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Investigation of widely used nanomaterials (TiO₂, Ag) and gold nanoparticles in standardised ecotoxicological tests

Summary

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Investigation of widely used nanomaterials (TiO₂, Ag) and gold nanoparticles in standardized ecotoxicological tests

Summary

by

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1 Introduction

In November 2007, the OECD's Working Party on Manufactured Nanomaterials (WPMN) launched a Sponsorship Programme involving OECD member countries, as well as non-member economies and stakeholders, to pool available expertise and to fund the safety testing of specific Manufactured Nanomaterials (MNs). In launching the Sponsorship Programme, the WPMN agreed on a priority list of 13 MNs for testing selected from a pool of nanomaterials that are in, or close to, commerce. They also agreed upon a list of endpoints for which the selected materials should be tested. Much valuable information on the safety of MNs can be derived by testing this representative set with respect to human health and environmental safety.

As a sponsor country for titanium oxide research and a co-sponsor for silver research, Germany – among others, is involved in the assessment of TiO₂ and silver with respect to potential effects on human health and the environment. Several months after starting the project the work programme was extended to include the nanomaterial gold. Since ecotoxicological data based on standardised test methods as requested for risk assessments are not available for these substances and information on modifications of standardised test procedures required for the testing of nanomaterials are lacking, the aim of the present project was to contribute to the following topics:

- Recommendations for the improvement of existing OECD Test Guidelines on the testing of nanomaterials
- Recommendations for the application of the investigated nanoparticles to the test medium
- Ecotoxicity of titanium oxide and silver with respect to
 - Earthworm reproduction
 - Respiration rate of soil microflora
 - Nitrification of soil microflora
 - Growth of plants
 - Reproduction of chironomids
 - Reproduction of daphnids
- Ecotoxicity of gold with respect to
 - Growth of algae
 - Immobilisation of daphnids
 - Development of fish embryos
 - Reproduction of chironomids

As a first step in the present project the German Federal Environment Agency selected several nanoparticles from the priority list of the OECD Sponsorship Programme, and the tests that should be performed with these nanoparticles were selected on the basis of available information and priority. The outcome is presented in following table (Table 1).

Table 1: Nanoparticles and test guidelines to be studied in the project

	Titanium dioxide					Silver	Gold
	Name of the product / code ¹ / producer /						
OECD Test Guideline	Aeroxid® P25 ² : Evonik	PC105 (NM-102): Crystal Global	Hombikat UV 100 (NM-101): Sachtleben	UV TITAN M212 (NM-104): Sachtleben	UV TITAN M262 (NM-103): Sachtleben	Ag Pure W10 (NM-300K)	Gold (NM 330): South Africa - MINTEK
201 (algae – growth)							x
202 (daphnids - immobilisation)							x
211 (daphnids - reproduction)	x						
219 (chironomids - emergence)	x		x			x	x
Draft – fish embryo test							x
222 (earthworms - reproduction)	x		x		x	x	
208 (plants - emergence, growth)	x						
216/217 (soil microflora – N-/C-transformation)	x						

¹ Terms in brackets: code of the materials according to the OECD Sponsorship Programme; ² P25 was distributed by Evonik; the OECD batch NM-105 is also the product AEROXIDE® TiO₂ P25, but stems from a different batch

2 Pre-tests

One essential step in ecotoxicity testing is the application of the test substance as bioavailability and subsequent toxicity can be influenced by the applied method. So far, documents referring specifically to the application of nanomaterials are unavailable.

Therefore, the application of the nanomaterials was studied in pre-tests with emphasis on terrestrial tests. For tests with daphnids and chironomids the method of Hund-Rinke et al. (2010 ¹) was used; supplementing studies on filtration and the use of stabilisers.. Moreover, the sorption of P25 to algae was investigated to obtain information on the feeding frequency of the daphnids in the reproduction test.

