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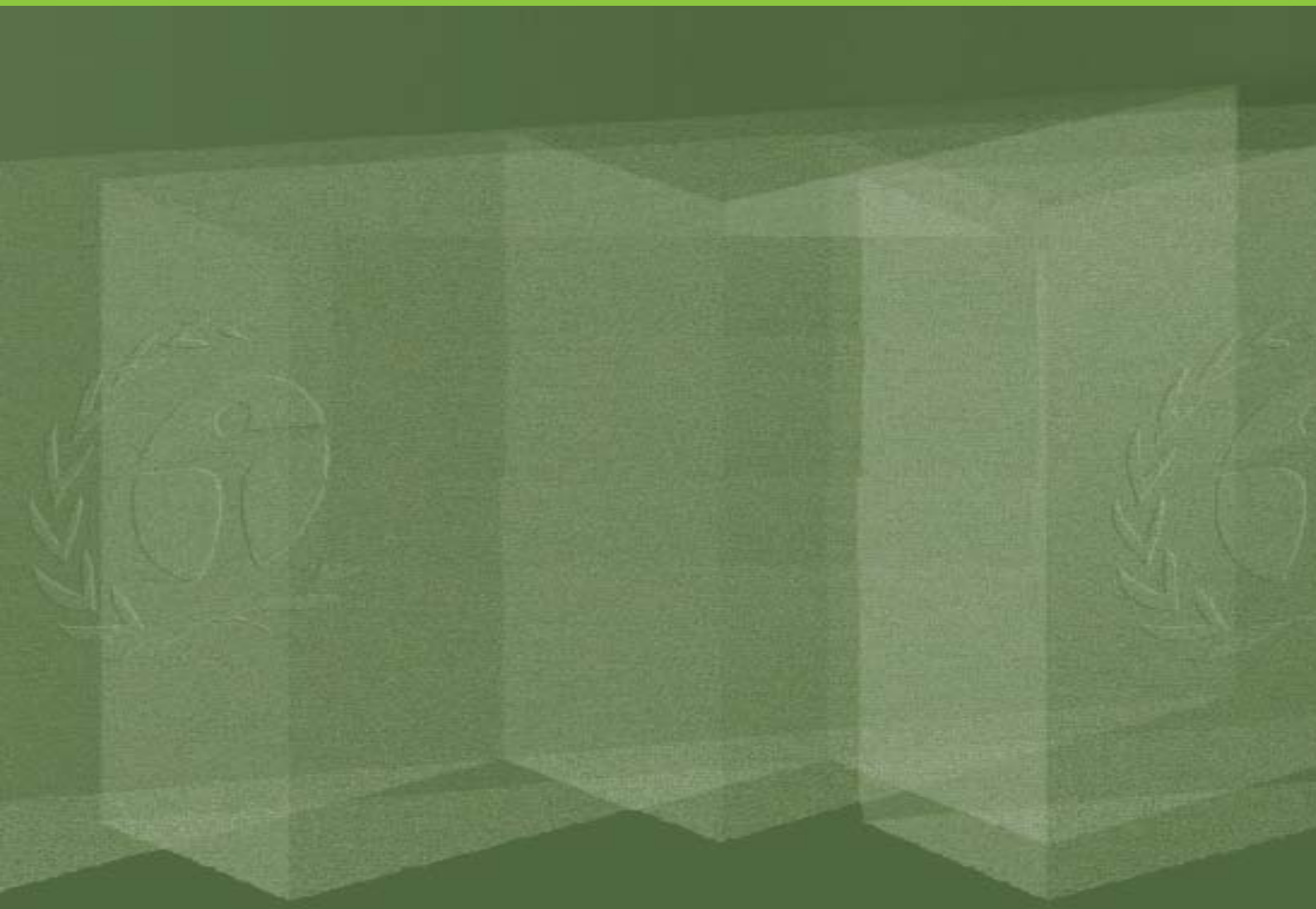

Investigation of widely used nanomaterials (TiO_2, Ag) and gold nanoparticles in standardised ecotoxicological tests

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Für Mensch und Umwelt

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Investigation of widely used nanomaterials (TiO_2, Ag) and gold nanoparticles in standardized ecotoxicological tests

by

Dr. Kerstin Hund-Rinke
Dr. Thorsten Klawonn

Fraunhofer Institute for Molecular Biology and Applied Ecology IME,
Schmallenberg, Germany

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Wörlitzer Platz 1
06844 Dessau-Roßlau
Germany
Phone: +49-340-2103-0
Fax: +49-340-2103 2285
Email: info@umweltbundesamt.de
Internet: http://www.umweltbundesamt.de
http://fuer-mensch-und-umwelt.de/

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

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Index of Abbreviations

CV:	coefficient of variation
DGT:	Diffusive gradients in thin films
dm:	Dry matter
ECx:	effect concentration (x %)
FW:	Fresh weight
GLP:	Good laboratory practice
LOEC:	No observed effect concentration
LOD:	Limit of detection
LOQ:	Limit of quantification
lux:	Lux
n.d.	not determined due to mathematical reasons or inappropriate data
NOEC:	No observed effect concentration
SD:	Standard deviation
TG:	Test guideline
WHC_{max}:	Maximum water holding capacity

1 Preliminary remark

Primarily, the research project aimed to extend the property data available for titanium dioxide (TiO_2) and silver (Ag). In a second approach, gold (Au) nanoparticles were investigated.

The results are presented separately for each type of nanoparticle.

Basic procedures were investigated in pre-tests. For these studies titanium dioxide and silver nanoparticles were applied. Due to the different modes of action and differing ecotoxicity, not every procedure was investigated with both types of materials. For a comprehensive conclusion results obtained for both types of materials are necessary. Therefore, all results of the pre-tests are presented together.

The responsibilities were as follows:

- Kerstin Hund-Rinke: ecotoxicological tests
- Thorsten Klawonn: chemical analyses

2 Introduction

At the nanoscale level, the physical, chemical, and biological properties of materials differ in fundamental, and often valuable, ways from the properties of individual atoms and molecules, or bulk matter. Research and development in nanotechnology is directed towards creating improved materials, devices, and systems that exploit the new properties. The specific properties of nanoparticles proved to be very useful for an increasing number of commercial applications, such as protective coatings, light-weight materials or self-cleaning clothing, for example.

As a consequence of their specific properties, nanoparticles differ from conventional chemicals with respect to their impact on human health and the environment. Therefore, traditional testing and assessment methods typically used to determine the safety of conventional chemicals are not necessarily (fully) applicable to nanoparticles.

In November 2007, OECD's Working Party on Manufactured Nanomaterials (WPMN) launched a Sponsorship Programme involving OECD member countries as well as non-member economies and other 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 selected for testing from a pool of nanomaterials that are in, or close to, commerce. The WPMN 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 of nanomaterials with respect to human health and environmental safety.

As a sponsor country supporting research into TiO_2 and a co-sponsor for Ag research, Germany, among others, is involved in assessing the potential effects of TiO_2 and Ag nanoparticles with respect to human health and the environment. Several months after starting the present project, the work programme was extended to include the nanomaterial gold. Since ecotoxicological data based on standardised test methods, as requested for risk assessment, are not available for these substances, and information on modifications to standardised procedures for testing nanoparticles is lacking, the aim of the present project was to contribute to the following topics:

- Recommendations concerning the improvement of existing OECD Test Guidelines for the testing of nanoparticles
- Recommendations on the application of the investigated nanoparticles to the test medium
- Ecotoxicity of TiO_2 and Ag nanoparticles 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

- o Immobilisation of daphnids
- o Development of fish embryos
- o 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 (Table 1).

Table 1: Nanoparticles and test guidelines selected for investigation within 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® TiO_2 P25, but stems from a different batch

Table 2 and Table 3 show the characteristics of the applied TiO_2 and Ag-nanoparticles. Gold nanoparticles were available as suspension. No further information was available.

Table 2: Properties of the applied TiO_2 nanoparticles.

Data from the Joint Research Centre, European Commission

Nanoparticles	NM-101	NM-103	NM-105 [1]
Crystal structure	Anatase	Rutile	Rutile - Anatase
Purpose	active component for photo catalytic reactions	UV screening agent in sunscreen	active component for photo catalytic reactions
Primary particle size (according to Scherrer)	8 nm	20 nm	21 nm
Composition	TiO_2: 91.7%	TiO_2: 89.0% Al2O3: 6.2%	TiO_2: > 99%
BET	> 250 m²/g	60 m²/g	60 m²/g
Coating	none	hydrophobic	none
Condition	solid, powder	solid, powder	solid, powder

[1] Data elaborated for NM-105 and not for the batch distributed by Evonik and used in this study

Table 3: Properties of the applied Ag nanomaterial.

Data from the Joint Research Centre, European Commission

Nanoparticles	NM-300K	NM-300KDIS
Condition	in dispersion	dispersion
Primary particle size (according to Scherrer)	15 nm	---

3 Structure of the report

One essential step in ecotoxicity testing is the application of the test substance into the test systems, as bioavailability and consequently toxicity can be influenced by the method of application. As recommendations for the application of nanoparticles were not available preliminary studies were performed. These experiments and conclusions on the performance of the main tests are presented in chapters 5 (terrestrial tests) and 6 (aquatic tests). In the following chapters the results obtained with the tests are presented. For the individual tests the structure of the IUCLID database is applied. The results are sorted with respect to the different test organisms, i.e. for every test organism the results obtained with the applied test substances are presented. This structure allows an easy transfer of the results into databases such as NanoHub, which is the adapted form of the IUCLID data base for nanomaterials. Repetitions, such as the description of the applied test procedure, are limited.

Some peculiarities of nanoparticle testing become obvious only when the total of the results obtained for all nanoparticles or test systems were considered. Such an approach is not considered in the IUCLID structure. Where a discussion of specific observations was necessary for a correct interpretation of the results, a subchapter "special considerations" was included in the respective results chapter (e.g. earthworm reproduction data for TiO_2 nanoparticles). A separate discussion chapter, which follows the presentation of all main tests, contains all discussions and conclusions of common relevance (e.g. proposal of spiking technique).

An example of the structure of the main tests is provided below.

Section: Main tests with the selected nanoparticles

Test organism 1 (example: earthworms)

- Test principle
- Materials and methods
  - Test guideline
  - GLP
  - Test material
    - Nanoparticle 1
    - Nanoparticle 2 - x (if more than one material is tested)
  - Analytical monitoring
  - Test item – Preparation protocol
  - Test species
- Study design
  - Study type
  - Test duration type and exposure period
  - Test substrate
  - Total exposure period
  - Post exposure period
- Test conditions
  - Environmental conditions
  - Test concentrations
    - Nanoparticle 1
    - Nanoparticle 2 - x (if more than one material is tested)
- Any other information on materials and methods
- Results
  - Nanoparticle 1
  - Nanoparticle 2 -x (if more than one material is tested)
  - Special considerations (presentation and discussion of special observations)
- Validity of the results
  - Nanoparticle 1
  - Nanoparticle 2 – x (if more than one material is tested)
- Data for the reference substance
- Conclusion
  - Nanoparticle 1
  - Nanoparticle 2 -x (if more than one material is tested)
- Executive summary
  - Nanoparticle 1
  - Nanoparticle 2 - x (if more than one material is tested)

Test organism 2

…

4 Methods for chemical analyses

For raw data examples, see chapters 21.1.2 (total Ag), 21.1.3 (Ag^+), 21.1.4 (Au)

For certificates of reference material and standards, see chapters 21.1.5 (Ti), 21.1.6 (Ag), 21.1.7 (Au)

4.1 Digestion of Titanium in aqueous samples and soils/sediment

4.1.1 Procedure

Soil/sediment samples

Approx. 200 mg of dried soil/sediment was weighed into a quartz digestion vessel and 5 mL of concentrated sulphuric acid was added. The subsequent digestion was performed by an Ultra Clave II Microwave (MLS GmbH, Leutkirch im Allgäu, Germany) using the following conditions:

Step 1: ramp 60 min to 250 °C

Step 2: 250 °C for 30 min

After cooling, the resulting solution was slowly and carefully brought to a volume of 20 mL with ultrapure water. For safety reasons the volumetric flask was put into an ice bath before adding water.

Aqueous samples

Prior to digestion, the aqueous sample containing TiO_2 was vigorously shaken (e. g. by a vortexer) for at least 1 min. Directly afterwards 4 mL were taken and 1 mL of a mixture of hydrochloric-, nitric-, and hydrofluoric acid in a ratio of 3:1:1 were carefully added. Of course, safety precautions for handling of chemicals and the risks of hydrofluoric acid were regarded.

The resulting sample was again placed on a vortex for at least 1 min followed by digestion in a standard laboratory ultrasonic bath (room temperature) for 30 minutes. Prior to the analytical measurement by ICP-OES the fluoride anions were complexed by addition of aqueous boronic acid.

4.1.2 Analytical measurement

All materials used for sample treatment were suitable for working with titanium at trace levels. The applied glassware (beakers and volumetric flasks) was cleaned with a Miele washer “Automatic Disinfector” combined with a water de-ioniser “Aquapurificator”, steamed with HNO_3 and rinsed three times with ultrapure water. The glassware was dried at approximately 60 °C. Additionally, digestions with quartz vessels filled only with concentrated nitric acid were performed in order to have thoroughly cleaned vessels available for the digestion of soil/sediment samples.

The pipettes used in variable volumes (50 – 250 µL, 200 – 1000 µL, 1000 – 5000 µL) were purchased from Gilson (Abimed, Langenfeld, Germany) and Eppendorf (Wesseling, Germany).

The water used for the analytical investigation was purified with a Pure Lab Ultra water purification system from ELGA LabWater, Celle, Germany. The purified water has a resistivity greater than 18 MΩ·cm. The applied acids were:

Nitric acid - "Supra" quality (ROTIPURAN® supplied by Roth, Karlsruhe, Germany)
Hydrochloric acid – "Instra-Analyzed" (supplied by Mallinckrodt Baker, Griesheim, Germany)
Hydrofluoric acid – "Suprapur" quality (supplied by Merck, Darmstadt, Germany)
Sulphuric acid – "Supra" quality (ROTIPURAN® supplied by Roth, Karlsruhe, Germany).

For ICP-OES measurements commercially available titanium standards containing 1000 mg/L Ti in ammonium hexafluorotitanate in water (CertiPUR, Merck, Darmstadt, Germany) were applied. With this standard solution appropriate stock solutions and subsequently calibration solutions were prepared.

(Certified) Reference materials (chapter 21.1.5) and verifying the method

To further determine the accuracy of the applied analytical method recalibration samples containing concentrations in the range of actual samples were also analysed.

Unfortunately, soil and/or sediment with certified values for Ti were not available. To verify the digestion as well as the analytical method the certified reference material BCR 142R with a not certified reference value for TiO_2 (4.5 g/kg) was digested and analysed along with the soil/sediment samples.

To further verify the used methods ultrapure water as well as sediment/soil was spiked with an exactly weighted amount of the nanoparticles. These mixtures also underwent the digestion procedures as well as the analytical measurements, and the recoveries were determined.

Soil/sediment samples

The supernatant was analysed by ICP-OES (Iris Intrepid II, Thermo Scientific, Dreieich, Germany) with a calibration adjusted to the sulphuric acid matrix. A raw data example is presented in chapter 21.1.1.

Aqueous samples

The ICP-OES calibration (Iris Intrepid II, Thermo Scientific, Dreieich, Germany) was adjusted to the HCl, HNO3, and complexed F- matrix. A raw data example is presented in chapter 21.1.1.

Quantification of nanoparticles added to the systems during the tests

To quantify the amount of added TiO_2-nanoparticles within the soils, sediments and aqueous test systems, additional control systems (same procedure, not spiked with nanoparticles) were analysed for their environmental titanium background. This background can then be straightforwardly subtracted from the measured Ti concentrations in test item-loaded samples. However, this approach will only provide reliable results if the added amount of nano-titanium dioxide clearly exceeds 25% of the previously determined natural background.

4.2 Digestion and quantification of TiO_2 in earthworms

4.2.1 Procedure

Cryogenic homogenisation

To ensure a complete digestion of *Eisenia fetida*, it was necessary to perform cryogenic homogenisation. To avoid contamination, homogenisation was performed under a laminar-flow hood. All materials used as well as the worms were cooled in liquid nitrogen. The samples were homogenised using a pestle and mortar. First a larger pestle was used for a coarser grinding followed by a smaller one. The resulting powder was transferred into cryo-proofed vials and stored directly above liquid nitrogen at approx. -150°C until lyophilisation.

Lyophilisation

The frozen samples were transferred into a Christ Alpha 1-2 freeze dryer (Martin Christ GmbH, Osterode am Harz, Germany) for lyophilisation. Freeze-drying was performed until samples reached constant weights.

Microwave digestion

Approx. 200 mg of homogenised and dried substance from each sample was weighed into a Teflon digestion vessel, and 5 mL of concentrated nitric acid (69%) was added followed by the microwave digestion.

The program was: heat for 60 min. to maximum temperature of 250°C, hold at 250°C for 30 min, initial pressure 40 bar.

After digestion 0.5 mL of hydrofluoric acid (40%) was added to the vessels and sonificated for 60 min. Prior to measurement the samples were filled up to an exact volume of 15 mL with 4% boronic acid; additionally, boronic acid was added to complex the fluoride ions.

Unfortunately a commercially available animal matrix reference material with a certified value for titanium dioxide is not available. Therefore, for quality assurance samples were spiked with an exactly weighed amount of TiO_2 nanoparticles prior to digestion.

4.2.2 Analytical measurement

Reagents for titanium analysis

Nitric acid (69%) was of "Rotipuran®" quality (supplied by Carl Roth, Karlsruhe).

The water used was purified using an ELGA Pure Lab Ultra water purification system (purified water resistivity >18 MΩ·cm).

Hydrofluoric acid (40%) was of Suprapur®" quality (supplied by VWR International, Darmstadt)

Boronic acid was of Suprapur®" quality (supplied by VWR International, Darmstadt)

For ICP-OES measurements a commercially available titanium standard containing 1000 mg/L Ti in ammonium hexafluorotitanate in water (CertiPUR, Merck, Darmstadt, Germany) was applied. With this standard solution appropriate stock solutions and subsequently calibration solutions were prepared.

Certified reference materials (chapter 21.1.5) and verifying the method

Unfortunately a commercially available animal matrix reference material with a certified value for titanium dioxide is not available. Therefore a few *Eisenia fetida* control samples were spiked with an exact amount of TiO_2 nanoparticles prior to digestion.

To additionally verify the analytical method a multi element CPI Standard (appropriately diluted to fit in the range of samples, purchased from CPI International, Amsterdam, The Netherlands) was analysed along with the samples to verify the measured results.

Furthermore, recalibration standards were analysed along with the samples.

Laboratory equipment

All materials used for sample treatment were suitable for analyses of titanium at trace levels. The glassware (beakers and volumetric flasks) was cleaned using a Miele washer "Automatic Disinfector" combined with a water de-ioniser "Aquapurificator", steamed out with HNO_3, rinsed with ultrapure water and dried at approximately 60°C. The pipettes used were adjustable to variable volumes (50 - 250 µL, 200 - 1000 µL, 1000 - 5000 µL) and were purchased from Gilson (Abimed, Langenfeld, Germany) and Eppendorf (Wesseling, Germany).

ICP-OES (raw data example : chapter 21.1.1)

Titanium concentrations of aqueous samples were measured using an IRIS Intrepid II ICP-OES (Thermo Electron, Dreieich, Germany). Titanium was detected at the wavelengths 334.941, 336.121,and 337.280 nm. Matrix adjusted calibrations were performed before each measurement. Depending on concentration range in samples the following calibration solutions were used: blank, 50, 100, 250, 500, 1000, and 2500 µg/L.

The calibration formula was calculated using the linear regression algorithm of the ICP-OES instrument software and was specific for the corresponding samples. The wavelength with the best correlation and recoveries for standards (337.280 nm) were used for calculating concentrations. Correlation coefficients (r) were at least 0.99941. For each sample, at least three internal measurements were performed and the mean was calculated and printed by the instrument software.

The applied LOD/LOQ (Limit of detection / Limit of quantification) calculations are:
LOD: 3 * method standard deviation from calibration line;
LOQ: 10 * method standard deviation from calibration line.

The information about the LOD/LOQ and correlation coefficient is compiled in Table 6.

A representative calibration line is shown in the raw data chapter 21.1.2

Coefficient of determination (r) for respective calibration functions were taken from ICP-OES instrument outputs.

The resulting values are reported in Table 4.

Table 4: TiO_2 in earthworms: LODs/LOQs, correlation.

Measurement date, description	LOD [µg/L]	LOQ [µg/L]	Correlation coefficient r
July 28, 2011 measurements of samples from May 19th	18	60 [1]	0.99990
July 14, 2011 measurements of samples from January25th and February 18th	65	218 [1]	0.99975
June 09, 2011 measurements of control worms for fortification	18	54 [1]	0.99941

[1] Internal LOQ calculation was performed with more digits

Instrumental and analytical set-up of the ICP-OES:

Thermo IRIS Intrepid II
Thermo Electron Corporation, Germany
Analytical conditions
-Nebuliser: Concentric glass nebuliser, Thermo Electron Corporation, Dreieich, Germany
-Spray chamber: Glass cyclonic spray chamber, Thermo Electron Corporation, Dreieich, Germany
-Nebuliser gas flow: 0.68 L/min
-Make-up gas flow: 0.5 L/min
-RF power: 1150 W
-Wavelengths: 334.941 nm, 336.121 nm, 337.280 nm (used for evaluation)

The mean recovery for CPI multi element solution (appropriately diluted) samples containing 500 µg Ti/L was 104 ± 7% (n = 6).

For further quality assurance, recalibration samples were analysed along with the samples and the mean accuracy was determined to 103 ± 3% (n = 6) for a Ti concentration of 500 µg/L.

For collecting validation information of the digestion procedure of samples as well as the analytical method several control worms were pooled and spiked with a weighed amount of TiO_2 nanoparticles.

Exactly 2698 µg TiO_2 nanoparticles (P25, 1617 µg Ti) were given to exactly 2164.0 mg of homogenised and dried worms resulting in a titanium amount of 747 µg/g. Without spiking, the *Eisenia fetida* material exhibited a mean titanium concentration of 44.9 ± 2.8 µg/g (n = 2). In conclusion the nominal value is calculated as 792 µg/L.

Spiked samples were digested and analysed along with actual samples, exhibiting a mean value of 659 ± 57 µg/L (n = 6), representing a mean recovery of 83.1 ± 7.2%. The quality requirements for the digestion and analysis of titanium in *Eisenia fetida* were set to 100 ± 25%, and were therefore fulfilled.

4.3 Digestion and quantification of silver and silver nanoparticles in soil (01A)

4.3.1 Preliminary remarks

The method for extraction/digestion of soil followed by analytical measurement of total Ag was developed by using silver nano powder (< 100 nm, Sigma-Aldrich, Schnelldorf, Germany) in the context of a nitrification test with *Eisenia fetida*.

An exact amount of the silver test item was introduced into the soil and samples were taken for analysis. The obtained recoveries in the measurement series verified the method.

The digestion and analytical methods are the same as for the determination of silver in sediment samples from the test with chironomids. In the latter, the nanosilver NM300K was applied and CRM026-050 sandy Loam (RT Corporation, Laramie, USA, reference value for Ag is 0.57 mg/kg) was successfully digested and analysed for its silver amount. Therefore, the developed method for the above mentioned silver nano powder can also be applied for NM-330K which was available as dispersion.

4.3.2 Procedure

Soil samples

The digestion procedure was performed according to DIN ISO 11466 and DIN EN 13346/DEV S7a. Therefore, prior to digestion the soil was dried at 105°C until constant weight for at least 12 h. Thereafter, approximately 3 g of the homogenised material was weighed and 28 g of *Aqua regia* was added. After 16 h at room temperature without agitation the mixture was heated under reflux for two hours. To avoid over-heating glass chips were added and foaming was avoided by adding a few drops of 1-octanole. The mixture was cooled to room temperature and then carefully brought to an exact volume of 100 mL. This *Aqua regia* extract was filtered (0.45 µm, Syringe Filter, Supor membrane, Pall Corporation, New York) and the silver concentration was determined by ICP-OES with a matrix-adjusted calibration.

4.3.3 Analytical measurement

Reagents for silver analysis

Nitric acid was of "Suprapur®" (supplied by Carl Roth, Karlsruhe) and hydrochloric acid of "instra-analysed" quality for trace metal analysis (supplied by Mallinckrodt Baker, Griesheim, Germany). The water used was purified using a Pure Lab Ultra water purification system (purified water resistivity >18 MΩ·cm).

A commercially available, multi element ICP-standard containing 1000 mg/L Ag in nitric acid 2-3% (lot no. HC957274, ICP Multi Element Standard Solution IV, CertiPUR®, Merck, Darmstadt, Germany) was used to prepare the appropriate stock solutions and respective calibration solutions. All prepared standard solutions had a final HNO_3 concentration of 3%.

(Certified) Reference materials and verifying of the method (certificate of reference material: chapter 21.1.6)

The analysed certified aqueous reference material was purchased from Environment Canada (TMDA-70, lot 0809, certified with 10.9 µg/L Ag, purchased from Environment Canada).

An exact amount of the nanosilver test item was introduced into the soil, and samples were taken for analysis. The obtained recoveries in the measurement series verified the method. It also confirmed the achieved homogeneity (except vessel 6- deviation may be due to homogeneity problems of the silver stock solution) for applying the nano silver in the soil for the *Eisenia fetida* test.

Additionally, recalibration samples were analysed along with actual samples and the recoveries were determined.

The silver concentrations in reagent blanks were always below the limit of detection.

Laboratory equipment

All materials used for sample treatment were suitable for the analysis of silver at trace levels. The glassware (beakers and volumetric flasks) was cleaned using a Miele washer "Automatic Disinfector" combined with a water de-ioniser "Aquapurificator", steamed out with HNO_3, rinsed

with ultrapure water and dried at approx. 60°C. The pipettes used were adjustable to variable volumes (50 - 250 µL, 200 - 1000 µL, 1000 - 5000 µL) purchased from Gilson (Abimed, Langenfeld, Germany) and Eppendorf (Wesseling, Germany).

ICP-OES (raw data example: chapter 21.1.2)

Silver concentrations of aqueous samples were measured using an IRIS Intrepid II ICP-OES (Thermo Electron, Dreieich, Germany). Silver was detected at the wavelength of 328.068 nm. Calibrations were performed before each measurement. Depending on the concentration range in the samples the following calibration solutions were used: blank, 1.0 µg/L, 2.5, 5.0, 10, 20, 25, 50, 100, 250, 500, 1000, and 2500 µg/L. The calibration formula was calculated using the linear regression algorithm of the ICP-OES instrument software and was specific for the correspondent samples. Correlation coefficients (r) were at least 0.9995. For each sample, at least three internal measurements were performed and the mean was calculated and printed by the instrument software.

The applied LOD/LOQ calculations are:
LOD: 3 * method standard deviation from calibration line
LOQ: 10 * method standard deviation from calibration line.

The information about the LOD/LOQ and correlation coefficient is compiled in Table 5.

A representative calibration line is shown in the raw data chapter 21.1.2.

Coefficients of determination (r) for respective calibration functions were taken from ICP-OES instrument outputs.

Table 5: Silver in soil: LODs/LOQs, correlation.

Measurement date, description	LOD [µg/L]	LOQ [µg/L]	Correlation coefficient r
March 29, 2010	3.7	12 [1]	0.9994
March 29, 2010	25	82 [1]	1.0000

[1] Internal LOQ calculation was performed with more digits

Instrumental and analytical set-up of the ICP-OES
-Thermo IRIS Intrepid II
-Thermo Electron Corporation, Germany
-Analytical conditions
-Nebuliser: Concentric glass nebulizer, Thermo Electron Corporation, Dreieich, Germany
-Spray chamber: Glass cyclonic spray chamber, Thermo Electron Corporation, Dreieich, Germany
-Nebuliser gas flow: 0.68 L/min
-Make-up gas flow: 0.5 L/min
-RF power: 1150 W
-Wavelength: 328.068 nm

Quality assurance measurements

The certified reference material TMDA-70 (certified with 10.9 µg Ag/L) was analysed as a quality assurance sample with solution samples from the test. In accordance with the quality assurance requirement, the silver recovery was in the range of ± 15% of the certified value. However, regarding Ag concentrations measured by ICP-OES, the mean recovery (accuracy) and precision of the non-digested CRM TMDA-70 measurements were 101 ± 2.9% (n = 4).

The recovery for Merck IV standard solution samples containing 50 µg/L was 101 ± 2.7%(n = 4) and 94.7 ± 0.7% for 500 µg/L. Analysis reagent blanks were always below the limit of detection of the respective measurement series.

An exact amount of the nano-silver test item was introduced into the soil and samples were taken for analysis. Samples from three test vessels were taken. According to the quality assurance requirement, the silver recovery was in the range of ± 25% for the silver in the soil from the respective vessels (10 mg/kg: 76.2 ± 8.8%; 100 mg/kg: 80.8 ± 1.7%; 100 mg/kg: 80.4 ± 3.1%).

4.4 Digestion and quantification of silver and silver nanoparticles in aqueous and sediment samples (OECD 219, test with chironomids)

4.4.1 Procedure

Aqueous samples

After thoroughly shaking the samples (vortex) 1 mL of the aqueous mixture was transferred into quartz digestion vessels and 2 mL of conc. nitric acid as well as 4 mL of Ultra-Pure water were added. The subsequent digestion was performed using an Ultra Clave II microwave (MLS GmbH, Leutkirch im Allgäu, Germany).

The following microwave program was applied:

Step 1: 25 min heating up to 220 °C

Step 2: 30 min at 220 °C

Thereafter, the digested samples were poured into volumetric flasks and filled up with ultrapure water to an exact volume of 15 mL. This final solution was analysed by ICP-OES for its amount of silver.

Sediment samples

The digestion procedure was performed according to DIN ISO 11466 and DIN EN 13346/DEV S7a. Therefore, prior to digestion the sediment was dried at 105 °C until constant weight for at least 12 h. Then approximately 3 g of the homogenised material was weighed and 28 g of *aqua regia* were added. After 16 h without agitation at room temperature this mixture was heated under reflux for two hours. To avoid over-boiling glass chips were added and foaming was avoided by adding a few drops of 1-octanole. The mixture was cooled to room temperature and then carefully brought to an exact volume of 100 mL. This *aqua regia* extract was filtered (0.45 µm, Syringe Filter, Supor membrane, Pall Corporation, New York) and the silver concentration was determined by ICP-OES with a matrix-adjusted calibration.

4.4.2 Analytical measurement

Reagents for silver analysis

Nitric acid was of "Suprapur®" (supplied by Carl Roth, Karlsruhe) and hydrochloric acid of "instra-analysed" quality (supplied by Mallinckrodt Baker, Griesheim, Germany). The water used was purified using a Pure Lab Ultra water purification system (purified water resistivity >18 MΩ·cm).

A commercially available silver ICP-standard containing 1000 mg/L Ag in nitric acid 2-3% (lot no. HC936000, CertiPUR®, Merck, Darmstadt, Germany) was used to prepare appropriate stock solutions and respective calibration solutions. All prepared standard solutions had a final HNO_3 concentration of 3%.

Certified reference materials (chapter 21.1.6) and verifying the method

The analysed certified aqueous reference material was purchased from Environment Canada (TMDA-70, lot 310). Unfortunately, the certified value is 10.9 µg Ag/L. For higher calibration ranges this certified value is above the limit of quantification because the LOD (limit of detection) and LOQ (limit of quantification) are strongly dependent on calibration. Therefore the measured silver concentrations in TMDA-70 could not be quantified reliably. Furthermore, a multi element Merck IV Standard (appropriately diluted to fit in the range of samples, lot HC957274, purchased from Merck, Darmstadt, Germany) was analysed along with the samples to verify the measured results. To verify the microwave procedure, Merck IV solution was also digested along with the aqueous test samples.

For sediment samples the certified reference material CRM026-050 Sandy Loam (RT Corporation, Laramie, USA, reference value for Ag is 0.57 mg/kg) was digested along with the sediment samples to additionally verify the microwave procedure.

The silver concentrations in digested and non-digested reagent blanks were always below the limit of quantification.

Laboratory equipment

All materials used for sample treatment were suitable for the analysis of silver at trace levels. The glassware (beakers and volumetric flasks) was cleaned using a Miele washer "Automatic Disinfector" combined with a water de-ioniser "Aquapurificator", steamed out with HNO_3, rinsed with ultrapure water and dried at approximately 60°C. The pipettes used were adjustable to variable volumes (50 - 250 µL, 200 - 1000 µL, 1000 - 5000 µL) and were purchased from Gilson (Abimed, Langenfeld, Germany) and Eppendorf (Wesseling, Germany).

ICP-OES (raw data example: chapter 21.1.2)

Silver concentrations of aqueous samples were measured using an IRIS Intrepid II ICP-OES (Thermo Electron, Dreieich, Germany). Silver was detected at the wavelengths 328.068 nm, and 338.289 nm. Calibrations were performed before each measurement. Depending on the concentration range in the samples the following calibration solutions were used: blank, 2.5 µg/L, 5.0 10, 25 50, 100, 250, 500, 1000, and 2000 µg/L. The calibration formula was calculated using the linear regression algorithm of the ICP-OES instrument software. Due to spectral interferences at the wavelength of 338.289 nm, especially in the sediment samples the obtained data from 328.068 were used for calculating concentrations. Correlation coefficients (r) were at least

0.9999. For each sample, at least three internal measurements were performed and the mean was calculated and printed by the instrument software.

The applied LOD/LOQ calculations are:
LOD: 3 * method standard deviation from calibration line
LOQ: 10 * method standard deviation from calibration line.

The information about the LOD/LOQ and correlation coefficient is compiled in Table 6.

A representative calibration line is shown in the raw data chapter.

Coefficients of determination (r) for respective calibration functions were taken from ICP-OES instrument outputs.

Table 6: Silver in aqueous and sediment samples: LODs/LOQs, correlation.

Measurement date, description	LOD [µg/L]	LOQ [µg/L]	Correlation coefficient r
February 18, 2011, digested aqueous samples 7d, 14d and 28d	1.9	6.3 1	0.9999
March 02, 2011, digested aqueous samples 0d, 1d	3.6	12 1	1.0000
March 02, 2011, digested sediment samples	3.9	13 1	1.0000

[1] Internal LOQ calculation was performed with more digits

Instrumental and analytical set-up of the ICP-OES
-Thermo IRIS Intrepid II
-Thermo Electron Corporation, Germany
-Analytical conditions
-Nebuliser: Concentric glass nebuliser, Thermo Electron Corporation, Dreieich, Germany
-Spray chamber: Glass cyclonic spray chamber, Thermo Electron Corporation, Dreieich, Germany
-Nebuliser gas flow: 0.68 L/min
-Make-up gas flow: 0.5 L/min
-RF power: 1150 W
-Wavelengths: 328.068 nm, 338.289 nm (not evaluated due to spectral interferences)

Quality assurance measurements

The certified reference material TMDA-70 (certified with 10.9 µg/L Ag) was analysed as quality assurance sample with solution samples from the test. According to the quality assurance requirement, the silver recovery was in the range of ± 15% of the certified value. However, regarding Ag concentrations measured by ICP-OES, the mean recovery (accuracy) and precision of the non-digested CRM TMDA-70 measurements were 104 ± 5.5% (n = 6).

The recovery for digested Merck IV standard solution samples containing 2.5 mg/L (to verify the digestion procedure for aqueous samples) was 105 ± 1.4% (n = 12). For non-digested Merck IV

samples the accuracy was determined to 106 ± 0.4% (n =2) for 500 µg/L and 101 ± 0.8% (n = 2) for 250 µg/L.

Analysis of acidified ultrapure water as reagent blanks as well as digestion and analysis of SiO_2 as blanks for sediment samples revealed silver concentrations which were always at least below the limit of quantification of the respective measurement series.

For further quality assurance, recalibration samples were analysed along with the samples and the mean accuracy was determined to 98.4 ± 1.2% (n = 3) for an Ag concentration of 100 µg/L and 99.2 ± 0.6% (n = 2) for 200 µg/L.

For collecting validation information on the digestion procedure of sediment samples as well as the analytical method, the mean recovery of silver in the certified reference material “026-050 Sandy loam” was determined to 114 ± 3.4% (n =3). Regarding the quality requirements the recoveries of metals in soils and sediments must be in the range of 100 ± 25%.

The validation information is summarised in Table 7.

Table 7: Silver in aqueous and sediment samples: method validation information.

Validation parameter	Results	Comment
Selectivity	two different wavelengths for ICP-OES method	interferences only observed at 338.289 nm
Linearity	applied calibration functions were linear	see Table 6 correlation coefficient (r) at least 0.9999
Limits of detection (LOD)	1.9 – 3.9 µg/L	see Table 6
Limits of quantification (LOQ)	6.3 – 13 µg/L	see Table 6
Reagent and sediment blanks	below < LOD = < 1.9 - < 3.9 µg/L; one UHQ sample from measurement series of March 02, 2011 < LOQ = < 11.9 µg/L	-
Accuracy and precision	mean recovery for TMDA-70: 104 ± 5.5% (n = 6)	for low concentration range of samples (10.9 µg/L)
Accuracy and precision	mean recovery for non digested Merck IV (500 µg/L): 106 ± 0.4% (n = 2)	corresponds to concentration range of samples (500 µg/L)
Accuracy and precision	mean recovery for non digested Merck IV (250 µg/L): 101 ± 0.8% (n = 2)	corresponds to concentration range of samples (250 µg/L)
Accuracy and precision	mean recovery for recalibration (100 µg/L): 97.2± 1.1% (n = 2)	corresponds to concentration range of samples (100 µg/L)
Accuracy and precision	mean recovery for recalibration (200 µg/L): 98.2 ± 1.0% (n = 2)	corresponds to concentration range of samples (200 µg/L)
accuracy and precision	mean recovery for digested Merck IV (2.5 mg/L): 105 ± 1.4% (n = 12)	corresponds to concentrations of applied loadings
Accuracy and precision	mean recovery for recalibration samples of 100 µg/L: 98.4 ± 1.2 (n = 3)	corresponds to concentration range of samples (100 µg/L)
Accuracy and precision	mean recovery for recalibration samples of 200 µg/L: 99.2 ± 0.6 (n = 2)	corresponds to concentration range of samples (200 µg/L)
Accuracy and precision	mean recovery for silver in digested CRM026-050: 114 ± 3.4 (n = 3)	Certified with 0.570 mg/kg
Reproducibility	mean recovery for TMDA-70: 104 ± 5.5% (n = 6)	for low concentration range of samples (10.9 µg/L)
Reproducibility	mean recovery for non digested Merck IV (500 µg/L): 106 ± 0.4% (n = 2)	corresponds to concentration range of samples (500 µg/L)
Reproducibility	mean recovery for non digested Merck IV (250 µg/L): 101 ± 0.8% (n = 2)	corresponds to concentration range of samples (250 µg/L)
Reproducibility	mean recovery for recalibration (100 µg/L): 97.2± 1.1% (n = 2)	corresponds to concentration range of samples (100 µg/L)
Reproducibility	mean recovery for recalibration (200 µg/L): 98.2 ± 1.0% (n = 2)	corresponds to concentration range of samples (200 µg/L)
Reproducibility	mean recovery for digested Merck IV (2.5 mg/L): 105 ± 1.4% (n = 12)	corresponds to concentrations of applied loadings
Reproducibility	mean recovery for recalibration samples of 100 µg/L: 98.4 ± 1.2 (n = 3)	corresponds to concentration range of samples (100 µg/L)
Reproducibility	mean recovery for recalibration samples of 200 µg/L: 99.2 ± 0.6 (n = 2)	corresponds to concentration range of samples (200 µg/L)
Reproducibility	mean recovery for silver in digested CRM026-050: 114 ± 3.4 (n = 3)	certified with 0.570 mg/kg

Presentation of the results

One millilitre of the samples were digested and to filled to 15 mL with ultrapure water (dilution factor 15). Prior to measurements the stock solution samples were additionally diluted 1:10 because of their higher concentration (dilution factor for stock solution samples is 150). When the measured value by ICP-OES without dilution factor was below the limit of detection (LOD) or quantification (LOQ) this is denoted in the tables. To obtain the measured silver concentrations of the instrument the values have to be divided by 15, and the data for the stock solutions by 150.

The amount of silver in dispersion was determined by UV-VIS measurements without preceding digestion. As a certified standard solution containing nano-Ag is not yet available, the calibration used for this method is performed with a silver standard. The quantification of total silver carried out after total digestion differs from the amount reported by the producer as the measurement performed by the producer was performed without matrix-adjusted calibration.

4.5 Quantification of silver in the extracts of DGTs, diffusive gradients in thin films

4.5.1 DGTs in general

DGT means diffusive gradients in thin films. DGT devices can collect the dissolved ions. The concentrations can then be determined by instrumental analysis.

“The simple device uses a layer of Chelex resin impregnated in a hydrogel to accumulate the metals. The resin-layer is overlain by a diffusive layer of hydrogel and a filter. Ions have to diffuse through the filter and diffusive layer to reach the resin layer. It is the establishment of a constant concentration gradient in the diffusive layer that forms the basis for measuring metal concentrations in solution quantitatively without the need for separate calibration” [Technical documentation], http://www.dgtresearch.com].

For detailed information please see the above mentioned technical documentation and references cited within.

4.5.2 Procedure

Preparation and application of DGTs

For preparation, the DGT devices were placed into a 0.01 mol/L NaCl solution. Argon was introduced into the solution for one hour. Afterwards the vessel containing the DGTs and the NaCl solution was tightly closed and remained for 24 h under inert gas. Thereafter the DGT devices were carefully introduced into the test setup with chironomids by pushing them on the sediment.

The devices remained in the test vessels for 48 h. After their removal they were thoroughly rinsed with ultrapure water and wrapped into polyethylene bags for storage at 4 °C for no longer than 4 days. The DGTs were broken up and the resin layer was extracted and directly transferred into 1.5 mL 1 mol/L nitric acid for elution of silver ions for at least 24 h. An exact volume of 1 mol/L nitric acid was added and the solution was sampled and analysed for its amount of silver by ICP-MS.

4.5.3 Analytical measurement

Reagents for silver analysis

Nitric acid was of "Suprapur®" (supplied by Carl Roth, Karlsruhe) and hydrochloric acid of "instra-analysed" quality (supplied by Mallinckrodt Baker, Griesheim, Germany). The water used was purified using a Pure Lab Ultra water purification system (purified water resistivity >18 MΩ·cm).

A commercially available multi element ICP-standard containing 1000 mg/L Ag in nitric acid 2-3% (lot no. HC957274, ICP Multi Element Standard Solution IV, CertiPUR®, Merck, Darmstadt, Germany; chapter 21.1.6) was used to prepare appropriate stock solutions and respective calibration solutions. All prepared standard solutions had a final HNO_3 concentration of 3%.

Certified reference materials (chapter 21.1.6) and verifying the method

The analysed certified aqueous reference materials – appropriately diluted to fit in the concentration range of samples - were purchased from Environment Canada (TMDA-70, lot 310, certified with 10.9 µg/L Ag and TMDWS2, certified with 9.97 µg/L; chapter 21.1.6).

Laboratory equipment

All materials used for sample treatment were suitable for the analysis of silver at trace levels. The glassware (beakers and volumetric flasks) was cleaned using a Miele washer "Automatic Disinfector" combined with a water de-ioniser "Aquapurificator", steamed out with HNO_3, rinsed with ultrapure water and dried at approx. 60 °C. The pipettes used were adjustable to variable volumes (50 - 250 µL, 200 - 1000 µL, 1000 - 5000 µL) and were purchased from Gilson (Abimed, Langenfeld, Germany) and Eppendorf (Wesseling, Germany).

ICP-MS (raw data example: chapter 21.1.3)

Silver concentrations of aqueous samples were measured using an Agilent 7500ce ICP-MS instrument (Agilent Technologies, Waldbronn, Germany). Silver was detected at the isotope 109 in the no-gas mode of the machine. Calibrations were performed prior to the measurement series. Depending on the concentration range in samples the following calibration solutions were used: 0.25, 0.50, 1.0, 2.5, 5.0, 10, and 25 µg/L. The calibration formula was calculated using the linear regression algorithm of the ICP-MS instrument software. Correlation coefficient (r) is 0.9999. For each sample, at least three internal measurements were performed and the mean was calculated and printed by the instrument software.

The applied LOD/LOQ calculations are:
LOD: 3 * method standard deviation from calibration line
LOQ: 10 * method standard deviation from calibration line.

The information about the LOD/LOQ and correlation coefficient is compiled in Table 8.

A representative calibration line is shown in the raw data chapter 21.1.2.

Coefficient of determination (r) for respective calibration function was taken from ICP-MS instrument outputs.

Table 8: Determination of silver ions: LODs/LOQs, correlation.

Measurement date, description	LOD [µg/L]	LOQ [µg/L]	Correlation coefficient r
March 4, 2011	0.0013	0.0039	0.9999

Instrumental and analytical set-up of the ICP-MS

-Agilent 7500i (Agilent Technologies, Germany)
-Analytical conditions
-Nebuliser: Micro Mist, Agilent Technologies, Germany
-Spray chamber: Scott Type, Agilent Technologies, Germany
-Nebuliser gas flow: 0.95 L/min
-Make-up gas flow: 0.12L/min
-RF power: 1500 W
-No-gas mode
I-sotope: ^{109}Ag

Quality assurance measurements (certificate of reference material chapter 21.1.6)

The certified reference materials TMDA-70 (certified with 10.9 µg/L Ag) and TMDWS2 were analysed as quality assurance sample with solution samples from the test. According to the quality assurance requirement, the silver recovery was in the range of ± 15% of the certified value. However, regarding Ag concentrations measured by ICP-MS, the mean recovery (accuracy) and precision of CRM TMDA-70 (dilution factor 10) measurements were 95.8 ± 4.0% (n = 5) and 97.5 ± 5.5% (n = 6) for CRM TMDWS2 (dilution factor 5).

The silver concentration in reagent blanks analysed along with the actual samples were mostly at least below the limit of quantification (LOQ = 0.039 µg/L, n = 10). Because of the low LOQ the measured values of three additional reagent blanks exhibited higher concentrations (0.004 µg/L, 0.020 µg/L and 0.005 µg/L). However, the latter concentrations were far below the measured amounts in actual samples and therefore did not influence the analytical measurement series.

The validation information is summarised in Table 9.

Table 9: Determination of silver ions: information on method validation.

Validation parameter	Results	Comment
Selectivity	Isotope ^{109}Ag for ICP-MS	interferences can be excluded for ^{109}Ag
Linearity	applied calibration functions were linear	see Table 6 correlation coefficient (r) at least 0.9999
Limit of detection (LOD)	0.013µg/L	see Table 6
Limit of quantification (LOQ)	0.039 µg/L	see Table 6
Reagent blanks	< LOQ = < 0.039 µg/L (n = 10); due to low LOQ three reagent blanks > LOQ: 0.004 µg/L, 0.020 µg/L, 0.005 µg/L (n = 3)	no influence on the analytical results
Accuracy and precision	mean recovery for TMDA-70: 95.8 ± 4.0% (n = 5)	diluted to 1.09 µg/L (factor 10) for low concentration range
Accuracy and precision	mean recovery for TMDWS2: 97.5 ± 5.5% (n = 6)	diluted to 1.99 µg/L (factor 5) for low concentration
Reproducibility	mean recovery for TMDA-70: 95.8 ± 4.0% (n = 5)	diluted to 1.09 µg/L (factor 10) for low concentration range
Reproducibility	mean recovery for TMDWS2: 97.5 ± 5.5% (n = 6)	diluted to 1.99 µg/L (factor 5) for low concentration

The amount of silver in dispersion in NM300K provided by the producer ´Rent a Scientist´ is determined by UV-VIS measurements without digestion. Because a certified standard solution containing nano-Ag is not available yet, the calibration used for this method was performed with a silver standard. Therefore, the analytical result that is provided by the producer was measured without matrix-adjusted calibration and may differ from the real value.

4.6 Quantification of silver in earthworms

4.6.1 Procedure

Cryogenic homogenisation

To ensure a complete digestion of *Eisenia fetida*, it was necessary to perform cryogenic homogenisation. To avoid contamination, homogenisation was performed under a laminar-flow hood. All materials used as well as the worms were cooled in liquid nitrogen. The samples were homogenised using a pestle and mortar. First a larger pestle was used for a coarser grinding followed by a smaller one. The resulting powder was transferred into cryo-proofed vials and stored directly above liquid nitrogen at approx. -150°C until lyophilisation.

Lyophilisation

The frozen samples were transferred into a Christ Alpha 1-2 freeze dryer (Martin Christ GmbH, Osterode am Harz, Germany) for lyophilisation. Freeze-drying was performed until samples reached constant weights.

Microwave digestion

Approximately 200 mg of homogenised and dried substance from each sample was weighed into a quartz digestion vessel, and 5 mL of concentrated nitric acid were added followed by the microwave digestion.

Program: heat for 25 min to maximum temperature of 220°C; hold at 220°C for 30 min; allow to cool for approx. 60 min; initial pressure 40 bar.

After digestion the vessels were filled up to an exact volume of 20 mL with ultrapure water.

A reference material (NIST 2977 Mussel Tissue) was digested along with the samples.

The digestion procedure is in accordance to the document 'Guidelines for Chemical Analysis, Digestion of Environmental Samples' from http://www.umweltprobenbank.de.

4.6.2 Analytical measurement

Reagents for silver analysis

Nitric acid was of "Suprapur®" (supplied by Carl Roth, Karlsruhe). The water used was purified using an ELGA Pure Lab Ultra water purification system (purified water resistivity >18 MΩ·cm).

A commercially available silver ICP-standard containing 1000 mg/L Ag in nitric acid 2-3% was used to prepare appropriate stock solutions and respective calibration solutions. All prepared standard solutions had a final HNO_3 concentration of 3%.

Certified reference materials (chapter 21.1.6) and verifying the method

The reference material NIST 2977 Mussel Tissue was digested and analysed along with the samples to verify the procedures (purchased from LGC Standards, Wesel, Germany). Unfortunately, only a non-certified reference value of 4.58 mg Ag / kg is provided for this material.

Furthermore, aqueous certified reference material TMDA-70 (purchased from Environment Canada, certified Ag conc. of 10.9 µg/L) was analysed along with the samples.

To additionally verify the analytical method a multi element Merck IV Standard (appropriately diluted to fit in the range of samples, lot HC957274, purchased from Merck, Darmstadt, Germany) was analysed along with the samples to verify the measured results.

The silver concentration in reagent blanks (n = 10) were below the limit of detection (< 2.6 µg/L), except one which was below the limit of quantification (< 8.8 µg/L)

Laboratory equipment

All materials used for sample treatment were suitable for analyses of silver at trace levels. The glassware (beakers and volumetric flasks) was cleaned using a Miele washer "Automatic Disinfector" combined with a water de-ioniser "Aquapurificator", steamed out with HNO3, rinsed with ultrapure water and dried at approx. 60°C. The pipettes used were adjustable to variable volumes (50 - 250 µL, 200 - 1000 µL, 1000 - 5000 µL) and were purchased from Gilson (Abimed, Langenfeld, Germany) and Eppendorf (Wesseling, Germany).

ICP-OES (raw data example: chapter 21.1.2)

Silver concentrations of aqueous samples were measured using an IRIS Intrepid II ICP-OES (Thermo Electron, Dreieich, Germany). Silver was detected at the wavelengths 328.068 nm, and 338.289 nm. Calibrations were performed before each measurement. Depending on concentration range in samples the following calibration solutions were used: blank, 1.0, 2.5, 5.0, 10, 25, 50, 100, and 250 µg/L.

The calibration formula was calculated using the linear regression algorithm of the ICP-OES instrument software. Due to spectral interferences at the wavelength of 338.289 nm, the obtained data from 328.068 were used for calculating concentrations. Correlation coefficient (r) was 0.99995. For each sample, at least three internal measurements were performed and the mean was calculated and printed by the instrument software.

The applied LOD/LOQ (Limit of detection / Limit of Quantification) calculations are:
LOD: 3 * method standard deviation from calibration line;
LOQ: 10 * method standard deviation from calibration line.

The information about the LOD/LOQ and correlation coefficient is compiled in Table 6.

The calibration line is shown in the raw data chapter 21.1.

Coefficient of determination (r) for respective calibration functions were taken from ICP-OES instrument outputs.

The resulting values are reported in Table 10.

Table 10: Silver in earthworms: LODs/LOQs, correlation.

Measurement date, description	LOD [µg/L]	LOQ [µg/L]	Correlation coefficient r
August 26, 2011	2.6	8.8*	0.99995

* Internal LOQ calculation was performed with more digits

Instrumental and analytical set-up of the ICP-OES:

-Thermo IRIS Intrepid II
-Thermo Electron Corporation, Germany
-Analytical conditions
-Nebuliser: Concentric glass nebulizer, Thermo Electron Corporation, Dreieich, Germany
-Spray chamber: Glass cyclonic spray chamber, Thermo Electron Corporation, Dreieich, Germany
-Nebuliser gas flow: 0.68 L/min
-Make-up gas flow: 0.5 L/min
-RF power: 1150 W
-Wavelengths: 328.068 nm, 338.289 nm (not evaluated due to spectral interferences)

Quality assurance measurements

The certified reference material TMDA-70 (certified as 10.9 µg Ag/L) was analysed as quality assurance sample with solution samples from the test. According to the quality assurance requirement, the silver recovery was in the range of ± 15% of the certified value. However, regarding Ag concentrations measured by ICP-OES, the mean recovery (accuracy) and precision of the non digested CRM TMDA-70 measurements were 109 ± 10% (n = 4).

The recovery for Merck IV solution samples containing 50 µg Ag / L was 104 ± 5% (n = 2).

For further quality assurance, recalibration samples were analysed along with the samples and the mean accuracy was determined to 101 ± 2% (n = 2) for an Ag concentration of 50 µg/L.

For collecting validation information of the digestion procedure of samples as well as the analytical method, the mean recovery of silver in the reference material NIST 2977 Mussel Tissue was determined as 73.5 ± 6.4% (n =3), although only a non-certified reference value was given in the certificate.

4.7 Dissolution and quantification of nano-Au in aqueous and sediment samples

4.7.1 Procedure

Dissolution of gold nanoparticles in aqueous samples

In order to dissolve gold nanoparticles within aqueous samples *Aqua regia* was applied. Therefore 1 mL of *Aqua regia* was added to 1 mL of the aqueous test sample. The mixture was carefully vortexed or shaken (for 5 min) and remained for at least for additional 24 h without agitation at room temperature prior to analytical determination.

Dissolution of gold nanoparticles in sediment samples

The digestion procedure was performed according to DIN ISO 11466 and DIN EN 13346/DEV S7a. Therefore, prior to digestion the sediment was dried at 105°C until constant weight for at least 12 h. Thereafter, approx. 3 g of the homogenised material were weighed and 28 g of *Aqua regia* were added. After 16 h at room temperature - without agitation - this mixture was heated under reflux for two hours. To avoid foaming and overboiling a few drops of 1-octanole and glass chips were added. The mixture was cooled to room temperature and then carefully brought to an exact volume of 100 mL. This *Aqua regia* extract was filtered (0.45 µm, Syringe Filter, Supor membrane, VWR, Darmstadt) and the gold concentration was determined by ICP-OES with a matrix-adjusted calibration.

4.7.2 Analytical measurement

Laboratory equipment and chemicals

All materials used for sample treatment were suitable for working with gold at trace levels. The applied glassware (beakers and volumetric flasks) was cleaned with a Miele washer “Automatic Disinfector” combined with a water de-ioniser “Aquapurificator”, afterwards washed three times with *aqua regia* (mixture of conc. hydchloric and conc. nitric acid in a ratio of 3 : 1) and rinsed three times with ultrapure water. The glassware was dried at approx. 60 °C.

The pipettes used in variable volumes (50 – 250 µL, 200 – 1000 µL, 1000 – 5000 µL) were purchased from Gilson (Abimed, Langenfeld, Germany) and Eppendorf (Wesseling, Germany).

The water used for the analytical investigation was purified with a Pure Lab Ultra water purification system from ELGA LabWater, Celle, Germany. The purified water has a resistivity greater than 18 MΩ·cm.

The applied acids were:
-Nitric acid - “Supra” quality (ROTIPURAN® supplied by Roth, Karlsruhe, Germany)

-Hydrochloric acid – “Instra-Analyzed®” (supplied by Mallinckrodt Baker, Griesheim, Germany)
-Aqua regia is not commercially available and was freshly prepared prior to usage. Therefore, concentrated hydrochloric and concentrated nitric acid were mixed in a ratio of 3:1.

For ICP-OES measurements a commercially available gold standard containing 1000 mg/L Au in tetrachloroaurat acid in 7% hydrochloric acid (CertiPUR®, Merck, Darmstadt, Germany) was applied. With this standard solution appropriate stock solutions and subsequently calibration solutions were prepared.

(Certified) Reference materials (chapter 21.1.7)

To determine the accuracy of the applied analytical method the NIST reference material 8011 (gold nanoparticles, nominal diameter 10 nm) was analysed along with aqueous samples of the test and the recovery was determined. For this material only an informative value of 51.56 ± 0.23 µg nano-gold /g was given. However, to verify the dissolution of nano-gold and the subsequent analytical investigation this reference material turned out to be very feasible. The amount of nano-gold corresponded to 51.56 mg/L and was therefore appropriately diluted with ultrapure water to fit in the concentration range of test samples.

In order to further verify the analytical method, recalibration samples were analysed along with test item samples and recoveries were determined.

ICP-OES (raw data example: chapter 21.1.4)

Gold concentrations of aqueous samples were measured using an IRIS Intrepid II ICP-OES (Thermo Electron, Dreieich, Germany). Gold was detected at wavelengths of 197.819, 208.209, and 242.795 nm. Calibrations were performed before each measurement. Depending on the concentration range in samples the following calibration solutions (matrix adjusted) were used: blank, 1.0, 2.5, 5.0, 10, 20L, 25, 50L, 100, and 250 µg/L. The calibration formula was calculated using the linear regression algorithm of the ICP-OES instrument software. The wavelength having the best correlation and recoveries for reference materials and recalibration samples were used for evaluation (197.819 nm). Correlation coefficients (r) were at least 0.9992. For each sample, at least three internal measurements were performed and the mean was calculated and printed by the instrument software.

The applied LOD/LOQ calculations are:
LOD: 3 * method standard deviation from calibration line
LOQ: 10 * method standard deviation from calibration line.

The information about the LOD/LOQ and correlation coefficient is compiled in Table 5.

A representative calibration line is shown in the raw data chapter 21.1.

Coefficients of determination (r) for respective calibration functions were taken from ICP-OES instrument outputs.

The resulting values are reported in Table 11.

Table 11: Au in daphnids and chironomids: LODs/LOQs.

Measurement date, description	LOD [µg/L]	LOQ [µg/L] [1]	Correlation coefficient r
Januar 19, 2012 sediment samples from chironomids test	2.7	9.0	0.999997
January 04, 2012 aqueous samples from chironomids test after 28 days	2.6	8.7	0.999168
December 19, 2012 aqueous samples from Daphnia test after 0 and 48 h and aqueous samples from chironomids test after 7 days	1.4	4.3	0.999922
November 30, 2011 aqueous samples from chironomids test after 0 and 1 days	1.6	5.4	0.999983

[1] internal LOQ calculation was performed with more digits

Instrumental and analytical set-up of the ICP-OES:

-Thermo IRIS Intrepid II
-Thermo Electron Corporation, Germany
-Analytical conditions
-Nebuliser: Concentric glass nebuliser, Thermo Electron Corporation, Dreieich, Germany
-Spray chamber: Glass cyclonic spray chamber, Thermo Electron Corporation, Dreieich, Germany
-Nebuliser gas flow: 0.68 L/min
-Make-up gas flow: 0.5 L/min
-RF power: 1150 W
-Wavelengths: 197.819 nm (used for evaluation), 208.209 nm and 242.795 nm.

Quality assurance measurements

The NIST reference material 8011 (gold nanoparticles, nominal diameter 10 nm, informative value of 51.56 mg/L, appropriately diluted to fit in concentration range of samples) was digested and analysed as quality assurance sample with samples from the test. According to the quality assurance requirement, the gold recoveries (accuracy and precision) were in the range of ± 15% of the certified value. However, regarding Au concentrations measured by ICP-OES, the mean recoveries (accuracy and precision) of NIST 8011 measurements were 97.4 ± 12.9% (n = 2, dilution factor 3750), 98.1 ± 2.6% (n = 2, dilution factor 750) and 97.8 ± 1.4 (n = 12).

To further verify the analytical determination the recoveries of recalibration standards were determined to 99.0 ± < 0.1% (n = 2) for 250 µg/L, 97.0 ± 2.6% (n = 6) for 50 µg/L and 107% (n = 1) for 12.5 µg/L.

5 Pre-tests - spiking of soil

So far, documents referring specifically to the application of nanoparticles for ecotoxicological tests are not available. Normally, the test substance is applied using a carrier. For test substances soluble in water; aqueous stock solutions are prepared for aquatic and terrestrial tests. For test substances insoluble in water several options exist. The guidelines ISO 14442 (2006) and OECD no. 23 (2000) provide guidance for aquatic tests including methods such as stirring (from several hours up to 6 weeks), ultrasonication, high-shear mixing, addition of solvents or emulsifying agents or the removal of non-dissolved test substances by filtration or centrifugation. The test guidelines dealing with side-effects on the soil microflora propose a mixture of silica sand and test substance. Organic solvents should be avoided as they can damage the soil microflora. In tests with organisms other than the soil microflora (e.g. plants or earthworms) the use of organic solvents is allowed.

As recommendations for the application of nanoparticles were not available preliminary studies had to be carried out with emphasis on terrestrial tests. The application form and the homogeneity of spiking were investigated. The tests were performed with earthworms and the soil microflora.

To obtain information on the influence of the application form several forms of spiking were investigated:

- Application by dispersion
- Application by dry powder
- Application in soil
- Application in food

Application in soil is an option for all terrestrial tests, whereas the application in food may be a suitable procedure for tests where the test organisms are fed, such as the earthworm reproduction test. For a homogenous distribution a solid carrier material such as dry soil or silica sand is recommended.

Homogeneity was documented by chemical analyses. Further information on homogeneity was obtained by microbial analyses. For nitrate analyses only a small amount (20 g) of test soil is required. The nitrate content was determined in several replicates sampled at different spots of the test soil. In addition to the chemical analyses the standard deviation of the values can be used as a further indicator for homogeneity.

The structure of the pre-tests is presented in the following graph (Figure 1).

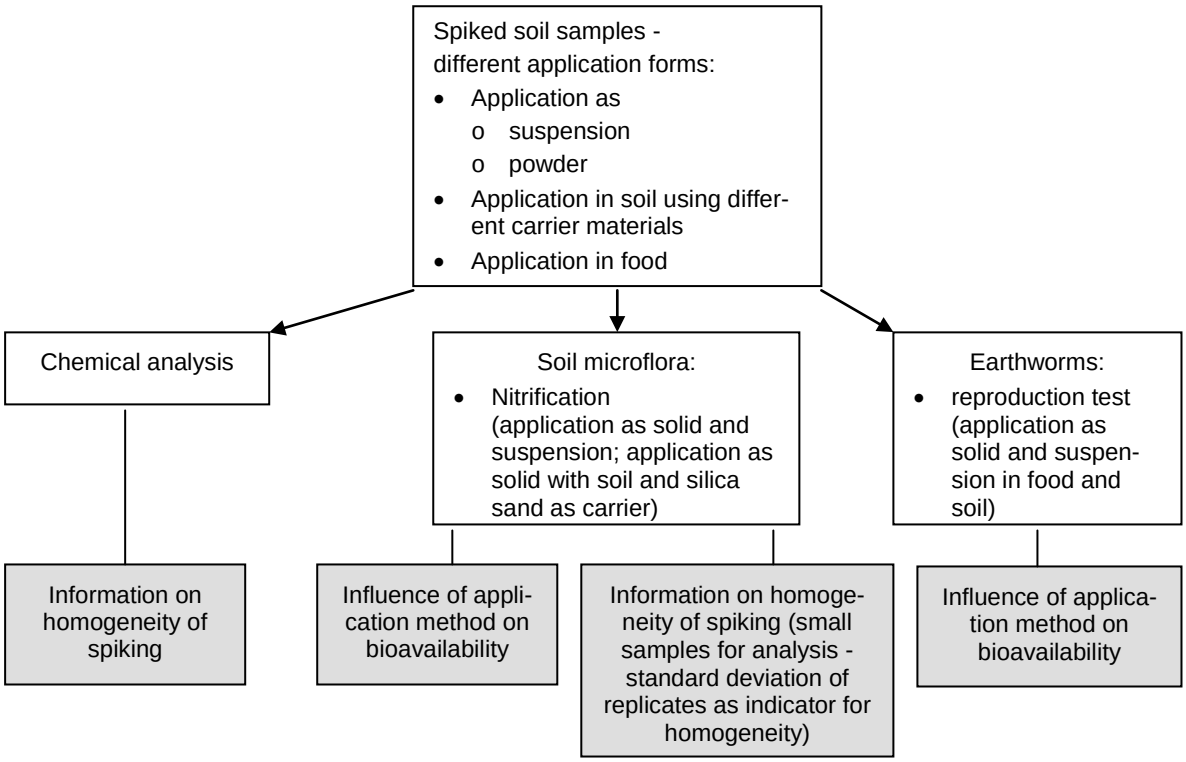


Figure 1: Structure of the pre-tests.

Carried out to elucidate the influence of the application form and the homogeneity of spiking

Spiking experiments were performed with P25 and silver. For silver no OECD-material was available at the time the pre-tests were started. Therefore, a commercially available silver nano-powder was used.

All tests were performed in a sandy field soil (described in 5.1.2).

5.1 Materials and methods

5.1.1 Nanomaterial

- P25 - distributed by Evonik for the OECD Sponsorship Programme. The properties should correspond to the properties of NM-105.
- Silver: Sigma-Aldrich, silver nano-powder, < 100 nm, 99.5% metals basis, order number 576832.

The test substances were stored in the dark at room temperature.

5.1.2 Test soil

The reference soil RefeSol 01A (sieved ≤2 mm; www.refesol.de), a loamy, medium-acidic, and very lightly humic sand, was used as the test medium,. Further characteristics of the soil are given in Table 12. The same soil was used for the main tests.

Table 12: Physico-chemical properties of the applied soil.

Physico-chemical soil properties	RefeSol 01A
pH	5.67
C_{org} [%]	0.93
N_{tot} [mg/kg]	882
CEC_{eff} [mmolc/kg]	37.9
Sand [%]	71
Silt [%]	24
Clay [%]	5
WHC_{max} [a] [ml H_2O/kg]	227

[a] WHC_{max}: maximum water-holding capacity

5.1.3 Application of the nanoparticles

Earthworm reproduction test

Spiking of soil with powder

In order to spike soil with the nanomaterial powder, the powder was mixed directly into the soil, whereby air-dried test soil or silica sand was used as a carrier (1% of the total amount). Silica sand is used in the building trade and is characterised by a particle size of 0 – 0.5 mm and a specific surface of 89 cm^2/g. The same silica sand is used when we perform tests in artificial soil (composition described, e.g. in OECD test guideline 222). Suitable amounts of nanomaterial powder to achieve the desired final soil content were mixed homogenously with the dry soil. Care was taken to avoid a modification of the TiO_2 crystalline structure. Uncontaminated test soil (between 20-30% of WHC_{max}) was spread on a plate, the carrier material with the powder was distributed onto the test soil, and the components mixed carefully. Then, the soil was adjusted to a water content of 55% of the maximum water-holding capacity (WHC_{max}).
Test concentration: 100 mg/kg soil dry matter (dm).

Spiking of food with powder

For the spiking of food with the nanomaterial, 40 g of air-dried ground cow manure was homogenously mixed with powder. The mixture was moistened with 120 ml deionised water. Test concentrations were: 6.38 mg/g food (dm), corresponding to 100 mg/kg soil (dm); introduction of 40 g moist food (10 g dry food and 30 ml deionised H_2O) on the surface of the 1-L test containers, each filled with 640 g soil (dm).

Spiking of soil with aqueous suspension

For the application of an aqueous suspension, a suspension was prepared with a magnetic flea (900 rpm; 1 min) and ultrasonication (3 min) in a bath sonicator (Hund-Rinke et al., 2010). Test soil was dried to about 10% of WHC_{max} and spread on a plate. Immediately after preparation, TiO_2 nanoparticle suspension was sprayed onto the soil by means of a syringe coupled with a cannula, and thoroughly mixed. Finally, the test soil was adjusted to a water content of 55% of the maximum water-holding capacity (WHC_{max}).

Test concentration: suspension with 100 mg/L deionised water; application of 250 ml test dispersion to 2.5 kg test soil (dm), corresponding to 10 mg/kg soil (dm).

Spiking of food with aqueous suspension

For spiking of food with suspension, a mixture of suspension and earthworm food was prepared, whereby 40 g of cow manure was mixed with 120 ml concentrated suspension.

Test concentration: suspension with 212 mg/L deionised water; application of 120 ml test suspension, corresponding to 10 mg/kg soil (dm).

Soil microflora - nitrification test

Spiking of soil with nanomaterial powder

For the first application, the nanomaterial powder was mixed directly into the soil, whereby air-dried test soil or silica sand (1% of the total amount) were used as carriers. Suitable amounts of powder to achieve the desired final soil content were mixed homogenously with the dry soil or the silica sand. Care was taken to avoid a modification of the crystalline structure of the nanoparticles. Uncontaminated test soil (between 20-30% of WHC_{max}) was spread on a plate, the carrier material with the powder distributed on the test soil, and all components mixed carefully. In the same way, 5 g/kg dm ground lucerne was mixed into the soil. Then, the soil was adjusted to a water-holding capacity of 55% of the maximum water-holding capacity (WHC_{max}).
Test concentrations: 9.3, 21, 45 and 100 mg/kg soil dry matter (dm)

Spiking of soil with aqueous nanomaterial dispersion

The method of the second trial was to spray a nanomaterial suspension (nanomaterial in deion. water) that had been prepared with a magnetic flea (900 rpm; 1 min) and ultrasonication (3 min) in a bath sonicator. Test soil was dried to about 10% of WHC_{max}, spread on a plate and 5 g/kg dm of ground lucerne was mixed into the soil. Immediately after preparation, TiO_2 nanoparticle suspension was sprayed onto the soil by means of a syringe coupled with a cannula and thoroughly mixed. Finally, the test soil was adjusted to a water-holding capacity of 50% of the maximum water-holding capacity. A maximum concentration of 260 mg/L application suspension was considered adequate for the tests. Higher concentrations would have sedimented rapidly which would have prevented a homogenous distribution of the nanomaterial in the soil. The maximum water content in the test soil should be about 50% of the maximum water-holding capacity. Due to these limitations, only soil contents of 9.3 and 21 mg/kg were tested. Higher concentrations achieved by several spiking – drying cycles were not studied since modification of the bioavailability of the nanomaterial by this process

could not be excluded.
Test concentrations: dispersion with 116.3 and 226.5 mg/L deionised water; application of 120 ml test dispersion to 1.5 kg test soil (dm), corresponding to 9.3 and 21 mg/kg soil (dm)

5.1.4 Ecotoxicological tests with earthworms

All tests were performed as described in the OECD Guideline 222 “Earthworm reproduction test with *Eisenia fetida*.” The earthworms were acclimatised to the test soil for 7 d prior to test start.

Adjusted to 55% of the maximum water-holding capacity, 640 g soil (dm) was added to containers at a depth of about 5 cm. All tests were performed in polypropylene containers (Bellaplast GmbH, Alf). The test was performed with four replicates for the control and four replicates for each test concentration.

For the experiments with spiked soil the contaminated soil was weighed in the test containers and 40 g (wet weight) of uncontaminated food was spread on the surface.

For the experiments with spiked food, the test containers were filled with uncontaminated soil and 40 g of contaminated food was spread on the surface.

The next day, 10 earthworms weighing between 250 mg and 600 mg were added to each container. The tests started with the introduction of *Eisenia fetida*. The containers were then incubated at 20°C ± 2°C, at a light/dark cycle of 16/8 h. Light intensity was 700 lux.

Once per week the water content was checked gravimetrically and evaporated water was replaced. Every 7 days 20 g (wet weight) of uncontaminated food per test container was spread on the soil surface. The adult earthworms were removed after 28 days. After 56 days (test end) the number of juveniles in each test container was counted.

5.1.5 Ecotoxicological tests with soil microflora

Three incubation containers (as described in 5.1.4) per treatment were filled with 658 g of spiked and lucerne meal-amended soil. A further three incubation containers were filled with 658 g of control soil. This soil was also amended with lucerne (5 g plant material per kilogram of soil (dry mass)).

The test was carried out in the dark at 20 ± 2°C for 28 days. The moisture content of the soil was maintained during the test at 40 - 60% of the WHC_{max} with a range at maximum of 5%. The mass in the test vessels was measured weekly. Evaporated water was supplemented by adding deionised water.

Two samples of each treated and control replicate were analysed for nitrate at the beginning (day 0) and at the end of the exposure period (28 days).

Nitrate analysis of soil samples:

Nitrate was extracted from soil by mixing samples (20 g dry mass) with 0.1 M KCl solution at a ratio of 5 mL of KCl solution per gram dry weight and shaking for 60 minutes at 150 rpm. The mixtures were filtered and the liquid phases photometrically analysed for nitrate (Spectroquant® NOVA 400). Analyses were performed immediately after extract preparation.

5.1.6 Chemical analyses

Particle size distribution was not determined in the dispersion, as the current methods are insufficient and the outcome does not provide information on the size distribution in soil or food. The state of the art methods are included in the publications of Fareé *et al.* (2011) and von der Kammer *et al.* (2012).

For information on homogeneity, the concentration of Ag nanoparticles in soil was determined. The only procedure for determining the TiO_2 nanoparticle concentration in soil was to use sulphuric acid which gave a combined result for Ti added as nanoparticles and Ti already present in soil. Due to high background values of Ti, added Ti was insufficiently detected. Therefore, only the results of Ag are presented. It is expected that the results of Ag can be transferred to the behaviour of TiO_2 nanoparticles. Although the chemical composition differs, the application procedure is the same: first, a suspension or a solid mixture of the is evenly distributed on a thin soil layer. Secondly,, soil and nanomaterial mixture (dispersion or solid mixture) are thoroughly mixed. Even if one nanomaterial sticks more to the soil than the other (e.g. by a different zeta potential), both materials should be distributed to a comparable extent due to the thoroughly mixing.

5.2 Results

5.2.1 Reproduction test with earthworms

The weight of the worms and the number of offspring are presented in Table 13 - Table 16.

The inhibition of reproduction and the inhibition of increase in weight affected by the test concentrations of P25 and Ag compared to the control are shown in Figure 2.

Table 13: Pre-tests: earthworm reproduction test with P25 and Ag (weight, test start).

Weight of earthworms per test vessel (10 earthworms) at test start

	Replicate 1 [g]	Replicate 2 [g]	Replicate 3 [g]	Replicate 4 [g]	Mean [g]	Standard deviation [g]
Control	3.11	3.06	3.21	2.93	3.08	0.12
P25 - soil application						
Suspension 10 mg/kg	3.82	3.62	3.70	3.73	3.72	0.08
Powder 100 mg/kg	2.99	2.86	2.78	2.76	2.85	0.11
P25 - food application						
Suspension 9.825 mg/kg	2.72	2.93	2.89	2.91	2.86	0.10
Powder 100 mg/kg	2.84	2.83	2.75	2.74	2.79	0.05
Silver - soil application						
Suspension 10 mg/kg	3.72	3.69	3.65	3.59	3.66	0.05
Powder 100 mg/kg dm	2.97	2.92	2.91	2.99	2.95	0.04
Silver - food application						
Suspension 9.840 mg/kg	2.82	2.86	3.01	2.96	2.91	0.09
Powder 100 mg/kg	3.09	3.25	3.23	2.78	3.09	0.22

Table 14: Pre-tests: earthworm reproduction test with P25 and Ag (weight, test end).

Weight of earthworms per test vessel (10 earthworms) at test end

	Replicate 1 [g]	Replicate 2 [g]	Replicate 3 [g]	Replicate 4 [g]	Mean [g]	Standard deviation [g]
Control	5.17	6.10	5.56	5.91	5.68	5.17
P25 - soil application						
Suspension 10 mg/kg	5.17	4.81	4.83	4.90	4.93	0.17
Powder 100 mg/kg	6.09	5.89	5.81	5.80	5.90	0.14
P25 - food application						
Suspension 9.825 mg/kg	5.35	5.68	5.63	5.66	5.58	0.15
Powder 100 mg/kg	5.25	5.22	5.21	5.14	5.20	0.05
Silver - soil application						
Suspension 10 mg/kg	4.85	4.90	4.73	4.71	4.80	0.09
Powder 100 mg/kg TM	5.89	5.48	6.35	6.26	6.00	0.40
Silver - food application						
Suspension 9.840 mg/kg	5.61	5.88	5.83	5.60	5.73	0.15
Powder 100 mg/kg	4.69	4.61	4.09	4.37	4.44	0.27

Table 15: Pre-tests: earthworm reproduction test with P25 and Ag (weight increase, test end).

Weight increase of earthworms per test vessel (10 earthworms) at test end

	Replicate 1 [g]	Replicate 2 [g]	Replicate 3 [g]	Replicate 4 [g]	Mean [g]	Standard deviation [g]	Statistical signifi-cance [1]
Control	2.06	3.04	2.35	2.98	2.61	0.48	
P25 - soil application							
Suspension 10 mg/kg	1.35	1.18	1.13	1.17	1.21	0.10	*
Powder 100 mg/kg	3.10	3.02	3.03	3.04	3.05	0.04	n.s.
P25 - food application							
Suspension 9.825 mg/kg	2.63	2.75	2.74	2.75	2.72	0.06	n.s.
Powder 100 mg/kg	2.40	2.39	2.46	2.40	2.41	0.03	n.s.
Silver - soil applica-tion							
Suspension 10 mg/kg	1.13	1.21	1.09	1.11	1.13	0.05	*
Powder 100 mg/kg dm	2.92	2.56	3.44	3.28	3.05	0.39	n.s.
Silver - food applica-tion							
Suspension 9.840 mg/kg	2.79	3.02	2.82	2.64	2.82	0.16	n.s.
Powder 100 mg/kg	1.61	1.36	0.87	1.60	1.36	0.35	*

[1] statistical significance: * 0.05 ≥ p > 0.01; ** 0.01 ≥ p > 0.001; * p ≤ 0.001; n.s. = not significant

Table 16: Pre-tests: earthworm reproduction test with P25 and Ag (number of offspring).

Number of offspring per test vessel (10 adult earthworms) at test end

	Replicate 1 [g]	Replicate 2 [g]	Replicate 3 [g]	Replicate 4 [g]	Mean [g]	Standard deviation [g]	Statistical signifi-cance [1]
Control	243	295	307	323	292.0	34.6	
P25 - soil application							
Suspension 10 mg/kg	264	270	283	221	259.5	26.9	n.s.
Powder 100 mg/kg	212	291	248	222	243.3	35.3	n.s.
P25 - food application							
Suspension 9.825 mg/kg	259	214	229	299	250.3	37.5	n.s.
Powder 100 mg/kg	208	242	229	204	220.8	17.9	*
Silver - soil applica-tion							
Suspension 10 mg/kg	291	292	224	246	263.3	33.8	n.s.
Powder 100 mg/kg dm	290	197	249	282	254.5	42.2	n.s.
Silver - food applica-tion							
Suspension 9.840 mg/kg	269	296	209	235	252.3	38.1	n.s.
Powder 100 mg/kg	161	172	178	179	172.5	8.3	*

[1] statistical significance: * 0.05 ≥ p > 0.01; ** 0.01 ≥ p > 0.001; * p ≤ 0.001; n.s. = not significant

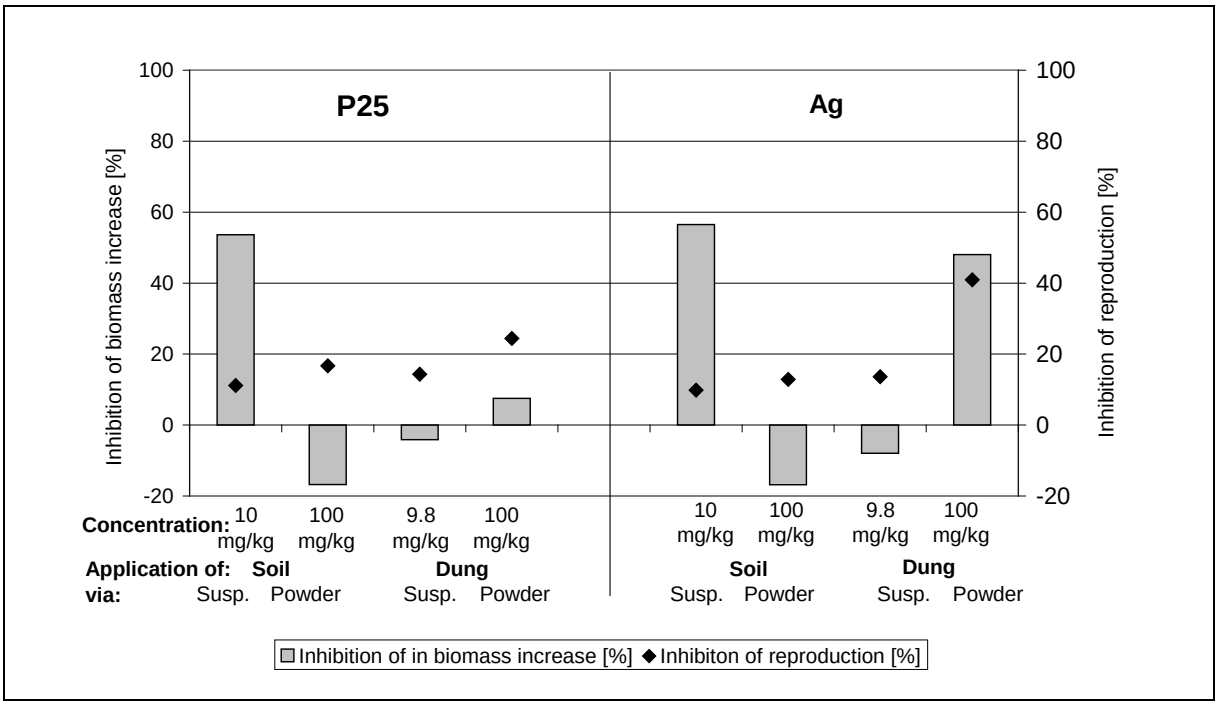


Figure 2: Pre-tests: earthworm reproduction test with P25 and Ag.

Percent inhibition of biomass increase and percent inhibition of reproduction compared to control (negative values indicate stimulation)

Effects on biomass and reproduction differ. Reproduction was inhibited at higher test concentrations, the increase in biomass showed no concentration dependency. Generally, the parameter biomass is difficult to assess. According to the guideline biomass has to be measured but it is no parameter for which ECx or NOEC values have to be reported.

No clear differences in effect were observed between the application in dung or soil and application via dispersion or powder.According to the guideline OECD 222 the relevant endpoint is reproduction. Inhibition of reproduction was only observed for the 100 mg/kg powder application (Ag and TiO_2 nanoparticles) in dung.

5.2.2 Soil microflora - nitrification activity

Experiments with silver nanoparticles

Three experiments differing in application forms (in silica sand, in soil, via suspension) and tested concentrations of nanoparticles were conducted.

1st experiment: Comparison of three spiking forms

- Spiking via soil and silica sand as solid carrier; two test concentrations (10 and 100 mg/kg)
- Spiking via dispersion; one test concentration resulting in a concentration of 10 mg/kg in soil; a concentration in soil of 100 mg/kg was not considered suitable; the concentration in the test suspension was considered to be too high and large agglomerates were expected.

2nd experiment: Comparison of two spiking forms – valdidation of the results for the most promising procedures of the 1st experiment

- Spiking via soil as solid carrier; two test concentrations (10 and 100 mg/kg); based on the results of the first experiment spiking using silica sand was considered less suitable.
- Spiking via dispersion (10 mg/kg)

3rd experiment: Comparison of two spiking forms – three test concentrations

- Spiking via soil as solid carrier; two test concentrations (10, 50, 100 mg/kg); based on the results of the first experiment spiking using silica sand was considered less suitable.
- Spiking via dispersion (10, 50, 100 mg/kg)

The microbial nitrification activity was used as indicator for ecotoxicity. A compilation of the results is shown in Figure 3. A comparison of the results obtained for the soil contents of 10 and 100 mg/kg indicates lowest effects for the application via silica sand, followed by the application via soil. At 10 mg/kg the highest effects are obtained with the application via suspension. A slightly increased toxicity is obtained at 50 mg/kg for the application via suspension. At 100 mg/kg no difference between an application via soil and suspension is obtained. Both application forms almost completely inhibit the nitrification activity. From these results it can be concluded that the bioavailability of the nanoparticles is reduced in the presence of silica sand. Therefore, this application form was not considered in the main experiments. Due to the comparable results for spiking via soil and dispersion in the 3rd experiment for the lowest test concentration (10 mg/kg) which did not correspond to the results of the 2nd experiment no information on the most suitable application form was achieved.

The standard deviation of the nitrification activity in the replicates was used as an indicator for the homogeneity of spiking. To determine the nitrification activity four soil samples (20 g each) were analysed. A high standard deviation of the activity values indicated a non-homogeneous distribution of the test substance. For this assessment only activity values that were in a medium inhibition range compared to the control were suitable as high inhibition results in low activity values. Even small differences in the activity values result in a comparable high percent standard deviation. Activity values which are only slightly reduced compared to the control are not applicable. In Figure 4 the standard deviation of suitable soil samples is presented. It is obvious that the application of dry powder as well as the application of suspensions can result in standard deviations which are in the range of the standard deviations of the control. However, for every application form outliers were observed (e.g.: experiment 1, application via silica sand results in a soil content of 100 mg/kg; experiment 3, application via suspension results in a soil content of 50 mg/kg). Therefore, both application techniques (application of dry powder and suspensions) are theoretically suitable. The reason for the outliers, however, is not yet understood.

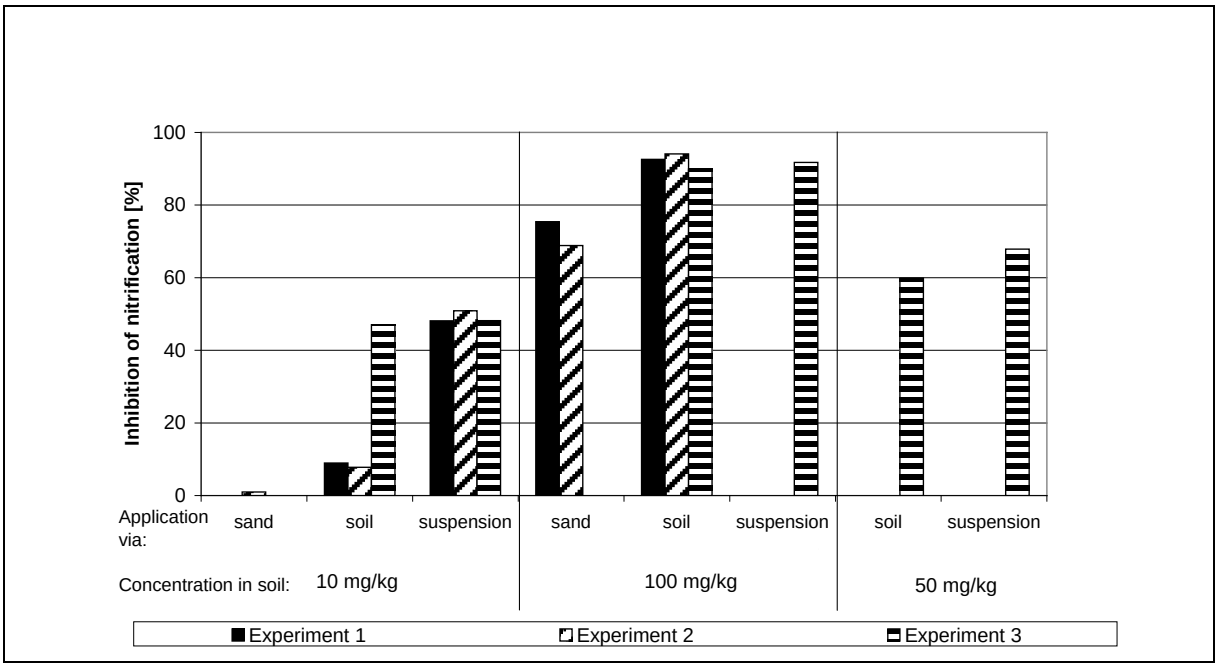


Figure 3: Pre-tests: effects of different application forms for Ag nanoparticles on nitrification activity.

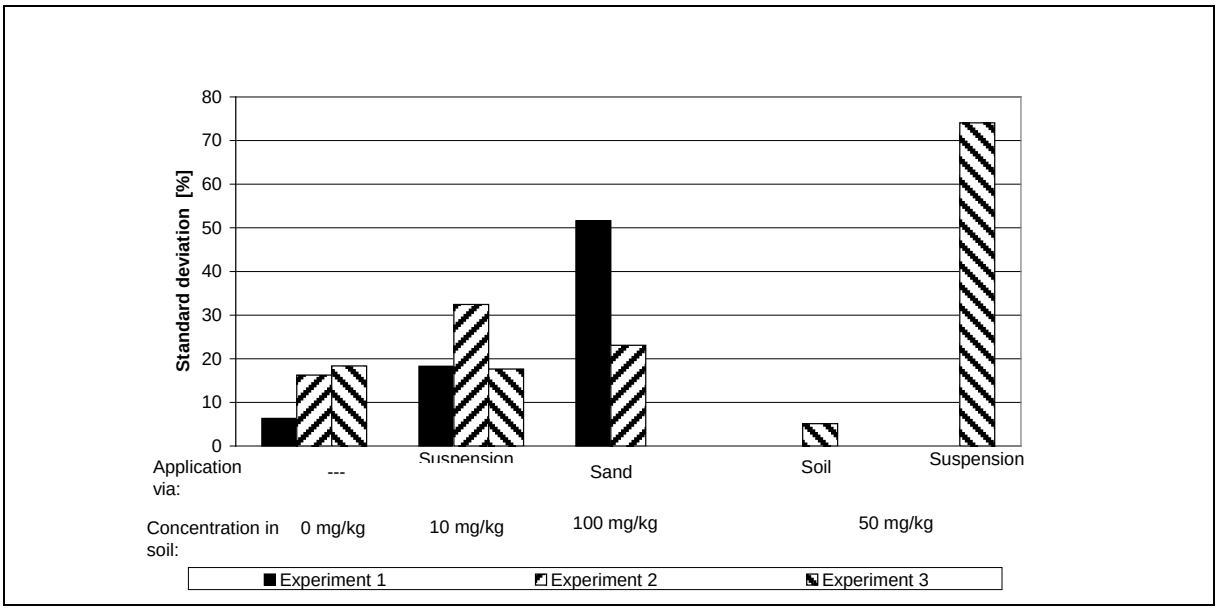


Figure 4: Pre-tests: standard deviation of nitrification activity in the nitrification tests with Ag resulting in ~50% inhibition.

Experiments with P25

The toxicity of P25 is much lower compared to the toxicity of silver (Figure 3 and 5). Therefore, information concerning suitable application techniques of P25 is limited. Nevertheless, it was observed that P25 application via soil achieved higher bioavailability compared to application via silica sand (Figure 5). Application via suspension resulted in no inhibition effect at all. It is assumed that the high concentration in the dispersions used for spiking showed a high agglomeration rate. The dispersions were very polydisperse and the particle size could

not be determined. From the results it is concluded that high test concentrations can be achieved by dry application whereas by wet application the maximum test concentration can be limited by the agglomeration behaviour of the nanoparticles in the dispersion.

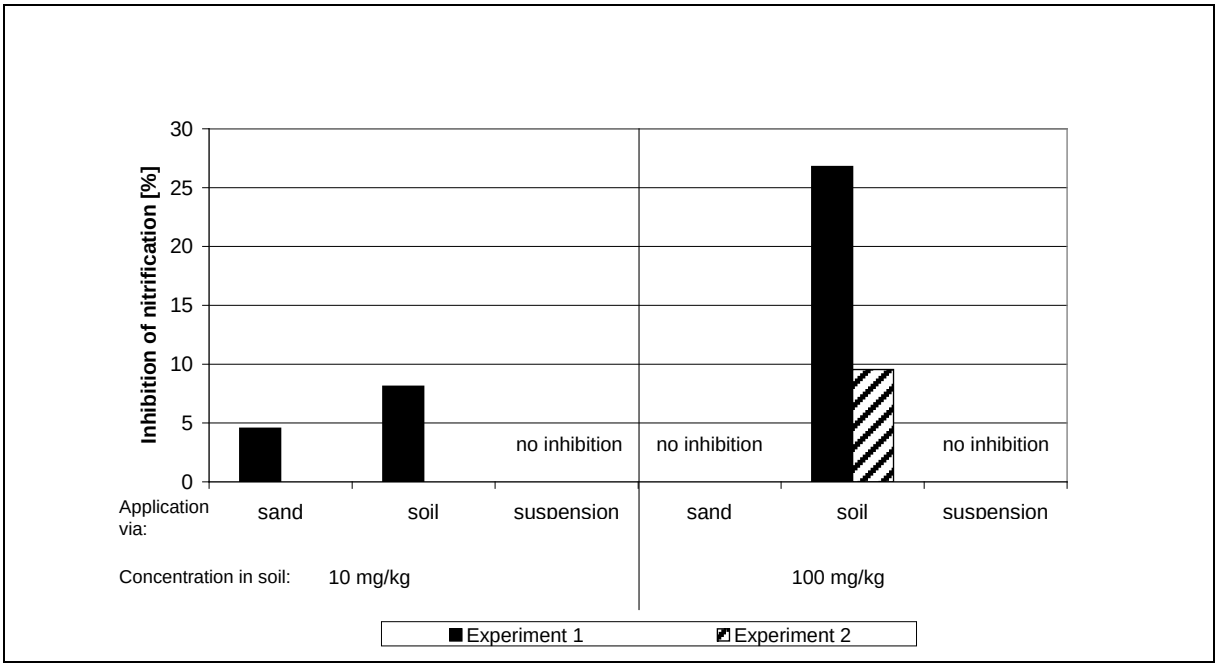


Figure 5: Pre-tests: effect of different application forms of P25 on nitrification activity.

5.2.3 Chemical analyses

Silver analyses NM-300K in the earthworm test

The pre-tests presented above were performed with material purchased from Sigma Aldrich and distributed as powder. This material was selectedas the OECD nanomaterial was not yet available. It had been expected that the OECD material would be available in solid form (powder). However, the NM-300K was received as a stabilised dispersion. To get information about the homogenous distribution of the NM-300K material soil samples from the earthworm test (see main test presented below) were used for chemical analyses. Soil spiked with NM-300K using a small amount of soil as carrier was investigated.

The results for the application via soil are presented in Table 17.

Table 17: Homogeneity of spiking: recovery of NM-300K in soil (earthworm test).

Five replicate samples, each measured twice

Application / sample		weighed for digestion [g]	measured Ag conc [µg/L]	dilution factor	calculated Ag conc. [mg/kg]	nominal Ag conc. [mg/kg]	recovery [%]	mean recovery ± SD [2]
Control	1	3.13	3.24	-	< LOD	-	-	-
	2	3.13	3.14	-	< LOD	-	-	
	3	3.08	3.28	-	< LOD	-	-	
	4	3.16	2.44	-	< LOD	-	-	
	5	3.16	3.04	-	< LOD	-	-	
Application via soil: 120 mg/kg	1-1	3.10	307	10	99.03	120	82.5	89.6 ± 4.4
	1-2	3.11	332	10	106.78	120	89.0	
	2-1	3.13	321	10	102.54	120	85.4	
	2-2	3.10	359	10	115.77	120	96.5	
	3-1	3.06	321	10	105.01	120	87.5	
	3-2	3.17	356	10	112.27	120	93.6	
	4-1	3.13	349	10	111.57	120	93.0	
	4-2	3.12	338	10	108.63	120	90.5	
	5-1	3.12	319	10	102.35	120	85.3	
	5-2	3.11	347	10	111.63	120	93.0	
Application via soil: 15 mg/kg	1-1	3.07	93.6	5	15.25	15	102	90.0 ± 14.5
	1-2	3.12	74.0	5	11.86	15	79.0	
	2-1	3.11	69.5	5	11.16	15	74.4	
	2-2	3.04	85.1	5	14.01	15	93.4	
	3-1	3.04	84.5	5	13.89	15	92.6	
	3-2	3.12	77.1	5	12.35	15	82.3	
	4-1	3.05	91.9	5	15.06	15	100	
	4-2	3.05	78.8	5	12.91	15	86.1	
	5-1	3.13	110	5	17.56	15	117	
	5-2	2.63	54.2	5	10.29	15	68.6	

[1] LOD = limit of detection (11.5 µg/L); [2] SD = standard deviation

With 90% each for the low and high test concentrations, recovery was satisfactory. Powder in the concentration of 120 mg/kg was more homogenously distributed than in concentrations of 15 mg/kg despite the same amount of carrier soil used for both concentrations. Nevertheless, 15% standard deviation was considered to be in an acceptable range.

After elaboration of the results of the main tests, we could also include biological variability in the considerations concerning the acceptance of the homogeneity of spiking. We selected 68 results (mean values and standard deviations) with either TiO_2 or Ag nanoparticles from randomly-selected tests of this project with different test organisms and test parameters, including earthworm reproduction, plant growth and microbial nitrogen and carbon transformation. The same criteria were applied to tests with conventional chemicals performed at the Fraunhofer-Institute (60 test results, mean values and standard deviations). Each standard deviation was expressed as a percentage of the respective mean value. The 90% percentile of the standard deviations for both sets of tests (nanoparticles and conventional chemicals) was

calculated. Ninety percent of the standard deviations in the nanomaterial tests were in the range 2–17% compared to 3–24% in the conventional chemical tests. The variability of the chemical analysis results was comparable to the variability of the ecotoxicological test results with nanoparticles. Furthermore, the variability of the nanomaterial tests based on dry spiking using soil as the carrier was comparable to the variability of the conventional substance tests spiked with aqueous solutions. We therefore concluded that the dry spiking procedure using soil as the carrier achieves adequate spiking homogeneity.

Silver analyses (Sigma Aldrich) in soil samples of the nitrification test

Due to the high background values of titanium in the applied soil only silver analyses of the soil samples are presented. Due to high concentration levels the samples had to be diluted prior to analysis to fit with the working and calibration range of the instrument (ICP-OES). The silver concentrations measured after digestion in the control soil samples were below the limit of detection (< 0.122 - < 0.819 mg/kg). Table 18 summarizes the measured silver concentrations in soil.

Table 18: Homogeneity of spiking: recovery of silver in soil (nitrification test).

Silver: Sigma Aldrich; six replicate samples

Application / sample		Weighed for digestion [g]	Measured Ag conc [µg/L]	Dilution factor	Calculated Ag conc. [mg/kg]	Nominal Ag conc. [mg/kg]	Recovery [%]	Mean recovery ± SD [2] [%]
Control		3.024	< LOD	2	< LOD [1]	-	-	-
Control		3.008	< LOD	2	< LOD [1]	-	-	-
Application via soil: 100 mg/kg	1	3.024	45.9	50	75.9	100	75.9	80.4 ± 3.1
	2	3.025	49.9	50	81.7	100	81.7	
	3	3.030	50.8	50	83.9	100	83.9	
	4	3.007	49.3	50	81.9	100	81.9	
	5	3.007	1184	2	78.7	100	78.7	
	6	3.043	1172	2	77.0	100	77.0	
Application via dispersion: 100 mg/kg	1	3.010	1229	2	81.7	100	81.7	80.6 ± 1.6
	2	3.035	1106	2	72.9	100	72.9	
	3	3.012	1206	2	80.1	100	80.1	
	4	3.002	1236	2	82.3	100	82.3	
	5	3.002	1230	2	81.9	100	81.9	
	6	2.999	1181	2	78.8	100	78.8	
Application via soil: 10 mg/kg	1	3.031	119	2	7.85	10	78.5	76.2 ± 8.8
	2	3.023	99.5	2	6.58	10	65.8	
	3	3.026	101	2	6.66	10	66.6	
	4	3.019	117	2	7.76	10	77.6	
	5	3.015	132	2	8.74	10	87.4	
	6	2.998	109	2	7.30	10	73.0	
Application via dispersion: 10 mg/kg	1	2.999	73.8	2	4.92	10	49.2	47.2 ± 1.6
	2	3.013	79.0	2	5.24	10	52.4	
	3	3.043	68.9	2	4.53	10	45.3	
	4	3.008	73.9	2	4.91	10	49.1	
	5	3.014	70.8	2	4.69	10	46.9	
	6	3.004	71.3	2	4.74	10	47.4	

[1] LOD = limit of detection (< 0.122 - < 0.819 mg/kg); [2] SD = standard deviation

The recovery determined for application via dispersion (10 mg/kg) was low. As the experiments focused on the characterisation of the homogeneity of spiking, no further sourcing concerning the low recovery was performed. Six replicates sampled at different spots of the soil were analysed. The standard deviation of the recovery was between 1.6 and 8.8 %. To receive information on the homogeneity, the recovery was considered to be 100 % and the standard deviation was calculated as percent of the recovery. Values between 2.0 and 11.5% were calculated (e.g.: 80.6 % was considered to be 100 %; 1.6 of 80.6 amounts to 2.0 %). A variability between 2.0 and 11.5 % is considered to be acceptable for biological analyses. The observed outliers in the nitrification test resulting in high standard deviation (Figure 4) cannot be explained by non-homogeneous spiking.

5.2.4 Conclusions

As the toxicity for silver was higher than that for P25, the following conclusions drawn for the most suitable application technique are based on the results obtained for silver:

- Based on the lower effects - indicating lower bioavailability – obtained upon the application via silica sand compared to the application via soil and suspension, the application via silica sand is considered less suitable.
- Application via powder allows a high variability of the test concentrations.
- Application via liquid suspension may cause a higher bioavailability of the elements.
- For earthworms, spiking of dung may result in a higher toxicity for earthworms compared to the spiking of soil. However, spiking of soil is the method described in the respective guidelines.

To obtain further results the following procedure was applied in the main tests:

- TiO_2 nanoparticles: application via suspension and via solid carrier (soil) in soil as well as via suspension (all tests) and directly in the form of powder in dung (tests with earthworms; due to the high amount of nanomaterial added to dung, no carrier was considered to be necessary)
- Ag nanoparticles: application via solid carrier (soil) in soil and directly in dung

6 Pre-tests - dispersion in aquatic tests

As mentioned in chapter 5 documents referring specifically to the application of nanoparticles for ecotoxicological tests are not available. For tests with daphnids and chironomids the method described by Hund-Rinke *et al*. (2010) was used, and supplementing studies concerning filtration and the use of stabilisers were performed.

