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Investigation of two widely used nanomaterials (TiO_2, Ag) for ecotoxicological long-term effects – adaption of test guidelines

Summary

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1 Introduction

As limited data is available about behavior, persistence and effects of nanomaterials in the environment, a comprehensive risk assessment is not possible to date [1–3]. One approach to reduce gaps of knowledge is the investigation of selected nanomaterials by OECD member states in the framework of the "*Working-Party on Manufactured Nanomaterials*". Germany is a sponsor for nanoparticulate titanium dioxide and co-sponsor for nano silver and is responsible for the creation of the risk assessment for those particles with respect to potential effects on the environment. Existing data are reviewed and areas of limited data are identified for further research. Availability of data from chronic ecotoxicological studies with nano titanium dioxide and nano silver is scarce. For this reason studies on the chronic toxicity of $nTiO_2$ on the sediment-dwelling worm *Lumbriculus variegatus* and the terrestrial mite *Hypoaspis aculeifer* were carried out in the course of the subproject presented in this report. A further subproject investigates the chronic toxicity of nAg on early life stages of the fish *Danio rerio*. To achieve a high comparability of the test results with other studies the investigations were performed on the basis of respective OECD guidelines. In addition investigations on the agglomeration behavior of the particles in the test media, concentrations used and uptake of the particles into the test organisms were carried out.

Test material

The tests described in this report were carried out with nanoparticulate titanium dioxide P25 (NM-105) and nano silver (NM-300 K).

The dispersions used for the application of NM-105 to the terrestrial and aquatic test systems were prepared by weighing the desired amount of test material and adding the respective volume of deionized water. The dispersion was stirred for 60 seconds on a magnetic stirrer (900 rpm) and subsequently treated in an ultra-sonic water bath for three minutes [13].

Measurement of the particle sizes and metal contents

During the exposure periods measurements regarding the particle behaviour (agglomeration) in the test matrices were performed. To check the used particle concentrations in water the total metal concentrations were analytically determined. The partitioning of agglomerate sizes was determined by means of dynamic light scattering (DLS). Moreover, test organisms used in the sediment contact test were investigated for the uptake of NM-105 into the organism.

2 Sediment-contact-test with *Lumbriculus variegatus*

To investigate the effect of NM-105 on the aquatic sediment-dwelling oligochaete *Lumbriculus variegatus* two definite tests according to OECD 225 [4] were carried out. In deviation from the respective guideline [4] NM-105 was applied via the water phase and not via sediment.

The test vessels were filled with sediment and 50% of the volume of the overlying water one day before beginning of exposure. The test organisms were introduced into the test vessels on the same day. The test vessels were incubated overnight under test conditions to allow the worms to bury themselves. On the following day, dispersions of the test substance were prepared and added to the test vessels for exposure to NM-105. After adding the dispersions the media were slightly stirred with a glass rod. The tests were incubated under the conditions described in Table 1.

Table 1: Exposure conditions in the sediment-contact test with *Lumbriculus variegatus.*

Test organism:	*Lumbriculus variegatus* (Müller), adult worms with synchronized reproduction cycle
Number of test organisms per vessel:	10 worms per test vessel; test start 10 d after synchronization
Water body:	For culturing of the worms and in the tests reconstituted water according to [5] was used. In the first definite test with *L. variegatus* besides reconstituted water the medium usually applied in tests at Fraunhofer IME was used. This is purified drinking water incl. filtration with activated charcoal, passage through a limestone column and aeration.
Sediment:	Artificial sediment according to [4]
Test duration:	28 days
Endpoints:	Survival, reproduction and biomass (dry weight) In addition the titanium concentration was determined in the worms from the second definite test.
Biological parameters:	Number of worms, dry weight of the worms in each replicate
Test vessels:	Glass vessels, 250 mL with plastic cap
Sediment per test vessel:	80 g fresh weight
Sediment height per test vessel:	approx. 1,5 cm
Volume of the water body:	180 mL
Aeration of the test vessels:	Continuous aeration during conditioning-, equilibration- and exposure phases; control: daily, on working days
Feeding during exposure:	Food in sediment (stinging nettel- and cellulose powder)
Change of water:	Static test system; regular equalization of evaporated water
Validity	Both definite tests with *L. variegatus* fulfilled all validity parameters mentioned in [4].
Temperature:	20,8 - 21,5°C (n = 54)
Light regime:	16 light : 8 dark