Terrestrial tests

Application forms that might be suitable for terrestrial tests and the homogeneity of spiking were investigated in tests carried out with earthworms and the soil microflora. Several forms of spiking were investigated:

^{1 1} Hund-Rinke, K., Schlich, K., Wenzel, A.: TiO₂ nanoparticles - Relationship between dispersion preparation method and ecotoxicity in the algal growth test. Umweltwiss. Schadst. Forsch. 22 (2010) 5 S. 517-528.

- Application as dispersion
- Application as solid (powder)
- Application in soil
- Application in food

On the basis of the obtained results the decision was made to apply the following procedure in the main tests:

- TiO₂ (available as insoluble, dry powder): application via suspension and via solid carrier (soil) in soil as well as via suspension and directly in the form of powder in dung. Due to the high amount of nanomaterial added to dung, no carrier was considered to be necessary.
- Ag - NM-300K (available as dispersion): application via solid carrier (soil) in soil and directly in dung.

For Au no terrestrial tests were performed.

3 Main tests

A short overview of the toxicity is shown in Table 2.

The **TiO₂ nanomaterials** were available as dry powder. The results are presented as mg TiO₂/kg or mg TiO₂/L. **Silver** was available as dispersion. Further to the total concentration of silver the concentrations of Ag ions in soil and sediment were determined via DGT (diffusive gradient in thin films). The results are presented as nominal concentrations and as Ag⁺ concentration. The **gold** concentration measured in NM-330 was lower than the value reported by the producer (expected: 0.01% corresponding to 100 mg/L; measured 43.8 mg/L). The NIST reference material 8011 (gold nanoparticles, nominal diameter 10 nm) was analysed along with the samples of the test; recovery amounted to about 100%. The recovery of the applied standard Au solution was about 100% as well. As details on the analytical method used by the producer of NM-330 are not known, the discrepancy between the results cannot be explained. Due to the discrepancy between measured and communicated values, the concentrations of the ecotoxicological analyses are presented as % NM-330 (v/v) in the test suspension.

Table 2: Summary of the effect values of the applied nanomaterials

Test	Guideline	Nanomaterial	Result
TiO₂			
Earthworm	OECD 222	P25, NM-101	Concentration-dependent stimulation in winter in tests performed with natural soil
		NM-103	Reproduction: no difference to the control up to the highest test concentration (NOEC ≥ 400 mg/kg)
Microflora – N-transformation	OECD 216	P25	Increased nitrogen transformation rate upon application via powder Application via powder: NOEC 9.3 mg/kg Application via dispersion: NOEC ≥ 21 mg/kg (highest test concentration)
Microflora – carbon transformation	OECD 217	P25	No effect up to the highest test concentration Application via powder: NOEC ≥ 100 mg/kg Application via dispersion: NOEC ≥ 21 mg/kg (highest test concentration)
Plant (bean, mustard, oat)	OECD 208	P25	<u>Germination, root length</u> : no effect on the three plants up to the highest test concentration (dry application 100 mg/kg, wet application 20 mg/kg) <u>Fresh weight</u> : no effect on mustard NOEC: oat: 67.0 mg/kg; bean: 44.0 mg/kg
Daphnids	OECD 211	P25	Change of the medium: 3 times per week or daily; sonication 3 min or 30 min: no effect up to the highest test concentration (5 mg/L)
Chironomids	OECD 219	P25, NM-101	No effect up to the highest test concentration (spiked water: 100 mg/L)
NM-300K			
Earthworm	OECD 222	NM-300K	Reproduction: NOEC < 15 mg/kg (lowest nominal concentration) 65.5 * 10 ⁻³ µg/kg (concentration of Ag ions)
Chironomids	OECD 219	NM-300K	Development: NOEC 0.625 mg/L (nominal concentration) 4 µg/L (concentration of Ag ions measured by DGT) Emergence: NOEC 1.250 mg/L (nominal concentration) 19 µg/L (concentration of Ag ions measured by DGT)
NM-330 / NM-330DIS			
Chironomids	OECD 219	NM-330	No inhibitory effect up to the highest test concentration Emergence, development (combined sexes): NOEC ≥ 50%
		NM-330DIS	Emergence rate (combined sexes): NOEC 10% Development time (combined sexes): NOEC 10%
Daphnids	OECD 202	NM-330	NOEC ≥ 50% (highest test concentration)
		NM-330DIS	NOEC 2.5%

continued

Table 2 continued.

