response curves shows a plateau for concentrations ≥ 169 mg/kg. Fig. 21 also presents the worm biomass without gut content. It is obvious, that in any test series no concentration response relationship is visible between the TCC concentration and the biomass without gut content, indicating that the loss of biomass is a consequence of a loss of gut content. Fig. 22 gives a better overview of the loss of gut content compared to the control for each test series. The maximum loss of gut content compared to the controls in each test series was around 74 – 92% for the test series I (at TCC 675 mg/kg), II (at TCC 337 mg/kg) and IV (at TCC 675 mg/kg). For test series III (at TCC 675 mg/kg) a loss of only 57% compared

to the control was observed. Significant difference between the test series I and the mixture test series were only observed between the test series I and test series III at 675 mg TCC/kg. Significant differences to the controls were observed for all test series, except the TCC + NM 102 test series III, in treatment groups with a TCC concentration greater than or equal to 169 mg/kg dw soil. The loss of gut content of the worms of the test series with TCC + NM 102 did not differ significantly from that of the specific controls. In general, the concentration response relationship was less pronounced for the test series with TCC and NM 102 than for the other test series. That is noticeable because the loss of gut content was e.g. 20-40% lower than that of worms exposed to the corresponding treatment groups with TCC alone. Significant differences between the corresponding treatment groups of these two test series were observed for the treatment groups with a TCC concentration of 42 and 338 mg/kg dw soil.

Worms exposed to the TiO_2 controls showed a biomass change which was comparable to that of control worms, when worm weights were based either on the weight with and without gut content (Fig. 21, B-C and Fig. 22).

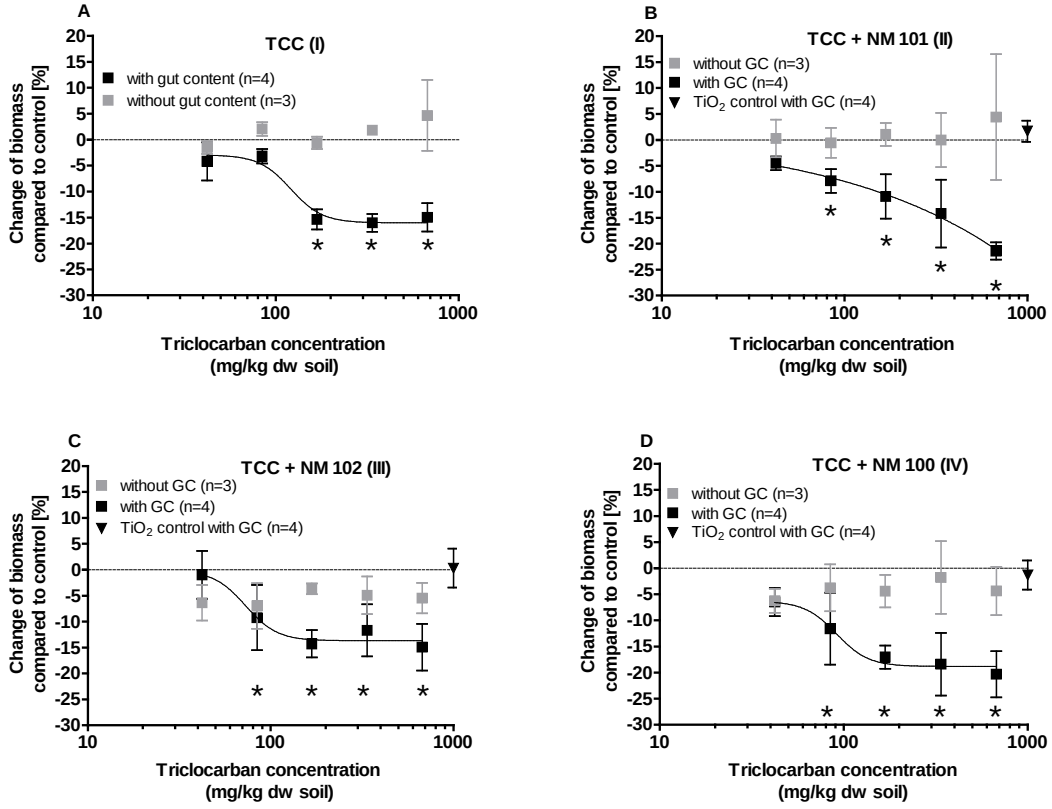

Fig. 21: Change of biomass of *E. fetida* compared to the control (%) based on either biomass with gut content (with GC, black) or without gut content (without GC, grey). Worms were exposed to either TCC (A), the TiO_2 materials (TiO_2 control, 1000 mg/kg dw soil) NM 101, NM 102 and NM 100, or mixtures of both (B, C, D) for 14 days. Subsequently, worms were left to purge their guts for 24 h. Error bars represent standard deviation derived from the replicates of one independently conducted experiment (n=3-4). Asterisks indicate significant differences to the control.

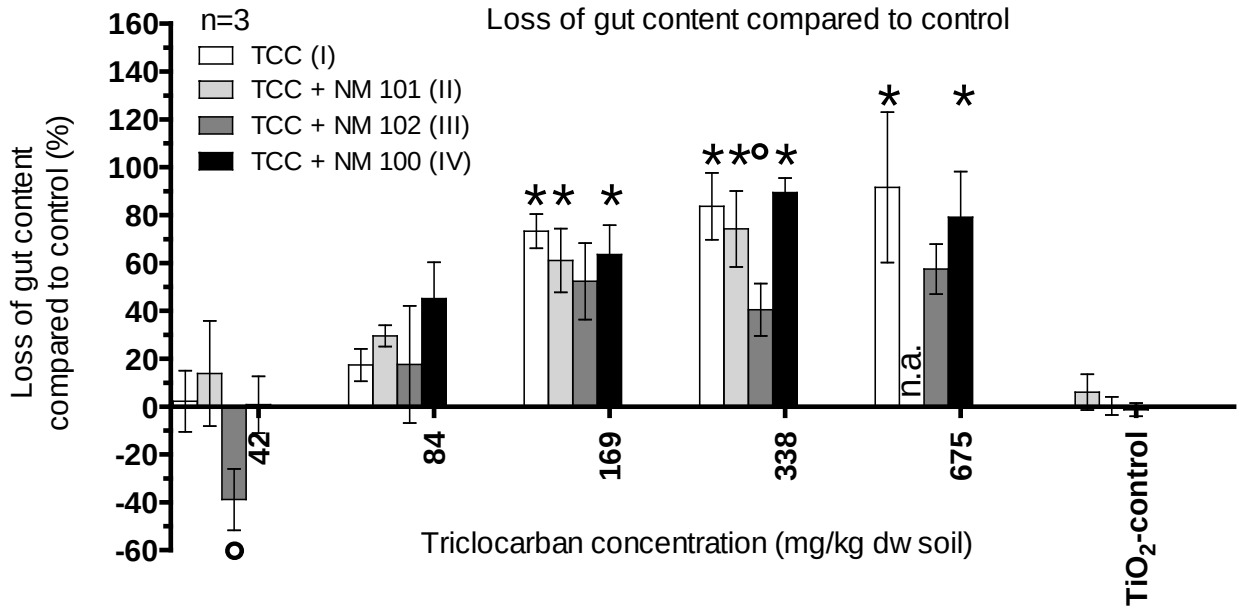

Fig. 22: Loss of gut content of *E. fetida* compared to the control (%). Worms were exposed to either TCC, the TiO_2 materials (TiO_2 control, 1000 mg/kg dw soil) NM 101, NM 102 and NM 100, or mixtures of both (II-IV) for 14 days. Error bars represent standard deviation derived from the replicates of one independently conducted experiment (n=3). Circles and asterisks indicate significant differences to the control respectively to the corresponding TCC treatment group.

8.2.3 Summary of the results of the earthworm, acute toxicity test

The results of the earthworm, acute toxicity test can be summarized in the following way:

- A positive concentration relationship between the TCC concentration and the mortality of *E. fetida* was observed (test series I, LC10 262 mg/kg dw soil).
- The mixture of TCC with NM 101 induced comparable effects on the survival of *E. fetida* (test series II, LC10 243 mg/kg dw soil), whereas the mixture of TCC with NM 102 (test series III, LC10 n.d) or NM 100 (test series IV, LC10 489 mg/kg dw soil) induced lower effects than detected in the TCC test series.
- TCC significantly reduced the biomass with gut content of *E. fetida* by 15% after an exposure period of 14 d in treatment groups with concentration ≥ 169 mg/kg dw soil in the test series I compared to the biomass change of control worms.
- For all test series except for that of TCC and NM 101 the concentration response curves regarding the endpoint biomass with gut content showed a plateau starting from a concentration of 169 mg/kg.
- Based on the worm biomass without gut content it is obvious that TCC had no effect on the biomass, but on the gut content of the worms.
- A negative concentration response relationship was observed between the TCC concentration and the loss of gut content of *E fetida* for all test series.

- In the test series III (NM 102), no significant differences were observed compared to the controls. In the treatment groups with 42 and 338 mg TCC/kg the loss of gut content was significantly less than in the corresponding treatment groups of the other TCC test series.
- All TiO_2 material controls showed no effect on the mortality, biomass or gut content of *E. fetida* under the conditions tested.

8.2.4 Earthworm, reproduction test (OECD 222)

In these experiments *Eisenia fetida* were exposed to spiked and unspiked RefeSol 01-A soil. The tests were analyzed by means of three endpoints: 1. After 28 days the mortality of the exposed adult worms was surveyed. 2. Biomass changes were detected by comparing worm weights at test initiation and termination. 3. After 56 days the reproduction rate was determined by counting the juvenile worms.

As explained before, the mixture experiments were done in two sequences. In test sequence A TCC and NM 101 were tested in the laboratories of IBACON GmbH and in test sequence B TCC, NM 102 and NM 100 were tested in the laboratories of the RWTH-Aachen.

Test sequence A (TCC and TCC + NM 101)

Test validity The reproduction of control worms of all test series was higher than 30 juveniles per 10 worms and mortality of adult worms was lower than 10% after 28 d of exposure (data not shown). For the TCC and the mixture test series with TCC and NM 101 (400 mg/kg dw soil) the coefficients of variation (CV), describing the variability of the reproduction of the control worms (n=8), were as low as (CV 17.4 and 13.0%, Tab. 17) requested by the OECD guideline 222 (≤ 30%). Only controls of the mixture test series with 1000 mg NM 101/kg showed a slightly higher CV value (CV 31.4%, Tab. 17).

Test conditions At test initiation pH values were in the range of 4.7-5.0 and slightly increased until test termination to pH values of around 5.5-6.3 (minimum and maximum values given). The water holding capacity at test termination (46.6%-68.3%) was comparable to that at test initiation (46.3%-58.4%, minimum and maximum values given).

Mortality No mortality higher than 10% was monitored in all treatment groups and controls except for the highest TCC treatment group (675 mg/kg) of all test series. Here 22.5 ± 5%, 20 ± 28.3% and 12.5±5% of the exposed worms died in the test series with TCC, with mixtures of TCC and NM 101 (400 mg/kg) and with mixtures of TCC and NM 101 (1000 mg/kg). Mortality in the latter treatment group was significantly lower compared to the corresponding TCC treatment group (data not shown).

Reproduction Fig. 23 demonstrates that in all test series negative concentration response relationships between the TCC concentration and the reproduction of *E. fetida* were observed. Significant differences to the controls were determined in the TCC test series for the treatment groups with TCC concentrations ≥ 84 mg/kg dw soil, whereas for the mixture test series (with NM 101 400 and 1000 mg/kg) this was only the case for treatment groups with TCC concentrations ≥ 168 and 338 mg/kg dw soil, respectively. The comparison of the different reproduction rates shows, that the reproduction in the mixture test series is higher in the lower concentrated TCC treatment groups (42-168 mg/kg dw soil) than those in the corresponding treatment groups of the test series with TCC alone. This is not the case for the higher TCC treatment groups (337 and 675 mg/kg dw soil). Here comparable reproduction rates are monitored. Significant differences between the treatment groups of the mixture test series and the corresponding TCC treatment groups of the TCC test series were determined for following TCC concentrations: 84 mg/kg TCC+NM 101 (400 mg/kg dw soil) as well as 42 and 169 mg/kg dw soil TCC + NM 101 (1000 mg/kg dw soil; Fig. 23). Tab. 17 summarizes the median effective concentrations (EC50) determined for the different test series which can be put in following increasing order:

243 mg/kg dw soil (TCC test series) > 308 mg/kg dw soil (TCC + NM 101 test series, 400 mg/kg) > 384 mg/kg dw soil (TCC + NM 101 test series, 1000 mg/kg).

Reproduction of the TiO_2 controls (400 and 1000 mg/kg; 112 ± 17% and 105 ± 28%) did not significantly alter from the reproduction of the specific controls and also not between themselves.

No effects on the biomass of worms were observed for all test series.

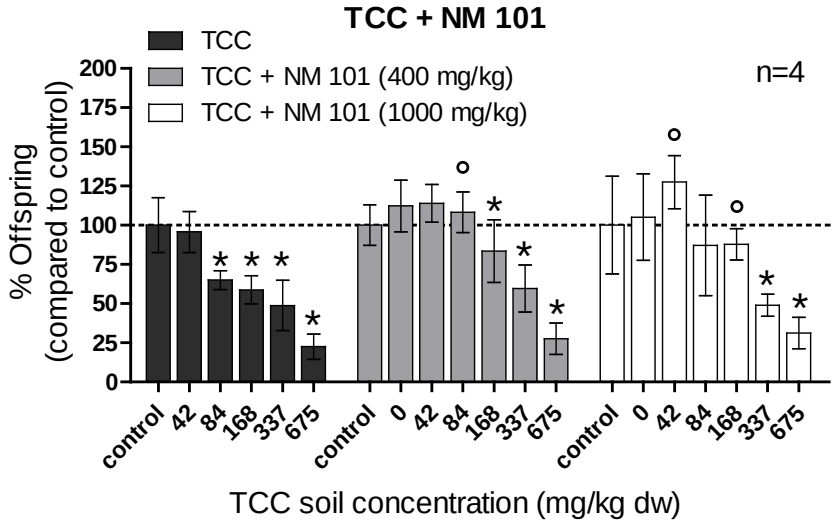

Fig. 23: Percentage offspring compared to the specific control group (%) of *Eisenia fetida* exposed to TCC (black bars), NM 101 (400 and 1000 mg/kg dw soil) and mixtures of both (grey and white bars) for 28 days. Error bars represent standard deviation derived from the replicates of one independently conducted experiment (n=4). Asterisks and circles indicate a significant difference to the control respectively corresponding TCC treatment group (*P<0.05).