Results from the definite tests with *L. variegatus*

At test start the agglomerate sizes measured in the sediment overlaying medium were between 402 and 1325 nm. Seven days after start of exposure the results from particle size measurements in the test media showed no difference to particle sizes in the controls, indicating complete sedimentation of NM-105.

In the first definite test the investigated nanomaterial NM-105 did not significantly influence the two considered endpoints in the worms up to the highest test concentration of 100 mg/l.

The second definite test should confirm the results obtained from the first definite test. Therefore, a limit test with the highest test concentration (100 mg/L) which had not shown an effect in the preceding test and a control were carried out. Chemical analysis of titanium concentrations at test start corresponds well with nominal concentrations. There was no significant difference between control and treatment (100 mg/L).

The endpoint “weight” was determined as fresh weight. Moreover, all worms from one concentration level were pooled before measurement and then directly analyzed for titanium concentrations. The results of chemical analysis of the worms are as follows: 100 ± 1 µg titanium/g worm (dry weight) in the controls and 112 ± 12 µg titanium/g worm (dry weight) in the treatment (mean values ± standard deviation). There was no significant difference between control and treatment.

3 Reproduction test with *Hypoaspis aculeifer*

The influence of NM-105 on survival and reproduction of the terrestrial predatory mite *Hypoaspis (Geolaelaps) aculeifer* CANESTRINI (Acari: Laelapidae) was investigated for 14 days each in a range finding test and two definite tests according to OECD 226 [6] (Table 2).

Table 2: Exposure conditions in the reproduction tests with *Hypoaspis aculeifer*.

Test organism:	*Hypoaspis aculeifer* (Canestrini), synchronized age
Number of test organisms per vessel:	10 adult, gravide females per test vessel, 28 – 35 days old
Substrate:	Artificial soil according to OECD 226 (2008)
Test duration:	14 days
Endpoints:	Reproduction, survival
Biological parameters:	Number of juvenile mites, number of adult mites in each replicate
Test vessels:	Glass vessels, 200 mL; covered tightly with perforated parafilm
Substrate per test vessel:	20 g (dry weight)
Feeding during exposure:	3, 7 and 10 days after start of exposure with mites of the species *Tyrophagus putrescentiae*
Validity:	In the range finding test and both definite tests with *H. aculeifer* all validity parameters mentioned in the guideline [6] were fulfilled.
pH value:	6,1 – 6,7
Soil humidity:	48,3 – 56,9% der WHCmax
Temperature:	20,2 – 21,3°C
Light regime:	16 : 8 h light-dark-rhythm Light intensity: 541 – 703 lx

The test concentrations 1 and 10 mg/kg were prepared by application of 20 mL and 50 mL dispersion, respectively. At the time of application the artificial soils were premoistened, the final moisture content of the artificial soils was adjusted to 40 – 60% of the maximum water holding capacity by application of the dispersion [8].The test concentrations 100 and 1000 mg/kg were prepared by mixing NM-105 into the substrate as a powder, soil moisture of 40 – 60% of the maximum water holding capacity was adjusted before application of the powder. The test concentration of 10 mg/kg was prepared with both methods of application, allowing a limited evaluation of the effect of the chosen method. Finally, each test vessel was filled with an amount of test substrate corresponding to 20 g dw, and ten adult females taken from a synchronized culture were added.