Test	Guideline	Nanomaterial	Result
NM-330 / NM-330DIS			
Algae	OECD 201	NM-330	Biomass, growth: NOEC 0.63%;
		NM-330DIS	Biomass, growth NOEC < 0.63% (lowest test concentration)
Fish embryo test	OECD Draft	NM-330	Development of embryos: LC ₁₀ > 50% (highest test concentration) Hatch: EC ₁₀ > 50% (highest test concentration)
		NM-330DIS	Development of embryos: LC ₁₀ 48% (48h); LC ₅₀ 10% (48h) Hatch: EC ₁₀ 1.2% (72 h); EC ₅₀ 10% (72 h); 96 h: EC values not calculable – 10% comparable to control; 50% dispersion 100% effect

Where relevant, specific information on individual tests is presented in the following. The results listed in the table were not mentioned again in this short report. Therefore, some tests don't appear in the following.

Test with earthworms – TiO₂

The control showed a reduced number of offspring in winter compared to experiments carried out in summer. This reduction in the number of juveniles was not observed for the uncoated nanomaterials P25 and NM-101, indicating a stimulation for P25 and NM-101 when the tests were performed in natural soil and in winter time. There are indications that the stimulation observed for P25 and NM-101 (uncoated nanomaterials) is due to a disturbance of the biological clock.

For the coated TiO₂ NM-103 no difference to the control was observed.

Test with earthworms – Ag

In addition to the number of offspring, the Ag concentration in the earthworms was determined. In the control worms and in the worms treated with the dispersant (the amount corresponded to the highest test concentration of silver) no silver was determined. In contrast, silver was detected in all worms incubated in soil with NM-300K (nanosilver). There was no obvious difference between experiments where the test substance was added via spiking of soil or spiking of food. A dependence on the concentration was not observed. It is therefore assumed that a steady state of silver uptake is already achieved at the lowest test concentration. Concentration-dependent effects are observed above the lowest test concentration. Although the applied test concentrations increased, the silver concentrations in the worms were the same. We assume that the fertility of adults is not affected but that the development of cocoons and the survival of juveniles in soil are sensitive life stages. We do not know yet whether the cocoons or the juvenile worms are the more susceptible life stage.

In none of the test designs the silver concentration in the worms exceeded the concentration in the test vessels. Therefore it is concluded that silver did not accumulate in the tissue of the worms. It is unclear whether the measured silver was located in the tissues or whether resi-

dues remained in the gut due to incomplete purging. We also do not know whether the determined silver occurs in its particle or in its ionic form.

Nitrogen transformation – TiO₂

The application via dispersion gave no clear information on its suitability for the application of nanomaterials. The difference in microbial activity of the spiked samples to the control was small and not statistically significant. It is assumed that an application via dispersion does not cause an increased bioavailability of TiO₂ nanoparticles for the soil microflora.

Test with plants – TiO₂

In addition to the endpoints mentioned in the test guideline (i.e. germination, biomass), the root length was determined. No statistically significant differences were observed for germination and root length. No phyto-pathological symptoms were observed up to a concentration of 100 mg/kg (application via powder) and 20 mg/kg (application via dispersion). The most sensitive endpoint was shoot fresh weight but only small effects were observed for *Avena sativa* (oat) and *Phaseolus aureus* (mung bean). Application via dispersion resulted in concentration-effect relationships that were highest for the low test concentration (10 mg/kg). It is assumed that the bioavailability of the nanoparticles decreased due to a higher agglomeration in the higher concentrated stock dispersion used for the high test concentration (20 mg/kg).