Tab. 17: Median effective concentration (EC50) and coefficients of variation (CV) of the controls (0 mg TiO_2/kg) and TiO_2 controls (400 or 1000 mg TiO_2/kg) of the reproduction tests with TCC and mixtures of TCC and NM 101.

Test series TiO_2, mg/kg soil dw)	EC50 (mg TCC/kg soil dw)	LOEC (mg TCC/kg soil dw)	CV control(%)	CV TiO_2 control(%)
TCC (0)	243	84	17.4	-
TCC + NM 101 (400)	308	169	13.0	14.8
TCC + NM 101 (1000)	384	338	31.4	26.1

Test sequence B (TCC, TCC + NM 102 and TCC + NM 100)

Test validity Reproduction of control worms of all test series was higher than 30 juveniles per 10 worms and mortality of adult worms was lower than 10% after 28 d of exposure (data not shown). For most of the controls (n=8) the coefficients of variation (CV), describing the variability of the reproduction , were higher (CV 32.8-39.3%, Tab. 18) than requested by the OECD guideline 222 (≤ 30%). Only controls of the mixture test series with 1000 mg NM 102/kg fulfilled this criterion (CV 9.4%). CVs of the different TiO_2 controls (TiO_2 without TCC) (n=4) were below 30% (Tab. 18).

Test conditions At test initiation pH values were in the range of 5.0-5.48 and slightly increased until test termination to pH values of around 5.56-6.17 (minimum and maximum values given). The water holding

capacity at test termination (22.3-44.7%) was slightly lower as that at test initiation (34.9-50.4%, minimum and maximum values given).

Mortality While no mortality was observed in the treatment groups of the mixture tests with TCC and NM 102, the mixture tests with TCC and NM 100 as well as the single substance test with TCC showed low mortality of maximum 7.5 % (data not shown).

Reproduction In the following the reproduction results are given for the different test series:

Worms exposed to a TCC soil concentration of 675 mg/kg dw showed a significantly lower reproduction compared to the reproduction of control worms (60% reproduction compared to the control, Fig. 24) resulting in a calculated EC50 of 956 mg/kg (Tab. 18).

In the mixture test series with TCC and 400 mg NM 102/kg the reproduction of worms exposed to 42-169 mg TCC/kg increased with the TCC soil concentration. Reproduction was maximal 55% (168 mg TCC/kg, Fig. 24) higher compared to that of control worms, however not significantly different based on statistics. Worms exposed to TCC soil concentrations higher than 169 mg/kg showed a reproduction that was comparable to that of control worms. The reproduction of worms exposed to NM 102 (400 mg/kg) as a single substance was also comparable to the reproduction of control worms. In general, no statistical difference between the reproduction of worms exposed to spiked and unspiked soil was found in the mixture test series with TCC and 400 mg NM 102/kg. Therefore no EC50 was calculable. Statistical differences were observed between the TCC and the mixture test series with TCC and 400 mg NM102/kg for treatment groups with 169 and 675 mg TCC/kg. Reproduction of worms compared to the control was higher in these treatment groups of the mixture test (155% and 87% compared to control) than in the corresponding treatment groups of the TCC test (93% and 62% compared to control, Fig 24).

In the mixture test series with 1000 mg NM 102/kg the reproduction of worms exposed to NM 102 and TCC was lower the higher the TCC soil concentration was (up to 50% lower reproduction compared to control worms, 675 mg/kg). Statistical significant differences between the reproduction of untreated worms and worms treated with a mixture of TCC and NM102 were observed for worms exposed to TCC concentrations greater than or equal to 338 mg TCC/kg (EC50 692 mg/kg, Tab. 18). No significant differences were determined between the TCC test series and the mixture test series with TCC and 1000 mg NM 102/kg. The reproduction of worms exposed to NM 102 (1000 mg/kg) as a single substance was comparable to the reproduction of control worms.

In the mixture test series with TCC and 400 mg NM 100/kg, worms exposed to a mixture of NM 100 and TCC in general had reproduction rates that were comparable to the reproduction rate of worms exposed to the corresponding TCC soil concentration. But in contrast to the test series with TCC alone, no significant difference to the controls was observed for the highest TCC concentration. The reproduction of worms exposed only to NM 100 (400 mg/kg) was comparable to that of worms exposed to untreated soil.

In the test series with TCC and 1000 mg NM 100/kg the effect on the reproduction of *E. fetida* was significantly different to that of control worms in the highest TCC concentration (EC50 494 mg/kg + NM100 (1000 mg/kg), Tab. 18). In this test series reproduction of worms exposed to NM 100 only (1000 mg/kg) was also comparable to the reproduction of control worms.

In conclusion, significant differences between the test series with TCC alone and the mixture test series were only observed for the test series with TCC and 400 mg/kg NM 102. For the other test series no significant differences were observed. Furthermore, the 95% confidence limits (CL) indicate for the mixture test series

with 1000 mg TiO_2/kg that their EC50 values are not different to that of the test series with TCC alone (Tab. 18).

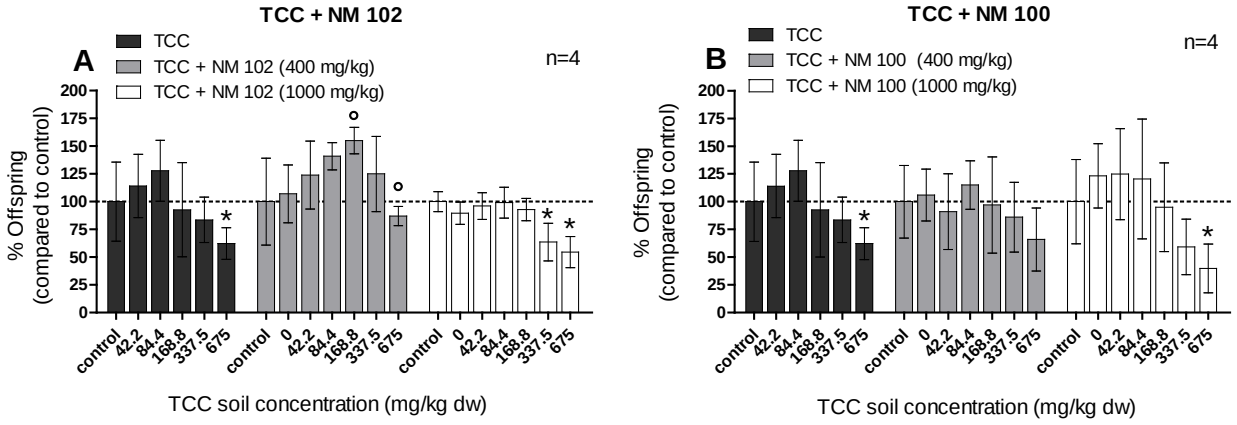

Fig. 24: Percentage offspring compared to the specific control group (%) of *Eisenia fetida* exposed to TCC (black bars), and mixtures of TCC and either NM 102 (A) or NM 100 (B; 400 and 1000 mg/kg dw soil; grey and white bars) for 28 days. Error bars represent standard deviation derived from the replicates of one independently conducted experiment (n=4). Asterisks and circles indicate a significant difference to the control respectively corresponding TCC treatment group (*P<0.05).

Tab. 18: Median effective concentration (EC50), coefficients of variation (CV) of the controls and CV of TiO_2 controls and 95% confidence limit (CL) of the reproduction tests with TCC, and mixtures of TCC and NM 102 or NM 100.

Test series (TiO_2, mg/kg soil dw)	EC50 (mg TCC/kg soil dw)	CV controls (%)	CV TiO_2 controls (%)	95% CL (lower/ upper)
TCC (0)	956	35.3	-	838/1176
TCC + NM 102 (400)	n.c.	39.3	24.2	n.c.
TCC + NM 102 (1000)	692	9.4	11.4	436/8296
TCC + NM 100 (400)	n.c.	32.8	21.9	n.c.
TCC + NM 100 (1000)	494	38.2	29.0	355/849

Biomass changes Biomass changes were calculated by first subtracting the worm biomass at test termination from that at test initiation. Thereafter the percentage of this difference from the initial biomass is calculated. Biomass changes of exposed worms were determined to assess e.g. differences in the ingestion of food during the experiments (Fig. 25). Figure 25 shows the biomass change of worms exposed to spiked soils compared to the biomass changes of control worms after 28 d. On average control worms in all experiments gained 28±7% weight during the tests. Values higher than 100% demonstrate a greater increase of biomass, whereas values lower than 100% demonstrate a smaller increase of biomass during the test compared to that of control worms.

In the tests with single substance exposures (TCC alone, TiO_2 alone), worms did not show a biomass change differing from that of control worms except for worms exposed to NM 100 itself (1000 mg NM 100/kg). Here worms showed a biomass change comparable to that of worms exposed to a mixture of TCC and NM 100.

In general, worms exposed to a mixture of NM 102 and TCC seem to increase their biomass more during the experiment than worms exposed to control soil: In the mixture test series with 400 mg NM 102/kg, the increase of biomass seems to depend on the TCC concentration. Exposure to higher TCC soil concentrations seems to lead to a greater increase in biomass than exposure to lower TCC soil concentrations. The maximum change of biomass was 40% greater than that of the control worms. On the contrary, in the mixture test series with 1000 mg NM 102/kg a biomass change of around 40% greater than that of the control worms was observed for worms of all TCC treatment groups. Worms exposed to NM 102 itself (1000 mg NM 102/kg dw) showed a biomass change comparable to that of control worms. However, no statistically significant differences between the control and treatments or between the earthworms of the tests with TCC alone and tests with TCC and NM 102 were observed.

In the mixture tests with NM 100 and TCC nearly the same trend as for the mixture tests with NM 102 and TCC were observed for the biomass changes of the exposed adult worms after 28 days. Fig. 25 shows that in general the increase of worm biomass in the test series with 400 mg NM 100/kg seems to have been greater than the increase of biomass of the controls. Furthermore, the increase in biomass was higher the higher the TCC soil concentration was. In the mixture test with 1000 mg NM 100/kg the biomass changes of worms exposed to TCC and NM 100 fluctuate around an average of 45% of the biomass change of the control worms for all tested TCC concentrations.

Thus, in all tests with TCC and TiO_2 materials of differing concentrations the biomass changes of the adult worms seem to have been higher than for control worms and higher for worms exposed to higher concentrations of TCC. However, the increase in biomass change was never higher than around 40-45% compared to that of the control worms (Fig. 25).

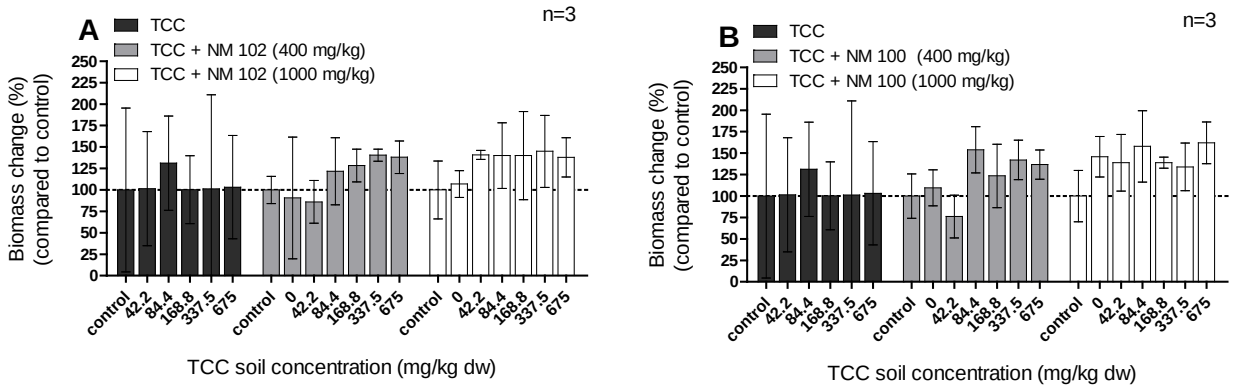

Fig. 25: Biomass changes of adult worms compared to that of control worms exposed to the single substances TCC, NM 102 and NM 100, as well as mixtures of TCC and NM 102 (A) and of TCC and NM 100 (B) for 28 days. Error bars represent standard deviation derived from the replicates of one independently conducted experiment (n=3). In general no statistical differences were observed.

8.2.5 Chemical analysis

Triclocarban analysis

The triclocarban (TCC) concentration was analyzed in soil samples (K1 and K2, Tab. 19) of the three test series of the test sequence A of the earthworm reproduction tests. Briefly, soil samples were taken at test initiation (0 d) and termination (56 d), extracted with acetone and subjected to LC-MS.

Tab. 19 presents the percentage of the nominal TCC concentration which was present in the soil samples at test initiation and termination. For all test series the measured values equate the nominal values, nearly 100% of the nominal values were recovered at test initiation and termination in the soil samples of all test series.

Tab. 19: Percentage of nominal TCC soil concentration (%) in soil samples (K1 and K2, 84 and 675 mg/kg dw soil) of the different test series of the earthworm reproduction test with TCC and mixtures of TCC and NM 101 (TiO_2: 400 and 1000 mg/kg) at test initiation (0 d) and termination (56 d).