6.1 Basic procedure

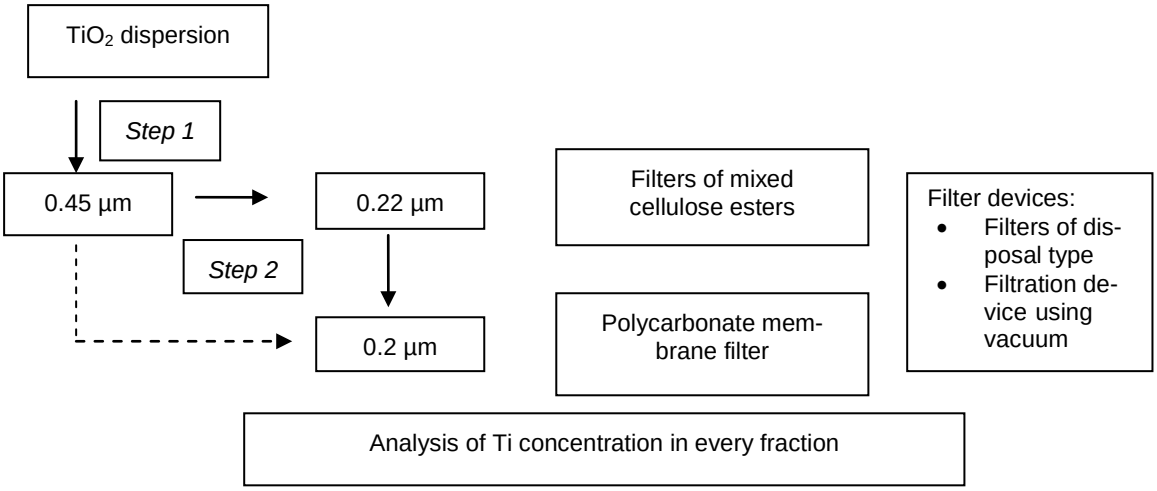
The method described by Hund-Rinke *et al*. (2010) was applied. For insoluble nanoparticles in powder form the required amounts were weighed in brown glass vessels (600 mL) using a suitable balance. Test medium was added, the mixture was stirred (magnetic stirrer, 900 rpm) and ultrasonified (3 min, 500 W) in a bath sonicator (Bandelin Sonorex RK 514 BH; 35 kHz; 215/860 W) filled with water to one third of the dispersion height in the bottles. For concentrations in the range of 5 - 100 mg/L every concentration was prepared individually. For concentrations below 5 mg/L concentrated stock suspensions were prepared in most cases. For the test with daphnids, a 20 mg/L stock dispersion was used.

For silver (NM-300K) stabilised in an aqueous medium suitable stock dispersions were prepared in the test medium. A homogenous distribution was achieved via stirring.

6.2 Filtration

The method to be applied for the exposure of daphnia was elaborated in pre-tests. According to the guideline, the test substance is dissolved in the exposure medium, the daphnia are added and the incubation is performed without any movement of the vessels or the exposure medium. This is a suitable procedure for soluble test substances. Nanoparticles, however, will sediment in tests without movement and the exposure concentration in the test media will decrease. Stirring or shaking during the incubation period is not advisable as daphnids and their reproduction rate are sensitive towards such procedures. Therefore, it was investigated whether a stabile dispersion can be achieved by elimination of the large agglomerates via filtration.

The following procedure was applied:



Two different filter types and two different filter devices were tested.

Filters: Mixed cellulose esters combined with filters of the disposal type

Polycarbonate membrane filter combined with a filtration device using vacuum

In a first step the dispersion of TiO_2 nanoparticles (P25; 10 mg/L) was filtered using a filter with a pore size of 0.45 µm (mixed cellulose esters). In a second step the filtrate was filtered again using a filter with a pore size of 0.2 (polycarbonate membrane filter) or 0.22 µm (mixed cellulose filter). In every fraction the Ti concentration was analysed.

In contrast, with mixed cellulose filters, almost no filtrate was obtained using polycarbonate membrane filters even when the filters were changed several times during the filtration process.

In Table 19, representative results from several filtration experiments with both filter types and filter devices are presented. In the first experiment different pore sizes were used for the two filtration processes in accordance with the scheme above. Although the primary particle diameter of P25 was 21 nm, almost no Ti was detected in the filtrate. Particles and agglomerates mainly remained above the filter. The maximum recovery in the filtrate of step 1 was 0.01%. Using the filtration device with vacuum, in step 2 a slightly higher Ti concentration was measured than in the filtrate of step 1. It is assumed that some agglomerates of the first filtration process remained in the filter device although the device was carefully cleaned before the second filtration process started. During the second filtration process the remaining agglomerates were rinsed in the new filtrate resulting in the increased Ti concentrations. For justification a second experiment was performed. In this experiment the dispersion was filtered twice with a filter of the same pore size. Again, a higher concentration of Ti was measured in the second filtrate when using vacuum filtration.

Between replicate samples the results can differ by a factor of 2 - 4. For ecotoxicological experiments with concentration-effect curves, concentrations differing by a factor of 2 - 3 have to be investigated. As the concentrations of the replicate samples differed by a factor of 2 - 4 no clear dose-response curves were expected by investigating the filtrates. Furthermore, filtration is very time consuming and does not seem to be suitable for routine testing. There-

fore, the testing of filtrates was cancelled for the test with daphnids and original dispersions were tested.

Table 19: Ti concentration in the filtrate of a TiO_2 dispersion after diverse filtration processes.

TiO_2 nanoparticles: 10 mg/L; Ti: 6 mg/L; filter material: mixed cellulose ester

	Filtration - Step 1	Filtration - Step 2
First experiment: step 1 and step 2: different pore sizes		
Filter	0.45 µm cellulose mixed ester	0.22 µm cellulose mixed ester
Filtration device using vacuum	Sample 1: 0.62 µg/L Sample 2: 0.33 µg/L	Sample 1: 1.27 µg/L Sample 2: 1.43 µg/L
Filter	0.45 µm cellulose mixed ester	0.2 µm polycarbonate membrane filter
Filters of the disposal type	Sample 1: 0.17 µg/L Sample 2: 0.17 µg/L	Sample 1: 0.26 µg/L Sample 2: 0.13 µg/L
Second experiment: step 1 and step 2: same pore sizes		
Filter	0.22 µm cellulose mixed ester	0.22 µm cellulose mixed ester
Filtration device using vacuum	Sample 1: 0.34 µg/L Sample 2: 1.39 µg/L	Sample 1: 2.87 µg/L Sample 2: 2.24 µg/L
Filter	0.2 µm polycarbonate membrane filter	0.2 µm polycarbonate membrane filter
Filters of the disposal type	Sample 1: 0.55 µg/L Sample 2: 0.22 µg/L	Sample 1: 0.17 µg/L Sample 2: 0.26 µg/L

6.3 Stabilisers

The investigation of stabilisers was not within the scope of this project as stabilisers are assumed toxic. However, due to many discussions in the scientific community, and especially the recommendations of scientists dealing with the preparation of homogenous test substances, the effect of the stabiliser sodium hexametaphosphate (0.01 %) was investigated. To give a complete overview on the experiments dealing with application methods for nanoparticles performed at Fraunhofer IME, the results are presented in the following. Experiments focusing on the preparation of homogenous test suspensions used concentrations of about 1 % (10 g/L). In the test guidelines for aquatic ecotoxicological tests a maximum concentration of 0.01 % is fixed for the use of solubilisers for organic chemicals insoluble in water. In our project, the same limit was applied for the stabilisers used for nanoparticles. The test was performed in Erlenmeyer flasks with TiO_2 nanoparticles and algae as a growth test according to the OECD test guideline 201 (Hund-Rinke *et al.*, 2010). The addition of the stabiliser resulted in stable suspensions. No sedimentation of the test substance occurred. Although the stabilizer was applied in the maximum tolerable concentration mentioned for stabilizers in the test guidelines a toxic effect was still observed and growth of algae was significantly reduced . However, in the presence of the stabiliser, TiO_2 nanoparticles (P25) affected the growth of algae less than without stabiliser (Figure 6). Bioavailability of P25 to algae is reduced in the presence of a stabiliser although the test suspension has a better homogeneity.

In the first step of hazard assessment, ecotoxicological tests must simulate a worst case scenario. The use of stabilisers at this stage should guarantee that the level of ecotoxicity does not decrease. As shown in Figure 6, the use of a stabiliser can reduce bioavailability. Therefore, the application and the benefit need to be justified.

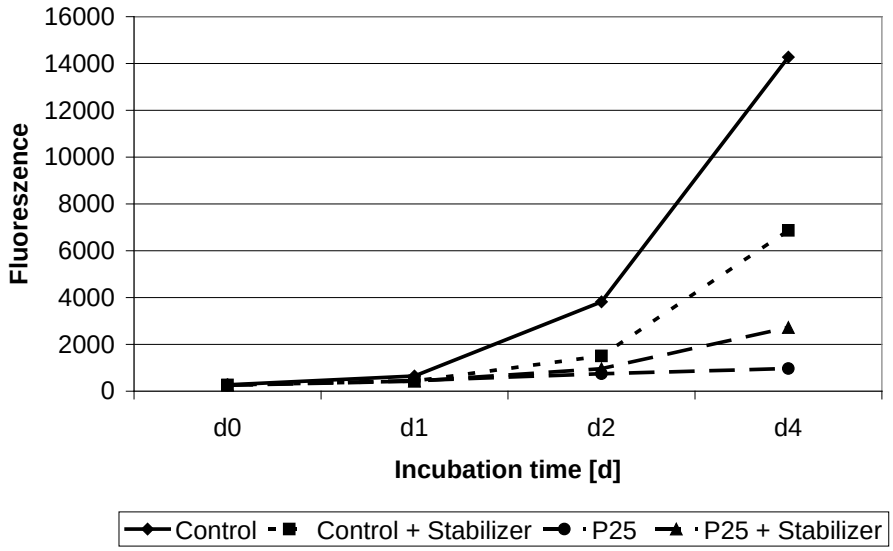


Figure 6: Effect of stabilisers (sodium hexametaphosphate, 0.01%) and P25 in the growth test with algae.

7 Reproduction Test with Earthworms (OECD TG 222) – TiO_2

7.1 Test principle

Adult earthworms of the species *Eisenia fetida* were placed in a defined soil containing different concentrations of the test item. The test item was applied once and the effects on biomass and mortality of the adult worms were determined after 28 days. After 56 days effects on reproduction were determined by counting the offspring.

In addition to the test guideline, accumulation in the adults was tested after the exposure period of 28 days.

7.2 Materials and methods

7.2.1 Test guideline

The test was performed according to:

OECD Guidelines for the Testing of Chemicals Test No. 222: Earthworm Reproduction Test (*Eisenia fetida, Eisenia andrei*) (2004).

7.2.2 GLP

The test was performed following the principles of GLP (OECD, 1998). In deviation to GLP no archiving of the raw data was performed and the quality assurance unit was not involved with respect to the inspection of the test, of the raw data, and the report. All laboratory equipment (e.g. balances, thermometers, pH-meters) was controlled and documented according to GLP.

7.2.3 Test material

- P25 - distributed by Evonik for the OECD Sponsorship Programme
  The properties should correspond to the properties of NM-105.
- NM-101
- NM-103

The test substances were stored in the dark at room temperature.

7.2.4 Analytical monitoring

Due to the high natural concentration of TiO_2 in the test soil no specific chemical analyses were performed for this medium.

Zeta potential was measured in the test dispersions using a Zetasizer Nano ZS. Following instrument settings were applied: (I) refractive index: 2.55; (II) adsorption: 0.073. The particle

size distribution was not determined due to: (i) the high concentrations of TiO_2 nanoparticles precluded a determination of the particle size distribution, (ii) size distribution in the dispersion would give no information on the size distribution in soil or feed. At present, measurement of the Zeta potential or particle size distribution in soil is not possible.

Ti was determined in the earthworms. Earthworms were incubated for 24 h on wet filter paper to purge their guts. Afterwards they were frozen (-20°C) until analysis.

7.2.5 Test item – preparation protocol

Four different modes of application were tested.

Spiking of soil with TiO_2 powder

For the first application the TiO_2 powder was mixed directly into the soil, whereby air-dried test soil (1% of the total amount) was used as a carrier. Suitable amounts of TiO_2 powder to achieve the desired final soil content were mixed homogenously with the dry soil. Care was taken to avoid a modification of the TiO_2 crystalline structure. Uncontaminated test soil (between 20 and 30% of WHC_{max}) was spread on a plate, the carrier material with the TiO_2 powder was distributed on the test soil, and all was mixed carefully. For the test with contaminated soil, the soil was adjusted to a water-holding capacity of 55% of the maximum water-holding capacity (WHC_{max}).

Test concentrations were: 50, 100 and 200 mg/kg soil dry matter (dm).

Spiking of feed with TiO_2 powder

The second application was the direct introduction of TiO_2 nanoparticles into the earthworm feed, which consisted of antibiotics-free cow manure. In all four replicates, 40 g of air-dried ground cow manure were homogenously mixed with TiO_2 powder. The mixture was moistened with 120 ml deionised water.

Test concentrations were: 3.19, 6.38 and 12.76 mg/g feed (dm) corresponding to 50, 100 and 200 mg/kg soil (dm); 40 g moist feed (10 g dry feed and 30 ml deionised H_2O) were applied on the surface of the 1-L test containers, each of which was filled with 640 g soil (dm).

Spiking of soil with aqueous TiO_2 dispersion

The third application trial was to spray a TiO_2 dispersion in deionised water onto the soil. The TiO_2 dispersion was prepared with a magnetic flea (900 rpm; 1 min) and ultrasonication (3 min) in a bath sonicator. Test soil was dried to about 10% of WHC_{max} and spread on a plate. Immediately after preparation, the TiO_2 dispersion was sprayed onto the soil by means of a syringe coupled with a cannula and then thoroughly mixed with the soil. Finally, the test soil was adjusted to a water-holding capacity of 55% of WHC_{max}. A maximum concentration of about 200 mg/L application dispersion of TiO_2 nanoparticles was considered as adequate for the tests. Higher concentrations would have sedimented rapidly preventing a homogenous distribution of the nanomaterial in the soil. Furthermore, it was assumed that higher concen-

trations in the application dispersion would result in larger agglomerates. The maximum water content in the test soil should be about 55% of the maximum water-holding capacity. Based on the present water content of the soil, 212 mg/L application dispersion had to be used. The suspension was continuously stirred to achieve homogeneity during spiking. Due to these limitations, only soil contents of 10 and 20 mg/kg were tested.

Test concentrations were: dispersion with 100 and 200 mg/L deionised water; application of 250 ml test dispersion to 2.5 kg test soil (dm) corresponding to 10 and 20 mg/kg soil (dm).

Spiking of feed with aqueous TiO_2 dispersion

The fourth and final type of application was a mixture of TiO_2 dispersion and earthworm feed, whereby 40 g of cow manure was mixed with 120 ml concentrated TiO_2 dispersion.

Test concentrations were: dispersion with 212 and 424 mg/L deionised water; application of 120 ml test dispersion corresponding to 10 and 20 mg/kg soil (dm)

7.2.6 Test species

The test organisms were synchronised adult earthworms of the species *Eisenia andrei* (Annelida, Oligochaeta), which were 2 - 12 months old, with a clitellum, and a wet mass between 250 mg and 600 mg.

Origin of the worms:	Regenwurmfarm Tacke, Klosterdiek 61, 46325 Borken. Specimens used in the test were bred in the laboratory of the Fraunhofer IME.
Breeding conditions:	Worms were bred in 1:1 mixtures of cow manure and sphagnum peat (dry mass basis) at 20°C ± 2°C.
Pre-treatment:	The worms were conditioned in the artificial soil for 7 days before use. The same feed as used in the test (see 9.3) was given in a sufficient amount.

7.3 Study design

7.3.1 Study type

Laboratory test

7.3.2 Test duration type and exposure period

Long-term test

The exposure period was 56 days.

- P25: 28 January – 25 March 2010; 19 May – 14 July 2010; 28 January – 25 March 2011

- NM-101: 18 February – 14 April 2010; 21 January - 18 March 2011
- NM-103: 7 April – 2 June 2010; 21 January – 18 March 2011

7.3.3 Test substrate

The soil used in the test was a natural sandy soil (certified RefeSol 01-A, batch IME-01: sand 71%, silt: 24%, clay: 5%, Org C: 0.93%, pH 5.7, clay: 5%). The soil was sieved to ≤ 2 mm. The soil was not sterilised and had been stored outdoors in high-grade stainless steel basins with drainage and ground contact at the test facility.

7.3.4 Total exposure period

56 days

7.3.5 Post exposure period

There was no post exposure period.

7.4 Test conditions

7.4.1 Environmental conditions

The incubation temperature was measured continuously with a thermograph. According to the guideline the permitted range is 20 ± 2 °C. A controlled light/dark cycle of 16 h:8 h was applied. The light intensity was measured using an illuminance meter (MINOLTA) with photometric sensor in Lux. According to the guideline the permitted value is about 600 lux. The test conditions are presented in Table 20.

Table 20: TiO_2: Incubation conditions in the reproduction test with earthworms.

	P25 first test	P25 second test	P25 third test	NM 101 first test	NM 101 second test	NM 103 first test	NM 103 second test
Incubation temperature [°C]	18 – 20	20 – 22	19 – 21	19 – 21	19 – 21	19 – 21	19 – 21
Light intensity [lux]	600 – 800	600 – 800	600 – 800	600 – 750	600 – 800	600 – 750	600 – 800
Soil dry mass [%]	81 - 89	79 - 90	80 - 90	79 - 90	81 - 89	80 - 90	81 - 88
pH (1 mol/L KCl) – test start	4.9	5.1	5.1 – 5.4	4.8 - 4.9	5.0	4.9 - 5.0	5.0
pH (1 mol/L KCl) – test end	6.4 – 6.5	6.5 – 6.6	6.7 – 6.9	6.2 – 6.4	6.7 – 6.9	6.2 – 6.6.	6.8 – 6.9

7.4.2 Test concentrations

TiO_2

The following nominal contents were applied in the test containers with TiO_2 nanoparticles:

50, 100, 200 mg/kg soil, dry mass (application via powder on soil)

50, 100, 200 mg/kg soil, dry mass (application via powder on feed)

10, 20 mg/kg soil, dry mass (application via dispersion on soil)

10, 20 mg/kg soil, dry mass (application via dispersion on feed)

Additionally, the following concentrations were investigated in the second and third test with P25:

50, 100, 200, 500, 750, 1000 mg/kg soil, dry mass (application via powder on soil)

The following concentrations were investigated in the second test with NM 101 and NM 103: 50, 100, 200, 400 mg/kg soil, dry mass (application via powder on soil).

7.4.3 Other information on materials and methods

Frequency of treatment

Treatment was applied once at test start.

Control group and treatment

The control consists of soil. Eight replicates per control were conducted.

Statistical method

Data evaluation

In this report numerical values are frequently rounded to a smaller degree of precision (number of digits) than used in the actual calculation. Minor differences in the results obtained from calculations with rounded values compared to results obtained with higher precision values are possible. They are, however, well within the limits of the experimental accuracy and of no practical concern.

Statistical calculations

For each concentration the percent mortality, the percent loss/increase in biomass of the adults, and the number of offspring produced in the test was determined.

Mortality, biomass and number of offspring were compared by a suitable test for multiple comparisons with a control after testing variance homogeneity. All statistical tests were performed with the computer software ToxRat Professional version 2.10.4.1 (ToxRat® Solutions GmbH).

Feed

Air-dried, finely ground cow manure was used as feed.

Test container

All tests were performed in polypropylene containers (Bellaplast GmbH, Alf). Adjusted to 55% of the maximum water-holding capacity, 640 g soil (dm) was filled into containers to a depth of about 5 cm. The containers were covered with transparent plastic lids to prevent worms from escaping and to guarantee access of light. The lids had several small holes to permit gaseous exchange between the medium and the atmosphere.

Test procedure

Soil and food were spiked. Test soil was filled in the test containers and an amount of 10 g air dried, finely ground cow manure per test container was spread on the soil surface and moistened with water. The next day (start of the test) batches of ten conditioned worms were weighed and placed into each container. Spiking of soil and food, respectively, filling of the test vessels and addition of the earthworms could not be performed at the same day due to high number of test variables and test concentrations.

Once a week the worms were fed according to their feed consumption. Feeding behaviour and the quantity of feed applied over the test period was recorded for each test container. The water content of the soil substrate in the test containers was maintained during the test period by weighing the test containers periodically and replenishing loss of water, if necessary.

The adult worms were kept in the substrate over a period of 4 weeks. At the end of this period, the adults were removed. For each container the total number and mass of living adult worms was recorded.

To allow the offspring to develop, the test containers were kept in the test environment for another period of 4 weeks. After this period the number of offspring per test container hatched from the cocoons was counted by hand selection.

The test was carried out at 20°C ± 2°C and a controlled light/dark cycle of 16 h:8 h with a light intensity of 400 lux to 800 lux.

7.5 Results

7.5.1 P25 - first experiment

(Raw data, chapter 21.2.1)

Zeta potential

Zeta potential of the stock dispersion (with deionised water) before application on feed and soil was -18 mV.

Effects

Effect concentrations

All earthworms survived. No effect on biomass increase was detected. Stimulated reproduction was observed. For the following test variants, statistically significant differences compared to the control were observed:

Spiking of feed, application of dry powder: 100 and 200 mg/kg

Spiking of soil, application of dry powder: 50 and 200 mg/kg

Spiking of feed, dispersion application: 20 mg/kg

Spiking of soil, dispersion application: 10 and 20 mg/kg

The effect values are summarised in Table 21.

Table 21: P25 – Test with earthworms (1st test): NOEC values.

	Application via powder on feed	Application via powder on soil	Application via dispersion on feed	Application via dispersion on soil
Mortality NOEC [mg/kg]	≥200	≥200	≥20	≥20
Biomass NOEC [mg/kg]	≥200	≥200	n.d. [1]	n.d. [1]
Reproduction NOEC [mg/kg]	50	< 50	10	<10

[1] n.d. = not determinable due to inconsistent concentration-effect curves (only two concentrations available)

Physical/pathological symptoms and changes in behaviour

No physical/pathological symptoms or changes in behaviour were observed. All specimens gave the impression of healthy condition.

Weight change

The results of weight change are presented in Table 22. No effect on biomass increase was detected. For raw data of the biomass see chapter 21.2.1.

Table 22: P25 – Test with earthworms (1st test): Mean weight at test start and weight change at test end

Concentrations given as nominal values

		Application via powder on feed [mg/kg]			Application via powder on soil [mg/kg]			Application via dispersion on feed [mg/kg]		Application via dispersion on soil [mg/kg]	
	Control	50	100	200	50	100	200	10	20	10	20
Mean weight at test start [g]	3.29	3.36	3.17	3.29	3.37	3.49	3.45	3.23	3.21	3.25	3.33
Standard deviation	0.24	0.25	0.26	0.27	0.43	0.13	0.14	0.09	0.16	0.12	0.25
CV	7.3	7.6	8.2	8.2	12.7	3.7	4.0	2.7	4.9	3.7	7.5
Mean weight change (increase) [%]	66.5	63.8	71.0	63.5	73.9	56.8	58.7	56.0*	64.0	58.3*	71.6
Standard deviation	7.0	12.5	8.3	6.7	19.2	7.7	4.7	3.2	5.5	4.6	14.0
CV	10.5	19.5	11.7	10.5	26.0	13.5	8.0	5.8	8.6	7.9	19.6

* Significant when compared with control: * $p > 0.05$

Mortality

No mortality was observed.

Reproduction

The results for reproduction are presented as mean values (Table 23). For single values of the replicates see chapter 21.2.1.

The test with powder-spiked soil showed concentration-dependant reproduction stimulation. The highest and lowest powder-spiked soil concentrations resulted in statistically significant differences in comparison to the control. Maximum reproduction stimulation was 49% (200 mg/kg), followed by 41% (100 mg/kg) and 39% (50 mg/kg).

The experiments with powder-spiked feed gave results comparable to the experiments with powder-spiked soil. Concentration-dependant stimulation of reproduction was observed in a range comparable to the experiments with powder-spiked soil.

The results from both soil tests with application via dispersion were comparable with regard to reproduction. We observed a significant difference ($P < 0.05$) of 54% (10 mg/kg) and 51% (20 mg/kg) compared to the control. The stimulation of reproduction was in a range comparable to the experiments with soil where the powder-spiked concentration was 10fold higher.

Experiments with aqueous dispersions added to feed gave results comparable to soil spiked with aqueous dispersions. No concentration-response relationships were observed.

Table 23: P25 – Test with earthworms (1st test): juveniles at test end.

Mean values and coefficient of variance (CV)

		Application via powder on feed [mg/kg]			Application via powder on soil [mg/kg]			Application via dispersion on feed [mg/kg]		Application via dispersion on soil [mg/kg]	
	Control	50	100	200	50	100	200	10	20	10	20
Juveniles	212.3	280.0	308.8	332.5	294.5	298.8	315.00	279.5	278.8	325.5	320.5
Standard deviation	45.8	84.4	67.5	70.6	43.9	73.9	42.4	51.7	12.1	73.9	14.2
CV	21.6	30.1	21.9	21.2	14.9	24.7	13.4	18.5	4.3	22.7	4.4
Inhibition [%]	---	-31.9 [1]	-45.5[1]	-56.7 [1]	-38.8 [1]	-40.8 [1]	-48.4 [1]	-31.7 [1]	-31.3 [1]	-53.3 [1]	-51.0 [1]
Statistical signifi-cance			* [2]	* [2]	* [2]		** [2]		* [2]	* [2]	* [2]

[1] negative values indicate stimulation [2] statistical difference (* 0.05 ≥ P ≥ 0.01; ** 0.01 ≥ P ≥ 0.001)

7.5.2 P25 - second experiment

(Raw data, chapter 21.2.2)

Zeta potential

Zeta potential of the stock dispersion (with deionised water) before application on feed and soil was -18 mV.

Effects

Effect concentrations

No mortality and no concentration-effect relationships for biomass increase and reproduction activity was observed. The NOEC and LOEC values are listed in Table 24.

Table 24: P25 – Test with earthworms (2nd test): NOEC values.

	NOEC [mg(kg]	LOEC [mg(kg]
Mortality	≥1000	>1000
Biomass	≥1000	>1000
Reproduction	≥1000	>1000

Physical/pathological symptoms and changes in behaviour

No physical/pathological symptoms or changes in behaviour were observed. All specimens gave the impression of healthy condition.

Weight change

The results of weight change are presented in Table 25. For raw data of the biomass see chapter 21.2.2. No effect on biomass increase was detected.

Table 25: P25 – Test with earthworms (2nd test): mean weight at test start and weight change at test end

Concentrations given as nominal values

	Control	50 mg/kg	100 mg/kg	200 mg/kg	500 mg/kg	1000 mg/kg
Mean weight at test start [g]	3.81	3.62	3.66	3.58	3.54	3.63
Standard deviation	0.30	0.10	0.11	0.11	0.08	0.23
CV	8.0	2.8	3.1	3.2	2.2	6.4
Mean weight change (increase) [%]	41.5	40.8	54.8 *	44.2	47.8	54.0
Standard deviation	7.5	4.2	7.3	7.5	8.6	11.1
CV	18.1	10.2	13.4	17.0	18.1	20.6

Significant when compared with control: * 0.05 ≥ P ≥ 0.01; ** 0.01 ≥ P ≥ 0.001

Mortality

No mortality was observed.

Reproduction

The results for reproduction are presented as mean values (Table 26). For single values of the replicates see chapter 21.2.2.

In contrast to the first test, no stimulation of reproduction was observed in the second test with P25 (Table 5). The main difference between the two tests was the timing: The first test began in January, the second in May. In the repeated test, a mean of 340 juveniles was counted in the control (standard deviation: 39 earthworms, corresponding to 11%). A slight reduction in offspring was detected for soil contents of 200 mg/kg (15% reduction) and 500 mg/kg (26%). Differences compared to the control, however, were not statistically significant; with a soil content of 1000 mg P25/kg the number of offspring was almost identical to the control.

Table 26: P25 – Test with earthworms (2nd test): juveniles at test end.

Mean values and coefficient of variance (CV)

	Control	50 mg/kg	100 mg/kg	200 mg/kg	500 mg/kg	1000 mg/kg
Juveniles	340.4	341.0	342.8	290.3	253.0	319.3
Standard deviation	38.8	32.5	28.2	24.3	61.8	42.5
CV	11.4	9.5	8.2	8.4	24.4	13.3
Inhibition [%]	0	-0.1	-0.7	14.7	25.7	7.1
Statistical significance [2]	-	-	-	-	*	-

[1] negative values indicate stimulation [2] statistical difference ([*] 0.05 ≥ P ≥ 0.01; [**] 0.01 ≥ P ≥ 0.001)

7.5.3 P25 - third experiment

(Raw data, chapter 21.2.3)

Effects

Effect concentrations

No concentration-dependant mortality was observed. In one vessel with concentrations of 500 and 1000 mg/kg only 9 worms were detected at day 28. No effect on biomass was detected whereas reproduction was stimulated. Statistically significant differences in reproduction were observed for test concentrations in the range of 50 – 1000 mg/kg. The NOEC and LOEC values are listed in Table 27.

Table 27: P25 – Test with earthworms (3rd test): NOEC-values

	NOEC [mg(kg]	LOEC [mg(kg]
Mortality	≥1000	>1000
Biomass	≥1000	>1000
Reproduction	<50	≤50

Physical/pathological symptoms and changes in behaviour

No physical/pathological symptoms or changes in behaviour were observed. All specimens gave the impression of healthy condition.

Weight change

The results of weight change are presented in Table 28. For raw data of the biomass see 21.2.3. For 200 mg/kg a significant difference in biomass increase compared to the control was observed. As there was no concentration-dependant effect, the statistical difference was not considered to be caused by the test substance.

Table 28: P25 – Test with earthworms (3rd test): mean weight at test start and weight change at test end.

Concentrations given as nominal values

	Control	50 mg/kg	100 mg/kg	200 mg/kg	500 mg/kg	750 mg/kg	1000 mg/kg
Mean weight at test start [g]	3.70	3.678	3.57	3.46	3.59	3.58	3.43
Standard deviation	0.26	0.16	0.20	0.09	0.22	0.12	0.18
CV	6.9	4.4	5.5	2.6	6.1	3.3	5.3
Mean weight change (increase) [%]	42.5	45.9	51.5	61.3 **	49.6	51.6	54.0
Standard deviation	9.3	5.9	10.9	4.9	9.4	5.4	10.3
CV	22.0	12.8	21.2	8.0	18.9	10.4	19.0

Significant when compared with control: * 0.05 ≥ P ≥ 0.01; ** 0.01 ≥ P ≥ 0.001

Mortality

No concentration-dependant mortality was observed. In one vessel with concentrations of 500 and 1000 mg/kg only 9 worms were detected at day 28. An influence on the number of offspring is not obvious. The standard deviations between the numbers of animals counted in the four replicates with the above concentrations are comparable to the other test concentrations.

Reproduction

The results obtained for reproduction are presented as mean values (Table 29). The single values of the replicates are presented in chapter 21.2.3.

Comparable to the first test with P25 a concentration-dependant stimulation of reproduction was observed. The effect, however, was less pronounced. The test was performed exactly one year after the first test.

Table 29: P25 – Test with earthworms (3rd test): juveniles at test end.

Mean values and coefficient of variance (CV)

	Control	50 mg/kg	100 mg/kg	200 mg/kg	500 mg/kg	750 mg/kg	1000 mg/kg
Juveniles	219.6	239.0	251.5	265.3	238.3	279.3	286.3
Standard deviation	33.3	22.3	15.5	31.3	11.5	27.0	21.3
CV	15.2	9.3	6.1	11.8	4.8	9.7	7.4
Inhibition [%]	0	-8,8	-14,5	-20,8	-8,5	-27,1	-30.3
Statistical significance [2]	-	*	**	**	*	**	**

[1] negative values indicate stimulation [2] statistical difference (* 0.05 ≥ P ≥ 0.01; ** 0.01 ≥ P ≥ 0.001)

7.5.4 NM-101 – first experiment

(Raw data, chapter 21.2.4)

Zeta potential

Zeta potential of the stock dispersion (with deionised water) before application on feed and soil was -18 mV.

Effects:

Effect concentrations

No mortality was detected. Statistically significant increased weight change was detected for variants with spiked soil via powder and via dispersion as well as for spiked feed with dispersion. Regarding the individual spiking variants, the increased values showed a concentration-effect relationship with a higher weight increase at higher test concentrations. Stimulation in reproduction increased with increasing test concentrations in all spiking variants. However, only the highest test concentration (200 mg/kg) of the test variant with spiked soil showed a statistically significant increase in the number of offspring. A compilation of the various NOEC values is shown in Table 30.

Table 30: NM-101 – Test with earthworms (1st test): NOEC values.

	Application via powder on feed	Application via powder on soil	Application via dispersion on feed	Application via dispersion on soil
Mortality NOEC [mg(kg]	≥200	≥200	≥20	≥20
Biomass NOEC [mg(kg]	≥200	<50	10	<10
Reproduction NOEC [mg(kg]	≥200	100	≥20	≥20

Physical/pathological symptoms and changes in behaviour

No physical/pathological symptoms or changes in behaviour were observed. All specimens gave the impression of healthy condition.

Weight change

The results of weight change are presented in Table 31. For raw data of the biomass see chapter21.2.4. Statistically significant increased weight change was detected for variants with spiked soil via powder and via dispersion as well as for spiked feed with dispersion.

Table 31: NM-101 – Test with earthworms (1st test): mean weight at test start and weight change at test end.

Concentrations given as nominal values

		Application via powder on feed [mg/kg]			Application via powder on soil [mg/kg]			Application via dispersion on feed [mg/kg]		Application via dispersion on soil [mg/kg]	
	Control	50	100	200	50	100	200	10	20	10	20
Mean weight at test start [g]	3.65	3.39	3.52	3.58	3.40	3.33	3.27	3.27	3.31	3.20	3.12
Standard deviation	0.21	0.16	0.34	0.19	0.18	0.32	0.14	0.14	0.28	0.14	0.19
CV	5.8	4.8	9.6	5.3	5.3	9.6	4.2	4.2	8.5	4.3	5.9
Mean weight change (increase) [%] [1]	66.1	79.1	74.7	75.1	83.4*	90.6**	90.9**	81.2	90.5***	86.3*	89.2**
Standard deviation	5.3	6.3	7.6	9.6	12.7	19.8	5.6	5.9	7.0	8.7	8.4
CV	8.1	8.0	10.2	12.7	15.3	21.8	6.1	7.3	7.7	10.0	9.5

[1] Significant when compared with control: * 0.05 ≥ P ≥ 0.01; ** 0.01 ≥ P ≥ 0.001; ***0.001 ≥ P

Mortality

No mortality was observed.

Reproduction

The results for reproduction are presented as mean values (Table 32). For single values of the replicates see chapter 21.2.4.

The test with powder-spiked soil showed a concentration-dependant stimulation of reproduction. Stimulation of reproduction was 6% (50 mg/kg) followed by 16% (100 mg/kg) and 24% (200 mg/kg) whereas only the highest test concentration was statistically different from the control.

The experiments with powder-spiked feed gave results comparable to the experiments with powder-spiked soil. Effects, however, were less pronounced. Concentration-dependant stimulation of reproduction was observed which, however, was not statistically significant.

In the tests with application on soil via dispersion 328 (10 mg/kg) and 334 (20 mg/kg) offspring was observed; in the control 303 juveniles were counted.

The reproduction rates in the experiments with aqueous dispersions added to feed were comparable to the tests with soil spiked with aqueous dispersions, yielding a higher number of offspring at the higher test concentration compared to the control and to the lower test concentration.

Table 32: NM-101 – Test with earthworms (1st test): juveniles at test end.

Mean values and coefficient of variance (CV)

		Application via powder on feed [mg/kg]			Application via powder on soil [mg/kg]			Application via dispersion on feed [mg/kg]		Application via dispersion on soil [mg/kg]	
	Control	50	100	200	50	100	200	10	20	10	20
Juveniles	302.8	296.8	319.8	336.75	321.5	352.5	374.8	327.8	333.8	307.8	337.8
Standard deviation	24.9	49.8	10.2	28.0	20.4	39.2	41.4	17.2	19.3	45.5	48.6
CV	8.2	16.8	3.2	8.3	6.3	11.1	11.0	5.2	5.8	14.8	14.4
Inhibition [%]	---	2.0	-5.6 [1]	-11.2 [1]	-6.2 [1]	-16.4 [1]	-23.8 [1]	-8.3 [1]	-10.2 [1]	-1.7 [1]	-11.6 [1]
Statistical signifi-cance		-	-	-	-	-	* [2]	-	-	-	-

[1] negative values indicate stimulation [2] statistical difference ([*] 0.05 ≥ P ≥ 0.01; [**] 0.01 ≥ P ≥ 0.001)

7.5.5 NM 101 - second experiment

(Raw data, chapter 21.2.5)

Effects

Effect concentrations

No mortality was detected. Weight change increased mainly with increasing concentrations of NM 101; but only in the highest test concentration (400 mg/kg) the difference was statistically significant. Concerning reproduction, no concentration-effect curves were obtained. The reproduction values obtained in the treated samples were not statistically different from the control. The LOEC for reproduction was above the highest test concentration (> 400mg/kg); the NOEC for reproduction was equal or above the highest test concentration (≥ 400mg/kg). All NOEC values are listed in Table 33.

Table 33: NM-101 – Test with earthworms (2nd test): NOEC values.

	NOEC [mg/kg]	LOEC [mg/kg]
Mortality	≥400	>400
Biomass	200	400
Reproduction	≥400	>400

Physical/pathological symptoms and changes in behaviour

No physical/pathological symptoms or changes in behaviour were obtained. All specimens gave the impression of healthy condition.

Weight change

The results of weight change are presented in Table 34. The raw data on biomass are presented in chapter 21.2.5. Only for the highest test concentration (400 mg/kg) a statistically significant difference in biomass increase was detected compared to the control.

Table 34: NM-101 – Test with earthworms (2nd test): mean weight at test start and weight change at test end.

Concentrations given as nominal values.

	Control	50 mg/kg	100 mg/kg	200 mg/kg	400 mg/kg
Mean weight at test start [g]	3.65	3.56	3.57	3.60	3.44
Standard deviation	0.22	0.28	0.22	0.24	0.15
CV	6.1	8.0	6.1	6.7	4.5
Mean weight change (increase) [%]	55.1	63.6	66.9	60.4	71.0 *
Standard deviation	7.2	3.1	11.7	4.7	8.9
CV	13.0	4.9	17.4	7.8	12.5

* Significant when compared with control: p ≥ 0.05

Mortality

No mortality was observed.

Reproduction

The results of reproduction are presented as mean values (Table 35). For single values of the replicates see chapter21.2.5.

No negative impact of NM 101 was observed in this test. In contrast to the first test no statistical difference was detected.

Table 35: NM-101 – Test with earthworms (2nd test): juveniles at test end.

Mean values and coefficient of variance (CV)

	Control	50 mg/kg	100 mg/kg	200 mg/kg	400 mg/kg
Juveniles	223.3	212.5	209.8	212.8	234.0
Standard deviation	15.1	22.3	15.5	46.8	20.3
CV	6.7	10.5	7.4	22.0	8.7
Inhibition [%]	0	4.8	6.0	4.7	-4.8
Statistical significance [2]	-	-	-	-	-

[1] negative values indicate stimulation [2] "-" = no statistical difference

7.5.6 NM -103 - first test

(Raw data, chapter 21.2.6)

Zeta potential

Zeta potential of the stock dispersion (with deionised water) before application on feed and soil was -18 mV.

Effects

Effect concentrations

No mortality occurred and no concentration-effect curves were obtained for biomass increase and number of offspring. The reproduction values obtained in the treated samples were not statistically different from the control. The LOEC for reproduction and weight change were above the highest test concentration (> 200mg/kg); the NOEC for reproduction and weight change were equal or above the highest test concentration (≥ 200mg/kg). The NOEC values are presented in Table 36.

Table 36: NM-103 – Test with earthworms (1st test): NOEC-values.

	Application via powder on feed	Application via powder on soil	Application via dispersion on feed	Application via dispersion on soil
Mortality NOEC [mg(kg]	≥200	≥200	≥20	≥20
Biomass NOEC [mg(kg]	≥200	≥200	≥20	≥20
Reproduction NOEC [mg(kg]	≥200	≥200	≥20	≥20

Physical/pathological symptoms and changes in behaviour

No physical/pathological symptoms or changes in behaviour were obtained. All specimens gave the impression of healthy condition.

Weight change

The results for weight change are presented in Table 37. For raw data of the biomass see chapter 21.2.6. The weight change increases with increasing test concentrations (exception: application via dispersion on feed). However, no statistically significant difference was observed.

Table 37: NM-103 – Test with earthworms (1st test): mean weight at test start and weight change at test end.

		Application via powder on feed [mg/kg]			Application via powder on soil [mg/kg]			Application via dispersion on feed [mg/kg]		Application via dispersion on soil [mg/kg]	
	Con-trol	50	100	200	50	100	200	10	20	10	20
Mean weight at test start [g]	3.86	3.73	3.89	3.75	3.86	3.83	3.78	3.56	3.80	3.78	3.46
Standard deviation	0.22	0.17	0.23	0.21	0.22	0.27	0.38	0.19	0.30	0.24	0.19
CV	5.6	4.5	5.8	5.6	5.6	7.2	10.0	5.3	7.9	6.4	5.4
Mean weight change (increase) [%]	44.2	38.6	44.7	51.7	43.7	51.7	56.1	53.0	50.6	52.0	60.5
Standard deviation	7.6	14.6	3.3	6.7	3.6	5.4	10.6	10.8	9.4	4.5	10.9
CV	17.3	37.7	7.3	13.0	8.3	10.4	19.0	20.4	18.6	8.7	18.1

Mortality

In one vessel (replicate 3, application of powder on soil, 100 mg/kg) one worm died. Apart from that, no mortality was observed.

Reproduction

The results of reproduction are presented as mean values (Table 38). For single values of the replicates see chapter 21.2.6.

The number of offspring was comparable to the controls for all application forms and treatments. No statistical differences were observed.

Table 38: NM-103 – Test with earthworms (1st test): juveniles at test end.

Mean values and coefficient of variance (CV)

		Application via powder on feed [mg/kg]			Application via powder on soil [mg/kg]			Application via dispersion on feed [mg/kg]		Application via dispersion on soil [mg/kg]	
	Control	50	100	200	50	100	200	10	20	10	20
Juveniles	365.1	332.3	345.3	365.0	337.5	371.8	342.8	341.5	325.5	340.5	365.1
Standard deviation	42.7	65.8	17.6	42.2	20.2	57.1	34.0	51.3	49.3	31.0	42.7
CV	11.7	19.8	5.1	11.5	6.0	15.3	9.9	15.0	15.1	9.1	11.7
Inhibition [%]	---	9.0	5.4	0.0	7.6	-1.8 [1]	6.1	6.5	10.9	6.7	7.6
Statistical significance [2]		-	-	-	-	-	-	-	-	-	-

[1] negative values indicate stimulation [2] "-" = no statistical difference

7.5.7 NM 103 - second experiment

(Raw data, chapter 21.2.7)

Effects

Effect concentrations

No concentration-effect curves were obtained. Therefore, no EC values were calculated. The reproduction values obtained in the treated samples were not statistically different from the control. The LOEC values for reproduction and weight change were above the highest test concentration (> 400mg/kg); the NOEC values for reproduction and weight change were equal or above the highest test concentration (≥ 400mg/kg). The NOEC and LOEC values are listed in Table 39.

Table 39: NM-103 – Test with earthworms (2nd test): NOEC-values.

	NOEC [mg(kg]	LOEC [mg(kg]
Mortality	≥400	>400
Biomass	≥400	>400
Reproduction	≥400	>400

Physical/pathological symptoms and changes in behaviour

No physical / pathological symptoms or changes in behaviour were obtained. All specimens gave the impression of healthy condition.

Weight change

The results of weight change are presented in Table 40. For raw data of the biomass see chapter 21.2.7. No effect on biomass increase was detected.

Table 40: NM-103 – Test with earthworms (2nd test): mean weight at test start and weight change at test end.

Concentrations given as nominal values

	Control	50 mg/kg	100 mg/kg	200 mg/kg	400 mg/kg
Mean weight at test start [g]	3.65	3.67	3.47	3.45	3.61
Standard deviation	0.22	0.33	0.27	0.13	0.22
CV	6.1	9.0	7.9	3.8	6.0
Mean weight change (increase) [%]	55.1	55.5	66.7 *	65.8	65.7
Standard deviation	7.2	13.3	7.5	13.7	9.7
CV	13.0	23.9	11.3	20.9	14.8

*Significant when compared with control: $p \geq 0.05$

Mortality

No mortality was observed.

Reproduction

The results of reproduction are presented as mean values (Table 41). For single values of the replicates see chapter 21.2.7.

No negative impact of NM 103 was observed in this test.

Table 41: NM-103 – Test with earthworms (2nd test): juveniles at test end.

Mean values and coefficient of variance (CV)

	Control	50 mg/kg	100 mg/kg	200 mg/kg	400 mg/kg
Juveniles	223.3	240.3	251.8	232.8	236.8
Standard deviation	15.1	30.6	42.1	39.7	37.6
CV	6.7	12.7	16.7	17.1	15.9
Inhibition [%]	---	-7.6	-12.8	-4.3	-6.0
Statistical significance [2]	-	-	-	-	-

[1] negative values indicate stimulation [2] "-" = no statistical difference

7.5.8 Considerations concerning the reproduction behaviour of earthworms in the presence of TiO_2 nanoparticles

Results from the control indicate that there is a circannual rhythm in the number of juveniles when the earthworms are incubated in natural soil. Figure 7 presents the number of juveniles in natural soil (RefeSol 01-A) obtained in control vessels (eight replicates). In winter the number of offspring (about 200) was lower than in summer (about 350 juveniles). The difference between the minimum and maximum values is about 75%. For P25 such a difference was not observed. The number of juveniles in the presence of a concentration TiO_2 nanoparticles of 200 mg/kg is presented in Figure 8. The difference between the maximum and minimum value is only 19%. For NM-103, in contrast, a circannual rhythm comparable to the control was observed resulting in stimulation in winter. Comparing the experiments performed in summer and winter, the difference between the maximum and minimum number of juveniles was about 60% (concentration of 200 mg/kg) for this material.

There is no clear effect-behaviour of NM-101. In the first experiment performed in winter 2010 a statistically significant stimulatory effect was observed for the highest test concentration (200 mg/kg), whereas a statistically significant stimulation was not detected in the second experiment performed in winter 2011. However, in 2011 the stimulatory effect was less pronounced (200 mg/kg: 48% in 2010; 21% in 2011) for P25 as well. Therefore, the missing effect for NM-101 in 2011 may result from a generally smaller effect.

There is an indication that the observed effect in natural soil is material-specific: The coated material (NM-103) showed a circannual rhythm comparable to the control, whereas a behaviour differing from the control was observed for the uncoated materials (NM-101 and P25). This may be explained by the fact that the coating prevents the direct contact of TiO_2 nanoparticles with the organism, whereas the earthworms are in direct contact with TiO_2 nanoparticles in the form of NM-101 and P25.

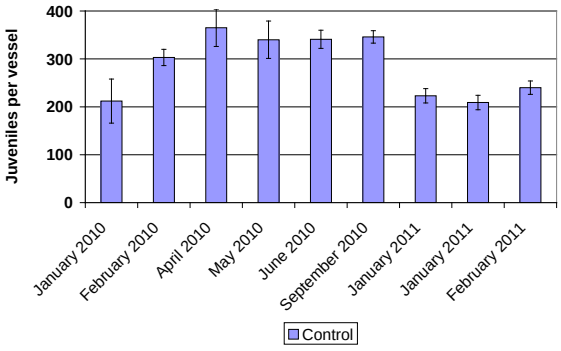


Figure 7: ***E. fetida*** **- Number of juveniles in natural soil (RefeSol 01-A) during the year.**

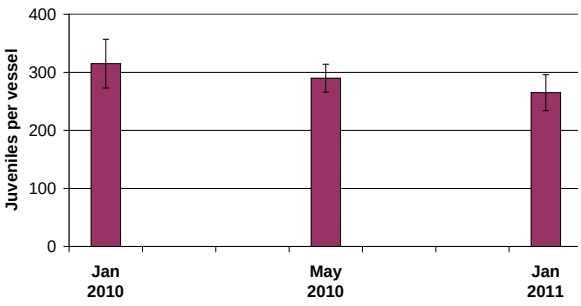


Figure 8: ***E. fetida*** **- Number of juveniles in natural soil spiked with P25 (200 mg/kg) (RefeSol 01-A) during the year.**

Circannual biological rhythms have been demonstrated for vertebrates and invertebrates, but the mechanisms generating them are still unclear (Nisimura and Numata, 2002). Rozen

(2003, 2006) collected earthworms of the species *Dendrobaena octaedra* in the field and cultured them in the laboratory under constant conditions. Even under constant conditions the reproduction rate of the laboratory-cultured worms was highest in spring and summer, and lowest in winter, indicating the existence of an internal reproduction regulation. It was shown that neurosecretory hormones regulate cyclical functions in earthworms, such as reproduction or secondary sex characteristics (Laverack, 1963 in Rozen, 2006). However, there is no information on whether these hormones are responsible for the circannual biological rhythm and whether TiO_2 nanoparticles potentially affects these hormones.

We assume that besides the metabolism of the earthworms substances occurring in the soil are involved in the biological rhythm of reproduction as observed in the control vessels. Soil collected in winter which was used for tests performed in summer resulted in a low reproduction rate in the controls and a stimulatory effect in the presence of P25 (17% stimulation in the presence of 200 mg/kg, 27% stimulation in the presence of 500 mg/kg). A test performed at the same time with freshly collected soil resulted in no stimulation (detailed data of both tests not shown). Furthermore, we observed that the circannual rhythm is less pronounced when the tests are performed in artificial soil (14 tests performed within a period of 4 years with tests starting nearly every month).

7.5.9 Concentration of Ti in earthworms

(Raw data, chapter 21.2.8)

In some of the tests the Ti concentration in earthworms was determined.

The results are compiled in the following tables (Table 42 - Table 45) and figures (Figure 9-Figure 12). In chapter 21.2.8 the results are shown in more detail. There are strong indications that Ti concentrations in the earthworms increase with increasing test concentrations. However, there seems to be a difference depending on whether the contamination is highly concentrated in food or distributed in soil. Contaminated food seems to result in higher concentrations in the earthworms showing an increase already at concentrations of 100 or 200 mg/kg, whereas for contaminated soil an increase is obvious only for a concentration of 1000 mg/kg. Obvious differences between the three nanoparticles were not observed. As only two replicates were carried out, no calculation was performed concerning the statistical difference. In none of the test designs the concentration in the earthworms exceeded the concentration in the test vessels. For P25-spiked soil the Ti concentration in the surrounding medium was calculated as 600 mg/kg (TiO_2 nanoparticles: 1000 mg/kg) and compared to the control. In the worms 88.0 µg/g (= 88.0 mg/kg) was measured. The difference between the Ti concentration in the earthworms exposed to contaminated medium and the earthworms exposed to the control soil (55.2 µg/g) was 33 µ/g (33 mg/kg), which is far below the concentration in the test medium (600 mg/kg). Therefore, it was concluded that accumulation in the worm tissue did not occur and that the measured Ti was still in the gut, possibly attached to remaining soil/food particles.

Table 42: P25 - Ti concentration in earthworms (1st test).

Sample	Mean Ti in dry weight ± SD [µg/g]
Control	58.4 ± 9.8
TiO_2 - 10 mg/kg; Ti – 6.0 mg/kg: spiked soil	54.2 ± 4.7
TiO_2 - 10 mg/kg; Ti – 6.0 mg/kg: spiked food	58.6 ± 4.3
TiO_2 - 20 mg/kg; Ti – 12 mg/kg: spiked soil	77.4 ± 2.9
TiO_2 - 20 mg/kg; Ti – 12 mg/kg: spiked food	64.6 ± 4.1
TiO_2 - 50 mg/kg; Ti – 30 mg/kg: spiked soil	76.4 ± 19.9
TiO_2 - 50 mg/kg; Ti – 30 mg/kg: spiked food	75.3 ± 6.1
TiO_2 - 100 mg/kg; Ti – 60 mg/kg: spiked soil	76.3 ± 12.7
TiO_2 - 100 mg/kg; Ti – 60 mg/kg: spiked food	101 ± 7.0
TiO_2 - 200 mg/kg; Ti – 120 mg/kg: spiked soil	72.4 ± 29.0
TiO_2 - 200 mg/kg; Ti – 120 mg/kg: spiked food	121 ± 31.0

Table 43: P25 - Ti concentration in earthworms (2nd test).

Sample	Mean Ti in dry weight ± SD [µg/g]
Control	55.2 ± 2.2
TiO_2 - 50 mg/kg; Ti – 30 mg/kg: spiked soil	49.2 ± 4.3
TiO_2 - 100 mg/kg; Ti – 60 mg/kg: spiked soil	43.7 ± 1.5
TiO_2 - 200 mg/kg; Ti – 120 mg/kg: spiked soil	50.1 ± 11.9
TiO_2 - 500 mg/kg; Ti – 300 mg/kg: spiked soil	61.0 ± 1.4
TiO_2 - 1000 mg/kg; Ti – 600 mg/kg: spiked soil	88.0 ± 21.7

Table 44: NM-101 - Ti concentration in earthworms (1st test).

Sample	Mean Ti in dry weight ± SD [µg/g]
Control	54.1
TiO_2 - 10 mg/kg; Ti – 6.0 mg/kg: spiked soil	49.7 ± 5.0
TiO_2 - 10 mg/kg; Ti – 6.0 mg/kg: spiked food	28.9 ± 4.9
TiO_2 - 20 mg/kg; Ti – 12 mg/kg: spiked soil	45.1 ± 3.7
TiO_2 - 20 mg/kg; Ti – 12 mg/kg: spiked food	38.1 ± 4.1
TiO_2 - 50 mg/kg; Ti – 30 mg/kg: spiked soil	59.4 ± 10.4
TiO_2 - 50 mg/kg; Ti – 30 mg/kg: spiked food	70.8 ± 7.5
TiO_2 - 100 mg/kg; Ti – 60 mg/kg: spiked soil	66.2 ± 1.7
TiO_2 - 100 mg/kg; Ti – 60 mg/kg: spiked food	53.1 ± 12.3
TiO_2 - 200 mg/kg; Ti – 120 mg/kg: spiked soil	107 ± 40
TiO_2 - 200 mg/kg; Ti – 120 mg/kg: spiked food	52.7 ± 8.6

Table 45: NM-103: Ti concentration in earthworms (1st test).

Sample	Mean Ti in dry weight ± SD [µg/g]
TiO_2 - 10 mg/kg; Ti – 6.0 mg/kg: spiked soil	22.6 ± 2.2
TiO_2 - 10 mg/kg; Ti – 6.0 mg/kg: spiked food	55.3 ± 21.1
TiO_2 - 20 mg/kg; Ti – 12 mg/kg: spiked soil	33.8 ± 6.3
TiO_2 - 20 mg/kg; Ti – 12 mg/kg: spiked food	30.1 ± 1.5
TiO_2 - 50 mg/kg; Ti – 30 mg/kg: spiked soil	43.6 ± 9.6
TiO_2 - 50 mg/kg; Ti – 30 mg/kg: spiked food	30.8 ± 1.5
TiO_2 - 100 mg/kg; Ti – 60 mg/kg: spiked soil	23.6 ± 3.0
TiO_2 - 100 mg/kg; Ti – 60 mg/kg: spiked food	32.9 ± 32.9
TiO_2 - 200 mg/kg; Ti – 120 mg/kg: spiked soil	56.1 ± 56.1
TiO_2 - 200 mg/kg; Ti – 120 mg/kg: spiked food	31.1 ± 31.1
TiO_2 - 10 mg/kg; Ti – 6.0 mg/kg: spiked soil	62.9 ± 62.9

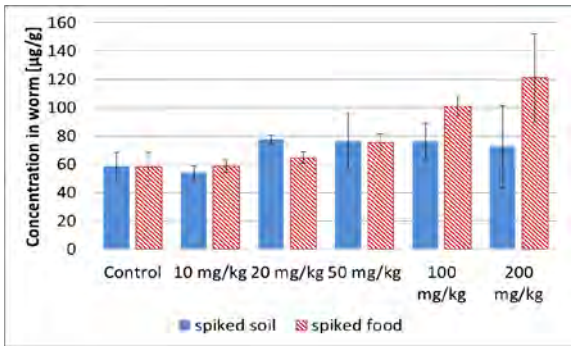


Figure 9: P25 – 1st test with earthworms: Ti concentration in earthworms (purged gut).

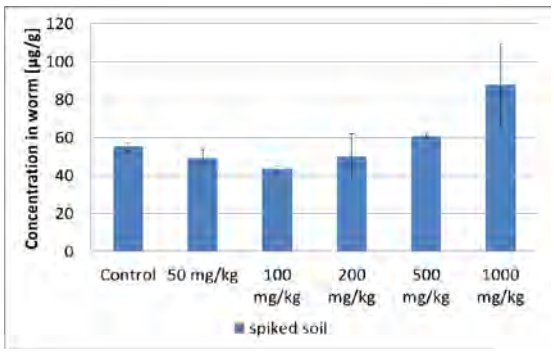


Figure 10: P25 – 2nd test with earthworms: Ti concentration in earthworms (purged gut).

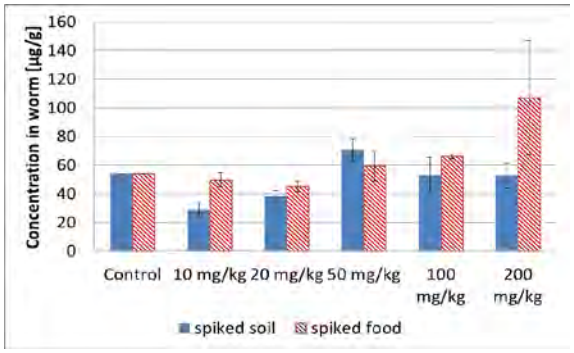


Figure 11: NM-101 – 1st test with earthworms: Ti concentration in earthworms (purged gut).

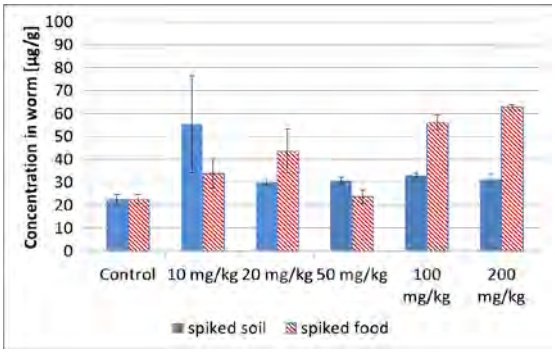


Figure 12: NM-103 – 1st test with earthworms: Ti concentration in earthworms (purged gut).

7.6 Validity

P25

The three earthworm reproduction tests fulfil the validity criteria of the guideline:

- With 212, 340 and 220 individuals the rate of reproduction in the control was ≥ 30 juveniles per test vessel.
- With 21.6%, 11.4% and 22.0% the CV of reproduction in the control did not exceed 30%.
- With 0% in all tests the percent mortality of the adults observed in the controls over the initial 4 weeks was ≤ 10%.

NM 101

Both earthworm reproduction tests fulfil the validity criteria of the guideline:

- With 303 and 223 individuals the rate of reproduction in the control was ≥ 30 juveniles per test vessel.
- With 8.2% and 6.7% the CV of reproduction in the control did not exceed 30%.
- With 0% in both tests the percent mortality of the adults observed in the controls over the initial 4 weeks was ≤ 10%.

NM 103

Both earthworm reproduction tests fulfil the validity criteria of the guideline:

- With 372 and 223 individuals the rate of reproduction in the control was ≥ 30 juveniles per test vessel.
- With 11.1% and 6.7% the CV of reproduction in the control did not exceed 30%.
- With 0% in both tests the percent mortality of the adults observed in the controls over the initial 4 weeks was ≤ 10%.

7.7 Data with the reference substance

As reference substance carbendazim was tested over the period February 11, 2010 - April, 8, 2010.

The following values were calculated for reproduction [mg/kg]; values in brackets indicate the confidence interval:

EC_{10}: 1.147 (1.118 -1.172)

EC_{20}: 1.309 (1.289 -1.328)

EC_{50}: 1.688 (1.670 -1.709)

LOEC: 1.500

NOEC: 0.750

All validity criteria mentioned in the guideline were fulfilled. According to the guideline significant effects should be observed between 1 and 5 mg/kg. This criterion was fulfilled.

7.8 Conclusion

The control showed a reduced number of offspring in winter compared to experiments carried out in summer. This reduction of the number of juveniles was not observed for the uncoated nanoparticles P25 and NM-101. Therefore, stimulation was observed 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 nanoparticles) is due to the disturbance of the biological clock.

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

7.9 Executive summary

TiO_2 nanoparticles (**NM-101, NM-103 and P25**) were tested in the OECD earthworm reproduction test. The particles were applied as powder and as aqueous dispersion in soil and in feed. The test substrate was a natural sandy soil. The experiments were performed several times.

The following test concentrations were investigated:

- Application via powder on feed: 50, 100, 200 mg/kg soil, dry matter
- Application via powder on soil: 50, 100, 200 mg/kg soil, dry matter
- Application via dispersion on feed: 10, 20 mg/kg soil, dry matter
- Application via dispersion on soil: 10, 20 mg/kg soil, dry matter.

In several tests performed only with powder-spiked soil a higher number of concentrations were investigated. The following approaches were studied:

- Application via powder on soil: 50, 100, 200, 400 mg/kg soil, dry matter (NM-101, NM-103)
- Application via powder on soil: 50, 100, 200, 500, 750, 1000 mg/kg soil, dry matter (P25).

The tested TiO_2 nanoparticles did not cause a reduction in the number of offspring. A stimulatory effect was observed, at least for the uncoated material P25, when the test was performed in winter. For the coated material NM-103 a stimulatory effect cannot be observed. The stimulatory effect is less pronounced for the second uncoated material (NM-101).

There are indications that the stimulation is due to a disturbance of the biological clock.

In some of the tests the Ti concentration was determined in the earthworms. There are strong indications that Ti concentrations in the worms increase with increasing test concentrations. However, there seems to be a difference depending on whether the contamination is highly concentrated in food or distributed in soil. Contaminated food seems to cause higher concentrations in the earthworms than contaminated soil, showing an increase at 100 or 200 mg/kg, whereas for contaminated soil an increase is obvious only for a concentration of 1000 mg/kg. Differences between the three nanoparticles were not observed. In none of the test designs the concentration in the worms exceeded the soil concentration in the test substrate. Therefore, it is concluded that the nanoparticles did not accumulate in the tissue of the worms but remained in the gut, possibly adsorbed to remaining soil/food particles.

8 Reproduction Test with Earthworms (OECD TG 222) – Ag

8.1 Test principle

Adult earthworms of the species *Eisenia fetida* were placed in a defined soil containing different concentrations of the test item. The test item was applied once and the effects on biomass and mortality of the adult worms are determined after 28 days. After 56 days effects on reproduction was determined by counting the offspring. In addition to the test guideline, accumulation in the adults was tested after the exposure period of 28 days.

According to the guideline, chemical effects are assessed based on the reproduction, although additionally biomass has to be measured. In the result sections below, biomass data and number of juveniles are presented, but ECx and NOEC values are only calculated for reproduction (number of juveniles).

8.2 Materials and methods

8.2.1 Test guideline

The test was performed according to:

OECD Guidelines for the Testing of Chemicals Test No. 222: Earthworm Reproduction Test (*Eisenia fetida, Eisenia andrei*) (2004)

8.2.2 GLP

The test was performed following the principles of GLP (OECD, 1998). In deviation to GLP no archiving of the raw data is performed and the quality assurance unit was not involved with respect to the inspection of the test, of the raw data and the report. All laboratory equipment (e.g. balances, thermometers, pH-meters) was controlled and documented according to GLP.

8.2.3 Test material

- NM-300K
- NM-300KDIS (Dispersant)

  The test substances were stored in the dark at room temperature.

8.2.4 Analytical monitoring

The concentration of silver ions in the test soil was measured by incubation of DGTs in the vessels. Two DGTs per test vessel were incubated for two days. The incubation started on day 0, day 26 and day 54.

The total content of Ag was determined in the soil and in the earthworms. Earthworms were incubated for 24 h on wet filter paper to purge their gut and then frozen at -20°C until analysis.

8.2.5 Test item – preparation protocol

NM-300K consists of silver nanoparticles stabilised in a dispersant. The stock dispersion contained 10% of silver. In contrast to TiO_2 nanoparticles we therefore tested only two different modes of application:

Spiking of soil

NM-300K was sprayed on 152.5 g soil (dry matter; 5% of WHC_{max}), and thoroughly mixed. The amount of NM-300K was weighed since exact pipetting was not possible due to the consistency of the material.

Uncontaminated test soil (about 20 – 30% of WHC_{max}) was spread on a plate, the carrier soil with the NM-300K was distributed on the test soil, and all was mixed carefully. The contaminated soil was adjusted to a water-holding capacity of 55% of WHC_{max}.

15 mg/kg: 0.430 g NM-300K in 3551.13 g soil (55% WHC_{max})

30 mg/kg: 0.861 g NM-300K in 3551.13 g soil (55% WHC_{max})

60 mg/kg: 1.721 g NM-300K in 3551.13 g soil (55% WHC_{max})

120 mg/kg: 3.443 g NM-300K in 3551.13 g soil (55% WHC_{max})

200 mg/kg: 5.738 g NM-300K in 3551.13 g soil (55% WHC_{max})

Additionally, a dispersant control was tested. The control achieved the amount of dispersant of the highest test concentration.

Spiking of feed

40 g of finely ground cow manure was mixed with stock dispersion of NM-300K to achieve the desired test content. Additionally, deionised water was added to achieve a final volume of 120 mL.

15 mg/kg: 0.480 mL NM-300K

30 mg/kg: 0.960 mL NM-300K

60 mg/kg: 1.922 mL NM-300K

120 mg/kg: 3.843 mL NM-300K

200 mg/kg: 6.405 mL NM-300K

Moreover, a dispersant control was tested which achieved the amount of dispersant of the highest test concentration.

8.2.6 Test species

The test organisms were synchronised adult earthworms of the species *Eisenia fetida andrei* (Annelida, Oligochaeta), which were 2 - 12 months old, with a clitellum, and a wet mass between 250 mg and 600 mg.

Origin of the worms:	Regenwurmfarm Tacke, Klosterdiek 61, 46325 Borken. Specimens used in the test were bred in the laboratory of the Fraunhofer IME.
Breeding conditions:	Worms were bred in 1:1 mixtures of cow manure and sphagnum peat (dry mass basis) at 20°C ± 2°C.
Pre-treatment:	The worms were conditioned in the artificial soil for 7 days before use. The same feed as used in the test (see 9.3) was given in a sufficient amount.

8.3 Study design

8.3.1 Study type

Laboratory test

8.3.2 Test duration type and exposure period

The test was long-term with an exposure period of 56 days.

- NM-300K: 9 June – 4 August 2010

8.3.3 Test substrate

The soil used in the test was a natural sandy soil (certified RefeSol 01-A, batch IME-01: sand 71%, silt: 24%, clay: 5%, Org C: 0.93%, pH 5.7, clay: 5%). The soil was sieved to ≤ 2 mm. The soil was not sterilised and had been stored outdoors in high-grade stainless steel basins with drainage and ground contact at the test facility.

8.3.4 Total exposure period

The exposure period lasted 56 days.

8.3.5 Post exposure period

There was no post exposure period.

8.4 Test conditions

8.4.1 Environmental conditions

The incubation temperature was measured continuously with a thermograph. According to the guideline the permitted range is 20 ± 2 °C. A controlled light/dark cycle of 16 h:8 h was applied. The light intensity was measured using an illuminance meter (MINOLTA) with photometric sensor in Lux. According to the guideline the permitted value is about 600 lux. The test conditions are presented in Table 46.

Table 46: NM-300K – Test with earthworms, incubation conditions.

	NM-300K
Incubation temperature [°C]	19 – 21
Light intensity [lux]	600 – 750
Soil dry mass [%]	78 - 90
pH [1 mol/L KCl] – test start	5.0 – 5.1
pH [1 mol/L KCl] – test end	6.8 – 7.1

8.4.2 Test concentrations

The following nominal contents were applied in the test containers with Ag:

- 15, 30, 60, 120, 200 mg/kg soil, dry mass (application on soil)
- 15, 30, 60, 120, 200 mg/kg soil, dry mass (application on feed).

8.4.3 Other information on materials and methods

Frequency of treatment

Treatment was performed once at test start.

Control group and treatment

For silver, eight controls (no addition of dispersant), four controls with dispersant applied in soil, and four controls with dispersant applied in feed were prepared (dispersant amount corresponded to the dispersant amount used in the highest test concentration).

Statistical method

Data evaluation

In this report numerical values are frequently rounded to a smaller degree of precision (number of digits) than used in the actual calculation. Minor differences in the results obtained from calculations with rounded values compared to results obtained with higher precision values are possible. They are, however, well within the limits of the experimental accuracy and of no practical concern.

Statistical calculations

For each concentration the percent mortality, the percent loss/increase in biomass of the adults, and the number of offspring produced in the test was determined.

Mortality, biomass and number of offspring were compared by a suitable test for multiple comparisons with a control after testing variance homogeneity. All statistical tests were performed with the computer software ToxRat Professional version 2.10.4.1 (ToxRat® Solutions GmbH).

Feed

Air-dried, finely ground cow manure was used as feed.

Test container

All tests were performed in polypropylene containers (Bellaplast GmbH, Alf). Adjusted to 55% of the maximum water-holding capacity, 640 g soil (dm) was filled into containers to a depth of about 5 cm. The containers were covered with transparent plastic lids to prevent worms from escaping and to guarantee access of light. The lids had several small holes to permit gaseous exchange between the medium and the atmosphere.

Test procedure

Soil and food were spiked. Test soil was added to the test containers and 10 g of air dried, finely ground cow manure per test container was spread onto the soil surface and moistened with water. The next day (start of the test) batches of ten conditioned worms were weighed and placed into each container. Spiking of soil and food, respectively, filling of the test vessels and addition of the earthworms could not be performed at the same day due to high number of test variables and test concentrations.

Once a week the worms were fed according to their feed consumption. Feeding behaviour and the quantity of feed applied over the test period was recorded for each test container. The water content of the soil substrate in the test containers was maintained during the test period by weighing the test containers periodically and replenishing lost water, if necessary.

The adult worms were kept in the substrate over a period of 4 weeks. At the end of this period, the adults were removed. For each container the total number and mass of living adult worms was recorded.

To allow the offspring to develop, the test containers were kept in the test environment for another period of 4 weeks. After this period the number of offspring per test container hatched from the cocoons was counted by hand.

The test was carried out at 20°C ± 2°C and a controlled light/dark cycle of 16 h:8 h with a light intensity of 400 - 800 lux.

8.5 Results

(Raw data, chapter 21.3)

8.5.1 NM-300K

Zeta potential

Zeta potential in dispersion with deionised water (20 g/L; 100 mg/L) was -12 mV.

Test item concentrations:

Total Ag-concentrations and the Ag^+-concentration (ion concentration of Ag) in soil were determined. Detailed results for the ion concentration are presented in chapter 21.3.1.

Total Ag-concentration

Two representative concentrations of total Ag in soil were determined. The results are presented in Table 47.

Recovery was 90% (15 mg/kg and 120 mg/kg). Therefore, the use of nominal concentrations for reporting the effects was considered acceptable.

Concentration of Ag^+

The results are presented in Table 48. In four concentrations (15 mg/kg, 60 mg/kg, 120 mg/kg, 200 mg/kg) the Ag^+ ions were determined via DGT. There was a small increase from the concentration at day 0 to the concentration at day 56. The concentrations of ions were in the range of 0.2 - 2.3 * 10^{-4}% of the nominal concentrations.

Table 47: NM-300K – Test with earthworms: Ag concentrations in spiked soil.

Five replicate samples, each measured twice

Application / sample		Weighed for digestion [g]	Measured Ag conc [µg/L]	Dilution factor	Calculated Ag conc. [mg/kg]	Nominal Ag conc. [mg/kg]	Recovery [%]	Mean recovery ± SD
Control	1	3.13	3.24	-	< LOD	-	-	-
	2	3.13	3.14	-	< LOD	-	-	
	3	3.08	3.28	-	< LOD	-	-	
	4	3.16	2.44	-	< LOD	-	-	
	5	3.16	3.04	-	< LOD	-	-	
Application via soil: 120 mg/kg	1-1	3.10	307	10	99.03	120	82.5	89.6 ± 4.4
	1-2	3.11	332	10	106.78	120	89.0	
	2-1	3.13	321	10	102.54	120	85.4	
	2-2	3.10	359	10	115.77	120	96.5	
	3-1	3.06	321	10	105.01	120	87.5	
	3-2	3.17	356	10	112.27	120	93.6	
	4-1	3.13	349	10	111.57	120	93.0	
	4-2	3.12	338	10	108.63	120	90.5	
	5-1	3.12	319	10	102.35	120	85.3	
	5-2	3.11	347	10	111.63	120	93.0	
Application via soil: 15 mg/kg	1-1	3.07	93.6	5	15.25	15	102	90.0 ± 14.5
	1-2	3.12	74.0	5	11.86	15	79.0	
	2-1	3.11	69.5	5	11.16	15	74.4	
	2-2	3.04	85.1	5	14.01	15	93.4	
	3-1	3.04	84.5	5	13.89	15	92.6	
	3-2	3.12	77.1	5	12.35	15	82.3	
	4-1	3.05	91.9	5	15.06	15	100	
	4-2	3.05	78.8	5	12.91	15	86.1	
	5-1	3.13	110	5	17.56	15	117	
	5-2	2.63	54.2	5	10.29	15	68.6	

[1] LOD = limit of detection (11.5 µg/L)

Table 48: NM-300K – Test with earthworms: concentration of Ag ions measured by DGTs in soil.

		Control	15 mg/kg	60 mg/kg	120 mg/kg	200 mg/kg
Day 0						
Replicate 1	[µg/kg] [1]	$0.141 * 10^{-3}$	$21.5 * 10^{-3}$	$19.6 * 10^{-3}$	$29.70 * 10^{-3}$	$33.6 * 10^{-3}$
Replicate 2	[µg/kg] [1]	$0.287 * 10^{-3}$	$36.1 * 10^{-3}$	$22.3 * 10^{-3}$	technical defect	$34.3 * 10^{-3}$
Mean value	[µg/kg] [1]	$0.214 * 10^{-3}$	$28.8 * 10^{-3}$	$21.0 * 10^{-3}$	$29.7 * 10^{-3}$	$34.0 * 10^{-3}$
Percentage [2]	[%]	---	$1.92 * 10^{-4}$	$0.35 * 10^{-4}$	$0.25 * 10^{-4}$	$0.17 * 10^{-4}$
Day 28						
Replicate 1	[µg/kg] [1]	$1{,}16 * 10^{-3}$	$41.9 * 10^{-3}$	$46.1 * 10^{-3}$	$56.7 * 10^{-3}$	$76.0 * 10^{-3}$
Replicate 2	[µg/kg] [1]	$0.696 * 10^{-3}$	$25.9 * 10^{-3}$	$37.6 * 10^{-3}$	$81.9 * 10^{-3}$	$64.1 * 10^{-3}$
Mean value	[µg/kg] [1]	$0.928 * 10^{-3}$	$33.9 * 10^{-3}$	$41.9 * 10^{-3}$	$69.3 * 10^{-3}$	$70.1 * 10^{-3}$
Percentage [2]	[%]	---	$2.26 * 10^{-4}$	$0.70 * 10^{-4}$	$0.58 * 10^{-4}$	$0.35 * 10^{-4}$
Day 56						
Replicate 1	[µg/kg] [1]	$1.23 * 10^{-3}$	$35.9 * 10^{-3}$	$75.5 * 10^{-3}$	$162 * 10^{-3}$	$86.2 * 10^{-3}$
Replicate 2	[µg/kg] [1]	$1.16 * 10^{-3}$	$95.0 * 10^{-3}$	$51.3 * 10^{-3}$	$140 * 10^{-3}$	$91.3 * 10^{-3}$
Mean value	[µg/kg] [1]	$1.20 * 10^{-3}$	$65.5 * 10^{-3}$	$63.4 * 10^{-3}$	$151 * 10^{-3}$	$88.8 * 10^{-3}$
Percentage [2]	[%]	---	$4.37 * 10^{-4}$	$1.06 * 10^{-4}$	$1.26 * 10^{-4}$	$0.44 * 10^{-4}$

[1] Ag^+ was measured in pore water as [µg/L]; for calculation in dry matter [µg/kg] a dry matter content of 88.9% was applied for the calculations at day 0 (mean value of the dry matter content of all test concentrations at day 0); at day 56 a dry matter content of 79.5% was applied (mean value of the dry matter content of all test concentrations at day 56); for day 28 a dry matter content of 84.2% was used (mean value of the measured dry matter contents at day 0 and 56)
[2] Recovery with respect to nominal concentration

Effects:

Effect concentrations:

No mortality was observed. Weight change of the adults varied, with both increases and decreases, compared to the control. For reproduction, dose-response curves were obtained (Figure 16, Figure 14) and ECx, NOEC and LOEC-values were calculated (Table 49). Two controls were tested: one control without addition of the stabiliser, and a control with dispersant (concentration of dispersant corresponded to the amount applied in the highest test concentration of NM 330-K). This concentration resulted in about 20% inhibition of reproduction. Inhibition of the dispersant was independent of the application via feed or via soil. Both control values were considered for the calculation of ECx, NOEC and LOEC. Considering the confidence intervals, both concentration-effect curves overlap. In a second experiment the effect of the dispersant was investigated again. In this experiment no difference between control and dispersant control was observed (number of juveniles: control 347 ± 27; dispersant control 329 ± 4). Therefore it is recommended to use ECx, NOEC and LOEC values referring to the control for the assessment of NM-300K.

Table 49: NM-300K – Test with earthworms: summary of effects on number of offspring [mg/kg].

	Spiked feed, control: without further additions	Spiked feed, control: dispersant control	Spiked soil, control: without further additions	Spiked soil, control: dispersant control
EC_{50} [1, 3] [mg/kg]	80.3 (58.5 - 113.4)	121.2 (85.3 - 183.8)	80.0 (33.6 - 413.3)	146.0 (85.8 - 741.4)
EC_{10} [1, 3] [mg/kg]	14.6 (4.6 - 24.8)	39.4 (7.5 - 62.9)	n.d. [2]	24.2 (0.2 - 50.7)
LOEC [3,] [mg/kg]	≤15.0	60.0	≤15.0	30.0
NOEC [3] [mg/kg]	<15.0	30.0	<15.0	15.0
LOEC [4] [µg/kg]	≤65.5 * 10^{-3}	63.4 * 10^{-3}	≤65.5 * 10^{-3}	--- [5]
NOEC [4] [µg/kg]	<65.5 * 10^{-3}	--- [5]	<65.5 * 10^{-3}	65.5 * 10^{-3}

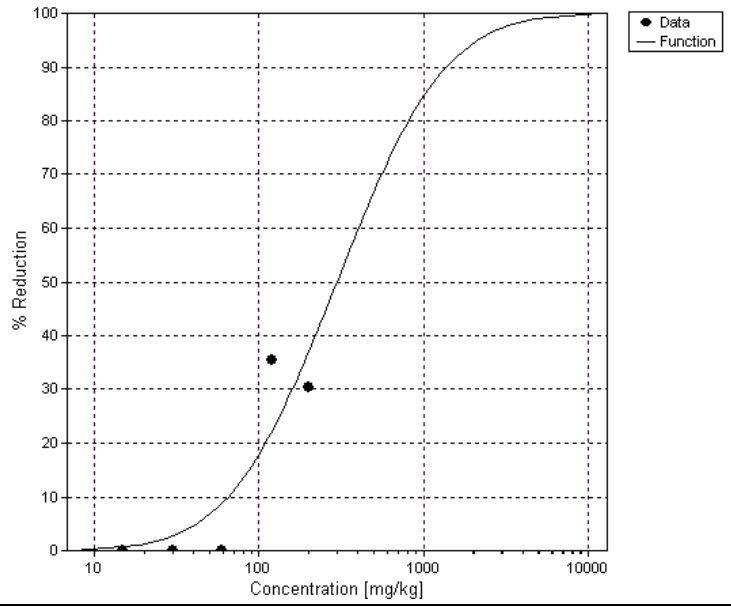
[1] values in brackets: confidence interval; [2] n.d. = not determined due to mathematical reasons or inappropriate data; [3] results refer to nominal values; [4] results refer to Ag ions measured at day 56; [5] ion concentration not measured in test vessels with 30 mg Ag/kg.

Physical/pathological symptoms and changes in behaviour

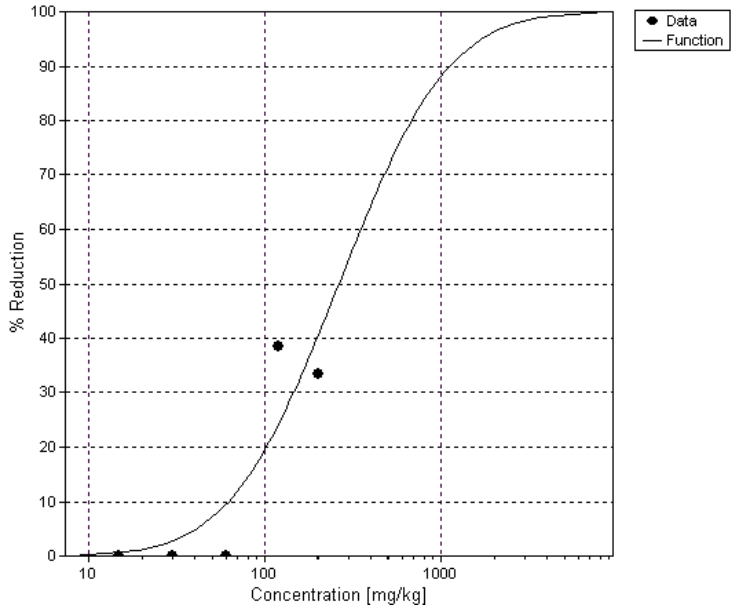
At the beginning of the test a high tendency of the worms to escape from the soil was observed in the tests with Ag-contents ranging from 60 to 200 mg/kg dm. As the vessels were covered with lids, in some of the test vessels the earthworms were observed at the lids and at the walls of the vessels (200 mg/kg: 2 vessels; 120 mg/kg: 2 vessels; 60 mg/kg: 1 vessel). After two days, the earthworms had moved into the soil again. After 28 days, neither physical / pathological symptoms nor changes in behaviour were observed. All specimens gave the impression of healthy condition.

Weight change of the adults

The results of weight change are presented in Figure 13, Figure 14 and Table 50. For raw data of the biomass see chapter 21.3.2. Due to feeding, the biomass of the worms increased in all test approaches during the incubation period. Compared to the control, the increases due to treatments were varied and ranged from a small change to large change in weight. Application in feed showed a concentration-effect relationship which did not occur for application in soil. The weight increase for application in soil exceeded the weight increase for the controls. Differences in weight between the three controls (control; control with dispersant on feed; control with dispersant on soil) during the incubation period were small. Therefore, the higher increase of the weight compared to the controls is assumed to be caused by the silver addition. The mode-of-action as well as the missing concentration-effect-relationship when soil was spiked cannot be explained so far.



Spiked feed, control: without further additions



Spiked feed, control: dispersant control

Figure 13: NM-300K – Test with earthworms: spiked feed, weight change - concentration-effect curve.

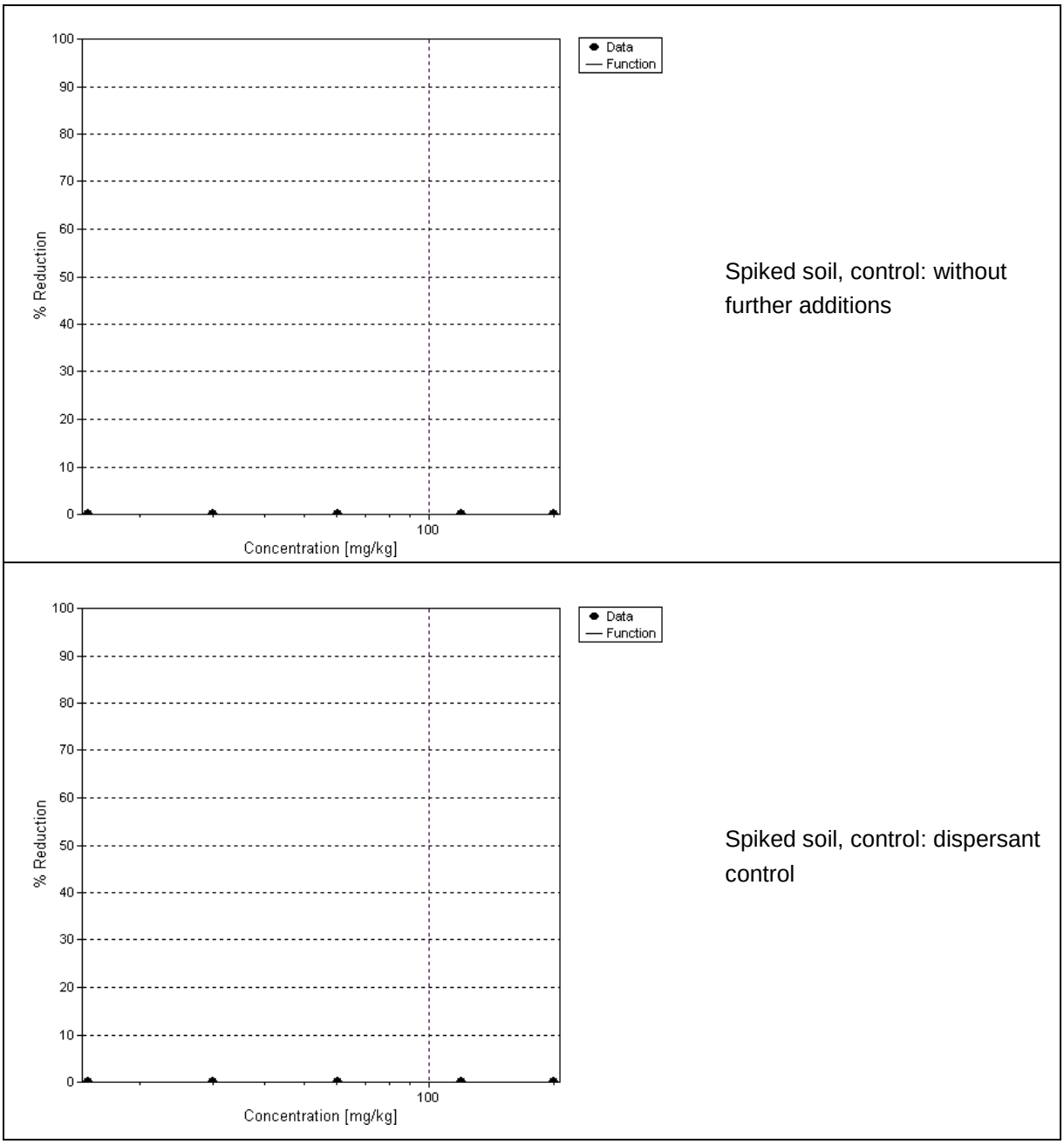


Figure 14: NM-300K – Test with earthworms: spiked soil, weight change - concentration-effect curve.

Table 50: NM-300K – Test with earthworms: mean weight at test start and weight change at test end.

Concentrations given as nominal values

	Control	Control with dispers-ant on feed	Control with dis-persant on soil	Application on feed [mg/kg]					Application on soil [mg/kg]				
				15	30	60	120	200	15	30	60	120	200
Mean weight at test start [g]	3.57	3.77	3.74	3.72	3.61	3.78	3.62	3.45	3.24	3.33	3.53	3.39	3.47
Standard deviation [g]	0.20	0.37	0.36	0.10	0.23	0.39	0.24	0.12	0.13	0.08	0.23	0.15	0.14
CV	5.5	9.8	9.7	2.7	6.3	10.5	6.7	3.5	3.9	2.3	6.5	4.3	4.0
Mean weight change [%]	40.1	42.0	35.8	51.6 *[1]	47.7	45.7	25.9	27.9 **	62.2 *	61.6	61.4 **	66.9 ***	62.4 **
Standard deviation [%]	7.4	1.6	8.6	7.1	3.4	4.4	12.4	2.9	19.7	17.8	9.0	4.8	5.9
CV	18.5	3.9	24.0	13.7	7.0	9.6	48.0	10.3	31.7	28.9	14.6	7.1	9.4

[1] Significant when compared with control (without dispersant): * 0.05 ≥ P ≥ 0.01; ** 0.01 ≥ P ≥ 0.001; ***0.001 ≥ P

Mortality:

No mortality was observed.

Reproduction:

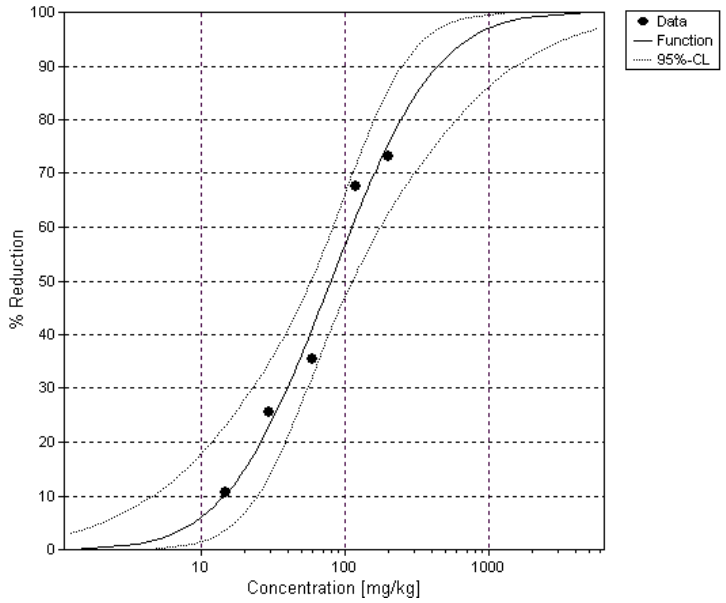
The results for reproduction are presented as mean values (Table 52). For single values of the replicates see chapter 21.3.2. Independent of whether the soil or feed was spiked, the dispersant had a small impact on reproduction, i.e. there were fewer juveniles in the control with dispersant (e.g. for soil spiking: 268) than in the control (e.g. for soil spiking: 341), representing a statistically significant 22% inhibition of reproduction. The small inhibitory effect was not reproducible. Therefore, it is concluded that the effect of the dispersant observed in this test reflects biological variability. Both control values were considered with respect to the effect of Ag-NPs and inhibition was calculated in comparison to both the control and the control with dispersant.

We found a concentration-effect relationship and observed strong inhibition of earthworm reproduction (Figure 15, Figure 16, Table 49). For the experiment with spiked soil, in comparison to the control, the inhibition of reproduction ranged from 26% at the lowest concentration to 72% at the highest concentration, yielding an EC50 of 80 mg/kg compared to the control and an EC50 of 146.0 mg/kg for the dispersant control. Soil spiking and feed spiking resulted in comparable effects and EC50 values.

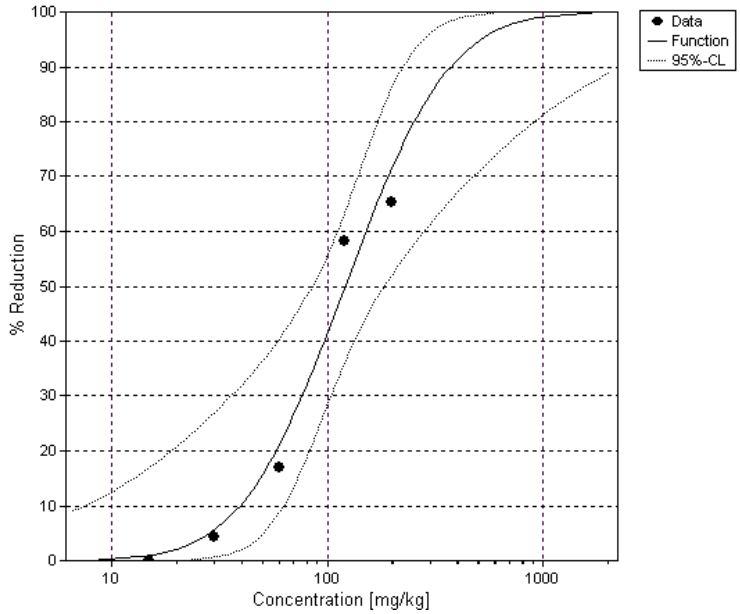
The size of the worms in the different treatments differed considerably. In the control, the worms were much smaller than in the samples treated with 120 and 200 mg/kg. The juve-

niles in the control worms showed the typical expected sizes. Ten representative worms were selected and are depicted in Figure 17. These worms were also weighed. As some of the selected worms escaped before weighing, the result is presented as mean weight per remaining worms (Table 51).

The effect was observed in all replicate test vessels. However, the effect was not reproducible when the test was repeated and different dung charges and grinding degrees were tested. Therefore, the reason for the increase in size is still unknown.

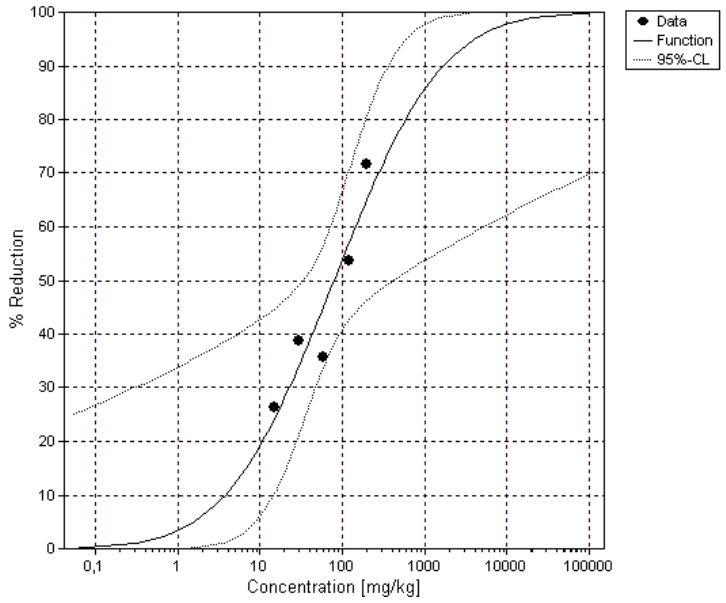


Spiked feed, control: without further additions

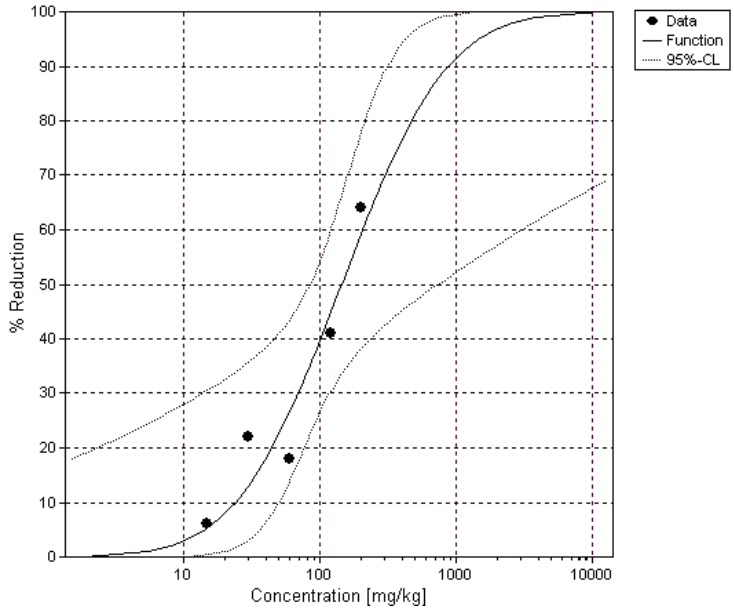


Spiked feed, control: dispersant control

Figure 15: NM-300K – Test with earthworms: spiked feed, reproduction - concentration-effect curve.



Spiked soil, control: without further additions



Spiked soil, control: dispersant control

Figure 16: NM-300K – Test with earthworms: spiked soil, reproduction - concentration-effect curve.

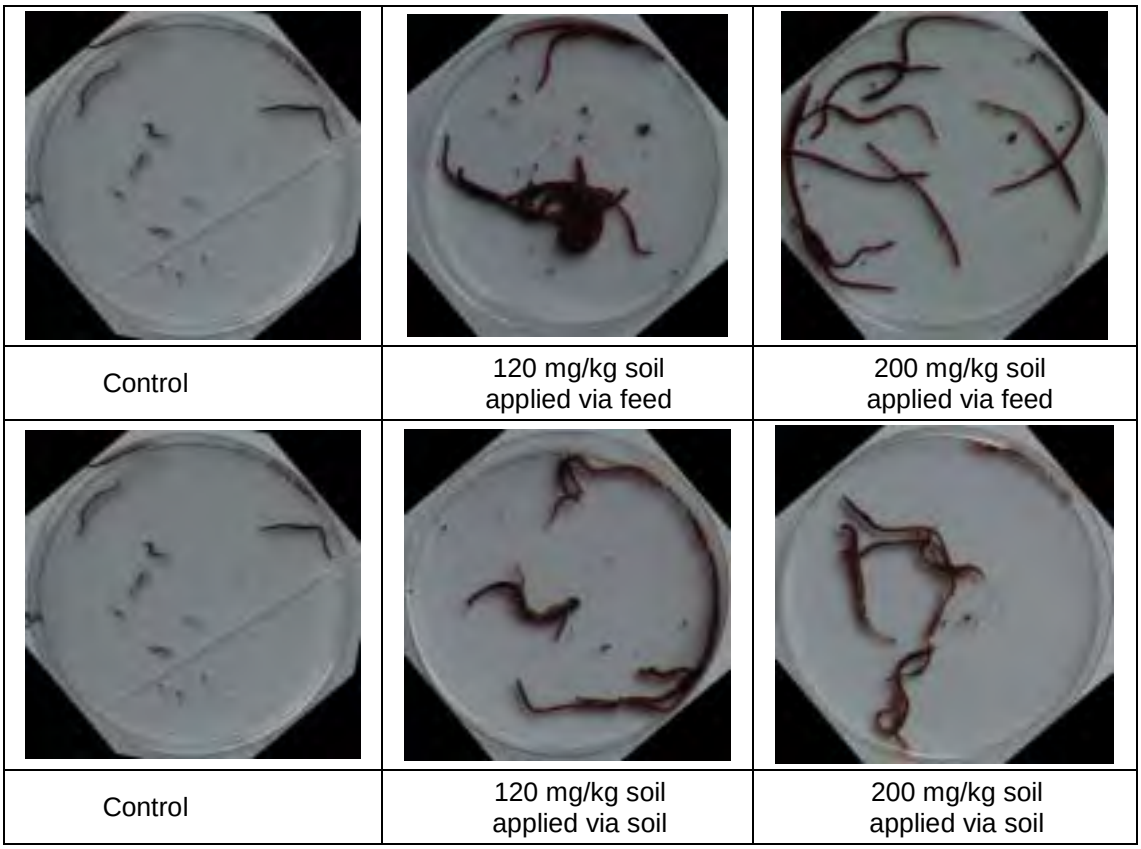


Figure 17: NM-300K - Representative sizes of earthworms after 56 days.

(Every photograph shows 10 worms; Ø of petri dishes: 100 mm; small black dots in petri dishes: gut content)

Table 51: NM-300K – Test with earthworms: mean weight of the offspring presented in Figure 17.

Content	Calculated weight per worm [mg]
Control	11
120 mg / kg (soil)	69.3
200 mg / kg (soil)	53.7
120 mg / kg (feed)	108.6
200 mg / kg (feed)	72.0

Table 52: NM-300K – Test with earthworms: number of juveniles at test end.

Mean values and coefficient of variance (CV)

	Control	Control with dispersant on feed	Control with dispersant on soil	Application on feed [mg/kg]					Application on soil [mg/kg]				
				15	30	60	120	200	15	30	60	120	200
Juveniles	341.4	265.5	268.0	304.8	253.8	220.5	111.0	91.8	251.5	208.8	219.5	158.0	96.5
Standard deviation	26.1	11.5	45.5	27.4	26.4	18.1	50.8	8.5	30.8	46.4	30.2	18.1	26.1
CV	7.6	4.3	17.0	9.0	10.4	8.2	45.8	9.3	12.2	22.2	13.7	11.4	27.0
Inhibition to control [%]	---	22 *	21 *	11	26 *	35 *	67 *	73 *	26 *	39 *	36 *	54 *	72 *
Inhibition to dispersant control [%]	---	---	---	-15	4	17	58 *	65 *	6	22	17	41 *	64 *

* statistical difference: $p > 0.05$ (* $0.05 \geq P \geq 0.01$; ** $0.01 \geq P \geq 0.001$)

8.5.2 Concentrations of silver in earthworms

In some of the test approaches the Ag concentration in the earthworms was determined.

The results are compiled in Table 53 and Figure 18. Chapter 21.3.1 presents the results in more detail.

In the control worms, and in the worms treated with the dispersant, no silver was determined. In contrast, silver was detected in all treated worms. There was no obvious difference between experiments with spiking of soil or spiking of food. A dependence on the concentration was not observed.

We observed a concentration-dependent effect on reproduction above the lowest test concentrations (15 mg/kg), but although the lowest and highest test concentrations differed by a factor of 13, the silver concentrations in the earthworms were comparable. We therefore assume that a steady state of silver uptake is already achieved at 30 mg/kg dm. The concentrations in the worms were below the concentration in the test vessels. It is unclear whether the measured silver is located in the tissues or whether residues remain in the gut due to incomplete purging. We also do not know whether the determined silver occurs in particle or ionic form. The comparable concentrations in earthworms exposed to soil concentrations greater than 30 mg/kg and the concentration-dependent inhibition of reproduction at concentrations of 30–200 mg/kg dm indicate that the silver content in the worms is not responsible for the observed effects. We assume that the fertility of adults is not affected but the development of cocoons and the juveniles in soil are Ag-sensitive life stages. Whether the cocoon or juvenile

worm life stage is more susceptible is still unknown. The number of cocoons is not an obligatory endpoint according to the guideline. Therefore, no special attention was placed on the remaining number of cocoons. Nevertheless, differences in the number of remaining cocoons at the various test concentrations were not obvious.

Table 53: NM-300K – Test with earthworms: Ag concentration in earthworms.