3.1 Results from the range finding tests with *Hypoaspis aculeifer*

The comparison of the results from exposure towards NM-105 with the control indicates significant effects of the test material on the number of juvenile mites in the highest test concentration (1000 mg/kg). The lowest test concentration (1 mg/kg) had significant effects on the number of juvenile mites and the survival of adult mites. For the mean test concentrations (10 and 100 mg/kg) no significant differences compared to the control were determined for the investigated endpoints.

3.2 Results from the definite tests with *Hypoaspis aculeifer*

In the first definite test no significant differences between control and treatments were determined for any of the considered endpoints.

The second definitive test was performed as a limit test to confirm the findings of the previous, first definitive test. Since the range finding test and the first definite test did not yield the same results, two test concentrations and a control were investigated in this test. A comparison of the results from the exposure towards NM-105 with the control shows significant effects of the test material on the number of juvenile mites in both test concentrations investigated (1000 and 1 mg). Compared to the control an effect of NM-105 on the survival of adult mites was not observed (Figure 1).

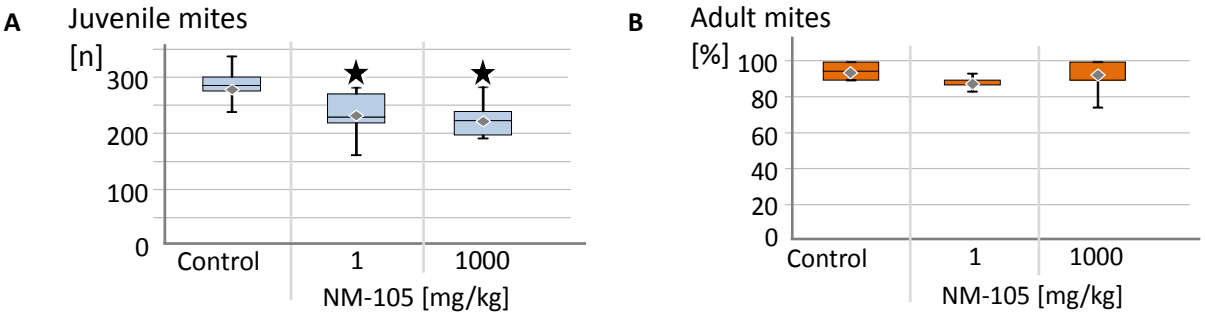

Figure 1: Results from the second definite test with *Hypoaspis aculeifer*.
A: Number of juvenile mites.
B: Survival of adult mites in percentage to the number of mites at test start.
Boxes represent the two mean quartiles, the median lies between the boxes; the bars mark minimal and maximum values; ♦: mean value; n = 8 (control and treatment).
★: Significant effect compared to control (Student t-Test; $p \leq 0.05$

4 Summary of the long-term tests with titanium dioxide

All tests described in this report fulfilled the validity criteria mentioned in the respective guidelines. The physical-chemical parameters measured during the exposure periods are within the recommended ranges. The particle size measurements performed in the single tests with the two organisms show that the application of the nanomaterial to the test system was reproducible. The concentrations measured at start of the sediment test are in good agreement with the nominal concentrations.

In the test with the aquatic sediment-dwelling worm *Lumbriculus variegatus* no significant effects on reproduction and biomass of the worms occurred after an exposure of 28 days. Analysis of titanium concentrations in the test organisms after test end did not show significant differences in worms that had been exposed to 100 mg/L NM-105 compared to the control.

The test with the terrestrial predatory mite *Hypoaspis aculeifer* did not yield clear results, since differing results were elaborated in the tests. In the range finding test, a significant lower number of juvenile mites was found at the lowest and highest investigated test concentration (1 and 1000 mg NM-105/kg). At 1 mg NM-105/kg artificial soil (dw) the survival of adult mites was lower than in the controls and any of the treatments. It was considered that the mites exposed to 1 mg NM-105/kg artificial soil (dw) might have been damaged during transfer into the test vessels and died before onset of reproduction. Since the reasons for the low number of juvenile mites at 1 mg/kg could not be ascertained, the first definitive test was conducted at the same test concentrations. No statistically significant difference between treatments

and control was detected for the endpoints survival and reproduction. The second definitive test was performed to confirm the findings of the previous, first definitive test. However, for the two investigated concentrations of 1 and 1000 mg/kg slight significant effects by NM-105 on the reproduction of the mites were observed. The difference in the number of juvenile mites compared to the control, however, is considerably less pronounced than in the range finding test. Contrary to the range finding test the survival rate of the adult mites was not significantly lower in this test than in the control or the other investigated test concentration.