Test series (TiO_2, mg/kg dw soil)	0 d		56 d	
	K1 % nominal ± SD[a]	K2 % nominal ± SD[a]	K1 % nominal ± SD[a]	K2 % nominal ± SD[a]
TCC	106 ± 2.7	110 ± 6.1	100 ± 2.7	103 ± 4.1
TCC + NM 101 (400)	111 ± 1.2	107 ± 4.9	101 ± 1.7	103 ± 3.7
TCC + NM 101 (1000)	105 ± 1.0	108 ± 7.0	98 ± 2.6	95 ± 3.1

[a] standard deviation

TiO_2 analysis

The TiO_2 concentration was analyzed in soil samples of the earthworm acute toxicity test. In detail, controls, the highest TCC treatment group without and with TiO_2 application (1000 mg/kg) and the treatment groups with only the TiO_2 material were sampled (three replicates per control group). Because the TiO_2 concentrations of the controls and the TCC treatment group without TiO_2 application were comparable to each other, replicates were pooled with the controls, resulting in 15 control replicates. The same was done for the TCC treatment group with TiO_2 application and the treatment groups containing only TiO_2, resulting in six replicates.

Fig. 26 presents the measured TiO_2 concentrations in the test soils. For the control soils a TiO_2 concentration of around 2800 mg/kg dw soil was detected, whereas a 1000 mg/kg dw soil higher value of around 3800 mg/kg dw soil was measured for the treatment groups applied with 1000 mg/kg of the TiO_2 material. The determined TiO_2 concentrations in the TiO_2 treatment groups were significantly higher than in the controls. The application efficiency (% of nominal value) was in the range of 87-96%. In general standard deviations are very low. The measured TiO_2 concentration of the additionally conducted TiO_2 controls (TiO_2 material directly weighed into the digestion vessel) corresponded to 87-103% of the nominal value. The determined Ti content (2.1%) and the already known Ti content (2.6%) of a rock sample were comparable to each other.

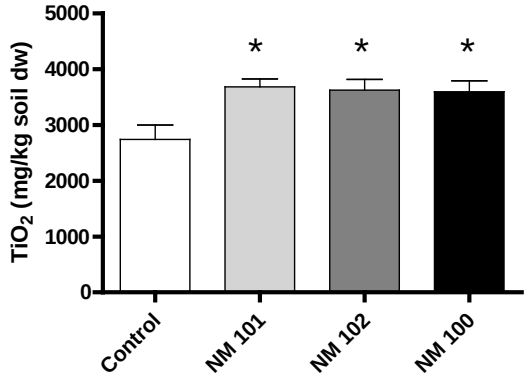

Fig. 26: TiO_2 concentrations in soil samples (controls, 0 mg/kg dw soil and TiO_2 controls, 1000 mg/kg dw soil) of the acute earthworm toxicity tests determined by means of ICP-OES. Error bars represent standard deviation derived from 6 replicates (TiO_2 controls) and 15 replicates (controls), respectively. Asterisks indicate a significant differences to the control group (*P<0.05).

TiO_2 concentrations of worm samples were measured by means of ICP-OES after the worms were digested with different acids (see section 6.1.7). First it has to be mentioned that the digestion method was not sufficient which is indicated by soil residues within the worm samples and TiO_2 residues in the TiO_2 controls after samples were digested. Consequently, results have to be interpreted carefully. However, digestion of all samples was carried out in the same way. Therefore, it is possible to compare the different treatment groups. Fig. 27 shows the TiO_2 concentration in the worm tissues. Apparently, in all test series the controls (no TiO_2 applied) show the highest TiO_2 tissue concentration in the range of 5-7.5 µg/kg dw tissue. The limit of quantification (LOQ) was determined as 0.85 µg/kg dw tissue. Dots in Fig. 27 mark the treatment groups in which 3-5 of the replicates had a TiO_2 concentration which was lower than the LOQ. For these replicates we assumed a TiO_2 concentration of 0 mg/kg. Therefore, these values represent estimated values. However they indicate that the TiO_2 concentration in these treatment groups was very low. Except for NM 102, for which the mixture as well as NM 102 control treatment groups are marked with a dot, only the mixture treatment groups are marked with a dot.

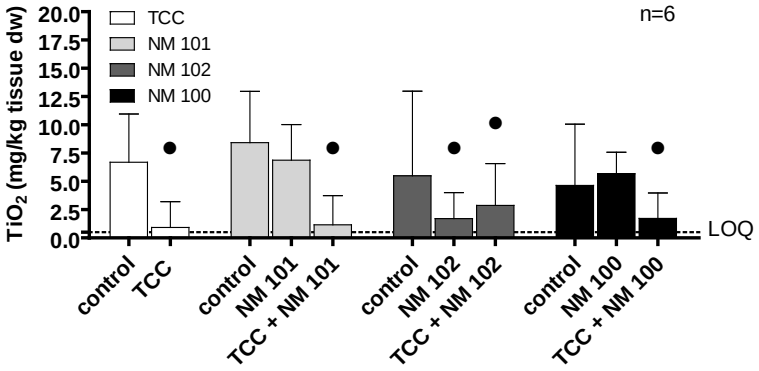

Fig. 27: TiO_2 concentrations in worm tissue (mg/kg tissue dw) of worms exposed to controls, TCC (675 mg/kg dw soil), NM 101/NM 102 and NM 100 (1000 mg/kg dw soil) and mixtures of both (675/1000 mg/kg dw soil) for 14 d. The dashed line marks the limit of quantification. Error bars represent standard deviation derived from 6 replicates. Dots mark treatment groups, for which in 3-5 replicates the limit of quantification was not reached.

8.3 Discussion

8.3.1 Characterization of the TiO_2 suspensions used in the earthworm tests

The DLS measurements indicate that the nanomaterials NM 102 and NM 101 formed large agglomerates in the test suspensions (NM 102: 873-1062 nm; NM 101 530-540 nm, Tab. 14). These values differ a lot from the primary particle sizes as indicated by the manufacturers (NM 102: 15-25 nm; NM 101: 7-10 nm). Opposite to this, the hydrodynamic diameters of the NM 100 particles in the suspension (242 nm) were similar to the primary particle size as indicated by the manufacturer (200-220 nm). Zeta potentials of NM 102 and NM 100 particles in the suspensions (9.4 and -5.5 mV, NM 102 and NM 100 suspensions) show that the suspensions were not stable and particles tend to agglomerate. However, the zeta potential of NM 101 (-33 mV) indicates a stable suspension.

8.3.2 Earthworm, acute toxicity test

In general, the acute toxicity tests revealed a concentration response relationship between the TCC concentration and the mortality of *Eisenia fetida* (LC_{10} 262 mg/kg, NOEC 338 mg/kg, t_{14}, Tab. 20). However, in the present study the observed acute toxicity of TCC to *E. fetida* was less pronounced than that documented in a study of Snyder et al. (LC50 40 mg/kg dw, after 28 d of exposure, Snyder et al. 2011). In the latter study worms were fed with TCC spiked sludge. In an additional experiment with 14C-radiolabeled TCC we showed that the bioaccumulation factor (BAF) of TCC was four times higher when earthworms were exposed to TCC via the food for eight days than via the soil (data not shown). Therefore, it is assumed that in the study of Snyder et al. (2011) the application of TCC via the food led to a higher bioavailability of TCC to the earthworms and consequently to more pronounced effects than in the present study.

Furthermore, the present study showed that TCC had a concentration dependent effect on the biomass of *E. fetida*. However, the biomass without gut content proofed that TCC had no direct effect on the biomass of *E. fetida* but an indirect effect on the gut content of the worms (up to 92% lower gut content compared to control worms, Fig. 22). It is suggested that TCC exhibits a narcotic effect on *E. fetida* resulting in a lower feeding rate and thereby lower gut content of the worms. This assumption is in line with observations showing that worms were less efficient to work through the soil at high TCC concentrations. The finding that TCC has an effect on the gut content of *E. fetida* also explains why the concentration response curves form a plateau between TCC concentrations of 169 and 675 mg/kg. At such high TCC concentrations worms nearly had no gut content any more. Therefore, higher concentrations do not result in more pronounced effects on the biomass of *E. fetida*.

The effect of TCC on the gut content of *E. fetida* may explain our finding, that the TiO_2 concentrations of worm tissue were lower in all worms when they were exposed simultaneously to TCC (Fig. 21): In the worm digest small soil residues were left in the test tube indicating that although worms were left to purge their guts for 24 h, small soil residues still remained in their guts. Considering that TCC had an effect on the gut content of the earthworms, it is assumed that the gut residue was less for worms exposed to TCC than for control worms or worms exposed to the TiO_2 material only. This would explain why the TiO_2 concentrations of worm tissue of worms exposed to TCC were in most of the cases below the LOQ. The results of the Ti tissue analysis further indicate that the TiO_2 materials were not taken up by the earthworms, because the concentration in the control worms was always higher than in the worms exposed to the TiO_2 test materials. However, this finding has to be verified by further experiments, because it was shown that the worm digestion was not complete. Ti analysis of the soil samples indicates that the method for applying the TiO_2

suspensions to soil resulted in a homogeneous distribution of the TiO_2 materials to the natural soil and resulted in a good application efficiency of 87-96% of the nominal value.

The mixture toxicity tests of TCC with the different sized TiO_2 materials demonstrate that the toxicity of TCC on the survival and biomass of *E. fetida* was influenced by the larger TiO_2 materials NM 102 and NM 100, whereas mixtures with the smallest material NM 101 resulted in comparable effects than in the TCC test series.

The effects in the mixture toxicity experiments clearly depend on the TiO_2 particle size: the addition of the smallest material (NM 101, primary particle size 7-10 nm) to the TCC spiked soil showed no influence, whereas the addition of the larger sized materials (NM 102 and NM 100, primary particle size15-25 nm and 200-220 nm) resulted in less pronounced effects on the survival of *E. fetida* compared to the TCC test series.

In conclusion, the present study shows that in the presence of the smallest particle (NM 101) the toxicity was comparable and in the presence of the larger particles (NM 102 and NM 100) the toxicity was lower than when no TiO_2 material was present in the TCC treatment groups. However, the results of this study do not explain the mechanisms which are responsible for the observed influence of the TiO_2 materials on the TCC toxicity. Possible explanations why lower effects on survival were observed are given in the next section. Furthermore, this study shows that none of the different sized materials affected the survival, biomass or gut content of *E fetida* under the conditions tested. These results are in line with other studies investigating the effect of TiO_2 nanomaterials on the survival of earthworms (Heckmann et al. 2011, Hu et al. 2010, McShane et al. 2012, Whitfield Åslund et al. 2011). In the study of McShane et al. (2012) e.g. TiO_2 exposures with concentrations of up to 9500 mg/kg for 14 days did not affect the survival of earthworms.

8.3.3 Earthworm, reproduction test

The coefficients of variation of the controls in test sequence A were only in one test series slightly higher (2%) than requested in the OECD guideline 222 ($\leq$ 30%), therefore the test was considered as valid.

For most of the test series of test sequence B the coefficients of variation (CV, Tab. 3) of the reproduction of the control worms were slightly higher (CV 32.8-39.3%) than requested in the OECD guideline 222 (CV $\geq$ 30%; 2004). However, CVs of the TiO_2 controls were below 30% for all tests (Tab. 3).

TCC experiments The TCC test series of test sequence B showed that worms exposed to TCC concentrations of 675 mg/kg dw had a significantly lower reproduction (EC50 956 mg TCC/kg dw, Tab. 18) than control worms. When compared to the results of the TCC test series of test sequence A (EC50 243 mg TCC/kg dw), it can be seen that in this test sequence B TCC had a lower chronic toxicity towards *E. fetida*. This discrepancy may be due to variations in test conditions between the two test series. For test sequence B lamps were placed in the door of the incubator which was vertically located to the test vessels, whereas in test sequence A lamps were placed above the test vessels. Therefore, the illumination in the test vessels might have been lower in test sequence B than in test sequence A although in both cases as high as recommended in the guideline. Because worms usually avoid strong illumination, worms in test sequence A might have spent more time below the soil surface than worms of test sequence B feeding on more of the food on the surface under lower illumination which was not contaminated with TCC. Consequently worms of test sequence A might have taken up more TCC than worms of test sequence B. However, both tests show that TCC has an effect on the reproduction of the worms and because in sequence A and B aTCC test was run, the mixture tests of each test sequence can be compared to the corresponding TCC test.

TCC concentrations of 51 mg/kg dw in sewage sludge were found in the environment (Heidler et al. 2006). Considering the EC50 value (956 mg/kg dw) of the TCC test series of the test sequence B it can be assumed that TCC does not pose a risk to *E. fetida*. Nevertheless, the TCC test series of test sequence A showed that significant effects on the reproduction of *E. fetida* already occurred at TCC soil concentrations close to sewage sludge concentrations of 51 mg/kg dw. As explained already above we observed that the BAF of TCC was four times higher when worms were exposed to TCC via the food than via soil. Considering additionally that in nature an exposure of TCC via the food e.g. sludge is more relevant than via soil, it has to be clarified, whether a TCC exposure via food (e.g. sludge) leads to greater effects on the reproduction of *E. fetida* than an exposure via soil.

TiO_2 material experiments None of the TiO_2 materials had an effect on the reproduction or biomass of *E. fetida* up to concentrations of 1000 mg/kg. Different results were observed for the reproduction of *E. andrei* in the presence of NM 101. In the study of Schlich et al. (2012) it was observed that NM 101 stimulated the reproduction by 23% compared to the control worms. However, our results are in line with results of Mc Shane et al. (2012) showing that TiO_2 concentrations of up to 10.000 mg/kg did not alter the reproduction of earthworms after a test period of 28 days.

Mixture experiments Because the extent of toxicity of TCC on the reproduction of *E. fetida* was different in test sequence A and B, the results of the mixture test series of the different test sequences will not be compared with each other.