Sample	Mean Ag in dry weight ± SD [µg/g]
Control	---
Vehicle soil	---
Vehicle food	---
15 mg/kg; spiked soil	6.99 ± <0.01
15 mg/kg; spiked food	9.54 ± 0.50
30 mg/kg; spiked soil	10.5 ± 0.4
30 mg/kg; spiked food	10.6 ± 0.4
60 mg/kg; spiked soil	11.1 ± 0.2
60 mg/kg; spiked food	11.7 ± 0.9
120 mg/kg; spiked soil	11.3 ± 0.4
120 mg/kg; spiked food	11.3 ± 0.3
200 mg/kg; spiked soil	11.2 ± 0.1
200 mg/kg; spiked food	13.2 ± 0.2

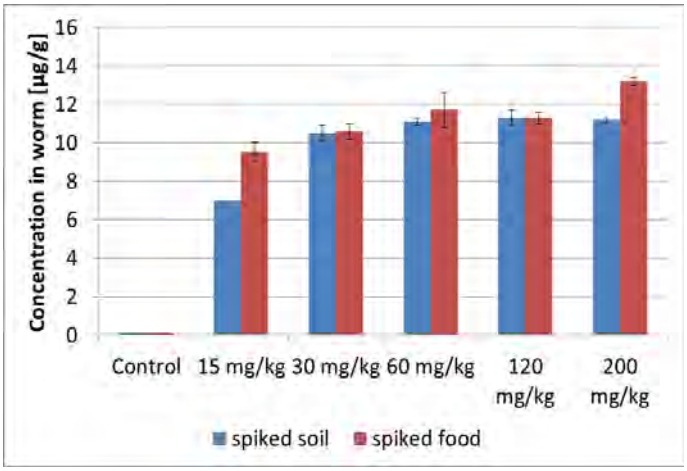


Figure 18: NM-300K – Test with earthworms: Ag concentration in earthworms.

8.6 Validity

NM-300K

The earthworm reproduction test fulfils the validity criteria of the guideline:

- With 341 individuals the rate of reproduction in the control was ≥ 30 juveniles per test vessel.
- With 7.6% the CV of reproduction in the control does not exceed 30%.
- With 0% in both tests the percent mortality of the adults observed in the controls over the initial 4 weeks is ≤ 10%.

8.7 Data with the reference substance

As reference substance carbendazim was tested.

Test period: February 11, 2010 - April, 8, 2010

The following values were calculated for reproduction [mg/kg]; values in brackets indicate the confidence interval:

EC_{10}: 1.147 (1.118 -1.172)

EC_{20}: 1.309 (1.289 -1.328)

EC_{50}: 1.688 (1.670 -1.709)

LOEC: 1.500

NOEC: 0.750

All validity criteria were fulfilled.

According to the guideline significant effects should be observed between 1 and 5 mg/kg. This criterion is fulfilled.

8.8 Conclusion

Silver nanoparticles (NM-300K) were tested in the earthworm reproduction test. The tested silver nanoparticles (NM-300K) caused a reduction of the reproduction rate.

Concerning reproduction, the ECx, NOEC and LOEC values presented in Table 54 were determined. The difference between application of the nanoparticles via feed and via soil seems to be negligible.

An obvious increase in weight of the juveniles was observed. However, the effect was not reproducible and comparable observations are not reported in the literature to the best of our knowledge.

8.9 Executive summary

Silver nanoparticles (**NM-300K**) and the dispersant in NM-300K (**NM-300KDIS**) were tested in the earthworm reproduction test. The particles were applied in soil and in feed. The test substrate was a natural sandy soil. The test concentrations were 15, 30, 60, 120, 200 mg/kg soil, dry matter.

No mortality was observed. For reproduction, dose-response curves were obtained. Two controls were considered: one control without addition of the dispersant, and a control with dispersant (concentration of dispersant corresponded to the amount applied in the highest test concentration of NM 330-K). This concentration resulted in about 20% inhibition of reproduction. Inhibition of the dispersant was independent of the application via feed or via soil. Both control values were considered for the calculation of ECx, NOEC and LOEC (Table 54). Considering the confidence intervals, both concentration-effect curves overlap. In a second experiment the effect of the dispersant was investigated again. In this experiment no difference between control and dispersant control was observed (number of juveniles: control 347 ± 27; dispersant control 329 ± 4). Therefore it is recommended to use ECx, NOEC and LOEC values referring to the control for the assessment of NM-300K.

Differences resulting from the exposure of the earthworms via feed and via soil seem to be negligible.

An increase in size and weight of the juveniles was observed. However, this observation was not reproducible.

The presented results are based on nominal concentrations. Based on the concentrations determined with DGTs in soil the effect values are lower by a factor about 10^{-4}. This illustrates that the basis of the calculation has to be clearly fixed for regulatory purposes.

Table 54: NM-300K - earthworm reproduction: summary of the effect values.

	Spiked feed, control: without further additions	Spiked feed, control: dispersant control	Spiked soil, control: without further additions	Spiked soil, control: dispersant control
EC_{50} [mg/kg] [1]	80.3 (58.5 - 113.4)	121.2 (85.3 - 183.8)	80.0 (33.6 - 413.3)	146.0 (85.8 - 741.4)
EC_{10} [mg/kg] [1]	14.6 (4.6 - 24.8)	39.4 (7.5 - 62.9)	n.d. [2]	24.2 (0.2 - 50.7)
LOEC [mg/kg]	≤15.0	60.0	≤15.0	30.0
NOEC [mg/kg]	<15.0	30.0	<15.0	15.0

[1] values in brackets: confidence interval;
[2] n.d. = confidence interval not determined due to mathematical reasons or inappropriate data

Additionally, the Ag concentration in the earthworms was determined. In the control worms and in the worms treated with the dispersant (concentration of dispersant corresponded to the amount applied in the highest test concentration of NM 330-K) no silver was determined. In contrast, silver was detected in all worms incubated in soil containing NM-300K and in the worms fed with spiked food. There was no obvious difference between the two experiments (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 achieved at the lowest test concentration.
Concentration-dependent effects are observed above the lowest test concentration. Although

the applied test concentrations increased, the silver concentration in the worms remained the same. We assume that the fertility of adults is unaffected but that the development of cocoons and the juveniles in soil are sensitive life stages. We do not know yet whether which life stage (cocoons or juvenile worms) is the most susceptible.

In none of the test designs did the silver concentration in the worms exceed 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 tissue or whether residues remained in the gut due to incomplete purging. We also do not know whether the determined silver occurred in particle or ionic form.

9 Microorganisms - Nitrogen Transformation Test (OECD TG 216)

9.1 Test principle

The effects of the test item on nitrogen transformation were determined in a natural soil. After mixing the test item into the soil, the soil was incubated in the dark at 20 ± 2°C for 28 days. Samples were taken at test start and after 28 days of incubation. The test item was applied once. To measure the nitrogen transformation the nitrate concentration in soil was determined after the soil had been amended with powdered plant material as a natural nitrogen source.

9.2 Materials and methods

9.2.1 Test guideline

The test was performed according to:

OECD Guidelines for the Testing of Chemicals Test No. 216: "Soil Microorganisms: Nitrogen Transformation Test" (2000).

9.2.2 GLP

The test was performed following the principles of GLP (OECD, 1998). In deviation to GLP no archiving of the raw data was performed and the quality assurance unit was not involved with respect to the inspection of the test, of the raw data and of the report. All laboratory equipment (e.g. balances, thermometers, pH-meters) were calibrated and documented according to GLP.

9.2.3 Test material

- P25 - distributed by Evonik for the OECD Sponsorship Programme
  The properties should correspond to the properties of NM-105.

The nanoparticles were stored in the dark at room temperature until use.

9.2.4 Analytical monitoring

Due to the high natural concentration of TiO_2 in the test soil no specific chemical analyses were performed in the soil.

The zeta potential was measured in the test dispersions using a Malvern Zetasizer Nano ZS. The particle size distribution in the dispersion was not determined. Doing so would give no information on the size distribution in soil. A measurement of the Zeta-potential or the particle size distribution in soil is not yet possible.

9.2.5 Test item – preparation protocol

We tested two different modes of application: spiking via powder and via dispersion.

The nominal concentrations of the test item in the test containers were 9.3, 21, 45 and 100 mg P25/kg soil, dry mass (application via powder) and 9.3 and 21 mg/kg (application via dispersion). Three replicates per concentration were conducted.

Spiking of soil with TiO_2 powder

For the first application the TiO_2 powder was mixed directly into the soil, whereby air-dried test soil (1% of the total amount) was used as a carrier for the TiO_2 powder. Amounts of TiO_2 powder suitable to achieve the desired final soil content were mixed homogenously with the dry soil. Care was taken to avoid a modification of the TiO_2 crystalline structure. Uncontaminated test soil (between 20-30% of WHC_{max}) was spread on a plate, the carrier material with the TiO_2 powder was distributed on the test soil, and all was mixed carefully. In the same way, 5 g/kg dm ground lucerne was mixed into the soil. For the test with contaminated soil the soil was adjusted to a water-holding capacity of 55% of (WHC_{max}).

Test concentrations were: 9.3, 21, 45 and 100 mg/kg soil dry matter (dm).

Spiking of soil with aqueous TiO_2 dispersion

The second application trial was to spray a TiO_2 dispersion (TiO_2 nanoparticles in deion. water) that had been prepared with a magnetic flea (900 rpm; 1 min) and ultrasonication (3 min) in a bath sonicator. Test soil was dried to about 10% of WHC_{max} and spread on a plate. 5 g/kg dm of ground lucerne was mixed into the soil. Immediately after preparation TiO_2 dispersion was sprayed on the soil by means of a syringe coupled with a cannula and then mixed thoroughly. Finally, the test soil was adjusted to a water-holding capacity of 55% of WHC_{max}. A maximum concentration of about 200 mg/L application dispersion of TiO_2 nanoparticles was considered as adequate for the tests. Higher concentrations would have sedimented rapidly preventing a homogenous distribution of the nanomaterial in the soil. Furthermore, it was assumed that higher concentrations in the application dispersion would result in larger agglomerates. Based on the water content of the soil, no more than 212 mg/L application dispersion could be used. The suspension was continuously stirred to achieve homogeneity during spiking. Due to these limitations, only the soil contents of 9.3 and 21 mg/kg were tested.

The test concentrations were: dispersion with 88 and 212 mg/L deionised water; application of 193 ml test dispersion to 1.8 kg test soil (dm) corresponding to 9.3; and application of 179 ml test dispersion to 1.8 kg test soil (dm) corresponding to 21 mg/kg soil (dm).

9.2.6 Test organism

A sandy soil with the individual soil microflora was investigated.

9.3 Study design

9.3.1 Total exposure period

The exposure period was 28 days:

June 17, 2010 - July 15, 2010

9.4 Test conditions

9.4.1 Environmental conditions

Physicochemical data

The incubation temperature was measured continuously with a thermograph. With 20 - 21°C the permitted range of 20 ± 2°C was kept. Incubation occurred in the dark. The soil dry mass was maintained during the whole test at 89.6% (controls), 89.6% (powder application: 9.3 mg/kg), 88.9% (powder application: 21 mg/kg), 89.7 % (powder application: 45 mg/kg), 89.2 % (powder application: 100 mg/kg), 89.4 % (dispersion application: 9.3 mg/kg) and 89.6% (dispersion application: 21 mg/kg).

9.4.2 Test soil

The test soil was a natural sandy soil (Certified RefeSol 01-A. batch IME-01: sand 71%, silt: 24%, clay: 5%, org C: 0.93%, pH 5.7, clay: 5%). Selected soil parameters are presented in Table 55. The soil was sieved to 2 mm. It was not sterilised and had been stored outdoors on the grounds of the test facility in high grade stainless steel basins with drainage, and ground contact.

For at least one year prior to soil sampling in the field, no plant protection products were applied to the sampling site. No organic or mineral fertilisers were applied to the soil for six and three months, respectively, prior to soil sampling.

Dates of the soil handling for the test are presented in Table 56.

Table 55: Test soil for microbial tests: soil parameters.

Soil name	RefeSol 01-A
Soil batch	IME-01
Soil texture	Loamy sand
Clay [%]	5
Silt [%]	24
Sand [%]	71
WHC [g H_2O/kg soil dry weight] [1]	264
CECeff [mmol/kg] [2]	37.9
pH	5.7
Total org. C [%]	0.93
Microbial biomass [mg C/kg dry mass soil], calculated from respiration activity	91
Microbial biomass [% of total org. C]	1.0
Total nitrogen [%]	0.09
NO_3^- [mg/kg dry weight]	81.7

[1] WHC = water holding capacity; [2] CECeff = effective cation exchange capacity

Table 56: Test soil for microbial tests: storage information.

Soil name	RefeSol 01-A
Soil batch	IME-01
Date of field sampling	11.06.2010
Start of indoor storage at room temperature to reduce the water content and to allow sieving; the soil was distributed in a thin layer; surface drying was prevented by periodically turning the soil.	11.-13.09.2008
Date of sieving for the study	13.06.2010
Start of soil conditioning [1]	13.06.2010
Date of application	17.06.2010

[1] Soil conditioning was performed at room temperature in the dark.

9.4.3 Concentration levels

For the application via powder, the nominal contents in the test containers with TiO_2 nanoparticles were 9.3, 21.0, 45.0, 100.0 mg/kg soil dry matter. For the application via dispersion, the nominal contents in the test containers with TiO_2 nanoparticles were 9.3 and 21.0 mg/kg soil dry matter. The concentrations differed by a factor of 2.2. Three replicates per concentration were conducted.

9.4.4 Other information on materials and methods

Frequency of treatment

The treatments were applied once at test start.

Control group and treatment

The control consisted of soil only without any nano-particle addition. Three replicates per control were conducted.

Statistical method

Data evaluation:

In this report numerical values were frequently rounded to a smaller degree of precision (number of digits) than used in the actual calculation. Minor differences in results obtained from calculations with rounded values in comparison to results obtained with higher precision values are possible. They are, however, well within the limits of the experimental accuracy and of no practical concern.

Statistical calculations:

For each concentration, the quantity of nitrate was determined. According to the guidelines for non-agrochemicals, the quantities of nitrate found in the treated samples after 28 days were compared to those found in the controls. Furthermore, the percent deviation values for the test concentrations were calculated. Means were compared by means of the STUDENT-t test and the U-Test (Wilcoxon, Whitney and Mann) for significant differences between control and study groups. EC_x,LOEC, and NOEC calculations were performed with the computer software ToxRat Professional version 2.10.4.1 (ToxRat® Solutions GmbH).

Test procedure

Sieved and spiked soil was amended with powdered plant material (lucerne-grass-green meal) at a plant/soil ratio of 5 g plant per kilogram of soil (dry mass). Three incubation containers per treatment were filled with 658 g of spiked soil. A further three incubation containers were filled with 658 g of control soil.

The test was carried out in the dark at 20 ± 2°C for 28 days. During the whole test the moisture content of the soil was maintained at 40 - 60% of WHC_{max} with a maximum of 5% outside this range. The mass in the test vessels was measured weekly. Evaporated water was supplemented by adding deionised water.

Samples of each treated and control replicate were analysed for nitrate at the beginning (3 h after application, in the following designated as day 0) and at the end of the exposure period (28 days).

Nitrate was extracted from soil by shaking samples (20 g dry mass) with 0.1 M KCl solution at a ratio of 5 mL of KCl solution per gram dry weight for 60 minutes at 150 rpm. The mix-

tures were filtered and the liquid phases analysed for nitrate photometrically (Spectroquant® NOVA 400). Analyses were performed immediately after preparing the extracts.

9.5 Results

9.5.1 Zeta potential

The zeta potential for P25 is presented in Table 57. A negative value of -18 mV was determined in deionised water.

Table 57: P25 - N-transformation: zeta potential in the stock dispersion used for application via dispersion.

Sample	Zeta potential [mV]
100 mg/L	-18 mV

9.5.2 Nitrogen transformation

Effect concentrations:

For the **application via powder** the following concentration-dependent effects were observed: concentration-dependent decreased nitrate values at day 0, increased nitrate values at day 28 and increased nitrogen transformation rates (difference of nitrate content between day 28 and day 0).

The application via dispersions gave no clear effect. Differences to the control were small and statistically not significant. There was no obvious effect dependant on concentration.

In Table 58 the effect values are presented. A prerequisite for calculating EC_x values is an inhibitory effect. Due to stimulation at day 28 an EC value could only be calculated at test start.

Table 58: P25 - N-transformation: summary of the effects.

Application via powder and dispersion

	Application via powder		Application via dispersion	
	Day 0 (= 3 h after application)	Day 28	Day 0	Day 28
Nitrate content				
EC_{10} [mg/kg]	23.6	n.d. [2]	n.d. [2]	n.d. [2]
EC_{25} [mg/kg]	108.3	n.d. [2]	n.d. [2]	n.d. [2]
LOEC [mg/kg]	21.0	100.0	> 21	> 21
NOEC [mg/kg]	9.3	45.0	≥ 21	≥ 21
Nitrogen transformation [1]				
EC_{10} [mg/kg]	n.d. [2]		n.d. [2]	
EC_{25} [mg/kg]	n.d. [2]		n.d. [2]	
LOEC [mg/kg]	21.0		> 21	
NOEC [mg/kg]	9.3		≥ 21	

[1] Nitrogen transformation: difference of nitrate content at day 28 and day 0; [2] n.d. = not determined due to mathematical reasons (only two concentrations)

Nitrate measurement

The results of the nitrate measurement are presented as mean values in Table 59 - Table 61. For single values of the replicates see Table 62.

Table 59: P25 - N-transformation: mean nitrate content [mg/kg].

			Application via powder [mg TiO_2/kg] [1]				Application via dispersion [mg TiO_2/kg]	
		Control	9.3	21	45	100	9.3	21
Day 0	Mean	27.3	27.2	24.7**	21.7***	21.5**	27.8	26.0
	Std.Dev.	1.1	2.8	1.7	2.6	3.4	2.8	4.1
	CV	4.0	10.3	6.7	12.0	15.9	10.0	15.8
Day 28	Mean	32.6	35.9	35.8	36.8	39.3**	36.2	34.1
	Std.Dev.	3.4	2.9	2.0	4.0	2.5	4.9	1.3
	CV	10.4	8.0	5.5	10.8	6.3	13.6	3.8

[1] statistical significance: * 0.05 ≥ P > 0.01; ** 0.01 ≥ P > 0.001; *** P <= 0.001

Table 60: P25 - N-transformation: mean nitrate content, [%] of control.

	Application via powder [mg TiO_2/kg]					Application via dispersion [mg TiO_2/kg]	
	Control	9.3	21	45	100	9.3	21
Day 0	100	99.6	90.5	79.5	78.8	101.7	95.2
Day 28	100	110.1	109.7	112.9	120.6	111.0	104.4

Table 61: P25 - N-transformation: N-transformation rate [mg/(kg*28 d)].

		Application via powder [mg TiO_2/kg]				Application via dispersion [mg TiO_2/kg]	
	Control	9.3	21	45	100	9.3	21
Difference of 2 determinations (d28-d0)	2.3	7.3	11.3	11.3	18.3	6.9	8.8
	6.7	9.0	12.5	14.2	11.9	7.3	11.5
	7.1	12.5	9.5	19.8	23.3	11.1	3.9
Mean	5.4	9.6	11.1*	15.1*	17.8*	8.3	8.1
Std.Dev	2.7	2.7	1.5	4.3	5.7	2.3	3.9
Deviation from control [%]		77.8	105.5	179.6	229.6	53.7	50.0

* 0.05 ≥ P > 0.01; ** 0.01 ≥ P > 0.001; *** P <= 0.001

Table 62: P25 - N-transformation: content of nitrate [mg/kg dry mass soil].

Single values of the replicates

Date of sampling	Replicate		Application via powder [mg TiO_2/kg]				Application via dispersion [mg TiO_2/kg	
		Control	9.3	21	45	100	9.3	21
Test start	1.1	27.5	27.2	22.3	18.8	21.5	27.7	22.9
	1.2	26.4	28.6	26.3	24.7	19.8	26.1	29.8
	2.1	26.5	31.4	24.0	23.3	23.1	31.2	21.7
	2.2	28.9	24.3	24.0	20.4	27.3	23.5	22.3
	3.1	28.3	23.9	24.7	24.0	19.9	28.0	28.5
	3.2	26.3	27.8	26.8	19.1	17.4	30.2	30.7
Day 28	1.1	26.2	35.4	36.5	33.5	37.4	31.3	36.7
	1.2	32.0	35.0	34.7	32.5	40.5	36.3	33.6
	2.1	33.3	35.9	35.9	37.2	38.0	33.7	33.5
	2.2	35.4	37.7	37.0	34.9	36.1	35.6	33.5
	3.1	35.3	31.4	38.0	39.9	41.9	45.6	33.4
	3.2	33.4	40.0	32.4	42.8	41.9	34.7	33.6

At test start the nitrate content decreased with increasing soil contents of TiO_2 nanoparticles. No clear difference between application forms was observed.

At day 28 the nitrate content increased with increasing soil contents of TiO_2 nanoparticles.

The transformation rate calculated during the incubation time of 28 days showed an increased nitrogen transformation rate with increasing contents of TiO_2 nanoparticles applied via powder. Application via dispersion did not cause an increased bioavailability of the nanomaterial compared to the application via dispersion. The transformation rates determined after application via dispersion were slightly lower than the transformation rates after application via powder.

9.6 Validity

A validity criterion has been formulated in the guideline for the testing of agrochemicals. The evaluation of the results with agrochemicals is based on relatively small differences (i.e. average value ± 25%) between the carbon dioxide released or the oxygen consumed in the control and the treated soil samples so that large variations in the controls can lead to false results. Therefore, the variation between replicate control samples should be less than ± 15%.

For non-agrochemicals concentration-effect relationships are relevant. Therefore a variation of 15% is of less importance. In this test the validity criteria for agrochemicals are fulfilled as the. CV was 4.0% (day 0) and 10.4% (day 28).

9.7 Reference substance

The guideline does not demand the investigation of a reference substance. A reference substance was not tested.

9.8 Conclusion

The effects of TiO_2 nanoparticles on earthworms were tested by:

- application via powder in soil: 9.3, 21.0, 45.0 and 100.0 mg/kg soil
- application via dispersion in soil: 9.3 and 21.0 mg/kg soil.

The application via powder caused concentration-dependent effects, namely concentration-dependent decreased nitrate values at day 0 (sampling of the soil three hours after application), increased nitrate values at day 28 and increased nitrogen transformation rates (difference of nitrate content between day 28 and day 0).

The application via dispersion gave no clear effect. The difference to the control was small and not statistically significant. It is assumed that an application via dispersion does not cause an increased bioavailability of TiO_2 nanoparticles for the soil microflora.

9.9 Executive summary

TiO_2 nanoparticles (**P25**) were tested in the nitrification assay (OECD TG No. 216). Soil was spiked with powder and with dispersion. As test substrate, a natural sandy soil was used. The following test concentrations were investigated:

- Application via powder in soil: 9.3, 21.0, 45.0, 100.0 mg/kg soil
- Application via dispersion in soil: 9.3, 21.0 mg/kg soil.

The nitrate content was determined photometrically at day 0 (sampling of the soil three hours after application) and at day 28.

The application via powder caused concentration-dependent effects, namely, decreased nitrate values at day 0 (sampling of the soil three hours after application), increased nitrate

values at day 28 and increased nitrogen transformation rates (difference in nitrate content between day 28 and day 0).

In Table 63 the NOEC and ECx values are summarised.

Table 63: P25 - N-transformation: summary of effects.

Application via powder and dispersion)

	Application via powder		Application via dispersion	
	Day 0 (= 3 h after application)	Day 28	Day 0	Day 28
Nitrate content				
EC_{10} [mg/kg] [2]	23.6	n.d.	n.d.	n.d.
EC_{25} [mg/kg] [2]	108.3	n.d.	n.d.	n.d.
LOEC [mg/kg]	21.0	100.0	> 21	> 21
NOEC [mg/kg]	9.3	45.0	≥ 21	≥ 21
Nitrogen transformation [1]				
EC_{10} [mg/kg] [2]	n.d.		n.d.	
EC_{25} [mg/kg] [2]	n.d.		n.d.	
LOEC [mg/kg]	21.0		> 21	
NOEC [mg/kg]	9.3		≥ 21	

[1] Nitrogen transformation: difference in nitrate content at day 28 and day 0;
[2] n.d. = not determined due to mathematical reasons or inappropriate data

The application via dispersion gave no clear effect. The difference to the control was small and not statistically significant. It is assumed that an application via dispersion does not cause an increased bioavailability of TiO_2 nanoparticles for the soil microflora.

10 Microorganisms - Carbon Transformation Test (OECD TG 217) – TiO_2

10.1 Test principle

The effects of the test item on carbon transformation were determined in a natural soil. After mixing the test item into the soil, the soil was incubated at 20 ± 2°C for 28 days in the dark. Samples were taken at test start and after 28 days of incubation. The test item was applied once. For measurement of carbon transformation a short-term respiration test (glucose-induced respiration rates) in soil was performed.

10.2 Materials and methods

10.2.1 Test guideline

The test was performed according to:

OECD Guidelines for the Testing of Chemicals Test No. 217: "Soil Microorganisms: Carbon Transformation Test" (2000).

10.2.2 GLP

The test was performed following the principles of GLP (OECD, 1998). In deviation to GLP no archiving of the raw data was performed and the Quality Assurance Unit was not involved with respect to the inspection of the test, of the raw data and of the report. All laboratory equipment (e.g. balances, thermometers, pH-meters) were controlled and documented according to GLP.

10.2.3 Test material

- P25 - distributed by Evonik for the OECD Sponsorship Programme
  The properties should correspond to the properties of NM-105.

The nanoparticles were stored in the dark at room temperature until use.

10.2.4 Test type

Static. laboratory test.

10.2.5 Analytical monitoring

Due to the high natural concentration of TiO_2 in the test soil no specific chemical analyses were performed in the soil.

The zeta potential was measured in the test dispersions using a Malvern Zetasizer Nano ZS. The particle size distribution in the dispersion was not determined. It would give no information on the size distribution in soil. A measurement of the Zeta-potential or the particle size distribution in soil is not yet possible.

10.2.6 Test item – preparation protocol

We tested two different modes of application: spiking via powder and spiking via dispersion.

The nominal concentrations of the test item in the test containers were 9.3, 21, 45, and 100 mg P25/kg soil, dry mass (application via powder) and 9.3 and 21 mg/kg (application via dispersion). Three replicates per concentration were conducted.

Spiking of soil with TiO_2 powder

For the first application the TiO_2 powder was mixed directly into the soil using air-dried test soil (1% of the total amount) as a carrier for the powder. Amounts of TiO_2 powder that were suitable to achieve the desired final soil content were mixed homogenously with the dry soil. Care was taken to avoid a modification of the TiO_2 crystalline structure. Uncontaminated test soil (between 20 and 30% of WHC_{max}) was spread on a plate, the carrier material with the TiO_2 powder was distributed onto the test soil, and all was mixed carefully. In the same way, 5 g/kg dm ground lucerne was mixed into the soil. For the test with contaminated soil, the soil was adjusted to a water-holding capacity of 55% of the maximum water-holding capacity (WHC_{max}).
Test concentrations were: 9.3, 21, 45 and 100 mg/kg soil dry matter.

Spiking of soil with aqueous TiO_2 dispersion

The second application trial was to spray a TiO_2 dispersion (TiO_2 nanoparticles in deionised water) that had been prepared with a magnetic flea (900 rpm; 1 min) and ultrasonication (3 min) in a bath sonicator. The test soil was dried to about 10% of WHC_{max} and spread on a plate. 5 g/kg dm of ground lucerne were mixed into the soil. Immediately after preparation, the TiO_2 dispersion was sprayed onto the soil by means of a syringe coupled with a cannula, and thoroughly mixed. Finally, the test soil was adjusted to a water-holding capacity of 55% of WHC_{max}.

A maximum concentration of about 200 mg/L application dispersion of TiO_2 nanoparticles was considered as adequate for the tests. Higher concentrations would have sedimented rapidly preventing a homogenous distribution of the nanomaterial in the soil. Furthermore, it was assumed that higher concentrations in the application dispersion would result in larger agglomerates. The maximum water content in the test soil should be about 55% of the maximum water-holding capacity. Based on the present water content of the soil, 202 mg/L application dispersion had to be used. The suspension was continuously stirred to achieve homogeneity during spiking. Due to these limitations only the soil contents of treatments 9.3 and 21 mg/kg were tested.

Test concentrations were: dispersion with 92 and 202 mg/L deionised water; application of 202 ml test dispersion to 2.0 kg test soil (dm) corresponding to 9.3 ml, and application of 208 ml test dispersion to 2.0 kg test soil (dm) corresponding to 21 mg/kg soil (dm)

10.2.7 Test organism

A sandy soil with the individual soil microflora was investigated.

10.3 Study design

10.3.1 Total exposure period

The exposure period was 28 days:
June 22, 2010 - July 20, 2010.

10.4 Test conditions

10.4.1 Environmental conditions

Physico-chemical data

The incubation temperature was measured continuously with a thermograph. With 20 - 21°C the permitted range of 20 ± 2°C was kept. Incubation occurred in the dark. During the whole test the soil dry mass was maintained at 88.7% (controls), 88.3% (powder application: 9.3 mg/kg), 89.4% (powder application: 21 mg/kg), 88.8% (powder application: 45 mg/kg), 88.8% (powder application: 100 mg/kg), 89.3% (dispersion application: 9.3 mg/kg) and 89.1% (dispersion application: 21 mg/kg).

10.4.2 Test soil

The test soil was a natural sandy soil (Certified RefeSol 01-A, batch IME-01: sand 71%, silt: 24%, clay: 5%, org C: 0.93%, pH 5.7, clay: 5%). Selected soil parameters are presented in Table 64. The soil was sieved to 2 mm. It was not sterilised and stored outdoors on the grounds of the test facility in high-grade stainless steel basins with drainage and ground contact. Dates of the soil handling for the test are shown in Table 65.

For at least one year prior to soil sampling in the field, no plant protection products were applied to the sampling site. No organic or mineral fertilisers were applied to the soil for six and three months, respectively, prior to soil sampling.

Table 64: Test soil for microbial tests: soil parameters.

Soil name	RefeSol 01-A
Soil batch	IME-01
Soil texture	Loamy sand
Clay [%]	5
Silt [%]	24
Sand [%]	71
WHC [g H_2O/kg soil dry weight]	264
CECeff [mmol/kg]	37.9
pH	5.7
Total org. C [%]	0.93
Microbial biomass [mg C/kg dry mass soil], calculated from respiration activity	92
Microbial biomass [% of total org. C]	1.0
Total nitrogen [%]	0.09
NO_3^- [mg/kg dry weight]	81.7

WHC = water holding capacity; CECeff = effective cation exchange capacity

Table 65: Test soil for microbial tests: storage information.

Soil name	RefeSol 01-A
Soil batch	IME-01
Date of field sampling	11.06.2010
Start of indoor storage at room temperature to reduce the water content and to allow sieving; the soil was distributed in a thin layer; surface drying was prevented by periodically turning the soil.	11.-13.09.2008
Date of sieving for the study	13.06.2010
Start of soil conditioning [1]	13.06.2010
Date of application	22.06.2010

[1] Soil conditioning was performed at room temperature in the dark.

10.4.3 Concentration levels

For the application via powder the nominal contents in the test containers with TiO_2 nanoparticles were 9.3, 21.0, 45.0 and 100.0 mg/kg soil dry matter.

For the application via dispersion the nominal contents in the test containers with TiO_2 nanoparticles were 9.3 and 21.0 mg/kg soil dry matter.

10.4.4 Other information on materials and methods

Frequency of treatment

The treatment was performed once at test start.

Control group and treatment

The control consisted of soil. Three replicates were conducted per control.

Statistical method

Data evaluation:

In this report numerical values were frequently rounded to a smaller degree of precision (number of digits) than used in the actual calculation. Minor differences in results obtained from calculations with rounded values in comparison to results obtained with higher precision values are possible. They are, however, well within the limits of the experimental accuracy and of no practical concern.

Statistical calculations:

For each concentration the quantity of consumed oxygen was determined. Performed according to the guidelines for non-agrochemicals, the glucose-induced respiration rates found in the treated samples after 28 days were compared to the respiration rates found in the controls. Furthermore, the percent inhibition value for the test concentrations was calculated. The percent deviation of the respiration rates were calculated in comparison to the control. ECx, LOEC and NOEC calculations were performed with the computer software ToxRat Professional version 2.10.4.1 (ToxRat® Solutions GmbH).

Test procedure

Three incubation containers per treatment were filled with 733 g moist spiked soil. A further three incubation containers were filled with 733 g of control soil.

The test was carried out in the dark at 20 ± 2°C for 28 days. During the test the moisture content of the soil was maintained at 40 - 60% of WHC_{max} with a maximum of 5% outside this range. The mass in the test vessels was measured weekly. Evaporated water was supplemented by adding deionised water.

Samples of each treated and control replicate were analysed for glucose-induced respiration at the beginning (day 0) and at the end of the exposure period (28 days).

The soil samples (100 g dry mass) were mixed with 4000 mg glucose per kg dry weight. The glucose concentration was based on a range finding test for the soil to achieve maximum activity. The glucose-amended soil samples were continuously incubated in an apparatus for the measurement of respiration rates (day 0: Sapromat® Voith Inc.; day 28: Sensomat, Aqualytik) at 20 ± 2°C. The oxygen consumed was measured consecutively for at least 12 h. Measurements started as soon as possible after glucose supplement. For evaluation the linear phase of oxygen consumption was used.

10.5 Results

10.5.1 Zeta potential

The zeta potential is presented in Table 66. A negative value of -18mV (100 mg/L) was determined in deionised water.

Table 66: P25 - C-transformation: zeta potential of the stock dispersion for application via dispersion.

Sample	Zeta potential [mV]
100 mg/L	-18 mV

10.5.2 Carbon transformation

Effect concentrations

For both application forms, inhibitory effects were not observed and no EC-values were calculated. There was no statistically significant difference between the treatments and the control. The NOEC was therefore higher than the highest test concentration (≥ 100 mg/kg).

Respiration measurement

For each treatment three replicate vessels were incubated. From each vessel one soil sample was taken for measurement. The results showed a large variation between the replicates. This was especially true for the measurement at day 28, where another measuring device than applied for day 0 had to be used due to technical reasons (day 0: Sapromat with continuous oxygen supply depending on respiration activity; the amount of oxygen supplied is the measure for microbial respiration activity; day 28: OxiTop (= respirometer without oxygen supply; a decrease in pressure is the measure for microbial respiration activity; 500 mL incubation vessels). In previous projects the comparability of both measuring devices was proven (joint project sponsored by BMBF: FKZ 0330303; Project: Biologische Testverfahren in der Vor-Ort-Analytik zur Beurteilung der Qualität von Böden und Bodenmaterialien; Teilvorhaben 2: Mikrobielle Atmungsaktivität).

Looking at the replicates, in several cases two values were identical or very similar, whereas one value differed obviously. In these cases a further assessment was performed after eliminating the "extreme" values. The results of the short-term respiration measurement are presented as mean values in Table 67 and for better visualisation in Figure 19. The evaluation based on all measured values and the evaluation based on the reduced number of values is listed. Table 68 shows the percentage deviation compared to the control. For single values of the replicates see Table 69.

In the test vessels with spiked soil a stimulation of respiration at day 28 was observed. Application via dispersion resulted in a slight inhibition of respiration activity. After elimination of the “extreme” values, the stimulation was less pronounced and the slight elimination vanished. No statistical significance was observed for either of the treatments and calculations.

Table 67: P25 - C-transformation: mean short-term respiration rate [mg O_2/(kg*h)].

			Application via powder [mg TiO_2/kg]				Application via dispersion [mg TiO_2/kg]	
		Control	9.3	21	45	100	9.3	21
Consideration of all values								
Day 0	Mean	3.3	3.8	3.6	3.5	3.8	3.8	3.7
	Std.Dev.	0.7	0.5	0.1	0.2	0.5	0.2	0.2
	CV	20.8	12.1	1.6	4.4	12.1	5.7	4.7
	Statistical significance	---	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Day 28	Mean	2.6	2.3	2.8	3.9	3.6	2.3	2.1
	Std.Dev.	0.5	0.8	0.9	0.8	0.9	0.8	0.5
	CV	18.0	34.8	32.6	19.4	24.1	34.8	22.7
	Statistical significance	---	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Elimination of extreme values								
Day 0	Mean	3.7	3.8	3.6	3.5	3.8	3.8	3.7
	Std.Dev.	0.2	0.5	0.1	0.2	0.5	0.2	0.2
	CV	5.4	12.1	1.6	4.4	12.1	5.7	4.7
	Statistical significance	---	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Day 28	Mean	2.3	2.3	2.3	3.9	3.1	2.3	2.3
	Std.Dev.	0.0	0.8	0.0	0.8	0.0	0.8	0.0
	CV	0.0	34.8	0.0	19.4	0.0	34.8	21.7
	Statistical significance	---	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

Table 68: P25 - C-transformation: mean short-term respiration rate; [%] of control.

	Application via powder [mg TiO_2/kg]					Application via dispersion [mg TiO_2/kg]	
	Control	9.3	21	45	100	9.3	21
Consideration of all values							
Day 0	100	115.2	109.1	106.1	109.1	109.1	112.1
Day 28	100	88.5	107.7	150.0	138.5	88.5	80.8
Elimination of extreme values							
Day 0	100	102.7	97.3	94.6	101.4	101.4	0
Day 28	100	0	0	169.6	134.8	0	0

Table 69: P25 - C-transformation: short-term respiration rate (SIR) [mg O_2/(kg*h)].

Single values of the replicates; values eliminated for the evaluation ("extreme" values are marked bold)

Date of sampling	Replicate		Application via powder [mg TiO_2/kg]				Application via dispersion [mg TiO_2/kg]	
		Control	9.3	21	45	100	9.3	21
Test start	1	**2.5**	3.9	3.5	3.5	3.3	-	3.6
	2	3.8	4.2	3.5	3.3	4.2	3.9	3.9
	3	3.5	3.3	3.6	3.6	-	3.6	3.6
Day 28	1	2.3	3.1	2.3	3.1	3.1	3.1	2.3
	2	**3.1**	1.5	2.3	3.9	3.1	2.3	**1.5**
	3	2.3	2.3	**3.9**	4.6	**4.6**	1.5	2.3

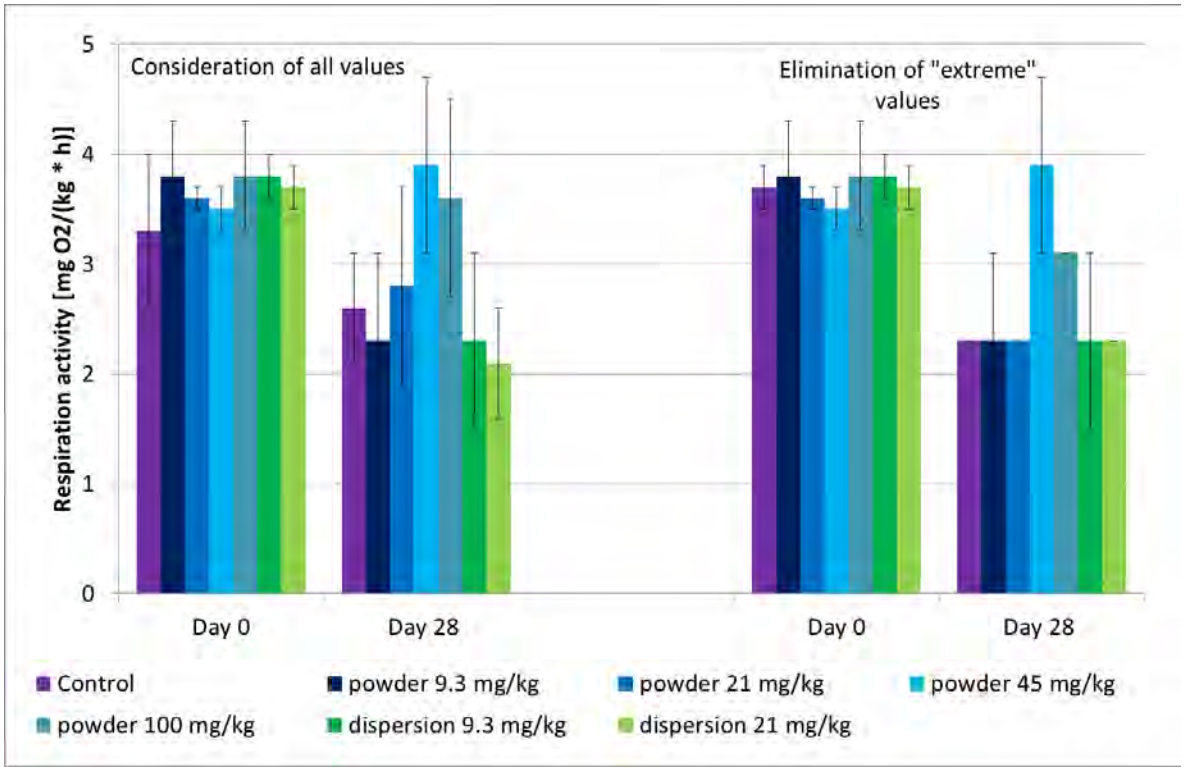


Figure 19: P25 – C-Tranformation: mean short-term respiration rate [(mg O_2/(kg*h)].

10.5.3 Further information

To confirm the results the test was repeated. The results are presented in Table 70, Table 71 and Figure 20.

Effect concentrations:

For both application forms, no inhibitory effects were observed and no EC-values were calculated.

There was no statistically significant difference between the treatments and the control after an incubation period of 28 d. The NOEC was ≥100 mg/kg (100 mg/kg: highest test concentration).

Respiration measurement:

Deviating from the first test, there was a small concentration-dependent inhibition at day 0; at day 28 a concentration-dependent stimulation was not measured. A statistical difference to the control was not observed. The results obtained at day 28 were independent of the application form (application of powder / application via dispersion). Therefore the conclusion drawn from the results from both tests (the first test and the repeated test) is the same: P25 does not affect the microbial respiration activity. A lower respiration activity after 28 days is well known. During the incubation period of 28 days the microorganisms consume nutrients. Depletion of the nutrients results in lower microbial biomass and consequently in a lower respiration activity at the end of the incubation period

Table 70: P25 - C-transformation: mean short-term respiration rate [mg O_2/(kg*h)].

			Application via powder [mg TiO_2/kg]				Application via dispersion [mg TiO_2/kg]	
		Control	9.3	21	45	100	9.3	21
Day 0	Mean	5.87	5.33	5.07	4.80	4.80	4.80	5.07
	Std.Dev.	0.46	0.92	0.46	0.00	0.00	0.00	0.46
	CV	7.9	17.3	9.1	0.0	0.0	0.0	9.1
	Statistical significance	---	n.s.	n.s.	* [1]	* [1]	* [1]	n.s.
Day 28	Mean	3.20	2.93	3.20	3.20	3.47	2.93	3.73
	Std.Dev.	0.00	0.46	0.00	0.00	1.22	0.46	0.46
	CV	0.0	15.7	0.0	0.0	35.3	15.7	12.4
	Statistical significance	---	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

[1] Statistical significance: p > 0.05

Table 71: P25 - C-transformation: mean short-term respiration rate, [%] of control.

	Application via powder [mg TiO_2/kg]					Application via dispersion [mg TiO_2/kg]	
	Control	9.3	21	45	100	9.3	21
Day 0	100	90.8	86.4	81.8	81.8	81.8	86.4
Day 28	100	91.6	0	0	108.4	91.6	116.6

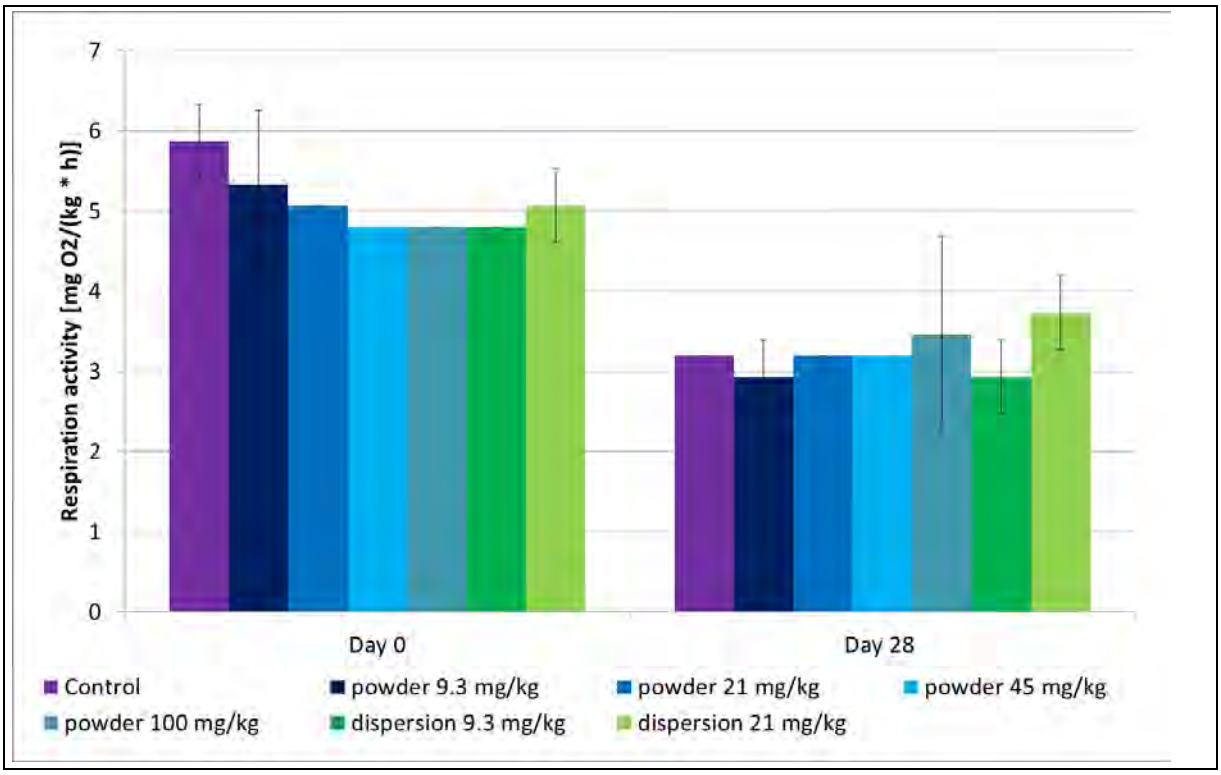


Figure 20: P25 – C-Transformation: mean short-term respiration rate [mg O_2/(kg*h)] (test repetition).

10.6 Validity

A validity criterion is only available in the guideline for the testing of agrochemicals. The evaluation of the results from the tests performed with agrochemicals is based on relatively small differences (i.e. average value ± 25%) between the carbon dioxide released or the oxygen consumed in control and treated soil samples; accordingly, large variations in the controls can lead to false results. Therefore, the variation between replicate control samples should be less than ± 15%.

For non-agrochemicals concentration-effect relationships are the relevant endpoint. Therefore, a variation of 15% is of minor importance. From the results it is obvious that there are no concentration-effect relationships and P25 does not affect microbial respiration activity. Nevertheless, the validity criteria for agrochemicals are fulfilled as the variation is 8% (day 0) and 0% (day 28).

10.7 Reference substance

In the guideline the investigation of a reference substance is not demanded. A reference substance was not tested.

10.8 Conclusion

The TiO_2 nanoparticles tested by means of

- Application via powder in soil: 9.3, 21.0, 45.0 and 100.0 mg/kg soil, dry matter,
- Application via dispersion in soil: 9.3 and 21.0 mg/kg soil, dry matter,

did not affect the carbon transformation activity.

10.9 Executive Summary

TiO_2 nanoparticles (**P25**) were tested in the microbial carbon transformation assay (OECD Test Guideline 217). Soil was spiked with the test item via powder and via dispersion. As the test substrate, a natural sandy soil was used. The following test concentrations were investigated:

- Application via powder in soil: 9.3, 21.0, 45.0 and 100.0 mg/kg soil, dry matter
- Application via dispersion in soil: 9.3 and 21.0 mg/kg soil, dry matter.

For each treatment three replicate vessels were incubated. From each vessel one soil sample was taken for measurement.

For both application forms, no inhibitory effect was observed and no EC-values were calculated. There is no statistically significant difference between the treatments and the control. The NOEC is higher than the highest test concentration (≥ 100 mg/kg). This result was confirmed by a repetition of the test.

11 Growth Tests with Plants (OECD TG 208) – TiO_2

11.1 Test principle

Seeds of different terrestrial plants were sown in control pots and in pots containing a natural soil and different concentrations of the test item. The test item was applied once. The selected test species were kept under the recommended growth conditions. Emergence and mass (fresh weight) of the shoots was determined at least 14 days after 50% of the control seedlings have emerged and compared with the shoots of the control plants.

11.2 Materials and methods

11.2.1 Test guideline

The test was performed according to OECD Guidelines for the Testing of Chemicals Test No. 208: Terrestrial Plant Test: Seedling Emergence and Seedling Growth Test (2006).

11.2.2 GLP

The test was performed following the principles of GLP. In deviation to GLP no archiving of the raw data was performed and the Quality Assurance Unit was not involved with respect to the inspection of the test, the raw data, and the report. All laboratory equipment (e.g. balances, thermometers, pH-meters) were controlled and documented according to GLP.

11.2.3 Test material

- P25 - distributed by Evonik for the OECD Sponsorship Programme
  The properties should correspond to the properties of NM-105.

The nanoparticles were stored in the dark at room temperature until use.

11.2.4 Analytical monitoring

Due to the high natural concentration of TiO_2 in the test soil no specific chemical analyses were performed in the test soil.

The zeta potential was measured in the test dispersions using a Malvern Zetasizer Nano ZS. The particle size distribution in the dispersion was not determined. Particle size gives no information on the size distribution in soil. A measurement of the zeta-potential or the particle size distribution in soil is not yet possible.

11.2.5 Test species

The plant species used in the test were *Avena sativa* (oat), *Sinapis alba* (mustard), and *Phaseolus aureus* (mung bean), which are representatives of monocotyledonous and dicotyledonous plants, respectively. The species are recommended by the guideline.

Origin of the seeds

Avena sativa:	Saaten-Union GmbH 30916 Isernhagen HB Date of receipt: April 26, 2010 Cultivar Freddy germination capacity: 92%
Sinapis alba:	Landesinstitut für Landwirtschaftliche Qualitätskontrolle; Producer country: Hungary Date of sealing (06.2005): certified seeds 1st generation. Cultivar Dr. Francks Hohenheimer gelb. Reference No.: H 4-51/632. Reference number of basis seeds: D/KA 3078590/4
Phaseolus aureus:	SPERLI Samen Carl Sperling & Co. Lüneburg, Germany D 6210 H; Charge 3650

Storage

The seeds were stored in the dark at room temperature (20 ± 5 °C) in the test facility and protected from moisture until use.

11.3 Study design

11.3.1 Study type

Laboratory test.

11.3.2 Test duration type

Short term test.

11.3.3 Test type

Seedling emergence toxicity test.

11.3.4 Substrate type

The soil used in the test was a natural sandy soil (Certified RefeSol 01-A, batch IME-01 composed of sand 71%, silt: 24%, clay: 5%, Org C: 0.93%, pH 5.7, clay: 5%). The soil was sieved to 2 mm. It was not sterilised and had been stored outdoors in high grade stainless steel basins with drainage and ground contact at the test facility.

11.3.5 Exposure period

The exposure period was 14 days starting after germination of 50% of the seeds in the control vessels.

- *Phaseolus aureus:* 2nd November 2009 - 17 November 2009
- *Avena sativa*: 5 January 2010 - 21 January 2010
- *Sinapis alba*: 5 January 2010 - 21 January 2010

11.3.6 Post exposure period

There was no post exposure period.

11.4 Test conditions

11.4.1 Environmental conditions

The test was carried out in a plant growth chamber at 20 ± 2 °C, 70 ± 25% humidity, and an illumination period of 16 h per day with a light intensity of > 7000 lux (light colour 25, universal white).

The incubation temperature was measured continuously with a thermograph. With 19 – 22 °C measured throughout the test, the permitted range of 20 ± 2°C was maintained.

The light intensity was measured in Lux using an illuminance meter (MINOLTA) with photometric sensor. With 9000 – 10,000 lux measured throughout the test, the permitted value of at least 7000 lux was maintained.

At 60 – 80% humidity, the permitted range of 70% ± 25% was maintained.

11.4.2 Concentration levels

The nominal concentrations in the test containers with TiO_2 nanoparticles were 10, 20, 30, 44, 67, 100 mg P25/kg soil, dry mass (application via powder) and 10 and 20 mg/kg (application via dispersion).

11.4.3 Other information on materials and methods

Frequency of treatment

The treatment was applied once at test start.

Control group and treatment

The control treatment group consisted of soil and plants. Four replicates were conducted per control.

Test item – Preparation protocol

Spiking of soil with TiO_2 powder

For the first application the TiO_2 powder was mixed directly into the soil using air-dried test soil (1% of the total amount) as a carrier for the TiO_2 powder. Suitable amounts of TiO_2 powder to achieve the desired final soil content were mixed homogenously with the dry soil. Care was taken to avoid a modification of the TiO_2 crystalline structure. Uncontaminated test soil (between 20 - 30% of WHC_{max}) was spread on a plate, the carrier material with the TiO_2 powder distributed on the test soil, and all was then mixed carefully. For the test with contaminated soil, the soil was adjusted to a water-holding capacity of 60% of the maximum water-holding capacity (WHC_{max}).

Spiking of soil with aqueous TiO_2 dispersion

The second application trial utilised a TiO_2 dispersion (TiO_2 nanoparticles in deion. water) that had been prepared with a magnetic flea (900 rpm; 1 min) and ultrasonication (3 min) in a bath sonicator. The test soil was dried to about 10% of WHC_{max} and spread on a plate. Immediately after preparation of the TiO_2 dispersion was sprayed onto the soil by means of a syringe coupled with a cannula, and then mixed thoroughly. Finally, the test soil was adjusted to a water-holding capacity of 60% of WHC_{max}. A maximum concentration of about 200 mg/L application dispersion of TiO_2 nanoparticles was considered adequate for the tests. Higher concentrations would have sedimented rapidly preventing a homogenous distribution of the nanomaterial in the soil. Furthermore, it was assumed that higher concentrations in the application dispersion would result in larger agglomerates. Based on the present water content of the soil, 165 mg/L application dispersion of TiO_2 nanoparticles was used in the test with *Phaseolus aureus*, and of 177 mg/L in the test with *Avena sativa* and *Sinapis alba.* The suspensions were continuously stirred to achieve homogeneity during spiking.

Due to these limitations, only the soil contents of the treatments 10 and 20 mg/kg were tested.
Test concentrations used in the test with *Avena sativa* and *Sinapis alba* were: dispersion with 89 and 178 mg/L deionised water; application of 225 ml test dispersion to 2.0 kg test soil (dm), corresponding to 10 and 20 mg/kg soil (dm).

Test concentrations in the test with *Phaseolus aureus* were: dispersion with 83 and 165 mg/L deionised water; application of 185 ml test dispersion to 1.5 kg test soil (dm), corresponding to 10 and 20 mg/kg soil (dm).

Fertiliser

Floragard Grünpflanzendünger was used as fertiliser., manufactured by Floragard Vertriebs GmbH für Gartenbau, P.O. Box 9006, 26138 Oldenburg, Germany. The concentration applied in the test was 1 mL fertiliser per litre water.

Nutrient content of Floragard Grünpflanzendünger:

Ammonium nitrogen 23 mg/L, iron (Chelate) 0.50 mg/L; nitrate nitrogen 23 mg/L; copper (chelate) 0.30 mg/L; phosphate 30 mg/L; manganese (chelate) 0.30 mg/L; potassium oxide 60 mg/L; molybdenum 0.01 mg/L; zinc (chelate) 0.05 mg/L; and boron 0.10 mg/L

Test containers

Round containers made of nonporous plastic with a diameter of 85 - 95 mm were used. A glass fibre wick originating from a water reservoir and passing through the bottom of the container was used to ensure consistent soil moisture. The containers were filled up with approximately 280 g moist soil.

Test procedure

For each plant test species a control and several concentrations were tested. Five seeds were planted in each replicate immediately after incorporation of the test item. For each test and each species seeds of the same size class were used. Twenty-four hours after test start, the glass fibre wicks passing through the bottom of the container were connected with a reservoir of fertiliser to ensure consistent soil moisture.

The test was carried out in a plant growth chamber at 20 ± 2°C, 70 ± 25% humidity and an illumination period of 16 h per day with a light intensity of > 7000 lux (light colour 25, universal white).

Watering/fertilisation: Continuous bottom watering of the test container via glass fibre wicks was performed. Fertiliser was used for watering.

The 14-day-growth-phase started when 50% of the seedlings in the control group had emerged. This day was determined as "growth day 1". On this day, the number of emerged seedlings of all containers was recorded. Observations concerning emergence and visual phytotoxicity and mortality were performed throughout the exposure period of 14 days. At "growth day 14", all seedlings were counted and the aboveground biomass was measured. The wet mass of the plants was measured immediately after harvesting and the length of the roots was determined. The roots were carefully rinsed with tap water. The length of the main root biomass and the shortest and longest root of individual roots were measured.

Statistical method

Data evaluation

Numerical values in this report are frequently rounded to a smaller degree of precision (number of digits) than were used in the actual calculation. Minor differences in results obtained

from calculations with rounded values compared to the results obtained with higher precision values are possible. They are, however, well within the limits of the experimental accuracy and of no practical concern.

Statistical calculations

The percentage inhibition of seedling emergence was calculated for each plant species as an absolute value and in comparison to the control. Survival of emerged seedlings was calculated as an absolute value. The percentage inhibition of fresh weight was calculated in comparison to the control. Germination and biomass were compared by a suitable test for multiple comparisons with a control after testing variance homogeneity. All statistical tests were performed with the computer software ToxRat Professional version 2.10.4.1 (ToxRat® Solutions GmbH).

11.5 Results

(Raw data, chapter 21.4)

11.5.1 Zeta potential

The zeta potential in deionised water is documented in Table 72. A negative zeta potential of -18 mV (in deion. water) was measured.

Table 72: P25 – Test with plants: zeta potential of the stock dispersion for application via dispersion.

Sample	Zeta potential [mV]
100 mg/L	-18 mV

11.5.2 Effects

Effect concentrations

The effect concentrations (EC_x, NOEC and LOEC values) are presented in Table 73.

Table 73: P25 – Plant test: summary of the effects.

Application via powder; critical effect and threshold concentrations [mg/kg]

	Avena sativa	*Phaseolus aureus*	*Sinapis alba*
Emergence			
EC_{10} [mg/kg]	32.4	n.d. [2]	n.d. [2]
EC_{50} [mg/kg]	n.d. [2]	n.d. [2]	n.d. [2]
LOEC [mg/kg]	> 100	n.d. [2]	> 100
NOEC [mg/kg]	≥ 100	n.d. [2]	≥ 100
Shoot fresh weight			
EC_{10} [mg/kg] [1]	51.7 (36.1 – 61.4)	n.d. [2]	n.d. [2]
EC_{50} [mg/kg] [1]	n.d. [2]	n.d. [2]	n.d. [2]
LOEC [mg/kg]	100.0	67	> 100
NOEC [mg/kg]	67.0	44	≥ 100
Root length:			
EC_{10} [mg/kg]	n.d. [2]	n.d. [2]	n.d. [2]
EC_{50} [mg/kg]	n.d. [2]	n.d. [2]	n.d. [2]
LOEC [mg/kg]	> 100	n.d. [2]	> 100
NOEC [mg/kg]	≥ 100	n.d. [2]	≥ 100

[1] values in brackets: confidence interval; [2] n.d. = not determined due to mathematical reasons or inappropriate data or considered as unreliable

Pathological symptoms

No pathological symptoms were observed during the test (Table 74).

Table 74: P25 – Plant test: pathological symptoms [% plants].

		Application via powder [mg TiO_2/kg]						Application via dispersion [mg TiO_2/kg]	
Test species	Control	10	20	30	44	67	100	10	20
Avena sativa	- [1]	-	-	-	-	-	-	-	-
Phaseolus aureus	-	-	-	-	-	-	-	-	-
Sinapis alba	-	-	-	-	-	-	-	-	-

[1] - = no visual symptom

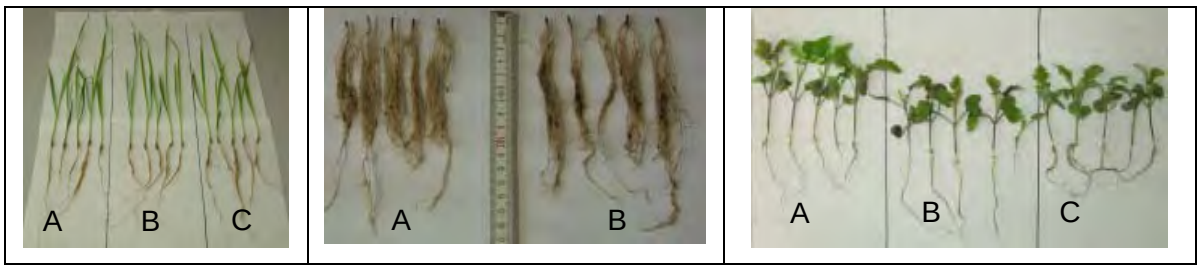
Seedling emergence and growth

The plants measured after the 14 days exposure periods are presented in Figure 21. No difference between control and highest test concentration is obvious. The results of emergence and growth inhibition are presented as mean values (Table 75 - Table 80, Figure 22). Raw data are presented in chapter 21.4 (Table 239 - Table 243). The most sensitive endpoint was the fresh shoot biomass. For *Phaseolus aureus* NOEC and LOEC values and for *Avena sativa* EC_{25} and EC_{50} values were calculated (Table 73). The calculated EC_{50} value was far beyond the highest test concentration (158.8 mg/kg with a confidence interval of 126.0 – 268.5 mg/kg). At higher test concentrations effects by nanoparticles can decrease due to agglomeration. According to our experience only EC values which are within the range of test

concentrations should be considered. Therefore the calculated EC_{50} is not considered reliable.

Effects obtained for lower concentrations that were higher than the effect at the highest test concentration of 100 mg/L (e.g. emergence rate of *Sinapis alba*) may be caused by a decreased bioavailability of the nanoparticles at the highest test concentration due to agglomeration. If the bioavailability differs at 10 and 20 mg/kg test substance applied via dispersion, this may also be the reason for slightly increased effects at a concentration of 10 mg/kg compared to 20 mg/kg (e.g. *Sinapis alba*: emergence root, shoot fresh weight).

Additionally to the conventional endpoints (emergence, shoot weight), root length was determined. Root length does not seem to be affected by P25. No concentration-effect relationships could be determined.



Avena sativa
A: control; B + C: highest test concentration - B: dry application; C: wet application

Phaseolus aureus
A: control; B: 100 mg/kg (dry application)
(unfortunately no photo for wet application available)

Sinapis alba
A: control; B + C: highest test concentration - B: dry application; C: wet application

Figure 21: P25 - Plants after the exposure period of 14 days.

Table 75: P25 – Plant test: emergence rate at test end [%].

		Application via powder [mg TiO_2/kg]						Application via dispersion [mg TiO_2/kg]	
Test species	Control	10	20	30	44	67	100	10	20
Avena sativa	100	95	95	90	90	80	80	90	95
Phaseolus aureus	100	100	100	100	100	100	95	100	100
Sinapis alba	95	90	80	85	85	75	95	70	95

Table 76: P25 – Plant test: emergence rate at test end compared to control [%].

		Application via powder [mg TiO_2/kg]						Application via dispersion [mg TiO_2/kg]	
Test species	Control	10	20	30	44	67	100	10	20
Avena sativa	100	95	95	90	90	80	80	90	95
Phaseolus aureus	100	100	100	100	100	100	95	100	100
Sinapis alba	100	95	84	89	89	79	100	74	100

Table 77: P25 – Plant test: fresh weight of the shoots; mean values ± SD [g].

		Application via powder [mg TiO_2/kg] [1]						Application via dispersion [mg TiO_2/kg]	
Test species	Control	10	20	30	44	67	100	10	20
Avena sativa	2.591 ± 0.286	2.571 ± 0.487	2.461 ± 0.279	2.567 ± 0.391	2.382 ± 0.329	2.200 ± 0.185	1.803 ± 0.204 [1]	2.130 ± 0.201 *	2.286 ± 0.368
Phaseolus aureus	3.851 ± 0.087	3.446 ± 0.166	3.472 ± 0.363	3.869 ± 0.414	3.674 ± 0.345	3.171 ± 0.377	3.127 ± 0.443	3.530 ± 0.400	3.271 ± 0.302±
Sinapis alba	2.411 ± 0.517	2.256 ± 0.398	2.195 ± 0.520	1.964 ± 0.486	2.243 ± 0.506	2.174 ± 0.868	1.984 ± 0.215	1.449 ± 0.468	2.040 ± 0.245

[1] *: significant when compared with control (p > 0.05)

Table 78: P25 – Plant test: growth inhibition related to control at test end [% FW].

		Application via powder [mg TiO_2/kg]						Application via dispersion [mg TiO_2/kg]	
Test species	Control	10	20	30	44	67	100	10	20
Avena sativa	0	1	5	1	8	15	30 * [1]	18	12
Phaseolus aureus	0	11	10	0	5	18 * [1]	19 * [1]	8	15
Sinapis alba	0	6	9	19	7	10	18	40	15

[1] *: significant when compared with control (p > 0.05)

Table 79: P25 – Plant test: mean root length of main root biomass [cm].

		Application via powder [mg TiO_2/kg]						Application via dispersion [mg TiO_2/kg]	
Test species	Control	10	20	30	44	67	100	10	20
Avena sativa	7.225 ± 0.386	7.800 ± 0.548	6.775 ± 0.506	7.525 ± 0.189	7.025 ± 0.556	6.925 ± 0.512	7.775 ± 0.386	7.375 ± 0.519	7.150 ± 0.569
Phaseolus aureus	12.0 ± 0.91	10.9 ± 0.85 [1]	10.6 ± 1.11	11.1 ± 1.32	10.2 ± 0.50 [1]	10.0 ± 0.41 [1]	11.0 ± 0.91 [1]	10.3 ± 0.29	9.9 ± 0.48
Sinapis alba	4.9 ± 1.88	5.1 ± 0.44	4.5 ± 0.90	3.8 ± 0.55	3.8 ± 0.30	3.9 ± 0.21	4.3 ± 0.32	4.3 ± 0.41	4.4 ± 0.30

[1] *: significant when compared with control (p > 0.05)

Table 80: P25 – Plant test: inhibition of mean root length of main root biomass [%].

		Application via powder [mg TiO_2/kg]						Application via dispersion [mg TiO_2/kg]	
Test species	Control	10	20	30	44	67	100	10	20
Avena sativa	---	-8 *)	6	-4	3	4	-8	-2	1
Phaseolus aureus	---	9 [1]	11	7	15 [1]	17 [1]	8 [1]	15	18
Sinapis alba	---	-3	9	23	23	22	13	13	12

[1] *: significant when compared with control ($p > 0.05$); [2] negative values indicate stimulation;

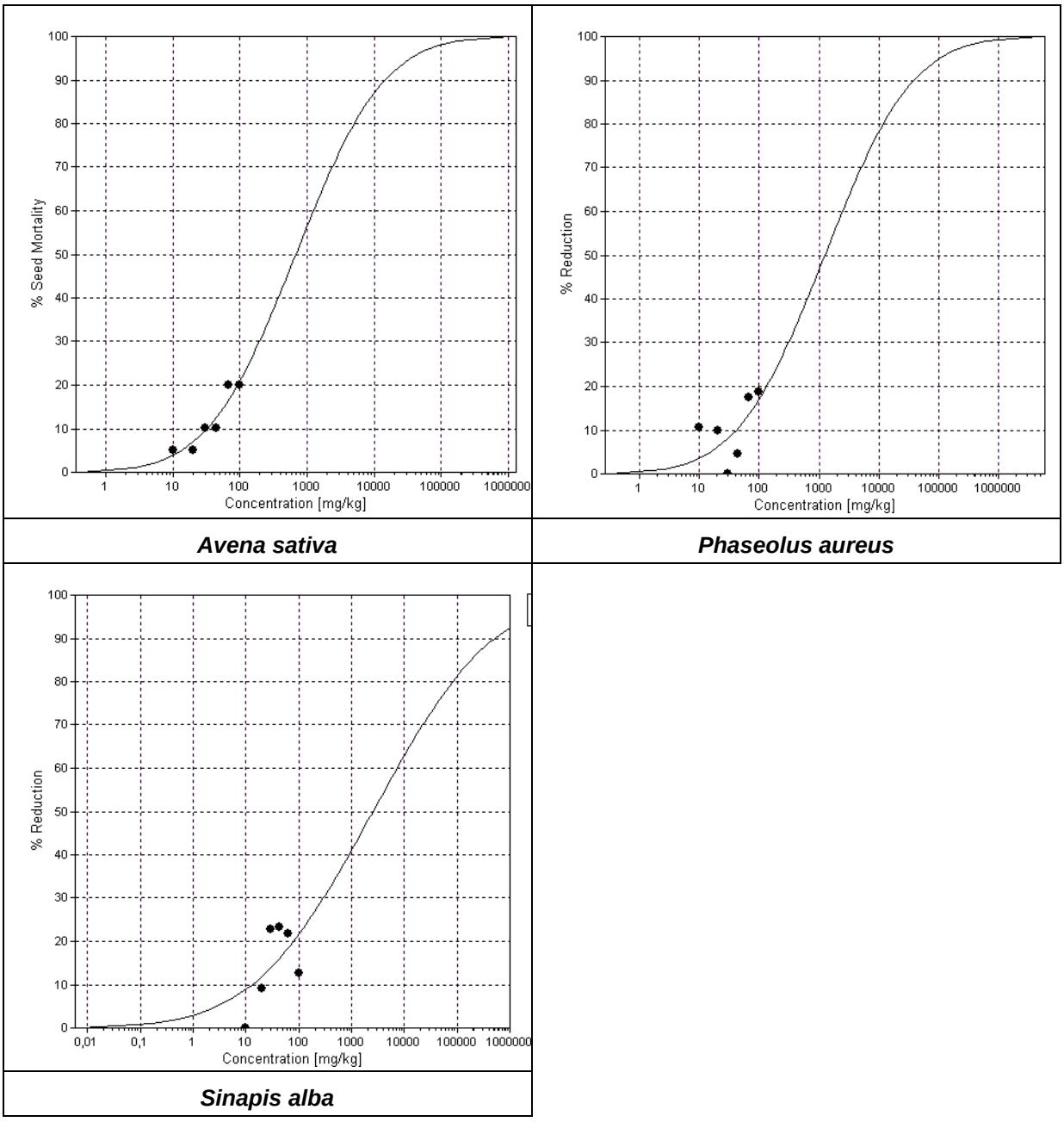


Figure 22: P25 – Test with plants: shoot fresh weight response curve (results for application via powder).

11.5.3 Results with reference substance

In order to confirm the reproducibility of the study as well as the germination capacity and sensitivity of the test species *Avena sativa*, *Phaseolus aureus* and *Latuca sativa*, the Terrestrial Plants Growth Tests with trichloroacetate (TCA) was performed. The results of the last reference study are in agreement with experienced EC_{50} values obtained at Fraunhofer IME.

Seedling emergence:

Avena sativa:	Control	85%	100 mg TCA/kg	75.0%
Phaseolus aureus:	Control	92.5%	100 mg TCA/kg	75.0%
Brassica/Sinapis alba:	Control	90.0%	100 mg TCA/kg	80.0%

EC_{50} values could not be calculated.

EC_{50} for growth:

Avena sativa:	6 mg/kg (fresh mass per plant)
Phaseolus aureus:	87 mg/kg (fresh mass per plant)
Brassica/Sinapis alba:	34 mg/kg (fresh mass per plant)

11.6 Validity

The test is considered to be valid as:

- The seedling emergence in the control exceeded 80% at the end of the test
- The control seedlings did not exhibit phytotoxic effects
- The mean survival of emerged control seedlings was at least 90% for the duration of the study
- Environmental conditions for a particular species were identical and growing media contained the same amount of soil matrix, support media, or substrate from the same source.

11.7 Conclusion

No statistical significant differences were observed for germination and root length. No phytopathological 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. Small effects showed *Avena sativa* and *Phaseolus aureus*. Application via powder resulted in concentration-effect relationships. The application via dispersion resulted in effects which were greater for the low test concentration (10 mg/kg). Effects obtained for lower concentrations that are greater than the effects at the highest test concentration may be caused by decreased bioavailability of the nanoparticles due to agglomeration in the stock dispersion used for the higher test concentration.

Avena sativa:

For fresh shoot weight the EC_{10}, NOEC, and LOEC were determined.

EC_{10}: 51.7 mg/kg (95% confidential interval: 36.1 – 61.4)

LOEC: 100.0 mg/kg

NOEC: 67.0 mg/kg

Phaseolus aureus:

For fresh shoot weight, the NOEC, and LOEC were determined.

LOEC: 67.0 mg/kg

NOEC: 44.0 mg/kg

Sinapis alba:

For fresh shoot weight, the NOEC, and LOEC were determined.LOEC: > 100.0 mg/kg

NOEC: ≥ 100.0 mg/kg

11.8 Executive summary

TiO_2 nanoparticles (**P25**) were tested in the seedling emergence test and in the growth test with plants (OECD test guideline 208). Soil was spiked via powder and via dispersion. As test substrate a natural sandy soil was used. Following test concentrations were investigated:

- Application via powder in soil: 10, 20, 30, 44, 67, 100 mg/kg soil, dry matter
- Application via dispersion in soil: 10, 20 mg/kg soil, dry matter.

The plant species used in the test were *Avena sativa* (oat), *Sinapis alba* (mustard) and *Phaseolus aureus* (mung bean), representative of monocotyledonous and dicotyledonous plants, respectively. Additionally 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. Phytopathological symptoms were not 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. Small effects were observed for *Avena sativa* and *Phaseolus aureus*. Application via dispersion resulted in effects which were greater 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).

A summary of the effect values is presented in Table 81.

Table 81: P25 – test with plants: summary of the effect values.

Application via powder; critical effect and threshold concentrations [mg/kg]

	Avena sativa	*Phaseolus aureus*	*Sinapis alba*
Emergence			
EC_{10} [mg/kg]	32.4	n.d. [2]	n.d. [2]
EC_{50} [mg/kg]	n.d. [2]	n.d. [2]	n.d. [2]
LOEC [mg/kg]	> 100	n.d. [2]	> 100
NOEC [mg/kg]	≥ 100	n.d. [2]	≥ 100
Shoot fresh weight			
EC_{10} [mg/kg] [1]	51.7 (36.1 – 61.4)	n.d. [2]	n.d. [2]
EC_{50} [mg/kg] [1]	n.d. [2]	n.d. [2]	n.d. [2]
LOEC [mg/kg]	100.0	67	> 100
NOEC [mg/kg]	67.0	44	≥ 100
Root length:			
EC_{10} [mg/kg]	n.d. [2]	n.d. [2]	n.d. [2]
EC_{50} [mg/kg]	n.d. [2]	n.d. [2]	n.d. [2]
LOEC [mg/kg]	> 100	n.d. [2]	> 100
NOEC [mg/kg]	≥ 100	n.d. [2]	≥ 100

[1] values in brackets: confidence interval;
[2] n.d. = not determined due to mathematical reasons or inappropriate data or considered unreliable

12 Emergence Test with Chironomids (OECD TG 219) – TiO_2

12.1 Test principle

Sediment-dwelling larvae (first instar) of the fresh water dipteran *Chironomus riparius* were placed in a sediment-water test system with defined artificial sediment. The overlaying water was spiked with the test item at a defined range of concentrations. The test item was applied once. Chironomid emergence was measured as the endpoint at the end of the test, i.e. after 28 days of incubation. Emergence rate, development time and rate, and sensitivity of the sexes in the treatment test systems and in the control were analysed for statistically significant differences using appropriate statistical methods.

12.2 Materials and methods

12.2.1 Test guideline

The test was performed according to:

OECD Guidelines for the Testing of Chemicals Test No. 219: Sediment-Water Chironomid Toxicity Using Spiked Water (2004)

12.2.2 GLP

The test was performed following the principles of GLP. In deviation to GLP no archiving of the raw data was performed and the Quality Assurance Unit was not involved with respect to the inspection of the test, of the raw data and of the report. All laboratory equipment (e.g. balances, thermometers, pH-meters) were controlled and documented according to GLP.

12.2.3 Test substances

- P25 - distributed by Evonik for the OECD Sponsorship Programme
  The properties should correspond to the properties of NM-105.
- NM-101

The test substances were stored in the dark at room temperature.

12.3 Analytical monitoring

Sampling

For the control and for each concentration one additional vessel was used for analytical measurements. The additional vessels were treated as the control vessels and the test vessels used for the assessment of the nanoparticles.

At several points of time aqueous samples (50 mL) were taken at four depths (about 2.0 cm; 4.0 cm; 5.5 cm; 6.5 cm). The samples were combined. About 20 mL was used for analysis and the remaining amount was carefully returned into the test vessels without disturbing the sediment.

Characterisation of application dispersion and test dispersion

Chemical analysis was performed in the samples collected from the additional vessels.

Furthermore, using a Malvern Zetasizer, the zeta potential was measured in one vessel of each concentration and of the control three hours after application of the nanoparticles. Particle size distribution was determined in the control and the test vessels with P25 at selected time points during the incubation period. No measurements were performed in the highly concentrated application dispersions as the particle size distributions were not representative for the particle size distribution in the test vessels. No measurements were performed for NM-101 due to the fast sedimentation and the high polydispersity at day 0 of P25.

Physical-chemical parameters (overlaying water)

In all vessels, temperature and pH were measured at test start and test end as well as once a week during the study. Dissolved oxygen was measured in one representative vessel per treatment at test start and twice a week during the course of the study, and in all test vessels at the end of the test. Hardness and ammonia were measured in the controls at the highest concentration in one test vessel at the start and the end of the study.

12.3.1 Details of sediment and water

Artificial sediment components

- Sphagnum peat, air-dried, finely ground 5%
- Kaolinite, air-dried 20%
- Industrial quartz sand, air-dried 75%

The test substrate was wetted with deionised water to reach a water content of about 25% - 30%. According to the guideline a water content between 30% and 50% is recommended. Our experience shows that lower water content results in a more homogenous distribution of the sediment in the individual vessels. Pulverised calcium carbonate of chemically pure quality ($CaCO_3$) was added to adjust the pH of the final mixture of the sediment to 7.0 ± 0.5. Organic carbon content of the final mixture was 1.5% (test with P25) and 2.1% (test with NM-101) which was in the demanded range of 2% ± 0.5%.

Water

- Purified tap water was used as test water.

12.3.2 Details of application

The nominal concentrations in the test containers with the test item were 15, 23, 39, 63, and 100 mg test item/L. Four replicates per concentration were conducted.

For each vessel, a 500 mL double concentrated stock dispersion of the nanomaterial was prepared in tap water. For the double concentrated dispersion of the final test concentration the respective amount of nanomaterial was weighed in brown glass vessels using a suitable balance. Five hundred millilitres of tap water was added, the mixture was stirred (magnetic stirrer, 900 rpm), followed by ultrasonic treatment in a water bath (3 min, 500 W). The double concentrated stock dispersion was added thoroughly to the water column (500 mL) in the test vessels 24 h after adding the test specimens. Due to the large amount of stock dispersion the dispersion mixed with the water in the test vessels while being added. There was no further mixing to avoid disturbance of the sediment.

12.4 Test organism

Test organisms were the first instar larvae from the dipteran *Chironomus riparius*.

Origin of the midges: Bayer Crop Science AG, 40789 Monheim, Germany. Specimens used in the test were bred in the laboratory of the Fraunhofer IME.

Breeding conditions: Purified tap water was added to a layer of diatomaceous earth. The dipterans were fed daily with powder of TetraMin® Hauptfutter (Tetra Werke, Melle, Germany).

Pre-treatment: Four to five days before adding the test organisms to the test vessels egg masses were taken from the cultures and placed in small aerated vessels with test water at about 20 °C. First instar larvae (one day post hatching) were used in the test. As the larvae were added one day before spiking, the age of the larvae was about 2 days at day 0 (day 0 = day of spiking the water phase).

12.5 Study design

12.5.1 Study type

Laboratory study.

12.5.2 Test duration type

Long-term.

12.5.3 Test type

Static.

12.5.4 Water media type

Fresh water.

12.5.5 Type of sediment

Artificial sediment.

12.5.6 Total exposure duration

The exposure period was 28 days.

- P25: April 21 - May 19, 2010
- NM-101: May 27 - June 24, 2010

No post-exposure observation period was performed.

12.5.7 Test conditions

<u>P25</u>

Hardness:	Test start 110 – 150 mg/L as $CaCO_3$ equivalents (demanded threshold value of 400 mg/L as $CaCO_3$ equivalents) Test end: 100 – 120 mg/L as $CaCO_3$ equivalents in the controls and 210 mg/L as $CaCO_3$ equivalents in one representative replicate of the highest test concentration
Test temperature:	20.3°C (permitted range: 20 ± 2°C)
pH:	7.9 – 8.4 (permitted range: pH 6 – 9)
Dissolved oxygen:	About 100% at test start and test end (demanded threshold value: 60%)
Ammonia:	Test start: 0.8 - 1.0 (control); 0.2 (highest test concentration)

	Test end: 8.0 - 10.2 (control); 9.2 (highest test concentration)
Nominal concentrations:	The nominal concentrations in the test containers with TiO_2 nanoparticles were 15, 23, 39, 63, and 100 mg test item/L.
Details on test conditions:	The light intensity was measured using an illuminance meter (MINOLTA) with photometric sensor in lux. With 748 – 850 lux the permitted range of about 500 - 1000 lux was kept.

NM-101

Hardness:	At test start 130 – 150 mg/L $CaCO_3$ equivalents in the control and 140 mg/L $CaCO_3$ equivalents in one representative replicate of the highest test concentration (demanded threshold value of 400 mg/L as $CaCO_3$ equivalents) Test end: 150 – 170 mg/L $CaCO_3$ equivalents in the control and 170 mg/L $CaCO_3$ equivalents in one representative replicate of the highest test concentration.
Test temperature:	20.3 °C -20.5°C (permitted range: 20 ± 2 °C)
pH:	7.8 – 8.7 (permitted range: pH 6 – 9)
Dissolved oxygen:	About 100% at test start and test end (demanded threshold value: 60%)
Ammonia:	Test start: 0.5 - 0.9 (control); 0.7 (highest test concentration) Test end: 0.1 - 7.5 (control); 0.6 (highest test concentration)
Nominal concentrations:	The nominal concentrations in the test containers with TiO_2 nanoparticles were 15, 23, 39, 63, and 100 mg test item/L.
Details on test conditions:	The light intensity was measured using an illuminance meter (MINOLTA) with photometric sensor in lux. With 771 – 826 lux the permitted range of about 500 – 1000 lux was kept.

Reference substance:

According to the guideline a test with a reference substance is not compulsory.
However, 2-chloracetamid was tested in a sediment-water chironomid toxicity test using spiked sediment (OECD 218).

12.5.8 Other information on materials and methods

Control treatment

The control consists of sediment, tap water and chironomids. Four replicates per control were conducted.

Statistical method

Data evaluation:

Numerical values in this report are frequently rounded to a smaller degree of precision (number of digits) than used in the actual calculation. Minor differences in the results obtained from calculations with the rounded values compared to the values obtained with higher precision values are possible. They are, however, well within the limits of the experimental accuracy and thus of no practical concern.

Statistical calculations:

The number of emerged males and females were determined. The results of the listed biological parameters (total, males, females) were compared by a suitable test for multiple comparisons with a control after testing variance homogeneity. All statistical tests were performed with the computer software ToxRat Professional version 2.10.4.1 (ToxRat® Solutions GmbH).

Food

TetraMin® Hauptfutter powder was used for feeding the larvae. According to the guideline the food ration for the first 10 days was 0.25 – 0.5 mg TetraMin® /larvae/day, from day 10 on the food ration was increased to 0.5 – 1.0 mg TetraMin® /larvae/day.

Test container

Round glass beakers (3L) were used as test vessels. The vessels were filled to a height of 2 cm with wet artificial sediment (corresponding to 370 g dry mass). The overlaying water was 8 cm high (ratio sediment:water about 1:4). The containers were covered with glass plates. After 10 days, emergence traps were placed on the test vessels, the glass plates remained on the emergence traps to avoid evaporation. Aeration of overlaying water was provided through a glass pipette fixed 2-3 cm above the sediment layer (at least 1 bubble /second).

Test procedure

Sediment was placed into the test vessels. Four hundred millilitres of tap water was added and the sediment-water system was left under gentle aeration for several days prior to adding the test organisms. Batches of twenty larvae were placed into each vessel.

After incubation for 24 h, 500 mL of the freshly prepared stock dispersion of the nanoparticles was added. A further 100 mL of tap water was used to rinse the vessels containing the stock dispersions. To avoid separation of sediment ingredients during addition of test water and stock dispersion, the surface of the water column was covered with a stainless steel disc

while water was poured onto it. The disc was removed immediately afterwards. Due to the large amount of treatment solution, the dispersion mixed while being added to the water column. There was no further mixing to avoid disturbance of the sediment.

The test was carried out at 20°C ± 2°C and at 16 h photoperiod (500 –1000 lux). The exposure duration was 28 days. Development time and total number of fully emerged male and female midges were determined. Test vessels were observed daily for visual assessment of abnormal behaviour. Emergence was counted daily. After identification the midges were removed from the test vessel. At test end, the test vessels were observed for visible pupae that had failed to emerge.

12.6 Results

12.6.1 P25

(Raw data, chapter 21.5.1)

Zeta potential

The zeta potential is presented in Table 82. The three highest test concentrations were determined. No significant difference between the concentrations was observed.

Table 82: P25 - Test with chironomids: zeta potential.

Sample	Zeta potential [mV]
39 mg/L	-20.7
63 mg/L	-18.8
100 mg/L	-19.4

Particle size distribution is presented in Table 83. At day 0 a difference between the control and the samples containing TiO_2 nanoparticles is observed at concentrations of 24 - 100 mg/L, where a large peak above 1000 nm was detected. The size of the peak increases with increasing concentration. It is assumed that the particles determined in the control originate from the sediment. At day 1 no difference between the control and the vessels containing TiO_2 nanoparticles was observed. It is assumed that the agglomerates measured at day 0 were sedimented. Therefore, no further measurements were performed. By chemical analysis, Ti was detected in the aqueous phase at day 1 (see below).

Table 83: P25 - Test with chironomids: particle size distribution (spiked water).

Concentration [mg/L]	Z-Average [nm] [1]	PDI [2]	Peak 1 [nm]	Peak 2 [nm]	Count rate [3] [kcps]	Measurement position [4]	Attenuation [5]
Day 0							
Control	1542	0.8	616	-	110	1.25	7
15	1101	0.5	737	-	162	4.65	7
24	2019	0.5	1070	-	184	4.65	6
39	2111	0.5	1208	-	79	4.65	5
63	2262	0.4	1364	-	32	1.25	4
100	2060	0.2	1659	-	486	1.05	6
Day 1							
Control	2551	1	302	-	198	4.65	8
15	970	0.6	481	-	289	4.65	9
24	1377	0.8	633	-	148	4.65	8
39	1871	0.9	525	-	67	4.65	7
63	1482	0.8	550	-	166	4.65	9
100	1971	1	535	-	149	4.65	9

[1] calculated value (cumulative mean); [2] increasing value indicates increasing polydispersity (maximum: 1); [3] best results with a count rate between 150 and 500 kilo counts per second (kcps); [4] measurement position in the middle of the measuring cell; [5] indicator for turbidity (high values indicate low turbidity; maximum: 11); [6] 1 mg/L samples below quantification limit; [7] prepared from 10 mg/L samples with 3 min of ultrasonic treatment; [8] prepared from 10 mg/L samples with 30 min of ultrasonic treatment

Test item concentrations

Titanium concentrations in the overlaying water at several measuring dates are presented in Table 84. There was sedimentation of TiO_2 nanoparticles resulting in Ti concentrations in the overlaying water below the detection limit at the end of the test. Information concerning the validity of the analyses is included in the annex. Due to the high background values (refer to Table 88 for NM-101) in this experimentTi was not analysed in the sediment.

Table 84: P25 - Test with chironomids: Ti concentration.

			Sample					
			Control	15 mg/L	24 mg/L	39 mg/L	63 mg/L	100 mg/L
Ti [mg/L]				9.0 [1]	14.4 [1]	23.4 [1]	37.8 [1]	59.9 [1]
Day 1								
Water	Replicate 1	[µg/L]	12.1	522.2	621.6	645.6	466.5	467.8
	Replicate 2	[µg/L]	11.6	515.2	632.7	656.7	466.3	474.6
	mean value	[µg/L]	11.8	518.7	627.2	651.2	466.4	471.2
Day 7								
Water	Replicate 1	[µg/L]	8.9	18.6	23.7	34.9	18.1	32.7
	Replicate 2	[µg/L]	8.6	18.2	23.0	34.3	16.0	36.1
	mean value	[µg/L]	8.8	18.4	23.4	34.6	17.1	34.4
Day 14								
Water	Replicate 1	[µg/L]	60.7	57.9	166.0	71.6	105.0	20.4
	Replicate 2	[µg/L]	58.5	56.0	171.0	69.2	102.0	19.9
	mean value	[µg/L]	59.6	57.0	168.5	70.4	103.5	20.2
Day 28								
Water	Replicate 1	[µg/L]	[2]	[2]	[2]	[2]	[2]	[2]
	Replicate 2	[µg/L]	[2]	[2]	[2]	[2]	[2]	[2]
	MW	[µg/L]						

[1] nominal values for Ti (calculated from TiO_2 concentrations); [2] < LOQ/LOD

Effects

Summarised results are presented in Table 85 and Table 86.

No significant effect was observed.

The NOEC (no observed effect concentration) of the biological endpoints was ≥ 100 mg/L. $EC_{10,}$ $EC_{20,}$ and EC_{50} values of the biological endpoints were all > 100 mg/L.

Physical/pathological symptoms and changes in behaviour

Neither physical nor pathological symptoms were obtained. All specimens gave the impression of healthy condition. Only one emerged animal died, in the second concentration (24 mg/L) at day 18.

Emergence rate

The results of emergence are presented in Table 85.

No concentration/effect dependency on emergence rate due to P25 was detected. The NOEC for the tested species *Chironomus riparius* was found to be > 100 mg/L for males, females, and the combined sexes.

Table 85: P25 - Test with chironomids: emergence.

Emergence: number of individuals; emergence rate: % of introduced larvae; concentrations given as nominal values

Control	15 mg/L	24 mg/L	39 mg/L	63 mg/L	100 mg/L
Emerged midges [Ind.]					
54	58	61	53	55	53
Emergence rate midges [%]					
67.5	72.5	76.2	66.2	68.7	66.2
Emerged midges [males]					
20	30	32	23	31	28
Emerged midges [females]					
34	28	29	30	24	25

Development time and rate

The results for development time and rate are presented as mean values (Table 86). No influence on emergence due to P25 was detected.

Table 86: P25 - Test with chironomids: development time [d] and rate [1/d].

Concentrations given as nominal values.

Control	15 mg/L	24 mg/L	39 mg/L	63 mg/L	100 mg/L
Development time midges					
18.5	18.5	17.5	17.5	17.2	18.5
Development rate midges					
0.054	0.054	0.057	0.057	0.058	0.054
Development time males					
17.5	17.5	16.4	16.4	16.4	16.9
Development rate males					
0.057	0.057	0.061	0.061	0.061	0.059
Development time females					
19.2	19.2	18.9	18.2	18.2	18.5
Development rate females					
0.052	0.052	0.053	0.055	0.055	0.054

12.6.2 NM-101

(Raw data, chapter 21.5.2)

Zeta potential

The zeta potential is presented in Table 87. The three highest test concentrations were determined. No significant difference between the concentrations was observed.

Table 87: Test with chironomids - NM-101: zeta potential.

Sample	Zeta potential [mV]
39 mg/L	-19.1
63 mg/L	-17.7
100 mg/L	-19.5

Test item concentrations

Ti concentrations in the overlaying water and in the sediment are presented in Table 88. There was sedimentation of TiO_2 nanoparticles resulting in decreasing Ti concentrations in the overlaying water. At test end nearly all of the TiO_2 was determined in the sediment. The test concentrations were low compared to the background of Ti in the sediment. Due to the high background values of Ti in the sediment, calculated recovery of the added Ti is not very precise. At least at the two highest test concentrations, recovery was within the desired range.

Table 88: NM-101 - Test with chironomids: Ti concentrations.

			Sample					
			Control	15 mg/L	24 mg/L	39 mg/L	63 mg/L	100 mg/L
		Ti [mg/L]		9.0 [1]	14.4 [1]	23.4 [1]	37.8 [1]	59.9 [1]
Day 1								
Water	Replicate 1	[µg/L]	6.6	825	1159	1626	1637	1310
	Replicate 2	[µg/L]	6.9	824	1167	1626	1641	1314
	mean value	[µg/L]	6.7	825	1163	1626	1639	1312
Day 7								
Water	Replicate 1	[µg/L]	32.8	56.3	46.8	54.5	83.5	95.9
	Replicate 2	[µg/L]	34.0	54.3	46.8	55.4	84.2	94.8
	mean value	[µg/L]	33.4	55.3	46.8	54.9	83.9	95.4
Day 14								
Water	Replicate 1	[µg/L]	51.4	58.2	61.7	60.9	134.6	101.6
	Replicate 2	[µg/L]	52.6	61.5	59.2	61.4	135.8	102.1
	mean value	[µg/L]	52.0	59.8	60.4	61.2	135.2	101.9

			Sample					
			Control	15 mg/L	24 mg/L	39 mg/L	63 mg/L	100 mg/L
		Ti [mg/L]		9.0 [1]	14.4 [1]	23.4 [1]	37.8 [1]	59.9 [1]
Day 28								
Water	Replicate 1	[µg/L]	2.82	1.83	0.979	0.266	1.35	3.49
	Replicate 2	[µg/L]	3.31	1.88	0.982	0.089	2.59	2.77
	MW	[µg/L]	3.07	1.86	0.981	0.177	1.97	3.13
Sediment		[mg/kg]	1084	1080	1163	1082	1161	1228
	Sample - control -	[mg/kg]	--	-4	79	-2	77	144
	Recovery [2]	[%]	--	--	203	--	77	150
	Sediment background + addition	[mg/kg]	1084	1099	1108	1123	1147	1184
	Recovery	[%]	--	98	105	96	101	104

[1] nominal values for Ti (calculated from TiO_2 concentrations); [2] Recovery considering the amount of sediment (370 g dry weight) in the vessel and assuming 100% of Ti in the sediment;

Exposure

Due to the activity of the chironomids in the sediment, settled TiO_2 nanoparticles were transferred into the sediment. During the exposure period of 28 days the settled white powder of TiO_2 on the surface of the sediment vanished. It is assumed that during their development the organisms were mainly exposed to TiO_2 nanoparticles present in the sediment. Exposure via the water phase after hatching and until leaving the aqueous compartment is considered to be negligible.

Effects

Summarised results are presented in Table 89 and Table 90.

No significant effect for the total midges, the males and females was observed.

The LOEC, $EC_{10,}$ $EC_{20,}$ and EC_{50} values of the biological endpoints were > 100 mg/L. The NOEC was ≥ 100 mg/L.

Physical/pathological symptoms and changes in behaviour

Neither physical nor pathological symptoms were obtained. All specimens gave the impression of healthy condition.

Emergence rate

The results of emergence are presented in Table 89.

No concentration/effect dependency on the emergence rate due to NM-101 was detected. The NOEC (no observed effect concentration) for the tested species *Chironomus riparius* was found to be > 100 mg/L for males, females, and the combined sexes.

Table 89: NM-101 - Test with chironomids: emergence.

Emergence: number of individuals; emergence rate: % of introduced larvae; concentrations given as nominal values

Control	15 mg/L	24 mg/L	39 mg/L	63 mg/L	100 mg/L
Emerged midges [Ind.]					
74	69	75	60	61	74
Emergence rate midges [%]					
92.5	86.25	93.75	75.0	76.25	92.5
Emerged midges [males]					
36	39	38	21	37	37
Emerged midges [females]					
38	30	37	39	24	37

Development time and rate

The results of development time and rate are presented as mean values (Table 90). No influence on emergence due to NM-101 was detected.

Table 90: NM-101 - Test with chironomids: development time [d] and rate [1/d].

Concentrations given as nominal values.

Control	15 mg/L	24 mg/L	39 mg/L	63 mg/L	100 mg/L
Development time midges					
18.5	20.3	18.3	18.7	18.1	18.0
Development rate midges					
0.055	0.050	0.056	0.055	0.057	0.057
Development time males					
17.0	19.3	17.0	17.1	17.6	16.5
Development rate males					
0.060	0.053	0.060	0.060	0.058	0.061
Development time females					
19.6	21.3	19.2	19.2	18.9	19.2
Development rate females					
0.051	0.047	0.052	0.052	0.053	0.052

12.7 Validity

12.7.1 P25

The test is considered of limited validity since:

- The mean emergence in the controls was 68% at test end. The minimum as stated in the guideline is 70%.
- The development time of most adults of *C. riparius* in the controls was between 16 and 23 days after their insertion into the test vessels. This is within the required range of 12 – 23 days. However, one further animal developed each time at days 25, 26 and 29.

All further criteria mentioned in the guidelines are fulfilled.

- At the end of the test the dissolved oxygen concentration was at least 60% of the air saturation level at the temperature used; the pH in the overlaying water was in a range of 6 – 9 in all test vessels.
- The water temperature differed by less than ± 1 °C between the vessels and was maintained within the temperature range of 20 ± 2 °C.

The test was not repeated as no difference between the controls and the treated samples was observed.

12.7.2 NM-101

The test is considered valid since:

- The mean emergence in the controls was 92.5% (corresponding to more than the minimum 70% mentioned in the guideline) at test end.

Furthermore:

- The development time of the adults of *C. riparius* in the controls was between 16 and 21 days after their insertion into the test vessels.
- At the end of the test the dissolved oxygen concentration was at least 60% of the air saturation level at the temperature used; the pH of the overlaying water was in a range from 6 – 9 in all test vessels.
- The water temperature differed not more than ± 1°C between the vessels and was maintained within the temperature range of 20 ± 2°C.

12.8 Additional experiments

We investigated whether it was suitable to mix the chironomid food into the sediment at test start to avoid a sorption of P25 into the food in the water phase. In the test with P25, an additional four control vessels and four vessels with the highest test concentration were used to apply ground nettle. In the control vessels very low hatching was observed with 3, 10, 5, 0 hatched animals. In the vessels with the highest test concentration only 36 animals in total hatched. Due to the low hatching rate compared to the vessels with periodical feeding over

the whole incubation time (e.g. control: 18 organisms vs. 54; highest concentration: 36 organisms vs. 53) mixing of food with sediment was not considered a suitable method.

12.9 Conclusion

P25: Up to a concentration of 100 mg/L, P25 resulted in no negative impact on the emergence of larvae in a spiked water sediment test with chironomids. The NOEC was ≥ 100 mg/L.

NM-101: Up to a concentration of 100 mg/L, NM-101 resulted in no negative impact on the emergence of larvae in a spiked water-sediment test with chironomids. The NOEC was ≥ 100 mg/L.

12.10 Executive summary

The OECD Test Guideline 219 (Sediment-Water Chironomid Toxicity Using Spiked Water) was applied using *Chironomus riparius* as test organism. The test required feeding of the larvae at least three times per week. When testing strongly adsorbing substances the amount of food necessary to ensure survival and natural growth of the organisms may be added to the sediment before the stabilisation period. Finely ground leaves (0.5% dry weight) may be used. As the sorption behaviour of the nanoparticles was unknown, both feeding variants were applied. Mixing the total amount of food into the sediment at test start to avoid a sorption of P25 into the food that was applied to the water phase, instead of periodical feeding, was not considered a suitable method as this caused reduced emergence even in the control.

For the tests with TiO_2 nanoparticles, the nominal concentrations in the test containers were 15, 23, 39, 63 and 100 mg test item/L.

There was strong sedimentation of TiO_2 nanoparticles resulting in Ti concentrations below the detection limit in the overlaying water. At test end nearly all of the applied TiO_2 nanoparticles were determined in the sediment.

P25: Concentrations up to 100 mg/L P25 did not cause a negative impact on the emergence of larvae in the sediment-water chironomid test using spiked water. The NOEC was ≥100 mg/L.

NM-101: NM-101 concentrations up to 100 mg/L did not have a negative impact on the emergence of larvae. The NOEC was ≥100 mg/L.

13 Emergence Test with Chironomids (OECD TG 219) - Ag

13.1 Test principle

Sediment-dwelling larvae (first instar) of the fresh water dipteran *Chironomus riparius* were placed in a sediment-water test system with defined artificial sediment. The overlaying water was spiked with the test item at a defined range of concentrations. The test item was applied once. Chironomid emergence was measured as the endpoint at the end of the test, i.e. after 28 days of incubation. Emergence rate, development time and rate, and sensitivity of the sexes in the treatment test systems and in the control were analysed for statistically significant differences using appropriate statistical methods.

13.2 Materials and methods

13.2.1 Test guideline

The test was performed according to:

OECD Guidelines for the Testing of Chemicals Test No. 219: Sediment-Water Chironomid Toxicity Using Spiked Water (2004)

13.2.2 GLP

The test was performed following the principles of GLP. In deviation to GLP no archiving of the raw data was performed and the Quality Assurance Unit was not involved with respect to the inspection of the test, of the raw data and of the report. All laboratory equipment (e.g. balances, thermometers, pH-meters) were controlled and documented according to GLP.

13.2.3 Test substances

- NM-300K
- NM-300KDIS (dispersant of NM-300K)

  The test substances were stored in the dark at room temperature.

13.3 Analytical monitoring

Sampling

For the control and for each concentration one additional vessel was used for analytical measurements. The additional vessels were treated as the control vessels and the test vessels used for the assessment of the nanoparticles.

At several points of time aqueous samples (5 mL) were taken at four depths (about 1.5 cm, 3.0 cm, 4.0 cm, and 5.5 cm). The samples were combined and used for analysis.

Characterization of application dispersion and test dispersion

Chemical analysis was performed in the samples collected from the additional vessels.

Furthermore, using a Malvern Zetasizer, the zeta potential was measured in one vessel of each concentration and of the control three hours after application of the nanoparticles. Particle size distribution was determined in the control and the test vessels with NM-300K at selected time points during the incubation period.

Physical-chemical parameters (overlaying water)

In all vessels temperature and pH were measured at test start and test end, as well as once a week during the study. Dissolved oxygen was measured in one representative vessel per treatment at test start and twice a week during the course of the study, and in all test vessels at the end of the test. Hardness and ammonia were measured in the controls and at the highest concentration in one test vessel at the start and the end of the study.