Test with chironomids – spiked water – TiO₂, Ag, Au

There was strong sedimentation of the tested nanomaterials resulting in concentrations below the detection limit in the overlaying water. At test end nearly all of the applied nanomaterials was determined in the sediment.

According to the guideline the test required feeding of the larvae at least three times per week. Two feeding regimes were tested as the sorption of the nanomaterials was unknown—feeding three times weekly and adding 0.5% dry weight of finely ground leaves to the sediment before the stabilisation period. Mixing the total amount of food into the sediment at test start to avoid a sorption of the nanomaterial to the food applied to the water phase instead of a periodical feeding was not considered a suitable method, as this caused reduced emergence even in the control.

Test with chironomids – Au

The addition of NM-330 and NM-330DIS caused coloured test suspensions. Changing colours during the incubation indicates a modification of the added NM-330 and NM-330DIS. Sedimentation of Au was demonstrated by chemical analysis. At day 28 of the incubation period 0.5% of the Au was detected in the water phase at the highest test concentration (50% test item). The Au concentrations determined at the lower test concentrations were below the detection limit.

The dispersant caused a concentration-dependent decrease of the oxygen concentration in the water phase. After three weeks the threshold concentration of 60% oxygen saturation was not achieved in the vessels with the highest concentration of the dispersant, not even

upon increased aeration. In the vessels with the highest concentration of the dispersant a very high microbial number was determined.

The oxygen supply was controlled qualitatively on a daily basis during the working week. The aeration was comparable for all vessels. A technical defect as the reason for the low oxygen concentration is unlikely. Therefore it is assumed that (i) microbial degradation of the dead larvae resulted in a decrease of the oxygen concentration and (ii) that the comparably low oxygen concentration is not the reason for the missing emergence.

Acute test with daphnids – Au

The dispersant caused a reduction of the pH and of the oxygen concentration. The pH was still in the accepted range of 6 – 9. All concentrations of the dispersant caused a reduction of the oxygen concentration below the threshold value of 3 mg/L. It is assumed that the low oxygen concentration does not affect toxicity, as the oxygen concentrations at the lowest and highest test concentrations were the same, although 0 (lowest test concentration) and 100% (highest test concentration) effect were achieved.

Test with algae – Au

As minor amounts of the test substance (NM-330) were available, the test was performed in multi-well plates (96-well plates and 24-well plates).

For the tests with NM-330 only the period of the test from 24 - 72 h was evaluated. Tests with NM-330DIS were assessed as described in the guideline (incubation period: 0 – 72) despite an evaluation of the 24 - 72 h that resulted in effect and threshold values comparable to those obtained for the complete test period. As an evaluation using the reduced and the normal incubation period gave comparable results for NM-330DIS, the results of both tests were compared despite the different incubation periods.

Tests using 96 well plates with a test volume of 200 µL per well can be used, if the available amount of test substance is strongly limited. However, using a small amount of test item resulted in fulfillment of only two of the three validity criteria mentioned in the guideline. The criteria “Mean of the replicate coefficients of variation in the section-by-section growth rate” was unfulfilled. A test volume of 2 mL (24-well plates) improved the validity of the tests. However, the use of 24 well plates decreases the number of variants which can be investigated in parallel.

Observed effect values were comparable for both test volumina.

Toxicity of dispersants .- Ag, Au

Silver and gold nanoparticles (NM-300K and NM-330) were available as dispersions. For both nanomaterials the dispersant was tested (NM-300KDIS; NM-330DIS). While the dispersant of silver (NM-300KDIS) revealed no toxicity, the dispersant of gold (NM-330DIS) was highly toxic. Toxicity was reduced in the presence of gold nanoparticles. It is assumed that active binding sites of the gold dispersant reacting with the organisms or with the nutrients are occupied by gold nanoparticles resulting in a reduced ecotoxicity. Experiments carried out to concentrate the gold nanoparticles in order to reduce the dispersant concentration in the test vessels failed.