Test sequence A

The results of this test sequence show that NM 101 had an influence on the effect of TCC on the reproduction of *E. fetida*. In detail, effects in the mixture test series in some treatment groups were significantly less pronounced than in the TCC test series. Furthermore, it was shown that this influence was dependent on the NM 101 concentration, because the observed inhibition of reproduction was lower the higher the applied NM 101 concentration was. This observation is reflected by the lowest observed effect concentrations (LOEC, Tab. 17) which are lower the higher the applied NM 101 concentration was (only TCC: 84 mg/kg dw soil; + 400 mg TiO_2/kg dw soil: 168,8 mg/kg dw soil; + 1000 mg TiO_2/kg dw soil: 337,5 mg mg TiO_2/kg dw soil). Additionally, the median lethal concentrations (EC50) can be put in following increasing order: + 1000 mg/kg (384 mg TCC/kg dw soil) > + 400 mg/kg (308 mg TCC/kg dw soil) > + 0 mg/kg (243 mg TCC/kg dw soil). The influence of NM 101 on the toxicity of TCC may be explained by different hypotheses:

1. Binding of TCC by NM 101: A study of Luo et al. (2011) demonstrated that the application of nano-TiO_2 to sediments increased the Brunauer Emmett Teller (BET) specific surface area of the sediment. As a consequence the phosphor binding potential of the sediment was higher compared to control sediments. Therefore, it can be assumed that in the present study the application of NM 101 to the soil increased the TCC binding potential of the soil and thereby lowered the bioavailability of TCC to *E. fetida*. Additionally, it has to be assumed that NM 101 which bound TCC is not taken up by the organisms, or that the bound TCC stays bound to NM 101 particles when they are taken up in the organism.

2. Degradation of TCC by NM 101: Nano-TiO_2 can be used for degrading organic contaminants in soils, e.g. p-nitrophenol can be degraded in presence of anatase TiO_2 when soils are either exposed to UV radiation or when a very high voltage is applied (Wang et al. 2011). This procedure results in

reactive oxygen species which are capable of degrading organic contaminants. However, it seems unlikely that the test soils of the present study are exposed to UV radiation.

3. Antagonism: NM 101 may have had an antagonistic effect to TCC and thereby may have lowered the effect of TCC at its target site.

4. NM 101 may have stimulated the feeding behavior of *E. fetida*, resulting in a lower uptake of TCC, because more uncontaminated food was eaten.

The LC-MS results show that a degradation of TCC did not occur. Consequently, the second hypothesis is disproved. The other hypotheses have to be verified in further studies.

Test sequence B

In the following, results of the TCC test series of test sequence B will be compared to the results of the mixture test series of test sequence B. First it has to be mentioned that biomass changes and reproduction varied a lot in the TCC test series as well as in the mixture test series (SD reproduction tests 8-54%; SD biomass change 5-95%). Additionally, in the TCC test series significant effects on reproduction compared to the control only occurred in one treatment group in which soil was applied with the highest TCC concentration. Therefore, comparison of the TCC test series with the mixture test series is difficult and has to be interpreted carefully.

Significant differences between the TCC and the mixture test series were only observed for the reproduction results of the test with TCC and 400 mg NM 102/kg dw. Here reproduction in some treatment groups (155% and 87% at 169 and 675 mg/kg dw, respectively) was significantly higher than in the corresponding treatment group of the TCC test series (93% and 62% at 169 and 675 mg/kg dw). Furthermore, for some treatment groups a stimulation of reproduction (maximum stimulation of 55% compared to control, 169 mg/kg dw) compared to control worms was observed. Therefore, it seems that NM 102 (400 mg/kg dw) applied to TCC spiked soil lowered the toxic effect of TCC. However, the mechanisms leading to lower effects are still unclear. Possible explanation might be the same as those suggested for the finding that NM 101 also lowered the toxicity of TCC. In the test series with TCC and 1000 mg NM102/kg inhibition of reproduction was comparable to that observed for the TCC only test series. Although the EC50 value of the TCC + NM 102 test series (1000 mg/kg; EC50: 692 mg/kg, 95% CL lower/ upper: 436/8296 mg/kg, tab. 18) was lower than that calculated for the TCC test series (EC50: 956 mg/kg, 95% CL lower/upper: 833/1176 mg/kg, tab. 18), the 95% CL indicate that the EC50 values are comparable to each other. Furthermore, no significant differences between the TCC and the TCC + NM 102 (1000 mg/kg) test series were documented.

The comparison of the reproduction results of the mixture tests with TCC and NM 100 with the TCC test series shows the same trends as the corresponding comparison with the NM 102 test series: In contrary to the TCC test series no significant effect on the reproduction of *E. fetida* was observed at all in the mixture test series with TCC and 400 mg NM 100/kg dw. This seems to indicate that NM 100 applied at 400 mg/kg dw to the soil may also have a lowering effect on the toxicity of TCC towards *E. fetida* leading to a higher reproduction than in the corresponding TCC only treatments. However, this influence cannot be seen in the mixture test series with 1000 mg NM 100/kg dw, because no difference in toxicity was observed compared to the TCC only test series: Although the EC50 value of the TCC + NM 100 (EC50 494 mg/kg dw, 95% CL lower/upper: 355/849 mg/kg, tab. 18) test series was lower than the TCC test series, the 95% confidence intervals indicate that the observed toxicity is comparable between both test series.

In conclusion, the chronic toxicity of TCC depends on the TiO_2 concentration in soil: Exposures of worms to a mixture of TCC and the lower TiO_2 material concentrations (400 mg/kg dw) led to lower chronic toxicity than for worms exposed to TCC only whereas higher TiO_2 material concentrations (1000 mg/kg dw) had no influence on the TCC toxicity. Furthermore this influence seems not to be connected to different characteristics of the investigated nanomaterials, because same trends were observed for the mixture test series with TCC and the non-nano reference NM 100 and the mixture test series with TCC and the nanomaterial NM 102.

After 28 d of exposure to untreated as well as to TCC treated soils (TCC test series) worms gained biomass of approximately 28% compared to their initial biomass.

Also the observed biomass changes indicate that the influence of the TiO_2 material on the toxicity of TCC depends on the TiO_2 material concentration in soil. The comparison of the results of the biomass changes of the TCC test series with those of the mixture test series shows: a) A concentration dependent increase of biomass up to 40-45% compared to the control for mixture test series with TCC and lower TiO_2 concentrations. b) A constant increase of biomass of approximately 40-45% compared to the control for test series with TCC and higher TiO_2 concentrations.

Nevertheless, the results of this study do not explain which mechanisms were responsible for the observed influence of the TiO_2 particles on the chronic toxicity of TCC for *E. fetida.* Possible explanations may be a) binding of TCC to TiO_2, b) degradation of TCC by NM 101, c) antagonism, d) Avoidance behavior (pronounced uptake of uncontaminated food). The reduced toxicity of TCC in TiO_2 spiked soil by degradation of the biocide can be excluded based on our analytical results (LC-MS).

8.4 Conclusion

The acute toxicity tests with TCC showed that TCC significantly lowered the survival rate and biomass of *E. fetida* (LC10 262 mg /kg dw soil, t_{14}). Furthermore, earthworm reproduction tests showed that TCC had a significant negative effect on the reproduction of *E. fetida* which was more pronounced in test sequence A (EC50 243 mg/kg dw soil) than in test sequence B (EC50 956 mg/kg dw soil). Acute mixture toxicity experiments revealed that the toxicity in the TCC treatment groups was comparable in the presence of the smallest particle (NM 101, LC10 243 mg/kg dw soil), whereas it was less pronounced in the presence of the larger particles (NM 102 and NM 100, LC10 not calculable and 489 mg/kg dw soil) compared to the treatment groups without presence of TiO_2 materials (LC10 262 mg/kg dw soil). In the mixture reproduction experiments based on LOEC values it was shown that in the presence of the smallest particle (NM 101) toxicity was lower the higher the applied NM 101 concentration was, compared to the TCC treatment groups without TiO_2 material application. TCC analysis of the soil samples of the reproduction test with TCC and TCC with NM 101 assured that no degradation of TCC through NM 101 occurred. As in the acute toxicity tests NM 102 and NM 100 lowered the effect of TCC in the reproduction mixture tests but this was only the case at the low application level of the materials (400 mg/kg). In presence of 1000 mg/kg similar effects were observed as in the TCC tests. Further research is necessary to investigate the mechanisms behind the observed influence of the TiO_2 materials on the toxicity of TCC. The TiO_2 analysis of the soil samples proof that the TiO_2 application method can be used to homogeneously and reproducibly apply TiO_2 materials to a natural soil.

8.5 Outlook

In further experiments the mechanisms which are responsible for the observed influence of the TiO_2 materials on the TCC toxicity have to be investigated: E.g. it has to be observed whether an adsorption of TCC on the TiO_2 materials occurs and whether the mixture of TCC and the TiO_2 materials stimulates the feeding behavior of *E. fetida*.

9 Activated sludge, respiration inhibition test (OECD 209)

Nano-TiO_2 is, among other products, present in personal care products (PCP) and paints for facades. During showering or strong rain events it may be washed down the drain or may be rinsed off the façade (Kaegi et al. 2008), finally ending up in WWTP. Only few studies exist which investigated the fate of TiO_2 in WWTP. Kiser et al. (2009) who measured the Ti concentration in different sections of a WWTP in Arizona showed that TiO_2 tends to adsorb to the biomass of activated sludge (100-1000 µg Ti/L). The latter was confirmed by them in a laboratory study with nano-TiO_2 showing a sludge adsorption potential of 70-85%. Because nano-TiO_2 exhibits such a high adsorption potential, it has to be evaluated whether nano-TiO_2 poses a risk to microorganisms of activated sludge.

Under real conditions activated sludge is not exposed to only one potential toxicant but to a whole variety of xenobiotics. Triclocarban (TCC), another ingredient of PCP e.g. is listed as a priority substance in a review of Clarke and Smith (2011) dealing with emerging pollutants in European sewage sludge and can be detected in very high concentrations in this matrix (51 mg/kg, Heidler et al. 2006, see also chapter 5). As it is known that nanomaterials mostly have a higher reactivity than their bulk form, it can be assumed that they also have a higher potential to interact with these co-contaminants. Therefore, it might be necessary to include investigations of the mixture toxicity of nano-TiO_2 and co-contaminants in the risk assessment of nano-TiO_2.

Thus, in the present study not only ecotoxicological effects of nano titanium dioxide (nano-TiO_2) alone but also combinatory effects of nano-TiO_2 in the presence of co-contaminants e.g. the antimicrobial substance TCC were considered while investigating the effect of nano-TiO_2 to activated sludge by using the respiration inhibition test (Fig. 27, OECD 2010). To the best of our knowledge no study was found in which the toxicity of TCC on the respiration of activated sludge microorganisms was investigated. In case that TCC would be none toxic to activated sludge microorganisms, it was planned to test 3,5 dichlorophenol (3,5-DCP) as co-contaminant with nano-TiO_2, which is used as the reference substance in the OECD 209 (OECD 2010) test and is known to inhibit the respiration of activated sludge microorganisms.

Further it was studied, whether nano-TiO_2 exhibits a photoxic potential to activated sludge while being illuminated simultaneously with SSR.

9.1 Material and methods

9.1.1 Chemicals

For a detailed description of the used TiO_2 materials see section 2.1.1. Triclocarban (3,4,4,-trichlorocarbanilide, CAS 101-20-2, chemical purity 99%, Sigma Aldrich, Steinheim, Germany) and 3,5-dichlorophenol (3,5-DCP; CAS 591-35-5, chemical purity 97%, Sigma Aldrich, Steinheim, Germany) were used as organic contaminants.

9.1.2 Test organism

Activated sludge was derived freshly from a domestic municipal waste water treatment plant (Bensheim, Germany or Rossdorf, Germany). After a settling period of 5-15 min coarse particles were removed from the sludge by decanting the upper water layer. The collected sludge was held in the lab for 2-3 days and was fed daily as described in the 'Mixture toxicity of TiO_2 materials and an organic compound to activated sludge in a respiration inhibition test.' (Annex 6). Activated sludge from the WWTP of Bensheim, Germany was used

for all tests except for the preliminary experiment with nano-TiO_2 and SSR. For this test activated sludge from the WWTP of Rossdorf, Germany was used.

9.1.3 Good laboratory praxis

Tests were performed in the laboratories of IBACON GmbH. Raw data was documented and balances as well as pipettes were used in the style of GLP. IBACON's SOP for the activated sludge respiration inhibition test was adapted to the testing with nanomaterials and to the performance of mixture experiments.

9.1.4 Activated sludge, respiration inhibition test (OECD 209)

The activated sludge, respiration inhibition test was performed according to the OECD guideline 209 (OECD 2010). As mixture toxicity experiments were conducted the test varied in some points from that described in the OECD guideline. A detailed description of the mixture toxicity experiments is given in the SOP 'Mixture toxicity of TiO_2 materials and an organic compound to activated sludge in a respiration inhibition test.' (Annex 6) and an overview of the performed tests can be seen in Fig. 28.

The preparation of the TiO_2 stock suspensions (1 g/L) according to the SOP 'Preparation of a NM 101, NM 102 or NM 100 nanomaterial suspension' occurred directly before they were applied to the specific treatment group. Apart from sonicating the materials, the application procedure was the same as described for solutions in the OECD guideline 209 (OECD 2010).

DLS and ELS measurements of the used stock suspensions were not performed as it was shown in previous studies (section 2.2.4) that the used dispersion method resulted in reproducible HD and ZP values for all materials in the stock suspension.

In general, each replicate contained activated sludge (3 g dry weight per liter), tap water and, depending on the concentration a mixture of different ratios of deionized water and TiO_2 stock suspension (TiO_2 control) or TiO_2 stock suspension and defined amount/dilution of the organic co-contaminants, respectively. Only deionized water was applied to the controls. Test vessels were aerated for 3 h (= exposure time) (Fig. 29), thereafter the oxygen consumption (respiration) of the activated sludge was measured in special flasks (Karlsruher flask) by means of an oxygen electrode over a time period of 11 min (Fig. 29).