13.3.1 Details of sediment and water

Artificial sediment components

- Sphagnum peat, air-dried, finely ground 5%
- Kaolinite, air-dried 20%
- Industrial quartz sand, air-dried 75%

The test substrate was wetted with deionised water to reach a water content of 25% - 30%. According to the guideline a water content between 30% and 50% is recommended. Our experience shows that lower water content results in a more homogenous distribution of the sediment in the individual vessels. Pulverised calcium carbonate of chemically pure quality ($CaCO_3$) was added to adjust the pH of the final mixture of the sediment to 7.0 ± 0.5. Organic carbon content of the final mixture was 2.0% which was within the demanded range of 2% ± 0.5%.

Water

- Purified tap water was used as test water.

13.3.2 Details of application

The nominal concentrations in the test containers with test item were 0.3125, 0.625, 1.25, 2.5, 5.0, and 10.0 mg test item/L. Four replicates per concentration were conducted.

A stock dispersion of 200 mg/L was prepared by mixing the respective amount of NM-300K with tap water. Fifty millilitres of the stock dispersion was used for the 10 mg/L concentration.

Appropriate amounts of the stock dispersion were diluted to obtain further concentrations. Each vessel was spiked with 50 mL of a suitable dispersion.

The dispersions were mixed by stirring.

13.4 Test organism

Test organisms were the first instar larvae from the dipteran *Chironomus riparius*.

Origin of the midges:	Bayer Crop Science AG, 40789 Monheim, Germany. Specimens used in the test were bred in the laboratory of the Fraunhofer IME.
Breeding conditions:	Purified tap water was added to a layer of diatomaceous earth. The dipterans were fed daily with powder of TetraMin® Hauptfutter (Tetra Werke, Melle, Germany).
Pre-treatment:	Four to five days before adding the test organisms to the test vessels, egg masses were taken from the cultures and placed in small aerated vessels with test water at about 20 °C. First instar larvae (one day post hatching) were used in the test. As the larvae were added one day before spiking, the age of the larvae was about 2 days at day 0 (day 0 = day of spiking the water phase).

13.5 Study design

13.5.1 Study type

Laboratory study.

13.5.2 Test duration type

Long-term.

13.5.3 Test type

Static.

13.5.4 Water media type

Fresh water.

13.5.5 Type of sediment

Artificial sediment.

13.5.6 Total exposure duration

The exposure period was 28 days.

- NM-300K: January 12 - February 9, 2011

No post-exposure observation period was performed.

13.5.7 Test conditions

NM-300K

Hardness:	At test start 100 – 120 mg/L $CaCO_3$ equivalent in the control and 110 mg/L $CaCO_3$ equivalents in one representative replicate of the highest test concentration (demanded threshold value of 400 mg/L as $CaCO_3$ equivalents). Test end: 140 – 170 mg/L $CaCO_3$ equivalents in the controls and 160 mg/L $CaCO_3$ equivalents in one representative replicate of the highest test concentration.
Test temperature:	20.3°C - 20.3°C (permitted range: 20 ± 2°C)
pH:	8.0 – 8.2 (permitted range: pH 6 – 9)
Dissolved oxygen:	93 - 97% at test start and 70 - 99% at test end (demanded threshold value: 60%)
Ammonia:	Test start: 0.3 - 0.5 (control); 0.4 (highest test concentration) Test end: 3.0 - 8.0 (control); 7.0 (highest test concentration)
Nominal concentrations:	The nominal concentrations in the test containers with Ag were 0.3125, 0.625, 1.25, 2.5, 5.0, and 10.0 mg test item/L.
Details on test conditions:	The light intensity was measured using an illuminance meter (MINOLTA) with photometric sensor in lux. With 621 – 682 lux the permitted range of about 500 – 1000 lux was kept.

Reference substance

According to the guideline a test with a reference substance is not necessary.
However, 2-chloracetamid was tested in a sediment-water chironomid toxicity test using spiked sediment (OECD 218).

13.5.8 Other information on materials and methods

Control treatment

The control consisted of sediment, tap water and chironomids. Four replicates per control were conducted. Additionally a dispersant control with the concentration of dispersant of the highest test concentration was tested.

Statistical method

Data evaluation:

Numerical values in this report are frequently rounded to a smaller degree of precision (number of digits) than used in the actual calculation. Minor differences in the results obtained from calculations with the rounded values compared to the values obtained with higher precision values are possible. They are, however, well within the limits of the experimental accuracy and thus of no practical concern.

Statistical calculations:

The number of emerged males and females were determined. The results of the listed biological parameters (total, males, females) were compared by a suitable test for multiple comparisons with a control after testing variance homogeneity. All statistical tests were performed with the computer software ToxRat Professional version 2.10.4.1 (ToxRat® Solutions GmbH).

Food

TetraMin® Hauptfutter powder was used for feeding the larvae. According to the guideline the food ration for the first 10 days was 0.25 – 0.5 mg TetraMin® /larvae/day, from day 10 on the food ration was increased to 0.5 – 1.0 mg TetraMin® /larvae/day.

Test container

Round glass beakers (3L) were used as test vessels. The vessels were filled to a height of 2 cm with wet artificial sediment (corresponding to 370 g dry mass). The overlaying water was 8 cm high (ratio sediment:water about 1:4). The containers were covered with glass plates. After 10 days, emergence traps were placed on the test vessels, the glass plates remained on the emergence traps to avoid evaporation. Aeration of overlaying water was provided through a glass pipette fixed 2-3 cm above the sediment layer (at least 1 bubble /second).

Test procedure

Sediment was put into the test vessels. Nine hundred and fifty millilitres of tap water was added and the sediment-water system was left under gentle aeration for several days prior to adding the test organisms. Batches of 20 larvae were placed into each vessel.

After an incubation of 24 h, 50 mL of the freshly prepared stock dispersion of the nanoparticles was added. To avoid separation of sediment ingredients during addition of test water and stock dispersion, the surface of the water column was covered with a stainless steel disc while water was poured onto it. The disc was removed immediately afterwards. There was no further mixing to avoid disturbance of the sediment.

The test was carried out at 20 C ± 2°C and over a 16 h photoperiod (500 –1000 lux). The exposure duration was 28 days. The development time and total number of fully emerged male and female midges were determined. The test vessels were observed daily for visual assessment of abnormal behaviour. Emergence was counted daily. After identification the midges were removed from the test vessel. At test end, the test vessels were observed for visible pupae that had failed to emerge.

13.6 Results

(Raw data, chapter 21.6)

In a first test (test concentrations 15, 24, 39, 63, 100 mg/L) all chironomid larvae died. Only the larvae in the control and in the dispersant control (NM-300KDIS) with the concentration of dispersant of the highest test concentration (100 mg/L) survived. The results are presented in Table 91. Emergence occurred between day 15 and 21 of the total incubation period (4 weeks). The total number of emerged midges in the four replicates, the total number and the ratio of females and males are shown. The difference between the control and the dispersant control was considered to be small. Whether the observed small difference indicates an effect caused by the dispersant or represents biological variability is unknown. On the basis of a range finder test (0.01, 0.1, 1.0, 10.0 mg/L) as test concentrations for the main test 0.3215, 0.625, 1.25, 2.5, 5.0, 10.0 mg/L were selected. In the following, the results of the second test are presented. As the highest test concentration, 10 mg/L was selected, which is lower than the highest concentration of for the first test by a factor of 10. As the amount of dispersant, corresponding to a silver concentration of 100 mg/L resulted in no or only a small effect, no dispersant control was included in the second test.

Table 91: Comparison of emergence of chironomids in the presence of NM-300KDIS (dispersant of NM-300K) and the control.

Date	19.10.		20.10.		21.10.		22.10.		23.10.		24.10.		25.10.		26.10.		Total		Ratio
Day	14		15		16		17		18		19		20.		21				
Sex	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	
Control	0	9	0	9	4	8	3	4	6	3	7	3	4	0	2	0	26	36	1.4
Dispersant (NM-300KDIS)	0	0	0	11	3	8	7	7	11	2	4	1	2	0	0	0	27	29	1.1

Zeta potential

A negative zeta potential of -16.1 mV was measured for NM-300K in (5 mg/L in test medium) (Table 92).

Table 92: NM-300K - Test with chironomids: zeta potential in test medium.

Sample	Zeta potential [mV]
5 mg/L	-16.1 mV

Particle size distribution

The particle size distribution in all treatments is presented in Table 93. Particles could be determined in the control indicating the measurement of sediment particles. In the vessels with NM-300K peak maximums were observed that differed from the peak maximums determined in the control. The peak maximums differed at the four measurement times and cannot be interpreted so far. The high attenuation values indicated obvious polydispersity.

Table 93: NM-300K - Test with chironomids: particle size distribution (spiked water).

Concentration [mg/L]	Z-Average [nm] [1]	PDI [2]	Peak 1 [nm]	Peak 2 [nm]	Count rate [3] [kcps]	Measurement position [4]	Attenuation [5]	Remarks
Day 0								
Control	1861	1	515	-	140	1.5	8	
0.3125	1543	1	208 [82%]	26 [18%]	73	1.5	7	count rate below recommended range
0.625	687	0.7	244 [66%]	42 [34%]	280	1.5	8	
1.25	337	0.5	269 [47%]	65 [45%]	156	1.5	7	3rd peak at 15 nm with 8%
2.5	299	0.4	67 [50%]	271 [40%]	94	1.5	6	3rd peak at 14 nm with 10%
5.0	174	0.3	62 [48%]	199 [45%]	379	1.5	7	3rd peak at 10 nm with 7%
10.0	87	0.4	116 [83%]	14 [8%]	309	1.5	6	artefact at 4912 nm with 8%
Day 7								
Control	1740	1	330	-	92	1.5	8	
0.3125	1028	0.9	367 [89%]	54 [11%]	211	1.5	9	
0.625	1052	0.7	415 [91%]	79 [9%]	91	1.5	7	
1.25	1315	0.9	300 [91%]	35 [9%]	262	1.5	8	
2.5	744	0.7	303 [64%]	71 [36%]	260	1.5	9	
5.0	930	0.8	199 [69%]	49 [31%]	158	1.5	8	
10.0	224	0.4	73 [63%]	419 [30%]	203	1.5	7	3rd peak at 14 nm with 7%
Day 14								
Control	1650	0.9	577	-	112	4.65	7	
0.3125	1290	0.9	368	-	92	4.65	7	
0.625	841	0.6	507	-	381	4.65	7	
1.25	825	0.6	492	-	355	4.65	7	
2.5	-	-	-	-	-	-	-	
5.0	1529	1	235 [94%]	21 [6%)	239	4.65	9	
10.0	1035	0.8	138	-	108	4.65	7	
Day 28								
Control	3488	1	158	-	151	1.5	10	
0.3125	2416	1	424	-	89	1.5	7	
0.625	868	0.7	646 [70%]	114 [30%]	213	1.5	11	
1.25	1314	0.8	453 [94%]	71 [6%]	336	1.5	8	
2.5	1890	1	379	-	88	1.5	7	
5.0	906	0.8	489 [93%]	87 [7%]	102	1.5	7	
10.0	929	0.8	406 [91%]	74 [9%]	142	1.5	7	

[1] calculated value (cumulative mean); [2] increasing value indicates increasing polydispersity (maximum: 1); [3] best results with a count rate between 150 and 500 kilo counts per second (kcps); [4] measurement position in the middle of the measuring cell; [5] indicator for turbidity (high values indicate low turbidity; maximum: 11); [6] 1 mg/L samples below quantification limit; [7] prepared from 10 mg/L samples with 3 min of ultrasonic treatment; [8] prepared from 10 mg/L samples with 30 min of ultrasonic treatment

Test item concentrations

Total Ag concentrations in water and sediment were determined. Furthermore the Ag^+ concentration (ion concentration of Ag) was determined in the sediment.

The Ag concentrations of the stock suspensions (20 mg/L; 200 mg/L) as well as the concentrations in the overlaying water in the vessels only used for chemical analyses were determined. Samples of 20 mL of the overlaying water were taken at three depths and combined. Only 20 mL was used for chemical analysis and the remaining volume was backfilled in the test vessels.

The results of the stock suspensions are presented in Table 94.

Recovery of the test item was between 80% (stock suspension 200 mg/L) and 92% (stock suspension 20 mg/L).

Table 94: NM-300K - Test with chironomids: Ag concentration of stock suspensions.

	Replicate		Measured value	Nominal value	Recovery
Stock suspension 20 mg/L	1	[µg/L]	18750	20000	93.8
Stock suspension 20 mg/L	2	[µg/L]	18015	20000	90.1
Stock suspension 200 mg/L	1	[µg/L]	162900	200000	81.5
Stock suspension 200 mg/L	2	[µg/L]	159750	200000	79.9

In Table 95, Figure 23, the measured silver concentrations in the aqueous phase of the test systems are summarised. The amount of silver in the sediment after 28 days is compiled in Table 96.

Table 95: NM-300K - Test with chironomids: Ag concentration in test.

			Control	0.325 mg/L	0.625 mg/L	1.25 mg/L	2.5 mg/L	5 mg/L	10 mg/L
Day 1									
Water	Replicate 1	[µg/L]	< LOD (3.6) [1]	208	402	854	1697	3110	7022
	Replicate 2	[µg/L]	< LOD (3.6) [1]	197	399	915	1622	3108	7106
	Mean value	[µg/L]	< LOD (3.6) [1]	203	401	885	1660	3109	7064
	Recovery [2]	[%]	---	64.8	64.1	70.8	66.4	62.2	70.6
Day 7									
Water	Replicate 1	[µg/L]	< LOD (1.9) [1]	90.3	85.7	164	287	355	1727
	Replicate 2	[µg/L]	< LOD (1.9) [1]	96.6	92.8	171	288	339	1715
	Mean value	[µg/L]	< LOD (1.9) [1]	93.5	89.2	168	288	347	1721
	Recovery [2]	[%]	---	29.9	14.3	13.4	11.5	6.9	17.2
Day 14									
Water	Replicate 1	[µg/L]	< LOD (1.9) [1]	< LOQ (6.3) [1]	< LOQ (6.3) [1]	< LOQ (6.3) [1]	166	192	671
	Replicate 2	[µg/L]	< LOD (1.9) [1]	< LOQ (6.3) [1]	< LOQ (6.3) [1]	< LOQ (6.3) [1]	177	179	666
	Mean value	[µg/L]	< LOD (1.9) [1]	< LOQ (6.3) [1]	< LOQ (6.3) [1]	< LOQ (6.3) [1]	171	186	668
	Recovery [2]	[%]	---	---	---	---	6.8	3.7	6.7
Day 28									
Water	Replicate 1	[µg/L]	< LOD (1.9) [1]	< LOD (1.9) [1]	< LOD (1.9) [1]	< LOD (1.9) [1]	< LOD (1.9) [1]	109	277
	Replicate 2	[µg/L]	< LOD (1.9) [1]	< LOD (1.9) [1]	< LOD (1.9) [1]	< LOD (1.9) [1]	< LOD (1.9) [1]	96	278
	Mean value	[µg/L]	< LOD (1.9) [1]	< LOD (1.9) [1]	< LOD (1.9) [1]	< LOD (1.9) [1]	< LOD (1.9) [1]	103	278
	Recovery [2]	[%]	---	---	---	---	---	2.1	2.8

[1] LOD: limit of detection; LOQ: limit of quantification; [2] Recovery referring to the nominal test concentration [mg/L];

Table 96: NM-300K - Test with chironomids: Ag concentration in dried sediment samples after 28 days.

Sample	Weighed for digestion [g]	Measured Ag conc. [µg/L]	Calculated Ag conc. in sediment [mg/kg]	Mean Ag conc. in sediment ± SD [mg/kg]	Ag recovery related to 370 g of dried sediment [%]
Sediment control	3.075	0.66	0.022	0.03 ± < 0.1	-
Sediment control	3.055	1.38	0.045		
Sediment 312.5 µg/L	3.082	24.1	0.783	0.77 ± < 0.1	91.2
Sediment 312.5 µg/L	3.081	23.4	0.758		
Sediment 625 µg/L	3.087	36.0	1.16	1.17 ± < 0.1	69.1
Sediment 625 µg/L	3.076	36.0	1.17		
Sediment 1250 µg/L	3.042	103	3.39	3.32 ± 0.1	98.4
Sediment 1250 µg/L	3.058	100	3.26		
Sediment 2500 µg/L	3.079	211	6.86	6.41 ± 0.6	94.8
Sediment 2500 µg/L	3.063	182	5.95		
Sediment 5000 µg/L	3.066	349	11.4	11.2 ± 0.3	82.6
Sediment 5000 µg/L	3.078	338	11.0		
Sediment 10000 µg/L	3.139	328 * 2 (dilution) = 656	20.9	21.0 ± 0.2	77.8
Sediment 10000 µg/L	3.112	329 * 2 (dilution) = 658	21.2		

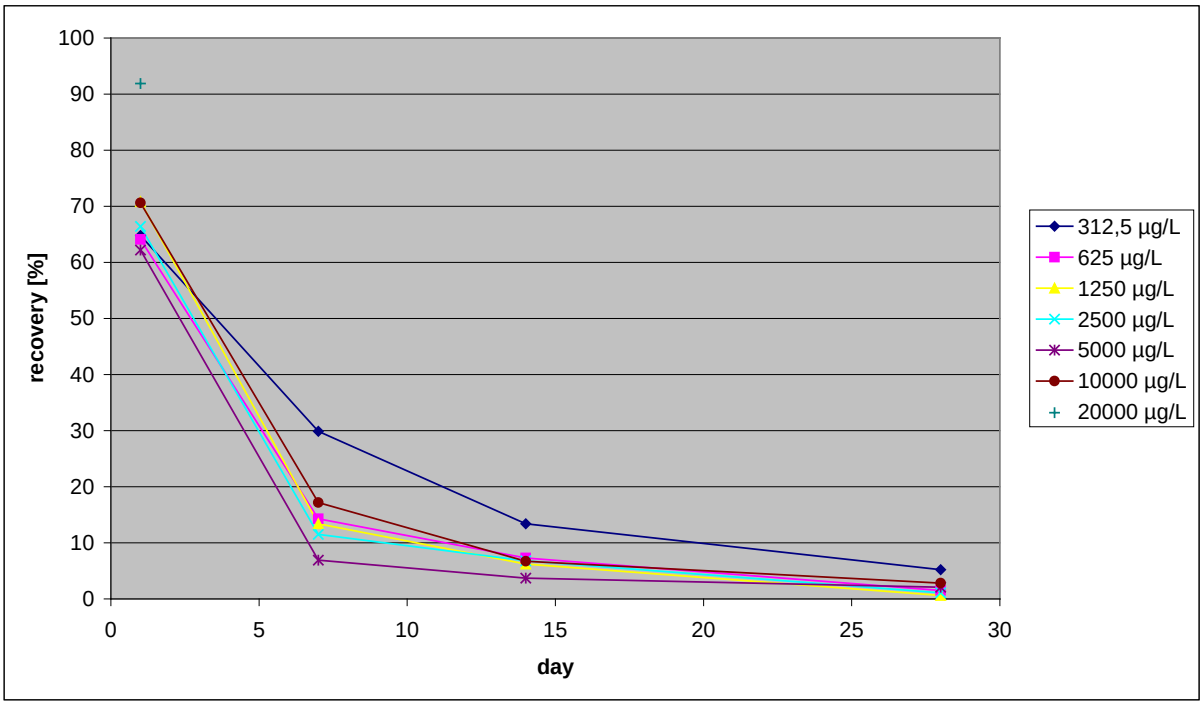


Figure 23: NM-300K – Test with chironomids: days versus recoveries in aqueous samples.

The calculated recoveries of measured values which were below LOD/ LOQ are also presented.

Table 97: NM-300K - Test with chironomids: concentration of Ag ions measured by DGTs in the sediment at test end.

		Control	0.325 mg/L	0.625 mg/L	1.25 mg/L	2.5 mg/L	5 mg/L	10 mg/L
Replicate 1	[µg/L]	0.0017	0.0024	0.0050	0.0050	0.0129	0.1364	0.4860
Replicate 2	[µg/L]	0.0015	0.0022	0.0035	0.0322	0.0122	0.4176	0.7590
Mean value	[µg/L]	0.0016	0.0023	0.0042	0.0186	0.0125	0.2770	0.6225
Recovery [1]	[%]	---	$0.22 * 10^{-3}$	$0.42 * 10^{-3}$	$1.4 * 10^{-3}$	$0.44 * 10^{-3}$	$5.5 * 10^{-3}$	$6.21* 10^{-3}$
Recovery [2]	[%]	---	$0.26 * 10^{-4}$	$0.50 * 10^{-4}$	$1.6 * 10^{-4}$	$0.52 * 10^{-4}$	$6.6 * 10^{-4}$	$7.4 * 10^{-4}$

[1] Recovery with respect to nominal concentration added to the test vessels; concentration in control sediment was considered; [2] Recovery assuming 100% of NM-300K in the sediment and considering the water in the sediment (370 g sediment, dry weight, 489.5 g fresh weight); concentration in control sediment was considered.

During the four week incubation period, sedimentation of NM-300K took place. It mainly occurred during the first week. At test end Ag could be determined in the water phase only at the two highest test concentrations. The remaining concentrations in the water phase were below the detection limit (0.325 – 2.5 mg/L) or low. In the test vessels with 5 and 10 mg/L, 2 and 3 % of the applied amount of NM-300K was detected. This calculation is based on the concentration in the stock suspension of 20 mg/L.

By chemical analysis, 69 - 98% of the expected NM-300K amount was detected in the sediment. The quality criterion for recovery in environmental samples is ± 25%. With the exception of the concentration of 0.625 mg/L, the samples were within this range. Compared to the total Ag concentration, the concentration of the Ag ions in the sediment was low. Depending on the referred value (concentration in overlaying water or concentration in water content of sediment) the percentage of the ions was about 10^{-3} or 10^{-4}% (Table 97). Chemical analysis revealed that silver nanoparticles sedimented. Therefore, the concentration in the water content of the sediment seems to be more suitable with respect to hazard assessment.

Further results concerning the validation of the chemical analyses are presented in chapter 21.5.1.

Effects

A summary of all endpoints is given in Table 98. Summarised results are presented in Table 99. The presented results are based on nominal concentrations. Based on the ion concentration determined with DGTs in the sediment, the effect values are lower by a factor of 10^{-3} – 10^{-4}. This makes it clear that the basis of the calculation has to be fixed for regulatory purposes.

A concentration/effect dependency on the emergence rate due to silver was detected.

Table 98: NM-300K - Test with chironomids: summary of the effects.

Concentrations given as nominal values.

	NOEC [mg/L] [2]	LOEC [mg/L] [2]	EC_{10} [mg/L] [1,2]	EC_{20} [mg/L] [1,2]	EC_{50} [mg/L] [1,2]
Emerged males and females	1.250	2.5	1.583 (1.350 - 1.750)	1.772 (1.566 - 1.926)	2.201 (2.041 - 2.357)
Emerged midges [males]	1.250	2.5	2.059 (n.d.)	2.175 (n.d.)	2.415 (n.d.)
Emerged midges [females]	1.250	2.5	1.055 (0.825 - 1.242)	1.276 (1.051 - 1.467)	1.835 (1.610 - 2.091)
Development rate of males and females	0.625	1.250	0.925 (n.d.)	1.897 (n.d.)	7.508 (n.d.)
Development rate of males	0.625	1.250	0.994 (n.d.)	1.824 (n.d.)	5.828 (n.d.)
Development rate of females	n.d.	n.d.	0.934 (n.d.)	2.443 (n.d.)	15.369 (n.d.)

[1] values in brackets: confidence interval; [2] n.d. = not determined due to mathematical reasons or inappropriate data

Physical/pathological symptoms and changes in behaviour

At a concentration of 5 mg/L, emerged organisms were smaller than the organisms in the control and in the concentrations ranging from 0.3125 - 0.625 mg/L. It is assumed that the size of the organisms was affected by NM-300K. In the next concentration no emergence was observed at all.

Emergence rate

The results of emergence are presented in Table 99.

A concentration/effect dependency on the emergence rate due to silver was detected. Furthermore a time delayed emergence was observed in concentrations ≥ 1.25 mg/L.

Table 99: NM-300K - Test with chironomids: emergence.

Emergence: number of individuals; emergence rate: % of introduced larvae; concentrations given as nominal values

Control	0.3125 mg/L	0.625 mg/L	1.25 mg/L	2.5 mg/L	5.0 mg/L	10.0 mg/L
Emerged midges [Ind.]						
67	76	75	67	21	0	0
Emergence rate midges [%]						
83.75	95.0	93.75	83.75	26.25	0	0
Emerged midges [males]						
33	31	39	35	12	0	0
Emerged midges [females]						
34	45	36	32	9	0	0

Development time and rate

The results of development time and rate are presented as mean values (Table 100). A concentration/response curve of emergence due to silver was detected.

Table 100: NM-300K - Test with chironomids: development time [d] and rate [1/d].

Concentrations given as initially measured values

Control	0.3125 mg/L	0.625 mg/L	1.25 mg/L	2.5 mg/L	5.0 mg/L	10.0 mg/L
Development time midges						
18.7	20.0	19.1	22.1	24.8	---	---
Development rate midges						
0.054	0.051	0.053	0.046 *	0.041 *	---	---
Development time males						
17.8	18.7	17.8	21.3	24.1	---	---
Development rate males						
0.057	0.054	0.057	0.047 *	0.042 *	---	---
Development time females						
19.6	20.8	20.3	22.9	24.5	---	---
Development rate females						
0.051	0.049	0.050	0.044 *	0.041	---	---

* Significant deviation when compared with control (total midges, males: Williams Multiple Sequential t-test, $p < 0.05$; one-sided; females: multiple sequentially rejective comparisons after Welch of treatments with "control" by the t test procedure, $p < 0.05$; one-sided)

Observations

The overlaying water changed colour during the incubation period and the sedimentation of NM-300K.

Day 2: The overlaying water of the vessels with NM-300K had a yellowish colour. The vessel with the highest test concentration was slightly brownish.

Day 5: A ginger colour of the water body at concentrations of 0.3125 - 5.0 mg/L was observed. At the highest test concentration the water body had turned to red.

Day 8: In all vessels the colour of the overlaying water was brown, with the intensity concentration dependant.

It is assumed that the change in colour was based on agglomeration and sedimentation. Due to the high polydispersity (see Table 93), DLS measurements have limited information and the process in the test vessels cannot be proven in detail.

13.7 Validity

The test is considered valid since:

- The mean emergence in the controls was 83.8% (corresponding to the minimum 70% mentioned in the guideline) at test end.

Furthermore:

- The development time of the adults of *C. riparius* in the controls was between 15 and 25 days after their insertion into the test vessels.
- At the end of the test the dissolved oxygen concentration was at least 60% of the air saturation level at the temperature used; the pH of the overlaying water was in a range from 6 – 9 in all test vessels.
- The water temperature differed by less than ± 1°C between the vessels and was maintained within the temperature range of 20 ± 2°C.

13.8 Additional experiments

In a pre-test, we investigated whether it was suitable to mix the total food in the sediment at test start to avoid a sorption of the test item to the food in the water phase. Those experiments were done only for TiO_2 nanoparticles. For complete information, the results are also mentioned in the chapter for silver. Four control vessels and four vessels with the highest test concentration were used to examine the effects of applying ground nettle. In the control vessels very low hatching was observed with 3, 10, 5, 0 hatched animals. In the vessels with the highest test concentration 36 animals hatched in total. Due to the low hatching rate compared to the vessels with periodical feeding over the whole incubation time (e.g. control: 18 organisms vs. 54; highest concentration: 36 organisms vs. 53) mixing of food with sediment was not considered a suitable method.

13.9 Conclusion

NM-300K showed a concentration-effect curve for the emergence of larvae in a spiked water sediment test with chironomids.
Based on nominal concentrations, the NOEC was 1.250 mg/L (total emergence and emerged males and females) and 0.625 mg/L (total development rate and development rates of males), respectively. For females no NOEC could be calculated as there was a statistically significant effect at 1.25 mg/L, whereas no effect was observed at 2.5 mg/L and a 100% effect was observed at 5 and 10 mg/L. Based on the ion concentration in the sediment determined with DGTs, the effect values are lower by a factor of 10^{-3} – 10^{-4}.

13.10 Executive summary

NM-300K was tested in the OECD Test Guideline 219 (Sediment-Water Chironomid Toxicity Using Spiked Water) using *Chironomus riparius* as the test organism. The organisms were fed three times per week. The nominal concentrations in the test containers with silver were 0.3125, 0.625, 1.25, 2.5, 5 and 10 mg test item/L.

There was strong sedimentation of silver resulting in Ag concentrations below the detection limit in the overlaying water. At test end nearly all of the applied Ag was identified in the sediment.

The concentration of the Ag ions in the sediment was determined using DGTs. Compared to the total Ag amount the concentration of the Ag ions in the sediment was low. Depending on

the referred value (nominal concentration in overlaying water or concentration in water content of sediment) the percentage of the ions was about 10^{-3} or 10^{-4}%.

The dispersant used for stabilising the Ag nanoparticles had no negative effect on the emergence of the chironomids.

The application of NM-300K resulted in a clear concentration-effect curve. The NOEC value for total emergence and for emerged males and females was 1.250 mg/L. The NOEC value for the total development rate and for the development rates of males was 0.625 mg/L. For females a NOEC could not be calculated as there was a statistically significant effect at 1.25 mg/L, whereas no effect was determined at 2.5 mg/L and a 100% effect was measured at 5 and 10 mg/L.

The presented results are based on nominal concentrations. Based on the ion concentration determined with DGTs in the sediment, the effect values are lower by a factor of 10^{-3} – 10^{-4}.

This illustrates that the basis of the calculation has to be clearly fixed for regulatory purposes.

A summary of the results obtained for all endpoints is presented in Table 101.

Table 101: NM-300K – test with chironomids: summary of effect values.

Concentrations given as nominal values.

	NOEC [mg/L] [2]	LOEC [mg/L] [2]	EC_{10} [mg/L] [1,2]	EC_{20} [mg/L] [1,2]	EC_{50} [mg/L] [1,2]
Emerged males and females	1.250	2.5	1,583 (1.350 - 1.750)	1.772 (1.566 - 1.926)	2.201 (2.041 - 2.357)
Emerged midges [males]	1.250	2.5	2.059 (n.d.)	2.175 (n.d.)	2.415 (n.d.)
Emerged midges [females]	1.250	2.5	1.055 (0.825 - 1.242)	1.276 (1.051 - 1.467)	1.835 (1.610 - 2.091)
Development rate of males and females	0.625	1.250	0.925 (n.d.)	1.897 (n.d.)	7.508 (n.d.)
Development rate of males	0.625	1.250	0.994 (n.d.)	1.824 (n.d.)	5.828 (n.d.)
Development rate of females	n.d.	n.d.	0.934 (n.d.)	2.443 (n.d.)	15.369 (n.d.)

[1] values in brackets: confidence interval;
[2] n.d. = not determined due to mathematical reasons or inappropriate data

14 Emergence Test with Chironomids (OECD TG 219) - Au

14.1 Test principle

Sediment-dwelling larvae (first instar) of the fresh water dipteran *Chironomus riparius* were placed in a sediment-water test system with defined artificial sediment. The overlaying water was spiked with the test item at a defined range of concentrations. The test item was applied once. Chironomid emergence was measured as the endpoint at the end of the test, i.e. after 28 days of incubation. Emergence rate, development time and rate, and sensitivity of the sexes in the treatment test systems and in the control were analysed for statistically significant differences using appropriate statistical methods.

14.2 Materials and methods

14.2.1 Test guideline

The test was performed according to OECD Guidelines for the Testing of Chemicals Test No. 219: Sediment-Water Chironomid Toxicity Using Spiked Water (2004).

14.2.2 GLP

The test was performed following the principles of GLP. In deviation to GLP no archiving of the raw data was performed and the Quality Assurance Unit was not involved with respect to the inspection of the test, of the raw data and of the report. All laboratory equipment (e.g. balances, thermometers, pH-meters) were controlled and documented according to GLP.

14.3 Test substances

- NM-330: gold nanoparticles in dispersant
- NM-330DIS: dispersant of the gold nanoparticles

14.4 Analytical monitoring

Sampling

For the control and for each concentration one additional vessel was used especially for analytical measurements. The additional vessels were treated as the control vessels and the test vessels used for the assessment of the nanoparticles.

At day 0 the pure substance and the double concentrated stock suspensions were measured. During the incubation period, 20 mL of test solution were taken from the middle of the water phase at day 1, 7 and 28 for chemical analysis. Furthermore the concentration in the sediment was determined at the end of the incubation period (day 28).

Characterisation of application dispersion and test dispersion

Zeta-potential and particle size distribution of the nanoparticles were measured with a Malvern Zeta-Sizer. At day 0 the applied suspensions and the purified tap water used for the control were characterised. Furthermore, particle size distribution was determined in the control and the test vessels with NM-330 at selected time points during the incubation period. The samples collected for the chemical analysis were used.

Physical-chemical parameters (overlaying water)

In all vessels temperature and pH were measured at test start and test end as well as once a week during the study. Dissolved oxygen was measured in one representative vessel per treatment at test start and twice a week during the course of the study, and in all test vessels at the end of the test. Hardness and ammonia were measured in the controls and at the highest concentration in one test vessel at the start and the end of the study.

14.4.1 Details on sediment and water

Artificial sediment components

Sphagnum peat, air-dried, finely ground	5%
Kaolinite, air-dried	20%
Industrial quartz sand, air-dried	75%

The test substrate was wetted with deionised water to reach a water content of 25% - 30%. According to the guideline a water content between 30% and 50% is recommended. Our experience shows that lower water content results in a more homogenous distribution of the sediment in the individual vessels. Pulverised calcium carbonate of chemically pure quality ($CaCO_3$) was added to adjust the pH of the final mixture of the sediment to 7.0 ± 0.5. Organic carbon content of the final mixture was 1.8% which was within the demanded range of 2% ± 0.5%.

Water

Purified tap water was used as test water.

14.4.2 Details on application

The nominal concentrations in the test containers with the test item (NM-330; NM-330DIS) were 50%, 10%, 1%, and 0.1% test item/L. Four replicates per concentration were conducted.

For each vessel a 135 mL stock dispersion of the nanomaterial was prepared in tap water. For the double concentrated stock dispersions of the final test concentrations a measuring cylinder was filled with the respective volume of nanomaterial and adjusted to 135 mL with

purified tap water. The stock dispersion was added thoroughly to the water column in the test vessels 24 h after adding the test specimens. An additional 10 mL of purified tap water was used to rinse the measuring cylinder. Due to the large amount of treatment solution, the dispersion mixed with the water column while being added. No further mixing was applied in order to avoid a disturbance of the sediment.

14.5 Test organism

Test organisms were the first instar larvae from the dipteran *Chironomus riparius*.

Origin of the midges: Bayer Crop Science AG, 40789 Monheim, Germany. Specimens used in the test were bred in the laboratory of the Fraunhofer IME.

Breeding conditions: Purified tap water was added to a layer of diatomaceous earth. The dipterans were fed daily with powder of TetraMin® Hauptfutter (Tetra Werke, Melle, Germany).

Pretreatment: Four to five days before adding the test organisms to the test vessels egg masses were taken from the cultures and placed in small aerated vessels with test water at about 20°C. First instar larvae (one day post hatching) were used in the test. As the larvae were added one day before spiking, the age of the larvae was about 2 days at day 0 (day 0 = day of spiking the water phase).

14.6 Study design

14.6.1 Study type

Laboratory study.

14.6.2 Test duration type

Long-term.

14.6.3 Test type

Static.

14.6.4 Water media type

Fresh water.

14.6.5 Type of sediment

Artificial sediment.

14.6.6 Total exposure duration

The exposure period was 28 days. November 24 – December 22, 2011.

No post-exposure observation period was performed.

14.6.7 Test conditions

NM 330

Hardness:	Test start: 110 – 130 mg/L as $CaCO_3$ equivalents in the controls and in one representative replicate of the highest test concentration (demanded threshold value of 400 mg/L as $CaCO_3$ equivalents). Test end: 100 – 140 mg/L as $CaCO_3$ equivalents in the controls and in one representative replicate of the highest test concentration
Test temperature:	20°C (permitted range: 20 ± 2°C)
pH:	Permitted range: pH 6 – 9 Test start: 7.3 -7.8 (in permitted range) Test end: *Control*: 7.9 – 8.5 (in permitted range) *Gold*:8.1 – 8.5 (in permitted range) *Dispersant*: 8.5 - 9.4 (in permitted range; one replicate of 50 % dispersant: 9.5 – just outside the permitted range) No peculiarities during the test
Dissolved oxygen:	Test start: about 100 % in all test vessels During the test: *Control*: 68 – 91 % *Gold*: 68 – 96 % (exception: December 13: 49 %) *Dispersant*: oxygen concentration in water phase dependant on concentration of dispersant 0.1 – 10 %: 62 – 93 % (exception: 10 %, November 29 36 %, aeration was increased; 1 %, December 12: 56 %, aeration was increased resulting in values above the threshold value of 60 % at the next measuring date) 50 %: 49 % at test start, aeration was increased resulting in values above the threshold value of 60 % at the next measuring date; values below the threshold value

	from December 12 on, increased aeration resulted in no improvement.
Ammonia:	Test start: 0.8 – 0.9 mg/L in the controls and in one representative replicate of the highest test concentration (one replicate in the control: 0.5) Test end : *Control*: 23 – 28 mg/L *Gold* (highest test concentration): 27 mg/L *Dispersant*: 0.1 mg/L
Nominal concentrations:	The nominal concentrations in the test containers with gold and dispersant were 50%, 10%, 1%, 0.1%. For gold the concentrations corresponded to 25, 5, 0.5 and 0.05 mg /L.
Details on test conditions:	The light intensity was measured using an illuminance meter (MINOLTA) with photometric sensor in Lux. With 523 – 577 lux the permitted range of about 500 - 1000 lux was kept.

Reference substance:

According to the guideline a test with a reference substance is not necessary. However, 2-chloracetamid was tested in a sediment-water chironomid toxicity test using spiked sediment (OECD 218).

14.6.8 Other information on materials and methods

Control treatment

The control consists of sediment, tap water and chironomids. Four replicates per control were conducted. Additionally a dispersant control with the concentration of dispersant of the highest test concentration was tested.

Statistical method

Data evaluation:

Numerical values in this report are frequently rounded to a smaller degree of precision (number of digits) than used in the actual calculation. Minor differences in the results obtained from calculations with the rounded values compared to the values obtained with higher precision values are possible. They are, however, well within the limits of the experimental accuracy and thus of no practical concern.

Statistical calculations:

The number of emerged males and females were determined. The results of the listed biological parameters (total, males, females) were compared by a suitable test for multiple comparisons with a control after testing variance homogeneity. All statistical tests were per-

formed with the computer software ToxRat Professional version 2.10.4.1 (ToxRat® Solutions GmbH).

Food

TetraMin® Hauptfutter powder was used for feeding the larvae. According to the guideline the food ration for the first 10 days was 0.25 – 0.5 mg TetraMin® /larvae/day, from day 10 on the food ration was increased to 0.5 – 1.0 mg TetraMin® /larvae/day.

Test container

Glass vessels (600 mL) were used as test vessels. The vessels were filled up to a height of 1.5 cm with 128.2 g wet artificial sediment (corresponding to 95 g dry mass). The overlaying water was 6 cm high (ratio sediment:water about 1:4). The containers were covered with glass plates. After 10 days, emergence traps were placed on the test vessels, the glass plates remained on the emergence traps to avoid evaporation. Aeration of overlaying water was provided through a glass pipette fixed 2-3 cm above the sediment layer (at least 1 bubble /second).

Test procedure

Sediment was put into the test vessels. One hundred and twenty five millilitres of tap water was added and the sediment-water system was left under gentle aeration for several days prior to adding the test organisms. Batches of 20 larvae were placed into each vessel.

After an incubation period of 24 h, 135 mL of the freshly prepared stock dispersion of the nanoparticles was added. A further 10 mL of tap water were used to rinse the vessels containing the stock dispersions. To avoid separation of sediment ingredients during addition of test water and stock dispersion, the surface of the water column was covered with a stainless steel disc while water was poured onto it. The disc was removed immediately afterwards. Due to the large amount of stock dispersion the dispersion admixed while being added to the water column. No further mixing was applied in order to avoid disturbance of the sediment.

The test was carried out at 20 C ± 2°C and at 16 h photoperiod (500 –1000 lux). The exposure duration was 28 days. Development time and total number of fully emerged male and female midges were determined. Test vessels were observed daily for visual assessment of abnormal behaviour. Emergence was counted daily. After identification the midges were removed from the test vessel. At test end, the test vessels were observed for visible pupae that had failed to emerge.

14.7 Results

The zeta potential is presented in Table 102. The values were measured in dilution water. The applied concentrations reflect the situation in the test. The highest concentration of NM-330 resulted in a zeta potential of -48 mV. With decreasing concentration the zeta potential

became less negative. The concentration of 10% NM-330 resulted in a zeta potential of -24 mV.

Table 102: NM-330 – Test with chironomids: zeta potential.

Concentrations given as percentage of NM-330 in dilution water

Sample	Zeta potential [mV]
10%	-24.2 (± 0.4)
50%	-48.0 (± 1.1)

Particle size distribution is presented in Table 103. Only reliable results are presented. In the distributed NM-330 suspension the particle size was about 45 – 50 nm. Dilution resulted in larger agglomerates and worse poly dispersity indices in the stock suspensions. Measurements below 2% NM-330 did not show acceptable results. It is assumed that the concentrations were too low for the measurement. On days 1, 7, 14 and 28 in one vessel per test concentration the particle size distribution was determined. However, at day 1 only in the highest test concentration acceptable results were obtained. All further measurements revealed no acceptable results. Sedimentation of NM-330 as well as re-suspension of sediment particles in the overlaying water resulted in values unsuitable for interpretation.

Table 103: Particle size distribution in the chironomid test (spiked water) with NM-330 (gold nanoparticles in dispersant).

Mean value of 10 measurements; concentration given as percentage of NM-330 in the medium (application suspension or in test vessels)

Concentration [mg/L]	Z-Average [nm] [1] (±SD) [4]	PDI [2] (±SD) [4]	Peak 1 [nm] (±SD) [4]	Peak 2 [nm] (±SD) [4]	Peak 1 [%]	Peak 2 [%]	Attenuation [3]	Remark
Day 0 Application suspension								
2.0%	118.1 (±6.9)	0.3 (±0.03)	159.0 (±9.5)	-	96	3	8	Peak 2 = artefact
20%	342.8 (±50.8)	0.4 (±0.05)	497.0 (±69.2)	36.8 (±46.9)	96	4	6	
100%	49.1 (±22.2)	0.2 (±0.03)	45.7 (±3.4)	8.2 (±1.0)	77	19	6	
Day 1 Test medium								
50%	82.3 (±2.7)	0.4 (±0.06)	108.5 (±8.9)	-	90	10	6	Peak 2 = artefact

[1] calculated value (cumulative mean); [2] increasing value indicates increasing polydispersity (maximum: 1); [3] indicator for turbidity (high values indicate low turbidity; maximum: 11); All presented results have a count rate between 150 and 500 kilo counts per second (kcps), which creates the best results stated by Malvern. Results presented are the best results out of ten in accordance to the quality report. For a better comparison of the results the measurement position was fixed at 1.5 mm from the wall of the cell for each measurement; [4] SD = standard deviation

The concentrations of Au are presented in Table 104. 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% also. Details of the analytical method used by the producer of NM-330 are unknown. Therefore, the discrepancy in 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.

Using the measured concentration as 100%, it is obvious that at day 0 the concentrations in the stock suspensions were above quantification and detection limits were in the range of the expected values (expected 2% - measured 1.7%; expected 20% - measured 18%). During the incubation period of 28 days sedimentation occurred. In the highest test concentration, at the end of the test only 0.6% of NM-330 was detected. The lower test concentrations were below the detection limit.

At the end of the incubation period the Au concentration in the sediment was determined. The results are presented in Table 105. The Au concentration in the sediment of the control samples and of the lowest test concentration were below the detection limit. The other test concentrations showed about 50% of the expected concentration. We cannot explain the missing 50% of the substance. Sorption of 50 % at the walls of the test vessels, independent of the test concentrations, is considered to be unreliable. The spacing factors between the concentrations that were above the detection limit fit, and the recovery of the reference for the chemical analyses was about 100%. Additionally, a nano-particular gold reference material (NIST standard) was applied. The concentrations of the stock suspensions were correct and the required volumes of the stock suspensions were added to the test vessels at test start according to the documentation of the procedure. The required volumes were 50% of the overlaying water. Therefore, the addition of half of the required volumes (corresponding to 50% recovery) would have been evident.

Table 104: Concentration of Au in the test vessels with NM-330 (overlaying water).

Concentrations given as percentage of NM-330 in the stock suspension (day 0) and in the test medium (day 1 – 28).

Sample	Nominal concentration [%]	Au concentration [µ/L]	Concentration with respect to NM-330 (pure substance) [%]
Day 0			
dispersant	--	-14.9 (< detection limit)	---
Dilution water (= control)	--	-22.0 (< detection limit)	---
NM-330 0.2%	0.2	53.1 (< quantification limit)	---
NM-330 2%	2	752	1.7
NM-330 20%	20	8010	18
NM-330 100 % (pure substance)	100	43840	100
Day 1			
Control	---	-1.69 (< detection limit)	---
NM-330 0.1%	0.1	34.5 (< quantification limit)	---
NM-330 1%	1	199	0.45
NM-330 10%	10	1251	2.2
NM-330 50%	50	11690	27
Day 7			
Control	---		
NM-330 0.1%	0.1	-0.986 (< detection limit)	---
NM-330 1%	1	38.9 (< quantification limit)	---
NM-330 10%	10	222	0.5
NM-330 100%	50	4559	10
Day 28 – overlaying water			
Control	---		
NM-330 0.1%	0.1	1.6 (< detection limit)	---
NM-330 1%	1	2.4 (< detection limit)	---
NM-330 10%	10	-9.6 (< detection limit)	---
NM-330 100%	50	254	0.6

Table 105: Concentration of Au in the test vessels with NM-330 (sediment).

Concentrations given as percentage of NM-330 in the stock suspension (day 0) and in the test medium (day 1 – 28).

Sample	Weighed for digestion [g]	Measured Ag conc. [µg/L]	Calculated Ag conc. in sediment [mg/kg]	Mean Ag conc. in sediment ± SD [mg/kg]	Ag recovery related to 95 g of dried sediment [1] [%]
Sediment control	3.061	-4.54	-0.148	< detection limit	---
Sediment control	3.022	-3.77	-0.125		
Sediment (0.1%)	3.058	-2.56	-0.084	< detection limit	---
Sediment (0. %)	3.042	-1.88	-0.062		
Sediment (1%)	3.085	18.7	0.606	0.541	43 %
Sediment (1%)	3.030	14.4	0.475		
Sediment (10%)	3.022	162	5.36	6.10	48 %
Sediment (10%)	3.017	207	6.85		
Sediment (50%)	3.046	906	29.75	28.78	46 %
Sediment (50%)	3.046	847	27.81		

[1] concentration of Au in NM-330 of 43,840 µg/L

Observations

Colour of test suspensions

The addition of NM-330 resulted in coloured test suspensions. At day 1 (Figure 24) the test suspensions with the highest test concentration were red. 25 % NM-330 resulted in blue-grey colour. The colours of the further test concentrations were comparable to the control and to the test vessels with dispersant (NM-330DIS). During the incubation period the colour of the test suspensions with 25% gold nanoparticles vanished. The red colour of the highest test concentration turned to grey indicating further agglomeration of the gold nanoparticles. The test vessels with dispersant turned to amber (Figure 25).

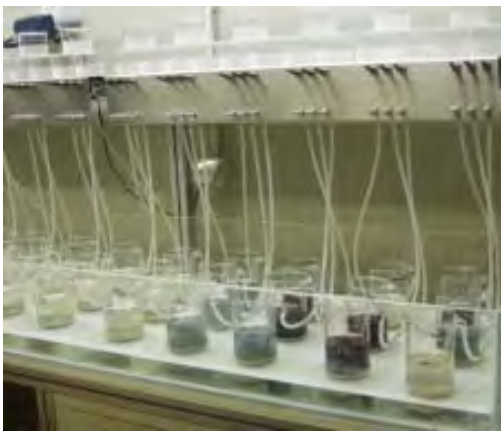


Figure 24: NM-300K – Test with chironomids: Colour of the test vessels at day 1

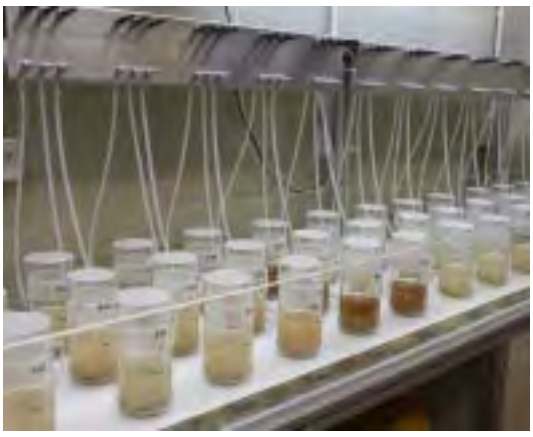


Figure 25: NM-300K – Test with chironomids: Colour of the test vessels at day 12

Oxygen concentration in the presence of dispersant (NM-330DIS)

The dispersant resulted in a decreased oxygen concentration in the water phase dependant on concentration of the dispersant. The threshold concentration of 60% oxygen saturation was not achieved in the vessels with the highest dispersant concentration after the third week even with increased aeration. In the vessels with the highest dispersant concentration a very high microbial number was determined.

As explanation for the observations, two different possibilities are listed:

Possibility 1:

Larvae are introduced → larvae die after an incubation period of several days due to a toxic effect → degradation of the larvae by microorganisms → an increased number of microorganisms due to the nutrients and low oxygen concentration measured in the test medium due to increased microbial activity

Possibility 2:

Larvae are introduced → low oxygen concentration → larvae die → increased number of microorganisms due to the nutrients.

The oxygen supply was controlled qualitatively on a daily basis during the working week. The aeration of all vessels was comparable. Therefore, possibility 2 is rejected. 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 decreased oxygen concentration and (ii) that the comparable low oxygen concentration is not the reason for the missing emergence.

Effects

A strong effect was observed for the dispersant (NM-330DIS) at the highest test concentration. The larvae were fully grown even though there was a delay in the development. However, no larvae emerged as the organisms died before hatching. In the presence of gold this effect did not occur.

For the concentrations resulting in emergence (NM-330: all test concentrations; NM-330DIS all test concentrations except the highest test concentration) no statistical difference between the treated vessels and the control was observed for the development time and the emergence rate. For the development rate a statistical difference was calculated. However, no concentration-effect relationship and no difference between the vessels with NM-330 (gold in dispersant) and NM-330DIS (dispersant) was obvious. Therefore, it was concluded that the statistical difference in the development rate is not substance related effect but indicates biological variability.

Physical/pathological symptoms and changes in behaviour

Neither physical nor pathological symptoms were observed in the presence of gold nanoparticles. All specimens gave the impression of healthy condition.
In the highest test concentration of the dispersant, full-grown larvae were observed after an incubation period of three weeks. After 28 days all larvae were dead on the surface of the sediment.

Emergence rate

The results of emergence are presented in Table 106.

For NM-330 (gold nanoparticles in dispersant) no concentration/effect dependency on emergence rate was detected. The NOEC for the tested species *Chironomus riparius* was found to be ≥ 50% for the combined sexes. For the dispersant NM-330DIS the highest test concentration (50%) resulted in no emergence at all. The lower test concentrations showed no statistical significant difference compared to the control. The NOEC for NM-330DIS was calculated to be 10%.

No difference between the sexes was observed.

Table 106: Emergence at test end. Emerged midges [Ind.] and emergence rate [% of introduced larvae]; concentrations given as nominal values.

	NM-330				NM-330DIS			
Control	0.1%	1.0%	10%	50%	0.1%	1.0%	10%	50%
Emerged midges [Ind.]								
75	76	75	77	73	75	68	73	0
Emergence rate midges [%]								
93.7	95.0	93.7	91.7	91.2	93.7	85.0	91.2	0
Emerged midges [males]								
36	33	42	34	40	34	31	36	0
Emerged midges [females]								
39	43	33	43	33	41	37	37	0

Development time and rate

The results for development time and rate are presented as mean values (Table 86).

For NM-330 (gold nanoparticles in dispersant) and the development time, no statistically significant difference was observed. The development rate showed a statistically significant difference (Williams multiple sequential t-test, $\alpha = 0.05$) for the combined sexes, males and females and all test concentrations, although no concentration-effect curves were obvious.

For the three lower test concentrations of the dispersant NM-330DIS the development time showed no significant difference compared to the control for the combined sexes, males and females. The development rate for the combined sexes and for males differed statistically significant from the control, although no concentration-effect curves were obtained. The development rate of the females was not statistically affected.

Table 107: Development time [d] and rate [1/d] of midges.

Concentrations given as nominal values.

	NM-330				NM-330DIS			
Control	0.1%	1.0%	10%	50%	0.1%	1.0%	10%	50%
Development time midges								
17.2	19.1	18.8	20.0	18.7	19.6	19.5	18.2	---
Development rate midges								
0.059	0.054 *	0.054 *	0.051 *	0.054 *	0.052 *	0.053 *	0.056 *	--- *
Development time males								
16.3	17.5	17.8	18.3	18.0	18.6	18.8	17.3	---
Development rate males								
0.062	0.058 *	0.057 *	0.056 *	0.056 *	0.055 *	0.055 *	0.059 *	--- *
Development time females								
18.0	20.3	19.9	21.3	19.4	20.3	19.9	19.2	---
Development rate females								
0.056	0.050 *	0.051 *	0.048 *	0.052 *	0.050	0.052	0.053	---

* Significant deviation when compared with control (Williams Multiple Sequential t-test, $p < 0.05$; one-sided)

14.8 Validity

The test is considered valid since:

- The mean emergence in the controls was 93.7% (corresponding to the minimum 70% mentioned in the guideline) at test end.
- The development time of the adults of *C. riparius* in the controls was 17.2 days after their insertion into the test vessels (threshold concentration in the test guideline: between 12 and 23 days).
- At the end of the test the dissolved oxygen concentration was at least 60% of the air saturation level at the temperature used; the pH of the overlaying water was in a range from 6 – 9 in all test vessels. The test vessels with 50% dispersant are an exception where a substance specific effect is assumed.
- The water temperature differed by less than ± 1°C between the vessels and was maintained within the temperature range of 20 ± 2°C.

14.9 Conclusion

The dispersant NM-330DIS in the highest test concentration showed an obvious effect. No emergence was observed. In contrast, NM-330 (gold nanoparticles in dispersant) showed no effect, even at the highest test concentration of 50%. Gold nanoparticles compensated the effect of the dispersant.

14.10 Executive summary

NM-330 and **NM-330DIS** were tested in the test with *Chironomus riparius* with spiked water (OECD 219).

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.

The nominal test concentrations in the vessels were 0, 0.1, 1, 10 and 50% of the test item/L. The test concentrations were verified by chemical analysis.

General observations

The addition of NM-330 and NM-330DIS caused coloured test suspensions. Changing colours during the incubation indicated 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 of all vessels was comparable. A technical defect as 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.

Effects

For the dispersant a strong effect was observed at the highest test concentration. The larvae were fully grown, even though their development was delayed. However, no larvae emerged as the organisms died before hatching. In the presence of gold this effect did not occur.

All effect values are summarised in Table 108. In contrast to the dispersant no effect was observed for the treatments with gold. Although statistically significant differences to the control were observed for the development rates, the differences were not considered to be an effect of the test substance, since they were not related to concentration-effect relationships.

Table 108: NM-330, NM-330DIS – test with chironomids: summary of the effects.

Effects given as percentage of NM-330 in the test medium

	NOEC [%]	LOEC [%]
	NM-330	
Emergence rate – combined sexes, males, females	≥ 50	> 50
Development time – combined sexes, males, females	≥ 50	> 50
Development rate – combined sexes	not evaluable [1]	not evaluable [1]
Development rate – males	not evaluable [1]	not evaluable [1]
Development rate – females	not evaluable [1]	not evaluable [1]
	NM-330DIS	
Emergence rate – combined sexes, males, females	10	1
Development time – combined sexes, males, females	10	1
Development rate – combined sexes	not evaluable [1]	not evaluable [1]
Development rate – males	not evaluable [1]	not evaluable [1]
Development rate – females	10	1

[1] There was a statistically significant difference to the control, but no concentration-effect relationship.

15 Reproduction Tests with Daphnids (OECD TG 211) – TiO_2

15.1 Test principle

Young female *Daphnia* (parent animals) aged less than 24 h at test start were exposed to the test item for 21 days under semi-static conditions. The test item was added to the water at a defined range of concentrations. The test solution was exchanged either daily or three times a week. At the end of the test, the total number of living offspring produced per parent animal and alive at test end was assessed. Immobilisation and reproduction rate in the treatments and in the control were analysed for statistically significant differences.

Three tests were performed:

Test 1: medium renewal three times a week

Test 2: medium renewal three times a week and daily

Test 3: medium renewal three times a week, sonication period 3 and 30 min

In the first test the results differed from those reported in the literature. Test 2 and 3 were performed for clarification of the discrepancies.

15.2 Materials and methods

15.2.1 Test guideline

The test was performed according to

OECD 211 (21.09.1998): OECD guideline for testing of chemicals – *Daphnia magna* Reproduction Test.

15.2.2 GLP

The test was performed following the principles of GLP. In deviation to GLP no archiving of the raw data was performed and the Quality Assurance Unit was not involved with respect to the inspection of the test, of the raw data and of the report. Any laboratory equipment (e.g. balances, thermometers, pH-meters) was controlled and documented according to GLP.

15.2.3 Test substances

$\underline{TiO_2}$

- P25 - distributed by Evonik for the OECD Sponsorship Programme.

The test substance was stored in the dark at room temperature.

15.3 Analytical monitoring

Test concentrations and particle size distribution were determined once per week.

15.3.1 Details on sampling

Samples for analyses were taken from the stock dispersion of each concentration before test start, and from the test vessels after the incubation periods listed below together, as well as at each medium renewal. Samples from the incubated test suspensions were withdrawn from the water phase. Care was taken that sedimented particles were not removed. Sampling times were as follows:

Medium renewal three times a week

- Day 0 (start of the test): stock dispersion and each test concentration
- Day 2: each test concentration after incubation (before medium renewal) in the test vessels with daphnids
- Day 7: stock dispersion and each test concentration
- Day 9: each test concentration after incubation in the test vessels with daphnids
- Day 14: stock dispersion and each test concentration
- Day 16: each test concentration after incubation in the test vessels with daphnids.

Medium renewal daily

- Day 0 (start of the test): stock dispersion and each test concentration
- Day 1: each test concentration after incubation (before medium renewal) in the test vessels with daphnids
- Day 7: stock dispersion and each test concentration
- Day 8: each test concentration after incubation in the test vessels with daphnids
- Day 14: stock dispersion and each test concentration
- Day 15: each test concentration after incubation in the test vessels with daphnids.

15.3.2 Details on analytical methods

Characterisation of the application dispersion and test dispersion

For test item concentrations see chapter 4.1

Zeta-potential and particle size distribution was measured using a Malvern Zetasizer NanoZS. Particle size distribution was measured only in suitable (higher) test concentrations.

15.3.3 Details on test suspensions

Purified tap water was used as test water and to prepare the test suspension. The stock dispersion was 20.0 mg/L. The test concentrations were achieved by dilution:

5.0 mg/L: 250 mg/L stock dispersion + 750 mL purified tap water

1.0 mg/L: 50 mg/L stock dispersion + 950 mL purified tap water

0.5 mg/L: 25 mg/L stock dispersion + 975 mL purified tap water

0.1 mg/L: 5 mg/L stock dispersion + 995 mL purified tap water

0.05 mg/L: 2.5 mg/L stock dispersion + 997.5 mL purified tap water

The stock dispersion and every test concentration was stirred (magnetic stirrer, 900 rpm) and treated with ultrasound in a water bath (3 min, 500 W). For the third test, 30 min of ultrasound was included in addition to the original 3 min ultrasound.

For the renewal of the medium the test suspensions were freshly prepared.

15.4 Test organism

The test organisms were young specimens of *Daphnia magna*, 4 – 24 h old at test start.

Origin of the daphnids:	German Federal Environment Agency, Institut für Wasser-, Boden- und Lufthygiene. Specimens used in the test were bred in the laboratory of the Fraunhofer IME.
Breeding conditions:	Adult *Daphnia*, at least 3 weeks old, were separated from the stock population by sieving. Batches of 30 to 50 animals were held at room temperature in approx. 1.8 L dilution water for one week. During this week the daphnids were fed daily with an algal suspension (*Desmodesmus subspicatus*) and Liquizell (HOBBY). Algae growing in the log-phase were centrifuged and the pellet was re-suspended in a few mL of medium. 30 mL of this suspension were given to 1 L Daphnia medium. The water was changed once per week. New born *Daphnia* were separated by sieving, the first generation was discarded.
Holding- and dilution-water:	Purified drinking water was used as holding- and dilution water. The purification included filtration with activated charcoal, passage through a lime-stone column and aeration. To avoid copper contamination, plastic water pipes were used in the purification system. The following water chemistry data, recorded regularly in the testing facility, were: pH, conductivity, dissolved oxygen content, content of nitrate, nitrite, ammonium ($NH4^+$), phosphate, calcium, magnesium, total hard-

	ness, alkalinity, DOC content, content of metals (copper, iron, manganese and zinc)
Food:	The daphnids were fed during the test with suspensions of the unicellular alga *Desmodesmus subspicatus*. The content of food in the test suspensions, measured as turbidity at 758 nm, was increased during the test from about 7 mg C/L equivalents to 15 mg C/L equivalents.

15.5 Study design

15.5.1 Study type

Reproduction, semi-static.

15.5.2 Water medium type

Fresh water.

15.5.3 Total exposure duration

21 d, for each test period;

- September 22, 2010 - October 13, 2010
- March 9, 2011 – March 30, 2011
- August 17, 2011 – September 7, 2011

No post-exposure observation period was performed.

15.5.4 Test conditions

<u>P25 – first test</u>

Total hardness:	1.1 mmol/L
Test temperature:	20.7 - 21.3°C (permitted range: 20 ± 2°C)
pH:	7.8 – 8.8 (permitted range: pH 6 – 9; variation less than 1.5)
Dissolved oxygen:	About 100% corresponding to about 8.6 mg/L (demanded threshold value: 3 mg/L)
Salinity:	304 - 337 µS/cm
Nominal concentrations:	The nominal concentrations in the test containers with TiO_2 nanoparticles were 0.05, 0.1, 0.5, 1.0, 5.0 mg test item/L.

Details on test conditions:

- Test vessel: glass beakers (60 mL) filled with 50 mL test suspension; covered with glass panes
- Aeration: no
- Renewal rate of test solution (frequency/flow rate): 3 times a week
- No. of organisms per vessel: 1
- No. of vessels per concentration (replicates): 10
- No. of vessels per control (replicates): 10

TEST MEDIUM / WATER PARAMETERS

The quality of the applied water is described in Table 109.

Table 109: Chemical parameter of the holding- and dilution-water in the first test

Conductivity [µS/cm]	Alkalinity [mmol/l]	Total hard-ness [mmol/l]	Ca hardness [mmol/l]	Mg hardness [mmol/l]	TC [mg/L]	IC [mg/L]
304 - 337	2.1 – 2.9	1.1	0.9	0.2	28.2	27.2
TOC [mg/L]	NO_3 [mg/L]	NO_2 [mg/L]	NH_4 [mg/L]	PO_4 [mg/L]	Cl [mg/L]	Cd [µg/L]
0.9	2.8 – 3.2	< 0.005 - 0.017	< 0.01 – 0.02	0.35 – 1.04	< 0.02	< LOQ
Cr [µg/L]	Cu [µg/L]	Fe [µg/L]	Mn [µg/L]	Ni [µg/L]	Pb [µg/L]	Zn [µg/L]
< LOQ	2.35 – 7.38	< LOQ – 4.6	< LOQ	< LOQ	< LOQ	4.7 – 5.8

OTHER TEST CONDITIONS

- Culture medium different from test medium: no
- Intervals of water quality measurement: once per month
- Adjustment of pH: no
- Photoperiod: light/dark cycle 16/8 h
- Light intensity: 563 - 591 lux

<u>P25 – second test</u>

Total hardness:	1.0 mmol/L
Test temperature:	19.9 - 20.6°C (permitted range: 20 ± 2°C)
pH:	7.9 – 8.7 (permitted range: pH 6 – 9; variation less than 1.5)
Dissolved oxygen:	About 100% corresponding to about 8.6 mg/L (demanded threshold value: 3 mg/L)
Salinity:	298 µS/cm

Nominal concentrations: The nominal concentrations in the test containers with TiO_2 nanoparticles were 1.0, 5.0 mg test item/L.

Details on test conditions:

- Test vessel: glass beakers (60 mL) filled with 50 mL test suspension; covered with glass panes
- Aeration: no
- Renewal rate of test solution (frequency/flow rate): 3 times a week and daily
- No. of organisms per vessel: 1
- No. of vessels per concentration (replicates): 10
- No. of vessels per control (replicates): 10

TEST MEDIUM / WATER PARAMETERS

The quality of the applied water is described in Table 110.

Table 110: Chemical parameter of the holding- and dilution-water in the second test

Conductivity [µS/cm]	Alkalinity [mmol/l]	Total hard-ness [mmol/l]	Ca hardness [mmol/l]	Mg hardness [mmol/l]	NPOC [a] [mg/L]	
298	2.1	1.0	0.8	0.2	0.76	
NO_3 [mg/L]	NO_2 [mg/L]	NH_4 [mg/L]	PO_4 [mg/L]	Cl [mg/L]	Cd [µg/L]	
4	< 0.005	< 0.01	1.18	< 0.02	< LOQ	
Cr [µg/L]	Cu [µg/L]	Fe [µg/L]	Mn [µg/L]	Ni [µg/L]	Pb [µg/L]	Zn [µg/L]
<1.96	<6.24	<8.57	<2.26	<1.26	<9.50	< 15.2

[a] NPOC = non purgeable organic carbon

OTHER TEST CONDITIONS

- Culture medium different from test medium: no
- Intervals of water quality measurement: once per month
- Adjustment of pH: no
- Photoperiod: light/dark cycle 16/8 h
- Light intensity: 560 - 607 lux

<u>P25 – third test</u>

Total hardness: 1.2 - 1.3 mmol/L

Test temperature: 20.5 - 21.3°C (permitted range: 20 ± 2°C)

pH: 8.0 – 8.8 (permitted range: pH 6 – 9; variation less than 1.5)

Dissolved oxygen: About 100% corresponding to about 8.6 mg/L (demanded threshold value: 3 mg/L)

Salinity: 326 µS/cm

Nominal concentrations: The nominal concentrations in the test containers with TiO_2 nanoparticles were 1.0, 5.0 mg test item/L.

Details on test conditions:

- Test vessel: glass beakers (60 mL) filled with 50 mL test suspension; covered with glass panes
- Aeration: no
- Renewal rate of test solution (frequency/flow rate): 3 times a week
- No. of organisms per vessel: 1
- No. of vessels per concentration (replicates): 10
- No. of vessels per control (replicates): 10

TEST MEDIUM / WATER PARAMETERS

The quality of the applied water is described in Table 111.

Table 111: Chemical parameter of the holding- and dilution-water in the third test

Conductivity [µS/cm]	Alkalinity [mmol/l]	Total hard-ness [mmol/l]	Ca hardness [mmol/l]	Mg hardness [mmol/l]	NPOC [a] [mg/L]	
314 - 326	2.1 – 2.4	1.2 - 1.3	0.9 – 1.0	0.2 - 0.4	0.45 - 0.80	
NO_3 [mg/L]	NO_2 [mg/L]	NH_4 [mg/L]	PO_4 [mg/L]	Cl [mg/L]	Cd [µg/L]	
2 - 3	< 0.005 - 0.009	0.01	0.28 – 0.61	< 0.02 – 0.02	< 3.12 [LOQ]	
Cr [µg/L]	Cu [µg/L]	Fe [µg/L]	Mn [µg/L]	Ni [µg/L]	Pb [µg/L]	Zn [µg/L]
<1.96	<6.24	<8.57	<2.26	<1.26	<9.50	5.16 – 10.5

[a] NPOC = non purgeable organic carbon

OTHER TEST CONDITIONS

- Culture medium different from test medium: no
- Intervals of water quality measurement: once per month
- Adjustment of pH: no
- Photoperiod: light/dark cycle 16/8 h
- Light intensity: 811 - 891 lux

VEHICLE CONTROL PERFORMED: No

Reference substance: According to the guideline a test with a reference substance is not compulsory necessary.

15.5.5 Other information on materials and methods

Test performance

Less than 24 h old *Daphnia magna* were exposed to five concentrations of the test item under semi-static conditions for a period of 21 days. Test suspensions were exchanged three times a week. Algae of a stock culture were added to achieve the desired amount in the test medium (21.8.1, Table 278; calibration curves: Figure 59, Figure 60). Afterwards, the test organisms were added.

Statistical method

Data evaluation

In this report numerical values are frequently rounded to a smaller degree of precision (number of digits) than have been used in the actual calculation. Minor differences in results obtained from calculations with rounded values compared to those obtained with higher precision values are possible. They are, however, well within the limits of the experimental accuracy and of no practical concern.

The parental mortality, the time to first brood and the number of offspring were used to calculate the intrinsic rate of population increase r as integrative parameter relevant for population effects.

Statistical calculations

The results of the listed biological parameters were compared by a suitable test for multiple comparisons with a control after testing variance homogeneity. All statistical tests were performed with the computer software ToxRat version 2.10.4.1 Professional (ToxRat® Solutions GmbH).

Pre-tests

In pre-tests the behaviour of P25 in the presence of algae used as food for the daphnids was studied. On the basis of these results the period for the renewal of the medium was determined. The experiments consisted of:

- Three concentrations of P25 (0.05 mg/L; 0.5 mg/L, 5.0 mg/L) and the control
- For each sampling time, three replicates per concentration and the control
- Four sampling times (0, 24, 48, 72 h)
- Concentration of algae: during the reproduction test as the parent animals grow the amount of algae added as feed had to increase. For the pre-test that investigated the behaviour of P25 in the presence of algae (e.g. sorption and sedimentation), a me-

dium concentration of algae (resulting in an extinction of 0,075 at 585 nm in the test vessels) was used.

TiO_2 concentrations were prepared as described above (1 min stirring, 3 min ultrasonic treatment) and the test medium was filled in the test vessels used for the main test. Algae were added. For each sampling time about 35 mL of the respective test vessels (three per concentration + control) were carefully removed and the TiO_2 concentration was determined. Removal of sedimented particles was avoided. The results of the analyses are presented in Table 112. Recovery of P25 used as standard was 100%.

Table 112: P25 – test with daphnids: concentration of TiO_2 determined in the supernatant of the test vessels.

Mean values of three replicates [µg/L] and standard deviation.

TiO_2 [mg/L]	Pure algae	Stock suspension		Day 0		Day 1		Day 2		Day 3	
		Mean [µg/L]	Standard deviation	Mean [µg/L]	Standard deviation	Mean [µg/L]	Standard deviation	Mean [µg/L]	Standard deviation	Mean [µg/L]	Standard deviation
0,05	< LOQ	56.7	3.2	48.8	1.4	26.8	2.3	21.6	1.8	18.7	2.4
0,5	< LOQ	65.2	2.7	51.8	2.0	22.5	0.8	19.3	0.6	16.7	1.5
5.0	< LOQ	72.3	2.8	56.9	0.9	18.5	0.7	11.9	0.2	8.9	0.2

From the results the following conclusions can be drawn:

- Due to the 100% recovery of the standard the lower values in the samples result from the incubation design.
- The main difference was between day 0 and day 1. This may be due to sedimentation of P25 agglomerates and P25 sorbed to algae.

In experiments with lumbriculus (UBA project FKZ: 3709 65 418) it was found, by particle measurement, that sedimentation mainly occurred within the first three hours. Due to their mobility, daphnids ingest sedimented as well as non-sedimented nanoparticles and algae. Therefore, a daily renewal of the medium with a temporarily higher concentration in the supernatant should not give a significant advantage compared to medium renewals at longer time intervals. Therefore it was decided to renew the medium three times a week. At every change of the medium, clean vessels are filled with freshly prepared dispersion and the daphnids are transferred. Therefore, an increase of the test concentrations due to remaining sedimented nanoparticles is impossible.

15.6 Results

15.6.1 P25 - First test

(Raw data, chapter 21.8.1)

Zeta potential

The zeta potential in the test medium is presented in Table 113. A negative value of -18 mV was achieved.

Table 113: P25 – 1st test with daphnids: zeta potential.

Sample	Zeta potential [mV]
P25 in tap water (application dispersion):	-18 mV

Particle size distribution

At day 0 and day 2 the particle size distribution was determined with the device Malvern Nano ZS (Table 114). The particle size distribution was measured only in the stock suspension and at concentrations of 1 and 5 mg/L. Even at a concentration of 1 mg/L the value corresponded to the value measured for the control. Measurements were done in freshly prepared and incubated samples. At present, not enough knowledge is available to interpret the results properly. As it can be assumed that knowledge concerning the measurement and interpretation of suspensions containing nanoparticles and their agglomerates will increase, the results obtained in this project can be potentially interpreted retrospectively. In Table 114 the measured results and applied parameters are presented.

Table 114: P25 – 1st test with daphnids: particle size distribution.

Concentration [mg/L]	Z-average [nm] [1]	PDI [2]	Peak 1 [nm] [6]	Peak 2 [nm] [6]	Count rate [3] [kcps]	Measurement position [4]	Attenuation [5]
Day 0							
Control	1421	0.821	506.3		50.5	4.65	11
20 mg/L (stock suspension)	1780	0.742	804.2	-	262.4	4.65	6
1 mg/L	1768	1.000	412.1	-	243.9	4.65	9
5 mg/L	1662	0.752	726.5	-	48.2	4.65	6
Day 2 - supernatant of the suspensions after incubation in the test vessels							
Control	1261	0.831	48.0 (86%)	74.87 (14%)	50.8	4.65	10
1 mg/L	659.3	0.580	553.4 (81%)	157.3 (19%)	66.1	4.65	8
5 mg/L	2661	1.000	652.9	-	246.1	4.65	9
Day 2 - freshly prepared suspensions							
Control	810.4	0.703	766.0 (72%)	205.7 (28%)	52.7	4.65	11
20 mg/L (stock suspension)	2320	0.652	1105	-	239.0	4.65	6
1 mg/L	641.0	0.568	426.4	-	72.4	4.65	8
5 mg/L	1255	0.550	797.6	-	148.3	4.65	7

[1] calculated value (cumulative mean); [2] increasing value indicates increasing polydispersity (maximum: 1); [3] best results with a count rate between 150 and 500 kilo counts per second (kcps); [4] measurement position in the middle of the measuring cell; [5] indicator for turbidity (high values indicate low turbidity; maximum: 11); [6] In the case of more than two peaks, value in brackets gives percentage of the single peak compared to all peaks (prerequisite: the peak increases 10%).

Test item concentrations

The concentrations of P25 are presented in Table 115 (percentage recovery) and Table 273 (chapter 21.8.1, measured concentrations). The stock suspensions had a recovery of about 90%. Dilution of the stock dispersions resulted in analytical concentrations which were about 64 - 86% of the nominal concentrations. Incubation for two days resulted in a decrease of the Ti concentration in the overlaying water.

Table 115: P25 – 1st test with daphnids: Ti recovery [%]

Concentration	d0 freshly prepared	d2 incubated for 2 days in test vessels	d7 freshly prepared	d9 incubated for 2 days in test vessels	d14 freshly prepared	d16 incubated for 2 days in test vessels
Test suspensions						
0.05 mg/L	68.1	17.7	64.0	3.1	65.3	17.4
0.1 mg/L	63.6	15.4	61.9	3.9	69.3	15.3
0.5 mg/L	64.9	14.6	66.9	2.8	59.9	14.0
1.0 mg/L	72.6	15.6	72.4	3.5	75.3	14.6
5.0 mg/L	84.4	6.9	76.6	4.4	86.0	18.0
Stock suspension						
20 mg/L	89.8		87.2		88.2	

Effects

Summarised results are presented in Table 116, Table 117 and Figure 26 - Figure 30.

No concentrations causing a modification of the mobility or reproduction of the adults were observed. No other clinical signs were detected in any replicate at any concentration tested.

The LOEC, $EC_{10,}$ $EC_{20,}$ and EC_{50} values of the biological endpoints (cumulative offspring per survivor, mobility) were > 5 mg/L. The NOEC was ≥ 5 mg/L.

For the body length the LOEC and NOEC were 0.5 mg/L and 0.1 mg/L.

Reproduction rate

The results of survival and reproduction are presented in Table 116 and Figure 26 - Figure 29.

Table 116: P25 – 1st test with daphnids: survival and reproduction data.

Number of *D. magna* per concentration: n = 10.

Concentration	Parental survival	Age at first brood	Cumulative offspring per female	Intrinsic rate of increase
[mg TiO_2./L]	[%]	Mean ± SD [days]	Mean ± SD [Ind.]	Mean ± SD [Ind./day]
Control	100	11.0 ± 1.65	90.4 ± 18.12	0.309 ± 0.032
0.05 (nominal)	100	10.9 ± 1.43	80.2 ± 16.22	0.290 ± 0.029
0.1 (nominal)	100	10.5 ± 1.56	86.9 ± 13.50	0.303 ± 0.030
0.5 (nominal)	100	12.1 ± 1.51	78.1 ± 14.88	0.296 ± 0.050
1.0 (nominal)	100	10.7 ± 1.32	84.0 ± 6.83	0.301 ± 0.025
5.0 (nominal)	100	11.1 ± 0.97	83.1 ± 12.58	0.288 ± 0.021

SD = standard deviation.

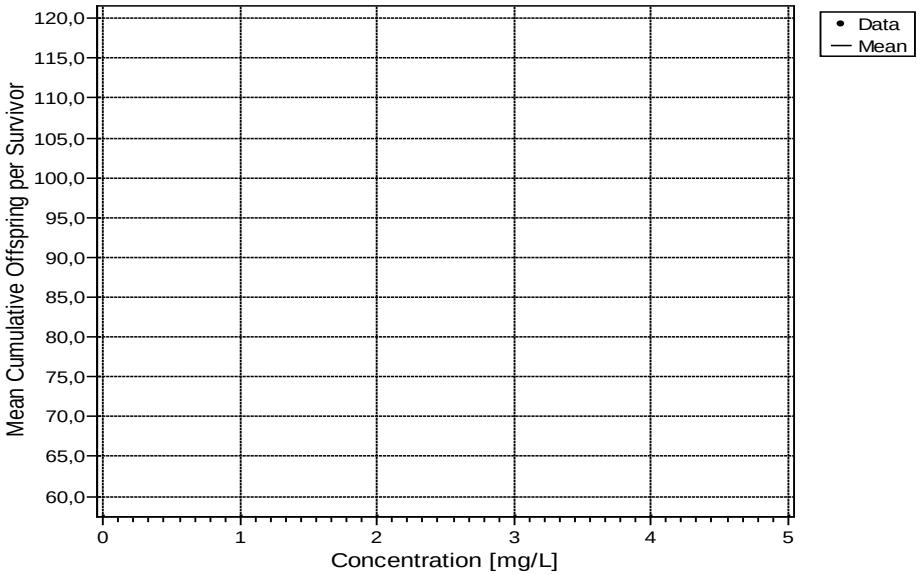


Figure 26: P25 – 1st test with daphnids: mean cumulative offspring per survivor of *Daphnia magna* after 21 d.

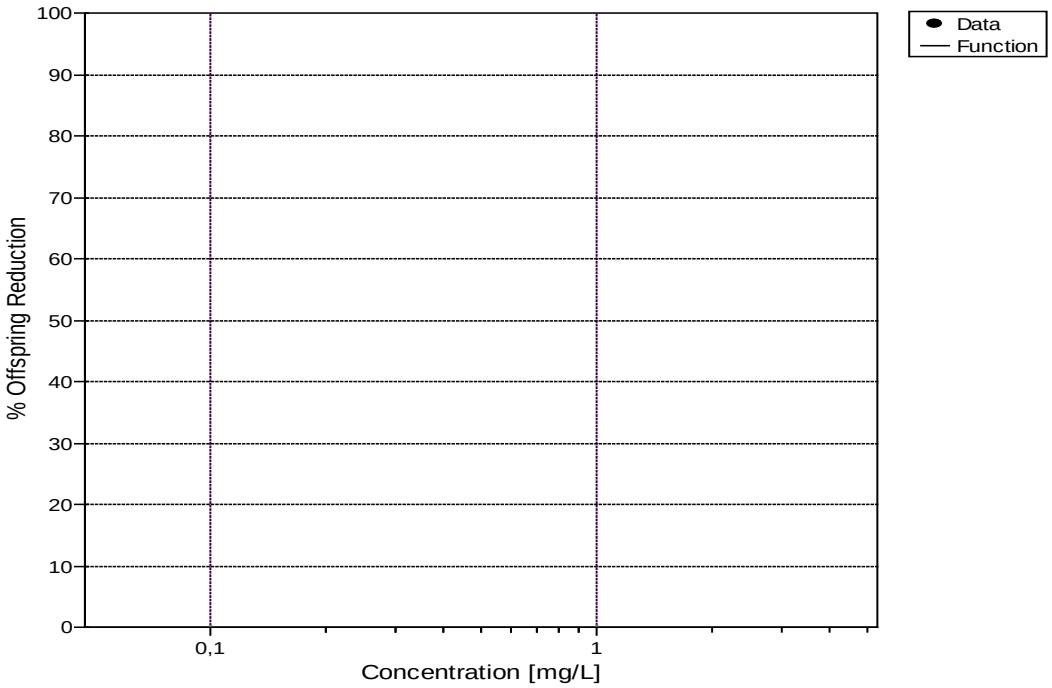


Figure 27: P25 – 1st test with daphnids: concentration-effect curve on mean cumulative offspring per survivor of *Daphnia magna* after 21 d.

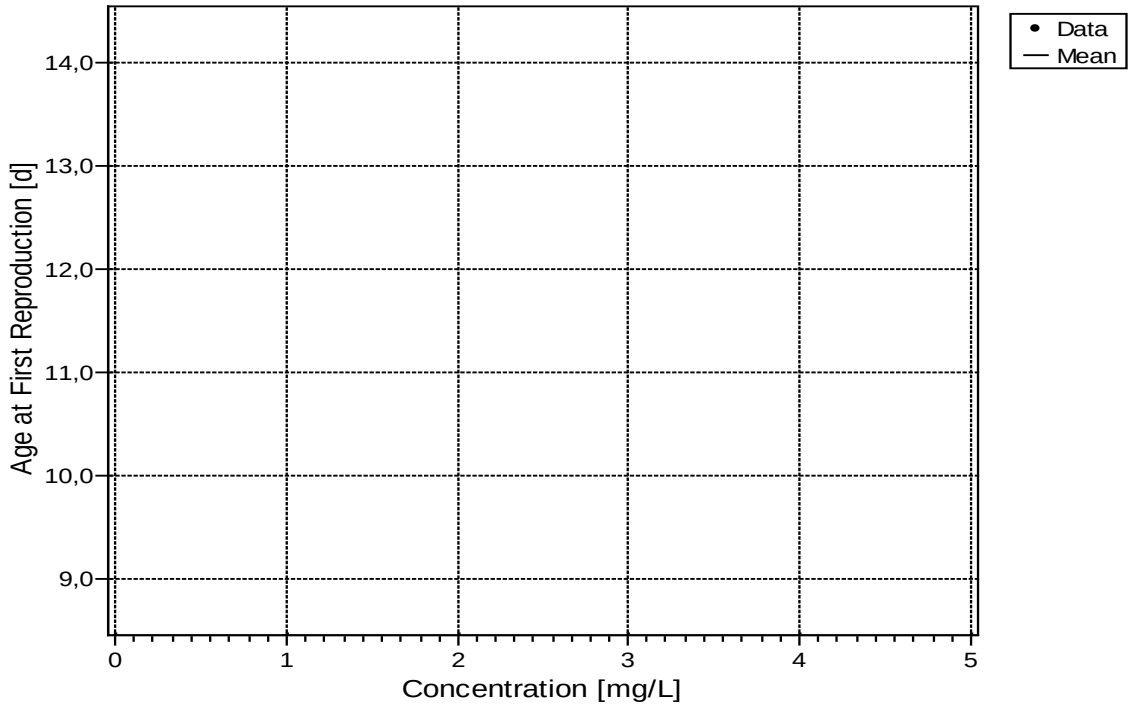


Figure 28: P25 – 1st test with daphnids: age at first reproduction of *Daphnia magna.*

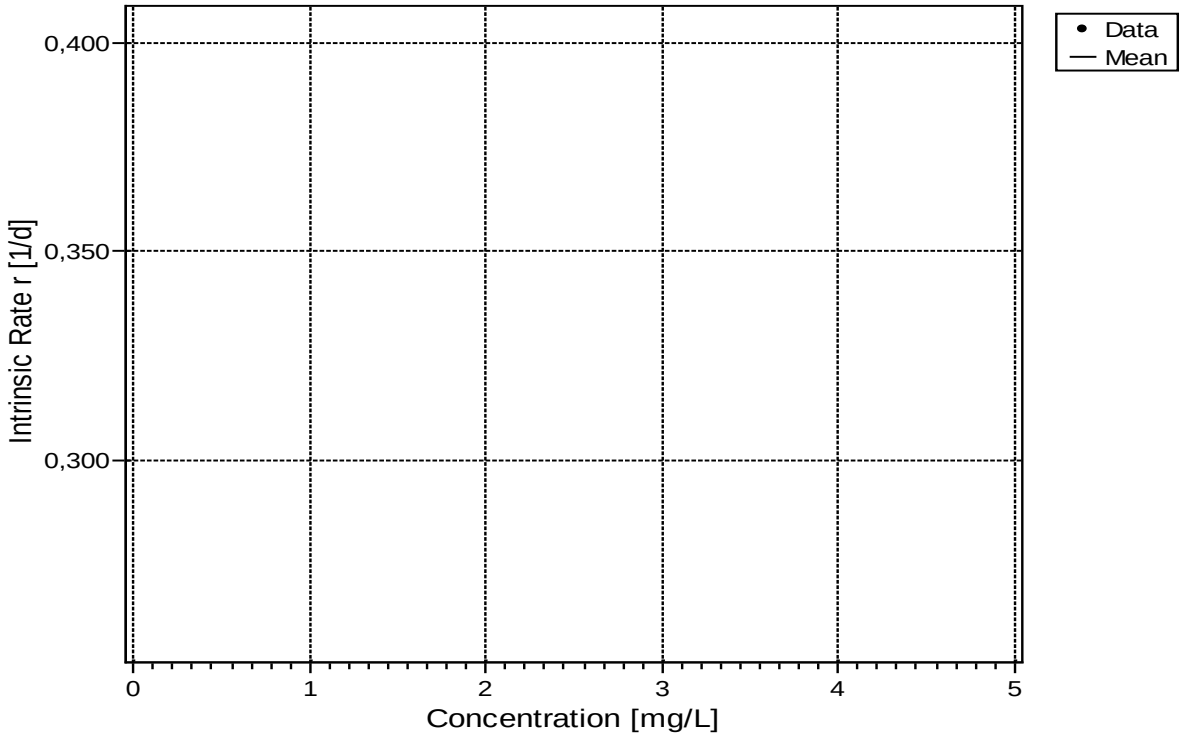


Figure 29: P25 – 1st test with daphnids: intrinsic rate of population increase r of *Daphnia magna* after 21 d.

No concentration/effect dependency on P25 on the reproduction rate was detected. The NOEC (no observed effect concentration) for the tested species *Daphnia magna* was found to be ≥ 5 mg/L for reproduction rate and survival.

Body length

The results of the body length are presented in Table 117 and Figure 30. The three highest test concentrations (0.5, 1.0, 5.0 mg/L) resulted in statistical significant differences to the control. However, no concentration effect relationship was observed. The length of the daphnids at 1.0 mg/L exceeded the length at 0.5 and 5.0 mg/L. The maximum effect was 10%. Therefore, it is concluded that P25 up to a concentration of 5 mg/L does not affect body length and the statistical difference is based on the very homogenous length and the high number of replicates.

Table 117: P25 – 1st test with daphnids: body length of the adult daphnids at day 21.

Replicate	Control	0.05 mg/L	0.1 mg/L	0.5 mg/L	1.0 mg/L	5.0 mg/L
1	4.79	4.65	4.09	4.69	4.43	4.76
2	4.66	4.96	4.87	4.48	5.07	4.39
3	4.89	4.49	4.72	4.46	4.67	4.41
4	4.84	4.52	4.83	4.62	4.56	4.03
5	4.87	4.99	5.04	4.77	4.22	4.53
6	4.90	4.59	4.83	4.82	4.41	4.26
7	5.10	4.60	4.79	4.34	4.63	4.34
8	4.96	4.72	5.04	4.44	5.10	4.39
9	5.44	5.00	5.01	4.59	4.96	4.63
10	4.66	4.67	5.29	4.95	5.05	4.99
Number	10	10	10	10	10	10
Mean	4.91	4.72	4.85	4.62 * [1]	4.71 * [1]	4.47 * [1]
Standard deviation	0.22	0.18	0.30	0.18	0.30	0.26

* [1] Significant different to the control; t-test procedure after Williams, α = 0.05, one-sided smaller

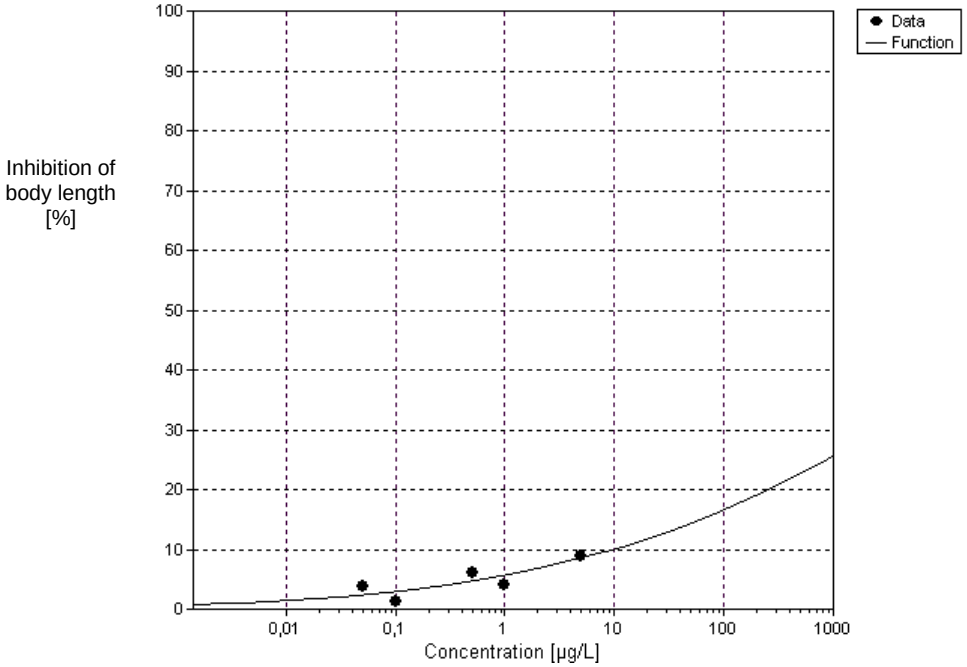


Figure 30: P25 – 1st test with daphnids: length response curve after 21 days.

15.6.2 P25 - Second test

(Raw data, chapter 21.8.2)

According to the literature (Zhu et al., 2010) and personal communication (University of Frankfurt) P25 caused toxicity in the reproduction test with daphnids. Effects were reported at concentrations of about 1 mg/L. Zhu et al. (2010) applied high concentrations of algae as food and fed daily. Therefore, two concentrations (1 mg/L and 5 mg/L) were tested with feeding and renewal of the medium daily and three times a week.

Zeta potential

The zeta potential in the test medium is presented in Table 118. A negative value of -18 mV was achieved.

Table 118: P25 – 2nd test with daphnids: zeta potential.

Sample	Zeta potential [mV]
P25 in tap water (application dispersion):	-18 mV

Particle size distribution

Particle size distribution was determined with the device Malvern Nano ZS in all samples which were used for the determination of the test concentrations (Table 119). It is already known that the available measuring devices are of limited suitability for polydispers samples. At present, not enough knowledge is available to interpret the results properly. As it can be assumed that knowledge concerning the measurement and interpretation of suspensions containing nanoparticles and their agglomerates will increase, the results obtained in this project potentially can be interpreted retroactively. In Table 83 measuring results and applied parameters are presented.

Table 119: P25 – 2nd test with daphnids: particle size distribution.

Concentration [mg/L]	Z-Average [nm] [1]	PDI [2]	Peak 1 [nm]	Peak 2 [nm]	Count Rate [3] [kcps]	Measurement Position [4]	Attenuation [5]
Day 0							
20 mg/L (stock suspension)	2066	0.768	895.5	-	73.3	4.65	5
5 mg/L (daily) [6]	2276	0.881	796.9	-	70.3	4.65	5
5 mg/L (3 x) [7]	1606	0.791	793.2	-	169.8	4.65	7
1 mg/L (daily)	1360	0.825	690.9	-	182.4	4.65	7
1 mg/L (3 x)	1021	0.853	428.5	-	283.5	4.65	9
Day 1 in samples with daily renewal of the medium							
5 mg/L	1272	0.752	524.9	-	188.1	4.65	8
1 mg/L	857.1	0.710	473.8	-	151.5	4.65	9

Continued

Table 83 (continued)

Day 2 in samples with renewal of the medium three times a week							
5 mg/L	1251	0.883	443.8	-	97.5	4.65	8
1 mg/L	1171	0.835	286.1	-	142.0	4.65	10
Day 7							
20 mg/L (stock suspension)	1621	0.462	1054	-	216.4	4.65	6
5 mg/L (daily) [6]	1982	0.684	995.9	-	233.0	4.65	6
5 mg/L (3 x) [7]	1036	0.769	498.1	-	53.9	4.65	6
1 mg/L (daily)	1017	0.610	641.5	-	144.8	4.65	7
1 mg/L (3 x)	1127	0.836	511.3 (84.2%) [8]	161.3 (15.8%) [8]	240.5	4.65	9
Day 8 in samples with daily renewal of the medium							
5 mg/L	939.4	0.657	502.4	-	99.5	4.65	7
1 mg/L	982.2	0.761	414.1	-	113.4	4.65	9
Day 9 in samples with renewal of the medium three times a week							
5 mg/L	650.7	0.611	442.8	-	229.4	4.65	8
1 mg/L	775.4	0.703	433.6	-	217.8	4.65	9
Day 14							
20 mg/L (stock suspension)	1547	0.495	1018	-	254.4	4.65	6
5 mg/L (daily) [6]	1694	0.559	961.2	-	188.1	4.65	6
5 mg/L (3 x) [7]	1107	0.709	608.7	-	147.6	4.65	7
1 mg/L (daily)	1438	0.795	523.2	-	200.6	4.65	7
1 mg/L (3 x)	947.2	0.885	358.3	-	184.9	4.65	9
Day 15 in samples with daily renewal of the medium							
5 mg/L	1268	0.721	551.9	-	104.9	4.65	7
1 mg/L	2482	0.711	343.5	-	68.3	4.65	7
Day 16 in samples with renewal of the medium three times a week							
5 mg/L	1116	0.680	528.9	-	143.3	4.65	7
1 mg/L	1768	0.934	553.4	-	223.5	4.65	8

[1] calculated value (cumulative mean); [2] increasing value indicates increasing polydispersity (maximum: 1); [3] best results with a count rate between 150 and 500 kilo counts per second (kcps); [4] measurement position in the middle of the measuring cell; [5] indicator for turbidity (high values indicate low turbidity; maximum: 11); [6] 1 mg/L samples below quantification limit; [7] prepared from 10 mg/L samples with 3 min of ultrasonic treatment; [8] prepared from 10 mg/L samples with 30 min of ultrasonic treatment; [6] samples with daily renewal of the medium; [7] samples with renewal of the medium three times a week; [8] In the case of more than two peaks, value in brackets gives percentage of the single peak compared to all peaks (prerequisite, the peak increases 10%)

Test item concentrations

The concentrations of P25 are presented in Table 120 (percentage recovery) and Table 273 (chapter 21.8.2, measured concentrations). The stock suspensions had a recovery between 85 and 101%. Dilution of the stock dispersions resulted in analytical concentrations between 82 - 103% of the nominal concentrations. The concentration in the supernatant decreased during incubation except the concentration in the vessel with TiO_2 nanoparticles of 1 mg/L. At day 16 a higher concentration compared to day 15 was detected. One sample was taken

which was analysed twice. The replicate determinations were identical. It is assumed that the sample was contaminated with sedimented nanoparticles.

Table 120: P25 – 2nd test with daphnids: Ti recovery [%].

Concentration	Test suspension 1 mg/L	Test suspension 5 mg/L	Stock suspension 20 mg/L
d0 freshly prepared	94.9	81.6	84.6
d1 incubated for 1 day in test vessels with daily medium renewal	30.4	22.6	
d2 incubated for 2 day in test vessels with medium renewal three times a week	10.8	8.6	
d7 freshly prepared	85.3	89.1	92.5
d8 incubated for 1 day in test vessels with daily medium renewal	42.4	37.6	
d9 incubated for 2 day in test vessels with medium renewal three times a week	38.4	27.6	
d14 freshly prepared	103.1	99.1	101.3
d15 incubated for 1 day in test vessels with daily medium renewal	49.3	42.4	
d16 incubated for 2 day in test vessels with medium renewal three times a week	(77.4) *	23.0	

* Sample presumably contaminated with sedimented nanoparticles

Effects

Summarised results are presented in Table 121 - Table 123 and Figure 31 - Figure 34.

No concentrations causing a modification of the mobility or reproduction of the adults were observed. No other clinical signs were detected in any replicate at any concentration tested.

The LOEC, $EC_{10,}$ $EC_{20,}$ and EC_{50} values of the biological endpoints (cumulative offspring per survivor, mobility, and body length) were > 5 mg/L. The NOEC was ≥ 5 mg/L.

Reproduction rate

The results obtained for survival and reproduction are presented in Table 121, Table 122 and Figure 31 - Figure 33.

From day 7 on in the control of the test design where the medium was replaced three times a week, a single daphnid containing algae in its brood pouch was observed throughout the test. This daphnid did not reproduce at all which might have been due to the algae. Therefore, this organism was not considered for the calculation of the reproduction.

A concentration/effect dependency on P25 for the reproduction rate was not detected. This was independent on the time interval for the renewal of the medium. The NOEC (no ob-

served effect concentration) for the tested species *Daphnia magna* was found to be ≥ 5 mg/L for reproduction rate and survival.

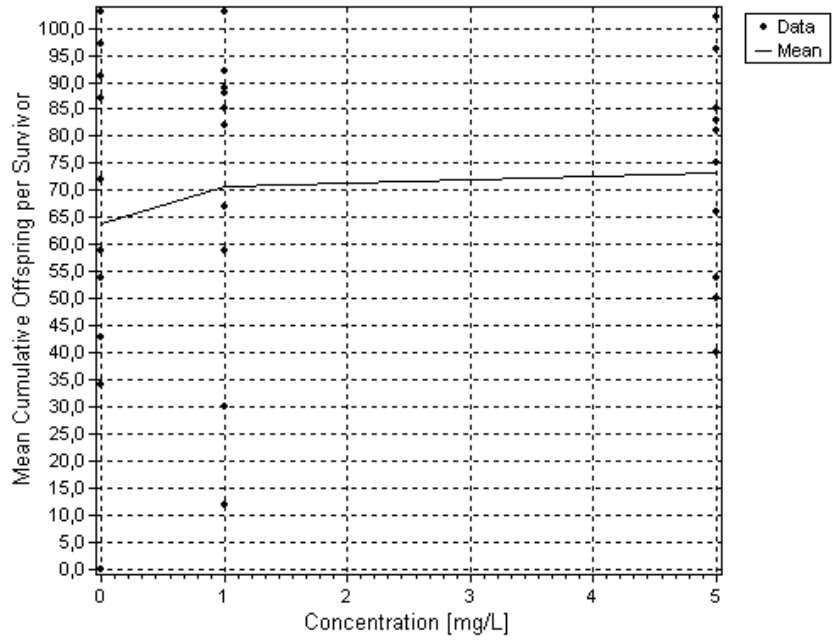
Table 121: P25 – 2nd test with daphnids: survival and reproduction data.

Number of *D. magna* per concentration: n = 10

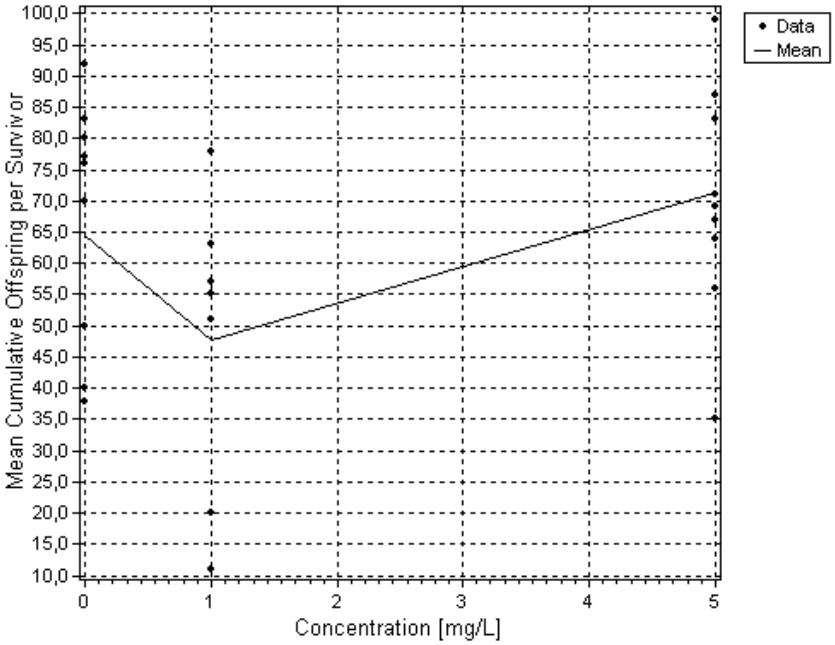
Concentration	Parental survival	Age at first brood	Cumulative offspring per female	Intrinsic rate of increase
[mg TiO_2./L]	[%]	mean ± SD [days]	mean ± SD[ind.]	mean ± SD[ind./day]
Medium renewal three times a week				
Control	100	11.9 ± 1.19	75.8 ± 22.02	0.267 ± 0.033
1.0 (nominal)	100	11.2 ± 1.16	70.7 ± 29.28	0.279 ± 0.032
5.0 (nominal)	100	11.8 ± 1.49	73.2 ± 20.30	0.271 ± 0.037
Daily medium renewal				
Control	100	10.7 ± 1.14	64.4 ± 20.74	0.287 ± 0.040
1.0 (nominal)	70	11.1 ± 1.51	47.9 ± 23.86	0.244 ± 0.059
5.0 (nominal)	100	10.3 ± 0.79	71.4 ± 18.00	0.297 ± 0.027

Table 122: P25 – 2nd test with daphnids: percent survival and reproduction.