The results obtained in the aquatic test with *L. variegatus* are comparable to the results from studies with *Chironomus riparius*, which were also carried out in sediment-water systems in the scope of the partner project (FKZ 3709 64 416).

The differences occurring for significant effects in the different test designs with *H. aculeifer* are assumed to be influenced by the test design. In all the three tests reproduction at 1 mg NM-105/kg is lower than in the respective controls. In the two definite tests the differences observed between reproduction in the control and reproduction at 1 mg/kg are quite similar. Use of eight replicates in the second definite test considerably increased the power of the test. As a result the statistical evaluation of the data revealed significant differences for variations that were smaller than the differences obtained in tests with a lower number of replicates. Since the standard design applied for the test fulfills all validity criteria, a NOEC of ≥ 1000 mg NM-105/L has to be assumed.

5 Fish Early Life Stage-Test with *Danio rerio* and nano silver

To determine the potential chronic impact of the test item nano silver (NM-300 K) on the eraly life stages of fish two definite tests were carried out with the zebra fish (*Danio rerio)* according to OECD 210 [9]. As dosing of nanoparticles in a flow through-system can be difficult depending on the dispersing, adsorptive and/or electrostatic properties, we decided to use semi-static exposure conditions in large aquaria (240 L continuously agitated test medium). Pseudo-replicate test cages were placed at the water surface of the aquaria, each containing an individual test group of 20 fertilized eggs. To minimize stress to the early life stages, exchange of test media was scheduled after 7, 14, 21 and 28 days by tranferring the fish to freshly prepared test aquaria. During a 7 days lasting orientation study the stability of the particle size (70-80% of the particles were determined constantly to a size of 50-60 nm during the first 5 days) and the homogeneity of the substance (centre of the water body, larvae cages: mean concentrations 82-114% of the nominal concentration) was confirmed.

Table 3: Test conditions for the Early Life Stage Tests with *Danio rerio a*nd nano silver.

Test organism:	*Danio rerio,* Cyprinidae, teleostei The used strain (origin: former West Aquarium GmbH, 37431 Bad Lauterberg, Germany) has been cultivated under inbreeding conditions at Fraunhofer IME for more than 20 years. It is comparably close to wild type, closely following embryonic and sexual development as described in Hisaoka et al. [10, 11] and Takahashi [12].
Test duration:	35 days
Test vessels:	Glass aquaria with 240 L test dispersion (unaerated)
Water body:	Purified drinking water, purification incl. filtration with activated charcoal, passage through a limestone column and aeration by 4 pumps placed at each corner at the bottom of each aquarium.
Temperature, light regime:	26 ± 1°C; 14 h light : 10 h darkness
Number of test organisms at test start:	20 freshly fertilized eggs per pseudoreplicate (larvae cage)
Change of water:	Every 7 days transfer of larvae cages in freshly prepared aquaria
Endpoints:	Hatch, survival rate, size at test end (length, weight)
Feeding during exposure:	From day 6 on: breeding food (Tetra, AZ 000) twice daily *ad libitum; from day* 16 on: ground TetraMin flake food twice daily *ad libitum; from day* 9 on: addition of brine shrimp nauplii (*Artemia salina)*
Range finding:	Fish embryo test over 48 h: NOEC survival: 200 µg nAg/L; NOEC heart beat frequency: 100 µg nAg/L
Test design:	Orientation test: 1 aquarium with 4 larvae cages; 400 µg nAg/L Definite test 1: 1 aquarium each with 4 larvae cages; 200, 100, 50, 25 and 12.5 µg nAg/L, control and dispergent control Definite test 2: 2 aquaria each with 6 larvae cages; 100, 50 and 12.5 µg nAg/L, control
Analytics of the water body:	Total silver in the centre of the cages before and after change of medium
Validity:	The validity criteria of OECD TG 210 [9] were totally fulfilled only in the second one of both definite tests. From the results of the first definite test, however, valuable information was obtained which confirmed the results of the second test.
Additional test:	Exposure of juvenile animals over 21 days at 25 and 100 µg nAg/L; investigation of uptake and distribution of silver in the tissue fractions 1) head and skin/gills, 2) stomach/intestine, 3) filet. Measurement of total silver and dissolved silver in water.