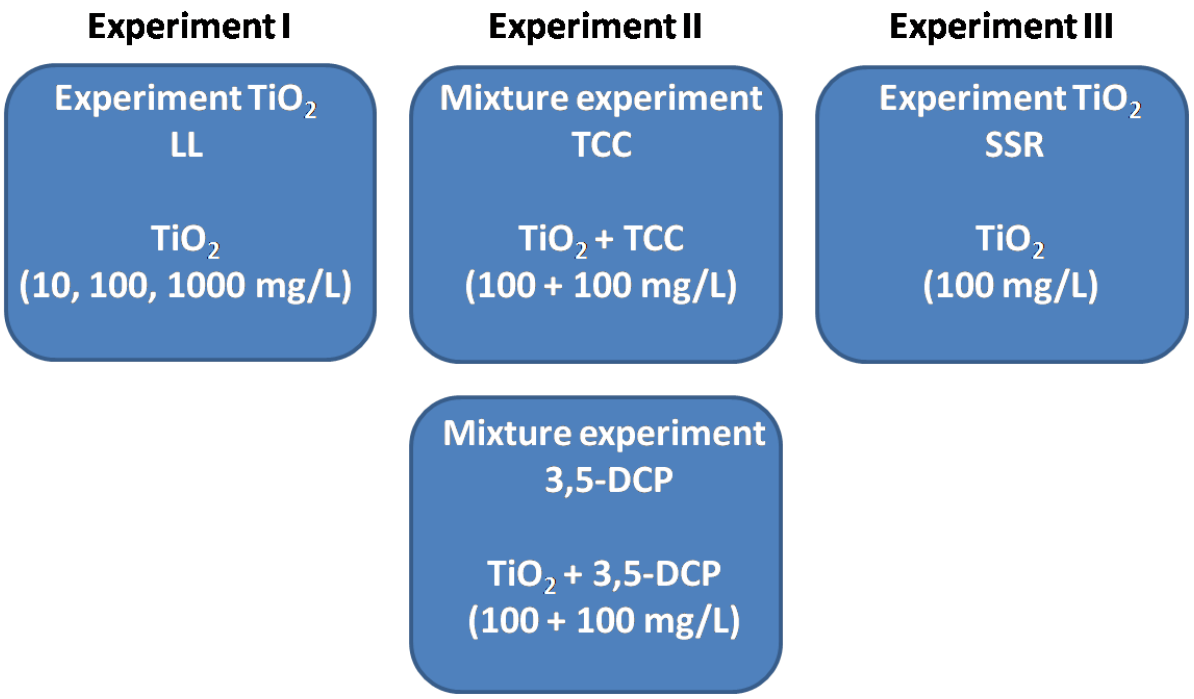

Fig. 28: Overview of the activated sludge, respiration inhibition tests (OECD 209) of the present study. *TCC* triclocarban, *3,5-DCP* 3,5 dichlorophenol, *LL* laboratory light *SSR* simulated solar radiation

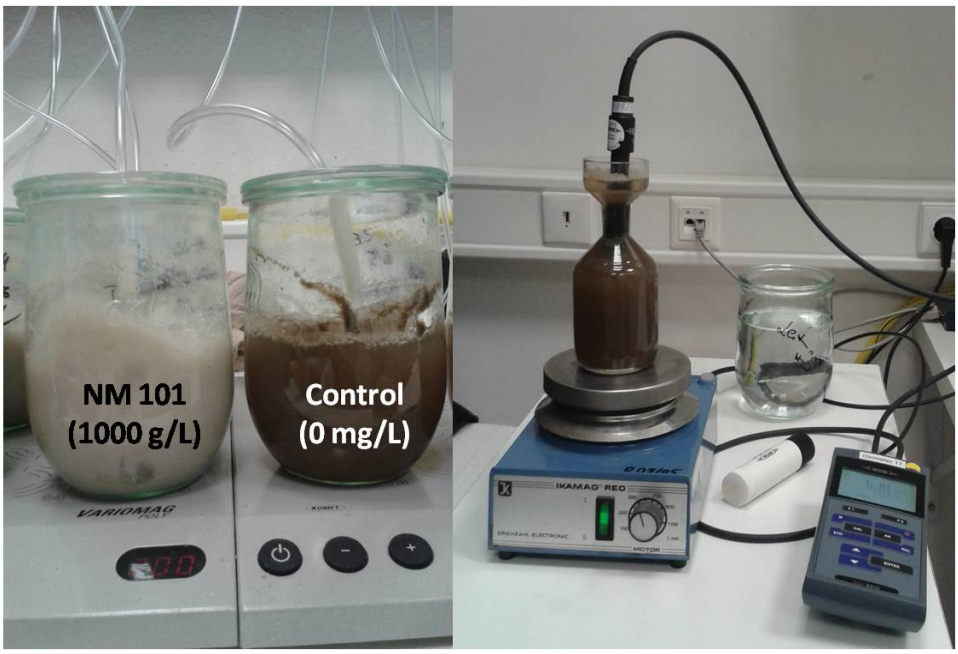

Fig. 29: Activated sludge, respiration inhibition test (OECD 209): Test vessels (left) and oxygen measurement in a Karlsruherflask (right) are shown.

Experiment I - TiO_2 materials with laboratory light (LL)

Before mixture experiments were performed it was investigated, in three independent experiments whether the nanomaterials had an effect on the respiration of the activated sludge. Briefly, test were conducted in which three different concentrations of the three TiO_2 materials (10, 100 and 1000 mg/L) and a blank control were tested. Further, three concentrations (3.2, 10, 32 mg/L) of 3,5-dichlorophenol (3,5-DCP) were applied in each test. 3,5-DCP served as a positive control. The inhibition of the nitrification rate was not investigated because this is not required when the test substance has no effect on the total respiration rate of the activated sludge (Umweltbundesamt 2012), which was the case for the tested TiO_2 materials.

Experiment II - Mixture experiments

Two different mixture experiments were accomplished with the same conditions and set up as in the TiO_2 material experiment as described above. In the first experiment a mixture of one of the three TiO_2 materials and the organic compound TCC was tested, whereas in the second experiment the positive control 3,5-DCP was used instead of TCC. Each test consisted of a mixture treatment group with the TiO_2 material (100 mg/L) and the organic compound (100 mg/L), a TiO_2 material control (100 mg/L), an organic compound control (100 mg/L), a blank control (0 mg/L) and three different concentrations of the positive control (3.2, 10 and 100 mg/L). Except for the blank control which consisted of six replicates, three replicates were tested per treatment group.

Experiment III – TiO_2 materials with simulated solar radiation (SSR)

Two parallel test series were run for a preliminary experiment with nano-TiO_2 and SSR. Both test series consisted of three controls (0 mg/L, SSR) and three TiO_2 treatment groups with NM 101, NM 102 and NM 100 (each 100 mg/L, SSR). One treatment group consisted of three replicates (100 mg/L). Further three control replicates were tested in the laboratory light test series. Additional to this positive controls (3,5-DCP) were used as usual.

SSR exposure of the aerated test vessels occurred in a SUNTEST XLS+ (Atlas, Linsengericht-Altenhaßlau, Germany, Fig. 30) in a tempered water bath (20 °C). This weathering instrument emits a spectrum comparable to that of sunlight and was used with an irradiance of 50 W/m² in the wavelength range of 300-400 nm. The corresponding spectrum is shown in Annex 7 and was provided by IBACON GmbH.

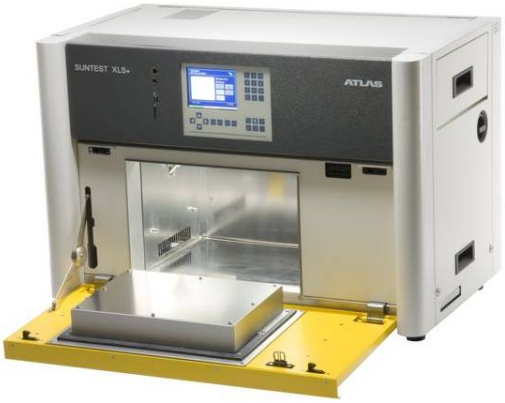

Fig. 30: SUNTEST XLS + (Atlas, Linsengericht-Altenhaßlau, Germany) without the water bath.

9.1.5 Analysis and statistics

Data was statistically analyzed with ToxRat® Professional (version 2.10, ToxRat solutions GmbH). Significant differences between the treatment groups and the controls were determined using student-t test for homogeneous variances (two sided, *P<0.05). Concentration response functions of 3,5-DCP were fitted to the data using probit analysis with linear max. likelihood regression. The median effective concentration (EC50) was calculated from this function.

9.2 Results

In general it was difficult to interpret the data of the activated sludge test, because often the respiration rate of the controls increased over time. This is probably due to the long time passing by the measurement of the first control and the last control. This long period is due to the large test set up. As a consequence, a feigned stimulation of respiration would have been observed if the test substance had no effect on the respiration rate of the activated sludge. To prevent a misinterpretation of the results, especially of the mixture experiments, the respiration of the treatment groups was, for most experiments, compared to the control respiration rate of the two control replicates which were chronologically closest instead to the mean control respiration rate.

9.2.1 Experiment I - TiO_2 materials with laboratory light (LL)

The respiration rate of the used activated sludge accounted to 24.6, 18.5 and 26.6 mg $O_2*h^{-1}*g^{-1}$ dry weight (dw) activated sludge (data not shown) and the coefficient of variation (CV) of the mean respiration rate was always ≤ 30% for the tests with NM 101, NM 102 and NM 100 (9.6%, 8.4% and 19.8%, data not shown). Therefore the respiration rate of the used activated sludge and the CV of the mean respiration rate of the sludge met the validity criteria given in the OECD guideline 209. The pH value at test initiation was comparable to the corresponding controls and ranged from 7.4 to 8.1. In the SSR test it was shown that the materials had no effect on the pH of the activated sludge after an exposure period of 3 h (section 7.2.4). The EC50 values of the positive controls were calculated as 3.56, 4.34 and 4.19 mg/L for the NM 101, NM 102 and NM 100 tests.

As described above the respiration rate of the different controls increased over time by around 22% in the NM 101 test. Consequently, the chronologically closest controls were compared with the specific treatment groups. These control groups, however, only consisted of two replicates so that no statistics could be performed for the NM 101 test. The mean inhibition of respiration ranges between -7.8 and 7.4% (Tab. 20).

The respiration rate of the lowest (10 mg/L) and highest NM 102 treatment group (1000 mg/L, Tab. 20) was significantly higher (almost 20%) than the mean control respiration rate. In this test the respiration rate of the last control replicates (21.4 $mg*L^{-1}*h^{-1}$) was lower than that of the controls which were measured in the middle of the test (24 $mg*L^{-1}*h^{-1}$, Tab. 20). When the respiration rate of these treatment groups is compared only to the two control replicates in the middle of the test the mean inhibition of the respiration rate accounts only to -8.1 ± 4.7% and -10.6 ± 7.0%.

No significant differences were observed between the respiration rate of the NM 100 treatment groups and the mean respiration rate of the controls. The respiration rate of the different control replicates of this test did not increase with time (Tab. 20).

Tab. 20: Mean O_2 consumption ($mg*L^{-1}*h^{-1}$) and inhibition of the respiration rate (%) for the treatment groups of the TiO_2 material experiment with laboratory light (LL, experiment I)

Treatment group	Mean O_2 consumption ± SD ($mg*L^{-1}*h^{-1}$)			Mean inhibition of respiration rate ± SD (%)		
NM material	101	102	100	101	102	100
Control (mean)	29.5 ± 2.8	22.2 ± 1.9[a]	31.9 ± 6.3[a]	-	-	-
Control (I+II)	26.6 ± 2.0[a]	21.2 ± 2.4	32.6 ± 9.7	-	-	-
3,5-DCP (3.2 mg/L)	15.1 ± 1.2[a]	12.2 ± 0.9[a]	17.9 ± 2.9[a]	43.4± 4.5	45.0 ± 4.1	43.8 ± 9.1
3,5-DCP (10 mg/L)	9.9 ± 0.4[a]	8.2 ± 1.4[a]	9.9 ± 2.3[a]	62.9 ± 1.4	63.2 ± 6.2	68.9 ± 7.2
3,5-DCP (32 mg/L)	4.1 ± 0.6[a]	3.4 ✣ 1.0[a]	4.7 ✣ 1.9[a]	84.7 ± 2.2	84.9 ± 4.4	85.3 ± 5.9
Control (III+IV)	29.4 ± 1.2[b]	24.0 ± 0.0	28.0 ± 5.7	-	-	-
NM (10 mg/L)	27.2 ± 0.7[a]	25.9 ± 1.1[a]*	31.2 ± 5.9[a]	7.4 ± 2.2	-16.9 ± 5.1*	2.0 ± 18.4
NM (100 mg/L)	30.6 ± 0.4[b]	22.5 ± 1.2[a]	35.1 ± 4.6[a]	-3.9 ± 1.4	-1.4 ± 5.4	-10.0 ± 14.3
NM (1000 mg/L)	34.9 ± 4.5[c]	26.5 ± 1.7[a]*	35.0 ± 2.9[a]	-7.8 ± 13.8	-19.7 ± 7.6*	-9.9 ± 9.0
Control (V+VI)	32.4 ± 0.6[c]	21.4 ± 1.2	35.0 ± 4.6	-	-	-

* significant difference to the corresponding control, [a,b,c] indicate the control group to which the treatment groups were compared to

9.2.2 Experiment II - Mixture experiment with TCC and TiO_2 materials

The respiration rate of the used activated sludge accounted to 21.8 mg O_2*h^{-1}*g^{-1} dry weight activated sludge (data not shown) and the coefficient of variation (CV) of the mean respiration rate of the activated sludge was 12.9% (data not shown). The pH value at test initiation in the different treatments was comparable to the control (7.5-7.8) and ranged from 7.5 to 7.9. In the SSR test it was shown that the materials had no effect on the pH of the activated sludge after an exposure period of 3 h (section 7.2.4). The EC50 value of the positive control was calculated as 6.27 mg/L.

The respiration rate of the controls increased over by 25%, from 24.3 mg*L^{-1}*h^{-1}in the first two replicates to 30.3 mg*L^{-1}*h^{-1} in the last two replicates. Almost 3.5 h lay between the measurement of the first and the last two controls. This might have allowed a further growth of the bacteria within the activated sludge inoculum used for the preparation of the controls and treatment groups and consequently to a higher respiration rate of the controls and treatment groups which were measured at the end of the test. Therefore, the respiration rate of the treatment groups was compared to the chronologically closest control group. Table 21 shows the mean inhibition of respiration rate of the different treatment groups and it can be seen that the inhibition of respiration rate of the TCC, NM and TCC+NM treatment groups was not higher or lower than 10/-10%.