Concentration	Parental survival	Cumulative offspring per female	Intrinsic rate of increase
[mg TiO_2./L]	[%]		
Medium renewal three times a week			
Control -	100	100	100
1.0 (nominal)	100	93	104
5.0 (nominal)	100	97	101
Daily medium renewal			
Control	100	100	100
1.0 (nominal)	70	74	85
5.0 (nominal)	100	111	103

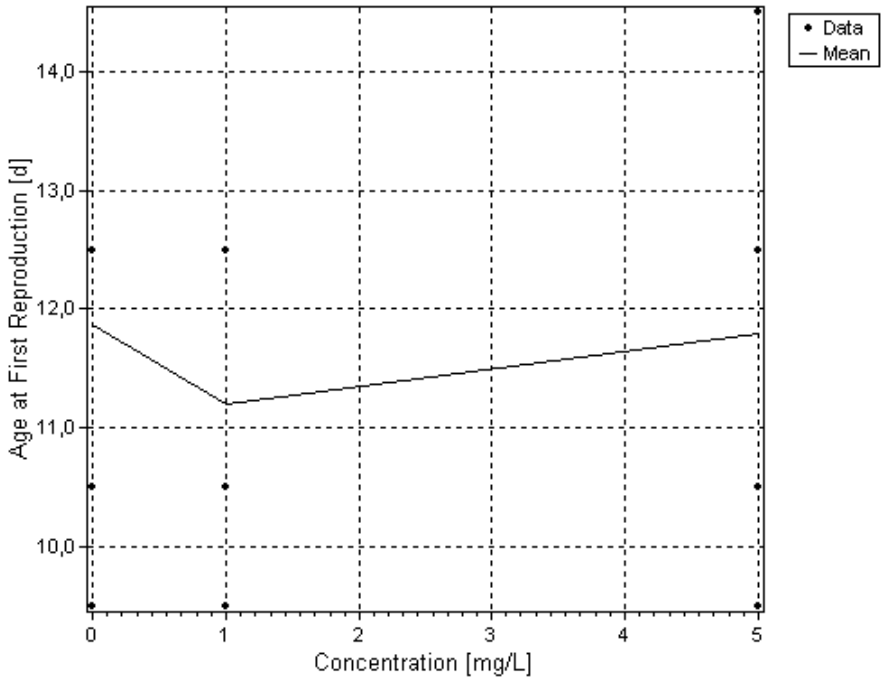


Medium renewal three times a week

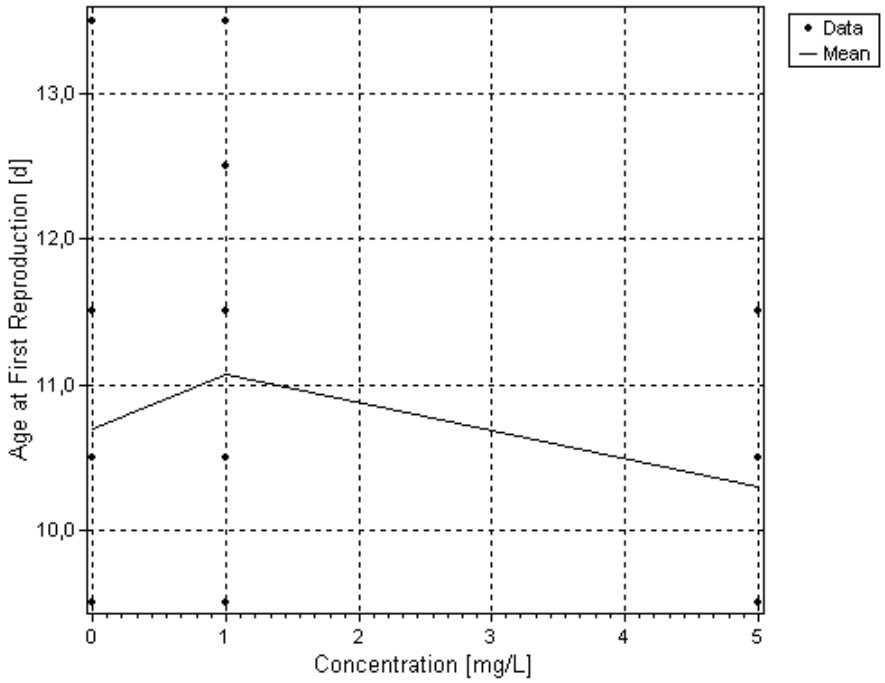


Daily medium renewal

Figure 31: P25 – 2nd test with daphnids: mean cumulative offspring per survivor of *Daphnia magna* after 21 d.

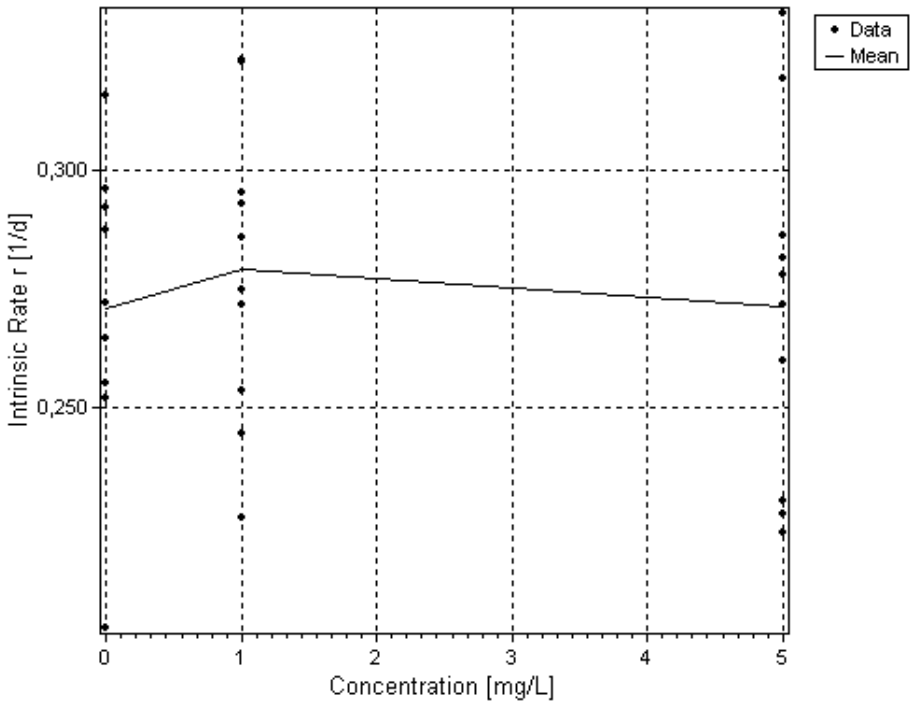


Medium renewal three times a week

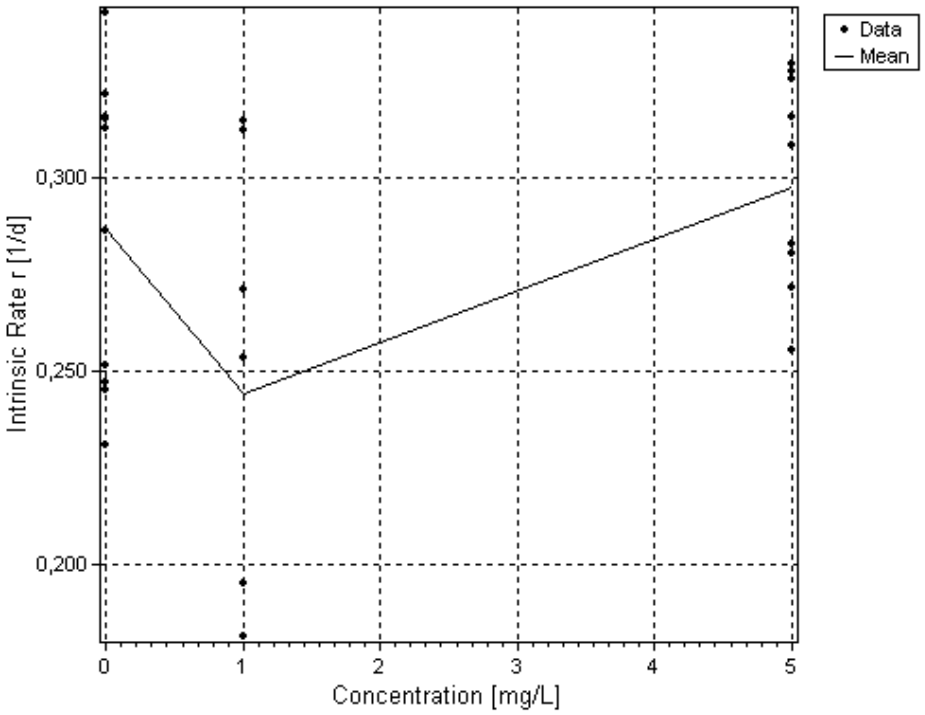


Daily medium renewal

Figure 32: P25 – 2nd test with daphnids: age at first reproduction of *Daphnia magna.*



Medium renewal three times a week



Daily medium renewal

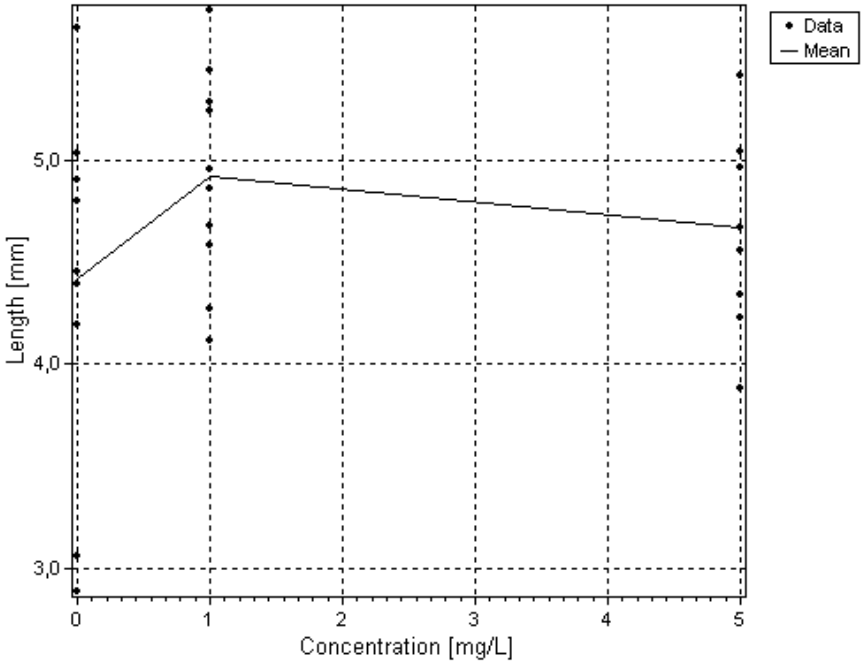
Figure 33: P25 – 2nd test with daphnids: intrinsic rate of population increase r of *Daphnia magna* after 21 days.

Body length

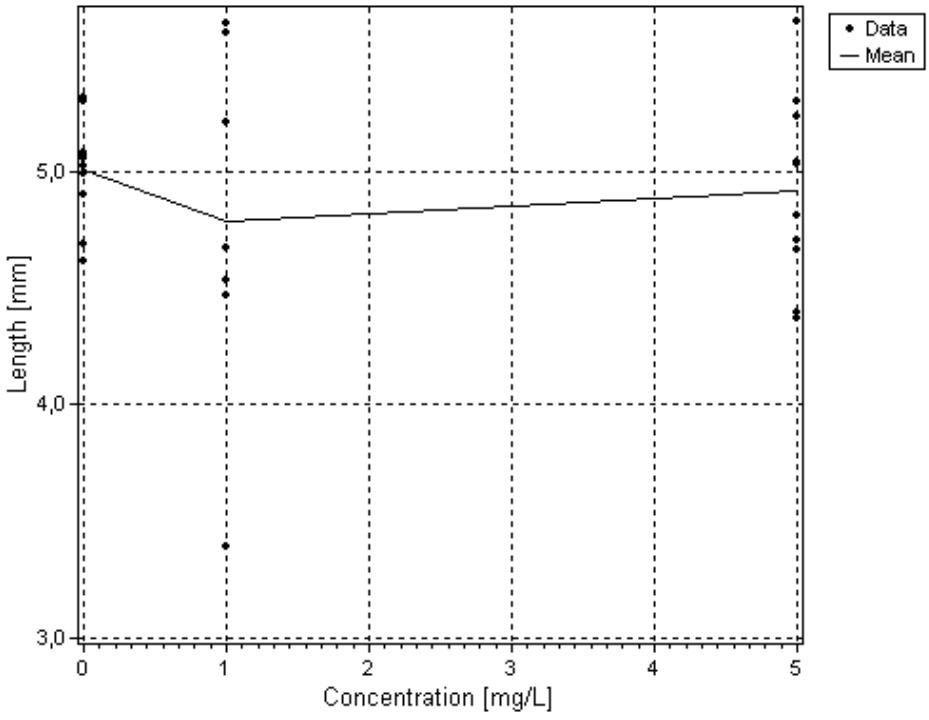
The results for the body length are presented in Table 123 and Figure 34. Neither the applied concentration nor the time interval of medium renewal affected the body length of the adult daphnids.

Table 123: P25 – 2nd test with daphnids: body length of the adult daphnids at day 21.

	Medium renewal three times a week			Daily medium renewal		
Replicate	Control	1 mg/L	5 mg/L	Control	1 mg/L	5.0 mg/L
1	4.39	5.73	4.67	4.99	5.21	5.30
2	3.06	4.12	4.96	4.69	---	5.23
3	4.80	5.44	5.04	5.30	3.39	4.66
4	4.45	5.24	4.67	5.31	4.47	4.70
5	5.64	4.68	5.41	5.02		4.37
6	4.90	4.86	4.56	5.08	5.59	5.03
7	4.80	4.27	3.88	5.05	4.53	5.64
8	5.03	4.58	4.23	5.06	5.63	4.81
9	4.19	4.95	4.34	4.90	---	4.39
10	2.89	5.28	4.96	4.61	4.67	5.04
Number	10	10	10	10	7	10
Mean	4.42	4.92	4.67	5.00	4.78	4.92
Standard deviation	0.86	0.52	0.45	0.23	0.78	0.41



Medium renewal three times a week



Daily medium renewal

Figure 34: P25 – 2nd test with daphnids: response curve of the body length after 21 days.

15.6.3 P25 – Third test

(Raw data, chapter 21.8.3)

In the experiments reported by Zhu et al. (2010) and those performed at the University of Frankfurt, periods of ultrasonication were applied that exceeded the period applied by Fraunhofer IME (IME: 3 min; Zhu: 10 min; University of Frankfurt: 60 min). To investigate whether a longer period of ultrasonic treatment would cause an effect, a further experiment with 3 min and 30 min ultrasonication was performed. Two concentrations (1mg/L and 5 mg/L) were tested. The medium was renewed three times a week.

Zeta potential

The Zeta potential in the test medium is presented in Table 124. A negative value of -18 mV was achieved.

Table 124: P25 – 3rd test with daphnids: zeta potential.

Sample	Zeta potential [mV]
P25 in tap water (application dispersion):	-18 mV

Particle size distribution

Particle size distribution was determined with the device Malvern Nano ZS in all samples which were used for the determination of the test concentrations. It is already known that the available measuring devices are of limited suitability for polydispers samples. At present, not enough knowledge is available to interpret the results properly. As it can be assumed that knowledge concerning the measurement and interpretation of suspensions containing nanoparticles and their agglomerates will increase, the results obtained in this project potentially can be interpreted retroactively. In Table 125 measuring results and applied parameters are presented.

The peak measured in the stock dispersion was larger than the peaks determined in the diluted test dispersions. The period of ultrasonication treatment of the stock suspensions showed no obvious tendency with respect to the size of peak 1 (e.g. it is not clear whether a 3 min ultrasonication treatment resulted in larger peaks than a 30 min ultrasonication treatment). For the freshly prepared test concentrations, 1 mg/L resulted in smaller particle sizes (location of the peak) compared to the concentrations of 5 mg/L. No tendency was obvious for the aged test suspensions of different concentrations. Furthermore, no tendency was obvious for freshly prepared and aged test suspensions with respect to the two ultrasonication periods.

Table 125: P25 – 3rd test with daphnids: particle size distribution.

Concentration [mg/L]	Z-Average [nm] [1]	PDI [2]	Peak 1 [nm]	Peak 2 [nm]	Count Rate [3] [kcps]	Measurement position [4]	Attenuation [5]
Day 0							
20 mg/L (stock suspension) – 3 min	1992	0.6	1058	-	189	4.65	6
20 mg/L (stock suspension) – 30 min	1671	0.4	1199	-	228	4.65	6
5 mg/L – 3 min	1479	0.9	711	-	159	4.65	7
1 mg/L - 3 min	823	0.7	390	-	207	4.65	9
5 mg/L – 30 min	858	0.6	617	-	205	4.65	7
1 mg/L - 30 min	370	0.5	280	-	277	4.65	9
Day 2							
5 mg/L – 3 min	3565	1	578	-	108	4.65	9
1 mg/L – 3 min	959	0.7	404	-	217	4.65	10
5 mg/L – 30 min	1373	0.9	706 (60%)	175 (40%)	254	4.65	11
1 mg/L - 30 min	1091	0.8	442	-	272	4.65	10
Day 7							
20 mg/L (stock suspension) – 3 min	2805	0.5	1312	-	219	4.65	6
20 mg/L (stock suspension) – 30 min	2750	0.5	1256	-	236	4.65	6
5 mg/L - 3 min	1586	0.8	630	-	180	4.65	7
1 mg/L – 3 min	907	0.7	453	-	204	4.65	9
5 mg/L – 30 min	1522	0.6	833	-	82	4.65	6
1 mg/L - 30 min	574	0.5	342	-	171	4.65	8
Day 9 in samples with renewal of the medium three times a week							
5 mg/L – 3 min	513	0.6	355	-	196	4.65	9
1 mg/L – 3 min	2127	1	346	-	140	4.65	8
5 mg/L – 30 min	477	0.6	357	-	146	4.65	8
1 mg/L - 30 min	563	0.6	403	-	179	4.65	9

Continued

Table 125 (continued)

Day 14							
20 mg/L (stock suspension) – 3 min	2309	0.7	1046	-	198	4.65	6
20 mg/L (stock suspension) – 30 min	2184	0.4	1303	-	224	4.65	6
5 mg/L - 3 min	1369	0.8	633	-	158	4.65	7
1 mg/L – 3 min	848	0.8	33	-	491	4.65	7
5 mg/L – 30 min	1298	0.5	905	-	221	4.65	7
1 mg/L - 30 min	414	0.5	341	-	304	4.65	9
Day 16 in samples with renewal of the medium three times a week							
5 mg/L – 3 min	594	0.6	324	75	148	4.65	8
1 mg/L – 3 min	496	0.6	472 (75%)	147 (25%)	186	4.65	9
5 mg/L – 30 min	450	0.4	421	-	240	4.65	8
1 mg/L - 30 min	949	0.8	322 (88%)	79 (12%)	120	4.65	8

[1] calculated value (cumulative mean); [2] increasing value indicates increasing polydispersity (maximum: 1); [3] best results with a count rate between 150 and 500 kilo counts per second (kcps); [4] measurement position in the middle of the measuring cell; [5] indicator for turbidity (high values indicate low turbidity; maximum: 11); [6] In the case of more than two peaks, value in brackets gives percentage of the single peak compared to all peaks (prerequisite, the peak exceeds 10%).

Test item concentrations:

The applied test concentrations were not validated. The test suspensions were prepared following the procedure applied for the first two tests. For these tests the recoveries were within an acceptable range.

Effects:

For mobility and body length the NOEC values were ≥ 5.0 mg/L, and the LOEC values were > 5.0 mg/L. For the mean cumulative offspring per female the NOEC was 1.0 mg/L and the LOEC 5.0 mg/L. No difference between ultrasonication periods of 3 min and 30 min was observed. No other clinical signs were detected in any replicate at any concentration tested. We recommend that the calculated differences for the cumulative offspring per female not be overestimated due to the fact that: (i) only two concentrations were tested and the reliability check via concentration-effect relationships was not possible; (ii) the results of 3 min and 30 min ultrasonication did not differ; and (iii) in contrast to the first and second test, only in the third test a LOEC was determined.

Detailed results are presented in Table 126 - Table 128 and Figure 35 - Figure 38.

Reproduction rate:

The results of survival and reproduction are presented in Table 126, Table 127 and Figure 35 - Figure 38.

At 5 mg/L statistically reduced reproduction activity was observed. The NOEC (no observed effect concentration) for the tested species *Daphnia magna* was found to be 1 mg/L for reproduction. For mobility no statistical difference was observed. For survival the NOEC was ≥ 5.0 mg/L. No statistical difference between the ultrasonication periods of 3 min and 30 min was detected. Neither the results for 1 mg/L with both sonication periods nor for 5 mg/L were different.

Table 126: P25 – 3rd test with daphnids: survival and reproduction data.

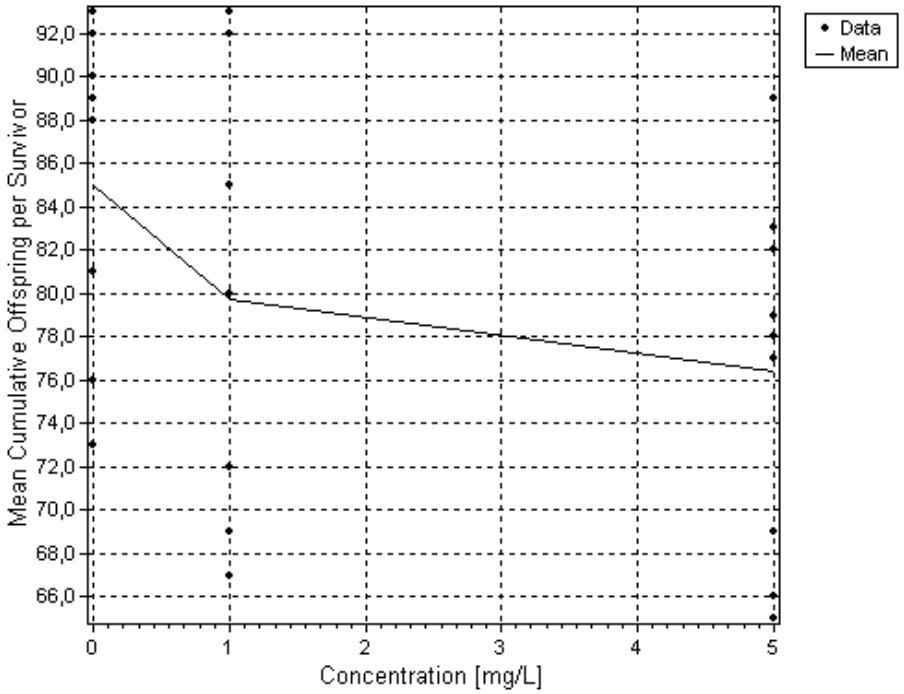
Number of *D. magna* per concentration: n = 10

Concentration	Parental survival	Age at first brood	Cumulative offspring per female	Intrinsic rate of increase
[mg TiO_2./L]	[%]	mean ± SD [days]	mean ± SD[ind.]	mean ± SD[ind./day]
Ultrasonication: 3 min				
Control	100	9.8 ± 0.95	85.0 ± 7.7	0.306 ± 0.021
1.0 (nominal)	80	9.6 ± 0.32	79.8 ± 9.9	0.304 ± 0.022
5.0 (nominal)	90	10.1 ± 1.35	76.4 ± 8.2 [1]	0.301 ± 0.033
Ultrasonication: 30 min				
Control	100	9.8 ± 0.95	85.0 ± 7.7	0.306 ± 0.021
1.0 (nominal)	100	9.8 ± 0.95	81.9 ± 8.9	0.314 ± 0.025
5.0 (nominal)	100	9.9 ± 0.97	71.2 ± 17.7 [1]	0.297 ± 0.021

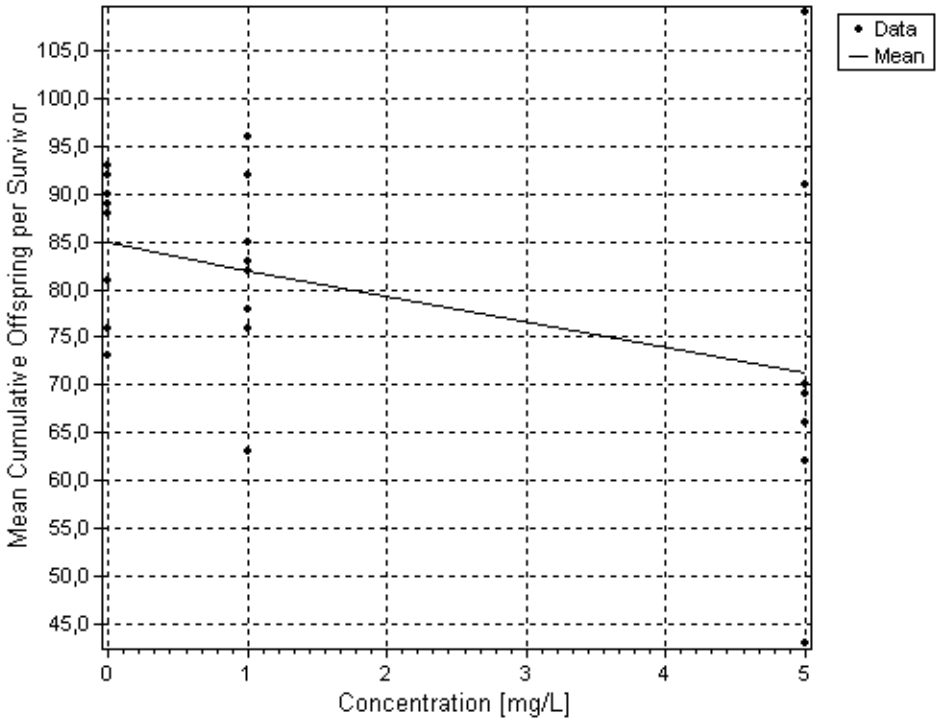
[1] statistical significance p> 0.05

Table 127: P25 – 3rd test with daphnids: percentage survival and reproduction.

Concentration	Parental survival	Cumulative offspring per female	Intrinsic rate of increase
[mg TiO_2./L]	[%]	[%]	[%]
Ultrasonication: 3 min			
Control -	100	100	100
1.0 (nominal)	80	94	99
5.0 (nominal)	90	90	98
Ultrasonication: 30 min			
Control	100	100	100
1.0 (nominal)	100	96	103
5.0 (nominal)	100	84	97

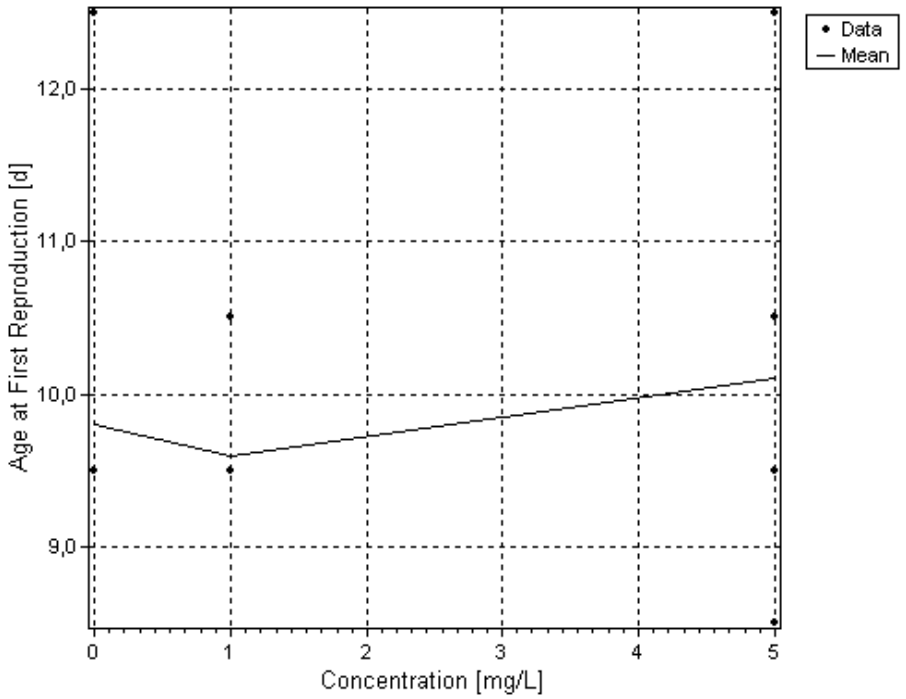


Ultrasonication period: 3 min

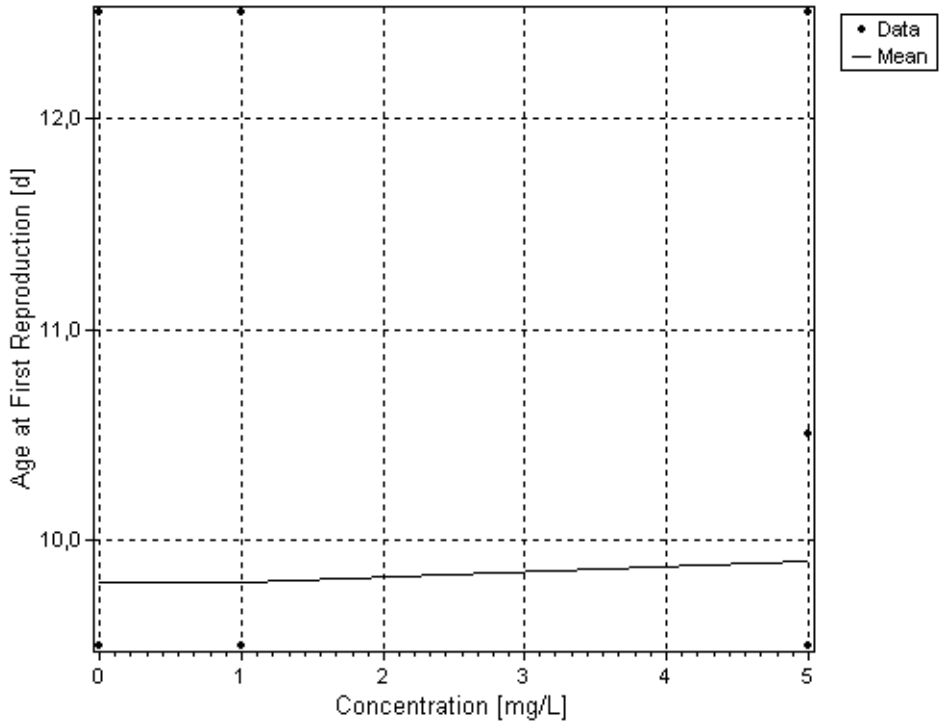


Ultrasonication period: 30 min

Figure 35: P25 – 3rd test with daphnids: mean cumulative offspring per survivor of *Daphnia magna* in presence of P25 after 21 d.

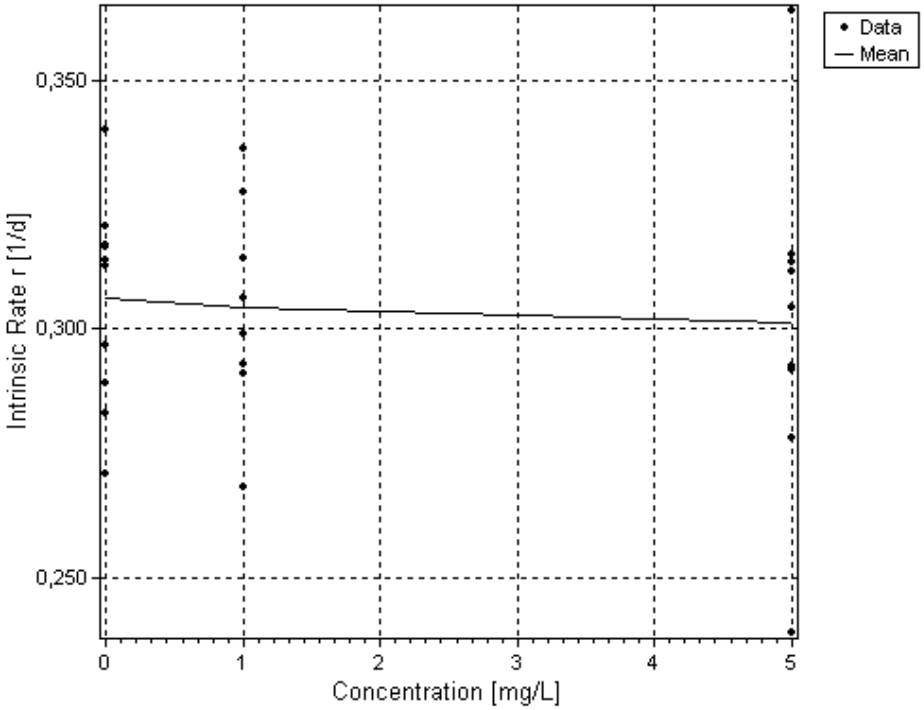


Ultrasonication period: 3 min

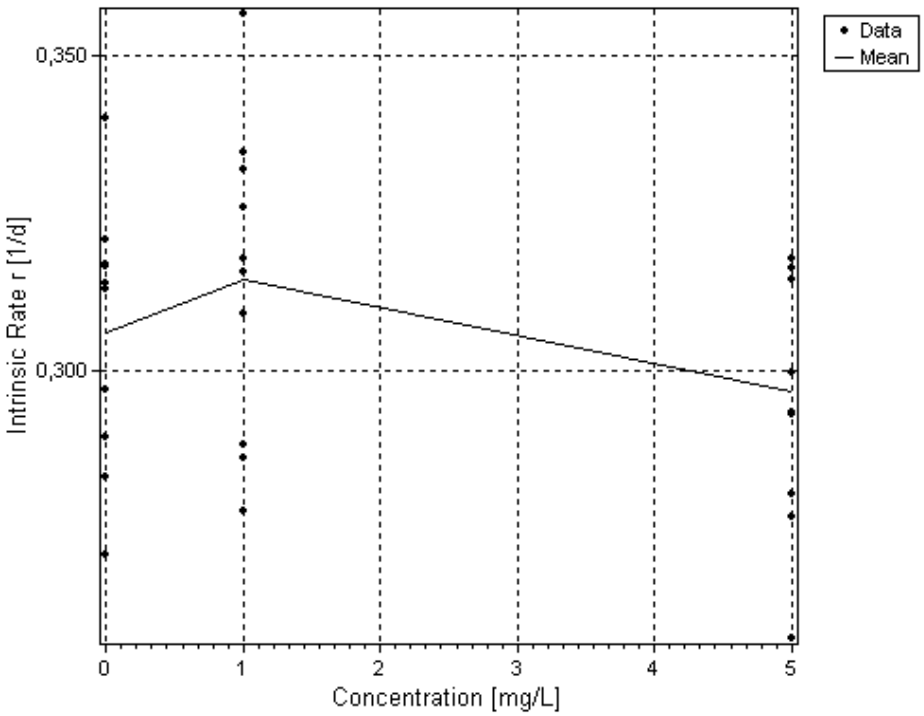


Ultrasonication period: 30 min

Figure 36: P25 – 3rd test with daphnids: age at first reproduction of *Daphnia magna* in presence of P25.



Ultrasonication period: 3 min



Ultrasonication period: 30 min

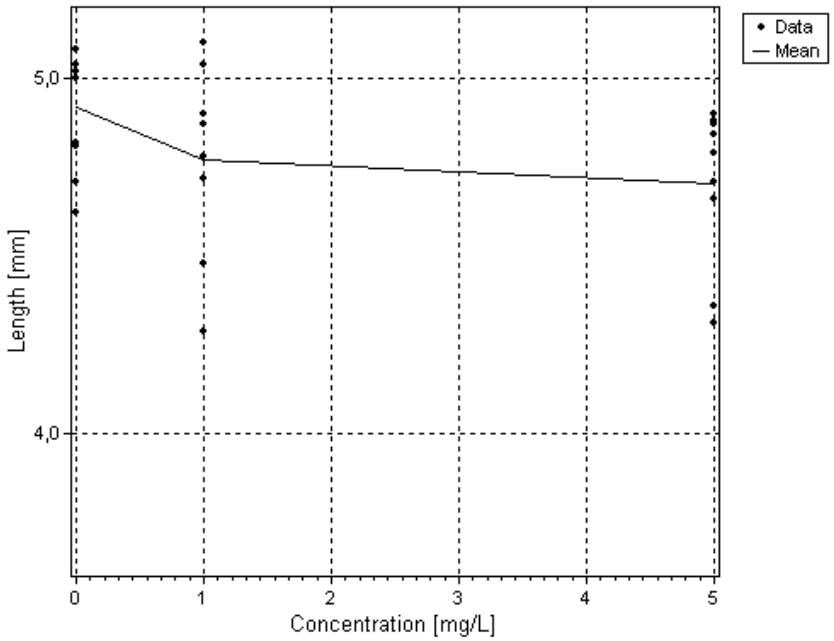
Figure 37: P25 – 3rd test with daphnids: intrinsic rate of population increase r of *Daphnia magna* after 21 days.

Body length

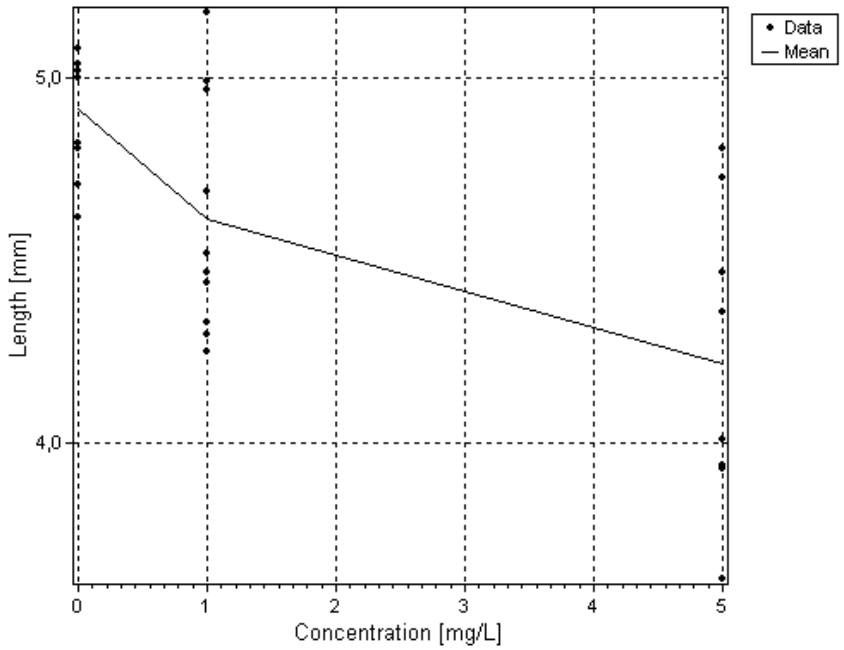
The results of the body length are presented in Table 128 and Figure 38. Neither the applied concentration nor the ultrasonication period resulted in a statistically significant difference in the body length of the adult daphnids compared to the control.

Table 128: P25 – 3rd test with daphnids: body length of the adult daphnids at day 21.

	Ultrasonication period: 3 min			Ultrasonication period: 30 min		
Replicate	Control	1 mg/L	5 mg/L	Control	1 mg/L	5.0 mg/L
1	5.00	4.90	4.71	5.00	4.25	3.63
2	5.08	---	4.84	5.08	4.47	4.01
3	5.04	5.10	4.88	5.04	4.99	4.47
4	4.82	4.87	4.31	4.82	4.52	4.36
5	4.71	5.04	4.66	4.71	4.69	4.81
6	4.62	---	4.90	4.62	4.44	4.36
7	5.08	4.78	---	5.08	5.18	4.73
8	4.81	4.72	4.87	4.81	4.97	3.94
9	5.02	4.29	4.79	5.02	4.30	3.94
10	5.00	4.48	4.36	5.00	4.33	3.93
Number	10	8	9	10	10	10
Mean	4.92	4.77	4.70	4.92	4.61	4.22
Standard deviation	0.16	0.27	0.22	0.16	0.33	0.39



Ultrasonication period: 3 min



Ultrasonication period: 30 min

Figure 38: P25 – 3rd test with daphnids: response curve of the body length after 21 days.

15.7 Validity

P25 - three tests:

The tests are considered valid since:

- survival in the control (100%) was above 80% (all tests: 100%)
- within the 21 days the mean number of offspring in the control was above the criterion of 60/female (first test: 90.4; second test (medium renewal three times a week): 75.8; second test (daily medium renewal): 64.4; third test: 85.0).

15.8 Conclusion

Three experiments were performed. The results concerning the NOEC differ slightly. A summary is presented in Table 129. The effect of P25 on the reproduction activity, mobility, and body length seems to be small up to the highest test concentration of 5 mg/L. The differences between the tests reflect the biological variability.

Table 129: P25 – 3rd test with daphnids: summary of the NOEC values.

Mean cumulative offspring per female, mobility and body length in the three tests

	1st test: ultrasonication period 3 min; medium renewal 3 times per week	2nd Test: medium renewal daily or 3 times per week	3rd test: ultrasonication period. 3 min and 30 min
Mean cumulative offspring per female			
NOEC [mg/L]	≥ 5.0	≥ 5	5.0
Mobility			
NOEC [mg/L]	≥ 5.0	≥ 5	≥ 5.0
Body length			
NOEC [mg/L]	0.1 mg/L	≥ 5	≥ 5.0

15.9 Executive summary

TiO_2 nanoparticles were tested in the reproduction test with daphnids (OECD 211). Three semi-static tests were carried out. In the first test the medium was renewed on days 2, 5, 7, 9, 12, 14, 16 and 19. The nominal concentrations of TiO_2 nanoparticles in the test containers were 0.05, 0.1, 0.5, 1.0 and 5.0 mg test item/L. The concentrations of the test item were measured in the freshly prepared test suspensions on days 0, 7 and 14. After two days of incubation the concentrations of the test item were measured in the incubation flasks (days 2, 9, 16). Sedimentation of TiO_2 nanoparticles resulted in a reduction of the Ti concentrations in the overlaying water after incubation.

In the second test two concentrations (1 mg/L, 5 mg/L) were investigated. Two periods for the renewal of the test medium were studied: three times per week and a daily.

In the third test two concentrations (1 mg/L, 5 mg/L) were investigated. Two ultrasonication periods (3 min and 30 min) were studied. The test medium was renewed three times per week.

The results concerning the NOEC differ slightly. A summary is presented in Table 130. The effect of P25 on reproduction activity, mobility and body length seems to be negligible up to the highest test concentration of 5 mg/L. The differences between the tests reflect the biological variability.

Table 130: P25 – Compilation of the tests with daphnids: summary of the NOEC values.

Mean cumulative offspring per female, mobility and body length in the three tests

	1st test: ultrasonication period 3 min; medium renewal 3 times per week	2nd test: medium renewal daily or 3 times per week	3rd test: ultrasonication period 3 min and 30 min
Mean cumulative offspring per female			
NOEC [mg/L]	≥ 5.0	≥ 5.0	5.0
Mobility			
NOEC [mg/L]	≥ 5.0	≥ 5.0	≥ 5.0
Body length			
NOEC [mg/L]	0.1 mg/L	≥ 5.0	≥ 5.0

16 Acute Immobilisation Tests with Daphnids (OECD TG 202) - Au

16.1 Test principle

Young female *Daphnia* (parent animals) aged less than 24 h at test start were exposed to the test item for 2 days under static conditions. The test item had been added to the water at a defined range of concentrations. Immobilisation was recorded after 24 and 48 h. Immobilisation in the treatments and in the control were analysed for statistically significant differences using appropriate statistical methods.

16.2 Materials and methods

16.2.1 Test guideline

The test was performed according to OECD 202 (13.04.2004): OECD guideline for testing of chemicals – *Daphnia sp.* Acute Immobilisation Test.

16.2.2 GLP

The test was performed following the principles of GLP. In deviation to GLP no archiving of the raw data was performed and the Quality Assurance Unit was not involved with respect to the inspection of the test, of the raw data and of the report. Any laboratory equipment (e.g. balances, thermometers, pH-meters) was controlled and documented according to GLP.

16.3 Test substances

- NM-330: gold nanoparticles in dispersant
- NM-330DIS: dispersant of the gold nanoparticles

16.4 Analytical monitoring

16.4.1 Details on sampling

Samples were taken from the pure substance (NM-330), from test concentrations at test start and in the test vessels after the incubation period.

16.4.2 Details on analytical methods

Characterization of the application dispersion and test dispersion

The zeta potential and particle size distribution was measured using a Malvern Zeta-Sizer NanoZS.

Chemical analysis

The applied chemical methods are described in chapter 6.7. Only the two highest test concentrations in the main test were analysed.

16.4.3 Details on test suspensions

Purified tap water was used as test water and to prepare the test suspension.

The pristine gold dispersion and the dispersing agent were used as stock dispersions. The test concentrations were achieved by dilution.

Pre-test

50.0%: 50 mL/L gold dispersion / dispersing agent per 100 mL with purified tap water

10%: 10 mL/L gold dispersion / dispersing agent per 100 mL with purified tap water

1.0%: 1 mL/L gold dispersion / dispersing agent per 100 mL with purified tap water

Main test

10%: 25 mL/L gold dispersion / dispersing agent per 250 mL with purified tap water

5.0%: 12.5 mL/L gold dispersion / dispersing agent per 250 mL with purified tap water

2.5%: 6.25 mL/L dispersing agent per 250 mL with purified tap water

1.25%: 3.125 mL/L dispersing agent per 250 mL with purified tap water

0.625%: 1.563 mL/L dispersing agent per 250 mL with purified tap water

For every test concentration a small amount of water was poured in a volumetric flask, the required gold dispersion and the dispersion agent resp. were added and the volume adjusted to 250 mL with purified tap water. The mixture was shaken several times.

16.5 Test organism

The test organisms were young specimens of *Daphnia magna*, 4 – 24 h old at test start.

Origin of the daphnids:	German Federal Environment Agency, Institut für Wasser-, Boden- und Lufthygiene. Specimens used in the test were bred in the laboratory of the Fraunhofer IME.
Breeding conditions:	Adult *Daphnia*, at least 3 weeks old, were separated from the stock population by sieving. Batches of 30 to 50 animals were held at room temperature in approx. 1.8 L dilution water for one week. During this week the daphnids were fed daily with an algal suspension (*Desmodesmus subspicatus*) and LiquizellR (HOBBY). Algae growing in the log-phase were centrifuged and the pellet was re-suspended in a few mL of medium. 30 mL of this suspension were given to 1 L Daphnia medium. The water

	was changed once per week. Newborn Daphnia were separated by sieving, the first generation was discarded.
Holding- and dilution-water:	Purified drinking water was used as holding- and dilution water. The purification included filtration with activated charcoal, passage through a lime-stone column and aeration. To avoid copper contamination, plastic water pipes were used. The following water chemistry data were regularly recorded in the testing facility, and were: pH, conductivity, dissolved oxygen content, content of nitrate, nitrite, ammonium ($NH4^+$), phosphate, calcium, magnesium, total hardness, alkalinity, DOC content, content of metals (copper, iron, manganese and zinc).
Food:	The daphnids were not fed during the test.

16.6 Study design

16.6.1 Study type

Short-term test, static.

16.6.2 Water medium type

Fresh water.

16.6.3 Total exposure duration

48 h

- Pre-test: November 29, 2011 - December 01, 2011
- Main test: December 06, 2011 – December 08, 2011

No post-exposure observation period was performed.

16.6.4 Test conditions

Pre-test

Total hardness:	1.2 mmol/L
Test temperature:	20.7 - 21.3 °C (permitted range: 20 ± 2 °C)
pH in control:	8.1 – 8.3 (permitted range: pH 6 – 9; variation less than 1.5)
Dissolved oxygen in control:	8.3 – 9.0 mg/L corresponding to 97 – 100% (demanded threshold value: 3 mg/L)
Salinity:	277 µS/cm

Nominal concentrations: The nominal concentrations in the test containers with gold dispersion / dispersant were 1.0, 10.0, 50.0 % (v/v).

Details on test conditions:

- Test vessel: glass beakers (60 mL) filled with 50 mL test suspension; covered with glass panes
- Aeration: no
- No. of organisms per vessel: 5
- No. of vessels per concentration (replicates): 2
- No. of vessels per control (replicates): 2

TEST MEDIUM / WATER PARAMETERS

The quality of the applied water is described in Table 131.

Table 131: Chemical parameter of the holding- and dilution-water in the pre-test

Conductivity [µS/cm]	Alcalinity [mmol/l]	Total hardness [mmol/l]	Ca hardness [mmol/l]	Mg hardness [mmol/l]	NPOC [a] [mg/L]	Cl [mg/L]
277	1.8	1.2	0.8	0.4	0.7332	0.0.
NO_3 [mg/L]	NO_2 [mg/L]	NH_4 [mg/L]	PO_4 [mg/L]	Cd [µg/L]	Cr [µg/L]	Cu [µg/L]
2.2	<0.005	<0.01	1.3	<3.05	<3.04	<5.07
Fe [µg/L]	Mn [µg/L]	Ni [µg/L]	Pb [µg/L]	Zn [µg/L]		
<22.1	<2.86	<2.37	<6.59	6.69		

[a] NPOC = non purgeable organic carbon

OTHER TEST CONDITIONS

- Culture medium different from test medium: no
- Intervals of water quality measurement: once per month
- Adjustment of pH: no
- Photoperiod: light/dark cycle 16/8 h
- Light intensity: 553 - 560 lux

Main test

Total hardness: 1.2 mmol/L

Test temperature: 20.5 - 21.5 °C (permitted range: 20 ± 2°C)

pH in control: 8.3 – 8.4 (permitted range: pH 6 – 9; variation less than 1.5)

Dissolved oxygen in control: 8.2 – 8.7 mg/L corresponding to 96 - 99%. (demanded threshold value: 3 mg/L)

Salinity: 277 µS/cm

Nominal concentrations: The nominal concentrations in the test containers with gold dispersion / dispersant were 0.625 – 10% (v/v)

Details on test conditions:

- Test vessel: glass beakers (60 mL) filled with 50 mL test suspension; covered with glass panes
- Aeration: no
- No. of organisms per vessel: 5
- No. of vessels per concentration (replicates): 4
- No. of vessels per control (replicates): 4

TEST MEDIUM / WATER PARAMETERS

The quality of the applied water is described in Table 132.

Table 132: Chemical parameter of the holding- and dilution-water in the main test

Conductivity [µS/cm]	Alcalinity [mmol/l]	Total hard-ness [mmol/l]	Ca hardness [mmol/l]	Mg hardness [mmol/l]	NPOC [a] [mg/L]	Cl [mg/L]
277	1.8	1.2	0.8	0.4	0.7332	0.0.
NO_3 [mg/L]	NO_2 [mg/L]	NH_4 [mg/L]	PO_4 [mg/L]	Cd [µg/L]	Cr [µg/L]	Cu [µg/L]
2.2	<0.005	<0.01	1.3	<3.05	<3.04	<5.07
Fe [µg/L]	Mn [µg/L]	Ni [µg/L]	Pb [µg/L]	Zn [µg/L]		
<22.1	<2.86	<2.37	<6.59	6.69		

[a] NPOC = non purgeable organic carbon

OTHER TEST CONDITIONS

- Culture medium different from test medium: no
- Intervals of water quality measurement: once per month
- Adjustment of pH: no
- Photoperiod: light/dark cycle 16/8 h
- Light intensity: 553 - 560 lux

VEHICLE CONTROL PERFORMED: No

Reference substance: A reference substance ($K_2Cr_2O_7$) is tested twice a year.
January 2011: EC_{50} – 24 h: 0.78 mg/L (0.68 – 0.89)
June 2011: EC_{50} – 24 h: 0.85 mg/L (0.74 – 0.96)
Results of an interlaboratory test (ISO 6341):
EC_{50} – 24 h: 0.6 - 2.1 mg/L

16.6.5 Other information on materials and methods

Control treatment

The control consisted of purified drinking water and daphnids.

Test performance

Less than 24 h old *Daphnia magna* were exposed to defined concentrations of the test item under static conditions for a period of 48 days. The daphnids were exposed without aeration. The daphnids were subjected to a light/dark cycle of 16/8 h. The test temperature during the test was 18 – 22 C. The temperature did not vary by more than 2°C within these limits. The light intensity did not exceed 15 -20 µE / (m2 * s) or 1125 - 1500 lux.

Statistical method

Data evaluation:

In this report numerical values are frequently rounded to a smaller degree of precision (number of digits) than have been used in the actual calculation. Minor differences in results obtained from calculations with rounded values compared to those obtained with higher precision values are possible. They are, however, well within the limits of the experimental accuracy and of no practical concern.

The parental immobility was used to calculate effects.

Statistical calculations:

The results of the immobilisation were compared by a suitable test for multiple comparisons with a control after testing variance homogeneity. All statistical tests were performed with the computer software ToxRat Professional version 2.10.4.1 (ToxRat® Solutions GmbH).

16.7 Results

Chemical analyses were performed only for the main tests. In range-finder-tests the determination of the test concentration is not obligatory according to the guideline. 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. Details on the analytical method used by the producer of NM-330 are unknown. Therefore, the discrepancy of 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.

16.7.1 Pre-test

Effects:

Summarised results are presented in Table 116.

A high toxicity was detected for the dispersant. In the control and in the test vessels with gold dispersion no immobilisation was observed. Based on these findings for the main test, following concentrations were selected:

NM-330 (gold nanoparticles): 10%, 5% (v/v)

NM-330DIS (dispersant): 10%, 5%, 2.5%, 1.25%, 0 625% (v/v)

Table 133: Immobilisation effects at 24 and 48 h.

Concentration	Immobilisation 24 h [%]	Immobilisation 48 h [%]
Control	0	5
NM-330 (gold nanoparticles)		
1% (nominal)	0	0
10% (nominal)	0	0
50% (nominal)	0	0
NM-330DIS (dispersant)		
1% (nominal)	100	10
10% (nominal)	100	100
50% (nominal)	100	100

Water parameters

Summarised results of the oxygen concentrations and pH-values are presented in Table 134.

The dispersant resulted in a decrease of the oxygen concentration during the test which was less pronounced in the presence of gold nanoparticles. The pH was lower in the vessels with dispersant. In the presence of gold nanoparticles the decrease was less pronounced.

Table 134: Oxygen concentration and pH values during the test.

Concentration	O_2 [mg/L]		O_2 [%]		pH	
	start	end	start	end	start	end
Control	9.0	8.3	100	97	8.1	8.3
NM-330 (gold nanoparticles)						
1%	9.1	8.4	103	99	8.0	8.3
10%	8.8	8.0	100	93	7.9	8.2
50%	8.8	5.5	99	63	6.9	7.8
NM-330DIS (dispersant)						
1%	8.8	4.8	102	54	6.7	8.0
10%	8.5	4.4	98	51	6.0	6.4
50%	7.9	5.0	90	58	5.8	5.8

16.7.2 Main test

Based on the findings in the pre-test, the following concentrations were selected for the main test:

NM-330 (gold nanoparticles): 10%, 5% (v/v)

NM-330DIS (dispersant): 10%, 5%, 2.5%, 1.25%, 0,625% (v/v)

Zeta potential

The zeta potential of NM-330 (10%) in purified tap water is presented in Table 118. A negative value of -24 mV was achieved.

Table 135: Zeta potential.

Sample	Zeta potential [mV]
10% NM-330 in purified tap water	-24

Particle size distribution

The particle size distribution of NM-330 in the test suspensions was determined at day 0 and day 48. However, the concentrations of NM-330 were too low to give acceptable values. Therefore, no results are presented.

Test item concentrations

The concentrations of Au are presented in Table 136. Only the two highest test concentrations were analysed. 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). As already mentioned above, 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. Details on the analytical method used by the producer of NM-330 are unknown. Therefore, the discrepancy of 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.

Using the measured concentration as 100% it is obvious that at day 0 the concentrations in the test suspensions were in the range of the expected values (expected 5% - measured 6%; expected 10% - measured 11%). During the incubation period of two days sedimentation occurred resulting in concentrations of gold of about 1% for both test concentrations.

Due to the uncertainty the concentrations of the ecotoxicological analyses are presented as % NM-330 in the test suspension.

Table 136: Concentration of Au in the test vessels with NM-330.

Concentrations given as percentage of NM-330 in the test medium.

Sample	Au concentration [µ/L]	Concentration with respect to NM-330 (pure substance) [%]
NM-330 (pure substance)	43840	100
Day 0		
Control	4.23 (< detection limit)	---
NM-330 5%	2680	6.1
NM-330 10%	4985	11.4
Day 2		
Control	6.70 (< detection limit)	---
NM-330 5%	427	0.97
NM-330 10%	538	1.2

Effects:

In Table 137 the effects for NM-330 and NM-330DIS after an incubation time of 48 h are summarised.

Table 137: Summarised effects for NM-330 and NM-330DIS.

Concentrations given as percentage of NM-330 and NM-330DIS in the test medium.

	NM-330 (gold nanoparticles)	NM-330DIS (dispersant)
EC50	---	3.24% (v/v)
LOEC	> 10% (v/v)	5.0% (v/v)
NOEC	≥ 10% (v/v)	2.5% (v/v)

Mobility

Concentration dependent toxicity (Table 138; Figure 39) was detected for the dispersant. In the control and in the test vessels with gold dispersion no immobilisation after an incubation period of 24 h and 5% immobilisation after an incubation period of 48 h were detected. For the dispersant immobilisation in a concentration of 1.25 % was higher than at 2.5 %. Based on the results the following effect values were calculated by the statistical programme ToxRat.

Based on these findings of both tests, following effect values were calculated:

NM-330 (gold nanoparticles): LOEC > 50% (v/v): NOEC ≥ 50% (v/v)

NM-330DIS (dispersant): EC50 (48 h) 3.24% (v/); LOEC 5.0% (v/v): NOEC 2.5% (v/v)

Table 138: Immobilisation of *Daphnia magna* in the presence of NM-330 and NM-330DIS.

Concentrations given as percentage of NM-330 and NM-330DIS in the test medium.

Concentration	Immobilisation 24 h [%]	Immobilisation 48 h [%]
Control	0	5
NM-330 (gold nanoparticles)		
10%	0	5
50%	0	5
NM-330DIS (dispersant)		
0.625%	0	0
1.25%	0	30
2.5%	0	15
5%	35	65
10%	80	100

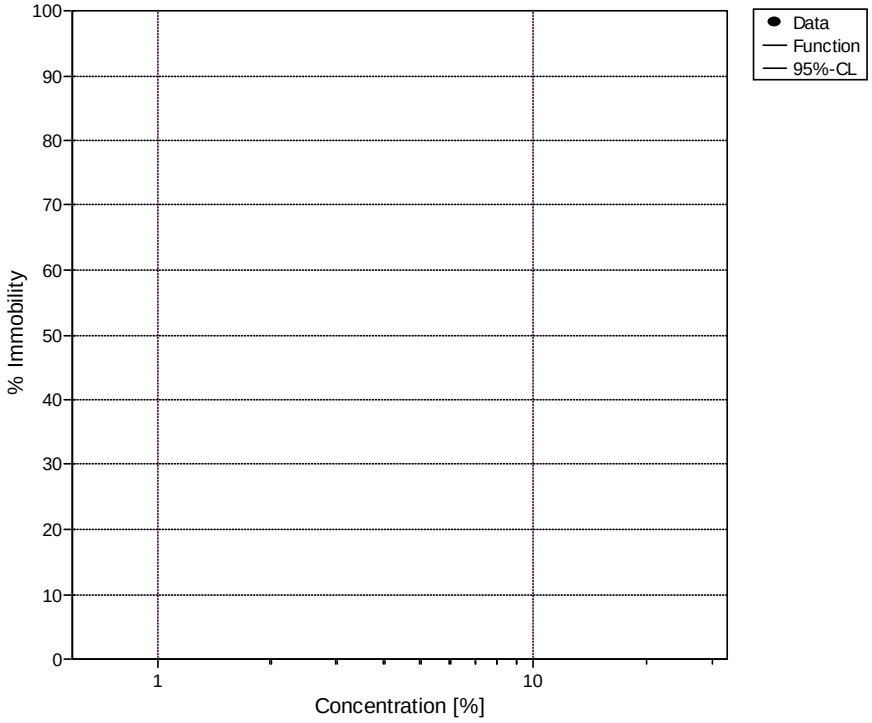


Figure 39: NM-330DIS – effect on mobility of *Daphnia magna.*

Incubation time: 24 h.

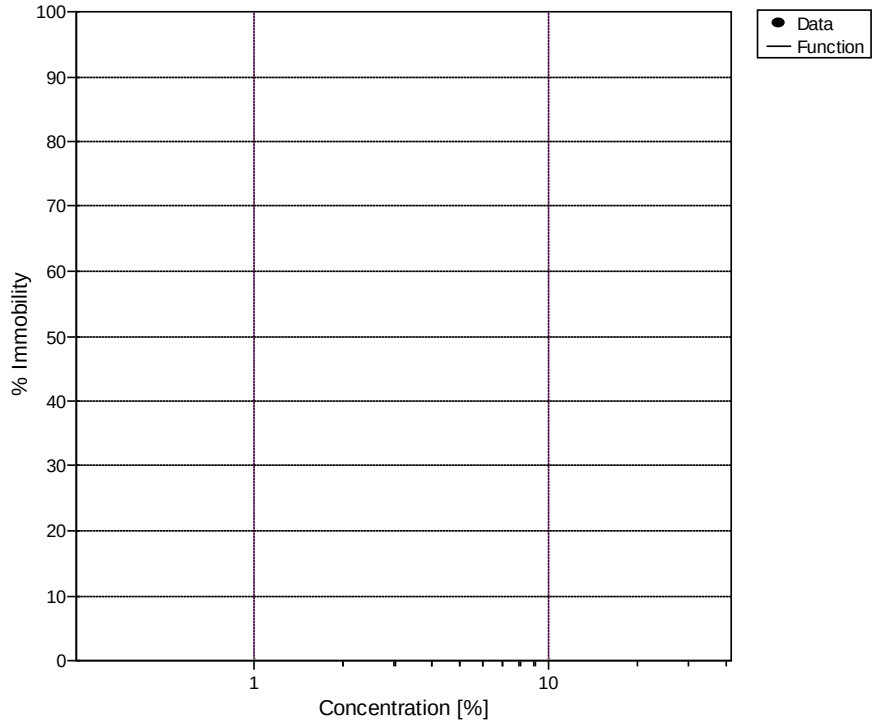


Figure 40: NM-330DIS –effect on mobility of *Daphnia magna*.

Incubation time: 48 h.

Water parameter

Summarised results of the oxygen concentrations and pH-values are presented in in Table 139.

Oxygen concentration

The dispersant resulted in a decrease of the oxygen concentration during the test which was much more pronounced than in the control and in the presence of gold nanoparticles. In the control and the vessels with gold nanoparticles, the validity criterion (O_2-concentration ≥ 3 mg/L at test end) was fulfilled. In all vessels with the dispersant the validity criterion was not fulfilled.

An influence of the low oxygen concentration on the mobility of the daphnids was not suspected. The oxygen concentration in the lowest and highest test concentration was comparable, although 0 and 100% effect was achieved.

pH value

At the test start there was a concentration dependent decrease of the pH value in the test vessels with dispersant. The value was within the accepted range of 6 – 9.

During the incubation period of 48 h the pH increased. Nevertheless, at test end the pH was below the values of the control samples.

In the presence of gold nanoparticles the pH was slightly lower than in the control samples at test start. At test end no difference compared to the control was observed.

Table 139: Oxygen concentration and pH values during the test.

Concentration	O_2 [mg/L]		O_2 [%]		pH	
	start	end	start	end	start	end
Control	8.7	8.2	99	96	8.3	8.4
NM-330 (gold nanoparticles)						
10% (nominal)	9.0	8.2	103	94	8.1	8.3
50% (nominal)	9.0	8.1	102	93	8.0	8.3
NM-330DIS (dispersant)						
0.625% (nominal)	9.0	1.3	102	14	7.2	7.9
1.25% (nominal)	9.0	1.2	101	13	6.8	7.8
2.5% (nominal)	9.1	4.9	104	55	6.5	8.0
5% (nominal)	9.1	0.9	102	11	6.3	7.0
10% (nominal)	9.1	1.9	103	14	6.1	6.4

16.8 Validity

The tests are considered valid since:

- survival in the control was above 90% (pre-test: 100%; main test: 95%)
- the dissolved oxygen concentration in the control and in the vessels with gold nanoparticles (NM-300) was ≥ 3 mg/L.
  In the vessels with dispersant the oxygen concentration was below the threshold concentration. This seems to be an effect of the chemical substance.

16.9 Conclusion

One preliminary (range finder) test and one main test were performed. The dispersant resulted in a significant toxicity. NM-330 (gold nanoparticles) compensated for the toxicity of the dispersant. No toxicity was observed in the presence of NM-330. In the presence of the dispersant alone, the oxygen concentration fell below the threshold value of 3 mg/L. However, it is assumed that the low oxygen concentration did not affect toxicity. The oxygen concentrations in the lowest and highest test concentration were comparable, although 0 and 100% immobilisation was achieved.

In Table 140 the effects for NM-330 and NM-330DIS are summarised.

Table 140: Summarised effects for NM-330 and NM-330DIS.

Concentrations given as percentage of NM-330 and NM-330DIS in the test medium.

	NM-330 (gold nanoparticles)	NM-330DIS (dispersant)
EC50	---	3.2% (v/v)
LOEC	> 50% (v/v)	5.0% (v/v)
NOEC	≥ 50% (v/v)	2.5% (v/v)

16.10 Executive summary

NM-330 (gold nanoparticles in dispersant) and **NM-330DIS** (dispersant of the gold nanoparticles) were tested in the acute test with *Daphnia magna* (OECD 202). Two static tests with different test concentrations were performed. The mobility of the daphnids was recorded after 24 h and 48 h.

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.

Due to the low concentration of the Au nanoparticles in NM-330 the particle size distribution could not be determined. The zeta potential determined for the highest test concentration (10%) in purified tap water (= test water) was -24 mV.

During the incubation period of two days sedimentation occurred, resulting in concentrations of gold in the overlaying water of 1% for both concentrations analysed (5 and 10%).

Concentration dependent toxicity was detected for the dispersant. In the control and in the test vessels containing the gold dispersion, no immobilisation was detected after an incubation period of 24 h; 5% immobilisation occurred after an incubation period of 48 h.

The dispersant caused a reduction of the pH-value and of the oxygen concentration. The pH was still within 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 did not affect toxicity, as the oxygen concentrations at the lowest and highest test concentrations were the same despite immobilisation effects of 0% in the lowest test concentration and of 100% in the highest test concentration.

Based on the findings of both tests, the following effect values were calculated:

NM-330 (gold nanoparticles): LOEC > 50% (v/v): NOEC ≥ 50% (v/v)

NM-330DIS (dispersant): EC_{50} (48 h) 3.24% (v/v); LOEC 5.0% (v/v): NOEC 2.5% (v/v).

17 Growth Inhibition Tests with Algae (OECD TG 201) - Au

17.1 Test principle

The purpose of the test was to determine the effects of a substance on the growth of fresh water microalgae and/or cyanobacteria. Exponentially growing test organisms were exposed to the test substance in batch cultures over a period of 72 h. In spite of the relatively brief test duration, effects over several generations were able to be assessed.

17.2 Materials and methods

17.2.1 Test guideline

The test was performed according to OECD 201 (23.03.2006): OECD guideline for testing of chemicals – Fresh water Alga and Cyanobacteria, Growth inhibition Test.

17.2.2 GLP

The test was performed following the principles of GLP. In deviation to GLP no archiving of the raw data was performed and the Quality Assurance Unit was not involved with respect to the inspection of the test, of the raw data and of the report. Any laboratory equipment (e.g. balances, thermometers, pH-meters) was controlled and documented according to GLP.

17.3 Test substances

- NM-330: gold nanoparticles in dispersant
- NM-330DIS: dispersant of the gold nanoparticles

17.4 Analytical monitoring

17.4.1 Details on sampling

The concentration of gold was determined in the pristine NM-330. The test concentrations were prepared in the multi-well-plates. As the volume was too small (i.e. below the level of quantification) no further chemical analyses were performed.

17.4.2 Details on analytical methods

<u>Characterisation of the application dispersion and test dispersion</u>

The particle size distribution was measured using a Malvern Zeta-Sizer Nano ZS.

17.4.3 Details on test suspensions

The mineral medium described in the OECD test guideline was used.

The pristine gold dispersion and the dispersing agent were used as stock dispersion. The test was performed in multi-well-plates and the different test concentrations were achieved by dilution in the respective plates.

96-well plates:

Blank:	180 µL ultrapure water
	20 µL 2 fold OECD algae medium
Control:	160 µL ultrapure water
	20 µL 2 fold OECD algae medium
	20 µL algae suspension (10^5 cells/mL)
Dilution:	160 µL test item
	20 µL 2 fold OECD algae medium
	20 µL algae suspension (10^5 cells/mL)

24-well plates:

Blank:	1800 µL ultrapure water
	200 µL 2 fold OECD algae medium
Control:	1600 µL ultrapure water
	200 µL 2 fold OECD algae medium
	200 µL algae suspension (10^5 cells/mL)
Dilution:	1600 µL test item
	200 µL 2 fold OECD algae medium
	200 µL algae suspension (10^5 cells/mL)

A dual dilution series was prepared. For the test requiring a volume of 200 µL, a 96-well plate was used. All vessels of the dilution wells were filled with 160 µL ultrapure water. For the highest test concentration the respective wells were filled with 320 µL prestine gold dispersion or dispersing agent and 160 µL were transferred to the wells for the next test concentration and mixed. This procedure is repeated for the further dilution concentrations. From the wells for the last test concentration 160 µL were depleted.

For test 1 the dilution of NM-330 was additionally performed using NM-330DIS instead of ultrapure water to achieve the same concentration of the dispersant in every test concentration.

For the test requiring a volume of 2000 µL, a 24-well plate was used for the dilution series. A comparable procedure was applied as described above.

17.5 Test organism

The green alga *Pseudokirchneriella subcapitata* (obtained from the Culture Collection of Algae at the University of Göttingen, Germany; SAG database no. 61.81) was used as the test organism.

17.6 Study design

17.6.1 Study type

72 h, static.

17.6.2 Water medium type

Fresh water.

17.6.3 Total exposure duration

72 h.

- Test 1: performed in 96-well-plates (test volume 200 µL) November 21, 2011 - November 24, 2011
- Test 2: performed in 96-well-plates (test volume 200 µL) November 29, 2011 - December 01, 2011
- Test 3: performed in 24-well-plates (test volume 2000 µL) December 19, 2011 - December 22, 2011

No post-exposure observation period was performed.

17.6.4 Test conditions

Test temperature / illumination:

All experiments were incubated at 22 ± 1°C with light intensity adjusted to ~7000 lux (95 µE m-2 s-1) provided by OSRAM L 36W/21-840 Plus Eco lamps. The light intensity was measured using an LI-189 luminance meter with radiation sensor (LI-COR, Lincoln, USA) with a cosine (2π) receptor in lux units.

- Test 1: Test temperature: 22.0°C (permitted range: 21 – 24°C, controlled by ± 2°C)
  Illumination: 7568 lux
- Test 2: Test temperature: 22.0°C (permitted range: 21 – 24°C, controlled by ± 2°C)
  Illumination: 7721 lux
- Test 3: Test temperature: 22.0°C (permitted range: 21 – 24°C, controlled by ± 2°C)
  Illumination: 7822 lux

Test concentrations:

Following test concentrations for NM-302 and NM-302DIS were tested:

- Test 1 + 2: 80, 40, 20, 10, 50, 25, 12.5, and 6.25%
- Test 3: 80 and 40%

Details on test conditions:

- Test vessel: black multi-well plates (96-well, 24-well)
- No. of replicates per concentration (replicates): 3
- No. of replicates per control (replicates): 6

VEHICLE CONTROL PERFORMED: No

Reference substance: A reference substance (3-5-dichlorophenol) is tested periodically at the Fraunhofer Institute IME.
March 2011: growth rate - EC_{50} – 72 h: 2.91 mg/L (2.35 – 3.63).

17.6.5 Other information on materials and methods

Test performance

Three days prior to testing, a pre-culture of the test alga *Pseudokirchneriella subcapitata* was established in sterile OECD growth medium, according to test guideline no. 201, to obtain exponentially growing algae. All stock solutions for the OECD medium were prepared with purified water processed using an ELGA “PURELAB Ultra”. Cell concentrations were calculated using an electronic particle counter (CASY 1 Model TT, Schärfe System, Reutlingen, Germany). The cultures were kept in suspension by rotary shaking at 100 rpm on a Multitron Incubation Shaker (INFORS, Switzerland).

In the test, algal biomass was determined after 0, 24, 48 and 72 h by recording the fluorescence intensity using a Tecan Spectrafluorplus microtiter plate reader. The fluorescence signal was converted into cell numbers using a calibration curve.

Statistical method

Data evaluation:

In this report numerical values are frequently rounded to a smaller degree of precision (number of digits) than have been used in the actual calculation. Minor differences in results obtained from calculations with rounded values compared to those obtained with higher precision values are possible. They are, however, well within the limits of the experimental accuracy and of no practical concern.

The cell number was used to calculate effects.

Statistical calculations:

Calculations were performed with the computer software ToxRat Professional version 2.10.4.1 (ToxRat® Solutions GmbH).

17.7 Results

17.7.1 Particle size distribution

The particle size distribution was only determined in the pristine NM-330 as the dilutions were performed in the micro-wells resulting in a volume that is too little for measuring. The results are presented in Table 141. The zeta-potential of the pristine NM-330 gives no information on the conditions in the test. Therefore, no zeta potential was determined.

Table 141: Particle size distribution of the NM-330 (gold nanoparticles in dispersant) (mean value of 10 measurements; SD = standard deviation)

Concentration given as percentage of NM-330 in the test medium

Concentration [mg/L]	Z-Average [nm] [1] (±SD)	PDI [2] (±SD)	Peak 1 [nm] (±SD)	Peak 2 [nm] (±SD)	Peak 1 [%]	Peak 2 [%]	Attenuation [3]	Remark
100% Au	49.1 (±22.2)	0.2 (±0.03)	45.7 (±3.4)	8.2 (±1.0)	77	19	6	

17.7.2 Test concentrations

The dilutions for the tests were performed in the multi-well plates. Due to the low volumes an insufficient amount of solution was available for further analyses. The concentrations of the ecotoxicological analyses are presented as % NM-330 (v/v) in the test suspension.

17.7.3 Test 1

In test 1 the toxicity of NM-330 and of NM-330DIS (dispersant of gold nanoparticles) was determined. NM-330 was investigated twice: the test concentrations were achieved by dilution with ultrapure water and with NM-330DIS. Dilution in NM330DIS was performed in order to obtain comparable concentrations of the dispersant in all test concentrations.The test concentrations of NM-330DIS were achieved by dilution with ultrapure water.

Effects:

In the presence of the two highest concentrations of NM-330 (gold nanoparticles in dispersant) and ultrapure water as diluent, the fluorescence at day 0 fell below the background value. Subtraction of the background values resulted in negative values. After 24 h fluorescence values above the background values were determined. In the highest test concentration the fluorescence did not change until the end of the test (Figure 41). It was assumed that

the low fluorescence values at test start were not an indicator for toxicity but were due to silencing of the signals by the test item. After sedimentation or agglomeration of the nanoparticles and no further silencing, toxicity becomes obvious and can be evaluated. This assumption is supported by the results for NM-330 diluted in the test with dispersant. In that experiment the concentration of the dispersant was comparable for all test concentrations. In all test concentrations high toxicity was observed for the incubation period between 24 and 72 h. Fluorescence below the background value was observed for the two highest test concentrations (Figure 42). In the test showing the toxicity of the dispersant only, a typical concentration-effect dependency is achieved (Figure 43). In this test no gold nanoparticles were included and no silencing below the background values was observed.
Therefore, for NM-330 (gold nanoparticles in dispersant) only the incubation period between 24 and 72 h was used for the evaluation.

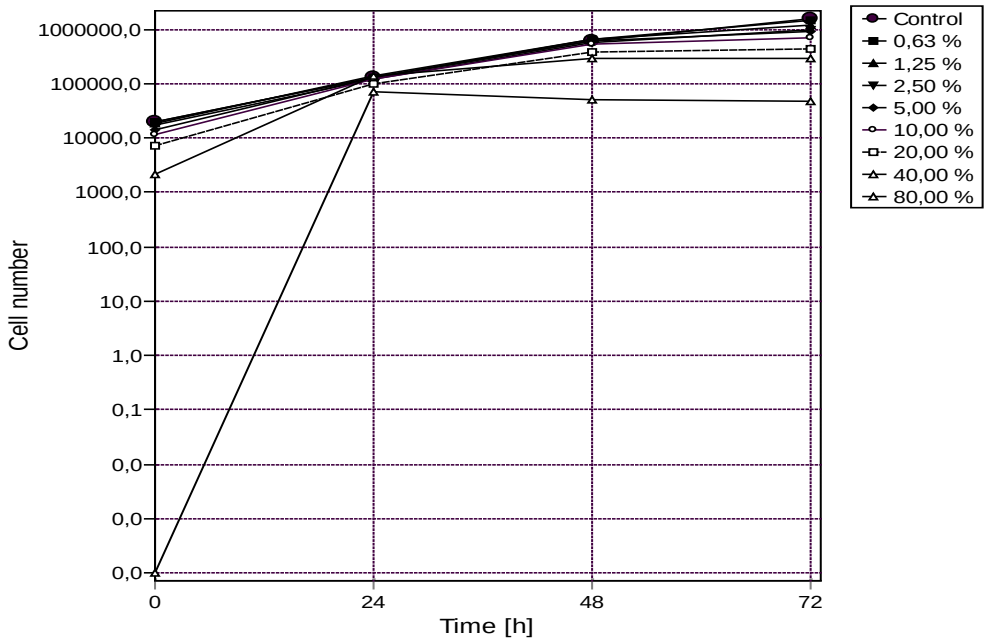


Figure 41: NM-330 - effect on cell number of *Pseudokirchneriella subcapitata.*

Test concentrations received by dilution with ultrapure water.

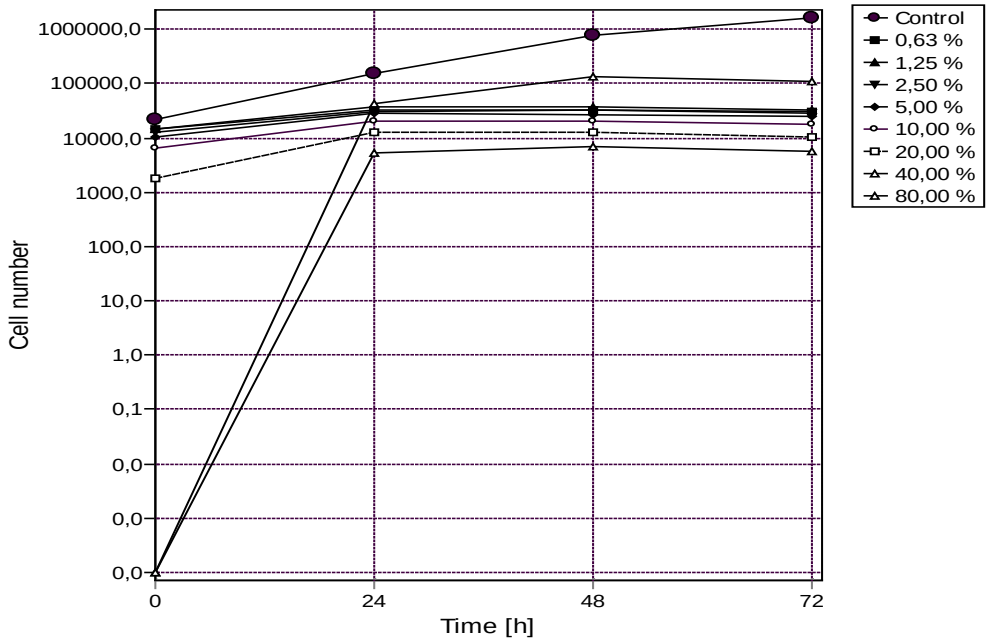


Figure 42: NM-330 - effect on cell number of *Pseudokirchneriella subcapitata*.

Test concentrations received by dilution with NM-330DIS.

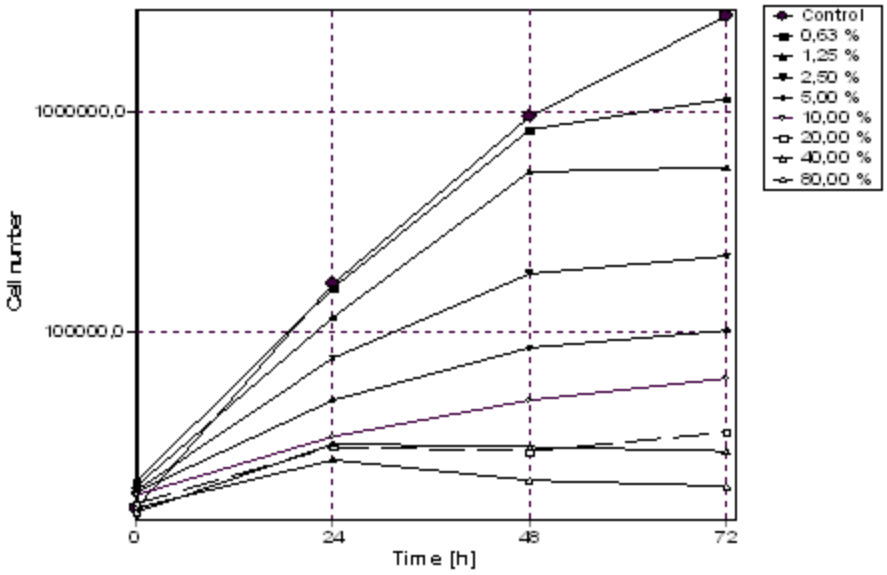


Figure 43: NM-330DIS - effect on cell number of *Pseudokirchneriella subcapitata.*

Test concentrations received by dilution with ultrapure water.

A summary of the effective concentration results are presented in Table 142.

In the test with NM-330DIS high toxicity was observed. The values are comparable independent of the evaluation period (24 – 72 h or 0 – 72 h). Therefore it was concluded that the results for NM-330DIS, calculated following the guideline, (0 – 72 h), can be compared with the results obtained for NM-330 (which can only be evaluated for the incubation period of 24 – 72 h). In the presence of gold nanoparticles toxicity decreased. It is assumed that gold nanoparticles cover chemical groups of the dispersant responsible for toxicity. The high toxicity of dispersant is proven by the test with NM-330 and preparation of the test concentrations by dilution with dispersant. In all test vessels the dispersant concentration was comparable, and high toxicity and no clear concentration-effect curves were obtained.

The concentration-effect curves are presented in Figure 44 - Figure 51.

Table 142: NM-330 – 1st test with algae: summary of the effects.

Effects given as percentage of NM-330 in the test medium.

	Biomass	Growth rate
	NM-330; concentrations achieved by dilution with **ultrapure water** **(evaluation period: 24 – 72 h)**	
NOEC [%]	0.63	0.63
LOEC [%]	1.25	1.25
EC50 [%] [1]	5.19 (4.43 – 6.07)	19.0 (15.3 – 23.9)
	NM-330; concentrations achieved by dilution with **dispersant** **(evaluation period: 24 – 72 h)**	
NOEC [%]	< 0.63	< 0.63
LOEC [%]	≤ 0.63	≤ 0.63
EC50 [%] [1]	No calculation possible due to quality of data	
	NM-330DIS; concentrations achieved by dilution with ultrapure water **(evaluation period: 24 – 72 h)**	
NOEC [%]	< 0.63	0.63
LOEC [%]	≤ 0.63	1.25
EC50 [%] [1]	0.46 (0.43 – 0.48)	1.61 (1.45 – 1.78)
	NM-330DIS; concentrations achieved by dilution with ultrapure water **(evaluation period: 0 – 72 h)**	
NOEC [%]	< 0.63	< 0.63
LOEC [%]	≤ 0.63	≤ 0.63
EC50 [%] [1]	0.48 (0.46 – 0.51)	2.42 (2.15 – 2.71)

[1] Values in brackets: confidence interval

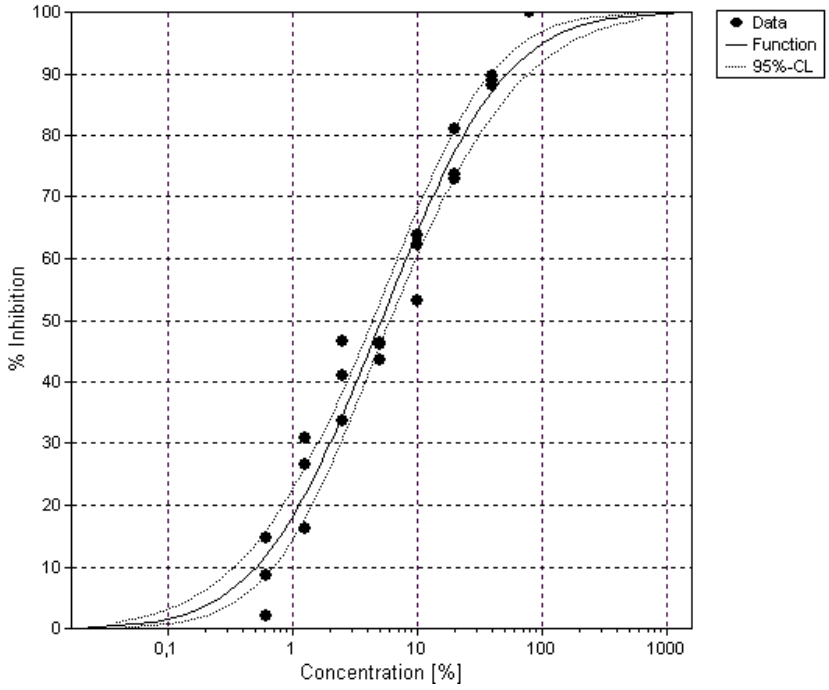


Figure 44: NM-330 - effect on yield of *Pseudokirchneriella subcapitata.*

Concentrations achieved by dilution with ultrapure water (evaluation period: 24 – 72 h).

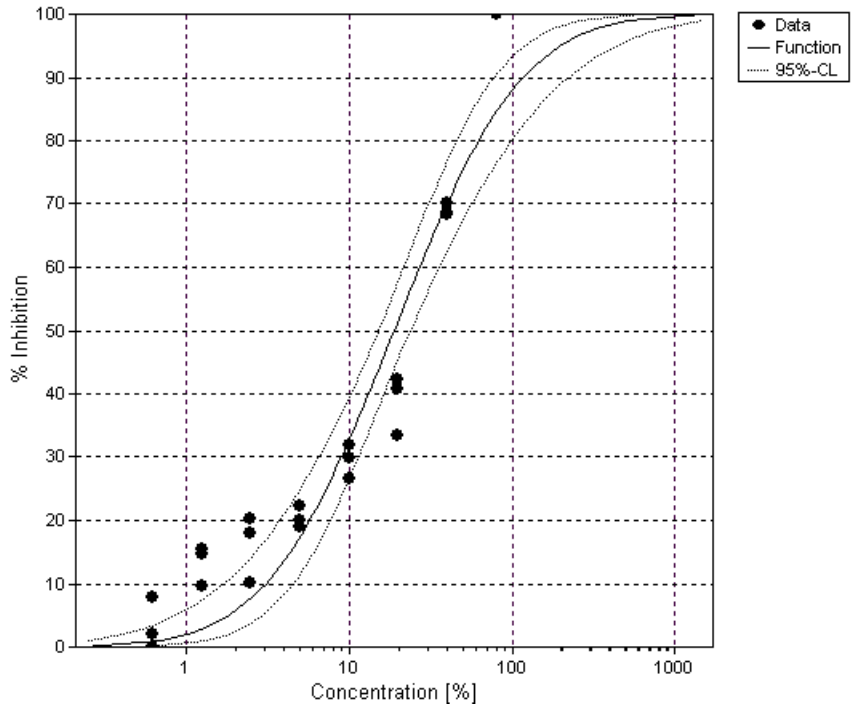


Figure 45: NM-330 - effect on growth rate of *Pseudokirchneriella subcapitata.*

Concentrations achieved by dilution with ultrapure water (evaluation period: 24 – 72 h).

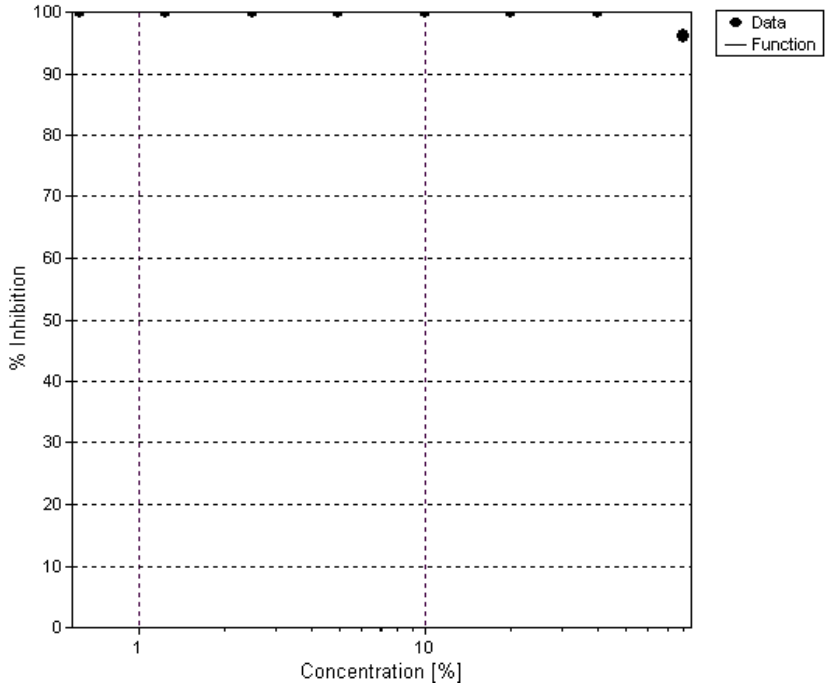


Figure 46: NM-330 - effect on yield of *Pseudokirchneriella subcapitata* .

Concentrations achieved by dilution with dispersant (evaluation period: 24 – 72 h).

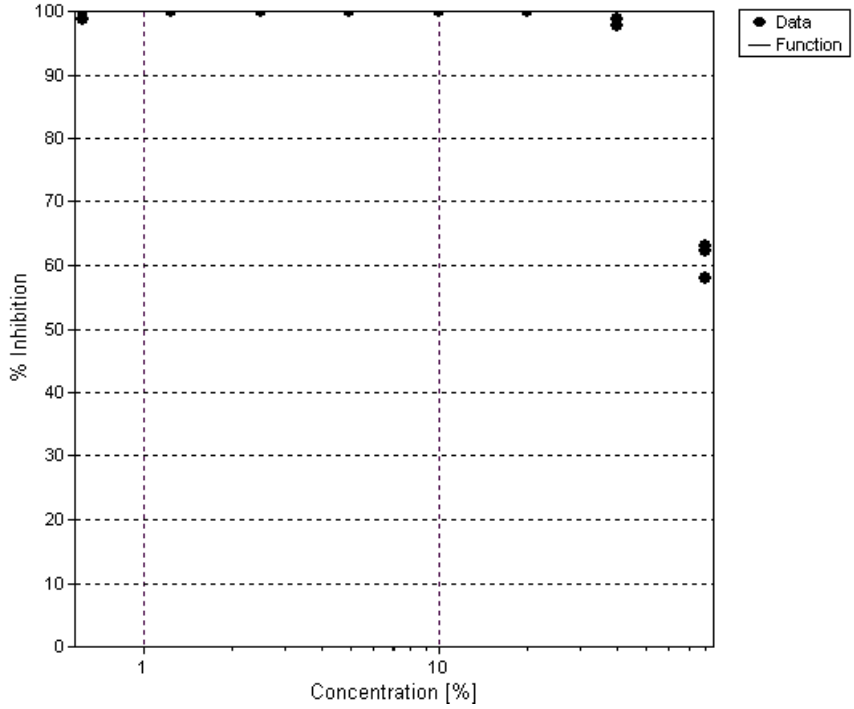


Figure 47: NM-330 - effect on growth rate of *Pseudokirchneriella subcapitata.*

Concentrations achieved by dilution with dispersant (evaluation period: 24 – 72 h).

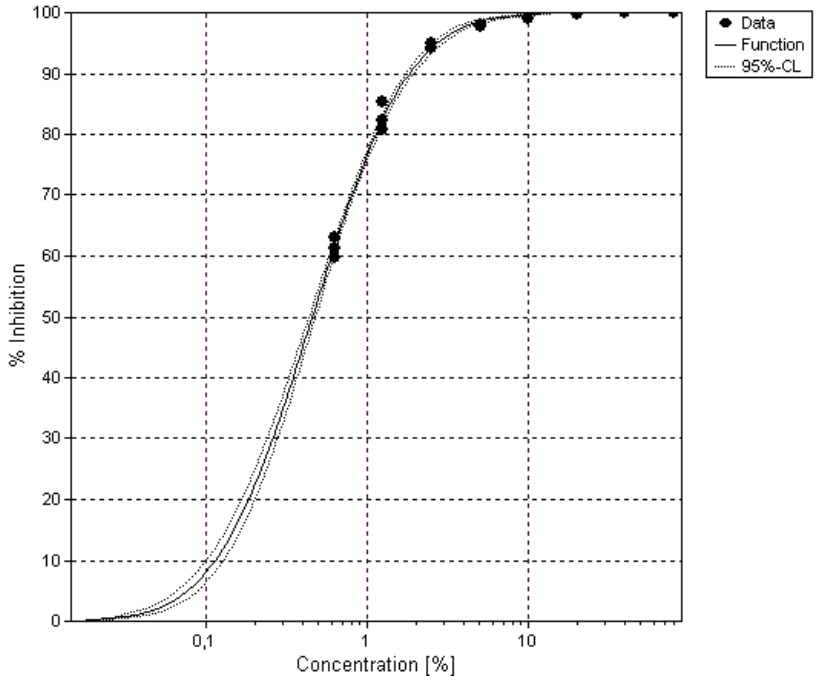


Figure 48: NM-330DIS –effect on yield of *Pseudokirchneriella subcapitata.*

Concentrations achieved by dilution with ultrapure water (evaluation period: 24 – 72 h).

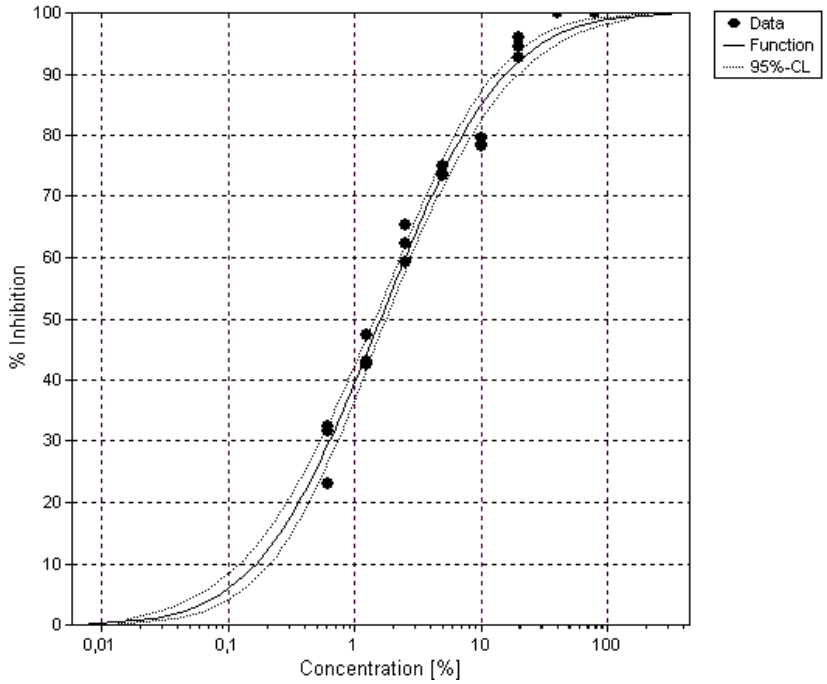


Figure 49: NM-330DIS – effect on growth rate of *Pseudokirchneriella subcapitata.*

Concentrations achieved by dilution with ultrapure water (evaluation period: 24 – 72 h).

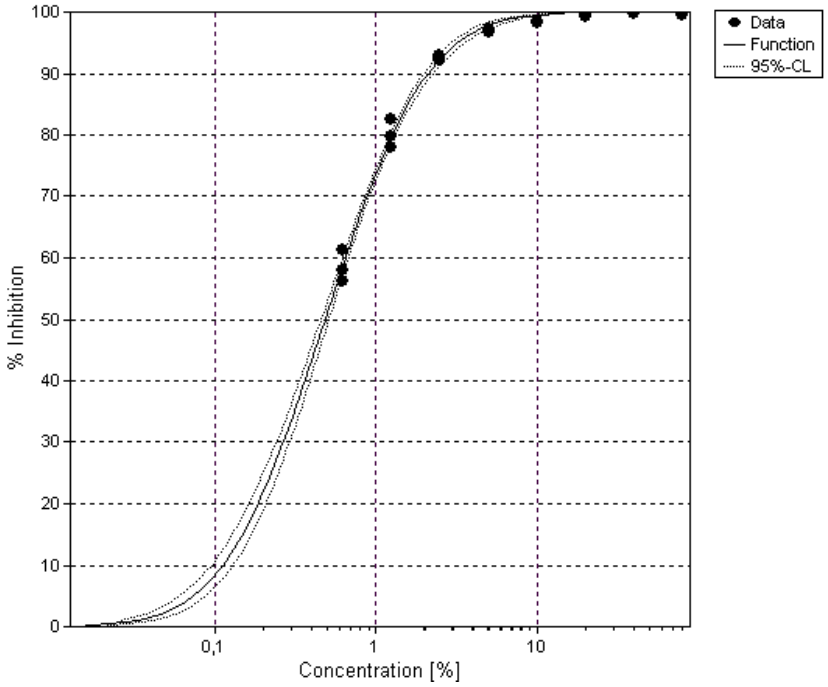


Figure 50: NM-330DIS – effect on yield of *Pseudokirchneriella subcapitata.*

Concentrations achieved by dilution with ultrapure water (evaluation period: 0 – 72 h).

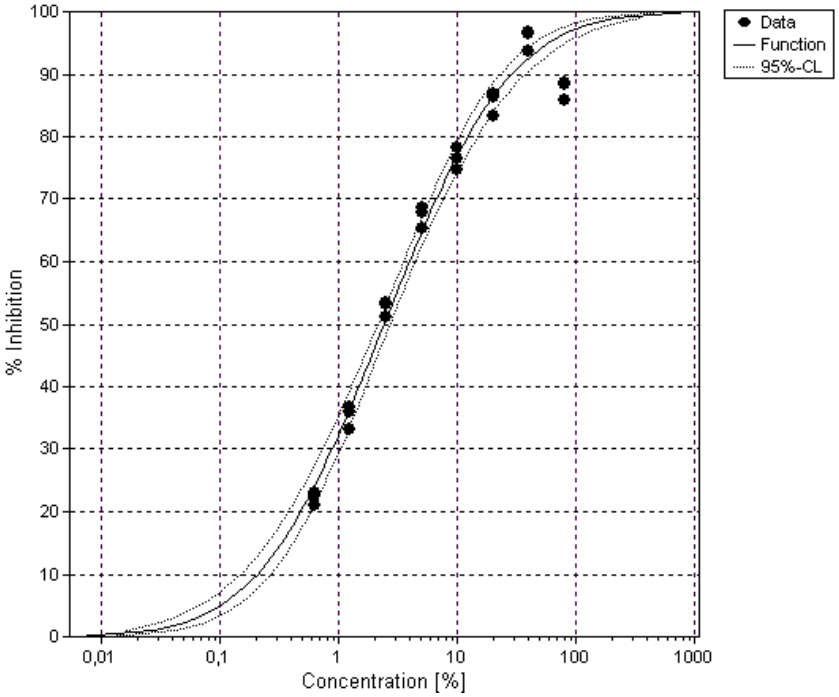


Figure 51: NM-330DIS – effect on growth rate of *Pseudokirchneriella subcapitata.*

Concentrations achieved by dilution with ultrapure water (evaluation period: 0 – 72 h).

17.7.4 Test 2

In test 2 the toxicity of NM-330 and NM-330DIS was determined to verify the results obtained in test 1. The test concentrations were achieved by dilution with ultrapure water.

Effects:

The compiled effect and threshold concentrations are presented in Table 143. The concentration-effect curves are shown in Figure 52- Figure 55. The results are comparable to test 1. Toxicity of NM-330 is smaller than toxicity of NM-330DIS. Therefore, it was concluded that gold nanoparticles reduce the toxicity of the dispersant.

Table 143: NM-330 – 2nd test with algae: summary of the effects.

Effects given as percentage of NM-330 in the test medium.