4 Recommendation for the test performance

4.1 Suitability of test guidelines

Our experiments showed that the following test guidelines

- OECD Test Guideline No. 222 (earthworm reproduction test)
- OECD Test Guideline No. 216 (soil microflora, nitrogen transformation test)
- OECD Test Guideline No. 217 (soil microflora, carbon transformation test)
- OECD Test Guideline No. 208 (plant test)
- OECD Test Guideline No. 219 (chironomid test with spiked water)
- OECD Test Guideline No. 211 (daphnia reproduction test)
- OECD Test Guideline No. 202 (daphnia immobilisation test)
- OECD Test Guideline No. 201 (algae growth test)
- OECD Draft – fish embryo test

can be used to test nanomaterials in powder form or dispersed nanomaterials. Modifications to the test performance do not seem to be necessary. However, recommendations for the application of the nanomaterials are necessary.

4.2 Application of nanomaterials to solid test media (soil)

The preparation of test materials in powder form using 1% dry soil as a carrier is a suitable method for the application of the investigated nanomaterials in solid test media. The application via dispersion using water as a dispersant seems to be less suitable. This recommendation is justified as follows: in most tests showing a concentration-effect curve after spiking the soil with the powder, concentration-dependent effects were not observed after the application of a dispersion. Examples are:

- Plant test with *Avena sativa* – growth
- Soil microflora – nitrogen / carbon transformation test
- Earthworm reproduction test

4.3 Spiking of soil versus spiking of feed

Although the same effects were observed for nanomaterials added directly to the soil and nanomaterials applied via feed, direct application to the soil is preferred as this method is described in an OECD guideline (earthworm reproduction test).

4.4 Application of insoluble nanomaterials in powder form to aquatic test media

The method described by Hund-Rinke *et al.* (2010) is suitable for the application of insoluble nanomaterials in powder form to aquatic test media:

- Weighing of the required amounts in glass vessels
- Addition of test medium

- Stirring of the mixture (1 min; magnetic stirrer; 900 rpm)
- Treatment with ultrasound (3 min, 500 W) in a bath sonicator (Bandelin Sonorex RK 514 BH; 35 kHz; 215/860 W).

Filtration (mixed cellulose ester, polycarbonate membrane filters; pore size 0.2 mm; filter of disposal type, filtration using vacuum) and the use of a synthetic stabiliser (sodium hexametaphosphate, 0.01%) are not recommended.

For nanomaterials stabilised in an aqueous medium (silver NM-300K; gold NM-330) a homogenous distribution in suitable stock dispersions can be achieved by stirring.

4.5 Sensitivity of the applied test systems

For TiO₂ we observed effects in terrestrial test systems (earthworms, soil microflora) whereas effects on aquatic test systems (daphnids, chironomids) were not observed. On the basis of these results we recommend the application of both aquatic and terrestrial tests within a comprehensive hazard / risk assessment. This is contrary to the approach described in the scope of REACH where terrestrial tests are required only for substances with high production volumes. However, it must be considered that a limited number of aquatic test organisms were investigated within this project. Further tests carried out with fish and algae may lead to modified conclusions, though published results do not indicate a high toxicity of the tested TiO₂ nanomaterials for these organisms.

4.6 Toxicity of dispersants

Silver and gold nanomaterials were available as dispersions and the whole product was tested. As a consequence, no information was obtained on the toxicity of the nanoparticles. For regulatory purposes it is recommended that the producers are obliged to additionally provide their nanomaterials without dispersant. It is expected that nanoparticles and dispersants separate in the environment, and information on fate and effect of the pure particles is needed.

4.7 Total concentration vs. ion concentration

For metals forming ions, such as silver, the results can be calculated on the basis of the total concentration or the ion concentration. The effect values differ significantly. In our study (here: chironomids: OECD 219; earthworms: OECD 222), for example, the effect values differ by a factor of 10³ – 10⁴ depending on whether the total concentration or the Ag⁺ concentration determined with DGTs in the sediment and soil resp. is used for the calculation of the endpoints (NOEC, ECx). This illustrates that the basis of the calculation has to be clearly fixed for regulatory purposes.