Tab. 21: Mean O_2 consumption (mg*L^{-1}*h^{-1}) and inhibition of the respiration rate (%) for the treatment groups of the mixture experiment with triclocarban (Experiment II)

Treatment group	Mean O_2 consumption ± SD (mg*L^{-1}*h^{-1})	Mean inhibition of respiration rate ± SD (%)
Control (mean)	26.2 ± 3.4	-
Control (I+II)	24.3 ± 0.4[a]	-
3,5-DCP (3.2 mg/L)	15.6 ± 1.1[a]	35.9 ± 4.5
3,5-DCP (10 mg/L)	10.0 ± 0.4[a]	58.7 ± 1.5
3,5-DCP (32 mg/L)	3.9 ± 0.3[a]	84.1 ± 1.3
Control (III+IV)	24.0 ± 0.8[b]	-
TCC (100 mg/L)	22.9 ± 1.5[b]	4.8 ± 6.3
NM 101 (100 mg/L)	25.0 ± 1.3 b	7.1 ± 2.4
NM 102 (100 mg/L)	28.0 ± 2.0[c]	-1.6 ± 14.6
NM 100 (100 mg/L)	29.9 ± 1.7[c]	1.2 ± 5.8
NM 101 + TCC (100/100 mg/L)	22.3 ± 0.6[b]	-4.0 ± 5.5
NM 102 + TCC (100/100 mg/L)	24.4 ± 3.5[c]	7.5 ± 6.5
NM 100 + TCC (100/100 mg/L)	33.5 ± 1.6[c]	-10.5 ± 5.4
Control (V+VI)	30.3 ± 2.4[c]	-

[a,b,c] indicate the control group to which the treatment groups were compared to

9.2.3 Experiment II - Mixture experiment with 3,5-DCP and TiO_2 materials

The respiration rate of the controls accounted to 21.8 mg O_2*h^{-1}*g^{-1} dry weight activated sludge (data not shown) and the coefficient of variation (CV) of the mean respiration rate was 12.1% (data not shown). The pH value at test initiation in the treatments was comparable to the control (7.5-8.0) and ranged from 7.6 to 8.0. In the SSR test it was shown that the materials had no effect on the pH of the activated sludge after an exposure period of 3 h (section 7.2.4). The EC50 value of the positive control was calculated as 5.28 mg/L.

The respiration rate of the controls increased during the testing period by around 30%, from 23.1 mg*L^{-1}*h^{-1}in the first two replicates to 29.7 mg*L^{-1}*h^{-1} in the last two replicates. This increase also occurred in experiment I and reasons for this increase are explained in section 7.2.2. Therefore, the respiration rate of the treatment groups was compared to the chronologically closest control group. Table 22 summarizes the mean inhibition of respiration rate of the different treatment groups compared to the corresponding controls. The inhibition of respiration rate of the TiO_2 controls ranged between 0.0 and 11.5%, whereas the respiration rate of the activated sludge exposed to the 3,5-DCP control (3.2 mg/L) experienced an inhibition of around 40%. A similar inhibition of the respiration rate was observed for the mixture treatment groups with NM and 3,5-DCP.

Tab. 22: Mean O_2 consumption (mg*L^{-1}*h^{-1}) and inhibition of the respiration rate (%) for the treatment groups of the mixture experiment with 3,5-dichlorophenol (Experiment II)

Treatment group	Mean O_2 consumption ± SD (mg*L^{-1}*h^{-1})	Mean inhibition of respiration rate ± SD (%)
Control (mean)	26.2 ± 3.2	-
Control (I+II)	23.1 ± 1.2[a]	-
3,5-DCP (3.2 mg/L)	13.4 ± 1.2 [a]	42.2 ± 5.3
3,5-DCP (10 mg/L)	9.7 ± 0.1 [a]	58.2 ± 0.3
3,5-DCP (32 mg/L)	3.5 ± 0.7 [a]	85.0 ± 3.0
Control (III+IV)	25.7 ± 1.6 [b]	-
NM 101 (100 mg/L)	25.7 ± 3.0 [b]	0.0 ± 11.8
NM 102 (100 mg/L)	27.8 ± 1.3 [c]	6.4 ± 4.4
NM 100 (100 mg/L)	26.3 ± 2.5 [c]	11.5 ± 8.4
NM 101 + DCP (100/3.2 mg/L)	14.7 ± 0.9[b]	43.0 ± 3.4
NM 102 + DCP (100/3.2 mg/L)	16.9 ± 0.3 [c]	43.1 ± 1.0
NM 100 + DCP (100/3.2 mg/L)	18.1 ± 0.3 [c]	39.1 ± 1.1
Control (V+VI)	29.7 ± 1.6[c]	-

[a,b,c] indicate the control group to which the treatment groups were compared to

9.2.4 Experiment III - TiO_2 materials with simulated solar radiation (SSR)

The respiration rate of the activated sludge used for the LL and SSR tests accounted to 24.9 and 21.4 mg O_2*h^{-1}*g^{-1} dry weight activated sludge (data not shown) and the coefficient of variation (CV) of the mean respiration rate of the activated sludge was 20.6% and 25.6% (data not shown). The pH value of the activated sludge applied with the different TiO_2 materials (8.1-8.4) at test termination was comparable to that of the controls (8.1-8.3) in the preliminary experiment with SSR. The EC50 value of the positive control was calculated as 18.67 mg/L. It is noteworthy that compared to the other tests the activated sludge was obtained from another WWTP, namely that of Rossdorf, Germany (see section 7.1.2). This may explain differences of the EC50 values of the positive control.

The respiration rate of the last two replicates of the control were lower than those two of the middle of the experiment, therefore the respiration rate of the treatment groups was compared to the mean respiration rate of the controls. Both test series (LL and SSR) revealed an inhibition of the respiration rate of the TiO_2 treatment groups which was lower than 13%, demonstrating that SSR did not induce an inhibition of the respiration rate of the activated sludge by the different TiO_2 materials. No differences in respiration rates of the different TiO_2 materials were recognized.

Tab. 23: Mean O_2 consumption (mg*L^{-1}*h^{-1}) and inhibition of the respiration rate (%) for the treatment groups of the TiO_2 material experiment with simulated solar radiation (SSR, experiment III); LL laboratory light

Light conditions	Treatment group	Mean O_2 consumption ± SD (mg*L^{-1}*h^{-1})	Mean inhibition of respiration rate ± SD (%)
LL	Control (mean)	29.9 ± 6.2[a]	-
	Control (I+II)	22.7 ± 2.6	-
	3,5-DCP (3.2 mg/L)	22.1 ± 0.9 [a]	2.7 ± 3.9
	3,5-DCP (10 mg/L)	17.7 ± 0.0 [a]	22.0 ± 0.0
	3,5-DCP (32 mg/L)	5.0 ± 0.2 [a]	77.9 ± 0.9
	Control (III+IV)	34.3 ± 4.8	-
	NM 101 (100 mg/L)	26.6 ± 1.2 [a]	11.0 ± 4.1
	NM 102 (100 mg/L)	25.9 ±0.3 [a]	13.2 ± 1.1
	NM 100 (100 mg/L)	27.9 ± 1.0 [a]	6.6 ± 3.3
	Control (V+VI)	32.5 ± 1.7 [a]	-
SSR	Control (mean)	25.7 ± 6.6[b]	-
	NM 101 (100 mg/L)	22.9 ± 1.7 [b]	11.1 ± 6.3
	NM 102 (100 mg/L)	25.5 ± 0.9 [b]	0.7 ± 3.4
	NM 100 (100 mg/L)	23.6 ± 2.3 [b]	8.2 ± 9.0

[a,b,c] indicate the control group to which the treatment groups were compared to

9.3 Discussion

Except for the NM 102 test with laboratory light all experiments fulfilled the validity criteria of the OECD guideline 209, namely a mean control respiration rate of 20 mg O_2*g dw^{-1}*h^{-1} and a coefficient of variation of ≤ 30% of the mean control respiration rate (section 7.2.1-7.2.4). Because the CV of the mean respiration rate of the controls of the NM 102 test was within the given range and the mean respiration rate of the controls was only slightly lower (18.5%) than indicated in the OECD guideline the test was deemed as valid.

All experiments which were performed with the different sized TiO_2 materials under LL or SSR, except for the NM 102 test with LL, revealed that they did not affect the respiration rate of the activated sludge by more than 13%. In the NM 102 test with LL significant differences compared to the control were observed for the NM 102 treatment groups with 10 mg/L and 1000 mg/L (LL, Tab. 20). Due to following reasons they have to be critically examined: Considering that in most of the other tests the respiration rate of the controls at the end of the test was 22-30% higher than at test start, it is assumed that a stimulation of the respiration rate by 20% is due to the time dependent increase of the respiration rate of the inoculum of the activated sludge and has to be critically considered. Consequently, the observed stimulation of the NM 102 treatment groups may be related to such stimulation, although a respiration rate of the controls of this height at the end of this test was not observed. It is suggested that the fact that the respiration rate of the controls of this tests did not increase over time was an exception and did not reflect the usual conditions of the activated sludge which was applied to the NM 102 treatment groups. These assumptions are confirmed by the comparison of the respiration rate of the NM 102 treatment groups to that of the controls of the middle of the test. Applying the respiration rates of these controls only, NM 102 treatment groups show only a stimulation of around 10%, which is comparable to the results observed for the other TiO_2 material treatment groups. Moreover, no stimulation was observed for the 100 mg/L treatment group (Tab. 20), indicating that the observed stimulation was not concentration dependent. Further, all other tests, in which the respiration rate of the 100 mg/L treatment group was repeatedly tested and compared to the chronologically closest control groups, revealed that no stimulation occurred (Tab. 21, 22 and 23). Therefore, we conclude that the significant differences observed for these two treatment groups are due to an artifact i.e. NM 102 has no effect on the respiration rate of the activated sludge. It is obvious that a concentration of 100 mg/L does not affect the respiration rate of activated sludge.

Summing up, none of the tests excerpts any effect on the respiration rate of activated sludge, not even if activated sludge was simultaneously exposed to SSR and the TiO_2 materials. The latter may be a result of the presence of natural organic matter (NOM) within the activated sludge which is on the one hand also capable of absorbing UV irradiation in the same wavelength region at which TiO_2 materials are photoactivated (Doll & Frimmel 2005). On the other hand NOM may adsorb on the surface of the TiO_2 material thereby reducing the formation of ROS.

In a study with an anaerobic (low dissolved oxygen) sequencing batch reactor in which activated sludge was exposed to TiO_2 (50 mg/L) either under acute (24 h) or chronic conditions (70 d) it was discovered that nano-TiO_2 had no acute effects on the nitrogen and phosphorus removal efficiency (Zheng et al. 2011). However, longer exposure resulted in a 60% lower nitrogen removal efficiency compared to the controls. This effect correlated with a significantly lower diversity of microbial community and an abundance of nitrifying bacteria.

Long term effects of nano-TiO_2 on microorganisms, as a reduced microbial biomass and a reduced bacterial diversity were also observed in a study of Ge et al. (2011) in which the influence of nano-TiO_2 (0-2 mg/kg grassland soil; 15-20 nm, 81% anatase and 19% rutile) on soil microbial communities was investigated.

These studies demonstrate that effects of nano-TiO_2 on microorganisms in environmental complex media as soil and sewage sludge seem to occur mainly after an exposure period of several weeks.

This may explain why no effects of the TiO_2 materials on the activated sludge were observed after an exposure period of 3 h in the present study. As the study of Zheng et al. (2011) was performed under anaerobic conditions, further studies are necessary to investigate whether nano-TiO_2 also reduces the abundance of nitrifying bacteria under aerobic conditions in activated sludge.

To the best of our knowledge no study was found in which the toxicity of TCC to activated sludge was investigated. However, Neumegen et al. (2005) tested the effect of triclosan, which has a comparable chemical structure as TCC, on activated sludge microorganisms by using a biochemical oxygen demand test (5 d) and observed an EC50 in the mg/L range (EC50 1.82 mg/L). A study of Lawrence et al. (2009) observed the effect of low levels of TCC (10 µg/L) on river biofilm communities and found that these levels altered the community composition, algal biomass, architecture and activity of those after a test period of 8 weeks.

In contrast to triclosan (Neumengen et al. 2005) TCC did not alter the respiration rate of activated sludge microorganisms in our study compared to control organisms. Maybe the differences in toxicity are related to the exposure duration which was only 3 h in our study compared to 5 days in their study.

The mixture experiments revealed that as in the single substance tests of the different sized TiO_2 materials and the organic compound (TCC) mixtures of both did not induce toxic effects on activated sludge microorganisms. Furthermore, the toxicity of the reference compound (3,5-DCP) towards the respiration rate of activated sludge was not altered under the conditions tested. As interaction of the organic compounds with the TiO_2 material is a prerequisite for the occurrence of mixture effects and as this is probably a time dependent process, it may be assumed that the test period was too short to observe potential mixture toxicity. This highlights the need for performing mixture experiments under prolonged exposure periods.

9.4 Conclusion

All materials did not affect the respiration rate of activated sludge when tested with both light conditions (LL and SSR). Furthermore, they did not change the extent of toxicity of an organic substance found to be none toxic to activated sludge (TCC) in our study and an organic substance known to be toxic to activated sludge (3,5-DCP).

9.5 Outlook

As other studies show that nano-TiO_2 has an influence on microbial communities in complex environmental media as soils and anaerobic sewage sludge after prolonged exposure periods (60-70 d) testing with longer exposure periods may be considered when investigating the influence of nano-TiO_2 on microbial communities in further studies. It might be also interesting to investigate the mixture toxicity under prolonged exposure periods as interactions between nanomaterials and organic compounds in complex environmental media may take place after a certain period of time.

10 Summary of the study

10.1 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) and zeta potential (ZP) 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 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. This strong agglomeration behavior is stated in the sedimentation experiment described in section 3.2.