	Biomass	Growth rate
	NM-330; concentrations achieved by dilution with **ultrapure water (evaluation period: 24 – 72 h)**	
NOEC [%]	0.63	0.63
LOEC [%]	1.25	1.25
EC50 [%] [1]	8.96 (6.62 – 12.2)	39.2 (35.1 – 43.6)
	NM-330DIS; concentrations achieved by dilution with **ultrapure water (evaluation period: 0 – 72 h)**	
NOEC [%]	< 0.625	< 0.625
LOEC [%]	≤ 0.625	≤ 0.625
EC50 [%] [1]	1.05 (1.00 – 1.10)	4.59 (3.51 – 5.97)

[1] Values in brackets: confidence interval

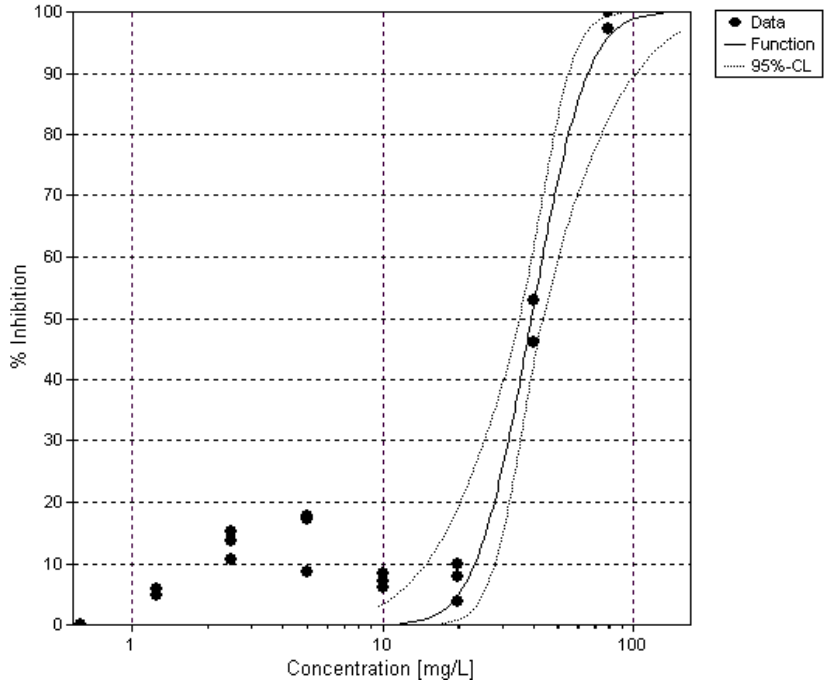


Figure 52: NM-330 – effect on yield of *Pseudokirchneriella subcapitata.*

Concentrations achieved by dilution with ultrapure water (evaluation period: 24 – 72 h)

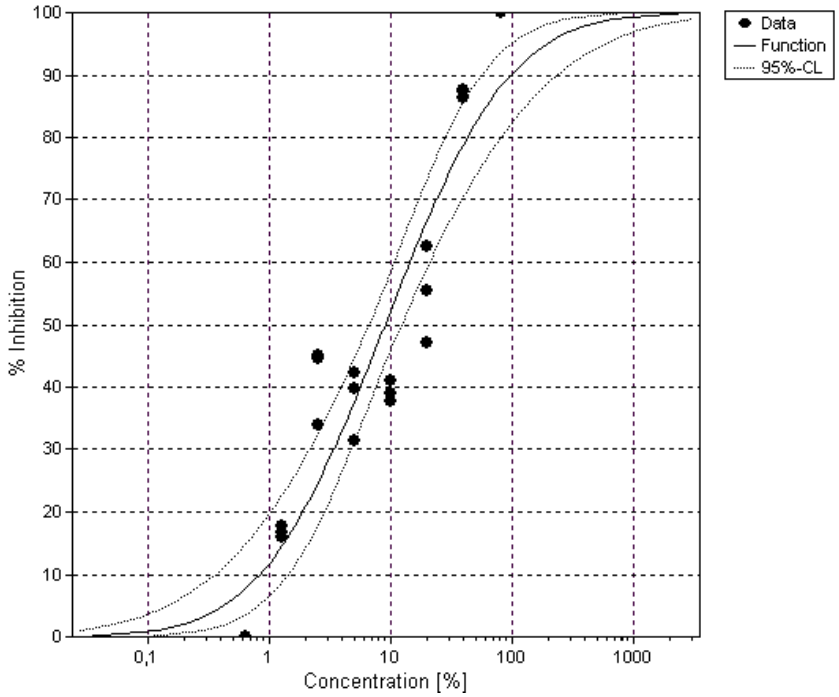


Figure 53: NM-330 – effect on growth rate of *Pseudokirchneriella subcapitata.*

Concentrations achieved by dilution with ultrapure water (evaluation period: 24 – 72 h).

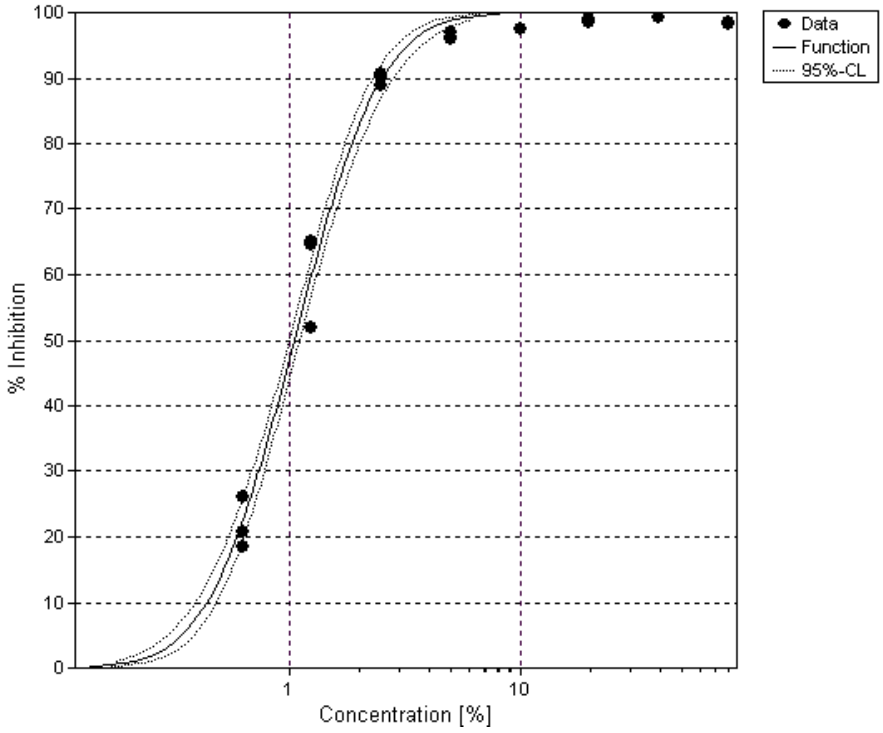


Figure 54: NM-330DIS – effect on yield of *Pseudokirchneriella subcapitata.*

Concentrations achieved by dilution with ultrapure water (evaluation period: 0 – 72 h).

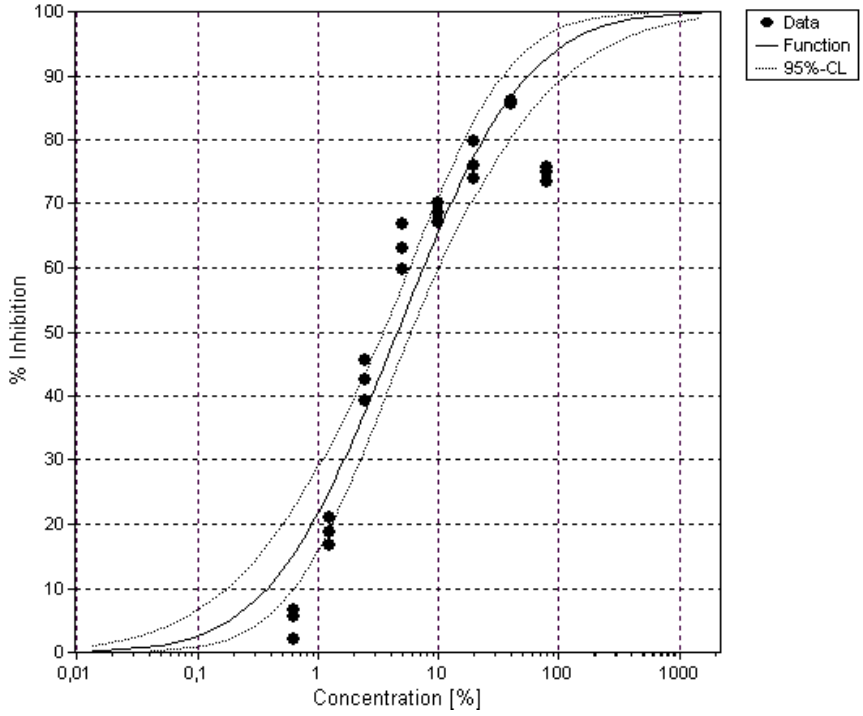


Figure 55: NM-330DIS - effect on growth rate of *Pseudokirchneriella subcapitata.*

Concentrations achieved by dilution with ultrapure water (evaluation period: 0 – 72 h).

17.7.5 Test 3

Tests 1 and 2 were performed in 96-well microplates. Due to this, a higher number of variants and test concentrations could be investigated at the same time. For four out of five experiments, one of the three validity criteria was not fulfilled. According to OECD 201, the mean coefficient of variation, measured in the control from 0 to 72 h, must not be higher than 35%. In most tests performed here this coefficient of variation was exceeded. Low incubation volume (200 µL) is the assumed reason. Therefore, a further test was performed in 24-well plates with a test volume of 2 mL and a reduced number of test concentrations (80 and 40% test item concentration in the test). The results are presented in Figure 56 and Figure 57.

For both test items (NM-330 and NM-330DIS) the validity criteria were fulfilled.

The percentage inhibition of the two test concentrations for NM-330 tested in all three tests were rather comparable (Table 144). Although only two concentrations were tested in test 3 the EC_{50} value was in a comparable magnitude of order as for Test 1 and Test 2. An EC_{50} of 53.01% (50.5 – 55.6) was calculated (Test 1: 19.0%; Test 2: 39.2%).

NM-330DIS showed expected high toxicity (about 100% effect for yield and growth rate for both test concentrations).

Based on the results it was concluded that tests performed in 96-well microplates can be used if the amount of test substance is very limited. Although one of three validity criteria was not fulfilled in most of the tests with a test volume of 200 µL the results are considered to be suitable for risk assessment.

Table 144: Summarised percentage inhibition of algae growth by the concentrations of NM-330 applied in all tests (evaluation period: 24 – 72 h).

Concentration of NM-330 given as percentage of NM-330 in the test medium.

	Inibition of growth rate [%]	
	40% of NM-330	80% of NM-330
Test 1	52.5	100
Test 2	50.6	100
Test 3	38.1	67.1

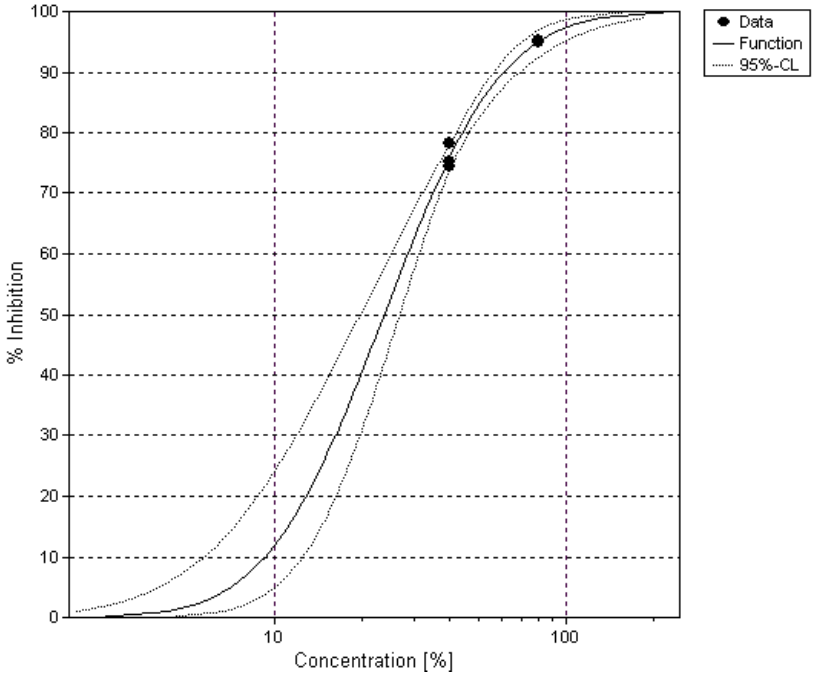


Figure 56: Concentration-effect curve showing the influence of NM-330on yield of the introduced *Pseudokirchneriella subcapitata.*

Concentrations achieved by dilution with ultrapure water (evaluation period: 0 – 72 h)

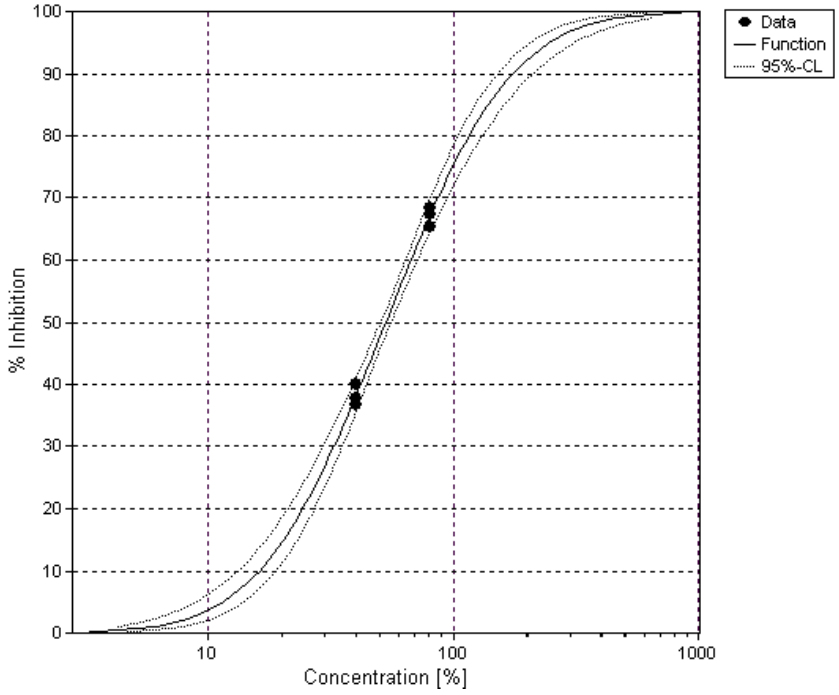


Figure 57: Concentration-effect curve showing the influence of NM-330 on growth rate of the introduced *Pseudokirchneriella subcapitata.*

Concentrations achieved by dilution with ultrapure water (evaluation period: 24 – 72 h).

17.8 Validity

The validity criteria refer to the control and an incubation period of 72 h. Therefore, for the validity check the whole incubation period was used, although for the tests with NM-330 the incubation period of 24 – 72 h was used for the evaluation of the effects (see 17.7.3).

Validity criteria according to OECD TG 201:

- Factor of the biomass parameter, measured in the control between 0 and 72 h, must be at least 16.
- Evaluation of the section-by-section growth rates: the mean coefficient of variation, measured in the control from 0 to 72 h, must not be higher than 35%.
- The coefficient of variation of the mean specific growth rate, measured in the control from 0 to 72 h, must not exceed 7%.

Test 1:

- NM-330, concentrations achieved by dilution with ultrapure water (evaluation period: 24 – 72 h): valid
    - Factor of the biomass parameter, measured in the control between 0 and 72 h: 79.8 (validity criterion fulfilled).
    - Evaluation of the section-by-section growth rates: Arithmetic means of the control replicates from 0 h to 72 h were: Replicate 1: 1.538; Replicate 2: 1.493; Replicate 3: 1.481; Replicate 4: 1.402; Replicate 5: 1.431; Replicate 6: 1.393. [1/d]. Coefficients of variation in control replicates from 0 to 72 h were: Replicate 1: 32.9%; Replicate 2: 32.5%; Replicate 3: 36.0%; Replicate 4: 32.5%; Replicate 5: 35.9%; Replicate 6: 34.1%. The mean of the replicate coefficients of variation in the section-by-section growth rate was 34.0% (validity criterion fulfilled).
    - The coefficient of variation of the mean specific growth rate replicates in the control between 0 and 72 h was 3.9% (validity criterion fulfilled).
- NM-330, concentrations achieved by dilution with dispersant (evaluation period: 24 – 72 h): limited validity
    - Factor of the biomass parameter, measured in the control between 0 and 72 h: 93.3 (validity criterion fulfilled).
    - Evaluation of the section-by-section growth rates: Arithmetic means of the control replicates from 0 h to 72 h were: Replicate 1: 1.530; Replicate 2: 1.544; Replicate 3: 1.493; Replicate 4: 1.500; Replicate 5: 1.490. [1/d]. Coefficients of variation in control replicates from 0 to 72 h were: Replicate 1: 39.1%; Replicate 2: 40.3%; Replicate 3: 42.9%; Replicate 4: 43.4%; Replicate 5: 41.0%. The mean of the replicate coefficients of variation in the section-by-section growth rate was: 41.3%.
      The test did not fulfil this validity criterion!
    - The coefficient of variation of the mean specific growth rate replicates in the control between 0 and 72 h was 1.6% (validity criterion fulfilled).
- NM-330DIS: limited validity
    - Factor of the biomass parameter: 172.0 (validity criterion fulfilled)

- Evaluation of the section-by-section growth rates: Arithmetic means of the control replicates from 0 h to 72 h were: Replicate 1: 1.755; Replicate 2: 1.712; Replicate 3: 1.703; Replicate 4: 1.705; Replicate 5: 1.742; Replicate 6: 1.679. [1/d]. Coefficients of variation in control replicates from 0 to 72 h were: Replicate 1: 40.3%; Replicate 2: 37.5%; Replicate 3: 36.9%; Replicate 4: 37.1%; Replicate 5: 38.3%; Replicate 6: 35.8%. The mean of the replicate coefficients of variation in the section-by-section growth rate was: 37.6%.
  The test did not fulfil this validity criterion!
- The coefficient of variation of the mean specific growth rate replicates in the control between 0 and 72 h was 1.6%. The test this (validity criterion fulfilled).

Test 2

- NM-330, concentrations achieved by dilution with ultrapure water (evaluation period: 24 – 72 h): limited validity
  - Factor of the biomass parameter: 85.3 (validity criterion fulfilled).
  - Evaluation of the section-by-section growth rates: Arithmetic means of the control replicates from 0 h to 72 h. [1/d]. Coefficients of variation in control replicates from 0 to 72 h were: Replicate 1: 42.9%; Replicate 2: 45.5%; Replicate 3: 44.0%; Replicate 4: 51.4%; Replicate 5: 47.4%; Replicate 6: 47.5%. The mean of the replicate coefficients of variation in the section-by-section growth rate was: 46.5%. According to OECD 201, the mean coefficient of variation, measured in the control from 0 to 72 h, must not be higher than 35%.
    The test did not fulfil this validity criterion!
  - The coefficient of variation of the mean specific growth rate replicates in the control between 0 and 72 h was 2.6% (validity criterion fulfilled).
- NM-330DIS: limited validity
  - Factor of the biomass parameter: 104.0 (validity criterion fulfilled).
  - Evaluation of the section-by-section growth rates: Arithmetic means of the control replicates from 0 h to 72 h were: Replicate 1: 1.567; Replicate 2: 1.574; Replicate 3: 1.540; Replicate 4: 1.536; Replicate 5: 1.553; Replicate 6: 1.520. [1/d]. Coefficients of variation in control replicates from 0 to 72 h were: Replicate 1: 42.8%; Replicate 2: 42.2%; Replicate 3: 45.1%; Replicate 4: 42.2%; Replicate 5: 41.4%; Replicate 6: 39.0%. The mean of the replicate coefficients of variation in the section-by-section growth rate was: 42.1%.
    The test did not fulfil this validity criterion!
  - The coefficient of variation of the mean specific growth rate replicates in the control between 0 and 72 h was 1.3% (validity criterion fulfilled).

Test 3

- NM-330, concentrations achieved by dilution with ultrapure water (evaluation period: 24 – 72 h): valid
  - Factor of the biomass parameter: 36.9 (validity criterion fulfilled).
  - Evaluation of the section-by-section growth rates: Arithmetic means of the control replicates from 0 h to 48 h were: Replicate 1: 1.761; Replicate 2:

1.809; Replicate 3: 1.778; Replicate 4: 1.888; Replicate 5: 1.804; Replicate 6: 1.801. [1/d]. Coefficients of variation in control replicates from 0 to 48 h were: Replicate 1: 1.0%; Replicate 2: 0.1%; Replicate 3: 0.5%; Replicate 4: 0.8%; Replicate 5: 0.9%; Replicate 6: 4.3%. The mean of the replicate coefficients of variation in the section-by-section growth rate was: 1.3% (validity criterion fulfilled).
    - The coefficient of variation of the mean specific growth rate replicates in the control between 0 and 48 h was 2.4% (validity criterion fulfilled).

- NM-330DIS: valid
    - Factor of the biomass parameter: 174.0 (validity criterion fulfilled).
    - Evaluation of the section-by-section growth rates: Arithmetic means of the control replicates from 0 h to 72 h were: Replicate 1: 1.760; Replicate 2: 1.723; Replicate 3: 1.683; Replicate 4: 1.658; Replicate 5: 1.751; Replicate 6: 1.735. [1/d]. Coefficients of variation in control replicates from 0 to 72 h were: Replicate 1: 0.7%; Replicate 2: 8.6%; Replicate 3: 9.9%; Replicate 4: 24.0%; Replicate 5: 5.3%; Replicate 6: 7.3%. The mean of the replicate coefficients of variation in the section-by-section growth rate was: 9.3% (validity criterion fulfilled).
    - The coefficient of variation of the mean specific growth rate replicates in the control between 0 and 72 h was 2.3% (validity criterion fulfilled).

17.9 Conclusion

The dispersant NM-330DIS showed a high toxicity. This toxicity was reduced in the presence of gold nanoparticles.

Tests investigating NM-330 toxicity to algae could only be evaluated for the 24 -72 h period of the test. In the presence of the two highest concentrations of NM-330 (gold nanoparticles in dispersant) and ultrapure water as diluent, the fluorescence at day 0 fell below the background value. Subtraction of the background values resulted in negative values. After 24 h fluorescence values above the background values were determined. It was assumed that the low fluorescence values at test start were not an indicator for toxicity but were due to silencing of the signals by the test item. After sedimentation or agglomeration of the nanoparticles and no further silencing, toxicity became obvious and could be evaluated. Tests with NM-330DIS were assessed according to the guideline (incubation period: 0 – 72 h) even though an evaluation just for the period from 24 - 72 h resulted in effect and threshold values comparable to those for the complete test period. As the reduced and the standard incubation periods resulted in comparable results for NM-330DIS, the results of both test substances were compared despite the different incubation periods.

Performing a test in 96 well plates with a test volume of 200 µL, one of the three validity criteria mentioned in the guideline was difficult to fulfil (mean of the replicate coefficients of variation in the section-by-section growth rate). Using 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.

The effect and threshold concentrations were comparable for both test volumes.

In Table 145 the effects of NM-330 and NM-330DIS on algal growth are summarised. In Table 146 the percent inhibition of algal growth by two tested concentrations of NM-330 of all tests are shown.

17.10 Executive summary

NM-330 (gold nanoparticles in dispersant) and **NM-330DIS** (dispersant of the gold nanoparticles) were tested in the growth test with green algae (OECD 201). Every 24 h, fluorescence was recorded as an indicator for algal growth. The fluorescence signal was converted into cell numbers using a calibration curve.

The gold concentration measured in NM-330 was lower than the value reported by the producer (0.01% corresponding to 100 mg/L expected; 43.8 mg/L measured). The NIST reference material 8011 (gold nanoparticles, nominal diameter 10 nm) was analysed along with the samples of the test and the 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.

As only minor amounts of the test substance (NM-330) were available for testing the test was performed in multi-well plates (96-well plates and 24-well plates). 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 such a small amount of test item resulted in fulfilment of only two of the three validity criteria mentioned in the guideline. The validity criterion “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.

The dispersant itself (NM-330DIS) showed a high toxicity. The toxicity is reduced in the presence of gold nanoparticles.

For the tests with NM-330 only the 24 - 72 h period of the tests could be evaluated. Tests with NM-330DIS were assessed as described in the guideline (incubation period: 0 – 72 h) but an evaluation restricted to the period from 24 - 72 h 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.

The effect and threshold concentrations are comparable for both test volumes.

In Table 167 the effects obtained for NM-330 and NM-330DIS are summarised. Table 168 shows the percent inhibition of algal growth for the concentrations of NM-330 applied in all tests.

Table 145: NM-330 and NM-330DIS – test with algae: summary of the effects.

Effects given as percentage of NM-330 and NM-330DIS in the test medium.

	NM-330 (gold nanoparticles) (evaluation period: 24 – 72 h)		NM-330DIS (dispersant) (evaluation period: 0 – 72 h)	
	Test 1			
	Biomass	Growth rate	Biomass	Growth rate
NOEC [%]	0.63	0.63	< 0.625	< 0.625
LOEC [%]	1.25	1.25	≤ 0.625	≤ 0.625
EC_{50} [%] [1]	5.19 (4.43 – 6.07)	19.0 (15.3 – 23.9)	0.48 (0.46 – 0.51)	2.42 (2.15 – 2.71)
	Test 2			
NOEC [%]	0.63	0.63	< 0.625	< 0.625
LOEC [%]	1.25	1.25	≤ 0.625	≤ 0.625
EC50 [%] [1]	8.96 (6.62 – 12.2)	39.2 (35.1 – 43.6)	1.05 (1.00 – 1.10)	4.59 (3.51 – 5.97)
	Test 3			
EC_{50} [%] (testing of two test concentrations: 40% and 80%)	---	53.01 (50.5 – 55.6)	Toxicity too high for evaluation	

[1] values in brackets: confidence interval

Table 146: Summarised percentage inhibition of algal growth for the concentrations of NM-330 applied in all tests (evaluation period: 24 – 72 h).

Concentrations given as percentage of NM-330 and NM-330DIS in the test medium.

	Inhibition of growth rate [%]	
	40% of NM-330	80% of NM-330
Test 1	52.5	100
Test 2	50.6	100
Test 3	38.1	67.1

18 Tests with Fish Embryos (OECD draft proposal) - Au

18.1 Test principle

The aim of the test was to elucidate representatively, at which concentrations of NM-330DIS and NM-330DIS the embryos of fish were affected significantly.

Fertilised eggs of zebra fish (*Danio rerio*) were exposed to five concentrations under static conditions in multi-well plates (individual exposure). Coagulated eggs and abnormalities in genesis were recorded.

18.2 Materials and methods

18.2.1 Test guideline

The test was performed according the OECD draft proposal for a new guideline “Fish Embryo Toxicity (FET) Test” (2006). In accordance with the presently discussed modified version, the test was extended to 96 h.

18.2.2 GLP

The test was performed following the principles of GLP. In deviation to GLP no archiving of the raw data was performed and the Quality Assurance Unit was not involved with respect to the inspection of the test, of the raw data and of the report. Any laboratory equipment (e.g. balances, thermometers, pH-meters) was controlled and documented according to GLP.

18.3 Test substances

- NM-330: gold nanoparticles in dispersant
- NM-330DIS: dispersant of the gold nanoparticles

18.4 Analytical monitoring

18.4.1 Details on test suspensions

ISO water (1/5 strength) was used as test water and to prepare the test suspension (58.8 mg $CaCl_2$ * 2 H_2O; 24.7 mg $MgSO_4$ * 7 H_2O; 13.0 mg $NaHCO_3$; 1.15 mg KCl)

The pristine gold dispersion and the dispersing agent were used as stock dispersion. The test concentrations were achieved by dilution.

50%: 50 mL gold dispersion / dispersing agent per 100 mL with ISO water

10.0%: 10 mL gold dispersion / dispersing agent per 100 mL with ISO water

1.0%: 1.0 mL gold dispersion / dispersing agent per 100 mL with ISO water

0.1%: 100 µL gold dispersion / dispersing agent per 100 mL with ISO water

0.01%: 10 µL gold dispersion / dispersing agent per 100 mL with ISO water

18.5 Test organism

Danio rerio (Teleostei, Cyprinidae; Hamilton-Buchanan 1822); laboratory breed

Origin of the fish:	West Aquarium GmbH PB 146 37431 Bad Lauterberg, Germany. Fertilised eggs for the test were obtained from individuals that were reared in the laboratory of the Fraunhofer Institute, Schmallenberg, Germany.
Breeding conditions:	Parental fish were held in 150 L aquaria.. At time of egg collection, parental fish were about 18 months old (maximum age for parental fish is 2 years). Stock density was approximately 80 fish per vessel. The holding temperature was 26°C ± 1°C. The light/dark cycle was 12 h/12 h. The flow through rate was adjusted to achieve a 2-fold exchange of water per day. Fish were fed daily *ad libitum* with TetraMinR Hauptfutter (Tetra Werke, Melle, Germany) and brine shrimp nauplii (*Artemia salina*). The broodstock were visually checked every working day for mortality, illness, parasites or abnormal behaviour. No prophylactic treatment of fish took place. Only healthy fish without diseases and abnormalities were used as parental fish for the production of fertilised eggs. Fertilisation rate was checked to fulfil the quality criterion of at least 50% for accepting the batch as parental fish for the production of fertilised eggs for a study.
Obtaining of eggs:	Eggs were collected with spawning-trays (made of glass) that were placed at the bottom of the holding vessels described above. The trays were covered with a lattice (stainless steel), to prevent the adults from predating on the eggs, and artificial plant substrate (modified method according to (5)) to stimulate spawning into the tray). Lighting (one neon lamp per vessel, light intensity approximately 1000 lux, measured 5 cm above the water surface in the middle of the test vessel) induced mating of fish and spawning. The collected eggs were transferred from the spawning-tray onto a sieve, rinsed with clean water in order to remove faeces and food waste, put into glass dishes and incubated at 26.0°C.
Holding water:	Purified drinking water was used as holding water. The purification included filtration with activated charcoal, passage through

a lime-stone column and aeration. To avoid copper contamination, plastic water pipes were used in the testing facilities. The following water chemistry data are recorded regularly in the testing facility: pH, conductivity, dissolved oxygen content, content of nitrate, nitrite, ammonium (NH^{4+}), phosphate, calcium, magnesium, total hardness, alkalinity, DOC content, content of metals (copper, iron, manganese and zinc). During preparation and performance of the test, all values were within the admissible ranges.

18.6 Study design

18.6.1 Study type

Short-term test, static.

18.6.2 Water medium type

Fresh water.

18.6.3 Total exposure duration

96 h.

- November 30, 2011 - December 04, 2011

No post-exposure observation period was performed.

18.6.4 Test conditions

Test conditions

Fertilised eggs were exposed under static conditions to the test substance for a period of 96 h. The test temperature during the test was adjusted to 26.0°C. Polystyrene multi-well dishes (24 wells; NUNC, Denmark) with a total volume of 5 mL per well and flat bottom were used as test vessels. After 24 h and 48 h coagulated eggs and abnormalities in genesis were recorded. After 72 and 96 h hatching behaviour was documented.

Test procedure

After collecting the eggs, a pool of 50 – 100 undifferentiated eggs was transferred with a widened and deburred pipette tip into each of the beakers prepared with test dispersion and control water to guarantee an exposure to the test substance in the early genesis state. Time from spawning until transfer into the test solutions did not exceed one hour. From these egg

pools, one fertilised egg (microscopic determination of early blastula stage) was then transferred in each well.

For each test concentration one multi well dish was used. The first vertical column of wells (4 wells) was reserved for the control and was filled with 2 mL ISO water (1/5 strength) per well. The other 20 wells were filled with the test dispersions (2 mL per well). After adding the eggs to the multi-well dishes the wells were covered with sealing tape and incubated in an incubator at 26.0 ± 1°C with a light/dark cycle of 12/12 h. The remaining test solutions and purified drinking water were filled into beakers and also incubated.

The oxygen concentrations (WTW OXI 196) and pH values (WTW, pH-Meter 535) were measured in the beakers directly before adding the eggs.

Observation and evaluation letter code

All eggs (20 in the control and 20 in every test concentration) were observed and evaluated every 24 h, using an inverse microscope. Normally developed embryos were indicated in the protocol by the letter code N; all abnormal developments were indicated by specific letters (Table 147). All listed abnormities are assumed to have lethal effects on the embryos. For the description of hatching the letter code is presented in Table 148.

Table 147: Letter code for observed effects.

Endpoints after 24 h	Endpoints after 48 h
N: normally developed Embryo	N: normally developed Embryo
K: coagulated	K: coagulated
S: no somites	H: no heart beat
C: tales not separated from the yolk sac	B: no blood cycle
A: no development of eyes	P: no pigmentation
T: no spontaneous movement	O: edema

Table 148: Letter code for observed effects on hatching behaviour.

Hatching behaviour after 72 and 96 h
G: hatched larvae, alive
R: not yet hatched, alive
M: hatched larvae, dead
U: not yet hatched, dead

9.3.5 Endpoint evaluation and statistical analysis

The sum of individuals per concentration meeting lethal endpoint criteria was used to calculate a concentration effect - relationship by using the probit analysis. From this the LC/EC50- and LC/EC10-values were derived.

Heartbeat frequency was counted for 10 seconds per embryo. Only embryos without abnormity were included in the statistical evaluation (t-test or U-test).

Statistical method

Statistical calculations:

Calculations were performed with the computer software ToxRat Professional version 2.10.4.1 (ToxRat® Solutions GmbH).

18.7 Results

The zeta potential of NM-330 in ISO water (1/5 strength) is presented in Table 118. A negative value was achieved.

Table 149: Zeta potential in ISO water (1/5 strength)

Sample	Zeta potential [mV]
10% NM-330	-26.1
50% NM-330	-39.1

The pH-values and oxygen saturation at test start are presented in Table 150.

Table 150: pH-values and oxygen saturation at test start.

Initial concentration [%]	Control	0.01	0.1	1	10	50
NM-330 (gold in dispersant)						
O_2 [%]	93	94	92	92	92	93
pH	7.8	7.2	7.2	7.1	7.0	5.9
NM-330DIS (dispersant)						
Observations after 72 h						
O_2 [%]	93	94	93	92	93	93
pH	7.8	7.3	6.8	6.0	6.2	6.0

The effect values are presented in Table 151.The **dispersant NM-330DIS** resulted in a concentration-effect relationship concerning abnormities of the embryos. The dispersant at 50% resulted in complete mortality of all embryos. In the presence of 10% dispersant the larvae hatched after a shorter period of time. Some of them showed a lower heartbeat or missing blood circulation. After 120 h the hatched larvae were dead. In contrast, **NM-330** (gold in dispersant) resulted in no abnormities after 24 and 48 h. The heartbeat was comparable to the control. Also the hatching behaviour was comparable to the control. An overview of the effects on the embryos is shown in Table 152. The hatching behaviour is presented in Table 153. The percentage of coagulated embryos and hatched organisms is summarised in Table 154 and Table 155.

Table 151: Effect concentrations of NM-330 and NM-330DIS.

Concentrations given as % of the product in the test (v/v).

	NM-330			NM-330DIS		
	48 h	72 h	96 h	48 h	72 h	96 h
Development of embryos (mortality)						
LC10 [%]	> 50% [1]	> 50% [1]	> 50% [1]	48	10	9
LC50 [%]	> 50% [1]	> 50% [1]	> 50% [1]	10	17	16
Hatching						
EC10 [%]	--- [3]	> 50 % [1]	> 50% [1]	--- [3]	1.2	nc [2]
EC50 [%]	--- [3]	> 50 % [1]	> 50% [1]	--- [3]	10	nc [2]

[1] highest test concentration; [2] not calculable (10%: comparable to control; 50% all are dead); [3] no hatching before 48 h, therefore, no EC value determinable

Table 152: Overview on effects of embryos (number of individuals) observed during the study.

K (coagulated) indicates clear lethality, the other indicators (referring to Table 147) are assumed to result in lethality. They may occur at the same time in one embryo.

Initial concentration [%]	Control	0.01	0.1	1	10	50
NM-330 (gold in dispersant)						
Effects after 24 h [3]						
K	0	1	1	0	0	0
Sum of affected embryos after 24 h	**0**	**1**	**1**	**0**	**0**	**0**
Effects after 48 h [3]						
K	0	1	1	0	0	Due to sedimentation of particles on eggs no evaluation of the embryos possible
BO	0	0	1	0	0	
Sum of affected embryos after 48 h	**0**	**1**	**2**	**0**	**0**	
Heartbeat frequency after 48 h. mean [1]	156.8	157.7	157.3	158	156.4	
Standard deviation	3.8	3.6	3.9	4.4	3.4	
NM-330DIS (dispersant)						
Effects after 24 h [3]						
K	1	0	0	1	1	6
SCAT	0	0	0	0	1	0
Sum of affected embryos after 24 h	**1**	**0**	**0**	**1**	**2**	**6**
Effects after 48 h [3]						
K	1	0	0	1	2	11 [2]
O	0	0	0	0	1	0
Sum of affected embryos after 48 h	**1**	**0**	**0**	**1**	**4**	**11** [2]
Heartbeat frequency after 48 h. mean [1]	156.6	157.8	157.8	158.1	156.7	155.6
Standard deviation	4.9	3.3	4.2	4.1	3.8	4.7

[1] per minute, embryos without abnormities only; [2] five of the embryos were completely developed, however dead, no heartbeat, no blood circulation; [3] K: coagulated ; S: no somites; C: tales not separated from the yolk sac; A: no development of eyes; T: no spontaneous movement; B: no blood circulation; O: edema

Table 153: Overview of hatching behaviour (number of individuals) observed during the study.

Initial concentration	Control	0.01%	0.1%	1%	10%	50%
NM-330 (gold in dispersant)						
Observations after 72 h [1]						
R	19	19	19	20	16	19
G	1				4	1
K		1	1			
Observations after 96 h						
R	3	4	3	2	2	5
G	17	15	16	18	18	15
K		1	1			
NM-330DIS (dispersant)						
Observations after 72 h [1]						
R	19	20	20	19	7	
G					11	
K	1			1	2	11
U						9
Observations after 96 h						
R	2	1	1	6		
G	17	12	17	12	17	
K	1	1		1	2	
M		2	2	1	1	
U		4				

[1] G: hatched larvae, alive; K: coagulated; M: hatched larvae, dead; R: not yet hatched, alive; U: not yet hatched, dead

Table 154: Coagulated embryos [%].

Initial concentration	Control	0.01%	0.1%	1%	10%	50%
NM-330 (gold in dispersant)						
24 h	0	5	5	0	0	0
48 h	0	5	5	0	0	n.d. [1]
72 h	0	5	5	0	0	0
96 h	0	5	5	0	0	0
NM-330DIS (dispersant)						
24 h	5	0	0	5	10	30
48 h	5	0	0	5	10	55
72 h	5	0	0	5	10	100
96 h	5	30	10	10	15	100

[1] Due to sedimentation of particles on eggs no evaluation of the embryos possible

Table 155: Hatched organisms [%].

Initial concentration	Control	0.01%	0.1%	1%	10%	50%
NM-330 (gold in dispersant)						
72 h	5	0	0	0	20	5
96 h	85	75	80	90	90	75
NM-330DIS (dispersant)						
72 h	0	0	0	5	55	0 (100% dead)
96 h	85	75	95	70	90	0 (100% dead)

18.8 Validity

No validity criteria are listed in the draft OECD test guideline (2006). Test acceptance criteria included in the version presently discussed were used as an alternative. According to these criteria, the test is considered to be valid as:

- The fertilisation rate of the eggs was ≥70% (about 90%).
- At the beginning of the test, test dissolved oxygen concentration in the negative control and highest test concentration was ≥80% of saturation (92 – 94%)
- Overall survival of embryos in the native control was ≥90% until the end of the exposure (test with NM-330: 95%; test with NM-330DIS: 100%)
- Hatching rate in the negative control was ≥80 % at the end of 96 h exposure (85%)

18.9 Conclusion

The dispersant itself (NM-330DIS) showed a high toxicity. This toxicity is reduced in the presence of gold nanoparticles.

The effect values are summarised in Table 156.

18.10 Executive summary

NM-330 (gold nanoparticles in dispersant) and **NM-330DIS** (dispersant of the gold nanoparticles) were tested in the fish embryo test using *Danio rerio* (OECD draft). The test period was 96 h. Qualitative observations on hatching, survival and abnormal behaviour were made daily.

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 and 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 concen-

trations of the ecotoxicological analyses are presented as % NM-330 (v/v) in the test suspension.

Due to the low concentration of Au nanoparticles in NM-330 the particle size distribution could not be determined. For the zeta potential in ISO water (1/5 strength) a negative value was determined. At 10% the zeta potential was -26 mV, whereas the test concentration of 50% resulted in a more negative value (-39 mV).

Tests with the dispersant NM-330DIS showed a concentration-effect relationship for abnormities of the embryos. At 50% dispersant all embryos died. In the presence of 10% dispersant the larvae hatched after a reduced embryo development period. Some of them showed a lower heartbeat or missing blood circulation. In contrast, NM-330 (gold in dispersant) caused no abnormities after 24 and 48 h. Heartbeat and hatching behaviour were comparable to the control.

The effect values are presented in Table 156.

Table 156: Effect concentrations of NM-330 and NM-330DIS.

Concentrations given as % of the product in the test (v/v).

	NM-330			NM-330DIS		
	48 h	72 h	96 h	48 h	72 h	96 h
Development of embryos (mortality)						
LC_{10} [%]	> 50% [1]	> 50% [1]	> 50% [1]	48	10	9
LC_{50} [%]	> 50% [1]	> 50% [1]	> 50% [1]	10	17	16
Hatching						
EC_{10} [%]	--- [3]	> 50% [1]	> 50% [1]	--- [3]	1.2	nc [2]
EC_{50} [%]	--- [3]	> 50% [1]	> 50% [1]	--- [3]	10	nc [2]

[1] highest test concentration; [2] not calculable (10%: comparable to control; 50% all embryos died); [3] no hatching before 48 h, therefore, no EC value determinable

19 Extended summary

19.1 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 TiO_2 and Ag nanoparticles 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 nanoparticles 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 nanoparticles
- 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 157). In Table 158 and Table 159 the properties of the applied nanoparticles are presented. For gold no information was available.

Table 157: 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® TiO_2 P25, but stems from a different batch

Table 158: Properties of the applied TiO_2 nanoparticles.

Data from the Joint Research Centre, European Commission.

Nanoparticles	NM-101	NM-103	NM-105[1]
Crystal structure	Anatase	Rutile	Rutile - Anatase
Purpose	active component for photocatalytic reactions	UV screening agent in sunscreen	active component for photo catalytic reactions
Primary particle size (according to Scherrer)	8 nm	20 nm	21 nm
Composition	TiO_2: 91.7%	TiO_2: 89.0% Al_2O_3: 6.2%	TiO_2: > 99%
BET	> 250 m²/g	60 m²/g	60 m²/g
Coating	none	hydrophobic	none
Condition	solid, powder	solid, powder	solid, powder

[1] Data elaborated for NM-105 and not for the batch distributed by Evonik and used in this study

Table 159: Properties of the applied silver nanomaterial.

Data from the Joint Research Centre, European Commission.

Nanoparticles	NM-300K	NM-300KDIS
Condition	in dispersion	dispersion
Primary particle size (according to Scherrer)	15 nm	---

19.2 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 nanoparticles are unavailable.

Therefore, the application of the nanoparticles was studied in pre-tests with emphasis on terrestrial tests. For tests with daphnids and chironomids the method of Hund-Rinke et al. (2010) 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:

- 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:

- TiO_2 nanoparticles (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 nanoparticles (available as dispersion): application via solid carrier (soil) in soil and directly in dung.

For Au no terrestrial tests were performed.

19.3 Main tests

A short overview of the toxicity for all test organisms is presented in Table 160.

The **TiO_2 nanoparticles** were available as dry powder. The results are presented as mg TiO_2/kg or mg TiO_2/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 160: Summary of the effect values of the applied nanoparticles.

Test	Guideline	Nanomaterial	Result
TiO_2			
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 (100 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	Germination, root length: no effect on the three plants up to the highest test concentration (dry application 100 mg/kg, wet application 20 mg/kg) Fresh weight: 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)

continued

Table 160 continued.

Test	Guideline	Nanomaterial	Result
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%
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

19.3.1 Tests with earthworms (OECD TG 222) – TiO_2, Ag

TiO_2

TiO_2 nanoparticles (NM-101, NM-103 and P25) were tested in the earthworm reproduction test. The particles were applied as powder and as aqueous dispersion in soil and in feed. The test substrate was a natural sandy soil. The experiments were performed several times.

The following test concentrations were investigated:

- Application via powder in feed: 50, 100, 200 mg/kg soil, dry matter
- Application via powder in soil: 50, 100, 200 mg/kg soil, dry matter
- Application via dispersion in feed: 10, 20 mg/kg soil, dry matter
- Application via dispersion in soil: 10, 20 mg/kg soil, dry matter.

In several tests performed only with powder-spiked soil a higher number of concentrations were investigated. Following approaches were studied:

- Application via powder in soil: 50, 100, 200, 400 mg/kg soil, dry matter (NM-101, NM-103)
- Application via powder in soil: 50, 100, 200, 500, 750, 1000 mg/kg soil, dry matter (P25).

The tested TiO_2 nanoparticles did not cause a reduction in the number of offspring. Stimulation of offspring production was observed for the earthworms in the uncoated material P25 treatment when the test was performed in winter. For the coated material NM-103 a stimulatory effect was not observed. The stimulatory effect was less pronounced for the second uncoated material (NM-101).

There were indications that the stimulation was due to a disturbance of the biological clock.

In some of the tests the Ti concentration was determined in the earthworms. There were strong indications that Ti concentrations in the worms increased with increasing test concentrations. However, there seemed to be a difference depending on whether the contamination was highly concentrated in food or distributed in soil. Contaminated food seemed to cause higher concentrations in the earthworms than contaminated soil giving an increase at 100 or 200 mg/kg concentrations, whereas for contaminated soil an increase was obvious only for the concentration of 1000 mg/kg. Differences between the three nanoparticles were not observed. In none of the test designs the concentration in the worms exceeded the soil concentration in the test substrate. Therefore, it was concluded that the nanoparticles did not accumulate in the tissue of the worms, but remained in the gut, possibly adsorbed to remaining soil/food particles.

Ag

Silver nanoparticles (NM-300K) and the dispersant in NM-300K (NM-300KDIS) were tested in the earthworm reproduction test. The particles were applied in soil and feed. The test substrate was a natural sandy soil. The test concentrations were 15, 30, 60, 120, 200 mg/kg soil, dry matter.

NM-300K caused a reduction of the reproduction rate, whereas the dispersant in NM-300K (NM-300KDIS) showed no effect.

Concerning reproduction, the ECx, NOEC and LOEC values presented in Table 161 were determined. Differences resulting from the exposure of the earthworms via feed and via soil seem to be negligible.

An increase in size and weight of the juveniles was observed. However, this observation was not reproducible.

Table 161: NM-300K - earthworm reproduction: summary of the effect values.

	Spiked feed, control: without further additions	Spiked feed, control: dispersant control	Spiked soil, control: without further additions	Spiked soil, control: dispersant control
EC_{50} [mg/kg] [1]	80.3 (58.5 - 113.4)	121.2 (85.3 - 183.8)	80.0 (33.6 - 413.3)	146.0 (85.8 - 741.4)
EC_{10} [mg/kg] [1]	14.6 (4.6 - 24.8)	39.4 (7.5 - 62.9)	n.d. [2]	24.2 (0.2 - 50.7)
LOEC [mg/kg]	≤15.0	60.0	≤15.0	30.0
NOEC [mg/kg]	<15.0	30.0	<15.0	15.0

[1] values in brackets: confidence interval;
[2] n.d. = confidence interval not determined due to mathematical reasons or inappropriate data

Additionally, the Ag concentration inside 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 containing NM-300K (nanosilver) and in the worms fed with spiked food. There was no obvious difference between the two experiments (spiking of soil or spiking of food). A dependence on the concentration was not observed. It was therefore assumed that a steady state of silver uptake was achieved already at the lowest test concentration. Concentration-dependent effects were 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 life stages involving development of cocoons and the juveniles in soil are sensitive. We do not know yet which life stage is more susceptible.

In none of the test designs the silver concentration in the worms exceeded the concentration in the test vessels. Therefore, it was concluded that the silver did not accumulate in the tissue of the worms. It is unclear whether the measured silver was located in the tissue or whether residues remained in the gut due to incomplete purging. We also do not know whether the determined silver occurred in its particle or ionic form.

19.3.2 Tests with microorganisms – nitrogen transformation test (OECD TG 216) – TiO_2

TiO_2 nanoparticles (P25) were tested in the nitrification assay. Soil was spiked with powder and with dispersion. As test substrate a natural sandy soil was used. The following test concentrations were investigated:

- Application via powder in soil: 9.3, 21.0, 45.0, 100.0 mg/kg soil
- Application via dispersion in soil: 9.3, 21.0 mg/kg soil.

The nitrate content was determined photometrically at day 0 (sampling of the soil three hours after application) and at day 28.

The application via powder caused concentration-dependent effects, namely, decreased nitrate values at day 0 (sampling of the soil three hours after application), increased nitrate values at day 28, and increased nitrogen transformation rates (difference in nitrate content between day 28 and day 0).

In Table 162 the NOEC and ECx values are summarised.

Table 162: P25 - Summary of the effect values for nitrogen transformation.

Application via powder and dispersion.

	Application via powder		Application via dispersion	
	Day 0 (= 3 h after application)	Day 28	Day 0 (= 3 h after application)	Day 28
Nitrate content				
EC_{10} [mg/kg] [2]	23.6	n.d.	n.d.	n.d.
EC_{25} [mg/kg] [2]	108.3	n.d.	n.d.	n.d.
LOEC [mg/kg]	21.0	100.0	> 21	> 21
NOEC [mg/kg]	9.3	45.0	≥ 21	≥ 21
Nitrogen transformation [1]				
EC_{10} [mg/kg] [2]	n.d.		n.d.	
EC_{25} [mg/kg] [2]	n.d.		n.d.	
LOEC [mg/kg]	21.0		> 21	
NOEC [mg/kg]	9.3		≥ 21	

[1] Nitrogen transformation: difference in nitrate content at day 28 and day 0;
[2] n.d. = not determined due to mathematical reasons or inappropriate data

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.

19.3.3 Tests with microorganisms – carbon transformation test (OECD TG 217) – TiO_2

TiO_2 nanoparticles (P25) were tested in the microbial carbon transformation assay (OECD Test Guideline 217). Soil was spiked with the test item via powder and via dispersion. As test substrate a natural sandy soil was used. The following test concentrations were investigated:

- Application via powder in soil: 9.3, 21.0, 45.0 and 100.0 mg/kg soil, dry matter
- Application via dispersion in soil: 9.3 and 21.0 mg/kg soil, dry matter.

For each treatment three replicate vessels were incubated. From each vessel one soil sample was taken for measurement.

For both application forms, no inhibitory effect was observed and no EC-values were calculated. There is no statistically significant difference between the treatments and the control. The NOEC is higher than the highest test concentration (≥ 100 mg/kg). This result was confirmed by a repetition of the test.

19.3.4 Tests with plants (OECD TG 208) – TiO_2

TiO_2 nanoparticles (P25) were tested in seedling emergence and growth tests with plants (OECD test guideline 208). Soil was spiked via powder and via dispersion. As test substrate a natural sandy soil was used. Following test concentrations were investigated:

- Application via powder in soil: 10, 20, 30, 44, 67, 100 mg/kg soil, dry matter
- Application via dispersion in soil: 10, 20 mg/kg soil, dry matter.

The plant species used in the test were *Avena sativa* (oat), *Sinapis alba* (mustard) and *Phaseolus aureus* (mung bean), which are representative of monocotyledonous and dicotyledonous plants, respectively. The endpoints mentioned in the test guideline (i.e. germination, biomass) and the root length were 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* and *Phaseolus aureus*. 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).

A summary is presented in Table 163.

Table 163: P25 – test with plants: summary of the effect values.

Application via powder; critical effect and threshold concentrations [mg/kg].

	Avena sativa	*Phaseolus aureus*	*Sinapis alba*
Emergence			
EC_{10} [mg/kg]	32.4	n.d. [2]	n.d. [2]
EC_{50} [mg/kg]	n.d. [2]	n.d. [2]	n.d. [2]
LOEC [mg/kg]	> 100	n.d. [2]	> 100
NOEC [mg/kg]	≥ 100	n.d. [2]	≥ 100
Shoot fresh weight			
EC_{10} [mg/kg] [1]	51.7 (36.1 – 61.4)	n.d. [2]	n.d. [2]
EC_{50} [mg/kg] [1]	n.d. [2]	n.d. [2]	n.d. [2]
LOEC [mg/kg]	100.0	67	> 100
NOEC [mg/kg]	67.0	44	≥ 100
Root length:			
EC_{10} [mg/kg]	n.d. [2]	n.d. [2]	n.d. [2]
EC_{50} [mg/kg]	n.d. [2]	n.d. [2]	n.d. [2]
LOEC [mg/kg]	> 100	n.d. [2]	> 100
NOEC [mg/kg]	≥ 100	n.d. [2]	≥ 100

[1] values in brackets: confidence interval;
[2] n.d. = not determined due to mathematical reasons or inappropriate data or considered unreliable

19.3.5 Tests with chironomids (OECD TG 219) – TiO_2, Ag, Au

The OECD Test Guideline 219 (Sediment-Water Chironomid Toxicity Using Spiked Water) was applied using *Chironomus riparius* as test organism. The test required feeding of the larvae at least three times per week. Two feeding regimes were tested as the sorption of the nanoparticles 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 P25 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.

<u>TiO_2</u>

The nominal concentrations of TiO_2 nanoparticles in the test containers were 15, 23, 39, 63 and 100 mg test item/L.

There was strong sedimentation of TiO_2 nanoparticles resulting in Ti concentrations below the detection limit in the overlaying water. At test end nearly all of the applied TiO_2 nanoparticles were determined in the sediment.

P25: Concentrations up to 100 mg/L P25 did not cause a negative impact on the emergence of larvae in the sediment/water chironomid test using spiked water. The NOEC was ≥ 100 mg/L.

NM-101: NM-101 concentrations up to 100 mg/L did not have a negative impact on the emergence of larvae. The NOEC was ≥ 100 mg/L.

Silver

NM-300K: The nominal concentrations in the test containers with silver were 0.3125, 0.625, 1.25, 2.5, 5 and 10 mg test item/L.

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

The concentration of the Ag ions in the sediment was determined using DGTs. Compared to the total Ag amount the concentration of the Ag ions in the sediment was low. Depending on the referred value (nominal concentration in overlaying water or concentration in water content of sediment) the percentage of the ions was about 10^{-3} or 10^{-4}%.

The dispersant used for stabilising the Ag nanoparticles had no negative effect on the emergence of the chironomids.

The application of NM-300K resulted in a clear concentration-effect curve. The NOEC value for total emergence and for emerged males and females was 1.250 mg/L. The NOEC value for the total development rate and for the development rates of males was 0.625 mg/L. For females a NOEC could not be calculated as there was a statistically significant effect at 1.25 mg/L but no effect at 2.5 mg/L and a 100% effect were at 5 and 10 mg/L.

The presented results are based on nominal concentrations. Based on the ion concentration determined with DGTs in the sediment, the effect values are lower by a factor of 10^{-3} – 10^{-4}.

This illustrates that the basis of the calculation has to be clearly fixed for regulatory purposes.

A summary of the results obtained for all endpoints is presented in Table 164.

Table 164: NM-300K – test with chironomids: summary of effect values.

Concentrations given as nominal values.

	NOEC [mg/L] [2]	LOEC [mg/L] [2]	EC_{10} [mg/L] [1,2]	EC_{20} [mg/L] [1,2]	EC_{50} [mg/L] [1,2]
Emerged males and females	1.250	2.5	1,583 (1.350 - 1.750)	1.772 (1.566 - 1.926)	2.201 (2.041 - 2.357)
Emerged midges [males]	1.250	2.5	2.059 (n.d.)	2.175 (n.d.)	2.415 (n.d.)
Emerged midges [females]	1.250	2.5	1.055 (0.825 - 1.242)	1.276 (1.051 - 1.467)	1.835 (1.610 - 2.091)
Development rate of males and females	0.625	1.250	0.925 (n.d.)	1.897 (n.d.)	7.508 (n.d.)
Development rate of males	0.625	1.250	0.994 (n.d.)	1.824 (n.d.)	5.828 (n.d.)
Development rate of females	n.d.	n.d.	0.934 (n.d.)	2.443 (n.d.)	15.369 (n.d.)

[1] values in brackets: confidence interval;
[2] n.d. = not determined due to mathematical reasons or inappropriate data

Gold

NM-330 and **NM-330DIS:** The nominal test concentrations in the vessels were 0, 0.1, 1, 10 and 50% test item/L. The test concentrations were verified by chemical analysis.

General observations

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.

Effects

For the dispersant a strong effect was observed at the highest test concentration. The larvae were fully grown, even though their development was delayed. However, no larvae emerged as the organisms died before hatching. In the presence of gold this effect did not occur.

All effect values are summarised in Table 165. In contrast to the dispersant no effect was observed for the treatments with gold. Although statistically significant differences to the control were observed for the development rates, the differences were not considered to be an effect of the test substance, since they were not related to concentration-effect relationships.

Table 165: NM-330, NM-330DIS – test with chironomids: summary of the effects.

Effects given as percentage of NM-330 in the test medium.

	NOEC [%]	LOEC [%]
	NM-330	
Emergence rate – combined sexes, males, females	≥ 50	> 50
Development time – combined sexes, males, females	≥ 50	> 50
Development rate – combined sexes	not evaluable [1]	not evaluable [1]
Development rate – males	not evaluable [1]	not evaluable [1]
Development rate – females	not evaluable [1]	not evaluable [1]
	NM-330DIS	
Emergence rate – combined sexes, males, females	10	1
Development time – combined sexes, males, females	10	1
Development rate – combined sexes	not evaluable [1]	not evaluable [1]
Development rate – males	not evaluable [1]	not evaluable [1]
Development rate – females	10	1

[1] There was a statistically significant difference to the control, but no concentration-effect relationship.

19.3.6 Tests with daphnids – reproduction (OECD TG 211) – TiO_2

TiO_2

TiO_2 nanoparticles were tested in the reproduction test with daphnids (OECD 211). Three semi-static tests were carried out. In the first test the medium was renewed on days 2, 5, 7, 9, 12, 14, 16, and 19. The nominal concentrations of TiO_2 nanoparticles in the test containers were 0.05, 0.1, 0.5, 1.0, and 5.0 mg test item/L. The concentrations of the test item were measured in the freshly prepared test suspensions on days 0, 7, and 14. After two days of incubation the concentrations of the test item were measured in the incubation flasks (days 2, 9, and 16). Sedimentation of TiO_2 nanoparticles resulted in a reduction of the Ti concentrations in the overlaying water after incubation.

In the second test two concentrations (1 mg/L, 5 mg/L) were investigated. Two periods for the renewal of the test medium were studied: three times per week and daily.

In the third test two concentrations (1 mg/L, 5 mg/L) were investigated. Two ultrasonication periods (3 min and 30 min) were studied. The test medium was renewed three times per week.

The results concerning the NOEC differ slightly. A summary is presented in Table 166. The effect of P25 on reproduction activity, mobility and body length seems to be negligible up to the highest test concentration of 5 mg/L. The differences between the tests reflect the biological variability.

Table 166: P25 – test with daphnids: summary of the NOEC values.

Mean cumulative offspring per female, mobility and body length in the three tests.

	1st test: ultrasonication period 3 min; medium renewal 3 times per week	2nd test: medium renewal daily or 3 times per week	3rd test: ultrasonication period 3 min and 30 min
Mean cumulative offspring per female			
NOEC [mg/L]	≥ 5.0	≥ 5.0	5.0
Mobility			
NOEC [mg/L]	≥ 5.0	≥ 5.0	≥ 5.0
Body length			
NOEC [mg/L]	0.1 mg/L	≥ 5.0	≥ 5.0

19.3.7 Tests with daphnids – immobilisation (OECD TG 202) - Au

Au

NM-330 (gold nanoparticles in dispersant) and **NM-330DIS** (dispersant of the gold nanoparticles) were tested in the acute test with *Daphnia magna* (OECD 202). Two static tests with

different test concentrations were performed. The mobility of the daphnids was recorded after 24 h and 48 h.

Due to the low concentration of the Au nanoparticles in NM-330 the particle size distribution could not be determined. The Zeta potential determined for the highest test concentration (10%) in purified tap water (= test water) was -24 mV.

During the incubation period of two days sedimentation occurred resulting in concentrations of gold in the overlaying water of about 1% for both concentrations analysed (5 and 10%).

Concentration-dependent toxicity was detected for the dispersant. In the control and in the test vessels containing gold dispersion no immobilisation was detected after an incubation period of 24 h. After 48 h, 5% immobilisation occurred.

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.

Based on the findings the following effect values were calculated:

NM-330 (gold nanoparticles): LOEC > 50% (v/v): NOEC ≥ 50% (v/v)

NM-330DIS (dispersant): EC_{50} (48 h) 3.24% (v/v); LOEC 5.0% (v/v): NOEC 2.5% (v/v)

19.3.8 Tests with algae (OECD TG 201) - Au

Au

NM-330 (gold nanoparticles in dispersant) and **NM-330DIS** (dispersant of the gold nanoparticles) were tested in the growth test with green algae (OECD 201). Every 24 hours, fluorescence was recorded as an indicator for algal growth. The fluorescence signal was converted into cell numbers using a calibration curve.

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

The dispersant itself (NM-330DIS) showed a high toxicity. The toxicity was reduced in the presence of gold nanoparticles.

For the tests with NM-330 only the period of the test from 24 - 72 h was evaluated. Tests with NM-330DIS was 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.

As only minor amounts of the test substance (NM-330) were available for testing, the test was performed in multi-well plates (96-well plates and 24-well plates). However, using such a small amount of test item resulted in fulfilment of only two of the three validity criteria men-

tioned in the guideline. The criterion “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. But for a test with at least 3 replicates of 5 test concentrations, plus controls and blanks, meant several plates were necessary and each required sufficient shaking devices for incubation. This can be a disadvantage.

The effect and threshold concentrations are comparable for both test volumes.

In Table 167 the effects of NM-330 and NM-330DIS on algal growth are summarised. Table 168 shows the percent inhibition of algal growth for the concentrations of NM-330 applied in all tests.

Table 167: NM-330 and NM-330DIS – test with algae: summary of the effects.

Effects given as percentage of NM-330 and NM-330DIS in the test medium.

	NM-330 (gold nanoparticles) (evaluation period: 24 – 72 h)		NM-330DIS (dispersant) (evaluation period: 0 – 72 h)	
	Test 1			
	Biomass	Growth rate	Biomass	Growth rate
NOEC [%]	0.63	0.63	< 0.625	< 0.625
LOEC [%]	1.25	1.25	≤ 0.625	≤ 0.625
EC_{50} [%] [1]	5.19 (4.43 – 6.07)	19.0 (15.3 – 23.9)	0.48 (0.46 – 0.51)	2.42 (2.15 – 2.71)
	Test 2			
NOEC [%]	0.63	0.63	< 0.625	< 0.625
LOEC [%]	1.25	1.25	≤ 0.625	≤ 0.625
EC50 [%] [1]	8.96 (6.62 – 12.2)	39.2 (35.1 – 43.6)	1.05 (1.00 – 1.10)	4.59 (3.51 – 5.97)
	Test 3			
EC_{50} [%] (testing of two test concentrations: 40% and 80%)	---	53.01 (50.5 – 55.6)	Toxicity too high for evaluation	

[1] values in brackets: confidence interval

Table 168: Summarised percentage inhibition of algal growth for the concentrations of NM-330 applied in all tests (evaluation period: 24 – 72 h).

Concentrations given as percentage of NM-330 and NM-330DIS in the test medium.

	Inhibition of growth rate [%]	
	40% of NM-330	80% of NM-330
Test 1	52.5	100
Test 2	50.6	100
Test 3	38.1	67.1

19.3.9 Tests with fish embryos (OECD draft) - Au

NM-330 (gold nanoparticles in dispersant) and NM-330DIS (dispersant of the gold nanoparticles) were tested in the fish embryo test with *Danio rerio* (OECD draft). The test period was

96 h. Qualitative observations on hatching, survival and abnormal behaviour were made daily.

Due to the low concentration of Au nanoparticles in NM-330 the particle size distribution could not be determined. For the zeta potential in ISO water (1/5 strength) a negative value was determined. At 10% the zeta potential was -26 mV, whereas the test concentration of 50% resulted in a more negative value (-39 mV).

Tests with the **dispersant NM-330DIS** showed a concentration-effect relationship for abnormities of the embryos. At 50% dispersant all embryos died. In the presence of 10% dispersant the larvae hatched after a reduced embryo development period. Some of them showed a lower heartbeat or missing blood circulation. In contrast, **NM-330** (gold in dispersant) caused no abnormities after 24 and 48 h. Heartbeat and hatching behaviour were comparable to the control.

The effect values are presented in Table 169.

Table 169: Effect concentrations of NM-330 and NM-330DIS.

Concentrations given as % of the product in the test (v/v)

	NM-330			NM-330DIS		
	48 h	72 h	96 h	48 h	72 h	96 h
Development of embryos (mortality)						
LC_{10} [%]	> 50% [1]	> 50% [1]	> 50% [1]	48	10	9
LC_{50} [%]	> 50% [1]	> 50% [1]	> 50% [1]	10	17	16
Hatching						
EC_{10} [%]	--- [3]	> 50% [1]	> 50% [1]	--- [3]	1.2	nc [2]
EC_{50} [%]	--- [3]	> 50% [1]	> 50% [1]	--- [3]	10	nc [2]

[1] highest test concentration; [2] not calculable (10%: comparable to control; 50% all embryos died); [3] no hatching before 48 h, therefore, no EC value determinable

19.4 Recommendations for the test performance

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

19.4.2 Application of nanoparticles 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 nanoparticles 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 directly 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

19.4.3 Spiking of soil versus spiking of feed

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

19.4.4 Application of insoluble nanoparticles in powder form to aquatic test media

The method described by Hund-Rinke *et al.* (2010) is suitable for the application of insoluble nanoparticles 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 nanoparticles stabilised in an aqueous medium (silver NM-300K; gold NM-330) a homogenous distribution in suitable stock dispersions can be achieved by stirring.

19.4.5 Sensitivity of the applied test systems

For TiO_2 nanoparticles 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 TiO_2 nanoparticles for these organisms.

19.4.6 Toxicity of dispersants

Silver and gold nanoparticles 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 nanoparticles 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.

19.4.7 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 10^3 – 10^4 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.

20 Literature

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

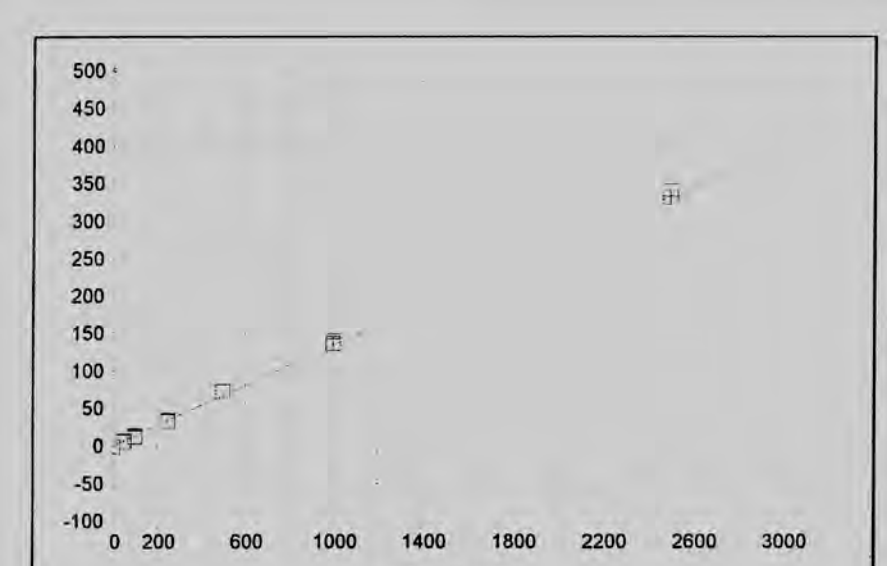
21 Annex

21.1 Raw data – methods for chemical analyses (chapter 4)

21.1.1 Raw data examples: Ti

Example for ICP-OES calibration - applied for determination of Ti-concentration

Calibration data from the measurement performed on July 14, 2011.



Ti 337,280 (99)

Regress.-Dat: 07/18/2011 08:59:48	Regress.-Typ Linear	Wichtung:	Keine
A0 (Schnittp.):	0,952766	Rekalibration	1,000000
A1 (Anstieg):	0,132640	Y-Schnittp:	0,000000
A2 (Krümmung):	0,000000		
n (Exponent):	1,000000		
Korrelation:	0,999754	Status:	OK.
Verfahr.-StdAbw:	21,776150		
NG:	65,328450		
BG:	217,761499		

Std.-Name	Gesetzte Konz.	Gefund. Konz.	Differenz	% Diff.	(S)IR	Std-Abw	Gewicht
Blank	0,00000	-11,590	-11,6	0,000	-0,58457	0,010	1
KalibStd-1	50,000	40,370	-9,63	-19,3	6,3074	0,093	1
KalibStd-2	100,00	84,879	-15,1	-15,1	12,211	0,705	1
KalibStd-3	250,00	244,63	-5,37	-2,15	33,401	0,693	1
KalibStd-4	500,00	537,97	38,0	7,59	72,309	0,319	1
KalibStd-5	1000,0	1016,7	16,7	1,67	135,80	3,10	1
KalibStd-6	2500,0	2487,1	-12,9	-0,517	330,84	8,18	1

Example for ICP-OES raw data printout - used for determination of total Ag-concentration

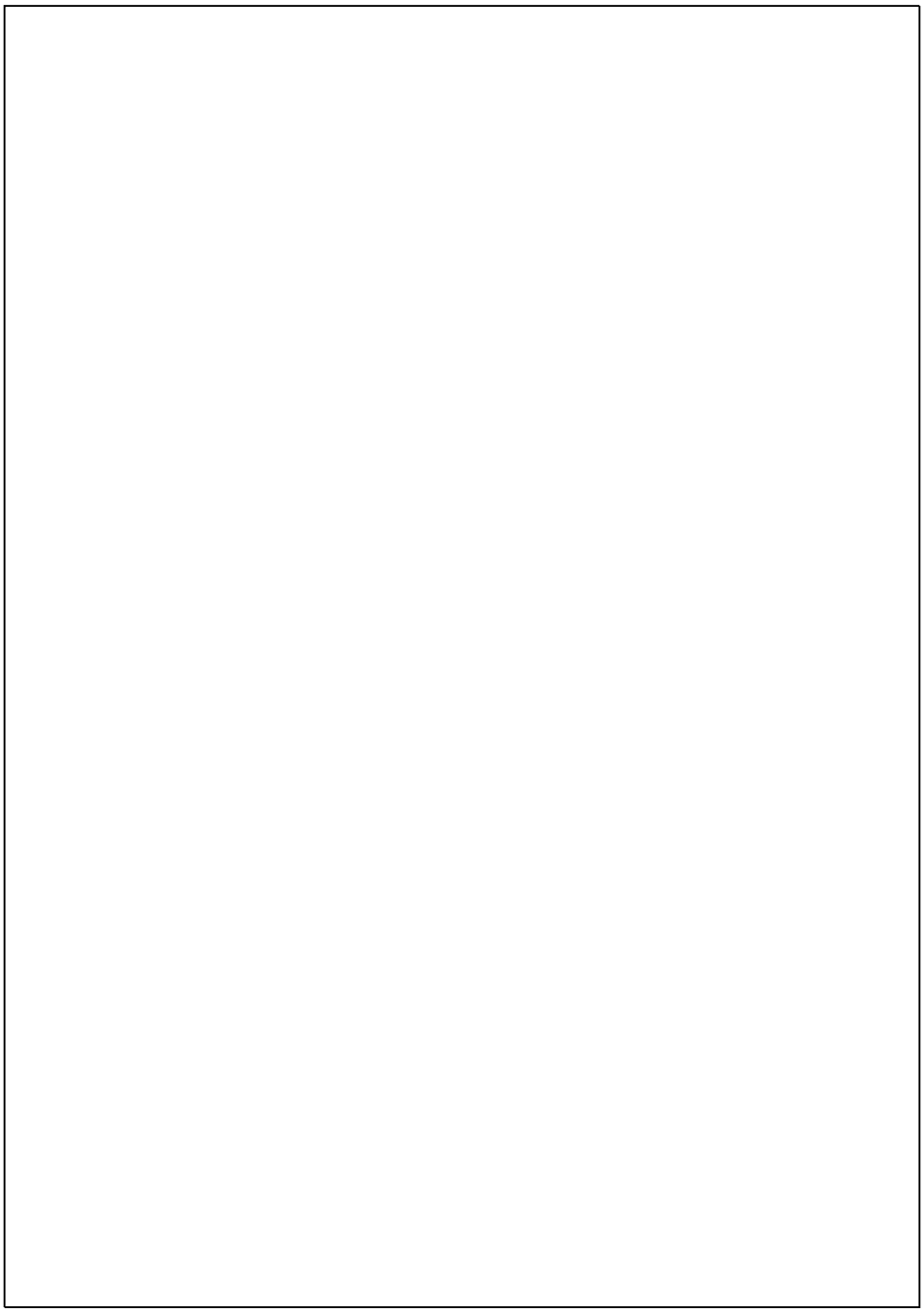
Example printout from the measurement performed on July 14, 2011.

21	Pro: 10mg/kg Boden a 07/14/2011 15:09:42 KONZ			
	Custom ID1:	Custom ID2:	Custom ID3:	
	Ti3349	Ti3361	Ti3372	
Einheit	µg/L	µg/L	µg/L	
Mittel	422,4	421,9	424,2	
StdAbw	7,7	7,1	7,4	
% RSD	1,825	1,691	1,736	
Mess.#1	427,1	425,7	429,0	
Mess.#2	426,6	426,3	427,8	
Mess.#3	413,5	413,6	415,7	
22	Pro: 10mg/kg Boden b 07/14/2011 15:11:55 KONZ			
	Custom ID1:	Custom ID2:	Custom ID3:	
	Ti3349	Ti3361	Ti3372	
Einheit	µg/L	µg/L	µg/L	
Mittel	326,5	324,8	324,4	
StdAbw	3,7	3,4	4,2	
% RSD	1,139	1,060	1,288	
Mess.#1	329,3	328,1	328,4	
Mess.#2	328,0	325,3	324,7	
Mess.#3	322,3	321,2	320,0	
23	Pro: 20mg/kg Futter a 07/14/2011 15:14:08 KONZ			
	Custom ID1:	Custom ID2:	Custom ID3:	
	Ti3349	Ti3361	Ti3372	
Einheit	µg/L	µg/L	µg/L	
Mittel	580,0	579,6	580,5	
StdAbw	7,2	8,0	9,1	
% RSD	1,236	1,382	1,559	
Mess.#1	588,2	588,7	590,8	
Mess.#2	576,7	576,3	576,6	
Mess.#3	575,1	573,8	574,1	
24	Pro: 20mg/kg Futter b 07/14/2011 15:16:21 KONZ			
	Custom ID1:	Custom ID2:	Custom ID3:	
	Ti3349	Ti3361	Ti3372	
Einheit	µg/L	µg/L	µg/L	
Mittel	614,7	612,4	613,0	
StdAbw	4,6	4,9	4,5	
% RSD	0,7547	0,8075	0,7384	
Mess.#1	609,5	607,0	607,8	
Mess.#2	618,4	616,6	616,2	
Mess.#3	616,2	613,6	615,0	

21.1.2 Raw data examples: total Ag

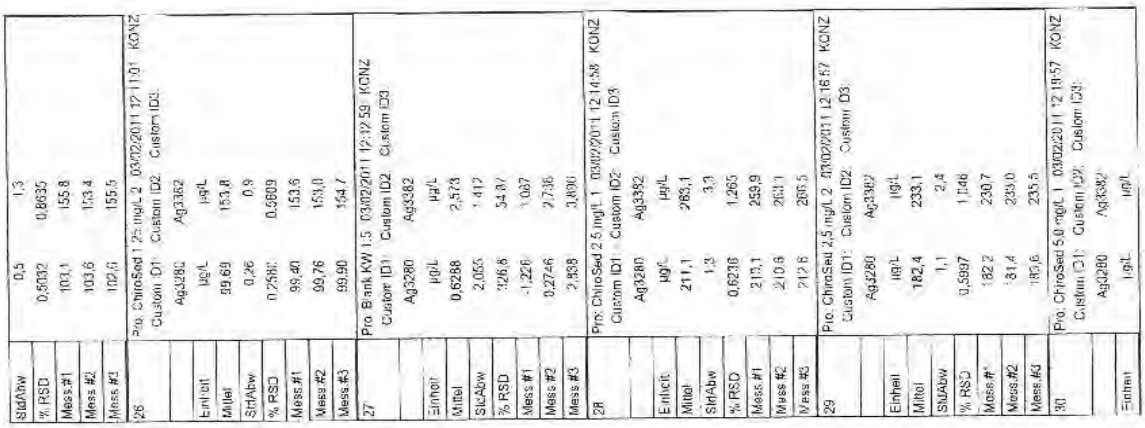
Example for ICP-OES calibration - applied for determination of total Ag-concentration

Calibration data from the measurement performed on March 2, 2011.

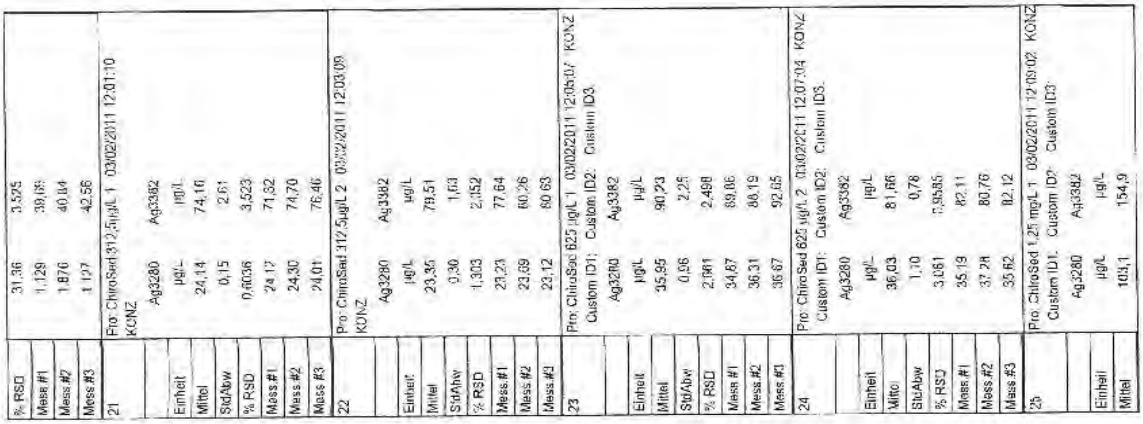


Example for ICP-OES raw data printout - used for determination of total Ag-concentration

Example printout from the measurement performed on March 2, 2011.



StdAbw	0,5	1,3
% RSD	0,5032	0,8635
Mess #1	103,1	155,8
Mess #2	103,6	153,4
Mess #3	102,6	155,5
26	Pro: ChiroSed 1,25 mg/L 2	03/02/2011 12:11:01 KONZ
	Custom ID1:	Custom ID2: Custom ID3:
	Ag3280	Ag3382
Einheit	µg/L	µg/L
Mittel	59,69	153,8
StdAbw	0,26	0,9
% RSD	0,4388	0,5809
Mess #1	99,40	153,6
Mess #2	99,76	153,0
Mess #3	99,90	154,7
27	Pro: Blank KW 1,5	03/02/2011 12:12:59 KONZ
	Custom ID1:	Custom ID2: Custom ID3:
	Ag3280	Ag3382
Einheit	µg/L	µg/L
Mittel	0,6288	2,573
StdAbw	2,055	1,412
% RSD	326,8	54,87
Mess #1	-1,226	1,087
Mess #2	0,2746	2,736
Mess #3	2,938	3,896
28	Pro: ChiroSed 2,5 mg/L 1	03/02/2011 12:14:58 KONZ
	Custom ID1:	Custom ID2: Custom ID3:
	Ag3280	Ag3382
Einheit	µg/L	µg/L
Mittel	211,1	263,1
StdAbw	1,3	3,3
% RSD	0,6216	1,265
Mess #1	213,1	259,9
Mess #2	210,6	263,1
Mess #3	212,6	266,5
29	Pro: ChiroSed 2,5 mg/L 2	03/02/2011 12:16:57 KONZ
	Custom ID1:	Custom ID2: Custom ID3:
	Ag3280	Ag3382
Einheit	µg/L	µg/L
Mittel	182,4	233,1
StdAbw	1,1	2,4
% RSD	0,5997	1,046
Mess #1	182,2	230,7
Mess #2	181,4	233,0
Mess #3	183,6	235,5
30	Pro: ChiroSed 5,0 mg/L 1	03/02/2011 12:18:57 KONZ
	Custom ID1:	Custom ID2: Custom ID3:
	Ag3280	Ag3382
Einheit	µg/L	µg/L

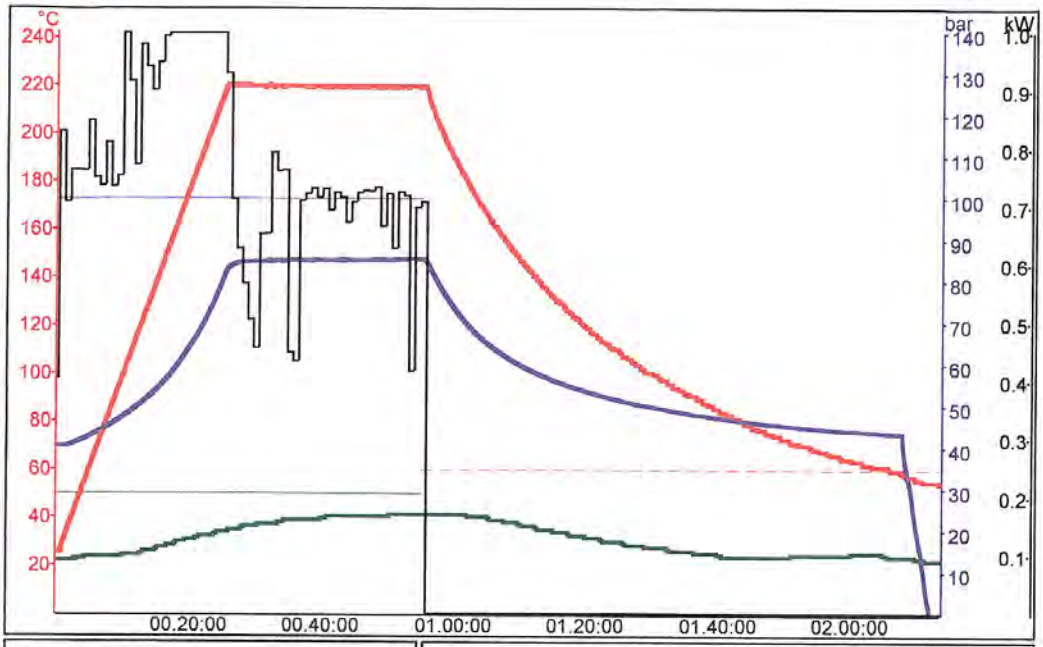


% RSD	31,36	3,525
Mess #1	1,129	39,05
Mess #2	1,876	40,04
Mess #3	1,197	42,56
21	Pro: ChiroSed 312,5µg/L 1	03/02/2011 12:01:10
	KONZ	
	Ag3280	Ag3382
Einheit	µg/L	µg/L
Mittel	24,14	74,16
StdAbw	0,15	2,61
% RSD	0,6036	3,523
Mess #1	24,17	71,32
Mess #2	24,30	74,70
Mess #3	24,01	76,46
22	Pro: ChiroSed 312,5µg/L 2	03/02/2011 12:03:09
	KONZ	
	Ag3280	Ag3382
Einheit	µg/L	µg/L
Mittel	23,35	79,51
StdAbw	0,30	1,63
% RSD	1,303	2,052
Mess #1	23,23	77,64
Mess #2	23,69	80,26
Mess #3	23,12	80,63
23	Pro: ChiroSed 625 µg/L 1	03/02/2011 12:05:07 KONZ
	Custom ID1:	Custom ID2: Custom ID3:
	Ag3280	Ag3382
Einheit	µg/L	µg/L
Mittel	35,95	90,93
StdAbw	0,96	2,25
% RSD	2,661	2,498
Mess #1	34,87	89,86
Mess #2	36,31	88,19
Mess #3	36,67	92,65
24	Pro: ChiroSed 625 µg/L 2	03/02/2011 12:07:04 KONZ
	Custom ID1:	Custom ID2: Custom ID3:
	Ag3280	Ag3382
Einheit	µg/L	µg/L
Mittel	36,03	81,66
StdAbw	1,10	0,78
% RSD	3,061	1,0585
Mess #1	35,19	82,11
Mess #2	37,24	80,76
Mess #3	35,62	82,12
25	Pro: ChiroSed 1,25 mg/L 1	03/02/2011 12:09:02 KONZ
	Custom ID1:	Custom ID2: Custom ID3:
	Ag3280	Ag3382
Einheit	µg/L	µg/L
Mittel	103,1	154,9

Printout of microwave program - used for determination of total Ag concentration

"NanoAg110127.dpr"

Erstellt von "Administrator"
27.01.2011 17:32:38



Parameter "UPB.mpr"

Stirrer	:	0 %
Start-Parameter	:	Aktuelle Werte
Ladedruck	:	40.0bar
Behälterkühlung	:	aktiviert
über	:	30°C
Druck nach Lauf	:	ablassen
Ablassrate	:	10bar/min
Temperatur unter	:	60°C
Behälter nach Lauf	:	öffnen
Rack	:	
Behälter-Typ	:	
Behälter-Zahl	:	1
Kühlung	:	

MW-Programm "UPB.mpr"

Nr	t	T1	T2	P	E
1	00:25:00	220°C	50°C	100.0bar	1000W
2	00:30:00	220°C	50°C	100.0bar	1000W

Grundlast

Bemerkungen

Nano-Ag

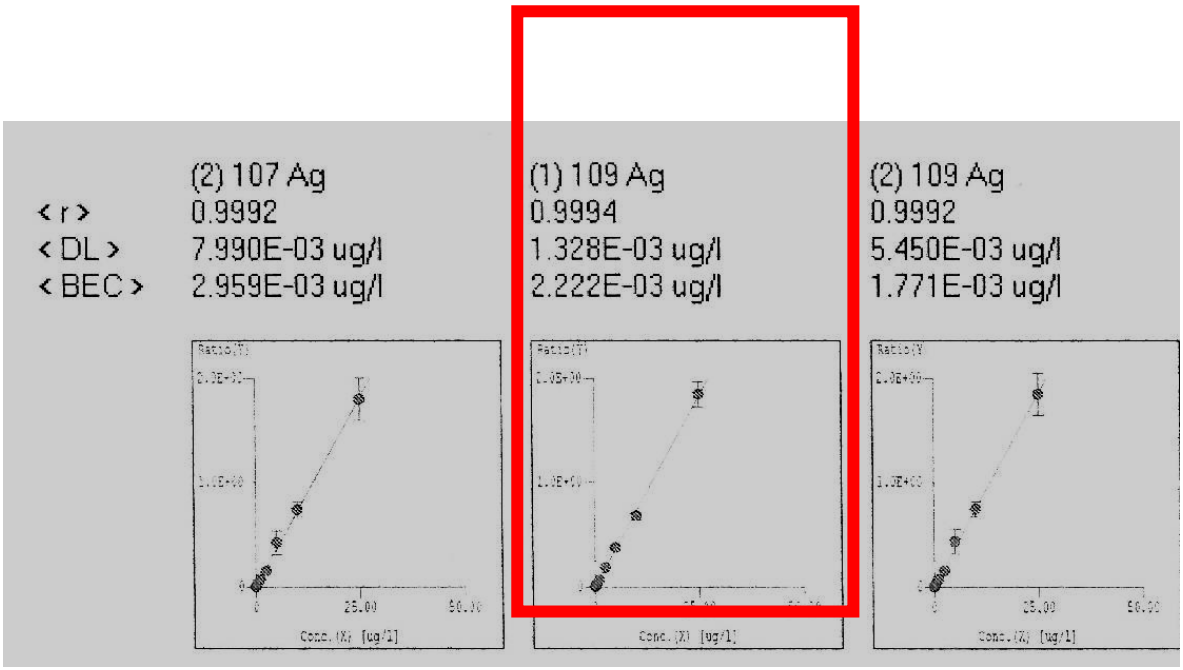
21.1.3 Raw data examples: Ag^+ (DGTs)

ICP-MS calibration - applied for determination of ion concentrations (DGTs)

Calibration data from the measurement performed on March 4, 2011. Calibration solutions used: 0.25 µg/L, 0.50 µg/L, 1.0 µg/L, 2.5 µg/L, 5.0 µg/L, 10 µg/L and 25 µg/L.

<r>: correlation coefficient

<DL> detection limit, limit of detection (LOD)



used for calculation

Example for ICP-MS raw data printout - used for determination of ion concentrations (DGTs)

Example printout from the measurement performed on March 4, 2011.

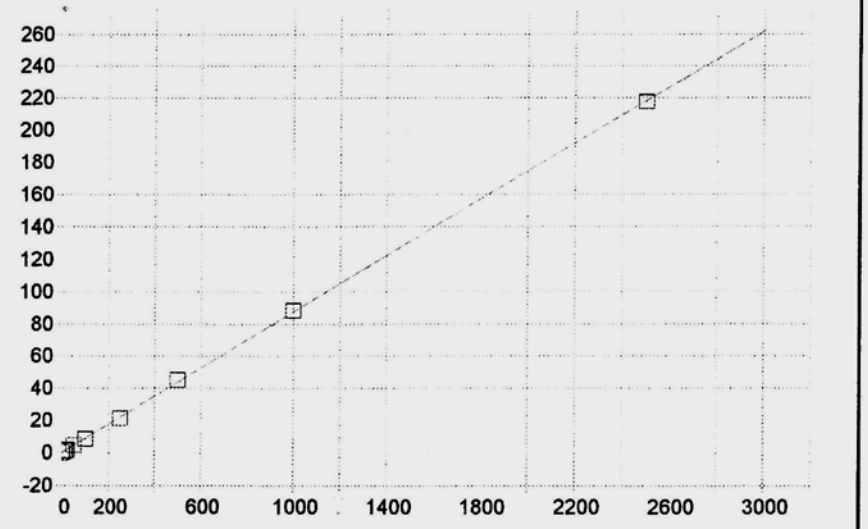
Quantitation Report - Summary

```
File Name      : SMPL010.D#
File Path      : C:\ICPCHEM\1\DATA\11C04L00.B\SMPL010.D\
Method         : C:\ICPCHEM\1\METHODS\Ag_DGT.M
Calibration    : C:\ICPCHEM\1\CALIB\AG_DGT.C
Acq Time       : Mar 4 2011  12:57 pm
Sample Name    : Chiro 0,3125mg B d28
Sample Type    : Sample
Comments       :
Prep Dilution  : 1.000
Auto Dilution  : Undiluted
Total Dilution : 1.000
Operator Name:
Acq Mode       : Spectrum
Cal Title      : DGT-Ag-Chiro-Regenw110304
Cal Type       : External Calibration Method
Last Calib     : Mar 04 2011  12:10 pm
Bkg File       : --------
Bkg Rejected Masses: --------
Interference Correction : ON
Blank File     : --------
VIS Fit        : Linear
Weighting Method: 1/(SD*SD)
Multi Tune     : #1 nogas.u
                 #2 he.u
```

Element	Mass	ISTD	Tune	CPS or Ratio		Conc.		RSD(%)	Time(sec)	Rep	VIS
Co	59	103	#2	0.007303254	P	1.119E-01	ug/l	1.92	0.50	3	
Ni	60	103	#1	0.01853044	P	6.514E-01	ug/l	3.12	0.20	3	
Ni	60	103	#2	0.009726885	P	4.513E-01	ug/l	14.27	0.20	3	
Rh	103		#1	407,511.6	P	[1.000	]	---	0.20	[3]	
Rh	103		#2	48,256.16	P	[1.000	]	---	0.20	[3]	
Ag	107	103	#1	9.491167E-4	P	1.005E-02	ug/l	11.22	0.20	3	
Ag	107	103	#2	0.001106351	P	1.228E-02	ug/l	6.50	0.20	3	
Ag	109	103	#1	8.832942E-4	P	9.857E-03	ug/l	13.22	0.20	3	
Ag	109	103	#2	0.001009237	P	1.182E-02	ug/l	23.55	0.20	3	

Example for ICP-OES calibration

Calibration data from the measurement performed on March 29, 2010.



Ag 328,068 (102)

Regress.-Dat: 03/29/2010 13:42:52		Regress.-Typ	Linear	Wichtung: Keine
A0 (Schnittp.):	0,212849		Rekalibration	1,000000
A1 (Anstieg):	0,087172		Y-Schnittp:	0,000000
A2 (Krümmung):	0,000000			
n (Exponent):	1,000000			
Korrelation:	0,999956		Status:	OK.
Verfahr.-StdAbw:	8,216515			
NG:	24,649544			
BG:	82,165147			

Std.-Name	Gesetzte Konz.	Gefund. Konz.	Differenz	% Diff.	(S)IR	Std-Abw	Gewicht
Blank	0,00000	-2,6222	-2,62	0,000	-0,01574	0,040	1
KalibStd-1	10,000	7,4661	-2,53	-25,3	0,86368	0,058	1
KalibStd-2	20,000	17,338	-2,66	-13,3	1,7243	0,042	1
KalibStd-3	50,000	50,389	0,389	0,777	4,6053	0,076	1
KalibStd-4	100,00	95,055	-4,94	-4,94	8,4990	0,072	1
KalibStd-5	250,00	243,04	-6,96	-2,78	21,399	0,052	1
KalibStd-6	500,00	515,34	15,3	3,07	45,136	0,125	1
KalibStd-7	1000,0	1010,2	10,2	1,02	88,278	0,109	1
KalibStd-8	2500,0	2493,8	-6,25	-0,250	217,60	0,180	1

Example for ICP-OES raw data printout

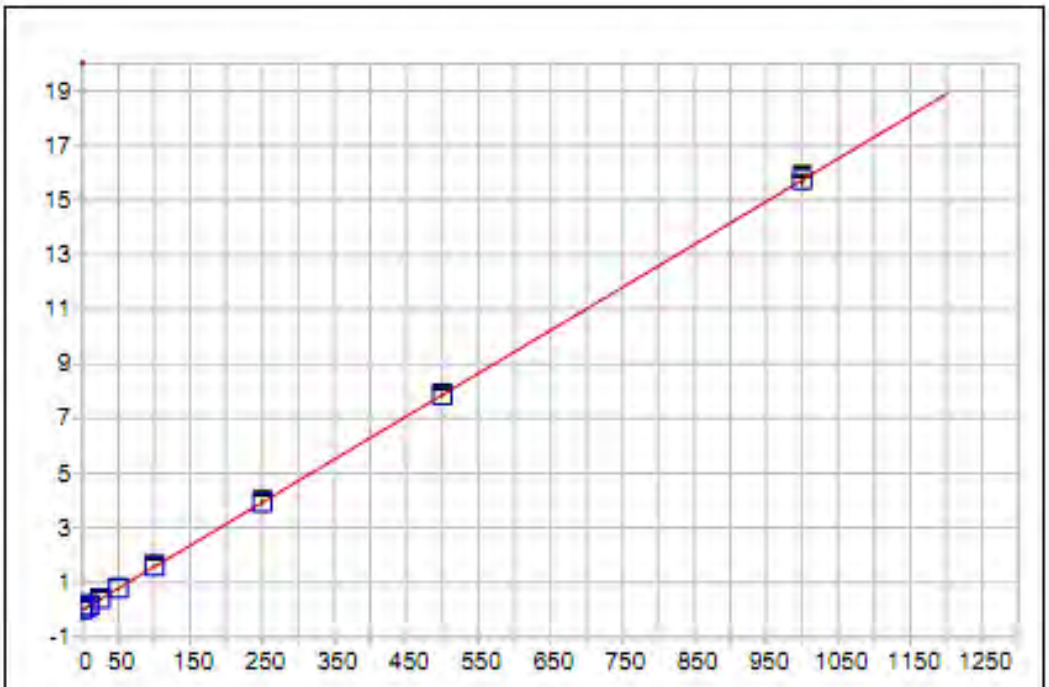
Example printout from the measurement performed on March 29, 2010.

Mess.#2	117,2
Mess.#3	116,9
36	Pro: 5.5 1:2 03/29/2010 12:34:45 KONZ Custom ID1: Custom ID2: Custom ID3:
	Ag3280
Einheit	µg/L
Mittel	131,7
StdAbw	0,5
% RSD	0,3734
Mess.#1	131,1
Mess.#2	131,9
Mess.#3	132,1
37	Pro: 5.6 1:2 03/29/2010 12:36:47 KONZ Custom ID1: Custom ID2: Custom ID3:
	Ag3280
Einheit	µg/L
Mittel	109,4
StdAbw	0,8
% RSD	0,7161
Mess.#1	108,5
Mess.#2	109,7
Mess.#3	110,0
38	Pro: Blank 03/29/2010 12:38:48 KONZ Custom ID1: Custom ID2: Custom ID3:
	Ag3280
Einheit	µg/L
Mittel	-2,707
StdAbw	0,533
% RSD	19,68
Mess.#1	-3,015
Mess.#2	-2,092
Mess.#3	-3,014
39	Pro: 6.1 1:2 03/29/2010 12:40:50 KONZ Custom ID1: Custom ID2: Custom ID3:
	Ag3280
Einheit	µg/L
Mittel	73,76
StdAbw	0,69
% RSD	0,9342
Mess.#1	73,15
Mess.#2	74,51
Mess.#3	73,63
40	Pro: 6.2 1:2 03/29/2010 12:42:52 KONZ Custom ID1: Custom ID2: Custom ID3:

21.1.4 Raw data examples: total Au

Example for ICP-OES calibration - applied for determination of total Au-concentration

Calibration data from the measurement performed on January 19, 2012.



Au 197,819 (170)

Regress.-Dat: 01/19/2012 16:15:19 Regress.-Typ Linear Wichtung: Keine

A0 (Schnittp.):	-0,013429	Rekalibration	1,000000
A1 (Anstieg):	0,015756	Y-Schnittp:	0,000000
A2 (Krümmung):	0,000000		
n (Exponent):	1,000000		
Korrelation:	0,999997	Status:	OK
Verfahr.-StdAbw:	0,904016		
NG:	2,712048		
BG:	9,040160		

Std.-Name	Gesetzte Konz.	Gefund. Konz.	Differenz	% Diff.	(S)IR	Std-Abw	Gewicht
Blank	0,00000	0,88433	0,884	0,000	0,00051	0,013	1
KalibStd-1	5,0000	3,7356	-1,26	-25,3	0,04544	0,009	1
KalibStd-2	10,000	9,8877	-0,112	-1,12	0,14236	0,029	1
KalibStd-3	25,000	24,650	-0,350	-1,40	0,37501	0,024	1
KalibStd-4	50,000	49,489	-0,511	-1,02	0,76643	0,009	1
KalibStd-5	100,00	101,69	1,69	1,69	1,5890	0,048	1
KalibStd-6	250,00	249,91	-0,087	-0,035	3,9247	0,077	1
KalibStd-7	500,00	499,71	-0,287	-0,057	7,8612	0,056	1
KalibStd-8	1000,0	1000,0	0,038	0,004	15,745	0,165	1

Example for ICP-OES raw data printout - used for determination of total Au-concentration

Example printout from the measurement performed on January 19, 2012.

12	Pro: NIST 8011 01/19/2012 14:05:08 KONZ		
	Custom ID1:	Custom ID2:	Custom ID3:
	Au1978	Au2082	Au2427
Einheit	µg/L	µg/L	µg/L
Mittel	**200,6**	**202,1**	**199,4**
StdAbw	2,4	1,3	4,9
% RSD	1,186	0,6458	2,459
Mess.#1	199,1	200,7	194,2
Mess.#2	199,5	203,3	203,9
Mess.#3	203,4	202,3	200,0
13	Pro: NIST 8011 1:250 01/19/2012 14:08:16 KONZ		
	Custom ID1:	Custom ID2:	Custom ID3:
	Au1978	Au2082	Au2427
Einheit	µg/L	µg/L	µg/L
Mittel	**199,0**	**203,5**	**199,4**
StdAbw	0,9	3,8	4,7
% RSD	0,4742	1,843	2,358
Mess.#1	198,1	199,5	197,0
Mess.#2	198,8	204,2	204,8
Mess.#3	200,0	206,9	196,4
14	Pro: Blank 10 % KW 01/19/2012 14:11:23 KONZ		
	Custom ID1:	Custom ID2:	Custom ID3:
	Au1978	Au2082	Au2427
Einheit	µg/L	µg/L	µg/L
Mittel	**0,8122**	**2,320**	**1,212**
StdAbw	1,086	1,088	6,923
% RSD	133,7	46,91	571,2
Mess.#1	-0,3253	3,480	8,892
Mess.#2	1,838	2,160	-4,547
Mess.#3	0,9243	1,321	-0,7095
15	Pro: ChiroSed Kontrolle a 01/19/2012 14:14:31 KONZ		
	Custom ID1:	Custom ID2:	Custom ID3:
	Au1978	Au2082	Au2427
Einheit	µg/L	µg/L	µg/L
Mittel	**-4,542**	**76,05**	**4,730**
StdAbw	1,781	3,19	7,207
% RSD	39,22	4,194	152,4
Mess.#1	-2,595	72,47	-2,632
Mess.#2	-6,091	78,60	11,77
Mess.#3	-4,940	77,07	5,052

21.1.5 Certificates of reference material and standard: Ti

Certified reference material TMDA70

Environment Canada Environnement Canada

Eingang: 28.09.10 - 4 x 500ml

CERTIFIED REFERENCE MATERIAL

TMDA-70, lot 0310

A high level fortified standard for trace elements

Trace element standards are made in filtered and diluted Lake Ontario water and are preserved with 0.2% nitric acid. This fortified bulk CRM has concentrations in the high range and is designed for calibration checks. Trace element reference materials are noted for their integrity and consistency, and are monitored in additional Proficiency Testing (PT) studies. "For Information" values indicate insufficient data exists to meet CRM certification criteria. The values and statistics for this CRM are derived from PT studies 83, 85, 87, 90, 93, and 95 dated March 2004, March 2005, January 2006, September 2007, March 2009, and March 2010 respectively. A more detailed report on the methods used in our PT studies for specific parameters is available upon request. Please note that expiry dates of 1 year from the date of shipping are not indicative of sample stability, but rather of sample transport, handling and storage. We strongly recommend that the CRM be tightly capped and refrigerated immediately after use.

Measurand	Value[a] in µg/L	±2σ[b]	C.I.[c]	Studies / Results (N)
Aluminum	415	38.3	2.79	6 / 181
Antimony	21.7	2.45	0.2	6 / 151
Arsenic	40.7	4.39	0.32	6 / 179
Barium	309	23.9	1.76	6 / 177
Beryllium	15.1	1.54	0.12	6 / 157
Bismuth	13.5	5.49	0.63	6 / 74
Cadmium	145	12.2	0.84	6 / 203
Chromium	389	31.9	2.3	6 / 192
Cobalt	285	23.2	1.7	6 / 181
Copper	398	32.4	2.3	6 / 196
Iron	368	37.1	2.7	6 / 183
Lead	444	37.6	2.7	6 / 193
Lithium	21.6	3.2	0.34	6 / 89
Manganese	302	23	1.6	6 / 191
Molybdenum	259	17.0	1.3	6 / 160
Nickel	327	28.1	2	6 / 192
Selenium	25.8	3.96	0.31	6 / 161
Silver	10.9	1.5	0.13	6 / 138
Strontium	441	32.7	2.5	6 / 171
Thallium	20	1.92	0.18	6 / 110
Tin	19.5	2.1	0.2	6 / 106
Uranium	55.9	5.24	0.45	6 / 129
Vanadium	312	21.7	1.6	6 / 171
Zinc	477	44.8	3.1	6 / 198
For information				
Boron	16	99 Results		
Rubidium	0.65	41 Results		
Titanium	0.39	46 Results		

[a] Outliers of > 3 std. dev. excluded and are calculated with 'Robust Analysis' Annex C, ISO DIS 13528:2005(E).
[b] 2-sigma limit for an individual measurement.
[c] 95% confidence interval on the population mean (σ x 1.96) ÷ √ N.