5.1 Results from the first definite test with *Danio rerio*

Mean concentrations of total silver were measured to 70% ± 2% for all treatments). In correspondence with the fish embryo test performed as range finding test, the NOEC for hatch was 200 µg nAg/L (nominal concentration). At 400 µg nAg/L effects occurred in the orientation test and in the fish embryo test. The lowest test concentration (12.5 µg nAg/L) and the dispersant control were lost due to a limited period of tap water contamination. This also influenced the survival rate (61%) of the larvae in the control, which is below the quality criterion of 70% given by the guideline [7]. The test concentrations from 25 to 200 µg nAg/L all showed 100% hatch, and a concentration-response relationship was observed regarding post-hatch success. In the highest test concentration (200 µg/L), all larvae died after being transferred to fresh test dispersion on day 7. At 100 µg Ag/L, unusual mortality started on day 14 and resulted in a survival rate of 45% at test end. At 50 µg/L, post hatch success was similar compared to the control (effect unclear), while 25 µg nAg/L did not have a negative impact on the survival rate. For growth (measured as

total length and weight) a clear reduction was observed already at 25 µg/L. However, it has to be stated that fish density was considerably higher than in the control.
As the first definitive test results were loaded with uncertainties, in the second definitive test three instead of the originally planned two concentrations were investigated in two true replicate vessels and six pseudo-replicates.

5.2 Results from the second definite test with *Danio rerio*

For all treatments the mean total silver concentrations were measured to 47% ± 6% of the nominal concentrations. During the intervals of media exchange, concentrations clearly decreased. Obviously, in the second test the pump performance had decreased. As in the first definitive test, hatch was complete in nearly all pseudo-replicate cages. Post-hatch success in all pseudo-replicates of the control and of all treatments except the highest test concentration was 75 – 100%. At 100 µg/L, mortality of 35 - 75% occurred in four cages of aquarium 1 after larvae transfer to fresh test dispersion on day 7. The other eight cages exhibited a post-hatch success comparable to the other treatments (70 – 95%). Due to the very high post-hatch success and the low variability in the other treatments, the mean survival rate of 70.3% at the highest concentration was statistically significantly lower. For length and weight a significant reduction was found already at 50 µg nAg/L (NOEC: 12,5 µg/L).

The surviving fish were analyzed for their total silver concentration. Increased total silver concentrations in water resulted in increased concentrations in fish. As for most metals, the relative accumulation decreased with increasing concentration in the surrounding medium.

Statistical evaluation of the endpoints was performed for the two definitive studies using mean measured concentrations (Table 4). The obviously enhanced stress in the first definitive test resulted in worse control performance (below validity criterion), but at the same time in more pronounced effects (length). The overall results are coincident with the second test. The statistical power for all endpoints was sufficient to identify deviations from controls of more than 7% as significant effects.

Table 4: Overview over effects in the Early Life Stage Tests with *Danio rerio* and nano silver. Calculations are based on measured mean concentrations (µg Ag/L).