10.2 Ecotoxicity tests

In the present study nano and non-nano scale TiO_2 materials were tested with standard OECD tests and also under consideration of relevant exposure scenarios as simulated solar radiation (SSR), mixture toxicity or embryonic development to investigate whether such exposure scenarios would influence 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 ecotoxicity is size dependent or nano specific with respect to the EU recommendation for a nanomaterial definition (European-Commission 2011/696/EU). Furthermore, it was of interest whether the standardized test guidelines are 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 ≥ 50 mg/L (≥ 50 mg/L for *D. magna* (mobility, 48 h), ≥ 100 mg/L for *Danio rerio* (mortality, 96 h), ≥ 1000 mg/L for activated sludge microorganisms (respiration rate, 3 h) and ≥ 1000 mg/kg for *E. fetida* (mortality and reproduction, 14 d, 56 d).

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 with daphnids 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 (Dabrunz et al. 2011, Wiench et al. 2009, Zhu et al. 2010), whereas others showed effects in this concentration range (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 endpoints 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.

We demonstrated that in the current standardized OECD tests the effects of TiO_2 nanomaterials were orders of magnitude lower (EC50 in µg/L range) when test set ups were modified by integrating relevant exposure scenarios, i.e. sunlight irradiation. Considering that the predicted environmental TiO_2 concentrations are assumed to be in the ng/L-µg/L (surface water/WWTP effluent) or µg/kg (soil) range (Gottschalk et al. 2009), neglecting alternative endpoints and realistic exposure scenarios of TiO_2 materials would result in an underestimation of the environmental risk of TiO_2 materials.

In our study, we did not consider alternative endpoints (e.g. behavior) of TiO_2 materials, but rather investigated the relevance of relevant exposure scenarios as e.g. I) solar radiation, II) mixture toxicity or III) embryonic development for ecotoxicity testing of 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, 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 believe that the SSR induced toxicity is related to the ROS formation of titanium dioxide known to be more pronounced for intermediate anatase particle sizes (Almquist & Biswas 2002). This may explain why in our study the nanoparticle with an intermediate size NM 102 (20-25 nm) exhibited the strongest SSR induced effect followed by the smallest nanoparticle NM 101 (7-10 nm) and the largest non-nano reference NM 100 (200-220 nm).

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 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 during the test period of 56 days was not degraded, i.e., lowering the TCC concentration by metabolization 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 and not at IBACON GmbH) 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 so that a direct comparison of the results is possible. As in the acute toxicity tests the addition 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 not taken up by the earthworms and thereby lowered the toxicity of TCC to the earthworms. 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 TiO_2 materials as shown especially for NM 102 in the *D. magna* immobilization test.

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 nano-materials 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 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 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 guideline (OECD 1984, 2004b) is applicable for testing TiO_2 nanomaterials as far as recommendations for the preparation and application of nanomaterial suspensions are given in the guideline.

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.

11 Overall 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. We suggest that phototoxicity was driven by a combination of factors as photoactivity, agglomeration state and particle/daphnia interaction.

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 photo activity 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 potential ROS formation potential also for other nanomaterials before conducting ecotoxicity tests.

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

12 Outlook

Several comments on necessary future studies have been mentioned in section 3.5, 4.5, 6.5, and 7.5. One of the main outcomes of our study is the requirement to perform more ecotoxicity test in the presence of simulated solar radiation. In our study it was shown that the SSR induced toxicity of the different sized TiO_2 materials was 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 even higher diversity of nanomaterials in general, it is recommended to establish a screening tool for photoactive substances which should be tested under simulated solar radiation for their ecotoxicity.

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.

13 References

(EG) K (2009): Nr. 1223/2009 Verordnung des europäischen Parlamentes und des Rates über kosmetische Mittel. Amtsblatt der europäischen Union L 342, 59 ff

Agency USEP (1990): Pesticide Fact Sheet Number 89.2: Avermectin B1. US EPA, Office of Pesticide Programs, Registration Div., Washington, DC

Ahn KC, Zhao B, Chen J, Cherednichenko G, Sanmarti E, Denison MS, Lasley B, Pessah IN, Kultz D, Chang DPY, Gee SJ, Hammock BD (2008): In vitro biologic activities of the antimicrobials triclocarban, its analogs, and triclosan in bioassay screens: Receptor-based bioassay screens. Environmental Health Perspectives 116, 1203-1210

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

Ancare Australia PL (2008): Material safety data sheet fenbendazole 1-5

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

Boyle D, Al-Bairuty GA, Ramsden CS, Sloman KA, Henry TB, Handy RD (2013): Subtle alterations in swimming speed distributions of rainbow trout exposed to titanium dioxide nanoparticles are associated with gill rather than brain injury. Aquatic Toxicology 126, 116-127

Brewer BN, Armbrust KL, Mead KT, Holmes WE (2004): Determination of abamectin in soil samples using high-performance liquid chromatography with tandem mass spectrometry. Rapid Communications in Mass Spectrometry 18, 1693-1696

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

Bundesamt für Strahlenschutz B (2012): Solare bodennahe UV-Strahlung in Deutschland: Jahresberichte über das solare UV-Messnetz in Deutschland (sUV-Mo-Net). Acessed 15.01.2014, www.bfs.de

Burrows LA, Edwards CA (2004): The use of integrated soil microcosms to assess the impact of carbendazim on soil ecosystems. Ecotoxicology 13, 143-161

BVL BfVuL (2011): http://www.bvl.bund.de/cln_007/nn_492012/DE/04__Pflanzenschutzmittel/02__ZugelassenePflanzenschutzmittel/02__OnlineDatenbank/psm__onlineDB__node.html__nnn=true. Online-database plant pesticides (03.02.11)

Calabrese EJ, Baldwin LA (2001): U-shaped dose-responses in biology, toxicology, and public health. Annu Rev Publ Health 22, 15-33

Cha J, Cupples AM (2009): Detection of the antimicrobials triclocarban and triclosan in agricultural soils following land application of municipal biosolids. Water Research 43, 2522-2530

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

Chen Z, Min H, Wu W, Chen M, Zhang F, Zhao B (2003): Effects of pesticide-contamination on population size and denitrification activity of denitrifying bacteria in paddy soils. The journal of applied ecology 14, 1765-9

Clarke BO, Smith SR (2011): Review of 'emerging' organic contaminants in biosolids and assessment of international research priorities for the agricultural use of biosolids. Environment International 37, 226-247

Company H-RA-V (1995): Environmental assessment NADA 137-600 Fenbendazole type a medicated article (PREMIX) in dairy cattle of breeding age

Crossland NO (1990): A review of the fate and toxicity of 3,4-dichloroaniline in aquatic environments. Chemosphere 21, 1489-1497

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

Das AC, Mukherjee D (1998): Insecticidal effects on soil microorganisms and their biochemical processes related to soil fertility. World Journal of Microbiology and Biotechnology 14, 903-909

Doll TE, Frimmel FH (2005): Photocatalytic degradation of carbamazepine, clofibric acid and iomeprol with P25 and Hombikat UV100 in the presence of natural organic matter (NOM) and other organic water constituents. Water Research 39, 403-411

Elanco Animal Health MSDS (2004): Maxiban Premix, Section 1 - Chemical Product and Company. Elanco Animal Health Division of Eli Lilly and Company 1-9

Ellis SR, Hodson ME, Wege P (2007): The influence of different artificial soil types on the acute toxicity of carbendazim to the earthworm Eisenia fetida in laboratory toxicity tests. European Journal of Soil Biology 43, S239-S245

Ensenbach U, Nagel R (1997): Toxicity of Binary Chemical Mixtures: Effects on Reproduction of Zebrafish (*Brachydanio rerio*). Archives of Environmental Contamination and Toxicology 32, 204-210

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

Froehner K, Backhaus T, Grimme LH (2000): Bioassays with *Vibrio fischeri* for the assessment of delayed toxicity. Chemosphere 40, 821-828

Garcia M, Roembke J, de Brito MT, Scheffczyk A (2008): Effects of three pesticides on the avoidance behavior of earthworms in laboratory tests performed under temperate and tropical conditions. Environmental Pollution 153, 450-456

Ge YG, Schimel JP, Holden PA (2011): Evidence for Negative Effects of TiO_2 and ZnO Nanoparticles on Soil Bacterial Communities. Environmental Science & Technology 45, 1659-1664

Gledhill WE (1975): Biodegradation of 3,4,4'-Trichlorocarbanilide, TCC, in sewage and activated sludge. Water Research 9, 649-654

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

Grela MA, Colussi AJ (1996): Kinetics of stochastic charge transfer and recombination events in semiconductor colloids. Relevance to photocatalysis efficiency. The Journal of Physical Chemistry 100, 18214-18221

Guerard JJ, Miller PL, Trouts TD, Chin Y-P (2009): The role of fulvic acid composition in the photosensitized degradation of aquatic contaminants. Aquatic Sciences 71, 160-169

Hansen M, Krogh KA, Bjorklund E, Halling-Sorensen B, Brandt A (2009): Environmental risk assessment of ionophores. TrAC Trends in Analytical Chemistry 28, 534-542

Hariharan C (2006): Photocatalytic degradation of organic contaminants in water by ZnO nanoparticles: Revisited. Applied Catalysis A: General 304, 55-61

Hartmann NB, Von der Kammer F, Hofmann T, Baalousha M, Ottofuelling S, Baun A (2010): Algal testing of titanium dioxide nanoparticles-Testing considerations, inhibitory effects and modification of cadmium bioavailability. Toxicology 269, 190-197

Haruta M (1997): Size-and support-dependency in the catalysis of gold. Catalysis Today 36, 153-166

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

Heidler J, Sapkota A, Halden RU (2006): Partitioning, persistence, and accumulation in digested sludge of the topical antiseptic triclocarban during wastewater treatment. Environmental Science & Technology 40, 3634-3639

Heidler J, Halden RU (2009): Fate of organohalogens in US wastewater treatment plants and estimated chemical releases to soils nationwide from biosolids recycling. JEM Journal of Environmental Monitoring 11, 2207-2215

Higgins CP, Paesani ZJ, Chalew TEA, Halden RU (2009): Bioaccumulation of triclocarban in *Lumbriculus variegatus*. Environmental Toxicology and Chemistry 28, 2580-2586

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

Hund-Rinke K (2010): Einfluss von nanoskaligem TiO_2 auf die Reproduktion von Regenwürmern (*Eisenia Andrei*). Frauenhofer IME, Geschäftsbericht 2010/2011, 70-71

Hurum DC, Agrios AG, Gray KA, Rajh T, Thurnauer MC (2003): Explaining the enhanced photocatalytic activity of Degussa P25 mixed-phase TiO_2 using EPR. The Journal of Physical Chemistry B 107, 4545-4549

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

ISO (2009): Water quality -- Determination of selected elements by inductively coupled plasma optical emission spectrometry (ICP-OES) ISO 11885:2009. ISO

IUPAC (05.02.2011): http://sitem.herts.ac.uk/aeru/iupac/314.htm. IUPAC, global availability of information on agrochemicals

Janicke W, Hilge G (1980): Messung der Bioeliminiation von Chloranilinen. gwf-wasser-abwasser 121 131-135

Jensen J, Diao X, Scott-Fordsmand JJ (2007): Sub-lethal toxicity of the antiparasitic abamectin on earthworms and the application of neutral red retention time as a biomarker. Chemosphere 68, 744-750

Kaegi R, Ulrich A, Sinnet B, Vonbank R, Wichser A, Zuleeg S, Simmler H, Brunner S, Vonmont H, Burkhardt M, Boller M (2008): Synthetic TiO_2 nanoparticle emission from exterior facades into the aquatic environment. Environmental Pollution 156, 233-239

Kim HJ, Lee HJ, Lee DS, Kwon JH (2009): Modeling the Fate of Priority Pharmaceuticals in Korea in a Conventional Sewage Treatment Plant.

Kiser MA, Westerhoff P, Benn T, Wang Y, Perez-Rivera J, Hristovski K (2009): Titanium Nanomaterial Removal and Release from Wastewater Treatment Plants. Environmental Science & Technology 43, 6757-6763

Kolar L, Kozuh Erzen N, Hogerwerf L, van Gestel CAM (2008): Toxicity of abamectin and doramectin to soil invertebrates. Environmental Pollution 151, 182-189

Kreuzig R, Bluemlein K, Hoeltge S (2007): Fate of the benzimidazole Antiparasitics flubendazole and fenbendazole in manure and manured solis. Clean-Soil Air Water 35, 488-494

Kuhlbusch TAJ, Nickel C, Hellack B, Gartiser S, Flach F, Schiwy A, Maes H, Schäffer A, Erdinger L, Gabsch S, Stintz M (2012): Fate and behaviour of TiO_2 nanomaterials in the environment, influenced by their shape, size and surface area. Federal Environment Agency Germany (Umweltbundesamt) Project No. (FKZ) 3709 65 417

Kühn R, Pattard M (1990): Results of the harmful effects of water pollutants to green algae (Scenedesmus subspicatus) in the cell multiplication inhibition test. Water Research 24, 31-38

Kumari B, Madan VK, Kathpal TS (2008): Status of insecticide contamination of soil and water in Haryana, India. Environmental monitoring and assessment 136, 239-244

Kupper T, Plagellat C, Braendli RC, de Alencastro LF, Grandjean D, Tarradellas J (2006): Fate and removal of polycyclic musks, UV filters and biocides during wastewater treatment. Water Research 40, 2603-2612

Kwon L-W, Armbrust KL, Xia K (2010): Transformation of Triclosan and Triclocarban in Soils and Biosolids-applied Soils. Journal of Environmental Quality 39, 1139-1144

Kwon S, Fan M, Cooper AT, Yang HQ (2008): Photocatalytic applications of micro- and Nano-TiO_2 in environmental engineering. Crit Rev Env Sci Tec 38, 197-226

Lawrence J, Zhu B, Swerhone G, Roy J, Wassenaar L, Topp E, Korber D (2009): Comparative microscale analysis of the effects of triclosan and triclocarban on the structure and function of river biofilm communities. Science of the Total Environment 407, 3307-3316

Lee KJ, Nallathamby PD, Browning LM, Osgood CJ, Xu X-HN (2007): In vivo imaging of transport and biocompatibility of single silver nanoparticles in early development of zebrafish embryos. ACS nano 1, 133-143