Last Updated: March 2010

Canada

Certificate of BCR-142R (in extracts, 2 pages)

EUROPEAN COMMISSION
JOINT RESEARCH CENTRE
Institute for Reference Materials and Measurements

Institute for Reference Materials and Measurements

CERTIFIED REFERENCE MATERIAL
BCR® – 142R

CERTIFICATE OF ANALYSIS

LIGHT SANDY SOIL			
Element	Mass fraction based on dry mass		Number of accepted sets of results p
	Certified value [1] [mg/kg]	Uncertainty [2] [mg/kg]	
Total content			
Cd	0.34	0.04	4
Co	12.1	0.7	6
Cu	69.7	1.3	8
Pb	40.2	1.9	4
Mn	970	16	9
Hg	0.067	0.011	6
Ni	64.5	2.5	7
Element	Mass fraction based on dry mass		Number of accepted sets of results p
	Certified value [3] [mg/kg]	Uncertainty [2] [mg/kg]	
Aqua regia soluble content			
Cd	0.249	0.010	4
Pb	25.7	1.6	7
Ni	61.1	1.5	9
Zn	93.3	2.7	9

1) Unweighted mean value of the means of p accepted sets of data, each set being obtained in a different laboratory and/or with a different method of determination. The certified values are traceable to the SI.

2) Half-width of the 95 % confidence interval of the mean defined in [1] or [3].

3) Unweighted mean value of the means of p accepted sets of data, each set being obtained in a different laboratory and/or with a different method of determination. The certified values are traceable to the aqua regia extraction method as described in the report (DIN 38414-S7).

Indicative Values

Element	Mass fraction	
	Indicative value [1] [mg/kg]	Uncertainty [2] [mg/kg]
Total content		
Zn	101	6
Aqua regia soluble content		
Co	10.2	0.6
Cu	69.8	1.0
Mn	800	50

[1] Mean value
[2] Standard deviation

Additional Material Information

Major Compounds	Mass fraction [g/kg]
SiO_2	620.5
CaO	93.5
MgO	15.0
Al_2O_3	75.0
TiO_2	4.5
Fe_2O_3	25.0
P_2O_5	3.25
K_2O	3.5

DESCRIPTION OF THE SAMPLE

The sample consists of about 50 g of powdered sandy soil (particles have passed a sieve with apertures < 90 µm) in brown glass bottles provided with a polyethylene insert and a screw cap. Additional information on the major composition, the preparation, the certified and the indicative values is given in the certification report.

ANALYTICAL METHOD USED FOR CERTIFICATION

A wide range of sample treatment methods was applied as necessary: amongst others digestion with mixtures of oxidising acids; addition of HF was mandatory for complete digestion of the material.

Methods of final determination were:

- Cold vapour atomic absorption spectrometry
- Cold vapour atomic fluorescence spectrometry
- Direct current plasma emission spectrometry
- Electrothermal atomic absorption spectrometry
- Energy dispersive X-ray fluorescence spectrometry
- Flame atomic absorption spectrometry
- Inductively coupled plasma atomic emission spectrometry
- Inductively coupled plasma mass spectrometry
- Instrumental neutron activation analysis
- Isotope dilution mass spectrometry
- Neutron activation analysis with radiochemical separation

21.1.6 Certificates of reference material and standard: Ag

Certificate of CRM026-050

Eingang 16.04.10 JH (2x)

Certificate of Analysis

NATURAL MATRIX CERTIFIED REFERENCE MATERIAL

Catalog No: CRM026-050
Lot No: BE026

METALS ON SOIL

ANALYTE CONCENTRATIONS

Element	Reference Value	S.D.	Confidence Interval	Prediction Interval
Aluminum, Al	17,730	5,772	14,611 - 20,848	4,962 - 30,497
Antimony, Sb	(<3.2)			
Arsenic, As	5.41	2.49	4.64 - 6.19	0.481 - 10.3
Barium, Ba	214	20.9	205 - 224	169 - 260
Beryllium, Be	18.0	1.99	17.0 - 19.0	13.7 - 22.3
Boron, B	(25.4)			
Cadmium, Cd	11.7	1.81	10.8 - 12.6	7.77 - 15.6
Calcium, Ca	6,221	738	5,798 - 6,644	4,573 - 7,870
Chromium, Cr	27.2	6.89	24.2 - 30.2	12.4 - 42.0
Cobalt, Co	6.77	1.41	6.02 - 7.52	3.68 - 9.86
Copper, Cu	18.8	1.62	17.3 - 19.7	14.8 - 22.7
Iron, Fe	21,906	3,187	20,153 - 23,658	14,849 - 28,962
Lead, Pb	25.6	3.93	23.5 - 27.7	17.0 - 34.2
Magnesium, Mg	2,837	801	2,421 - 3,254	1,058 - 4,617
Manganese, Mn	633	64.5	600 - 666	493 - 774
Mercury, Hg	2.42	0.32	2.16 - 2.46	1.30 - 3.32
Molybdenum, Mo	(1.25)			
Nickel, Ni	14.4	3.22	12.7 - 16.0	7.38 - 21.4
Potassium, K	3,600	1,055	3,057 - 4,142	1,299 - 5,900
Selenium, Se	(1.86)			
Silicon, Si	(166)			
Silver, Ag	(0.57)			
Sodium, Na	119	28.4	104 - 134	55.0 - 182
Strontium, Sr	38.4	4.03	35.6 - 41.1	28.7 - 48.1
Thallium, Tl	(<4.8)			
Vanadium, V	32.0	9.83	27.5 - 36.5	10.7 - 53.3
Zinc, Zn	140	14.8	132 - 147	108 - 172

All values except pH are expressed in mg/Kg (parts per million) on a dry weight basis, values in ( ) are not certified and are listed for information only. The Reference Values were determined by USEPA SW846 (3rd edition) Methods 3050 and 6010, except for Mercury (Method 7471). The sample is suitable for other 3000-series metals digestion procedures and 7000-series spectroscopic methods. The Confidence Interval (C.I.) range is the 95% C.I. for the Reference Value. The Prediction Interval (P.I.) is the 95% P.I. around the Reference Value. Measurements should fall within the P.I. 19 of 20 times. The Certifed Reference Values were established through extensive interlaboratory testing. All values were calculated using the USEPA BIWEIGHT Method.

"THIS PRODUCT WAS DESIGNED, PRODUCED, AND VERIFIED FOR ACCURACY AND STABILITY IN ACCORDANCE WITH USEPA/AALA RM-03 AND ISO GUIDES 34 AND 35."

Certifying Officer

RTC
2931 Soldier Springs Road
Laramie, WY 82070
Phone: 800.576.5690 or 307.742.5452
Fax: 307.745.7936
Web: www.RT-Corp.com

Store at 10-30°C

MSDS Available Upon Request

Certified reference material TMDWS2

Environment Canada Environnement Canada

CERTIFIED REFERENCE MATERIAL

TM-DWS.2, lot 0809

A trace element fortified sample

Trace element standards are made in filtered and diluted Lake Ontario water and are preserved with 0.2% nitric acid. This fortified bulk CRM has concentration ranges appropriate to drinking water analyses. Trace element standards are noted for their integrity and consistency, and are monitored in additional Proficiency Testing (PT) studies. "For Information" values indicate insufficient data exists to meet CRM certification criteria. The values and statistics for this CRM are derived from PT studies 87, 89, 90, and 93 dated January 2006, January 2007, September 2007, and March 2009 respectively. A more detailed report on the methods used in our PT studies for specific parameters is available upon request. Please note that expiry dates of 1 year from the date of shipping are not indicative of sample stability, but rather of sample transport, handling and storage. We strongly recommend that the CRM be tightly capped and refrigerated immediately after use.

Measurand	Value[a] in µg/L	±2σ[b]	C.I.[c]	Studies / Results (N)
Aluminum	58.6	6.27	0.545	4 / 127
Antimony	3.23	0.485	0.0485	4 / 98
Arsenic	4.19	0.639	0.0582	4 / 118
Barium	147	9.65	0.839	4 / 127
Beryllium	13.4	1.34	0.122	4 / 117
Boron	81.4	8.34	0.861	4 / 92
Cadmium	4.21	0.549	0.0453	4 / 141
Chromium	44.4	3.62	0.300	4 / 140
Cobalt	64.4	5.01	0.429	4 / 131
Copper	168	14.1	1.15	4 / 146
Iron	224	25.0	2.12	4 / 133
Lead	7.84	1.07	0.0949	4 / 122
Lithium	20.2	2.26	0.295	4 / 59
Manganese	47.3	3.33	0.279	4 / 137
Molybdenum	66.9	5.21	0.479	4 / 116
Nickel	82.5	5.73	0.478	4 / 138
Selenium	8.68	1.51	0.145	4 / 107
Silver	9.97	0.932	0.0927	4 / 99
Strontium	244	16.5	1.45	4 / 124
Thallium	8.34	0.847	0.0968	4 / 75
Tin	12.2	1.31	0.150	4 / 75
Titanium	15.2	1.25	0.125	4 / 98
Uranium	14.2	1.08	0.118	4 / 82
Vanadium	44.4	3.73	0.337	4 / 120
Zinc	380	33.4	2.80	4 / 136
For information				
Bismuth	14.3	54 Results		
Gallium	0.045	15 Results		
Rubidium	0.42	28 Results		

[a] Outliers of > 3 std. dev. excluded and are calculated with 'Robust Analysis' Annex C, ISO DIS 13528:2005(E).
[b] 2-sigma limit for an individual measurement.
[c] 95% confidence interval on the population mean (σ x 1.96) ÷ √ N.

Last Updated: 11 August 2009

Canada

ICP standard solution for silver

Certificate of Analysis
CertiPUR® Reference Material

Silver ICP Standard 1000 mg/l Ag CertiPUR®

1.70352.0100 **Lot No.: HC936000**

This Certificate of Analysis is based on the data from the Merck Calibration Laboratory for ICP-OES, according to DIN EN ISO / IEC 17025.
Accredited by the DKD (Deutscher Kalibrierdienst).

DAR Reg.-No.: DKD-K-14302
Ref. Calibration Certificate: 338/DKD-K-14302/09-02

Composition: Silver nitrate in nitric acid Suprapur® 2-3%

Assay: **989 mg/kg** **Analysis: ICP-OES**
1002 mg/l (calculated)

Measurement Uncertainty: **± 4 mg/kg (± 0.4%)**
This value represents the expanded uncertainty (*U*) for a coverage probability of 95%. Refer to page 2 for further details.

Traceability: This ICP Standard has been measured applying high precision ICP-OES in comparison to the corresponding **NIST SRM® 3151, lot 992212**

Trace impurities µg/ml:

Ag	*	Cr	<0.02	In	<0.02	Ni	<0.02	Sb	<0.02	Tl	<0.02
Al	<0.05	Cu	<0.02	Ir	<0.02	Os	<0.20	Sc	<0.02	Tm	<0.02
As	<0.20	Dy	<0.02	K	<0.20	P	<0.20	Se	<0.20	U	<0.02
Au	<0.02	Er	<0.02	La	<0.02	Pb	<0.05	Si	<0.20	V	<0.02
B	<0.05	Eu	<0.02	Li	<0.02	Pd	<0.02	Sm	<0.02	W	<0.05
Ba	<0.02	Fe	<0.05	Lu	<0.02	Pr	<0.02	Sn	<0.02	Y	<0.02
Be	<0.02	Ga	<0.02	Mg	<0.02	Pt	<0.02	Sr	<0.02	Yb	<0.02
Bi	<0.20	Gd	<0.02	Mn	<0.02	Rb	<0.02	Ta	<0.05	Zn	<0.02
Ca	<0.05	Ge	<0.02	Mo	<0.02	Re	<0.02	Tb	<0.02	Zr	<0.02
Cd	<0.02	Hf	<0.02	Na	<0.10	Rh	<0.02	Te	<0.20		
Ce	<0.02	Hg	<0.02	Nb	<0.05	Ru	<0.02	Th	<0.02		
Co	<0.02	Ho	<0.02	Nd	<0.02	S	<0.20	Ti	<0.05		

Date of release: 2009-02-24

Minimum shelf life: 2012-02-28

A. Yildirim

Dipl.Ing.Ayfer Yildirim
(responsible laboratory manager quality control)

Multi element standard solution Merck IV

Certificate of Analysis
CertiPUR® Reference Material

ICP Multi Element Standard Solution IV CertiPUR®

1.11355.0100 **Lot.-No. HC957274**

This product is intended for use as a reference material in atomic spectrometry. It has been produced from hi purity salts, using gravimetric procedures. The concentration of the elements in this solution is analysed by IC OES real-time internal standardization using NIST standard reference materials for calibration.

Composition and concentration:			***NIST Standard Reference Material***
Ag (Silver)	1007 ± 10	mg/l	SRM 3151 Batch Code 992212
Al (Aluminium)	993 ± 10	mg/l	SRM 3101a Batch Code 060502
B (Boron)	999 ± 10	mg/l	SRM 3107 Batch Code 070514
Ba (Barium)	999 ± 10	mg/l	SRM 3104a Batch Code 070222
Bi (Bismuth)	999 ± 10	mg/l	SRM 3106 Batch Code 991212
Ca (Calcium)	992 ± 10	mg/l	SRM 3109a Batch Code 050825
Cd (Cadmium)	990 ± 10	mg/l	SRM 3108 Batch Code 060531
Co (Cobalt)	997 ± 10	mg/l	SRM 3113 Batch Code 000630
Cr (Chromium)	1004 ± 10	mg/l	SRM 3112a Batch Code 030730
Cu (Copper)	995 ± 10	mg/l	SRM 3114 Batch Code 011017
Fe (Iron)	999 ± 10	mg/l	SRM 3126a Batch Code 051031
Ga (Gallium)	995 ± 10	mg/l	SRM 3119a Batch Code 890709
In (Indium)	1010 ± 10	mg/l	SRM 3124a Batch Code 991219
K (Potassium)	998 ± 10	mg/l	SRM 3141a Batch Code 051220
Li (Lithium)	995 ± 10	mg/l	SRM 3129a Batch Code 000505
Mg (Magnesium)	1000 ± 10	mg/l	SRM 3131a Batch Code 050302
Mn (Manganese)	1005 ± 10	mg/l	SRM 3132 Batch Code 050429
Na (Sodium)	989 ± 10	mg/l	SRM 3152a Batch Code 010728
Ni (Nickel)	999 ± 10	mg/l	SRM 3136 Batch Code 000612
Pb (Lead)	994 ± 10	mg/l	SRM 3128 Batch Code 030721
Sr (Strontium)	996 ± 10	mg/l	SRM 3153a Batch Code 990906
Tl (Thallium)	995 ± 10	mg/l	SRM 3158 Batch Code 993012
Zn (Zinc)	994 ± 10	mg/l	SRM 3168a Batch Code 080123

Matrix: Nitric acid 1 mol/l
Density: 1.090 g/cm³ (20°C)
Package: 100 ml PE-bottles

It is recommended to shake the solution thoroughly prior to use. Never pipet directly from the bottle. Prepare working solutions upon requirement by dilution with 1 molar nitric acid.

Date of release: ***2009-11-12***

Minimum shelf life: ***2012-11-30***

A. Yildirim

Dipl.-Ing. Ayfer Yildirim
(responsible laboratory manager quality control)

21.1.7 Certificates of reference material and standard: Au

Reference material NIST 8011 (extracted pages 1, 2)

National Institute of Standards & Technology

Report of Investigation

Reference Material 8011

Gold Nanoparticles, Nominal 10 nm Diameter

This Reference Material (RM) is intended primarily to evaluate and qualify methodology and/or instrument performance related to the physical/dimensional characterization of nanoscale particles used in pre-clinical biomedical research. The RM may also be useful in the development and evaluation of in vitro assays designed to assess the biological response (e.g., cytotoxity, hemolysis) of nanomaterials, and for use in interlaboratory test comparisons. RM 8011 consists of nominally 5 mL of citrate-stabilized Au nanoparticles in an aqueous suspension, supplied in hermetically sealed pre-scored glass ampoules sterilized by gamma irradiation. A unit consists of two 5 mL ampoules. The suspension contains primary particles (monomers) and a small percentage of clusters of primary particles.

Expiration of Material: The reference values for RM 8011 are valid, within the measurement uncertainties specified, until **31 December 2012**, provided the RM is handled in accordance with the instructions given in this report (see "Instructions for Use"). However, the size distribution may be altered and the RM invalidated if the material is contaminated or handled improperly.

Maintenance of Reference Values: NIST will monitor representative samples from this RM lot over the period of its validity. If substantive changes occur that affect the reference values before the expiration date, NIST will notify the purchaser. Registration (see attached sheet) will facilitate notification.

The overall technical coordination for material procurement, processing and measurement activities was conducted by V.A. Hackley and J.F. Kelly of the NIST Ceramics Division.

Reference and informational value measurements were performed at NIST by the following: NIST Analytical Chemistry Division: T.A. Butler, R. Case, K.W. Pratt, L.C. Sander and M.R. Winchester. NIST Ceramics Division: A.J. Allen, T.J. Cho, J. Grobelny, V.A. Hackley, D.-I. Kim and P. Namboodiri. NIST Metallurgy Division: J.E. Bonevich and A.J. Shapiro. NIST Polymers Division: M.L. Becker, D.L. Ho, A. Karim and B.M. Vogel. NIST Precision Engineering Division: B. Ming and A.E. Vladár. NIST Process Measurements Division: L.F. Pease III, M.J. Tarlov, D.H. Tsai, M.R. Zachariah and R.A. Zangmeister.

Statistical consultation on measurement design and analysis of the reference value data were performed by A.I. Avilés of the NIST Statistical Engineering Division.

Additional technical and coordination aspects were provided by the following: R.F. Cook, W.K. Haller and D.L. Kaiser of the NIST Ceramics Division.

Support aspects involved in the preparation and issuance of this RM were coordinated through the NIST Measurement Services Division.

RM 8011 was developed at the request of the National Cancer Institute (NCI). Development and production costs were subsidized by NCI.

Debra L. Kaiser, Chief
Ceramics Division

Gaithersburg, MD 20899
Report Issue Date: 13 December 2007

Robert L. Watters, Jr., Chief
Measurement Services Division

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Reference Values: Reference values are a best estimate of the true value provided by NIST where all known or suspected sources of bias have not been fully investigated by NIST [1]. Dimensional reference values (mean particle diameter in solution, as an aerosol and deposited on a substrate) are reported and are based on the following measurement techniques: atomic force microscopy (AFM), scanning electron microscopy (SEM), transmission electron microscopy (TEM), electrospray-differential mobility analysis (ES-DMA), dynamic light scattering (DLS) and small-angle x-ray scattering (SAXS). The corresponding reference values and expanded uncertainties are provided in Table 1. A synopsis of the methods used to generate reference values is provided starting on page 6.

Table 1. Reference Value Mean Size and Expanded Uncertainty [a]
Average Particle Size (Diameter), in nm

Technique	Analyte Form	Particle Size (nm)
Atomic Force Microscopy	dry, deposited on substrate	8.5 ± 0.3
Scanning Electron Microscopy	dry, deposited on substrate	9.9 ± 0.1
Transmission Electron Microscopy	dry, deposited on substrate	8.9 ± 0.1
Differential Mobility Analysis	dry, aerosol	11.3 ± 0.1
Dynamic Light Scattering	liquid suspension	13.5 ± 0.1
Small-Angle X-ray Scattering	liquid suspension	9.1 ± 1.8

[a] The expanded uncertainties, U, are calculated as $U = ku_c$, where u_c is intended to represent, at the level of one standard deviation, the combined standard uncertainty calculated according to the ISO and NIST Guides [2]. The coverage factor, k, for 95 % expanded uncertainty intervals is based on a t multiplier with the appropriate associated degrees of freedom.

Information Values: Additional measurements and data were obtained to further characterize the material and are provided as information values. NIST information values are considered to be of interest to the RM user, but insufficient information is available to assess adequately the uncertainty associated with the values or a limited number of analyses were performed. Information values and associated measurement uncertainties for chemical and electrochemical properties unrelated to particle size are presented in Table 2. An optical absorbance spectrum and asymmetric-flow field flow fractionation (AFFF) trace are provided in Figures 1 and 2, respectively. Material sterility was assessed. Electron microscopy images are provided in Figure 3. Particle size histograms are provided in Figures 4 through 7.

Table 2. Information Value Mean and Measurement Uncertainty [a]
Chemical and Electrochemical Properties

Measurement	Value
Au mass fraction ($\mu g\ g^{-1}$) [b]	51.56 ± 0.23
Cl^- ion mass fraction ($\mu g\ g^{-1}$) [c]	35.0 ± 4.6
citrate ion mass fraction ($\mu g\ g^{-1}$) [c]	1.7 ± 0.4
Na mass fraction ($\mu g\ g^{-1}$) [d]	95
pH [e]	7.19 ± 0.33
electrolytic conductivity, κ ($\mu S\ cm^{-1}$) [f]	417.9 ± 7.2
zeta potential (mV) [g]	--

[a] For pH, conductivity and Au mass fraction, the expanded uncertainty (95 % confidence interval) is calculated according to the ISO and NIST Guides [2]. Other reported uncertainties are two times the standard deviation of replicate measurements.

[b] Au bound into nanoparticles was determined from separate measurements of total Au and Au dissolved in the solution matrix. Both measurements were made using inductively-coupled plasma optical emission spectrometry (ICP-OES). Total Au was measured after digestion of the particles with a mixture of nitric and hydrochloric acids. Solution matrix Au was measured after removal of Au particles by ultracentrifugation, and was undetectable at the 3σ detection limit corresponding to 0.07 $\mu g\ g^{-1}$ in the undiluted supernatant. The Au mass fraction in the matrix was estimated as 0.5 time the 3σ limit and subtracted from the total Au mass fraction to obtain the reported value for the bound Au mass fraction.

[c] Levels of Cl^- and citrate ($C_3H_5O(COO)_3^{3-}$) ions were determined in native suspensions by ion chromatography with a conductivity detector. Chloride and citrate ions were identified based on the retention times of reference standards. Chloride levels in the water blank used to prepare calibrants were insignificant for this analysis.

ICP standard solution for gold

Eingang 15.02.2011

MERCK

Certificate of Analysis
CertiPUR® Reference Material

Gold ICP Standard 1000 mg/l Au CertiPUR®

1.70321.0100 **Lot No.: HC000178**

This Certificate of Analysis is based on the data from the Merck Calibration Laboratory for ICP-OES, according to DIN EN ISO / IEC 17025.
Accredited by the DKD (Deutscher Kalibrierdienst).

DAR Reg.-No.: DKD-K-14302
Ref. Calibration Certificate: 480/DKD-K-14302/10-11

Composition: Tetrachlorogoldacid in hydrochloric acid Suprapur® 7%

Assay: **967 mg/kg** **Analysis: ICP-OES**
999 mg/l (calculated)

Measurement Uncertainty: **± 3 mg/kg (± 0.3%)**
This value represents the expanded uncertainty (*U*) for a coverage probability of 95%. Refer to page 2 for further details.

Traceability: This ICP Standard has been measured applying high precision ICP-OES in comparison to the corresponding **NIST SRM® 3121, lot 991806**

Trace impurities µg/ml:

Ag	<0.02	Cr	<0.02	In	<0.02	Ni	<0.02	Sb	<0.05	Tl	<0.02
Al	<0.05	Cu	<0.02	Ir	<0.02	Os	<0.20	Sc	<0.02	Tm	<0.02
As	<0.20	Dy	<0.02	K	<0.20	P	<0.20	Se	<0.20	U	<0.02
Au	*	Er	<0.02	La	<0.02	Pb	<0.05	Si	<0.20	V	<0.02
B	<0.05	Eu	<0.02	Li	<0.02	Pd	<0.02	Sm	<0.02	W	<0.05
Ba	<0.02	Fe	<0.05	Lu	<0.02	Pr	<0.02	Sn	<0.02	Y	<0.02
Be	<0.02	Ga	<0.02	Mg	<0.02	Pt	<0.02	Sr	<0.02	Yb	<0.02
Bi	<0.20	Gd	<0.02	Mn	<0.02	Rb	<0.02	Ta	<0.05	Zn	<0.02
Ca	<0.05	Ge	<0.02	Mo	<0.02	Re	<0.02	Tb	<0.02	Zr	<0.02
Cd	<0.02	Hf	<0.02	Na	<0.10	Rh	<0.02	Te	<0.20		
Ce	<0.02	Hg	<0.02	Nb	<0.05	Ru	<0.02	Th	<0.02		
Co	<0.02	Ho	<0.02	Nd	<0.02	S	<0.20	Ti	<0.05		

Date of release: 2010-11-10

Minimum shelf life: 2013-11-30

A. Yildirim

Dipl.-Ing. Ayfer Yildirim
(responsible laboratory manager quality control)

21.2 Raw data – Reproduction test with earthworms – TiO_2 (chapter 7)

21.2.1 P25 - First test

Table 170: P25 (1st test) – earthworm test: living worms after 28 days [Individuals].

Single values of the parallel test pots

Replicate No.	1	2	3	4	5	6	7	8
Control	10	10	10	10	10	10	10	10
Application via powder on feed								
50 mg/kg	10	10	10	10	-	-	-	-
100 mg/kg	10	10	10	10	-	-	-	-
200 mg/mg/kg	10	10	10	10	-	-	-	-
Application via powder on soil								
50 mg/kg	10	10	10	10	-	-	-	-
100 mg/kg	10	10	10	10	-	-	-	-
200 mg/mg/kg	10	10	10	10	-	-	-	-
Application of P25 via dispersion on feed								
10 mg/kg	10	10	10	10	-	-	-	-
20 mg/kg	10	10	10	10	-	-	-	-
Application of P25 via dispersion on soil								
10 mg/kg	10	10	10	10	-	-	-	-
20 mg/kg	10	10	10	10	-	-	-	-

Table 171: P25 (1st test) – earthworm test: biomass of worm batches at test start [g].

Single values of the replicate test pots

Replicate No.	1	2	3	4	5	6	7	8
Control	3.808	3.024	3.331	3.164	3.109	3.405	3.242	3.244
Application via powder on feed								
50 mg/kg	3.260	3.734	3.259	3.178	-	-	-	-
100 mg/kg	3.218	3.087	2.872	3.492	-	-	-	-
200 mg/mg/kg	3.665	3.251	3.024	3.232	-	-	-	-
Application via powder on soil								
50 mg/kg	3.008	2.993	3.764	3.717	-	-	-	-
100 mg/kg	3.430	3.534	3.649	3.353	-	-	-	-
200 mg/mg/kg	3.428	3.524	3.264	3.577	-	-	-	-
Application of P25 via dispersion on feed								
10 mg/kg	3.204	3.320	3.121	3.274	-	-	-	-
20 mg/kg	3.063	3.169	3.434	3.168	-	-	-	-
Application of P25 via dispersion on soil								
10 mg/kg	3.392	3.235	3.101	3.278	-	-	-	-
20 mg/kg	3.594	3.076	3.161	3.492	-	-	-	-

Table 172: P25 (1st test) – earthworm test: biomass of worm batches after 28 days [g].

Single values of the replicate test pots

Replicate No.	1	2	3	4	5	6	7	8
Control	5.835	5.001	5.634	5.118	5.092	5.936	5.558	5.615
Application via powder on feed								
50 mg/kg	5.004	5.699	5.643	5.590	-	-	-	-
100 mg/kg	5.661	5.485	4.918	5.559	-	-	-	-
200 mg/mg/kg	5.843	5.347	5.216	5.090	-	-	-	-
Application via powder on soil								
50 mg/kg	5.627	5.793	5.999	5.782	-	-	-	-
100 mg/kg	5.468	5.347	5.473	5.582	-	-	-	-
200 mg/mg/kg	5.567	5.506	5.317	5.487	-	-	-	-
Application of P25 via dispersion on feed								
10 mg/kg	5.123	5.049	4.889	5.092	-	-	-	-
20 mg/kg	5.222	5.262	5.421	5.116	-	-	-	-
Application of P25 via dispersion on soil								
10 mg/kg	5.146	5.139	5.002	5.294	-	-	-	-
20 mg/kg	5.846	5.774	5.646	5.496	-	-	-	-

Table 173: P25 (1st test) – earthworm test: number of offspring at day 56.

Replicate No.	1	2	3	4	5	6	7	8
Control	174	152	164	210	256	280	228	234
Application via powder on feed								
50 mg/kg	225	191	347	357	-	-	-	-
100 mg/kg	266	288	272	409	-	-	-	-
200 mg/mg/kg	275	302	318	435	-	-	-	-
Application via powder on soil								
50 mg/kg	302	243	284	349	-	-	-	-
100 mg/kg	360	227	365	243	-	-	-	-
200 mg/mg/kg	297	267	365	331	-	-	-	-
Application of P25 via dispersion on feed								
10 mg/kg	221	259	342	296	-	-	-	-
20 mg/kg	278	272	269	296	-	-	-	-
Application of P25 via dispersion on soil								
10 mg/kg	286	255	337	424	-	-	-	-
20 mg/kg	327	320	301	334	-	-	-	-

Table 174: P25 (1st test) – earthworm test: soil dry mass [%].

Single values of the replicate test pots [%]; concentrations given as nominal values [mg/kg]

		Application via powder on feed			Application via powder on soil			Application via dispersion on feed		Application via dispersion on soil	
	Control	50	100	200	50	100	200	10	20	10	20
Test start	81.0	81.3	80.9	80.8	79.6	80.1	81.6	80.9	80.4	81.2	79.6
Test end	89.1	89.1	89.1	89.1	89.1	89.1	89.1	88.8	88.8	88.8	88.8

Table 175: P25 (1st test) – earthworm test: soil moisture [% WHC].

single values of the replicate test pots; since WHC is affected by feed adding, no calculation for test end can be applied; concentrations given as nominal values [mg/kg]

		Application via powder on feed			Application via powder on soil			Application via dispersion on feed		Application via dispersion on soil	
	Control	50	100	200	50	100	200	10	20	10	20
Test start	54.1	54.1	54.1	54.1	54.1	54.1	54.1	55.5	55.5	55.5	55.5

Table 176: P25 (1st test) – earthworm test: soil pH.

Single values of the parallel test pots; concentrations given as nominal values

		Application via powder on feed			Application via powder on soil			Application via dispersion on feed		Application via dispersion on soil	
	Control	50	100	200	50	100	200	10	20	10	20
Test start	4.9	4.9	4.9	4.9	4.9	4.9	4.9	4.9	4.9	4.9	4.9
Test end	6.5	6.5	6.4	6.5	6.5	6.5	6.4	6.5	6.5	6.5	6.5

21.2.2 P25 - Second test

Table 177: P25 (2nd test) – earthworm test: living worms after 28 days.

Single values of the parallel test pots [Individuals]

Replicate No.	1	2	3	4	5	6	7	8
Application via powder on soil								
Control	10	10	10	10	10	10	10	10
50 mg/kg	10	10	10	10	-	-	-	-
100 mg/kg	10	10	10	10	-	-	-	-
200 mg/mg/kg	10	10	10	10	-	-	-	-
500 mg/kg	10	10	10	10	-	-	-	-
1000 mg/kg	10	10	10	10	-	-	-	-

Table 178: P25 (2nd test) – earthworm test: biomass of worm batches at test start [g].

Single values of the replicate test pots

Replicate No.	1	2	3	4	5	6	7	8
Application via powder on soil								
Control	4.024	4.274	3.937	3.947	3.585	3.358	3.501	3.819
50 mg/kg	3.683	3.520	3.719	3.540	-	-	-	-
100 mg/kg	3.671	3.706	3.497	3.763	-	-	-	-
200 mg/mg/kg	3.669	3.560	3.675	3.432	-	-	-	-
500 mg/kg	3.463	3.619	3.493	3.596	-	-	-	-
1000 mg/kg	3.952	3.404	3.555	3.593	-	-	-	-

Table 179: P25 (2nd test) – earthworm test: biomass of worm batches after 28 days [g].

Single values of the replicate test pots

Replicate No.	1	2	3	4	5	6	7	8
Application via powder on soil								
Control	5.684	5.687	5.060	5.783	5.254	4.792	5.308	5.398
50 mg/kg	5.276	4.813	5.130	5.148	-	-	-	-
100 mg/kg	5.324	6.035	5.451	5.844	-	-	-	-
200 mg/mg/kg	5.327	4.849	5.660	4.848	-	-	-	-
500 mg/kg	4.701	5.479	5.173	5.606	-	-	-	-
1000 mg/kg	5.711	5.712	5.173	5.679	-	-	-	-

Table 180: P25 (2nd test) – earthworm test: number of offspring at day 56.

Replicate No.	1	2	3	4	5	6	7	8
Application via powder on soil								
Control	326	417	297	347	315	311	338	372
50 mg/kg	379	350	301	334	-	-	-	-
100 mg/kg	308	377	343	343	-	-	-	-
200 mg/mg/kg	261	294	320	286	-	-	-	-
500 mg/kg	174	319	279	240	-	-	-	-
1000 mg/kg	307	373	326	271	-	-	-	-

Table 181: P25 (2nd test) – earthworm test: soil dry mass [%].

Single values of the replicate test pots [%]; concentrations given as nominal values [mg/kg]

	Control	50	100	200	500	1000
Test start	89.4	89.3	89.3	89.7	89.8	89.7
Test end	79.0	82.1	83.1	81.4	80.9	80.3

Table 182: P25 (2nd test) – earthworm test: Soil moisture [% WHC]

Single values of the replicate test pots; since WHC is affected by feed adding; No calculation for test end can be applied; concentrations given as nominal values [mg/kg]

	Control	50	100	200	500	1000
Test start	52.14	52.77	52.77	50.5	50.03	50.42

Table 183: P25 (2nd test) – earthworm test: soil pH.

Single values of the parallel test pots; concentrations given as nominal values

	Control	50	100	200	500	1000
Test start	5.11	5.1	5.1	5.09	5.09	5.09
Test end	6.6	6.6	6.6	6.6	6.6	6.5

21.2.3 P25 - Third test

Table 184: P25 (3rd test) – earthworm test: living worms after 28 days.

Single values of the parallel test pots [Individuals]

Replicate No.	1	2	3	4	5	6	7	8
Application via powder on soil								
Control	10	10	10	10	10	10	10	10
50 mg/kg	10	10	10	10	-	-	-	-
100 mg/kg	10	10	10	10	-	-	-	-
200 mg/mg/kg	10	10	10	10	-	-	-	-
500 mg/kg	10	9	10	10	-	-	-	-
750 mg/kg	10	10	10	10	-	-	-	-
1000 mg/kg	10	9	10	10	-	-	-	-

Table 185: P25 (3rd test) – earthworm test: biomass of worm batches at test start [g].

Single values of the replicate test pots

Replicate No.	1	2	3	4	5	6	7	8
Application via powder on soil								
Control	3.846	3.631	4.068	3.560	3.904	3.565	3.242	3.776
50 mg/kg	3.650	3.459	3.766	3.828	-	-	-	-
100 mg/kg	3.357	3.476	3.631	3.809	-	-	-	-
200 mg/mg/kg	3.443	3.479	3.560	3.341	-	-	-	-
500 mg/kg	3.728	3.538	3.781	3.299	-	-	-	-
750 mg/kg	3.619	3.560	3.423	3.708	-	-	-	-
1000 mg/kg	3.352	3.235	3.655	3.492	-	-	-	-

Table 186: P25 (3rd test) – earthworm test: biomass of worm batches after 28 days [g].

Single values of the replicate test pots

Replicate No.	1	2	3	4	5	6	7	8
Application via powder on soil								
Control	5.225	5.244	5.543	4.492	5.711	5.137	5.033	5.714
50 mg/kg	5.444	5.278	5.297	5.410	-	-	-	-
100 mg/kg	5.357	5.438	5.614	5.160	-	-	-	-
200 mg/mg/kg	5.557	5.853	5.633	5.262	-	-	-	-
500 mg/kg	5.790	4.812	5.703	5.159	-	-	-	-
750 mg/kg	5.491	5.240	5.082	5.901	-	-	-	-
1000 mg/kg	5.498	4.518	5.685	5.474	-	-	-	-

Table 187: P25 (3rd test) – earthworm test: number of offspring at day 56.

Replicate No.	1	2	3	4	5	6	7	8
Application via powder on soil								
Control	219	293	207	192	197	237	218	194
50 mg/kg	268	214	234	240	-	-	-	-
100 mg/kg	243	238	273	252	-	-	-	-
200 mg/mg/kg	232	302	248	279	-	-	-	-
500 mg/kg	237	253	238	225	-	-	-	-
750 mg/kg	313	276	281	247				
1000 mg/kg	299	261	277	308	-	-	-	-

Table 188: P25 (3rd test) – earthworm test: soil dry mass [%].

Single values of the replicate test pots [%]; concentrations given as nominal values [mg/kg]

	Control	50	100	200	500	750	1000
Test start	89.3	89.3	88.9	89	89.7	89.2	88.6
Test end	82.8	80.2	82.9	82.7	81.9	81.8	81.9

Table 189: P25 (3rd test) – earthworm test: soil moisture [% WHC].

Single values of the replicate test pots; since WHC is affected by feed adding, no calculation for test end can be applied; concentrations given as nominal values [mg/kg]

	Control	50	100	200	500	750	1000
Test start	53.02	52.53	55.12	54.55	50.53	53.28	56.93

Table 190: P25 (3rd test) – earthworm test: soil pH.

Single values of the parallel test pots; concentrations given as nominal values

	Control	50	100	200	500	750	1000
Test start	5.4	5.3	5.2	5.3	5.2	5.1	5.2
Test end	6.9	6.9	6.7	6.7	6.8	6.8	6.9

21.2.4 NM-101 - First test

Table 191: NM-101 (1st test) – earthworm test: living worms after 28 days.

Single values of the parallel test pots [Individuals]

Replicate No.	1	2	3	4	5	6	7	8
Control	10	10	10	10	10	10	10	10
Application via powder on feed								
50 mg/kg	10	10	10	10	-	-	-	-
100 mg/kg	10	10	10	10	-	-	-	-
200 mg/mg/kg	10	10	10	10	-	-	-	-
Application via powder on soil								
50 mg/kg	10	10	10	10	-	-	-	-
100 mg/kg	10	10	10	10	-	-	-	-
200 mg/mg/kg	10	10	10	10	-	-	-	-
Application of P25 via dispersion on feed								
10 mg/kg	10	10	10	10	-	-	-	-
20 mg/kg	10	10	10	10	-	-	-	-
Application of P25 via dispersion on soil								
10 mg/kg	10	10	10	10	-	-	-	-
20 mg/kg	10	10	10	10	-	-	-	-

Table 192: NM-101 (1st test) – earthworm test: biomass of worm batches at test start [g].

Single values of the replicate test

Replicate No.	1	2	3	4	5	6	7	8
Control	3.742	3.791	3.945	3.498	3.569	3.361	3.448	3.852
Application via powder on feed								
50 mg/kg	3.150	3.430	3.523	3.543	-	-	-	-
100 mg/kg	3.449	3.191	3.993	3.434	-	-	-	-
200 mg/mg/kg	3.378	3.687	3.787	3.460	-	-	-	-
Application via powder on soil								
50 mg/kg	3.595	3.254	3.654	3.440	-	-	-	-
100 mg/kg	3.271	3.658	3.363	3.290	-	-	-	-
200 mg/mg/kg	3.332	3.127	3.785	3.091	-	-	-	-
Application of P25 via dispersion on feed								
10 mg/kg	3.250	3.312	3.413	3.087	-	-	-	-
20 mg/kg	3.694	3.230	3.020	3.304	-	-	-	-
Application of P25 via dispersion on soil								
10 mg/kg	3.120	3.234	3.382	3.069	-	-	-	-
20 mg/kg	3.131	3.380	2.958	3.025	-	-	-	-

Table 193: NM-101 (1st test) – earthworm test: biomass of worm batches after 28 days [g].

Single values of the replicate test pots

Replicate No.	1	2	3	4	5	6	7	8
Control	6.261	6.312	6.148	5.721	6.192	5.744	5.774	6.304
Application via powder on feed								
50 mg/kg	5.687	6.223	5.988	6.368	-	-	-	-
100 mg/kg	5.938	5.738	6.588	6.238	-	-	-	-
200 mg/mg/kg	6.076	6.367	6.170	6.403	-	-	-	-
Application via powder on soil								
50 mg/kg	6.262	6.127	6.580	5.892	-	-	-	-
100 mg/kg	6.351	6.233	6.180	6.471	-	-	-	-
200 mg/mg/kg	5.999	6.284	6.762	5.906	-	-	-	-
Application of P25 via dispersion on feed								
10 mg/kg	5.653	5.947	6.267	5.799	-	-	-	-
20 mg/kg	6.745	6.272	5.983	6.176	-	-	-	-
Application of P25 via dispersion on soil								
10 mg/kg	6.013	6.202	5.881	5.731	-	-	-	-
20 mg/kg	5.999	5.971	5.752	5.869	-	-	-	-

Table 194: NM-101 (1st test) – earthworm test: .umber of offspring at day 56.

Replicate No.	1	2	3	4	5	6	7	8
Control	286	267	289	309	340	286	315	330
Application via powder on feed								
50 mg/kg	248	269	361	309	-	-	-	-
100 mg/kg	328	318	306	327	-	-	-	-
200 mg/mg/kg	315	311	367	354	-	-	-	-
Application via powder on soil								
50 mg/kg	319	351	309	307	-	-	-	-
100 mg/kg	353	317	333	407	-	-	-	-
200 mg/mg/kg	345	425	392	337	-	-	-	-
Application of P25 via dispersion on feed								
10 mg/kg	327	349	328	307	-	-	-	-
20 mg/kg	340	310	356	329	-	-	-	-
Application of P25 via dispersion on soil								
10 mg/kg	371	266	286	308	-	-	-	-
20 mg/kg	392	329	354	276	-	-	-	-

Table 195: NM-101 (1st test) – earthworm test: soil dry mass [%].

Single values of the replicate test pots [%]; concentrations given as nominal values [mg/kg]

		Application via powder on feed			Application via powder on soil			Application via dispersion on feed		Application via dispersion on soil	
	Control	50	100	200	50	100	200	10	20	10	20
Test start	88.6	89.1	89.3	88.8	89.6	89.6	90.0	89.2	89.2	89.3	89.2
Test end	80.3	78.6	79.8	78.5	81.0	80.2	81.1	81.6	80.5	79.8	81.0

Table 196: NM-101 (1st test) – earthworm test: soil moisture [% WHC].

Single values of the replicate test pots; since WHC is affected by feed adding, no calculation for test end can be applied; concentrations given as nominal values [mg/kg]

		Application via powder on feed			Application via powder on soil			Application via dispersion on feed		Application via dispersion on soil	
	Control	50	100	200	50	100	200	10	20	10	20
Test start	56.5	54.1	52.7	55.8	51.3	51.0	49.2	53.3	53.6	52.73	53.10

Table 197: NM-101 (1st test): – earthworm test: soil pH.

Single values of the parallel test pots; concentrations given as nominal values [mg/kg]

		Application via powder on feed			Application via powder on soil			Application via dispersion on feed		Application via dispersion on soil	
	Control	50	100	200	50	100	200	10	20	10	20
Test start	4.8	4.8	4.8	4.8	4.8	4.8	4.8	4.8	4.8	4.8	4.9
Test end	6.3	6.2	6.3	6.3	6.3	6.3	6.3	6.3	6.3	6.4	6.4

21.2.5 NM-101 - Second test

Table 198: NM-101 (2nd test) – earthworm test: living worms after 28 days.

Single values of the parallel test pots [Individuals]

Replicate No.	1	2	3	4	5	6	7	8
Application via powder on soil								
Control	10	10	10	10	10	10	10	10
50 mg/kg	10	10	10	10	-	-	-	-
100 mg/kg	10	10	10	10	-	-	-	-
200 mg/mg/kg	10	10	9	10	-	-	-	-
400 mg/kg	10	10	10	10	-	-	-	-

Table 199: NM-101 (2nd test) – earthworm test: biomass of the worm batches at test start [g].

Single values of the replicate test pots

Replicate No.	1	2	3	4	5	6	7	8
Application via powder on soil								
Control	3.882	3.595	3.574	3.755	3.371	3.299	3.851	3.840
50 mg/kg	3.572	3.395	3.325	3.961	-	-	-	-
100 mg/kg	3.833	3.623	3.504	3.310	-	-	-	-
200 mg/mg/kg	3.902	3.701	3.383	3.433	-	-	-	-
400 mg/kg	3.434	3.650	3.386	3.287	-	-	-	-

Table 200: NM-101 (2nd test) – earthworm test: biomass of the worm batches after 28 days [g].

Single values of the replicate test pots

Replicate No.	1	2	3	4	5	6	7	8
Application via powder on soil								
Control	6.075	5.761	5.847	5.493	5.459	5.208	5.686	5.635
50 mg/kg	5.915	5.642	5.435	6.309	-	-	-	-
100 mg/kg	5.873	5.926	6.346	5.619	-	-	-	-
200 mg/mg/kg	5.986	6.027	5.468	5.617	-	-	-	-
400 mg/kg	6.272	6.027	5.860	5.360	-	-	-	-

Table 201: NM-101 (2nd test) – earthworm test: number of offspring at day 56.

Replicate No.	1	2	3	4	5	6	7	8
Control	215	236	236	242	222	199	228	208
50 mg/kg	243	198	215	194	-	-	-	-
100 mg/kg	226	206	190	217	-	-	-	-
200 mg/mg/kg	278	213	171	189	-	-	-	-
400 mg/kg	252	251	214	219	-	-	-	-

Table 202: NM-101 (2nd test) – earthworm test: soil dry mass [%].

Single values of the replicate test pots [%]; concentrations given as nominal values [mg/kg]

	Control	50	100	200	400
Test start	88.1	88.9	89.1	89.1	88.5
Test end	80.5	80.7	81.2	81.2	81.5

Table 203: NM-101 (2nd test) – earthworm test: soil moisture as [% WHC].

Single values of the replicate test pots; since WHC is affected by feed adding, no calculation for test end can be applied; concentrations given as nominal values [mg/kg]

	Control	50	100	200	400
Test start	59.48	55.08	54.13	54.04	57.22

Table 204: NM-101 (2nd test) – earthworm test: soil pH.

Single values of the parallel test pots; concentrations given as nominal values

	Control	50	100	200	400
Test start	5.0	5.0	5.0	5.0	5.0
Test end	6.9	6.9	6.8	6.7	6.8

21.2.6 NM-103 - First test

Table 205: NM-103 (1st test) – earthworm test: living worms after 28 days.

Single values of the parallel test pots [Individuals]

Replicate No.	1	2	3	4	5	6	7	8
Control	10	10	10	10	10	10	10	10
Application via powder on feed								
50 mg/kg	10	10	10	10	-	-	-	-
100 mg/kg	10	10	10	10	-	-	-	-
200 mg/mg/kg	10	10	10	10	-	-	-	-
Application via powder on soil								
50 mg/kg	10	10	10	10	-	-	-	-
100 mg/kg	10	10	9	10	-	-	-	-
200 mg/mg/kg	10	10	10	10	-	-	-	-
Application of P25 via dispersion on feed								
10 mg/kg	10	10	10	10	-	-	-	-
20 mg/kg	10	10	10	10	-	-	-	-
Application of P25 via dispersion on soil								
10 mg/kg	10	10	10	10	-	-	-	-
20 mg/kg	10	10	10	10	-	-	-	-

Table 206: NM-103 (1st test) – earthworm test: biomass of the worm batches at test start [g].

Single values of the replicate test pots

Replicate No.	1	2	3	4	5	6	7	8
Control	3.779	3.810	3.886	3.990	3.994	3.924	3.390	4.104
Application via powder on feed								
50 mg/kg	3.710	3.496	3.878	3.826	-	-	-	-
100 mg/kg	3.580	4.111	3.978	3.873	-	-	-	-
200 mg/mg/kg	3.824	3.617	3.546	4.014	-	-	-	-
Application via powder on soil								
50 mg/kg	3.454	3.953	3.828	4.094	-	-	-	-
100 mg/kg	3.866	3.461	3.508	4.279	-	-	-	-
200 mg/mg/kg	3.915	3.465	3.555	3.708	-	-	-	-
Application of P25 via dispersion on feed								
10 mg/kg	3.832	3.461	3.421	3.519	-	-	-	-
20 mg/kg	3.858	4.185	3.708	3.466	-	-	-	-
Application of P25 via dispersion on soil								
10 mg/kg	3.557	3.866	4.089	3.620	-	-	-	-
20 mg/kg	3.697	3.502	3.405	3.252	-	-	-	-

Table 207: NM-103 (1st test) – earthworm test: biomass of the worm batches after 28 days [g].

Single values of the replicate test pots

Replicate No.	1	2	3	4	5	6	7	8
Control	5.273	5.430	5.661	5.581	5.903	5.234	5.403	5.953
Application via powder on feed								
50 mg/kg	5.590	5.273	5.097	4.652	-	-	-	-
100 mg/kg	5.225	6.115	5.678	5.479	-	-	-	-
200 mg/mg/kg	5.714	5.179	5.572	6.305	-	-	-	-
Application via powder on soil								
50 mg/kg	4.913	5.677	5.369	6.090	-	-	-	-
100 mg/kg	5.876	5.484	5.097	6.455	-	-	-	-
200 mg/mg/kg	5.866	5.621	5.953	5.359	-	-	-	-
Application of P25 via dispersion on feed								
10 mg/kg	5.453	5.732	4.991	5.559	-	-	-	-
20 mg/kg	5.434	6.266	6.059	5.144	-	-	-	-
Application of P25 via dispersion on soil								
10 mg/kg	5.560	5.985	6.159	5.296	-	-	-	-
20 mg/kg	6.129	5.979	5.443	4.732	-	-	-	-

Table 208: NM-103 (1st test) – earthworm test: number of offspring at day 56.

Replicate No.	1	2	3	4	5	6	7	8
Control	318.00	376.00	374.00	412.00	394.00	307.00	326.00	414.00
Application via powder on feed								
50 mg/kg	382.00	375.00	333.00	239.00	-	-	-	-
100 mg/kg	341.00	332.00	371.00	337.00	-	-	-	-
200 mg/mg/kg	412.00	389.00	326.00	333.00	-	-	-	-
Application via powder on soil								
50 mg/kg	308.00	344.00	344.00	354.00	-	-	-	-
100 mg/kg	450.00	362.00	313.00	362.00	-	-	-	-
200 mg/mg/kg	392.00	330.00	314.00	335.00	-	-	-	-
Application of P25 via dispersion on feed								
10 mg/kg	306.00	412.00	301.00	347.00	-	-	-	-
20 mg/kg	357.00	345.00	348.00	252.00	-	-	-	-
Application of P25 via dispersion on soil								
10 mg/kg	327.00	384.00	339.00	312.00	-	-	-	-
20 mg/kg	395.00	349.00	279.00	326.00	-	-	-	-

Table 209: NM-103 (1st test) – earthworm test: soil dry mass content [%].

Single values of the replicate test pots [%]

		Application via powder on feed			Application via powder on soil			Application via dispersion on feed		Application via dispersion on soil	
	Control	50	100	200	50	100	200	10	20	10	20
Test start	89.2	89.0	89.5	89.1	89.7	89.3	89.9	89.6	89.0	89.3	89.7
Test end	79.9	81.9	80.9	79.5	79.5	81.9	81.3	81.2	81.4	82.0	81.9

Table 210: NM-103 (1st test) – earthworm test: soil moisture as [% WHC].

Single values of the replicate test pots; since WHC is affected by feed adding, no calculation for test end can be applied; concentrations given as nominal values [mg/kg]

		Application via powder on feed			Application via powder on soil			Application via dispersion on feed		Application via dispersion on soil	
	Control	50	100	200	50	100	200	10	20	10	20
Test start	53.2	55.1	51.5	54.3	51.1	53.5	50.1	50.8	54.5	53.3	50.6

Table 211: NM-103 (1st test) – earthworm test: soil pH.

Single values of the parallel test pots; concentrations given as nominal values

		Application via powder on feed			Application via powder on soil			Application via dispersion on feed		Application via dispersion on soil	
	Control	50	100	200	50	100	200	10	20	10	20
Test start	5.0	5.0	5.0	5.0	5.0	5.0	5.0	4.9	4.9	5.0	5.0
Test end	6.5	6.4	6.5	6.4	6.5	6.5	6.4	6.4	6.5	6.5	6.2

21.2.7 NM-103 - Second test

Table 212: NM-103 (2nd test) – earthworm test: living worms after 28 days.

Single values of the parallel test pots [Individuals]

Replicate No.	1	2	3	4	5	6	7	8
Application via powder on soil								
Control	10	10	10	10	10	10	10	10
50 mg/kg	10	10	10	10	-	-	-	-
100 mg/kg	10	10	10	10	-	-	-	-
200 mg/mg/kg	10	10	9	10	-	-	-	-
400 mg/kg	10	10	10	10	-	-	-	-

Table 213: NM-103 (2nd test) – earthworm test: biomass of the worm batches at test start [g].

Single values of the replicate test pots

Replicate No.	1	2	3	4	5	6	7	8
Application via powder on soil								
Control	3.882	3.595	3.574	3.755	3.371	3.299	3.851	3.840
50 mg/kg	3.843	3.398	3.389	4.054	-	-	-	-
100 mg/kg	3.413	3.622	3.729	3.110	-	-	-	-
200 mg/mg/kg	3.522	3.480	3.539	3.253	-	-	-	-
400 mg/kg	3.559	3.334	3.823	3.736	-	-	-	-

Table 214: NM-103 (2nd test) – earthworm test: biomass of the worm batches after 28 days [g].

Single values of the replicate test pots

Replicate No.	1	2	3	4	5	6	7	8
Application via powder on soil								
Control	6.075	5.761	5.847	5.493	5.459	5.208	5.686	5.635
50 mg/kg	5.846	5.493	5.743	5.620	-	-	-	-
100 mg/kg	5.813	6.091	5.808	5.366	-	-	-	-
200 mg/mg/kg	5.916	6.255	5.198	5.437	-	-	-	-
400 mg/kg	6.406	5.460	6.131	5.931	-	-	-	-

Table 215: NM-103 (2nd test) – earthworm test: number of offspring at day 56.

Replicate No.	1	2	3	4	5	6	7	8
Control	215	236	236	242	222	199	228	208
50 mg/kg	223	216	238	284	-	-	-	-
100 mg/kg	217	221	307	262	-	-	-	-
200 mg/mg/kg	214	230	289	198	-	-	-	-
400 mg/kg	274	208	264	201	-	-	-	-

Table 216: NM-103 (2nd test) – earthworm test: soil dry mass [%].

Single values of the replicate test pots [%]; concentrations given as nominal values [mg/kg]

	Control	50	100	200	400
Test start	88.1	88.4	88.1	88.2	88.3
Test end	80.5	81.2	81.6	81.6	81.2

Table 217: NM-103 (2nd test) – earthworm test: Soil moisture [% WHC].

Single values of the replicate test pots; since WHC is affected by feed adding, no calculation for test end can be applied; concentrations given as nominal values [mg/kg]

	Control	50	100	200	400
Test start	59.5	57.9	59.4	59.0	58.6

Table 218: NM-103 (2nd test) – earthworm test: soil pH.

Single values of the parallel test pots; concentrations given as nominal values

	Control	50	100	200	400
Test start	5.0	5.0	5.0	5.0	5.0
Test end	6.8	6.8	6.8	6.9	6.8

21.2.8 Ti concentration in earthworms

P25

The information about the LOD/LOQ and correlation coefficient are compiled in Table 6.

Coefficient of determination (r) for respective calibration functions were taken from ICP-OES instrument outputs.

Table 219: P25 (1st test) – Ti concentration in earthworms: LODs/LOQs, correlation.

Measurement date, description	LOD [µg/L]	LOQ [µg/L]	Correlation coefficient r
July 28, 2011 measurements of samples from May 19th	18	60*	0.99990
July 14, 2011 measurements of samples from January 25th and February 18th	65	218*	0.99975
June 09, 2011 measurements of control worms for fortification	18	54*	0.99941

* Internal LOQ calculation was performed with more digits

The recovery for CPI multi element solution (appropriately diluted) samples containing 500 µg Ti/L was 104 ± 7% (n = 6).

For further quality assurance, recalibration samples were analysed along with the samples and the mean accuracy was determined to 103 ± 3% (n = 6) for a Ti concentration of 500 µg/L. For collecting validation information of the digestion procedure of samples as well as the analytical method several control worms were pooled and spiked with a weighed amount of TiO_2 nanoparticles.

Therefore accurately weighted 2698 µg TiO_2 nanoparticles (P25, 1617 µg Ti) were given to exactly 2164.0 mg of homogenized and dried worms. This titanium amount represents 747 µg/g. Without spiking the *Eisenia fetida* material exhibited a mean titanium concentration of 44.9 ± 2.8 µg/g (n = 2). In conclusion the nominal value is calculated to 792 µg/L.

Spiked samples were digested and analysed along with actual samples, exhibiting a mean value of 659 ± 57 µg/L (n = 6), representing a mean recovery of 83.1 ± 7.2%. The quality requirements for the digestion and analysis of titanium in *Eisenia fetida* were set to 100 ± 25%, and were therefore fulfilled.

Analytical results

The titanium amounts in samples from two tests with P25 were quantified:

Loadings (food and soil), January 25th: control, 10 mg/kg, 20 mg/kg, 50 mg/kg, 100 mg/kg, 200 mg/kg (Table 18)

Loadings (soil), May 19th: 50 mg/kg, 100 mg/kg, 200 mg/kg, 500 mg/kg, 1000 mg/kg (Table 221)

The measured values in µg/L are calculated to µg/g:

Amount of Ti in dry weight = measured value / [1000 / 15 (final volume in mL)] / weight (mg) *1000

Table 220 - Table 221 summarize the measured titanium concentrations in the samples.

Table 220: P25 (1st test): Ti concentration in earthworms.

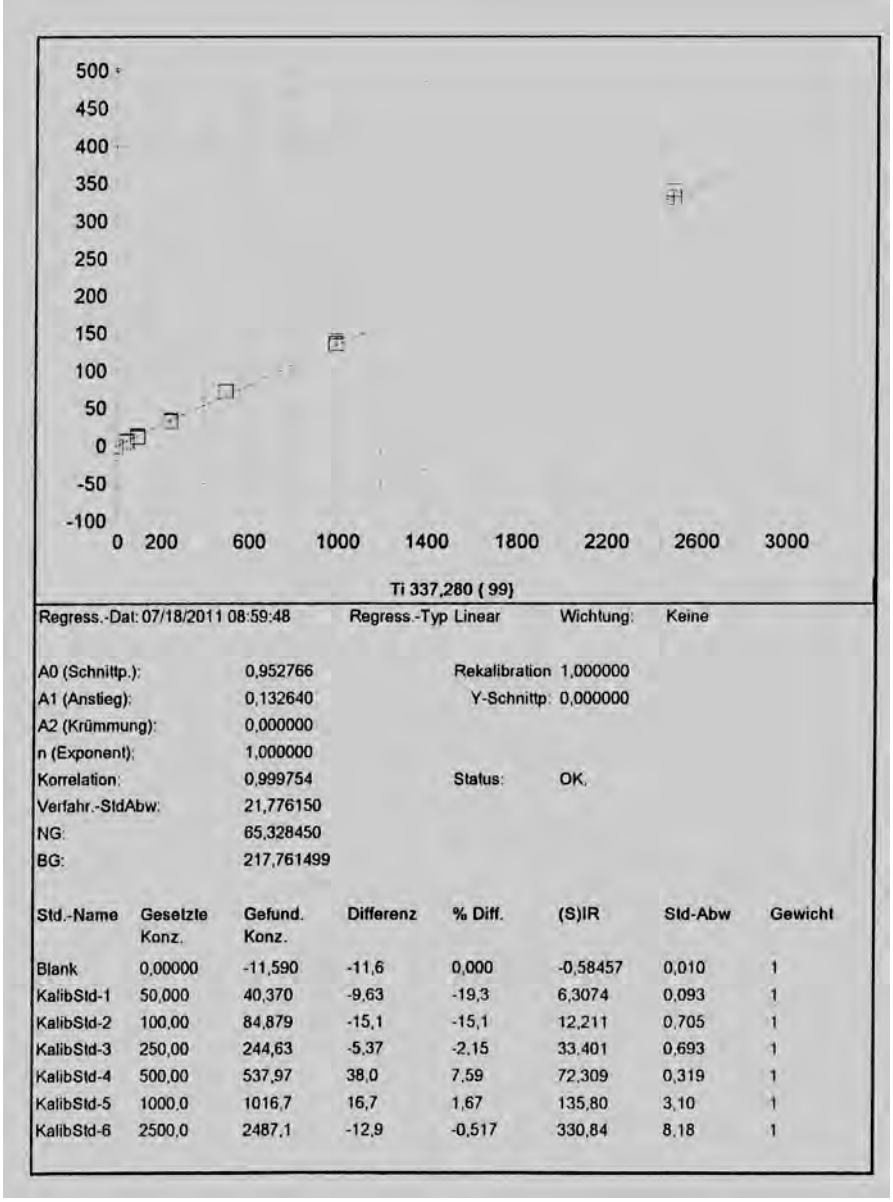
Sample name	Weighted sample [mg]	Measured value [µg/L]	Ti in dry weight [µg/g]	Mean Ti in dry weight ± SD [µg/g]
Control c	193.8	844	65.3	58.4 ± 9.8
Control d	202.2	694	51.5	
10 mg/kg (soil) c	209.8	711	50.8	54.2 ± 4.7
10 mg/kg (soil) d	206.5	792	57.5	
10 mg/kg (food) c	197.9	733	55.5	58.6 ± 4.3
10 mg/kg (food) d	198.8	817	61.7	
20 mg/kg (soil) c	196.8	989	75.3	77.4 ± 2.9
20 mg/kg (soil) d	192.5	1021	79.6	
20 mg/kg (food) c	195.7	804	61.6	64.6 ± 4.1
20 mg/kg (food) d	201.1	905	67.5	
50 mg/kg (soil) c	196.6	873	66.6	76.4 ± 19.9
50 mg/kg (soil) d	200.2	1152	86.3	
50 mg/kg (food) c	198.1	1051	79.6	75.3 ± 6.1
50 mg/kg (food) d	197.0	933	71.0	
100 mg/kg (soil) c	189.1	1076	85.3	76.3 ± 12.7
100 mg/kg (soil) d	212.6	954	67.3	
100 mg/kg (food) c	203.5	1302	96.0	101 ± 7
100 mg/kg (food) d	204.5	1452	106	
200 mg/kg (soil) c	199.5	1236	92.9	72.4 ± 29.0
200 mg/kg (soil) d	202.1	699	51.9	
200 mg/kg (food) c	195.0	1280	98.5	121 ± 31
200 mg/kg (food) d	205.6	1955	143	

Table 221: P25 (2nd test): Ti concentration in earthworms.

Sample name	Weighted sample [mg]	Measured value [µg/L]	Ti in dry weight [µg/g]	Mean Ti in dry weight ± SD [µg/g]
Control a	202	722	53.6	55.2 ± 2.2
Control b	201.7	763	56.7	
50 mg/kg (soil) a	202.5	705	52.2	49.2. ± 4.3
50 mg/kg (soil) b	202.2	621	46.1	
100 mg/kg (soil) a	203.7	579	42.6	43.7 ± 1.5
100 mg/kg (soil) b	200.6	599	44.8	
200 mg/kg (soil) a	201.9	788	58.6	50.1 ± 11.9
200 mg/kg (soil) b	201.4	560	41.7	
500 mg/kg (soil) a	201	804	60.0	61.0 ± 1.4
500 mg/kg (soil) b	201.5	834	62.0	
1000 mg/kg (soil) a	202.4	981	72.7	88.0 ± 21.7
1000 mg/kg (soil) b	201.7	1390	103	

Raw data examples

Example for ICP-OES calibration: Calibration data from the measurement performed on July 14, 2011



Ti 337,280 (99)

Regress.-Dat: 07/18/2011 08:59:48		Regress.-Typ Linear	Wichtung:	Keine
A0 (Schnittp.):	0,952766		Rekalibration	1,000000
A1 (Anstieg):	0,132640		Y-Schnittp:	0,000000
A2 (Krümmung):	0,000000			
n (Exponent):	1,000000			
Korrelation:	0,999754		Status:	OK.
Verfahr.-StdAbw:	21,776150			
NG:	65,328450			
BG:	217,761499			

Std.-Name	Gesetzte Konz.	Gefund. Konz.	Differenz	% Diff.	(S)IR	Std-Abw	Gewicht
Blank	0,00000	-11,590	-11,6	0,000	-0,58457	0,010	1
KalibStd-1	50,000	40,370	-9,63	-19,3	6,3074	0,093	1
KalibStd-2	100,00	84,879	-15,1	-15,1	12,211	0,705	1
KalibStd-3	250,00	244,63	-5,37	-2,15	33,401	0,693	1
KalibStd-4	500,00	537,97	38,0	7,59	72,309	0,319	1
KalibStd-5	1000,0	1016,7	16,7	1,67	135,80	3,10	1
KalibStd-6	2500,0	2487,1	-12,9	-0,517	330,84	8,18	1

Example for ICP-OES raw data printout: Example printout from the measurement performed on July 14, 2011

65	Pro: 100 mg/Kg Boden d 07/14/2011 16:48:24 KONZ		
	Custom ID1:	Custom ID2:	Custom ID3:
	Ti3349	Ti3361	Ti3372
Einheit	µg/L	µg/L	µg/L
Mittel	949,6	953,2	954,4
StdAbw	9,6	7,3	8,1
% RSD	1,012	0,7649	0,8441
Mess.#1	957,1	959,4	959,3
Mess.#2	953,0	954,9	958,7
Mess.#3	938,8	945,2	945,1
66	Pro: 100mg/Kg Futter c 07/14/2011 16:50:41 KONZ		
	Custom ID1:	Custom ID2:	Custom ID3:
	Ti3349	Ti3361	Ti3372
Einheit	µg/L	µg/L	µg/L
Mittel	1299,	1300,	1302,
StdAbw	9,	11,	13,
% RSD	0,6833	0,8760	1,003
Mess.#1	1304,	1310,	1312,
Mess.#2	1303,	1302,	1305,
Mess.#3	1289,	1288,	1287,
67	Pro: 100mg/Kg Futter d 07/14/2011 16:52:58 KONZ		
	Custom ID1:	Custom ID2:	Custom ID3:
	Ti3349	Ti3361	Ti3372
Einheit	µg/L	µg/L	µg/L
Mittel	1449,	1451,	1452,
StdAbw	6,	7,	8,
% RSD	0,4056	0,4643	0,5250
Mess.#1	1448,	1446,	1453,
Mess.#2	1455,	1458,	1459,
Mess.#3	1444,	1448,	1444,
68	Pro: 200mg/Kg Boden c 07/14/2011 16:55:13 KONZ		
	Custom ID1:	Custom ID2:	Custom ID3:
	Ti3349	Ti3361	Ti3372
Einheit	µg/L	µg/L	µg/L
Mittel	1244,	1245,	1236,
StdAbw	3,	4,	2,
% RSD	0,2212	0,3098	0,1531
Mess.#1	1242,	1240,	1235,
Mess.#2	1242,	1246,	1236,
Mess.#3	1247,	1247,	1239,

NM-101

The information about the LOD/LOQ and correlation coefficient are compiled in Table 222.

Coefficients of determination (r) for respective calibration functions were taken from ICP-OES instrument outputs.

Table 222: NM-101 - Ti concentration in earthworms: LODs/LOQs, correlation.

Measurement date, description	LOD [µg/L]	LOQ [µg/L]	Correlation coefficient r
July 14, 2011 measurements of samples	65	218*	0.99975
June 09, 2011 measurements of control worms for fortification	18	54*	0.99941

* Internal LOQ calculation was performed with more digits

The recovery for CPI multi element solution (appropriately diluted) samples containing 500 µg Ti/L was 107 ± 5% (n = 4).

For further quality assurance, recalibration samples were analysed along with the samples and the mean accuracy was determined to 105 ± 3% (n = 4) for a Ti concentration of 500 µg/L.

For collecting validation information on the digestion procedure of samples as well as the analytical method several control worms were pooled and spiked with a weighed amount of TiO_2 nanoparticles.

Accurately weighted 2698 µg TiO_2 nanoparticles (P25, 1617 µg Ti) were given to exactly 2164.0 mg of homogenized and dried worms. This titanium amount represents 747 µg/g. Without spiking the *Eisenia fetida* material exhibited a mean titanium concentration of 44.9 ± 2.8 µg/g (n = 2). In conclusion the nominal value is calculated to 792 µg/L.

Spiked samples were digested and analysed along with actual samples, exhibiting a mean value of 685 ± 51 µg/L (n = 4), representing a mean recovery of 86.5 ± 6.5%. The quality requirements for the digestion and analysis of titanium in *Eisenia fetida* were set to 100 ± 25%, and were therefore fulfilled.

Analytical results

In this chapter the measurement results of NM-101 in samples from *Eisenia fetida* are compiled.

The measured values in µg/L are calculated to µg/g:

Amount of Ti in dry weight = measured value/[1000 / 15 (final volume in mL)]/weighted sample (mg) *1000

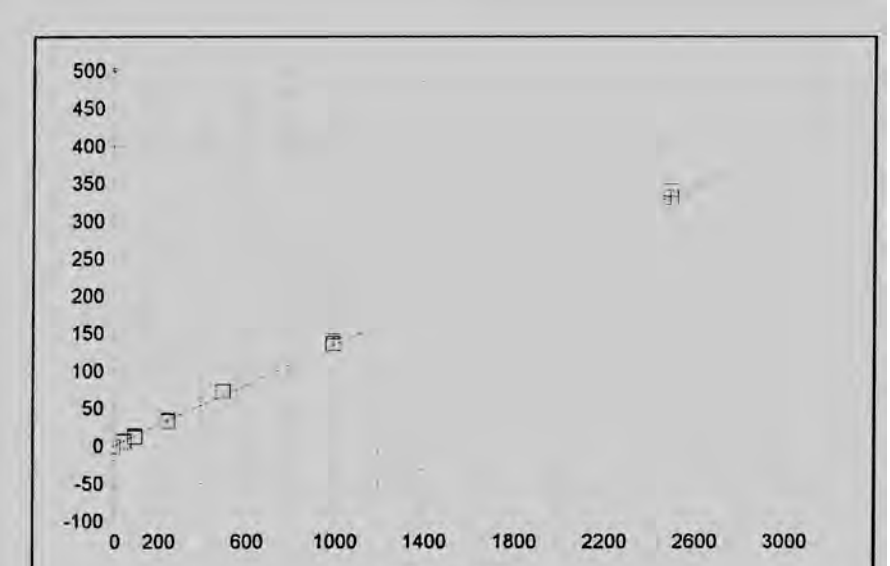
Table 223 summarizes the measured titanium concentrations in the samples.

Table 223: NM-101: Ti concentration in earthworms.

Sample name	Weighted sample [mg]	Measured value [µg/L]	Ti in dry weight [µg/g]	Mean Ti in dry weight ± SD [µg/g]
Control c	-	-	-	-
Control d	185.9	670	54.1	54.1
10 mg/kg (food) a	210.1	647	46.2	49.7 ± 5.0
10 mg/kg (food) b	205.8	730	53.2	
10 mg/kg (soil) a	196.7	424	32.3	28.9 ± 4.9
10 mg/kg (soil) b	191.4	324	25.4	
20 mg/kg (food) a	204.8	581	42.5	45.1 ± 3.7
20 mg/kg (food) b	192.8	613	47.7	
20 mg/kg (soil) a	203.1	477	35.2	38.1 ± 4.1
20 mg/kg (soil) b	190.8	522	41.0	
50 mg/kg (food) d	202.3	900	66.7	59.4 ± 10.4
50 mg/kg (food) e	207.9	721	52.0	
50 mg/kg (soil) d	207.2	904	65.4	70.8 ± 7.5
50 mg/kg (soil) e	190.7	968	76.1	
100 mg/kg (food) d	200.9	903	67.4	66.2 ± 1.7
100 mg/kg (food) e	205.6	891	65.0	
100 mg/kg (soil) d	206.4	851	61.8	53.1 ± 12.3
100 mg/kg (soil) e	210.3	623	44.5	
200 mg/kg (food) d	204.7	1844	135	107 ± 40
200 mg/kg (food) e	197.4	1037	78.8	
200 mg/kg (soil) d	203.6	797	58.7	52.7 ± 8.6
200 mg/kg (soil) e	190.7	592	46.6	

Raw data examples

Example for ICP-OES calibration: Calibration data from the measurement performed on July 14, 2011



Ti 337,280 (99)

Regress.-Dat: 07/18/2011 08:59:48	Regress.-Typ Linear		Wichtung:	Keine
A0 (Schnittp.):	0,952766		Rekalibration	1,000000
A1 (Anstieg):	0,132640		Y-Schnittp:	0,000000
A2 (Krümmung):	0,000000			
n (Exponent):	1,000000			
Korrelation:	0,999754		Status:	OK.
Verfahr.-StdAbw:	21,776150			
NG:	65,328450			
BG:	217,761499			

Std.-Name	Gesetzte Konz.	Gefund. Konz.	Differenz	% Diff.	(S)IR	Std-Abw	Gewicht
Blank	0,00000	-11,590	-11,6	0,000	-0,58457	0,010	1
KalibStd-1	50,000	40,370	-9,63	-19,3	6,3074	0,093	1
KalibStd-2	100,00	84,879	-15,1	-15,1	12,211	0,705	1
KalibStd-3	250,00	244,63	-5,37	-2,15	33,401	0,693	1
KalibStd-4	500,00	537,97	38,0	7,59	72,309	0,319	1
KalibStd-5	1000,0	1016,7	16,7	1,67	135,80	3,10	1
KalibStd-6	2500,0	2487,1	-12,9	-0,517	330,84	8,18	1

Example for ICP-OES raw data printout: Example printout from the measurement performed on July 14, 2011

21	Pro: 10mg/kg Boden a 07/14/2011 15:09:42 KONZ			
	Custom ID1:	Custom ID2:	Custom ID3:	
	Ti3349	Ti3361	Ti3372	
Einheit	µg/L	µg/L	µg/L	
Mittel	422,4	421,9	424,2	
StdAbw	7,7	7,1	7,4	
% RSD	1,825	1,691	1,736	
Mess.#1	427,1	425,7	429,0	
Mess.#2	426,6	426,3	427,8	
Mess.#3	413,5	413,6	415,7	
22	Pro: 10mg/kg Boden b 07/14/2011 15:11:55 KONZ			
	Custom ID1:	Custom ID2:	Custom ID3:	
	Ti3349	Ti3361	Ti3372	
Einheit	µg/L	µg/L	µg/L	
Mittel	326,5	324,8	324,4	
StdAbw	3,7	3,4	4,2	
% RSD	1,139	1,060	1,288	
Mess.#1	329,3	328,1	328,4	
Mess.#2	328,0	325,3	324,7	
Mess.#3	322,3	321,2	320,0	
23	Pro: 20mg/kg Futter a 07/14/2011 15:14:08 KONZ			
	Custom ID1:	Custom ID2:	Custom ID3:	
	Ti3349	Ti3361	Ti3372	
Einheit	µg/L	µg/L	µg/L	
Mittel	580,0	579,6	580,5	
StdAbw	7,2	8,0	9,1	
% RSD	1,236	1,382	1,559	
Mess.#1	588,2	588,7	590,8	
Mess.#2	576,7	576,3	576,6	
Mess.#3	575,1	573,8	574,1	
24	Pro: 20mg/kg Futter b 07/14/2011 15:16:21 KONZ			
	Custom ID1:	Custom ID2:	Custom ID3:	
	Ti3349	Ti3361	Ti3372	
Einheit	µg/L	µg/L	µg/L	
Mittel	614,7	612,4	613,0	
StdAbw	4,6	4,9	4,5	
% RSD	0,7547	0,8075	0,7384	
Mess.#1	609,5	607,0	607,8	
Mess.#2	618,4	616,6	616,2	
Mess.#3	616,2	613,6	615,0	

NM-103

The information about the LOD/LOQ and correlation coefficient are compiled in Table 224.

Coefficient of determination (r) for respective calibration functions were taken from ICP-OES instrument outputs.

Table 224: NM-103 - Ti concentration in earthworms: LODs/LOQs, correlation.

Measurement date, description	LOD [µg/L]	LOQ [µg/L]	Correlation coefficient r
July 28, 2011 measurements of samples from May 19th	18	60*	0.99990
June 09, 2011 measurements of control worms for fortification	18	54*	0.99941

* Internal LOQ calculation was performed with more digits

The recovery for CPI multi element solution (appropriately diluted) samples containing 500 µg Ti/L was 98.1 ± 1.2% (n = 3).

For further quality assurance, recalibration samples were analysed along with the samples and the mean accuracy was determined to 101 ± 2% (n = 3) for a Ti concentration of 500 µg/L.

For collecting validation information of the digestion procedure of samples as well as the analytical method several control worms were pooled and spiked with a weighed amount of TiO_2 nanoparticles.

Therefore accurately weighted 2698 µg TiO_2 nanoparticles (P25, 1617 µg Ti) were given to exactly 2164.0 mg of homogenized and dried worms. This titanium amount represents 747 µg/g. Without spiking the *Eisenia fetida* material exhibited a mean titanium concentration of 44.9 ± 2.8 µg/g (n = 2). In conclusion the nominal value is calculated to 792 µg/L.

Spiked samples were digested and analysed along with actual samples, exhibiting a mean value of 658 ± 60 µg/L (n = 4), representing a mean recovery of 83.0 ± 7.6%. The quality requirements for the digestion and analysis of titanium in *Eisenia fetida* were set to 100 ± 25%, and were therefore fulfilled.

Analytical results

In this chapter the measurement results of NM-103 in samples from *Eisenia fetida* are compiled. The titanium amounts in samples from three tests were quantified.

The measured values in µg/L are calculated to µg/g:

Amount of Ti in dry weight = measured value/[1000 / 15 (final volume in mL)]/weighted sample (mg) *1000.

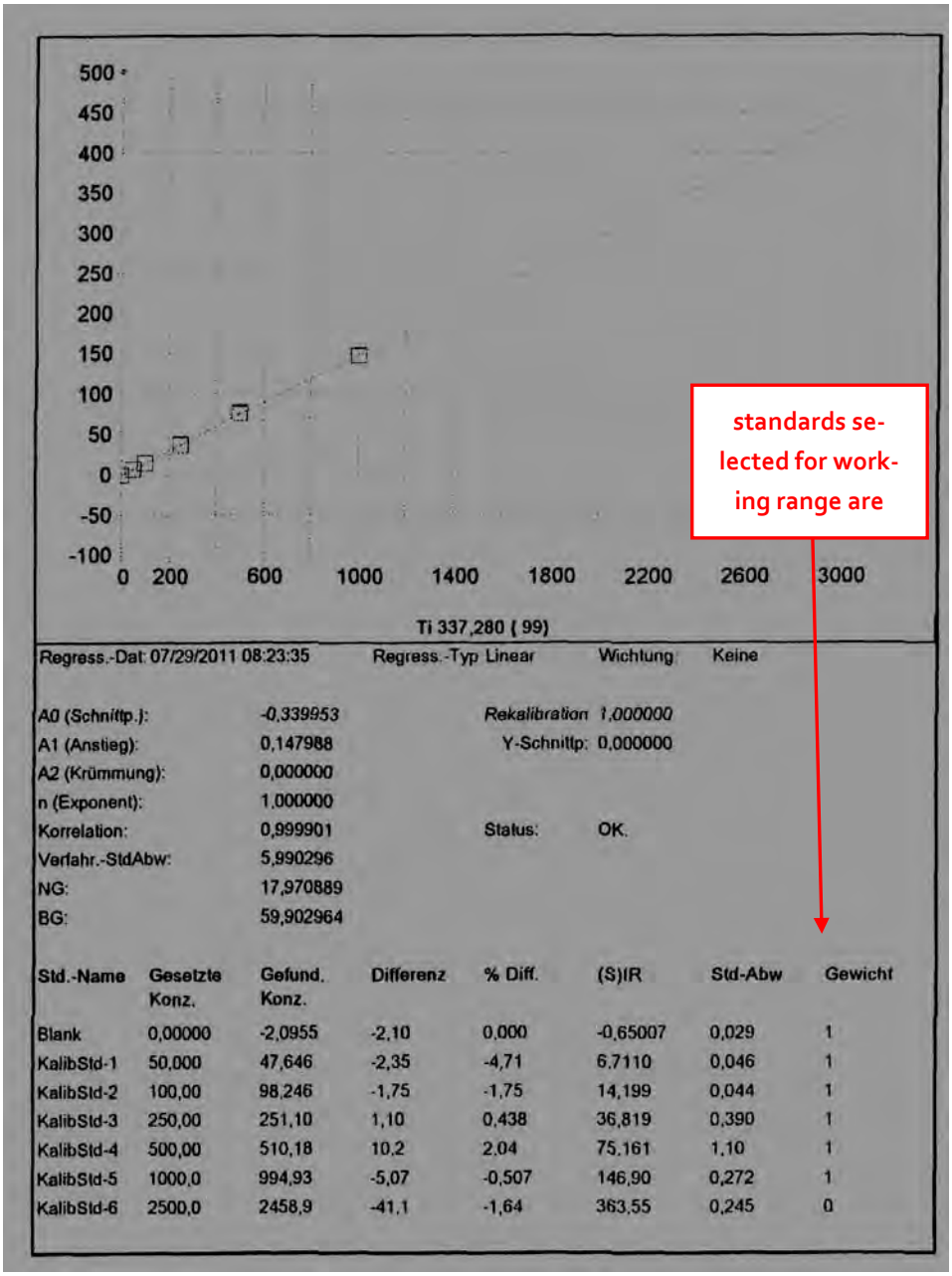
Table 225 summarizes the measured titanium concentrations in the samples.

Table 225: NM-103: Ti concentration in earthworms.

Sample name	Weighted sample [mg]	Measured value [µg/L]	Ti in dry weight [µg/g]	Mean Ti in dry weight ± SD [µg/g]
Control a	202.8	285	21.1	22.6 ± 2.2
Control b	201.9	325	24.1	
10 mg/kg (soil) a	202.6	949	70.3	55.3 ± 21.1
10 mg/kg (soil) b	200.9	541	40.4	
10 mg/kg (food) a	203.7	519	38.2	33.8 ± 6.3
10 mg/kg (food) b	202.5	396	29.3	
20 mg/kg (soil) c	202.2	391	29.0	30.1 ± 1.5
20 mg/kg (soil) d	202.0	420	31.2	
20 mg/kg (food) c	202.4	498	36.9	43.6 ± 9.6
20 mg/kg (food) d	201.8	678	50.4	
50 mg/kg (soil) c	202.2	401	29.7	30.8 ± 1.5
50 mg/kg (soil) d	203.2	432	31.9	
50 mg/kg (food) c	203.8	350	25.8	23.6 ± 3.0
50 mg/kg (food) d	201.7	289	21.5	
100 mg/kg (soil) c	202.1	454	33.7	32.9 ± 1.1
100 mg/kg (soil) d	203.4	436	32.2	
100 mg/kg (food) c	202.1	724	53.7	56.1± 3.4
100 mg/kg (food) d	202.2	789	58.5	
200 mg/kg (soil) c	201.9	397	29.5	31.1 ± 2.3
200 mg/kg (soil) d	201.3	439	32.7	
200 mg/kg (food) c	202.9	861	63.6	62.9 ± 1.0
200 mg/kg (food) d	202.6	840	62.2	

Raw data examples

Example for ICP-OES calibration: Calibration data from the measurement performed on July 28, 2011



Ti 337,280 (99)

Regress.-Dat: 07/29/2011 08:23:35		Regress.-Typ Linear	Wichtung	Keine
A0 (Schnittp.):	-0,339953	Rekalibration	1,000000	
A1 (Anstieg):	0,147988	Y-Schnittp:	0,000000	
A2 (Krümmung):	0,000000			
n (Exponent):	1,000000			
Korrelation:	0,999901	Status:	OK.	
Verfahr.-StdAbw:	5,990296			
NG:	17,970889			
BG:	59,902964			

Std.-Name	Gesetzte Konz.	Gefund. Konz.	Differenz	% Diff.	(S)IR	Std-Abw	Gewicht
Blank	0,00000	-2,0955	-2,10	0,000	-0,65007	0,029	1
KalibStd-1	50,000	47,646	-2,35	-4,71	6,7110	0,046	1
KalibStd-2	100,00	98,246	-1,75	-1,75	14,199	0,044	1
KalibStd-3	250,00	251,10	1,10	0,438	36,819	0,390	1
KalibStd-4	500,00	510,18	10,2	2,04	75,161	1,10	1
KalibStd-5	1000,0	994,93	-5,07	-0,507	146,90	0,272	1
KalibStd-6	2500,0	2458,9	-41,1	-1,64	363,55	0,245	0

Example for ICP-OES raw data printout: Example printout from the measurement performed on July 28, 2011

	Ti3349	Ti3361	Ti3372
Einheit	µg/L	µg/L	µg/L
Mittel	394,5	390,1	395,7
StdAbw	0,8	1,2	1,7
% RSD	0,2008	0,3102	0,4180
Mess.#1	394,8	390,4	396,2
Mess.#2	395,0	391,2	397,0
Mess.#3	393,5	388,8	393,8
41	Pro: 20mg/kg Futter a 07/28/2011 14:59:58 KONZ Custom ID1: Custom ID2: Custom ID3:		
	Ti3349	Ti3361	Ti3372
Einheit	µg/L	µg/L	µg/L
Mittel	392,0	387,2	391,0
StdAbw	1,2	1,2	1,6
% RSD	0,2984	0,3206	0,4164
Mess.#1	393,1	388,3	392,4
Mess.#2	392,1	387,4	391,3
Mess.#3	390,8	385,8	389,2
42	Pro: 20mg/kg Futter b 07/28/2011 15:02:14 KONZ Custom ID1: Custom ID2: Custom ID3:		
	Ti3349	Ti3361	Ti3372
Einheit	µg/L	µg/L	µg/L
Mittel	419,6	416,9	419,7
StdAbw	2,6	3,8	3,8
% RSD	0,6267	0,9007	0,8971
Mess.#1	416,6	412,7	415,4
Mess.#2	420,9	418,1	422,4
Mess.#3	421,4	419,9	421,3
43	Pro: Blank HNO3/H3BO3 1:2 07/28/2011 15:04:30 KONZ		
	Ti3349	Ti3361	Ti3372
Einheit	µg/L	µg/L	µg/L
Mittel	-0,6081	-0,2857	-1,136
StdAbw	0,0816	0,1071	0,237
% RSD	13,42	37,49	20,91
Mess.#1	-0,5190	-0,2393	-0,9027
Mess.#2	-0,6261	-0,4082	-1,128
Mess.#3	-0,6792	-0,2096	-1,377
44	Pro: 20mg/kg Boden a 07/28/2011 15:06:45 KONZ Custom ID1: Custom ID2: Custom ID3:		
	Ti3349	Ti3361	Ti3372
Einheit	µg/L	µg/L	µg/L
Mittel	491,0	491,4	497,5
StdAbw	5,3	6,3	6,3
% RSD	1,086	1,272	1,275
Mess.#1	493,9	494,5	500,9
Mess.#2	484,8	484,2	490,2
Mess.#3	494,2	495,5	501,4

21.3 Raw data – Reproduction test with earthworms – Ag (chapter 8)

21.3.1 Chemical analysis

Silver concentration in earthworms

The information about the LOD/LOQ and correlation coefficient are compiled in Table 226.

Coefficients of determination (r) for respective calibration functions were taken from ICP-OES instrument outputs.

Table 226: NM-300K - Ag concentration in earthworms: LODs/LOQs, correlation.

Measurement date, description	LOD [µg/L]	LOQ [µg/L]	Correlation coefficient r
August 26, 2011	2.6	8.8*	0.99995

* Internal LOQ calculation was performed with more digits

The certified reference material TMDA-70 (certified with 10.9 µg/L Ag) was analysed as quality assurance sample with solution samples from the test. According to the quality assurance requirement the silver recovery was in the range of ± 15% of the certified value. However, regarding Ag concentrations measured by ICP-OES, the mean recovery (accuracy) and precision of the non-digested CRM TMDA-70 measurements were 109 ± 10% (n = 4).

The recovery for Merck IV solution samples containing 50 µg Ag/L was 104 ± 5% (n = 2).

For further quality assurance, recalibration samples were analysed along with the samples and the mean accuracy was determined to 101 ± 2% (n = 2) for an Ag concentration of 50 µg/L.

For collecting validation information of the digestion procedure of samples as well as the analytical method, the mean recovery of silver in the reference material NIST 2977 Mussel Tissue was determined to 73.5 ± 6.4% (n =3), although only a reference value is given in the certificate.

The amount of silver in dispersion in NM300K provided by the producer ´Rent a Scientist´ is determined by UV-VIS measurements without prior digestion. Because a certified standard solution containing nano-Ag is not available yet, the calibration used for this method is performed with a silver standard. The quantification of total silver after total digestion may differ because the amount which was provided by the producer was measured there without matrix-adjusted calibration.

The measured values in µg/L are calculated to µg/g:

Amount of Ag in dry weight = measured value * weighed volume/weighted * 1.089

1.089 corresponds to the density of the nitric acid after digestion and filled up to 20 mL.

Analytical results

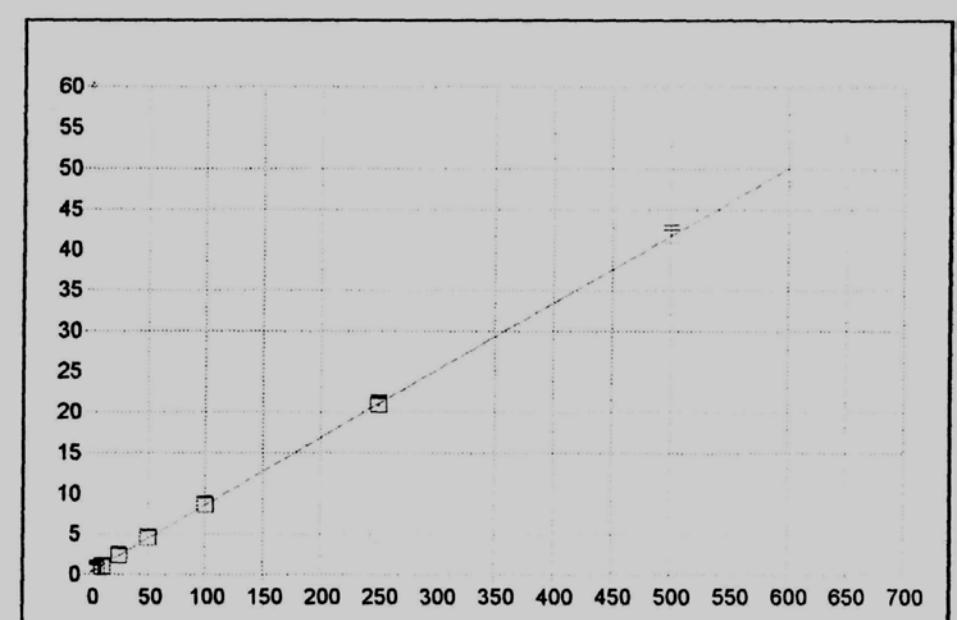
Table 227 summarizes the measured silver concentrations in the samples.

Table 227: NM-300K: Ag concentration in earthworms.

Sample name	Weighted sample [mg]	Brought to mass of [g]	Measured value [µg/L]	Ag in dry weight [µg/g]	Mean Ag in dry weight ± SD [µg/g]
Control a	203.5	21.426	-0.9251	-0.11	
Control b	201.9	21.207	-2.265	-0.26	
Vehicle (food) a	203.0	21.263	1.487	0.17	
Vehicle (food) b	203.4	21.302	-0.9253	-0.11	
Vehicle (soil) a	202.0	21.338	-0.2231	-0.03	
Vehicle (soil) b	201.6	21.289	-3.034	-0.35	
15 mg/kg (food) a	201.8	21.336	79.8	9.19	9.54 ± 0.50
15 mg/kg (food) b	204.7	21.301	87.3	9.90	
15 mg/kg (soil) a	204.8	21.276	61.8	6.99	6.99 ± <0.01
15 mg/kg (soil) b	203.0	21.315	61.2	6.99	
30 mg/kg (food) a	202.0	21.300	90.2	10.4	10.6 ± 0.4
30 mg/kg (food) b	201.9	21.324	94.8	10.9	
30 mg/kg (soil) a	202.0	21.294	89.0	10.2	10.5 ± 0.4
30 mg/kg (soil) b	201.5	21.233	94.3	10.8	
60 mg/kg (food) a	204.7	21.315	109	12.3	11.7 ± 0.9
60 mg/kg (food) b	204.7	21.351	97.2	11.0	
60 mg/kg (soil) a	204.8	21.277	96.8	10.9	11.1 ± 0.2
60 mg/kg (soil) b	202.0	21.283	97.8	11.2	
120 mg/kg (food) a	203.4	21.263	97.4	11.1	11.3 ± 0.3
120 mg/kg (food) b	202.1	21.300	100	11.5	
120 mg/kg (soil) a	201.8	21.252	101	11.6	11.3 ± 0.4
120 mg/kg (soil) b	201.3	21.266	95.3	11.0	
200 mg/kg (food) a	201.9	21.247	117	13.4	13.2 ± 0.2
200 mg/kg (food) b	202.1	21.211	115	13.1	
200 mg/kg (soil) a	201.4	21.284	97.9	11.3	11.2 ± 0.1
200 mg/kg (soil) b	202.5	21.340	96.5	11.1	

Raw data examples

Example for ICP-OES calibration: Calibration data from the measurement performed on August 26, 2011



Regress.-Dat: 08/26/2011 13:04:17		Regress.-Typ Linear	Wichtung:	Keine
A0 (Schnittp.):	0,254279		Rekalibration	1,000000
A1 (Anstieg):	0,082893		Y-Schnittp:	0,000000
A2 (Krümmung):	0,000000			
n (Exponent):	1,000000			
Korrelation:	0,999950		Status:	OK.
Verfahr.-StdAbw:	0,879086			
NG:	2,637259			
BG:	8,790863			

Std.-Name	Gesetzte Konz.	Gefund. Konz.	Differenz	% Diff.	(S)IR	Std-Abw	Gewicht
Blank	0,00000	0,41267	0,413	0,000	0,28849	0,042	1
KalibStd-1	1,0000	0,48085	-0,519	-51,9	0,29414	0,084	1
KalibStd-2	2,5000	2,0227	-0,477	-19,1	0,42195	0,106	1
KalibStd-3	5,0000	4,5630	-0,437	-8,74	0,63252	0,108	1
KalibStd-4	10,000	8,9808	-1,02	-10,2	0,99873	0,073	1
KalibStd-5	25,000	25,278	0,278	1,11	2,3497	0,142	1
KalibStd-6	50,000	51,509	1,51	3,02	4,5240	0,081	1
KalibStd-7	100,00	100,88	0,877	0,877	8,6163	0,141	1
KalibStd-8	250,00	249,38	-0,624	-0,250	20,926	0,206	1
KalibStd-9	500,00	501,49	1,49	0,299	41,825	0,280	0

Example for ICP-OES raw data printout: Example printout from the measurement performed on August 26, 2011

NanoAg Ag_Regenwürmer_110826 Hansknecht
6

StdAbw	7,49	8,71
% RSD	26,54	10,85
Mess.#1	36,86	90,24
Mess.#2	24,52	75,08
Mess.#3	23,32	75,25
26	Pro: Blank 10 % HNO3 08/26/2011 11:32:21 KONZ	
	Custom ID1:	Custom ID2: Custom ID3:
	Ag3280	Ag3382
Einheit	µg/L	µg/L
Mittel	-0,8579	8,536
StdAbw	0,4014	0,771
% RSD	46,79	9,037
Mess.#1	-1,258	9,409
Mess.#2	-0,4553	7,947
Mess.#3	-0,8603	8,251
27	Pro: 11KSA0404 a 08/26/2011 11:34:20 KONZ	
	Custom ID1:	Custom ID2: Custom ID3:
	Ag3280	Ag3382
Einheit	µg/L	µg/L
Mittel	-0,9251	78,15
StdAbw	1,160	3,41
% RSD	125,4	4,359
Mess.#1	-2,265	79,60
Mess.#2	-0,2531	74,26
Mess.#3	-0,2575	80,59
28	Pro: 11KSA0404 b 08/26/2011 11:36:18 KONZ	
	Custom ID1:	Custom ID2: Custom ID3:
	Ag3280	Ag3382
Einheit	µg/L	µg/L
Mittel	-2,265	60,02
StdAbw	0,702	3,80
% RSD	31,02	6,337
Mess.#1	-2,265	62,97
Mess.#2	-1,562	61,37
Mess.#3	-2,967	55,73
29	Pro: 11KSA0405 a 08/26/2011 11:38:17 KONZ	
	Custom ID1:	Custom ID2: Custom ID3:
	Ag3280	Ag3382
Einheit	µg/L	µg/L
Mittel	1,487	45,39
StdAbw	0,061	0,37
% RSD	4,088	0,8142
Mess.#1	1,455	44,98
Mess.#2	1,448	45,71
Mess.#3	1,557	45,48
30	Pro: 11KSA0405 b 08/26/2011 11:40:16 KONZ	
	Custom ID1:	Custom ID2: Custom ID3:
	Ag3280	Ag3382
Einheit	µg/L	µg/L

sample control a

sample control b

vehicle (food) a

Table 228: NM-300K: measured silver concentration in DGT extracts and calculated estimated average Ag concentration in matrix - day 0.

Sample ID	Measured extract Ni Concen-tration (µg/L)	Extract volume (L)	Mass extracted (µg)	Extraction factor	Calculated mass in DGT section (µg)	Deploy-ment time (h)	DGT area sampled (cm^2)	Metal ion flux µg/cm^2	DGT boundary thickness (cm)	Metal diffu-sion coeffi-cient (cm^2/sec)	Average water Ag concentration (mg/L)	Ag µg/L
Day 0												
Control A 10.06	0.005	0.015	0.00007	0.93	0.00008	48	3.142	1.4776E-10	0.094	1.23E-05	0.0000011	**0.001**
Control B 10.06	0.010	0.015	0.00015	0.93	0.00016	48	3.142	3.0004E-10	0.094	1.23E-05	0.0000023	**0.002**
Ag15mg A 10.06	0.758	0.015	0.01137	0.93	0.01223	48	3.142	2.2518E-08	0.094	1.23E-05	0.0001723	**0.172**
Ag15mg B 10.06	1.271	0.015	0.01907	0.93	0.02050	48	3.142	3.7758E-08	0.094	1.23E-05	0.0002889	**0.289**
Ag60mg A 10.06	0.690	0.015	0.01036	0.93	0.01114	48	3.142	2.051E-08	0.094	1.23E-05	0.0001569	**0.157**
Ag60mg B 10.06	0.787	0.015	0.01181	0.93	0.01270	48	3.142	2.3391E-08	0.094	1.23E-05	0.0001790	**0.179**
Ag120mg A 10.06	1.048	0.015	0.01572	0.93	0.01690	48	3.142	3.1133E-08	0.094	1.23E-05	0.0002382	**0.238**
Ag120mg B 10.06	23.570	0.015	0.35355	0.93	0.38016	48	3.142	7.0019E-07	0.094	1.23E-05	0.0053577	**5.358** [1]
Ag200mg A 10.06	1.183	0.015	0.01775	0.93	0.01908	48	3.142	3.5143E-08	0.094	1.23E-05	0.0002689	**0.269**
Ag200mg B 10.06	1.208	0.015	0.01812	0.93	0.01948	48	3.142	3.5886E-08	0.094	1.23E-05	0.0002746	**0.275**

Table 229: NM-300K: measured silver concentration in DGT extracts and calculated estimated average Ag concentration in matrix – day 28.

Sample ID	Measured extract Ni Concen-tration (µg/L)	Extract volume (L)	Mass extracted (µg)	Extraction factor	Calculated mass in DGT section (µg)	Deploy-ment time (h)	DGT area sampled (cm^2)	Metal ion flux µg/cm^2	DGT boundary thickness (cm)	Metal diffu-sion coeffi-cient (cm^2/sec)	Average water Ag concentration (mg/L)	Ag µg/L
Day 28												
Control A 10.07	0.027	0.015	0.00041	0.93	0.00044	48	3.142	8.0862E-10	0.094	1.23E-05	0.0000062	**0.006**
Control B 10.07	0.016	0.015	0.00024	0.93	0.00026	48	3.142	4.8452E-10	0.094	1.23E-05	0.0000037	**0.004**
Ag15mg A 10.07	0.982	0.015	0.01472	0.93	0.01583	48	3.142	2.9157E-08	0.094	1.23E-05	0.0002231	**0.223**
Ag15mg B 10.07	0.608	0.015	0.00912	0.93	0.00981	48	3.142	1.8062E-08	0.094	1.23E-05	0.0001382	**0.138**
Ag60mg A 10.07	1.081	0.015	0.01622	0.93	0.01744	48	3.142	3.2113E-08	0.094	1.23E-05	0.0002457	**0.246**
Ag60mg B 10.07	0.882	0.015	0.01322	0.93	0.01422	48	3.142	2.619E-08	0.094	1.23E-05	0.0002004	**0.200**
Ag120mg A 10.07	1.330	0.015	0.01995	0.93	0.02145	48	3.142	3.951E-08	0.094	1.23E-05	0.0003023	**0.302**
Ag120mg B 10.07	1.920	0.015	0.0288	0.93	0.03097	48	3.142	5.7037E-08	0.094	1.23E-05	0.0004364	**0.436**
Ag200mg A 10.07	1.782	0.015	0.02673	0.93	0.02874	48	3.142	5.2938E-08	0.094	1.23E-05	0.0004051	**0.405**
Ag200mg B 10.07	1.502	0.015	0.02253	0.93	0.02423	48	3.142	4.462E-08	0.094	1.23E-05	0.0003414	**0.341**

[1] Not considered for the assessment; technical defect of the DGT

Table 230: NM-300K: measured silver concentration in DGT extracts and calculated estimated average Ag concentration in matrix - day 56.