	LOEC (effect)	NOEC (effect)	EC_{50}	95%-confidence interval	EC_{10}
Hatch					
Orientation test	**400** (80%)	n.p.	n.p.	n.p.	n.p.
1st Definite test	n.p.	**136** (0%)	n.p.	n.p.	n.p.
2nd Definite test	n.p.	> 47 (0%)	n.p.	n.p.	n.p.
Survival rate					
1st Definite test	69 (27%)	34 (0%)	72	67-77	53
2 nd Definite test	**47** (20%)	**23** (0%)	62	56-68	41
Length					
1st Definite test	18 (19%)	n.p.	n.p.	n.p.	n.p.
2 nd Definite test	**23** (8%)	**5,9** (2%)	n.p.	n.p.	n.p.
Weight					
1st Definite test	18 (23%)	n.p.	n.p.	n.p.	n.p.
2 nd Definite test	**23** (26%)	**5,9** (7%)	n.p.	n.p.	n.p.

n.p.: not practicable

5.3 Results from the additional uptake and distribution experiment with *Danio rerio*

To investigate the main uptake route and distribution of nano silver in fish, an additional test was set up in the test systems using bigger juvenile fish exposed each to 25 und 100 µg Ag/L to differentiate total Ag residues in the different tissues. After 21 days exposure, of 15 fish per treatment tissue fractions of three fish each were pooled and investigated for their total silver content (Table 5).

Table 5: Additional uptake test with *Danio rerio: total silver* concentrations in different tissue fractions (µg/kg).

	Control			25 µg nAg/L			100 µg nAg/L		
	Head & skin	**Stomach & gut**	**Inner fish**	**Head & skin**	**Stomach & gut**	**Inner fish**	**Head & skin**	**Stomach & gut**	**Inner fish**
MV	18,7	63,7	11,3	134	5003	116	559	20334	415
SD	4,0	23,2	3,8	28,4	1210	120	71,2	11816	190
CV %	21,6	36,4	33,6	21,2	24,2	104	12,7	58,1	45,8

MW: mean value; SD: standard deviation; CV: variation coefficient

For the tissue portions „head and skin“ and “inner fish” including filet and inner organs, similar total silver concentrations were determined within each treatment. Stomach and guts exhibit approximately four times higher total silver concentrations than the other tissues in control fish, and approximately 45 times higher concentrations compared to other tissues in fish treated with nano silver. Thus, total silver concentrations in fish as measured in the second definitive test are for a small part due to uptake as dissolved silver and a major part taken up as (or associated with) particles via ingestion. The latter fraction mainly remains in the guts without entering the inner fish but may contribute to the slight silver accumulation by providing dissolved silver ions.

5.4 Summary of the long-term tests with nano silver, conclusions

A NOEC of 5.9 µg/L of nano silver (measured total silver concentration) was determined in the valid second test, based on an effect on growth, measured as total lengths. The threshold concentration for acute mortality of sensitive yolk sac larvae, most probably resulting from exposure to dissolved silver directly after renewal of the test dispersions (low complexation by organic carbon from feed and faeces), was determined in the 100 µg/L-treatment, total silver measured to be approximately 60 µg/L. The fish embryo test used as range finding test was able to predict the sensitivity of hatch (NOEC: 100 µg/L), but not that of following life stages.

A comparison with literature data on fish early life stage or juvenile sensitivity to silver nanoparticles (e.g. no significant effects on growth at 6.2 µg/L in Sheepshead minnow [14]) indicates that the elaborated data present the most sensitive endpoints relevant for population dynamics. Therefore, the described setup (static test with moving water, 7d – renewal of test dispersion, zebrafish as test species) seems to be suitable for the testing of nanomaterials.

Measurement of total silver concentrations in the fish showed significantly increasing concentrations with increasing Ag concentration in the test medium. In an additional experiment it was shown that most of the accumulated silver was located in the guts as a result of ingestion.

Pseudo-replicate cages located in one vessel per test concentration only may run into the risk of a bias by uneven conditions (see first definite test). Thus, at least two true replicates (containing pseudo-replicate chambers) are preferred in the static system, which can be statistically compared and combined if they do not differ significantly (see second definite test).

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