Liess M, Schulz R, Liess MHD, Rother B, Kreuzig R (1999): Determination of insecticide contamination in agricultural headwater streams. Water Research 33, 239-247

Liu W, Zhu L-s, Wang J, Xie H, Wang J-h (2009): Acute Toxicological Dosages of Chlorpyrifos, Malathion and Fenvalerate on Earthworm Eisenia foetida. Asian Journal of Ecotoxicology 4, 597-601

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

Luo ZX, Wang ZH, Wei QS, Yan CZ, Liu F (2011): Effects of engineered nano-titanium dioxide on pore surface properties and phosphorus adsorption of sediment: Its environmental implications. Journal of Hazardous Materials 192, 1364-1369

Ma H, Brennan A, Diamond SA (2012a): 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

Ma H, Brennan A, Diamond SA (2012b): Phototoxicity of TiO_2 nanoparticles under solar radiation to two aquatic species: *Daphnia magna* and *Japanese medaka*. Environmental Toxicology and Chemistry 31, 1621-1629

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

Milenkovski S, Baath E, Lindgren P-E, Berglund O (2010): Toxicity of fungicides to natural bacterial communities in wetland water and sediment measured using leucine incorporation and potential denitrification. Ecotoxicology 19, 285-294

Miller GC, Zisook R, Zepp R (1980): Photolysis of 3,4-dichloroaniline in natural waters. J. Agric. Food Chem. 28, 1053-1056

Miller TR, Heidler J, Chillrud SN, DeLaquil A, Ritchie JC, Mihalic JN, Bopp R, Halden RU (2008): Fate of triclosan and evidence for reductive dechlorination of triclocarban in estuarine sediments. Environmental science & technology 42, 4570-4576

Mottier ML, Alvarez LI, Pis MA, Lanusse CE (2003): Transtegumental diffusion of benzimidazole anthelmintics into Moniezia benedeni: correlation with their octanol-water partition coefficients. Experimental parasitology 103, 1-7

Neumegen RA, Fernández-Alba AR, Chisti Y (2005): Toxicities of triclosan, phenol, and copper sulfate in activated sludge. Environmental toxicology 20, 160-164

Nickel C, Hellack B, Nogowski A, Babick F, Stintz M, Maes H, Schäffer A, Kuhlbusch TAJ (2013): Mobility, fate and behavior of TiO_2 nanomaterials in different environmental media. Federal Environment Agency Germany (Umweltbundesamt) Project No. (FKZ) 3710 65 414

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

Pan Z, Lee W, Slutsky L, Clark RAF, Pernodet N, Rafailovich MH (2009): Adverse Effects of Titanium Dioxide Nanoparticles on Human Dermal Fibroblasts and How to Protect Cells. Small 5, 511-520

Pelaez M, Nolan NT, Pillai SC, Seery MK, Falaras P, Kontos AG, Dunlop PSM, Hamilton JWJ, Byrne JA, O'Shea K, Entezari MH, Dionysiou DD (2012): A review on the visible light active titanium dioxide photocatalysts for environmental applications. Applied Catalysis B: Environmental 125, 331-349

Piccinno F, Gottschalk F, Seeger S, Nowack B (2012): Industrial production quantities and uses of ten engineered nanomaterials in Europe and the world. Journal of Nanoparticle Research 14, 1-11

Plagellat C, Kupper T, de Alencastro LF, Grandjean D, Tarradellas J (2004): Biocides in sewage sludge: Quantitative determination in some Swiss wastewater treatment plants. Bulletin of Environmental Contamination and Toxicology 73, 794-801

Robichaud CO, Uyar AE, Darby MR, Zucker LG, Wiesner MR (2009): Estimates of Upper Bounds and Trends in Nano-TiO_2 Production As a Basis for Exposure Assessment. Environmental Science & Technology 43, 4227-4233

Romer I, White TA, Baalousha M, Chipman K, Viant MR, Lead J (2011): Aggregation and dispersion of silver nanoparticles in exposure media for aquatic toxicity tests. Abstr Pap Am Chem S 241

Sabljic A, Guesten H, Verhaar H, Hermens J (1995): QSAR modelling of soil sorption. Improvements and systematics of log K-oc vs. log K-ow correlations. Chemosphere 31, 4489-4514

Sandmann H (2001): Über die ultraviolette Sonnenstrahlung. Der mathematische und naturwissenschaftliche Unterricht (MNU) 54, 260-265

Saxena A, Bartha R (1983): Microbial mineralisation of humic acid-3,4-dichloroaniline complexes. Soil Biol. B. 15, 59-62

Snyder EH, O'Connor GA, McAvoy DC (2011): Toxicity and bioaccumulation of biosolids-borne triclocarban (TCC) in terrestrial organisms. Chemosphere 82, 460-467

Szprengier-Juszkiewicz T, Olejnik M, Jedziniak P, Frischke-Krajewska J (2008): Environmental contaminants—Occurrence of coccidiostats in poultry manure. Toxicology Letters 180S, 203

Thiele-Bruhn S (2005): Microbial inhibition by pharmaceutical antibiotics in different soils - Dose-response relations determined with the iron(III) reduction test. Environmental Toxicology and Chemistry 24, 869-876

Tišler T, Kožuh Eržen N (2006): Abamectin in the aquatic environment. Ecotoxicology 15, 495-502

Tong T, Shereef A, Wu J, Binh CTT, Kelly JJ, Gaillard J-F, Gray KA (2013): Effects of Material Morphology on the Phototoxicity of Nano-TiO_2 to Bacteria. Environmental science & technology 47, 12486-12495

Umweltbundesamt (2012): Frage und Antwort Dokument des Umweltbundesamtes zur neuen Richtlinie OECD 209 "Activated Sludge, Respiration Inhibition Test (Carbon and Ammonium Oxidation)". 1-6

van Tonder EC, de Villiers MM, Handford JS, Malan CEP, du Preez JL (1996): Simple, robust and accurate high-performance liquid chromatography method for the analysis of several anthelmintics in veterinary formulations. Journal of Chromatography A 729, 267-272
Viswanathan R, Scheunert I, Kohli J, Klein W, Korte F (1978): Long-term studies on the fate of 3,4-dichloroaniline-^{14}C in a plant-soil-system under outdoor conditions. J. Environ. Sci. Health 13, 243-259
Wang C-C, Zhang Z, Ying JY (1997): Photocatalytic decomposition of halogenated organics over nanocrystalline titania. Nanostructured Materials 9, 583-586
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 TC, Lu N, Li J, Wu Y (2011): Plasma-TiO_2 Catalytic Method for High-Efficiency Remediation of p-Nitrophenol Contaminated Soil in Pulsed Discharge. Environmental Science & Technology 45, 9301-9307
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
Wegman RCC, De Korte GAL (1981): Aromatic amines in surface waters of the netherlands. Wat. Res. 15, 391-394
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
Wightwick A, Allinson G (2008): Compilation of agrochemicals registered for use in Victoria and their physical-chemical properties. The State of Victoria, Department of Primary Industries, 1-31
Yarden O, Katan J, Aharonson N, Ben-Yephet Y (1985): Delayed and enhanced degradation of benomyl and carbendazim in disinfested and fungicide-treated soils. Phytopathology 75, 763-767
Yasin M, Baugh PJ, Bonwick GA, Davies DH, Hancock P, Leinoudi M (1996): Analytical method development for the determination of synthetic pyrethroid insecticides in soil by gas chromatography-mass spectrometry operated in negative-ion chemical-ionization mode. Journal of Chromatography A 754, 235-243
Ying G-G, Yu X-Y, Kookana RS (2007): Biological degradation of triclocarban and triclosan in a soil under aerobic and anaerobic conditions and comparison with environmental fate modelling. Environmental Pollution 150, 300-305
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

14 Acknowledgments

The study was funded by the Federal Environment Agency Germany within the framework of the UFOPlan 2010 of the Federal Ministry for the Environment, nature conservation and nuclear safety Germany.

Access to nanoparticles characterization (ICP OES, XRD and BET) was provided at the Natural History Museum London within the QualityNano scheme funded by the European Commission under FP7 Capacities Programme Grant Agreement No: 262163

Access to nanoparticle imaging (TEM) was provided by the Ernst Ruska-Centre for Microscopy and Spectroscopy of Electrons of the Forschungszentrum Jülich and RWTH Aachen University. We were kindly trained by Dr. Marc Heggen.

Radiometer measurements were carried out at the Federal Institute for Materials Research and Testing in Berlin together with Anne-Kathrin Barthel.

Annex

Annex I – Results of the literature study – compounds of high interest

Table 1a: Results of the literature study - compounds of high interest

Substance	CAS-Number	Usage	Occurrence/ Medium	Effect		
				Bacteria	Earthworm (LC_{50} [mg/kg dw.])	Earthworm (Literature and other)
Abamectin	71751-41-2	acaricide, nematicide, insecticide	- manure; 0,8 mg/kg dw. (Jensen et al. 2007)	- 1 mg/L no effect on activated sludge (Tišler & Kožuh Eržen 2006)	18	- LC_{50} 18 mg/kg dw. (eisenia andrei, Kolar et al. 2008) - NOEC 10 mg/kg for effects on body weight (Kolar et al. 2008)
Carbendazim	10605-21-7	- fungicide, - biocide used as film-preservative in painting, coatings and roof ceiling reach WWTPs (Waste water treatment plants) by leaching	- WWTP and urban water systems; 6.8 µg/kg dw.; no significant difference between hygienized and non hygienized sludge (Plagellat et al. 2004) -does not bind strongly to sludge (Kupper et al. 2006)	- little effect in the leucine sediment assay with EC_{50} values > 100 mg/L (Milenkovski et al. 2010) -significantly decreased population size and denitrifying activity (Chen et al. 2003)	16	- LC_{50} 6-16 mg/kg (Ellis et al. 2007)
Fenbendazole	43210-67-9	anthelmintic	-potentially in sewage treatment plants (Kim et al. 2009)	- microbial iron reduction in soil no effect up to conc. of 3.3 mg/kg (Thiele-Bruhn 2005)	180	- LC_{50} 180 mg/kg dw. - NOEC 56 mg/kg dw. (in, Hansen et al. 2009)
Fenvalerate	51630-58-1	insecticide, acaricide	- 6,2 µg/L agricultural runoff (Liess et al. 1999) -0.001-0.002 µg/ g soil Inida (Kumari et al. 2008)	- Staphylococcus, Nocardia and Fusarium with fenvalerate were inhibited, others stimulated (Das & Mukherjee 1998)	37,5	37.5 mg/kg d.w. (Liu et al. 2009)
Narasin	55134-13-9	coccidiostat, antibacterial agent (Ionophore)	- manure 0,2-9,6 mg/kg (Szprengier-Juszkiewicz et al. 2008) -soil after manure addition	- NOEC 17 mg/kg (soil respiration, Hansen et al. 2009) -EC_{50} 19.6 µM soil bacteria (Hansen et al. 2009)	46,4	- LC_{50} 46.4 mg/kg dw. (Eisenia andrei, in Hansen et al. 2009)
Triclocarban (TCC)	101-20-2	antimicrobial substance	51 mg/kg dw in hygenized sewage sludge (Heidler et al. 2006)	-	40,0	- 40 mg/kg (Snyder et al. 2011)

Table 1b: Results of the literature study - compounds of high interest

Substance	Analysis	log Kow (2-4)	Photodegradation	Half-life	^{14}C-availability	Characteristics
Abamectin	- ASE, LC-MS MS (Brewer et al. 2004)	4.4 (Wightwick & Allinson 2008)	yes in water; not in soil (2-8 weeks, Jensen et al. 2007)	1-60 d (Wightwick & Allinson 2008) -2 weeks-2 months (Agency 1990)	no (Hartman Analytic)	- chloride channel inhibitor which makes it likely to affect the membrane stability; neutral red retention test good method for tox in E. fetida (korystov in Jensen et al. 2007) -
Carbendazim	- Methanol extraction ; HPLC-DAD (soil, Burrows & Edwards 2004) - worm extraction (Burrows & Edwards 2004)	1.52 (Sabljic et al. 1995)	- photosensitive pesticide	- 20 days (Yarden et al. 1985) -in earthworm test stable over 28 days (Burrows & Edwards 2004) -	no (Hartman Analytic) - yes interisotop - yes Isotops. Co	- metabolite of other benzimidazole pesticides - registered in Germany until 2014 (BVL 2011) -data for avoidance test available (Garcia et al. 2008) - N-Heterocycle (main compound benzimidazole)
Fenbendazole	- HPLC-DAD (van Tonder et al. 1996) - Horizontal shaker (Kreuzig et al. 2007)	3.93 (Mottier et al. 2003)	-< 1 d(Company 1995)	- 5-70 d (Ancare Australia 2008) - < 1 d in water + UV (Company 1995) - 9 days (DT50) clay + manure; 54 d (DT50) clay; sand longer (Kreuzig et al. 2007)	no (Hartman Analytic)	-sorptive removal 20% in sewage treatment plants (Kim et al. 2009) -fenbendazole sulfoxide is formed as main metabolite (Kreuzig et al. 2007)
Fenvalerate	-Hexane DCM extraction, Florisil Clean-up; GC-NICI-MS (Yasin et al. 1996)	5.01 (IUPAC 05.02.2011)	on soil surface 2-18 d	35-77 d (IUPAC 05.02.2011)	no (Hartman Analytic)	-endocrine disrupter (IUPAC 05.02.2011)
Narasin	- no detailed information found -ionophores with ASE, HPLC (see above)	4.85 (pH 8); >6.2 (HPLC) (Elanco Animal Health 2004)	1.5 d Photolysis half life (Elanco Animal Health 2004)	-21-49 d soil (Elanco Animal Health 2004) -8.8 d in soil (as above)	no (Hartman Analytic)	
Triclocarban (TCC)	-Acetone extraction; HPLC-DAD (Ying et al. 2007)	4.9 (Ying et al. 2007)	in water 24 hr (Guerard et al. 2009)	-100 d 53-71 % biodegraded, slower if biosolids were added (Kwon et al. 2010)	yes (Hartman-Analytics)	Heidler et al. 2006 suggests that almost 3/4 of the used TCC is spread to fields through application of sewage sludge to fields.

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