Sample ID	Measured Extract Ni Concen-tration (µg/L)	Extract volume (L)	Mass Extracted (µg)	Extraction Factor	Calculated Mass in DGT section (µg)	Deploy-ment Time (h)	DGT area sampled (cm2)	Metal Ion Flux µg/cm2	DGT Boundary Thickness (cm)	Metal Dif-fusion Coefficient (cm2/sec)	Average water Ag concentration (mg/L)	**Ag µg/L**
Day 56												
Control A	0.0210	0.015	0.00031	0.93	0.00034	48	3.142	6.2374E-10	0.094	1.23E-05	0.0000048	**0.005**
Control B	0.0198	0.015	0.0003	0.93	0.00032	48	3.142	5.8961E-10	0.094	1.23E-05	0.0000045	**0.005**
15mg A	0.6131	0.015	0.0092	0.93	0.00989	48	3.142	1.8212E-08	0.094	1.23E-05	0.0001394	**0.139**
15mg B	1.6200	0.015	0.0243	0.93	0.02613	48	3.142	4.8126E-08	0.094	1.23E-05	0.0003683	**0.368**
60mg A	1.2878	0.015	0.01932	0.93	0.02077	48	3.142	3.8258E-08	0.094	1.23E-05	0.0002927	**0.293**
60mg B	0.8755	0.015	0.01313	0.93	0.01412	48	3.142	2.6008E-08	0.094	1.23E-05	0.0001990	**0.199**
120mg A	2.7636	0.015	0.04145	0.93	0.04457	48	3.142	8.2098E-08	0.094	1.23E-05	0.0006282	**0.628**
120mg B	2.3846	0.015	0.03577	0.93	0.03846	48	3.142	7.084E-08	0.094	1.23E-05	0.0005421	**0.542**
200mg A	1.4711	0.015	0.02207	0.93	0.02373	48	3.142	4.3703E-08	0.094	1.23E-05	0.0003344	**0.334**
200mg B	1.5578	0.015	0.02337	0.93	0.02513	48	3.142	4.6277E-08	0.094	1.23E-05	0.0003541	**0.354**

The calculations were performed according to the Technical documentation on http://www.dgtresearch.com and references cited within.

The following arithmetic statements were applied:

1. Extracted mass [µg] = measured extract Ag conc. [µg/L] *extract volume [L]
2. Extraction factor = 0.93 according to literature mentioned above
3. Calculated mass in DGT section [µg] = extracted mass [µg] / extraction factor
4. Metal ion flux [µg/s*cm^2] = calculated mass in DGT section [µg] / deployment time [s] * sampled DGT area [cm^2]
5. Estimated average Ag conc. in matrix [mg/L] = metal ion flux [µg/s*cm^2] *DGT boundary thickness [cm] / metal diffusion coefficient [cm^2 / s]
6. Estimated average Ag conc. in matrix [µg/L] = estimated average Ag conc. in matrix [mg/L] * 1000

21.3.2 Ecotoxicological test

Table 231: NM-300K – earthworm test: living worms after 28 days.

Single values of the parallel test pots [Individuals]

Replicate No.	1	2	3	4	5	6	7	8
Control	10	10	10	10	10	10	10	10
Control with dispersant on feed	10	10	10	10	-	-	-	-
Control with dispersant on soil	10	10	10	10	-	-	-	-
Application on feed								
15 mg/kg	10	10	10	10	-	-	-	-
30 mg/kg	10	10	10	10	-	-	-	-
60 mg/kg	10	10	10	10	-	-	-	-
120 mg/kg	10	10	10	10	-	-	-	-
200 mg/kg	10	10	9	10	-	-	-	-
Application on soil								
15 mg/kg	10	10	10	10	-	-	-	-
30 mg/kg	10	10	10	10	-	-	-	-
60 mg/kg	10	10	10	10	-	-	-	-
120 mg/kg	10	10	10	10	-	-	-	-
200 mg/kg	10	10	10	10	-	-	-	-

Table 232: NM-300K – earthworm test: biomass of the worm batches at test start.

Single values of the replicate test pots

Replicate No.	1	2	3	4	5	6	7	8
Control	3.57	3.85	3.87	3.43	3.52	3.60	3.35	3.39
Control with dispersant on feed	3.38	3.54	4.14	4.03	-	-	-	-
Control with dispersant on soil	3.58	3.31	4.11	3.94	-	-	-	-
Application on feed								
15 mg/kg	3.65	3.63	3.83	3.79	-	-	-	-
30 mg/kg	3.90	3.43	3.67	3.42	-	-	-	-
60 mg/kg	4.19	3.71	3.94	3.26	-	-	-	-
120 mg/kg	3.64	3.49	3.96	3.41	-	-	-	-
200 mg/kg	3.32	3.42	3.61	3.44	-	-	-	-
Application on soil								
15 mg/kg	3.14	3.31	3.12	3.38	-	-	-	-
30 mg/kg	3.39	3.32	3.39	3.22	-	-	-	-
60 mg/kg	3.35	3.86	3.46	3.44	-	-	-	-
120 mg/kg	3.42	3.46	3.18	3.52	-	-	-	-
200 mg/kg	3.46	3.59	3.55	3.28	-	-	-	-

Table 233: NM-300K – earthworm test: biomass of the worm batches after 28 days.

Single values of the replicate test pots

Replicate No.	1	2	3	4	5	6	7	8
Control	4.83	5.02	5.06	4.87	4.94	5.29	4.80	5.12
Control with dispersant on feed	4.85	5.03	5.78	5.76	-	-	-	-
Control with dispersant on soil	4.73	4.65	5.96	4.95	-	-	-	-
Application on feed								
15 mg/kg	5.77	5.17	5.99	5.66	-	-	-	-
30 mg/kg	5.57	5.16	5.46	5.11	-	-	-	-
60 mg/kg	6.05	5.64	5.60	4.70	-	-	-	-
120 mg/kg	4.92	4.13	4.46	4.69	-	-	-	-
200 mg/kg	4.36	4.40	4.54	4.32	-	-	-	-
Application on soil								
15 mg/kg	4.90	5.15	5.96	4.94	-	-	-	-
30 mg/kg	4.67	5.72	5.37	5.73	-	-	-	-
60 mg/kg	5.71	5.79	5.52	5.70	-	-	-	-
120 mg/kg	5.52	5.86	5.47	5.78	-	-	-	-
200 mg/kg	5.43	6.14	5.73	5.25	-	-	-	-

Table 234: NM-300K – earthworm test: number of offspring at day 56.

Replicate No.	1	2	3	4	5	6	7	8
Control	355	341	316	319	371	382	312	335
Control with dispersant on feed	261	254	266	281	-	-	-	-
Control with dispersant on soil	264	206	310	292	-	-	-	-
Application on feed								
15 mg/kg	281	328	281	329	-	-	-	-
30 mg/kg	279	232	230	274	-	-	-	-
60 mg/kg	206	233	239	204	-	-	-	-
120 mg/kg	119	64	82	179	-	-	-	-
200 mg/kg	100	81	89	97	-	-	-	-
Application on soil								
15 mg/kg	296	247	236	227	-	-	-	-
30 mg/kg	145	209	254	227	-	-	-	-
60 mg/kg	202	222	193	261	-	-	-	-
120 mg/kg	161	157	179	135	-	-	-	-
200 mg/kg	129	73	106	78	-	-	-	-

Table 235: NM-300K – earthworm test: soil dry mass content [%].

Single values of the replicate test pots [%]; concentrations given as nominal values [mg/kg]

				Application on feed					Application on soil				
	Con-trol	Control with dispers-ant on feed	Control with dis-persant on soil	15	30	60	120	200	15	30	60	120	200
Test start	88.3	88.6	88.7	88.7	88.9	88.5	88.5	89.1	89.5	88.7	89.3	89.2	89.4
Test end	78.3	79.6	79.6	81.4	79.6	79.5	79.2	78.8	80.2	79.5	79.4	79.4	78.5

Table 236: NM-300K – earthworm test: soil moisture [% WHC].

Single values of the replicate test pots; since WHC is affected by feed adding, no calculation for test end can be applied; concentrations given as nominal values [mg/kg]

				Application on feed					Application on soil				
	Con-trol	Control with dispers-ant on feed	Control with dis-persant on soil	15	30	60	120	200	15	30	60	120	200
Test start	58.6	56.7	56.3	55.9	54.9	57.1	57.5	53.7	51.6	56.1	53.0	53.1	52.0

Table 237: NM-300K – earthworm test: soil pH.

Single values of the parallel test pots; concentrations given as nominal values [mg/kg]

			Application on feed					Application on soil				
	Control	Control with disper-sant	15	30	60	120	200	15	30	60	120	200
Test start	5.0	4.9	5.0	5.0	4.9	4.9	5.0	5.0	5.0	5.0	5.0	5.1
Test end	6.8	6.8	6.8	7.0	7.1	6.9	6.8	7.0	7.1	7.0	7.0	6.8

21.4 Raw data - Test with plants (chapter 11)

Table 238: P25 – Test with plants: pathological symptoms [plants].

			Application via powder [mg TiO_2/kg]						Application via dispersion [mg TiO_2/kg]	
Test species	Replicate	Control	10	20	30	44	67	100	10	20
Avena sativa	- [1]	-	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	-	-	-	-
Phaseolus aureus	-	-	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	-	-	-	-
Sinapis alba	-	-	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	-	-	-	-

[1] - = no visual symptom

Table 239: P25 – Test with plants: emergence at test end [number of plants].

Single values of the replicate test pots

			Application via powder [mg TiO_2/kg]						Application via dispersion [mg TiO_2/kg]	
Test species	Replicate	Control	10	20	30	44	67	100	10	20
Avena sativa	1	5	5	5	5	5	4	3	4	4
	2	5	5	5	5	4	4	4	4	5
	3	5	4	4	4	5	4	4	5	5
	4	5	5	5	4	4	4	5	5	5
Phaseolus aureus	1	5	5	5	5	5	4	5	5	5
	2	5	5	5	5	5	5	5	5	5
	3	5	5	5	5	5	5	5	5	5
	4	5	5	5	5	5	5	5	5	5
Sinapis alba	1	5	4	3	4	4	2	4	2	5
	2	5	5	5	5	3	4	5	4	4
	3	5	5	4	4	5	4	5	5	5
	4	4	4	4	4	5	5	5	3	5

Table 240: P25 – Test with plants: fresh mass per plant [g].

Single values of the parallel test pots [g]

			Application via powder [mg TiO_2/kg]						Application via dispersion [mg TiO_2/kg]	
Test species	Replicate	Control	10	20	30	44	67	100	10	20
Avena sativa	1	2.966	2.627	2.467	2.972	2.633	2.103	1.522	1.915	1.885
	2	2.401	3.15	2.461	2.82	2.037	2.474	1.79	2.002	2.619
	3	2.657	1.961	2.115	2.325	2.691	2.144	1.99	2.313	2.578
	4	2.338	2.547	2.799	2.152	2.166	2.078	1.909	2.288	2.062
Phaseolus aureus	1	3.824	3.411	3.282	3.799	3.630	2.826	2.511	3.415	3.084
	2	3.865	3.684	3.963	3.628	4.106	3.036	3.550	4.114	3.424
	3	3.753	3.388	3.514	4.473	3.264	3.115	3.298	3.206	3.616
	4	3.961	3.299	3.130	3.577	3.695	3.705	3.150	3.385	2.960
Sinapis alba	1	2.101	2.804	1.688	2.388	2.365	1.437	2.076	1.220	2.358
	2	2.053	2.212	2.903	2.129	1.499	1.555	1.716	1.698	1.770
	3	3.165	2.153	2.221	1.264	2.603	2.398	1.927	1.959	1.968
	4	2.324	1.854	1.968	2.076	2.506	3.306	2.218	0.918	2.065

Table 241: P25 – Test with plants: root length - *Avena sativa* [cm].

	Replicate	Number of plants	Length of main root biomass	Maximum and minimum length of roots different to main root biomass	Remark
Control	I	5	6.8	12.0 – 21.5	-
	II	5	7.6	9.6 – 20.4	-
	III	5	7.5	11.2 – 16.6	-
	IV	5	7.0	16.6 – 19.5	-
Application via powder: 10 mg/kg	I	5	7.7	14.5 – 21.2	-
	II	5	8.0	12.8 – 29.4	-
	III	4	8.4	14.0 – 23.4	-
	IV	5	7.1	14.8 – 20.4	-
Application via powder: 20 mg/kg	I	5	6.2	11.4 – 19.6	-
	II	5	7.2	13.6 – 27.1	-
	III	4	7.2	12.6 – 23.4	-
	IV	5	6.5	15.0 – 19.2	-
Application via powder: 30 mg/kg	I	5	7.4	15.7 – 27.2	-
	II	5	7.5	13.0 – 23.1	-
	III	4	7.4	17.0 – 21.7	-
	IV	4	7.8	9.4 – 25.2	-
Application via powder: 44 mg/kg	I	5	6.6	10.9 – 24.4	-
	II	4	7.1	13.1 – 19.2	-
	III	5	7.6	16.8 – 22.5	-
	IV	4	6.5	16.6 – 22.2	-
Application via powder: 63 mg/kg	I	4	6.4	14.4 – 17.9	-
	II	4	7.0	16.7 – 25.2	-
	III	4	6.7	13.1 – 23.7	-
	IV	4	7.6	17.2 – 22.4	-
Application via powder: 100 mg/kg	I	3	8.0	22.2 – 32.2	-
	II	4	7.2	10.9 – 26.4	-
	III	4	8.0	14.4 – 21.3	-
	IV	5	7.9	12.7 – 16.8	-
Application via dispersion: 10 mg/kg	I	4	8.0	15.1 – 19.4	-
	II	4	7.6	16.4 – 23.5	-
	III	5	6.9	10.8 – 23.8	-
	IV	5	7.0	10.8 – 23.4	-
Application via dispersion: 20 mg/kg	I	4	7.4	8.4 – 18.7	-
	II	5	6.9	9.7 – 22.1	-
	III	5	6.5	7.4 – 25.6	-
	IV	5	7.8	12.6 – 25.3	-

Table 242: P25 – Test with plants: root length - *Sinapis alba* [cm].

	Replicate	Number of plants	Length of main root biomass	Maximum and minimum length of roots different to main root biomass	Remark
Control	I	5	3.4	4.6 – 10.4	-
	II	5	3.2	5.7 – 11.9	-
	III	5	6.7	8.4 – 13.6	-
	IV	4	6.4	11.4 – 14.6	-
Application via powder: 10 mg/kg	I	4	5.7	8.6 – 15.2	-
	II	5	4.7	5.0 – 9.3	-
	III	5	4.9	6.0 – 14.2	-
	IV	4	4.9	5.4 – 10.2	-
Application via powder: 20 mg/kg	I	3	5.2	7.7 – 14.4	-
	II	5	5.0	7.5 – 13.4	-
	III	4	3.2	5.2 – 7.9	-
	IV	4	4.5	4.7 – 12.6	-
Application via powder: 30 mg/kg	I	4	4.6	6.6 – 12.1	-
	II	5	3.7	5.7 – 16.8	-
	III	4	3.5	11.9 – 15.9	-
	IV	4	3.4	4.4 – 14.6	-
Application via powder: 44 mg/kg	I	4	3.7	5.2- 9.4	-
	II	3	3.9	5.4 – 11.4	-
	III	5	4.1	3.6 – 9.4	-
	IV	5	3.4	7.0 – 9.3	-
Application via powder: 63 mg/kg	I	2	3.9	4.1 – 6.7	-
	II	4	3.6	4.2 – 7.1	-
	III	4	4.1	5.2 – 15.1	-
	IV	5	3.8	4.4 – 11.4	-
Application via powder: 100 mg/kg	I	4	4.6	6.1 – 10.1	-
	II	5	4.5	5.9 – 12.2	-
	III	5	4.2	1.6 – 15.2	-
	IV	5	3.9	3.5 – 8.9	-
Application via dispersion: 10 mg/kg	I	2	4.2	10.1 – 14.7	-
	II	4	4.9	6.1 – 10.1	-
	III	5	4.0	0.9 – 8.4	-
	IV	3	4.1	4.4 – 13.2	-
Application via dispersion: 20 mg/kg	I	5	4.5	6.7 – 12.2	-
	II	4	4.5	5.2 – 9.6	-
	III	5	4.5	6.4 – 11.2	-
	IV	5	3.9	4.6 – 7.2	-

Table 243: P25 – Test with plants: root length - *Phaseolus aureus* [cm].

	Replicate	Number of plants	Length of main root biomass	Maximum and minimum length of roots different to main root biomass	Remark
Control	I	5	11.0	13.0 – 18.0	-
	II	5	13.0	3.0 – 9.0	-
	III	5	11.5	4.0 – 10.0	-
	IV	5	12.5	15.0 – 19.0	-
Application via powder: 10 mg/kg	I	5	10.5	12.5 – 22.0	-
	II	5	11.0	14.0 – 21.0	-
	III	5	10.0	13.0 – 22.0	-
	IV	5	12.0	15.5 – 21.0	-
Application via powder: 20 mg/kg	I	5	9.5	14.0 – 21.0	-
	II	5	10.0	13.5 – 21.0	-
	III	5	12.0	11.5 – 19.0	-
	IV	5	11.0	14.0 – 21.0	-
Application via powder: 30 mg/kg	I	5	10.0	13.0 – 17.0	-
	II	5	11.0	11.0 – 27.0	-
	III	5	13.0	13.0 – 22.0	-
	IV	5	10.5	14.5 -21.0	-
Application via powder: 44 mg/kg	I	5	9.5	12.0 – 20.0	-
	II	5	10.5	13.0 – 21.0	-
	III	5	10.5	16.0 – 23.0	-
	IV	5	10.5	11.0 – 19.0	-
Application via powder: 63 mg/kg	I	5	9.5	15.0 – 22.0	-
	II	5	10.5	11.0 – 17.0	-
	III	5	10.0	18.0 – 22.0	-
	IV	5	10.0	16.5 – 22.0	-
Application via powder: 100 mg/kg	I	4	12.0	14.0 – 18.0	-
	II	5	10.5	15.5 – 22.0	-
	III	5	11.5	14.5 – 22.0	-
	IV	5	10.0	15.0 – 22.0	-
Application via dispersion: 10 mg/kg	I	5	10.0	11.0 – 19.0	-
	II	5	10.5	11.0 – 22.0	-
	III	5	10.0	16.0 – 19.0	-
	IV	5	10.5	11.0 – 17.5	-
Application via dispersion: 20 mg/kg	I	5	10.5	15.0 – 23.0	-
	II	5	9.5	12.0 – 16.5	-
	III	5	10.0	14.0 – 25.0	-
	IV	5	9.5	15.0 – 19.0	-

21.5 Raw data – Emergence test with chironomids – TiO_2 (chapter 12)

21.5.1 P25

Table 244: P25 – Chironomid test: quality control/validation of chemical analyses.

	Sample description	Ti [µg/L] - measured	Ti [µg/L] - nominal	Recovery [%]
Samples of day 0 and day 1				
Positive control (aqueous samples with P25)	3.2 mg TiO_2/100mL	20197	19177.6	105
	3.2 mg TiO_2/100mL	20223	19177.6	105
	2.6 mg TiO_2/100mL	15300	15581.8	98.2
	2.6 mg TiO_2/100mL	14477	15581.8	92.9
	2.6 mg TiO_2/100mL	14587	15581.8	93.6
	2.6 mg TiO_2/100mL	14545	15581.8	93.3
Reference standard CPI	Ti 500 µg/L	504.4	500	101
	Ti 500 µg/L	505.57	500	101
	Ti 500 µg/L	505.23	500	101
	Ti 500 µg/L	506.57	500	101
Recalibration standard (commercially available Ti standard with soluble Ti)	Ti 500 µg/L	501.03	500	100
	Ti 500 µg/L	507.1	500	101
Samples of day 7				
Positive control (aqueous samples with P25)	208 mg TiO_2/L	128900	125140	103
	208 mg TiO_2/L	127850	125140	102
	26 mg TiO_2/L	18385	15582	118
	26 mg TiO_2/L	18030	15582	116
Reference standard CPI	Ti 25 µg/L	24.2	25	96.9
	Ti 25 µg/L	24.8	25	99.1
Reference standard (commercially available Ti standard with soluble Ti)	25 µg/L	24.5	25	98.1
	25 µg/L	24.1	25	96.4
Samples of day 7 and day 14				
Positive control (aqueous samples with P25)	208 mg TiO_2/L	127575	125140	102
	208 mg TiO_2/L	128400	125140	103
	26 mg TiO_2/L	13605	15581.8	87.3
	26 mg TiO_2/L	12975	15581.8	83.3
Reference standard CPI	Ti 25 µg/L	25.8	25	103
	Ti 25 µg/L	25.4	25	101
Reference standard (commercially available Ti standard with soluble Ti)	Ti 25 µg/L	26.6	25	106
	Ti 25 µg/L	25.3	25	101

Continued

Table 244: P25 – Chironomid test: quality control/validation of chemical analyses. (continued)

Samples of day 28				
Positive control (aqueous samples with P25)	208 mg TiO_2/L	122650	125140	98.0
	208 mg TiO_2/L	123050	125140	98.3
	26 mg TiO_2/L	16455	15582	106
	26 mg TiO_2/L	16270	15582	104
Reference standard CPI	Ti 25 µg/L	22.8	25	91.2
	Ti 25 µg/L	23.9	25	95.5
Reference standard (commercially available Ti standard with soluble Ti)	Ti 25 µg/L	24.0	25	96.0
	Ti 25 µg/L	24.8	25	99.1

Table 245: P25 – Chironomid test: physico-chemical test parameters.

Water hardness (TH): 1 mmol corresponds to 100 mg $CaCo_3$ equiv.

		Test start						Test end					
		O_2 %	Temp °C	pH	TH mmol/l	NH_4 mg/L	Light lux	O_2 %	Temp °C	pH	TH mmol/l	NH_4 mg/L	Light lux
Control	1	99.5	20.3	8.19	1.1	0.9	748-850	93.6	20.3	8.08	1.0	8.0	749-798
	2	99.3		8.20	1.1	0.9		97.8		8.26	1.0	10.8	
	3	96.5		8.12	1.3	1.0		94.2		8.22	1.2	10.0	
	4	96.8		8.04	1.3	0.8		88.3		8.15	1.1	10.0	
15 mg/L	1	97.2		7.93				103.8		8.39			
	2	99.3		8.16				96.8		8.28			
	3	97.1		8.15				102		8.36			
	4	95.8		8.12				99.9		8.34			
24 mg/L	1	97.1		7.90				100.4		8.41			
	2	98.2		8.07				100.3		8.34			
	3	99.3		8.27				100.1		8.29			
	4	95.2		8.04				99.9		8.33			
39 mg/L	1	99.1		8.20				100.1		8.36			
	2	97.5		8.16				96.4		8.32			
	3	99.0		8.24				100.1		8.35			
	4	99.4		8.15				100.2		8.37			
63 mg/L	1	96.0		7.93				97.1		8.27			
	2	95.7		7.99				100.3		8.36			
	3	98.6		8.08				98.7		8.25			
	4	100.0		8.15				99.6		8.35			
100 mg/L	1	96.6		8.05	1.5	0.2		101.1		8.36	1.3	9.2	
	2	99.9		8.12				99.4		8.33			
	3	97.5		8.08				68.1		8.28			
	4	100.4		8.05				96.6		8.32			
Control with nettle	1	99.1		8.01	1.2	0.6		104.4		8.65	2.1	10.8	
	2	91.1		7.98				103.5		8.65			
	3	100.8		8.02				97.2		8.41			
	4	99.4		8.08				96		8.44			
Control with nettle + 100 mg/L	1	89.4		8.02				101.4		8.64			
	2	92.9		8.02				104.1		8.70	2.2	8.8	
	3	92.3		8.05				103.8		8.66			
	4	87.1		7.97				94.5		8.41			
Vessels used for chemical analysis	K	98.2		7.75				98.7		8.22			
	1	99.0		7.94				97.5		8.05			
	2	98.2		7.99				100.8		8.21			
	3	99.4		8.26				100.2		8.45			
	4	99.6		8.26				95.4		8.24			
	5	99.2		8.32				85.8		8.16			

Table 246: P25 – Chironomid test: addition of food (TetraMin grinded).

Date		20.4			23.4			26.4			29.4	30.4			3.5
		Day -1	Day 0	Day 1	Day 2	Day3	Day 4	Day 5	Day6	Day 7	Day 8	Day 9	Day 10	Day 11	Day 12
		mg	mg	mg	mg	mg	mg	mg	mg	mg	mg	mg	mg	mg	mg
Control	1	15	-	-	24	-	-	26	-	-	10	48	-	-	32
	2	15	-	-	24	-	-	26	-	-	10	48	-	-	32
	3	15	-	-	24	-	-	26	-	-	10	48	-	-	32
	4	15	-	-	24	-	-	26	-	-	10	48	-	-	32
15 mg/L	1	15	-	-	24	-	-	26	-	-	10	48	-	-	32
	2	15	-	-	24	-	-	26	-	-	10	48	-	-	32
	3	15	-	-	24	-	-	26	-	-	10	48	-	-	32
	4	15	-	-	24	-	-	26	-	-	10	48	-	-	32
24 mg/L	1	15	-	-	24	-	-	26	-	-	10	48	-	-	32
	2	15	-	-	24	-	-	26	-	-	10	48	-	-	32
	3	15	-	-	24	-	-	26	-	-	10	48	-	-	32
	4	15	-	-	24	-	-	26	-	-	10	48	-	-	32
39 mg/L	1	15	-	-	24	-	-	26	-	-	10	48	-	-	32
	2	15	-	-	24	-	-	26	-	-	10	48	-	-	32
	3	15	-	-	24	-	-	26	-	-	10	48	-	-	32
	4	15	-	-	24	-	-	26	-	-	10	48	-	-	32
63 mg/L	1	15	-	-	24	-	-	26	-	-	10	48	-	-	32
	2	15	-	-	24	-	-	26	-	-	10	48	-	-	32
	3	15	-	-	24	-	-	26	-	-	10	48	-	-	32
	4	15	-	-	24	-	-	26	-	-	10	48	-	-	32
100 mg/L	1	15	-	-	24	-	-	26	-	-	10	48	-	-	32
	2	15	-	-	24	-	-	26	-	-	10	48	-	-	32
	3	15	-	-	24	-	-	26	-	-	10	48	-	-	32
	4	15	-	-	24	-	-	26	-	-	10	48	-	-	32
Vessels used for chemical analysis	K	15	-	-	24	-	-	26	-	-	10	48	-	-	32
	1	15	-	-	24	-	-	26	-	-	10	48	-	-	32
	2	15	-	-	24	-	-	26	-	-	10	48	-	-	32
	3	15	-	-	24	-	-	26	-	-	10	48	-	-	32
	4	15	-	-	24	-	-	26	-	-	10	48	-	-	32
	5	15	-	-	24	-	-	26	-	-	10	48	-	-	32

Continued

Table 246: P25 – Chironomid test: addition of food, continued.

Date			5.5.		7.5			10.5		12.5		14.5			17.5
		Day 13	Day 14	Day 15	Day 16	Day 17	Day 18	Day 19	Day 20	Day 21	Day 22	Day 23	Day 24	Day 25	Day 26
		mg	mg	mg	mg	mg	mg	mg	mg	mg	mg	mg	mg	mg	mg
Control	1	-	32	-	46.8	-	-	16	-	12	-	18	-	-	12
	2	-	32	-	46.8	-	-	24	-	18	-	27	-	-	18
	3	-	32	-	52	-	-	28	-	20	-	27	-	-	16
	4	-	32	-	41.6	-	-	22	-	20	-	30	-	-	20
15 mg/L	1	-	32	-	44.2	-	-	24	-	14	-	21	-	-	14
	2	-	32	-	46.8	-	-	30	-	22	-	27	-	-	16
	3	-	32	-	52	-	-	22	-	14	-	6	-	-	4
	4	-	32	-	46.8	-	-	16	-	14	-	15	-	-	10
24 mg/L	1	-	32	-	52	-	-	22	-	12	-	15	-	-	8
	2	-	32	-	46.8	-	-	14	-	10	-	12	-	-	8
	3	-	32	-	36.4	-	-	16	-	8	-	9	-	-	6
	4	-	32	-	44.2	-	-	18	-	18	-	27	-	-	16
39 mg/L	1	-	32	-	49.4	-	-	30	-	20	-	21	-	-	14
	2	-	32	-	44.2	-	-	30	-	30	-	45	-	-	30
	3	-	32	-	39.0	-	-	10	-	16	-	15	-	-	10
	4	-	32	-	52	-	-	18	-	4	-	3	-	-	-
63 mg/L	1	-	32	-	49.4	-	-	32	-	20	-	30	-	-	20
	2	-	32	-	49.4	-	-	2	-	-	-	-	-	-	-
	3	-	32	-	36.4	-	-	16	-	16	-	24	-	-	16
	4	-	32	-	41.6	-	-	22	-	16	-	21	-	-	14
100 mg/L	1	-	32	-	41.6	-	-	16	-	10	-	15	-	-	10
	2	-	32	-	44.2	-	-	18	-	18	-	24	-	-	16
	3	-	32	-	49.4	-	-	30	-	24	-	18	-	-	10
	4	-	32	-	49.4	-	-	24	-	20	-	27	-	-	18
Vessels used for chemical analysis	K	-	32	-	44.2	-	-	18	-	12	-	9	-	-	6
	1	-	32	-	52	-	-	18	-	14	-	15	-	-	10
	2	-	32	-	49.4	-	-	18	-	8	-	6	-	-	4
	3	-	32	-	41.6	-	-	16	-	4	-	6	-	-	4
	4	-	32	-	46.8	-	-	12	-	12	-	18	-	-	12
	5	-	32	-	52	-	-	36	-	36	-	36	-	-	14

Table 247: P25 – Chironomid test: number of hatched midges and sex.

Date		5.05. 2010		6.05. 2010		7.05. 2010		8.05. 2010		9.05. 2010		10.5. 2010		11.5. 2010	
		♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂
Control	1			-	1	1	-	-	-	4	2	3	1	1	-
	2			-	-	-	-	-	1	-	2	1	2	1	-
	3			-	1	-	-	-	1	2	2	2	1	1	-
	4			-	-	2	-	-	1	-	1	3	1	1	-
15 mg/L	1			-	1	-	2	-	2	2	-	1	-	3	-
	2			-	-	1	1	-	1	-	1	1	-	1	-
	3			-	-	-	-	-	-	1	6	1	1	1	-
	4			-	-	-	2	-	1	3	4	1	1	-	-
24 mg/L	1			-	-	-	-	-	5	-	2	2	-	1	1
	2			-	1	-	1	1	1	4	2,1†	2	-	-	1
	3			-	3	-	3	-	3	1	-	2	-	3	-
	4			-	1	-	2	-	3	4	-	-	1	-	-
39 mg/L	1			-	-	1	1	-	-	1	-	1	1	2	-
	2		1	-	1	-	-	1	-	2	-	-	-	-	-
	3			-	2	1	2	-	1	3	4	2	-	-	-
	4			-	-	-	-	-	3	3	2	2	1	3	1
63 mg/L	1			-	-	-	1	-	1	-	1	1	-	3	-
	2			-	-	1	-	1	8	3	3	3	-	1	-
	3			-	2	-	4	2	-	2	2	-	-	-	-
	4			-	2	-	2	1	1	1	2	-	-	2	-
100 mg/L	1			-	3	1	-	-	1	3	3	1	-	1	-
	2			-	1	1	2	-	3	2	-	1	1	-	-
	3			-	-	1	1	-	1	1	1	-	-	1	-
	4			-	-	-	1	-	2	2	3	-	-	-	1
Control with nettle	1			-	-	-	-	-	-	-	1	-	-	-	-
	2			-	-	-	-	-	-	1	5	-	-	1	1
	3			-	-	1♀†, 1 (sex)?		1	1	1	-	-	-	-	-
	4			-	-	-	-	-	-	-	-	-	-	-	-

Continued

Table 247: P25 – Chironomid test: number of hatched midges and sex. . continued

Control with nettle + 100 mg/L	1			2	1	1	-	1	2	1	-	-	-	-	-
	2			1	1	-	-	-	-	-	1	1	-	-	-
	3			2	1	2	-	6	-	-	-	1	-	-	-
	4			-	1	-	2	4	-	1	2	-	-	-	-
Vessels used for chemical analysis	K			1 (sex?)		1♂, 1 (sex?)		-	2	-	3	2♀, 1?		2	-
	1			-	-	-	-	1♂, 1?		4♀, 1♂, 3 (sex?)		-	1	1 (sex?)	
	2			-	-	-	1	2♂, 2 (sex?)		1♀, 1♂, 1 (sex?)		2♀, 1 (sex?)		2	-
	3			-	-	3♂, 1?		-	1	3	1	3	-	3	-
	4			-	-	2?		1♂, 1♀, 4?		3	-	3	-	-	-
	5			-	-	-	-	-	-	1	1	-	-		

Table 248: P25 – Chironomid test: number of hatched midges and sex.

Date		12.5.2010		13.5.2010		14.5.2010		15.5.2010		16.5.2010		17.5.2010		18.5.2010	
		♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂
Control	1	1	-	-	-	-	-	-	-	-	-	-	-	-	-
	2	2	1	-	-	1	-	1	-	-	-	-	-	1	-
	3	1	-	-	2	-	-	-	-	-	-	-	-	-	-
	4	-	-	3	-	1	-	-	-	-	-	-	-	-	-
15 mg/L	1	1	1	-	-	-	-	-	-	-	-	-	-	-	-
	2	3	-	1	1	-	-	1	-	-	-	-	-	-	-
	3	1	2	3	2	-	-	-	-	-	-	-	-	-	-
	4	-	1	-	-	2	-	-	-	-	-	-	-	-	-
24 mg/L	1	3	-	1	-	-	-	-	-	1	-	-	-	-	-
	2	-	1	-	-	1	-	-	-	-	-	-	-	-	-
	3	1	-	1	-	-	-	-	-	-	-	-	-	-	-
	4	-	-	-	-	-	-	1?	-	-	-	-	-	-	-
39 mg/L	1	2	1	2	1	-	-	-	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	4	2	1	1	-	-	-	1	-	-	-	-	-	-	-
63 mg/L	1	2	1	-	-	-	-	-	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	4	-	1	-	-	1	-	-	-	-	-	-	-	-	-
100 mg/L	1	2	-	-	-	-	-	-	-	-	-	-	-	-	-
	2	-	-	1	-	-	-	-	-	-	-	-	-	-	-
	3	-	2	3	-	3	-	-	1	-	-	-	-	-	-
	4	1	-	-	1	-	-	-	-	-	-	-	-	-	-
Vessels used for chemical analysis	K	1	-	2	1	-	-	-	-	-	-	-	-	-	-
	1	-	1	2	-	-	-	-	-	-	-	-	-	-	-
	2	2	1	2	-	-	-	-	-	-	-	-	-	-	-
	3	3	-	-	-	-	-	-	-	-	-	-	-	-	-
	4	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	5	-	-	4	1	1	-	3	-	2	-	-	-	1	-

Continued

Table 248: P25 – Chironomid test: number of hatched midges and sex. continued

Date		12.5. 2010		13.5. 2010		14.5. 2010		15.5 2010		16.5. 2010		17.5. 2010		18.5 2010	
		♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂
Control with nettle	1	-	1	1	-	-	-	-	-	-	-	-	-	-	-
	2	1 did not hatch		1	1	-	-	-	-	-	-	-	-	-	-
	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	4	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Control with nettle + 100 mg/L	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	3	1	-	-	-	-	-	-	-	-	-	-	-	-	-
	4	-	-	-	-	-	-	-	-	1	-	-	-	-	-

Table 249: P25 – Chironomid test: oxygen concentration [%].

Date		27.4	30.4	3.5	7.5	11.5	14.5
		O_2 %	O_2 %	O_2 %	O_2 %	O_2 %	O_2 %
Control	1	92.1	94.4	93.6	94.4	76.8	96.3
	2	90.4	89.7	93.4	83.2	88.0	96.2
	3	87.4	95.0	92.8	96.2	81.3	92.1
	4	90.2	93.5	90.9	95.2	73.9	86.6
15 mg/L	1	84.7	98.0	92.2	74.7	85.8	99.9
	2	98.0	96.6	94.4	72.7	88.0	94.2
	3	99.2	97.5	97.7	85.5	89.0	91.5
	4	98.2	97.9	96.0	86.0	92.0	98.3
24 mg/L	1	93.5	97.8	94.8	72.3	94.4	100.2
	2	92.6	89.4	98.3	79.3	89.2	99.4
	3	98.5	98.5	98.8	91.4	92.6	100.0
	4	97.6	99.4	96.3	84.7	91.2	96.4
39 mg/L	1	69.6	98.8	78.1	77.3	92.2	98.2
	2	96.6	98.1	95.7	81.5	86.6	96.8
	3	97.0	99.2	97.2	86.8	94.5	97.0
	4	99.0	97.4	95.7	84.7	82.1	98.8
63 mg/L	1	96.0	99.5	98.3	80.3	83.7	95.1
	2	98.3	96.7	95.7	82.7	93.1	100.1
	3	98.8	99.9	99.1	81.3	93.4	98.7
	4	95.7	96.4	92.1	77.2	90.4	96.2
100 mg/L	1	98.6	98.2	94.6	70.7	89.1	99.5
	2	79.7	98.2	95.0	83.5	90.4	98.7
	3	86.3	99.4	91.7	96.9	74.1	90.4
	4	100	99.8	97.5	72.3	83.3	97.3
Control with nettle	1	73.3	84.6	90.8	85.1	95.9	101.5
	2	95.3	95.5	94.1	99.9	97.8	100.5
	3	94.6	94.1	96.4	100	100.9	100.4
	4	87.0	89.1	92.4	95.8	94.9	99.9
Control with nettle + 100 mg/L	1	89.6	84.1	92.5	99.6	97.3	98.9
	2	80.4	89.4	89.8	97.9	95.3	102.1
	3	92.7	92.7	94.6	98.3	100.2	103.1
	4	94.1	91.9	92.6	97.7	98.5	101.6
Vessels used for chemical analysis	K	72.1	87.6	94.2	95.1	80.3	97.1
	1	90.0	92.3	77.3	96.4	73.2	92.3
	2	63.4	98.7	92.7	99.2	77.3	96.0
	3	62.5	99.0	97.7	100	92.3	101.1
	4	92.0	95.8	93.5	94.0	88.1	99.9
	5	87.6	94.8	93.1	96.8	71.5	92.2

Table 250: P25 – Chironomid test: temperature (°C) and pH value.

Date		28.04.2010		5.05.2010		12.5.2010	
		Temp °C	pH	Temp °C	pH	Temp °C	pH
Control	1	20.3	8.30	20.5	8.21	20.3	8.34
	2		8.31		8.08		8.36
	3		8.34		8.12		8.35
	4		8.09		8.02		8.27
15 mg/L	1		8.23		7.97		8.33
	2		8.31		8.18		8.34
	3		8.37		8.24		8.34
	4		8.31		8.19		8.39
24 mg/L	1		8.31		8.14		8.40
	2		8.35		8.24		8.34
	3		8.38		8.24		8.33
	4		8.37		8.22		8.34
39 mg/L	1		8.34		8.15		8.39
	2		8.37		8.11		8.33
	3		8.42		8.19		8.39
	4		8.40		8.22		8.25
63 mg/L	1		8.44		8.24		8.29
	2		8.40		8.22		8.36
	3		8.43		8.25		8.41
	4		8.30		8.11		8.36
100 mg/L	1		8.34		8.18		8.37
	2		8.38		8.18		8.35
	3		8.20		7.73		8.25
	4		8.34		8.09		8.34
Control with nettle	1		8.12		8.15		8.66
	2		8.48		8.51		8.72
	3		8.64		8.58		8.78
	4		8.58		8.57		8.70
Control with nettle + 100 mg/L	1		8.58		8.57		8.71
	2		8.30		8.44		8.67
	3		8.46		8.55		8.75
	4		8.58		8.55		8.71
Vessels for chemical analysis	K		8.53		8.26		8.38
	1		8.47		8.05		8.27
	2		8.53		8.12		8.27
	3		8.52		8.19		8.39
	4		8.53		8.20		8.38
	5		8.58		7.90		8.23

21.5.2 NM-101

Table 251: NM-101 – Chironomid test: quality control / validation of chemical analyses.

	Sample description	Ti [µg/L] - measured	Ti [µg/L] - nominal	Recovery [%]
Samples of day 0 and day 1				
Recalibration standard (commercially available Ti standard with soluble Ti)	500 µg/L	503.9	500	106
Reference standard CPI	500 µg/L	512.1	500	102
	500 µg/L	519.7	500	104
Positive control (aqueous samples with NM-101)	790 mg/L	74680	79000	94.5
	790 mg/L	74620	79000	94.5
Samples of day 7				
Positive control (aqueous samples with NM-101)	TiO_2 79 µgTi/L	69920	79000	88.5
	TiO_2 79 µgTi/L	70190	79000	88.8
	TiO_2 79 µgTi/L	71320	79000	90.3
	TiO_2 79 µgTi/L	71540	79000	90.6
Reference standard CPI	25 µg/L	26.39	25	106
	25 µg/L	26.25	25	105
Recalibration standard (commercially available Ti standard with soluble Ti)	Ti 50 µg/L	51.61	50	103
	Ti 50 µg/L	51.61	50	103
Samples of day 14				
Positive control (aqueous samples with NM-101)	TiO_2 79 µgTi/L	68810	79000	87.1
	TiO_2 79 µgTi/L	69580	79000	88.1
	TiO_2 79 µgTi/L	68870	79000	87.2
	TiO_2 79 µgTi/L	69240	79000	87.6
Reference standard CPI	25 µg/L	23.86	25	95.4
	25 µg/L	23.1	25	92.4
Recalibration standard (commercially available Ti standard with soluble Ti)	Ti 50 µg/L	47.56	50	95.1
	Ti 50 µg/L	47.02	50	94.0
Samples of day 28 – aqueous samples				
Positive control (aqueous samples with NM-101)	TiO_2 79 µgTi/L	70460	79000	89.2
	TiO_2 79 µgTi/L	70430	79000	89.2
	TiO_2 79 µgTi/L	74160	79000	93.9
	TiO_2 79 µgTi/L	74710	79000	94.6
Reference standard CPI	25 µg/L	24.92	25	99.2
	25 µg/L	26.85	25	107
Recalibration standard (commercially available Ti standard with soluble Ti)	Ti 50 µg/L	48.97	50	97.9
	Ti 50 µg/L	51.89	50	104

Continued

Table 251: NM-101 – Chironomid test: quality control / validation of chemical analyses. continued

Samples of day 28 - sediment				
Recalibration standard (commercially available Ti standard with soluble Ti)	Ti 500µg/L	516	500	103
	Ti 500µg/L	518	500	104
	Ti 500µg/L	538	500	108
	Ti 500µg/L	537	500	107
Positive control (soil samples with NM-101)	4500 mg/kg	3819	4500	85
	4500 mg/kg	4012	4500	89
	4500 mg/kg	4023	4500	89

Table 252: NM-101 – Chironomid test: physico-chemical test parameters.

		Test start						Test end					
		O_2 %	Temp °C	pH	TH mmol/l	NH_4 mg/L	Light lux	O_2 %	Temp °C	pH	TH mmol/l	NH_4 mg/L	Light lux
Control	1	104.6	20.3	8.28	1.4	0.9	771-826	109.9	20.3	8.44	1.6	0.8	770
	2	106.0		8.37	1.5	0.6		102.2		8.37	1.6	4.9	
	3	104.8		8.32	1.5	0.9		99.9		8.46	1.6	7.5	
	4	104.3		8.30	1.3	0.7		106.4		8.46	1.6	0.5	
15 mg/L	1	103.8		8.31				83.1		8.46			
	2	104.9		8.27				101.4		8.41			
	3	104.4		8.34				97.0		8.46			
	4	105.7		8.39				109.6		8.52			
24 mg/L	1	106.9		8.43				111.7		8.53			
	2	104.6		8.31				101.9		8.49			
	3	103.9		8.28				97.0		8.36			
	4	104.4		8.30				106.2		8.40			
39 mg/L	1	104.6		8.25				103.9		8.51			
	2	105.3		8.29				99.5		8.45			
	3	104.6		8.28				109.0		8.59			
	4	103.2		8.27				103.1		8.52			
63 mg/L	1	105.7		8.28				99.0		8.45			
	2	103.4		8.28				107.4		8.52			
	3	104.4		8.34				109.4		8.56			
	4	103.6		8.31				108.3		8.52			
100 mg/L	1	105.2		8.35				104.8		8.52			
	2	103.7		8.31	1.4	0.7		113.5		8.59	1.7	0.6	
	3	104.8		8.31				107.3		8.52			
	4	104.8		8.28				114.3		8.56			
Vessels used for chemical analysis	K	104.8		8.17	1.4	0.8		124.5		8.75	1.6	0.1	
	1	104.0		8.16				111.2		8.61			
	2	105.5		8.25				105.8		8.51			
	3	105.9		8.35				99.6		8.41			
	4	104.9		8.27				105.7		8.45			
	5	104.0		8.27	1.4	0.7		112.2		8.75	1.6	0.1	

Water hardness (TH): 1 mmol corresponds to 100 mg $CaCo_3$ equivalent.

Table 253: NM-101 – Chironomid test: addition of Food (TetraMin grinded).

Date		9.6.		11.6			14.6		16.6		18.6			21.6	
		Day -1	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11	Day 12
		mg	mg	mg	mg	mg	mg	mg	mg	mg	mg	mg	mg	mg	mg
Control	1	10	-	21	-	-	16	-	18	-	42	-	-	32	-
	2	10	-	21	-	-	16	-	18	-	42	-	-	32	-
	3	10	-	21	-	-	16	-	18	-	42	-	-	32	-
	4	10	-	21	-	-	16	-	18	-	42	-	-	32	-
15 mg/L	1	10	-	21	-	-	16	-	18	-	42	-	-	32	-
	2	10	-	21	-	-	16	-	18	-	42	-	-	32	-
	3	10	-	21	-	-	16	-	18	-	42	-	-	32	-
	4	10	-	21	-	-	16	-	18	-	42	-	-	32	-
24 mg/L	1	10	-	21	-	-	16	-	18	-	42	-	-	32	-
	2	10	-	21	-	-	16	-	18	-	42	-	-	32	-
	3	10	-	21	-	-	16	-	18	-	42	-	-	32	-
	4	10	-	21	-	-	16	-	18	-	42	-	-	32	-
39 mg/L	1	10	-	21	-	-	16	-	18	-	42	-	-	32	-
	2	10	-	21	-	-	16	-	18	-	42	-	-	32	-
	3	10	-	21	-	-	16	-	18	-	42	-	-	32	-
	4	10	-	21	-	-	16	-	18	-	42	-	-	32	-
63 mg/L	1	10	-	21	-	-	16	-	18	-	42	-	-	32	-
	2	10	-	21	-	-	16	-	18	-	42	-	-	32	-
	3	10	-	21	-	-	16	-	18	-	42	-	-	32	-
	4	10	-	21	-	-	16	-	18	-	42	-	-	32	-
100 mg/L	1	10	-	21	-	-	16	-	18	-	42	-	-	32	-
	2	10	-	21	-	-	16	-	18	-	42	-	-	32	-
	3	10	-	21	-	-	16	-	18	-	42	-	-	32	-
	4	10	-	21	-	-	16	-	18	-	42	-	-	32	-
Vessels used for chemical analysis	K	10	-	21	-	-	16	-	18	-	42	-	-	32	-
	1	10	-	21	-	-	16	-	18	-	42	-	-	32	-
	2	10	-	21	-	-	16	-	18	-	42	-	-	32	-
	3	10	-	21	-	-	16	-	18	-	42	-	-	32	-
	4	10	-	21	-	-	16	-	18	-	42	-	-	32	-
	5	10	-	21	-	-	16	-	18	-	42	-	-	32	-

Continued

Table 253: NM-101 – Chironomid test: addition of Food (TetraMin grinded). continued

Date		9.6.		11.6			14.6		16.6		18.6			21.6	
		Day 13	Day 14	Day 15	Day 16	Day 17	Day 18	Day 19	Day 20	Day 21	Day 22	Day 23	Day 24	Day 25	Day 26
		mg	mg	mg	mg	mg	mg	mg	mg	mg	mg	mg	mg	mg	mg
Control	1	32	-	56	-	-	20	-	8	-	6	-	-	4	-
	2	32	-	56	-	-	22	-	12	-	15	-	-	10	-
	3	32	-	56	-	-	34	-	20	-	24	-	-	12	-
	4	32	-	50.4	-	-	18	-	12	-	15	-	-	10	-
15 mg/L	1	32	-	56	-	-	38	-	38	-	39	-	-	20	-
	2	32	-	56	-	-	28	-	4	-	3	-	-	2	-
	3	32	-	56	-	-	38	-	38	-	51	-	-	32	-
	4	32	-	47.6	-	-	18	-	8	-	12	-	-	6	-
24 mg/L	1	32	-	44.8	-	-	18	-	4	-	3	-	-	2	-
	2	32	-	56	-	-	36	-	34	-	36	-	-	20	-
	3	32	-	50.4	-	-	20	-	8	-	6	-	-	4	-
	4	32	-	44.8	-	-	16	-	2	-	3	-	-	2	-
39 mg/L	1	32	-	56	-	-	36	-	26	-	27	-	-	16	-
	2	32	-	53.2	-	-	30	-	22	-	27	-	-	16	-
	3	32	-	47.6	-	-	22	-	14	-	21	-	-	14	-
	4	32	-	50.4	-	-	24	-	22	-	33	-	-	22	-
63 mg/L	1	32	-	56	-	-	36	-	30	-	45	-	-	28	-
	2	32	-	47.6	-	-	14	-	8	-	12	-	-	8	-
	3	32	-	53.2	-	-	24	-	12	-	18	-	-	10	-
	4	32	-	47.6	-	-	14	-	6	-	6	-	-	4	-
100 mg/L	1	32	-	56	-	-	20	-	12	-	9	-	-	6	-
	2	32	-	50.4	-	-	20	-	6	-	9	-	-	4	-
	3	32	-	56	-	-	24	-	14	-	18	-	-	12	-
	4	32	-	44.8	-	-	14	-	10	-	9	-	-	4	-
Vessels used for chemical analysis	K	32	-	50.4	-	-	24	-	4	-	6	-	-	4	-
	1	32	-	50.4	-	-	20	-	16	-	18	-	-	12	-
	2	32	-	50.4	-	-	18	-	12	-	18	-	-	12	-
	3	32	-	50.4	-	-	18	-	10	-	15	-	-	10	-
	4	32	-	47.6	-	-	14	-	6	-	6	-	-	4	-
	5	32	-	56	-	-	28	-	24	-	33	-	-	22	-

Continued

Table 253: NM-101 – Chironomid test: addition of Food (TetraMin grinded). continued

Date		23.6	
		Day 27	Day 28
		mg	mg
Control	1	-	-
	2	4	-
	3	4	-
	4	4	-
15 mg/L	1	6	-
	2	-	-
	3	12	-
	4	2	-
24 mg/L	1	-	-
	2	8	-
	3	-	-
	4	2	-
39 mg/L	1	6	-
	2	6	-
	3	10	-
	4	20	-
63 mg/L	1	16	-
	2	8	-
	3	8	-
	4	2	-
100 mg/L	1	2	-
	2	2	-
	3	6	-
	4	2	-
Vessels used for chemical analysis	K	8	-
	1	4	-
	2	12	-
	3	10	-
	4	4	-
	5	12	-

Table 254: NM-101 – Chironomid test: oxygen concentration [%].

Date		31.5	4.6	8.6	11.6	15.6	18.6	22.6			
		O_2 %	O_2 %	O_2 %	O_2 %	O_2 %	O_2 %	O_2 %	O_2 %	O_2 %	O_2 %
Control	1	102.6									
	2					90.9					
	3							85.4			
	4			91.4			92.9				
15 mg/L	1				87.7						
	2		98.1			90.0					
	3	99.7					79.8				
	4			93.5				72.2			
24 mg/L	1		101.2			93.8					
	2	99.7						90.4			
	3				82.5						
	4			95.9			98.1				
39 mg/L	1	101.6					88.2				
	2				81.8			88.4			
	3		98.5								
	4			93.9		90.3					
63 mg/L	1				89.7						
	2		96.4			86.8					
	3	102.4						95.9			
	4			91.1			96.4				
100 mg/L	1		97.7								
	2	102.6				93.8		101.1			
	3				96.1						
	4			94.6			92.2				
Vessels used for chemical analysis	1	103.3	97.8	86.7	96.6	87.0	93.2	106.1			
	2	102.1	97.5	97.8	92.5	89.1	93.3	94.5			
	3	102.4	96.0	90.6	92.6	88.7	91.7	92.1			
	4	104.7	98.0	95.6	95.1	90.5	95.1	94.4			
	5	102.8	98.7	91.7	93.4	89.6	94.1	96.6			

Table 255: NM-101 – Chironomid test: temperature (°C) and pH value.

Date		4.6.2010		11.06.2010		18.06.2010	
		Temp °C	pH	Temp °C	pH	Temp °C	pH
Control	1		8.30		8.00		8.49
	2		8.37		8.07		8.59
	3		8.33		8.18		8.49
	4		8.34		8.15		8.54
15 mg/L	1		8.32		8.13		8.42
	2		8.34		8.21		8.57
	3		8.34		8.25		8.39
	4		8.39		8.21		8.54
24 mg/L	1		8.38		8.31		8.66
	2		8.32		7.78		8.43
	3		8.30		8.13		8.54
	4		8.31		8.11		8.56
39 mg/L	1		8.29		7.78		8.42
	2		8.29		8.24		8.43
	3	20.3	8.36		8.20		8.61
	4		8.35	20.5	8.23	20.3	8.47
63 mg/L	1		8.35		8.28		8.19
	2		8.35		8.34		8.54
	3		8.31		8.33		8.54
	4		8.30		8.15		8.54
100 mg/L	1		8.36		8.26		8.56
	2		8.33		8.39		8.56
	3		8.32		8.26		8.58
	4		8.30		8.31		8.49
Vessels for chemical analysis	1		8.27		8.30		8.51
	2		8.30		8.32		8.56
	3		8.32		8.26		8.57
	4		8.36		8.27		8.61
	5		8.36		8.37		8.58

Table 256: NM-101 – Chironomid test: number of hatched midges and sex.

Date		11.06.2010		12.06.2010		13.06.2010		14.06.2010		15.06.2010		16.06.2010		17.06.2010	
		♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂
Control	1	-	-	1	4	-	1	2	2	3	-	2	1	-	-
	2	-	-	1	4	1	2	1	-	2	1	1	1	2	-
	3	-	-	-	-	-	2	-	1	-	1	2	4	1	2
	4	-	2	1	6	1	-	1	-	-	-	3	-	2	-
15 mg/L	1	-	-	-	-	-	-	-	1	-	-	-	-	-	-
	2	-	-	-	2	1	2	-	1	5	3	3	1	-	-
	3	-	-	-	-	-	-	-	1	-	-	-	-	1	3
	4	-	3	-	2	1	2	-	3	2	1	2	-	2	-
24 mg/L	1	-	4	-	5	1	-	1	-	2	1	4	-	1	-
	2	-	-	-	1	-	-	-	1	-	-	-	1	2	1
	3	-	2	1	2	1	3	-	1	2	2	1	1	1	-
	4	1	3	-	3	1	2	2	-	3	3	1	-	-	-
39 mg/L	1	-	-	-	1	-	1	-	-	1	2	1	1	1	-
	2	-	1	-	-	1	2	1	-	2	1	1	-	3	-
	3	1	2	1	1	1	2	1	-	1	-	2	1	1	-
	4	1	1	-	1	1	1	3	-	-	-	1	-	1	-
63 mg/L	1	-	-	-	2	-	-	-	-	-	-	1	2	-	1
	2	-	3	1	5	-	2	1	1	1	-	2	-	-	-
	3	1	-	1	3	1	-	-	2	1	2	2	1	1	-
	4	-	3	1	2	1	2	4	-	-	-	2	2	-	-
100 mg/L	1	-	-	1	2	2	5	-	-	2	2	-	-	2	-
	2	-	2	-	2	1	1	3	1	2	-	2	3	-	1
	3	-	-	-	4	-	2	1	1	2	1	2	-	3	-
	4	-	4	-	-	-	6	3	-	-	-	2	-	1	-
Vessels for chemical analysis	K	-	2	-	2	1	1	2	-	3	1	4	2	-	-
	1	-	2	1	2	-	1	3	1	1	1	-	-	1	-
	2	-	2	1	1	1	2	4	-	2	1	-	-	-	-
	3	-	2	-	3	2	2	2	-	2	-	1	1	-	-
	4	-	3	-	1	3	1	2	3	-	1	3	-	-	-
	5	-	-	-	4	-	1	-	1	-	1	1	-	5	-

Continued

Table 256: NM-101 – Chironomid test: number of hatched midges and sex. continued

Date		18.06.10		19.06.10		20.06.10		21.06.10		22.06.10		23.06.10		24.06.10	
		♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂
Control	1	1	1	2	-	-	-	-	-	-	-	-	-	-	-
	2	1	-	-	-	1	-	-	-	-	-	-	-	-	-
	3	1	1	-	-	1	-	2	-	-	-	-	-	-	-
	4	1	-	-	-	1	-	-	-	-	-	-	-	-	-
15 mg/L	1	1	5	1	1	2	1	2	1	1	-	-	-	-	-
	2	-	1	-	-	-	1	-	-	-	-	-	-	-	-
	3	-	2	-	-	-	2	1	-	4	-	-	-	-	-
	4	-	-	-	-	-	-	1	-	-	-	-	-	-	-
24 mg/L	1	1	-	-		-	-	-	-	-	-	-	-	-	-
	2	4	1	2	-	1	-	1	1	-	-	-	-	-	-
	3	2	-	1	-	-	-	-	-	-	-	-	-	-	-
	4	-	-	-	-	-	-	-	-	-	-	-	-	-	-
39 mg/L	1	3	1	1	-	-	1	1	-	2	-	-	-	-	-
	2	1	1	2	-	1	-	1	-	-	-	-	-	-	-
	3	-	-	1	-	-	-	-	-	-	-	-	-	-	-
	4	-	-	-	-	-	-	-	-	-	-	-	-	-	-
63 mg/L	1	-	-	-	-	1	1	-	1	1	-	-	-	-	-
	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	3	-	-	-	-	-	-	-	1	-	-	-	-	-	-
	4	-	1	-	-	1	-	-	-	-	-	-	-	-	-
100 mg/L	1	3	-	-	-	-	-	-	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	1	-	-	-	-	-	-	-
	3	1	-	-	-	-	-	-	-	-	-	-	-	-	-
	4	2	-	-	-	-	-	1	-	-	-	-	-	-	-
Vessels for chemical analysis	K	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	1	2	-	-	1	1	-	-	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	4	1	-	-	-	-	-	-	-	-	-	-	-	-	-
	5	1	-	-	-	-	-	-	-	-	-	-	-	-	-

21.6 Raw data – Emergence test with chironomids – Ag (chapter 13)

21.6.1 Chemical analysis

Table 257: NM-300K – Chironomid test: quality control / validation of chemical analyses.

	Sample description	Ag [µg/L] - measured	Ag [µg/L] - nominal	Recovery [%]
Samples of day 0 and day 1				
Recalibration Merck IV (commercial available silver standard with soluble silver)	2.5 mg/L sample A	2649	2500	106
	2.5 mg/L sample B	2655	2500	106
	2.5 mg/L sample C	2654	2500	106
	500 µg/L sample A	530	500	106
	500 µg/L sample B	527	500	105
Positive control (aqueous samples with NM-300K)	200 µg/L sample A	197	200	98.7
	200 µg/L sample B	199	200	99.6
Samples of day 28 - sediment				
CRMC026-sandy loam9_1	0.570 mg/kg	20.0	0.648	114
CRMC026-sandy loam9_2	0.570 mg/kg	19.7	0.628	110
CRMC026-sandy loam9_3	0.570 mg/kg	20.6	0.666	117

Table 258: NM-300K – Chironomid test: measured silver concentrations in DGT extracts and calculated estimated average Ag concentration in matrix.

Sample	measured extract Ag conc. [µg/L]	extract volume [L]	extracted mass [µg]	extraction factor	calculated mass in DGT section [µg]	Deployment time [h]	sampled DGT area [cm^2]	metal ion flux [$µg/s*cm^2$]	DGT boundary thickness [cm]	metal diffusion coefficient [cm^2/s]	estimated average Ag conc. in matrix [mg/L]	estimated average Ag conc. in matrix [µg/L]
control A	0.008	0.015	0.00011	0.93	0.00012	48	3.142	2.2607E-10	0.094	1.23E-05	0.0000017	0.0017
control B	0.007	0.015	0.00010	0.93	0.00011	48	3.142	1.9963E-10	0.094	1.23E-05	0.0000015	0.0015
0.3125 mg A	0.011	0.015	0.00016	0.93	0.00017	48	3.142	3.1549E-10	0.094	1.23E-05	0.0000024	0.0024
0.3125 mg B	0.010	0.015	0.00015	0.93	0.00016	48	3.142	2.9282E-10	0.094	1.23E-05	0.0000022	0.0022
0.625 mg A	0.022	0.015	0.00033	0.93	0.00035	48	3.142	6.4731E-10	0.094	1.23E-05	0.0000050	0.0050
0.625 mg B	0.015	0.015	0.00023	0.93	0.00025	48	3.142	4.5184E-10	0.094	1.23E-05	0.0000035	0.0035
1.25 mg A	0.022	0.015	0.00033	0.93	0.00035	48	3.142	6.5355E-10	0.094	1.23E-05	0.0000050	0.0050
1.25 mg B	0.142	0.015	0.00212	0.93	0.00228	48	3.142	4.2065E-09	0.094	1.23E-05	0.0000322	0.0322
2.5 mg A	0.057	0.015	0.00085	0.93	0.00092	48	3.142	1.6856E-09	0.094	1.23E-05	0.0000129	0.0129
2.5 mg B	0.054	0.015	0.00080	0.93	0.00086	48	3.142	1.5911E-09	0.094	1.23E-05	0.0000122	0.0122
5.0 mg A	0.600	0.015	0.00900	0.93	0.00968	48	3.142	1.7824E-08	0.094	1.23E-05	0.0001364	0.1364
5.0 mg B	1.837	0.015	0.02756	0.93	0.02963	48	3.142	5.4572E-08	0.094	1.23E-05	0.0004176	0.4176
10 mg A	2.138	0.015	0.03207	0.93	0.03448	48	3.142	6.3514E-08	0.094	1.23E-05	0.0004860	0.4860
10 mg B	3.339	0.015	0.05009	0.93	0.05385	48	3.142	9.9192E-08	0.094	1.23E-05	0.0007590	0.7590

The calculations were performed according to the Technical Documentation on http://www.dgtresearch.com and references cited within.

The following arithmetic statements were applied:

1. extracted mass [µg] = measured extract Ag conc. [µg/L] *extract volume [L]
2. extraction factor = 0.93 according to literature mentioned above
3. calculated mass in DGT section [µg] = extracted mass [µg] / extraction factor
4. metal ion flux [µg/s*cm^2] = calculated mass in DGT section [µg] / deployment time [s] * sampled DGT area [cm^2]
5. estimated average Ag conc. in matrix [mg/L] = metal ion flux [µg/s*cm^2] *DGT boundary thickness [cm] / metal diffusion coefficient [cm^2 / s]
6. estimated average Ag conc. in matrix [µg/L] = estimated average Ag conc. in matrix [mg/L] * 1000

Table 259: NM-300K – Chironomid test: mean estimated average Ag concentration in matrix ± SD [µg/L].

Sample	Estimated average Ag conc. in matrix [µg/L]	Mean [µg/L]	SD [µg/L]
control A	0.0017		
control B	0.0015	0.0016	0.0001
0.3125 mg A	0.0024		
0.3125 mg B	0.0022	0.0023	0.0001
0.625 mg A	0.0050		
0.625 mg B	0.0035	0.0042	0.0011
1.25 mg A	0.0050		
1.25 mg B	0.0322	0.0186	0.0192
2.5 mg A	0.0129		
2.5 mg B	0.0122	0.0125	0.0005
5.0 mg A	0.1364		
5.0 mg B	0.4176	0.2770	0.1988
10 mg A	0.4860		
10 mg B	0.7590	0.6225	0.1930

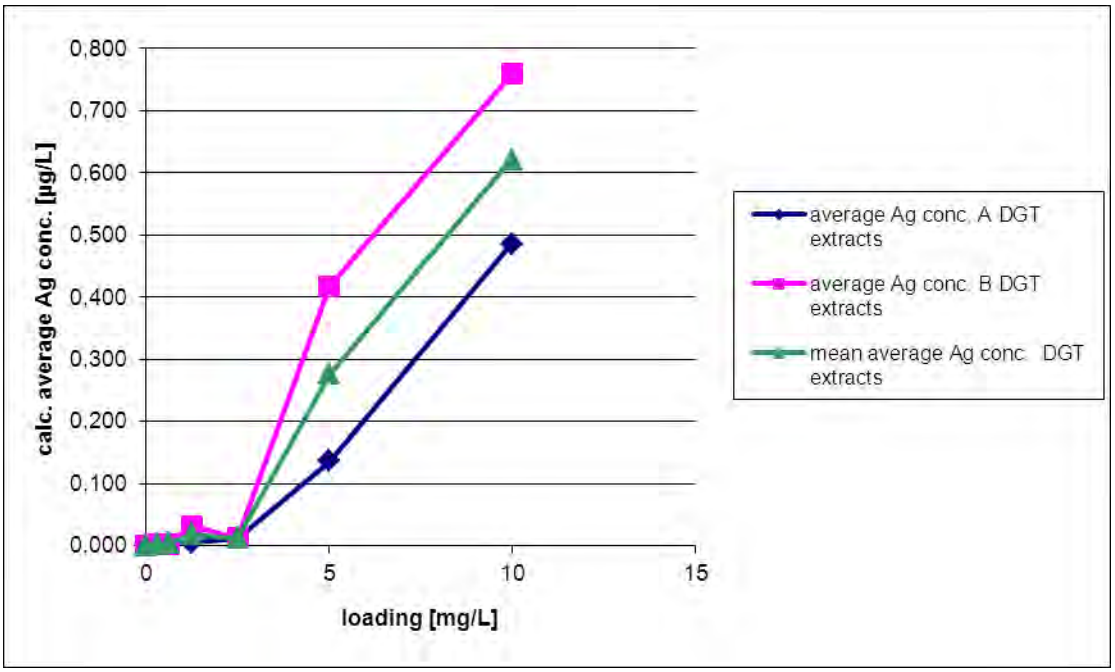


Figure 58: NM-300K - Test with chironomids: weighed concentration vs. estimated average Ag concentration in matrix obtained from the DGT extracts.

21.6.2 Ecotoxicological test

Table 260: Physico-chemical test parameters.

		Test start						Test end					
		O_2 %	Temp °C	pH	TH mmol/l	NH_4 mg/L	Light lux	O_2 %	Temp °C	pH	TH mmol/l	NH_4 mg/L	Light lux
Control	1	95.6		8.16	1.1	0.4		95.9		8.45	1.4	8.0	
	2	95.7		8.15	1.2	0.4		94.7		8.42	1.7	6.0	
	3	97.3		8.20	1.1	0.5		86.3		8.34	1.4	6.0	
	4	96.7		8.14	1.2	0.3		87.7		8.32	1.6	6.0	
0.3125 mg/L	1	95.3		8.08				94.9		8.45			
	2	95.0		8.07				92.6		8.44			
	3	93.8		8.00				93.0		8.42			
	4	95.0		8.05				90.9		8.48			
0.625 mg/L	1	95.9		8.11				90.3		8.48			
	2	96.1		8.15				76.5		8.19			
	3	94.2		8.12				93.2		8.48			
	4	92.8		8.05			636 - 682	93.3		8.42			
1.25 mg/L	1	92.6		8.01				69.8		8.26			621 - 657
	2	95.4		8.05				85.0		8.32			
	3	94.8		8.08				92.7		8.44			
	4	96.8		8.19				93.4		8.40			
2.5 mg/L	1	96.1		8.13				81.7		8.31			
	2	95.6	20.3	8.12				82.6	20.3	8.33			
	3	95.7		8.13				79.7		8.32			
	4	95.2		8.12				89.8		8.42			
5 mg/L	1	93.4		8.11				93.4		8.45			
	2	94.5		8.01				94.5		8.48			
	3	94.2		8.01				94.2		8.48			
	4	94.0		8.01				94.0		8.48			
10 mg/L	1	97.1		8.22				97.1		8.46			
	2	95.2		8.15	1.1	0.4		95.2		8.50	1.6	7.0	
	3	96.3		8.23				96.3		8.50			
	4	95.8		8.17				95.8		8.48			
Vessels used for chemical analysis	K	95.8		8.13	1.2	0.4		95.8		8.16	1.5	8.0	
	1	94.3		8.11				94.3		8.38			
	2	95.9		8.15				95.9		8.19			
	3	97.0		8.22				97.0		8.52			
	4	96.3		8.21				96.3		8.40			
	5	96.5		8.19				96.5		8.47			
	6	94.9		8.15	1.1	0.5		94.9		8.49	1.6	7.0	

Water hardness (TH): 1 mmol corresponds to 100 mg $CaCo_3$ equivalent.

Table 261: NM-300K – Chironomid test: addition of Food (TetraMin grinded).

Date		11.1.		13.1.	14.1.			17.1.		19.1.		21.1.			24.1.
		Day -1	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11	Day 12
		mg	mg	mg	mg	mg	mg	mg	mg	mg	mg	mg	mg	mg	mg
Control	1	10	-	5	24	-	-	16	-	20	-	42	-	-	32
	2	10	-	5	24	-	-	16	-	20	-	42	-	-	32
	3	10	-	5	24	-	-	16	-	20	-	42	-	-	32
	4	10	-	5	24	-	-	16	-	20	-	42	-	-	32
0.3125 mg/L	1	10	-	5	24	-	-	16	-	20	-	42	-	-	32
	2	10	-	5	24	-	-	16	-	20	-	42	-	-	32
	3	10	-	5	24	-	-	16	-	20	-	42	-	-	32
	4	10	-	5	24	-	-	16	-	20	-	42	-	-	32
0.625 mg/L	1	10	-	5	24	-	-	16	-	20	-	42	-	-	32
	2	10	-	5	24	-	-	16	-	20	-	42	-	-	32
	3	10	-	5	24	-	-	16	-	20	-	42	-	-	32
	4	10	-	5	24	-	-	16	-	20	-	42	-	-	32
1.25 mg/L	1	10	-	5	24	-	-	16	-	20	-	42	-	-	32
	2	10	-	5	24	-	-	16	-	20	-	42	-	-	32
	3	10	-	5	24	-	-	16	-	20	-	42	-	-	32
	4	10	-	5	24	-	-	16	-	20	-	42	-	-	32
2.5 mg/L	1	10	-	5	24	-	-	16	-	20	-	42	-	-	32
	2	10	-	5	24	-	-	16	-	20	-	42	-	-	32
	3	10	-	5	24	-	-	16	-	20	-	42	-	-	32
	4	10	-	5	24	-	-	16	-	20	-	42	-	-	32
5 mg/L	1	10	-	5	24	-	-	16	-	20	-	42	-	-	32
	2	10	-	5	24	-	-	16	-	20	-	42	-	-	32
	3	10	-	5	24	-	-	16	-	20	-	42	-	-	32
	4	10	-	5	24	-	-	16	-	20	-	42	-	-	32
10 mg/L	1	10	-	5	24	-	-	16	-	20	-	42	-	-	32
	2	10	-	5	24	-	-	16	-	20	-	42	-	-	32
	3	10	-	5	24	-	-	16	-	20	-	42	-	-	32
	4	10	-	5	24	-	-	16	-	20	-	42	-	-	32
Vessels used for chemical analysis	K	10	-	5	24	-	-	16	-	20	-	42	-	-	32
	1	10	-	5	24	-	-	16	-	20	-	42	-	-	32
	2	10	-	5	24	-	-	16	-	20	-	42	-	-	32
	3	10	-	5	24	-	-	16	-	20	-	42	-	-	32
	4	10	-	5	24	-	-	16	-	20	-	42	-	-	32
	5	10	-	5	24	-	-	16	-	20	-	42	-	-	32
	6	10	-	5	24	-	-	16	-	20	-	42	-	-	32

Continued

Table 261: NM-300K – Chironomid test: addition of Food (TetraMin grinded). continued

Date			26.1.		28.1.			31.1		2.2.		4.2.			7.2
		Day 13	Day 14	Day 15	Day 16	Day 17	Day 18	Day 19	Day 20	Day 21	Day 22	Day 23	Day 24	Day 25	Day 26
		mg	mg	mg	mg	mg	mg	mg	mg	mg	mg	mg	mg	mg	mg
Control	1	-	32	-	44.2	-	-	10	-	4	-	6	-	-	2
	2	-	32	-	49.4	-	-	20	-	14	-	12	-	-	6
	3	-	32	-	52	-	-	24	-	14	-	18	-	-	10
	4	-	32	-	46.8	-	-	20	-	12	-	12	-	-	8
0.3125 mg/L	1	-	32	-	52	-	-	34	-	22	-	12	-	-	4
	2	-	32	-	52	-	-	28	-	8	-	6	-	-	2
	3	-	32	-	52	-	-	26	-	10	-	12	-	-	-
	4	-	32	-	46.8	-	-	18	-	6	-	6	-	-	2
0.625 mg/L	1	-	32	-	52	-	-	18	-	8	-	12	-	-	6
	2	-	32	-	46.8	-	-	32	-	16	-	9	-	-	4
	3	-	32	-	49.4	-	-	18	-	4	-	3	-	-	-
	4	-	32	-	52	-	-	24	-	8	-	3	-	-	-
1.25 mg/L	1	-	32	-	52	-	-	34	-	26	-	12	-	-	6
	2	-	32	-	„	-	-	36	-	28	-	21	-	-	8
	3	-	32	-	„	-	-	36	-	28	-	30	-	-	10
	4	-	32	-	„	-	-	40	-	38	-	39	-	-	10
2.5 mg/L	1	-	32	-	„	-	-	40	-	40	-	60	-	-	38
	2	-	32	-	„	-	-	40	-	40	-	60	-	-	38
	3	-	32	-	„	-	-	40	-	40	-	57	-	-	28
	4	-	32	-	„	-	-	38	-	34	-	39	-	-	22
5 mg/L	1	-	32	-	„	-	-	-	-	-	-	-	-	-	-
	2	-	32	-	„	-	-	-	-	-	-	-	-	-	-
	3	-	32	-	„	-	-	-	-	-	-	-	-	-	-
	4	-	32	-	„	-	-	-	-	-	-	-	-	-	-
10 mg/L	1	-	32	-	„	-	-	-	-	-	-	-	-	-	-
	2	-	32	-	„	-	-	-	-	-	-	-	-	-	-
	3	-	32	-	„	-	-	-	-	-	-	-	-	-	-
	4	-	32	-	„	-	-	-	-	-	-	-	-	-	-
Vessels used for chemi-cal analysis	K	-	32	-	52	-	-	24	-	12	-	9	-	-	8
	1	-	32	-	52	-	-	38	-	36	-	27	-	-	14
	2	-	32	-	49.4	-	-	22	-	10	-	15	-	-	6
	3	-	32	-	52	-	-	32	-	20	-	18	-	-	2
	4	-	32	-	52	-	-	40	-	34	-	33	-	-	20
	5	-	32	-	52	-	-	-	-	-	-	-	-	-	-
	6	-	32	-	52	-	-	-	-	-	-	-	-	-	-

Table 262: NM-300K – Chironomid test: oxygen concentration [%].

Date		18.1.	21.1.	25.1.	28.1.	1.2.	4.2.
		O_2 %	O_2 %	O_2 %	O_2 %	O_2 %	O_2 %
Control	1	84.0	87.6	83.5			
	2	90.7	92.0		83.6		
	3	88.0	91.2				82.4
	4	82.7	95.7				
0.3125 mg/L	1	89.4	90.6	77.2			
	2	87.0	87.6		83.2		
	3	88.5	88.9			65.2	
	4	86.3	82.5				91.6
0.625 mg/L	1	91.5	94.5		84.3		
	2	93.2	94.7			67.1	
	3	91.3	91.4	82.3			91.2
	4	85.0	85.7				
1.25 mg/L	1	86.3	86.3			66.4	
	2	79.5	85.1	69.8			
	3	90.7	93.4		90.2		
	4	91.5	95.2				81.0
2.5 mg/L	1	83.2	89.9	74.2			
	2	83.6	91.4			75.2	
	3	77.2	92.8		86.4		
	4	80.8	89.0				92.6
5 mg/L	1	96.3	95.6			85.1	
	2	91.4	91.9	81.7			
	3	92.8	94.9		88.5		
	4	91.4	92.3				91.1
10 mg/L	1	91.7	82.9	81.2	74.4	69.3	90.9
	2	82.1	89.2	83.6	67.2	65.3	83.4
	3	88.0	91.2	81.9	75.2	64.7	86.4
	4	76.3	88.7	80.2	73.7	65.2	92.5
Vessels used for chemical analysis	1	91.7	82.9	81.2	74.4	69.3	90.9
	2	82.1	89.2	83.6	67.2	65.3	83.4
	3	88.0	91.2	81.9	75.2	64.7	86.4
	4	76.3	88.7	80.2	73.7	65.2	92.5
	5	88.8	91.4	85.2	87.0	66.2	90.5
	6	91.8	94.7	87.8	79.3	79.3	94.9

Table 263: NM-300K – Chironomid test: temperature (°C) and pH value.

Date		21.1.11		28.1.11		4.2.11	
		Temp °C	pH	Temp °C	pH	Temp °C	pH
Control	1	20.3	8.17	20.3	8.29	20.3	8.38
	2		8.17		8.31		8.45
	3		8.17		8.29		8.34
	4		8.28		8.28		8.32
0.3125 mg/L	1		8.32		8.35		8.35
	2		8.29		8.31		8.35
	3		8.26		8.32		8.38
	4		8.24		8.30		8.44
0.625 mg/L	1		8.35		8.29		8.36
	2		8.35		8.34		8.26
	3		8.32		8.31		8.45
	4		8.24		8.35		8.35
1.25 mg/L	1		8.17		8.30		8.27
	2		8.17		8.33		8.32
	3		8.23		8.41		8.32
	4		8.28		8.38		8.29
2.5 mg/L	1		8.30		8.36		8.30
	2		8.30		8.32		8.28
	3		8.31		8.29		8.30
	4		8.30		8.35		8.32
5 mg/L	1		8.31		8.35		8.41
	2		8.24		8.31		8.45
	3		8.23		8.39		8.48
	4		8.25		8.32		8.50
10 mg/L	1		8.36		8.39		8.48
	2		8.35		8.41		8.44
	3		8.36		8.41		8.51
	4		8.35		8.41		8.48
Vessels for chemical analysis	1		8.23		7.99		8.46
	2		8.14		8.00		8.40
	3		8.28		8.21		8.41
	4		8.32		8.20		8.51
	5		8.28		8.32		8.46
	6		8.31		8.29		8.66

Table 264: NM-300K – Chironomid test: number of hatched midges and sex.

Date		27.1.11		28.1.11		29.1.11		30.1.11		31.1.11		1.2.11		2.2.11	
Day		15		16		17		18		19		20		21	
		♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂
Control	1	-	-	-	3	-	3	-	5	3	1	2	-	-	1
	2	-	-	-	1	1	2	1	3	1	1	2	-	1	-
	3	-	-	-	-	2	1	-	1	2	2	3	1	-	1
	4	-	-	-	2	-	-	1	1	4	2	1	1	2	-
0.3125 mg/L	1	-	-	-	-	-	-	1	-	-	2	1	-	3	2
	2	-	-	-	-	-	-	-	2	1	3	6	2	1	1
	3	-	-	-	-	1	2	-	1	-	3	1	2	5	-
	4	-	-	-	2	-	1	1	2	3	2	4	-	1	1
0.625 mg/L	1	-	-	-	-	-	4	1	2	1	3	2	2	1	-
	2	-	2	-	-	-	-	-	1	-	1	2	1	4	1
	3	-	-	-	1	-	3	-	6	1	-	5	1	1	-
	4	-	-	-	-	-	2	-	3	1	2	4	3	1	-
1.25 mg/L	1	-	-	-	-	-	-	-	2	-	1	1	-	-	3
	2	-	-	-	-	-	-	-	1	-	1	-	2	-	2
	3	-	-	-	-	-	-	-	-	-	2	1	1	1	1
	4	-	-	-	-	-	-	-	-	-	-	-	-	-	1
2.5 mg/L	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	4	-	-	-	-	-	-	-	-	-	1	1	-	-	1
5 mg/L	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	4	-	-	-	-	-	-	-	-	-	-	-	-	-	-
10 mg/L	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	4	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Vessels for chemical analysis	K	-	-	-	-	-	2	-	3	2	1	2	1	2	1
	1	-	-	-	-	-	-	-	1	-	-	-	-	-	1
	2	-	-	-	1	-	2	-	4	-	2	1	2	2	1
	3	-	-	-	-	-	1	-	2	-	1	2	2*	1	1
	4	-	-	-	-	-	-	-	-	-	-	-	-	-	3
	5	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	6	-	-	-	-	-	-	-	-	-	-	-	-	-	-

* One male not completely hatched (organism laid on the water surface).

Continued

Table 264: NM-300K – Chironomid test: number of hatched midges and sex. continued

Date		3.2.11		4.2.11		5.2.11		6.2.11		7.2.11		8.2.11		9.2.11	
Day		22		23		24		25		26		27		28	
		♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂
Control	1	-	-	-	-	-	-	1	-	-	-	-	-	-	-
	2	3	-	-	-	-	-	-	1	-	-	-	-	-	-
	3	1	-	-	-	-	-	1	-	-	-	-	-	-	-
	4	1	-	1	-	-	-	-	-	-	-	-	-	-	-
0.3125 mg/L	1	1	3	3	-	1	-	1	-	-	-	-	-	-	-
	2	1	-	1	-	-	-	-	-	1	-	-	-	-	-
	3	-	-	1	-	1	-	1	-	2	-	-	-	-	-
	4	-	-	1	-	1	-	-	-	-	-	-	-	-	-
0.625 mg/L	1	-	-	-	-	-	-	**	-	-	-	-	-	-	
	2	1	1	4	-	-	-	1	-	-	-	-	-	-	-
	3	1	-	-	-	1	-	-	-	-	-	-	-	-	-
	4	3	-	-	-	1	-	-	-	-	-	-	-	-	-
1.25 mg/L	1	3	2	3	1	1	-	-	-	-	-	-	-	-	-
	2	2	1	2	2	-	1	1	1	-	-	-	-	-	-
	3	-	1	3	-	1	-	4	-	-	-	1	2	-	-
	4	-	2	2	2	1	1	4	2	-	-	1	-	-	-
2.5 mg/L	1	-	-	-	-	-	-	-	-	-	1	-	-	-	-
	2	-	-	-	-	-	-	-	-	1	-	-	-	2	-
	3	-	-	-	1	-	-	2	1	1	1	-	1	-	1
	4	1	2	-	1	-	-	1	1	-	-	-	-	-	-
5 mg/L	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	4	-	-	-	-	-	-	-	-	-	-	-	-	-	-
10 mg/L	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	4	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Vessels for chemical analysis	K	1	-	1	-	-	-	-	-	-	-	-	-	-	-
	1	1	2	2	4	1	-	1	-	-	-	1	2	1	-
	2	-	-	-	-	-	-	1	-	1	-	-	-	-	-
	3	1	1	2	-	-	-	3	2	-	-	-	-	-	-
	4	-	-	1	5	1	-	-	-	-	-	1	-	1	-
	5	-	-	-	-	-	-	-	-	-	-	1	-	-	-
	6	-	-	-	-	-	-	-	-	-	-	-	-	-	-

** One larva did not hatch (larva laid on the water surface

21.7 Raw data – Emergence test with chironomids (Au nanoparticles)

21.7.1 Chemical analysis

Day 0, Day 1

	dilution	Measured value considering the different wave length for determination			measured value * dilution		
		Au1978 [3] µg/L	Au2082 µg/L	Au2427 µg/L	Au1978 [3] µg/L	Au2082 µg/L	Au2427 µg/L
Day 0 – stock suspensions							
Control	10	-1.49 [1]	0.12 [1]	-3.06 [1]	-14.9 [1]	1.22 [1]	-30.6 [1]
Dispersant	10	-2.20 [1]	1.18 [1]	9.67 [1]	-22.0 [1]	11.8 [1]	96.7 [1]
NM-330 0.2%	10	5.31 [2]	9.95 [2]	0.42 [1]	53.1 [2]	100 [2]	4.2 [1]
NM-330 2 %	10	75.2	78.0	67.0	752	780	670
NM-330 20%	50	160	163	160	8010	8125	8005
NM-330 100%	200	219	221	205	43840	44100	41040
Day 1							
NM-330DIS	10	-0.17 [1]	2.78 [1]	-2.48 [1]	-1.69 [1]	27.8 [1]	-24.8 [1]
NM-330 0.1%	10	3.45 [2]	4.24 [2]	1.00 [1]	34.5 [2]	42.4 [2]	10.0 [1]
NM-330 1 %	10	19.9	25.9	18.4 [2]	199	259	184 [2]
NM-330 10 %	10	125	130	119	1251	1299	1190
NM-330 50 %	100	117	120	115	11690	12020	11500

[1]Limit of detection; [2]Limit of determination; [3]used for calculation of concentration

Calibration for day 0, day 1

	dilu-tion	nomi-nal	Measured value considering the different wave length for determination			measured value * dilution			Recovery		
			Au1978 [1] µg/L	Au2082 µg/L	Au2427 µg/L	Au1978 [1] µg/L	Au2082 µg/L	Au2427 µg/L	Au1978 [1] rec %	Au2082 rec %	Au2427 rec %
nano Gold reference material (value not certified)											
NIST 8011 A mg/L	250	51.56	205	211	202	51325	52850	50450	99.5	102.5	97.8
NIST 8011 B mg/L	250	51.56	204	204	201	50975	50900	50300	98.9	98.7	97.6
NIST 8011 A mg/L	250	51.56	204	205	197	51075	51250	49300	99.1	99.4	95.6
NIST 8011 B mg/L	250	51.56	203	204	205	50625	50950	51150	98.2	98.8	99.2
recalibration standard											
Standard Au 50 µg/L		50	48.1	51.0	49.2				101	103	96.9
Standard Au 50 µg/L		50	47.0	49.3	51.8				99.5	103	103
Limit of detec-tion			50.4	51.7	48.4						
Limit of deter-mination			49.7	51.3	51.3						

[1] used for calculation of concentration

Day 7

	dilution	Measured value considering the different wave length for determination			measured value * dilution		
		Au1978 [3] µg/L	Au2082 µg/L	Au2427 µg/L	Au1978 [3] µg/L	Au2082 µg/L	Au2427 µg/L
Day 7							
NM-330 0.1%	10	-0.099 [1]	7.42 [2]	-4.28 [1]	-0.986 [1]	74.2 [2]	-42.8 [1]
NM-330 1 %	10	3.89 [2]	11.9	5.56 [2]	38.9 [2]	119	55.6 [2]
NM-330 10 %	10	22.2	30.0	24.9 [2]	222	300	249 [2]
NM-330 50 %	50	91.2	94.5	90.8	4559	4724	4541

[1]Limit of detection; [2]Limit of determination; [3]used for calculation of concentration

Calibration for day 7

	dilu-tion	nomi-nal	Measured value considering the different wave length for determination			measured value * dilution			Recovery		
			Au1978 [1] µg/L	Au2082 µg/L	Au2427 µg/L	Au1978 [1] µg/L	Au2082 µg/L	Au2427 µg/L	Au1978 [1] rec %	Au2082 rec %	Au2427 rec %
nano Gold reference material (value not certified)											
NIST 8011 A mg/L	250	51.56	201	203	203	50325	50625	50675	97.6	98.2	98.3
NIST 8011 B mg/L	250	51.56	200	202	198	49950	50450	49600	96.9	97.8	96.2
NIST 8011 A mg/L	250	51.56	207	205	204	51725	51200	50900	100	99.3	98.7
NIST 8011 B mg/L	250	51.56	202	202	203	50425	50550	50650	97.8	98.0	98.2
recalibration standard											
Standard Au 50 µg/L		50	48.1	51.0	49.2				96.2	102	98.4
Standard Au 50 µg/L		50	47.0	49.3	51.8				94.0	98.6	104
Limit of detec-tion			4.29	3.21	7.50						
Limit of deter-mination			14.3	10.7	25.0						

[1]used for calculation of concentration

Day 28

	dilution	Measured value considering the different wave length for determination			measured value * dilution		
		Au1978 [3] µg/L	Au2082 µg/L	Au2427 µg/L	Au1978 [3] µg/L	Au2082 µg/L	Au2427 µg/L
Day 28							
NM-330 0.1%	10	0.156 [1]	4.15 [2]	-2.81 [1]	1.556 [1]	41.5 [2]	-28.1 [1]
NM-330 1 %	10	0.242 [1]	5.14 [2^^]	-1.81 [1]	2.42 [1]	51.4 [2]	-18.1 [1]
NM-330 10 %	10	-0.958 [1]	3.11 [2]	0.190 [1]	-9.58 [1]	31.1 [2]	2 [1]
NM-330 50 %	10	25.4	29.0	26.1	254	290	261

[1]Limit of detection; [2]Limit of determination; [3]used for calculation of concentration

Calibration for day 28

	dilu-tion	nomi-nal	Measured value considering the different wave length for determination			measured value * dilution			Recovery		
			Au1978 [1] µg/L	Au2082 µg/L	Au2427 µg/L	Au1978 [1] µg/L	Au2082 µg/L	Au2427 µg/L	Au1978 [1] rec %	Au2082 rec %	Au2427 rec %
nano Gold reference material (value not certified)											
NIST 8011 A mg/L	750	66.2	67.5	70.1	750	49650	50640	52545	96.3	98.2	102
NIST 8011 B mg/L	750	68.7	68.2	69.7	750	51518	51158	52290	99.9	99.2	101
NIST 8011 A mg/L	3750	12.1	11.7	16.8	3750	45525	43838	63075	88.3	85.0	122
NIST 8011 B mg/L	3750	14.6	14.1	11.5	3750	54900	52763	43125	106	102	83.6
recalibration standard											
Standard Au 50 µg/L			47.7	46.9	52.7				95.4	93.8	105
Standard Au 50 µg/L			48.0	47.4	48.1				95.9	94.9	96
Standard Au 12.5 µg/L			13.4	11.9	12.5				107	95.3	100
Limit of detec-tion			2.62	1.67	2.07						
Limit of deter-mination			8.73	5.58	6.91						

[1]used for calculation of concentration

Day 28 - sediment

	dilution	Measured value considering the different wave length for determination			measured value per kg sediment		
		Au1978 [3] µg/L	Au2082 µg/L	Au2427 µg/L	Au1978 [3] mg/kg	Au2082 mg/kg	Au2427 mg/kg
Control A	3.061	-4.54 [1]	76.1	4.73 [1]	-0.148 [1]	2.484	0.15 [1]
Control B	3.022	-3.77 [1]	77.4	7.93 [1]	-0.125 [1]	2.562	0.26 [1]
NM-330 0.1% A	3.058	-2.56 [1]	68.3	8.25 [1]	-0.084 [1]	2.235	0.27 [1]
NM-330 0.1% B	3.042	-1.88	80.6	10.5 [2]	-0.062 [1]	2.648	0.34 [2]
NM-330 1 % A	3.085	18.7	104	23.9 [2]	0.606	3.368	0.78 [2]
NM-330 1 % B	3.030	14.4	98.9	26.5 [2]	0.475	3.264	0.87 [2]
NM-330 10 % A	3.022	162	219	182	5.36	7.26	6.03
NM-330 10 % B	3.017	207	276	235	6.85	9.13	7.78
NM-330 50 % A	3.046	906	993	972	29.75	32.59	31.90
NM-330 50 % B	3.046	847	934	923	27.81	30.67	30.29
Limit of detection	3.043	2.71	2.0	10.3	0.089	0.066	0.338
Limit of determination	3.043	9.04	6.68	34.5	0.297	0.220	1.13

[1]Limit of detection; [2]Limit of determination; [3]used for calculation of concentration

Calibration for day 28

	dilution	nominal	Measured value considering the different wave length for determination			measured value * dilution			Recovery		
			Au1978 [1] µg/L	Au2082 µg/L	Au2427 µg/L	Au1978 [1] µg/L	Au2082 µg/L	Au2427 µg/L	Au1978 [1] rec %	Au2082 rec %	Au2427 rec %
nano Gold reference material (value not certified)											
NIST 8011 A mg/L	250	51560	201	202	199	50150	50525	49850	97.3	98.0	96.7
NIST 8011 B mg/L	250	51560	199	204	199	49750	50875	49850	96.5	98.7	96.7
NIST 8011 A mg/L	250	51560	197	201	197	49125	50175	49175	95.3	97.3	95.4
NIST 8011 B mg/L	250	51560	199	200	199	49775	50100	49675	96.5	97.2	96.3
recalibration standard											
Standard Au 50 µg/L		250	248	249	242				99.0	99.5	96.7
Standard Au 50 µg/L		250	248	248	204				99.0	99.2	81.7

[1]used for calculation of concentration

21.7.2 Ecotoxicological test

Table 265: Physico-chemical test parameters: control, NM-330DIS (dispersant).

		Test start						Test end					
		O_2 %	Temp °C	pH	TH mmol/l	NH_4 mg/L	Light lux	O_2 %	Temp °C	pH	TH mmol/l	NH_4 mg/L	Light lux
Control	1	99.5	20	7.65	1.1	0.85	531 - 568	86.2	20	8.43	1.4	28	523 - 577
	2	96.6		7.30	1.3	0.49		93.4		8.49	1.0	23	
	3	98.3		7.51	1.2	0.81		96.6		8.52	1.0	24	
	4	99.4		7.52	1.2	0.82		92.6		8.50	1.1	25	
Dispersant 0.1%	1	98.5		7.60				90.1		8.49			
	2	96.7		7.42				87.1		8.49			
	3	99.0		7.47				89.2		8.49			
	4	96.0		7.43				87.7		8.46			
Dispersant 1.0%	1	95.8		7.49				86.9		8.60			
	2	95.4		7.50				90.8		8.65			
	3	99.2		7.66				91.3		8.68			
	4	98.7		7.54				93.0		8.68			
Dispersant 10%	1	90.1		7.71				89.8		9.26			
	2	95.6		7.63				94.5		9.37			
	3	97.7		7.57				92.9		9.33			
	4	98.3		7.69				92.0		9.32			
Dispersant 50%	1	98.4		7.79				0.7		9.42			
	2	97.4		7.64				0.3		9.45			
	3	91.8		7.26				1.4		9.47			
	4	97.8		7.63	1.3	0.77		2.0		9.43	1.1	0.1	

continued

Water hardness (TH): 1 mmol corresponds to 100 mg $CaCo_3$ equivalent.

Table 265: Physico-chemical test parameters: control, NM-330DIS (dispersant). continued

		Test start						Test end					
		O_2 %	Temp °C	pH	TH mmol/l	NH_4 mg/L	Light lux	O_2 %	Temp °C	pH	TH mmol/l	NH_4 mg/L	Light lux
Gold 0.1%	1	97.8	20	7.63			531 - 568	94.3	20	8.43			523 - 577
	2	96.7		7.61				95.5		8.47			
	3	98.4		7.73				89.4		7.86			
	4	90.0		7.34				87.1		8.47			
Gold 1.0%	1	94.5		7.64				76.1		8.06			
	2	97.7		7.75				85.7		8.42			
	3	98.0		7.72				84.7		8.43			
	4	94.6		7.72				88.3		8.50			
Gold 10%	1	95.8		7.61				87.0		8.48			
	2	93.2		7.76				89.1		8.50			
	3	96.2		7.49				80.3		8.40			
	4	95.0		7.70				87.1		8.50			
Gold 50%	1	94.9		7.64				84.4		8.46			
	2	95.6		7.79				83.7		8.45			
	3	96.0		7.75				86.9		8.47			
	4	87.1		7.48	1.3	0.83		84.4		8.38	0.9	27	
Vessels used for chemical analysis	1	91.5		7.41				80.3		8.33			
	2	95.5		7.57				79.5		8.45			
	3	88.4		7.34				79.9		8.38			
	4	93.0		7.37				80.1		8.46			

Water hardness (TH): 1 mmol corresponds to 100 mg $CaCo_3$ equivalent.

Table 266: Addition of Food (TetraMin grinded): control, NM-330DIS (dispersant).

		Day -1	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11	Day 12
		mg	mg	mg	mg	mg	mg	mg	mg	mg	mg	mg	mg	mg	mg
Control	1	10	-	21	-	-	16	-	18	-	36	-	-	32	-
	2	10	-	21	-	-	16	-	18	-	36	-	-	32	-
	3	10	-	21	-	-	16	-	18	-	36	-	-	32	-
	4	10	-	21	-	-	16	-	18	-	36	-	-	32	-
Dispersant 0.1%	1	10	-	21	-	-	16	-	18	-	36	-	-	32	-
	2	10	-	21	-	-	16	-	18	-	36	-	-	32	-
	3	10	-	21	-	-	16	-	18	-	36	-	-	32	-
	4	10	-	21	-	-	16	-	18	-	36	-	-	32	-
Dispersant 1.0%	1	10	-	21	-	-	16	-	18	-	36	-	-	32	-
	2	10	-	21	-	-	16	-	18	-	36	-	-	32	-
	3	10	-	21	-	-	16	-	18	-	36	-	-	32	-
	4	10	-	21	-	-	16	-	18	-	36	-	-	32	-
Dispersant 10%	1	10	-	21	-	-	16	-	18	-	36	-	-	32	-
	2	10	-	21	-	-	16	-	18	-	36	-	-	32	-
	3	10	-	21	-	-	16	-	18	-	36	-	-	32	-
	4	10	-	21	-	-	16	-	18	-	36	-	-	32	-
Dispersant 50%	1	10	-	21	-	-	16	-	18	-	36	-	-	32	-
	2	10	-	21	-	-	16	-	18	-	36	-	-	32	-
	3	10	-	21	-	-	16	-	18	-	36	-	-	32	-
	4	10	-	21	-	-	16	-	18	-	36	-	-	32	-
Date		23.11		25.11			28.11		30.11		2.12			5.12	

continued

Table 266: Addition of Food (TetraMin grinded): control, NM-330DIS (dispersant). continued

		Day 13	Day 14	Day 15	Day 16	Day 17	Day 18	Day 19	Day 20	Day 21	Day 22	Day 23	Day 24	Day 25	Day 26	Day 27
		mg	mg	mg	mg	mg	mg	mg	mg	mg	mg	mg	mg	mg	mg	mg
Control	1	32	-	45.6	-	-	28	-	14	-	6	-	-	2	-	1
	2	32	-	40.8	-	-	10	-	4	-	6	-	-	4	-	2
	3	32	-	40.8	-	-	6	-	6	-	6	-	-	4	-	2
	4	32	-	40.8	-	-	12	-	6	-	0	-	-	0	-	0
Dispersant 0.1%	1	32	-	48	-	-	26	-	14	-	9	-	-	2	-	1
	2	32	-	45.6	-	-	32	-	24	-	12	-	-	2	-	0
	3	32	-	45.6	-	-	24	-	12	-	12	-	-	6	-	2
	4	32	-	48	-	-	34	-	24	-	24	-	-	4	-	1
Dispersant 1.0%	1	32	-	48	-	-	30	-	26	-	33	-	-	16	-	4
	2	32	-	43.2	-	-	28	-	24	-	30	-	-	16	-	7
	3	32	-	48	-	-	30	-	18	-	15	-	-	4	-	1
	4	32	-	43.2	-	-	16	-	10	-	9	-	-	4	-	2
Dispersant 10%	1	32	-	45.6	-	-	20	-	14	-	18	-	-	6	-	3
	2	32	-	45.6	-	-	18	-	10	-	6	-	-	2	-	1
	3	32	-	48	-	-	28	-	14	-	12	-	-	8	-	2
	4	32	-	40.8	-	-	18	-	6	-	3	-	-	2	-	0
Dispersant 50%	1	32	-	48	-	-	40	-	40	-	*	-	-	*	-	*
	2	32	-	48	-	-	40	-	40	-		-	-		-	
	3	32	-	48	-	-	40	-	40	-		-	-		-	
	4	32	-	48	-	-	40	-	40	-		-	-		-	
Date		7.12		9.12			12.12		14.12		16.12.			19.12		21.12

* No further feeding as organisms were dead

Continued

Table 266: Addition of Food (TetraMin grinded): control, NM-330DIS (dispersant). continued

		Day -1	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11	Day 12
		mg	mg	mg	mg	mg	mg	mg	mg	mg	mg	mg	mg	mg	mg
Gold 0.1%	1	10	-	21	-	-	16	-	18	-	36	-	-	32	-
	2	10	-	21	-	-	16	-	18	-	36	-	-	32	-
	3	10	-	21	-	-	16	-	18	-	36	-	-	32	-
	4	10	-	21	-	-	16	-	18	-	36	-	-	32	-
Gold 1.0%	1	10	-	21	-	-	16	-	18	-	36	-	-	32	-
	2	10	-	21	-	-	16	-	18	-	36	-	-	32	-
	3	10	-	21	-	-	16	-	18	-	36	-	-	32	-
	4	10	-	21	-	-	16	-	18	-	36	-	-	32	-
Gold 10%	1	10	-	21	-	-	16	-	18	-	36	-	-	32	-
	2	10	-	21	-	-	16	-	18	-	36	-	-	32	-
	3	10	-	21	-	-	16	-	18	-	36	-	-	32	-
	4	10	-	21	-	-	16	-	18	-	36	-	-	32	-
Gold 50%	1	10	-	21	-	-	16	-	18	-	36	-	-	32	-
	2	10	-	21	-	-	16	-	18	-	36	-	-	32	-
	3	10	-	21	-	-	16	-	18	-	36	-	-	32	-
	4	10	-	21	-	-	16	-	18	-	36	-	-	32	-
Vessels used for chemical analysis	1	10	-	21	-	-	16	-	18	-	36	-	-	32	-
	2	10	-	21	-	-	16	-	18	-	36	-	-	32	-
	3	10	-	21	-	-	16	-	18	-	36	-	-	32	-
	4	10	-	21	-	-	16	-	18	-	36	-	-	32	-
Date		23.11		25.11			28.11		30.11		2.12			5.12	

continued

Table 266: Addition of Food (TetraMin grinded): control, NM-330DIS (dispersant). continued

		Day 13	Day 14	Day 15	Day 16	Day 17	Day 18	Day 19	Day 20	Day 21	Day 22	Day 23	Day 24	Day 25	Day 26	Day 27
		mg	mg	mg	mg	mg	mg	mg	mg	mg	mg	mg	mg	mg	mg	mg
Gold 0.1%	1	32	-	45.6	-	-	24	-	14	-	12	-	-	2	-	1
	2	32	-	43.2	-	-	14	-	8	-	6	-	-	0	-	0
	3	32	-	48	-	-	32	-	24	-	18	-	-	4	-	1
	4	32	-	48	-	-	18	-	14	-	12	-	-	4	-	1
Gold 1.0%	1	32	-	48	-	-	16	-	6	-	3	-	-	0	-	0
	2	32	-	45.6	-	-	28	-	12	-	9	-	-	4	-	2
	3	32	-	48	-	-	28	-	20	-	12	-	-	4	-	2
	4	32	-	45.6	-	-	26	-	18	-	9	-	-	0	-	0
Gold 10%	1	32	-	45.6	-	-	32	-	18	-	15	-	-	0	-	0
	2	32	-	40.8	-	-	26	-	10	-	9	-	-	2	-	0
	3	32	-	48	-	-	32	-	26	-	30	-	-	16	-	7
	4	32	-	45.6	-	-	32	-	16	-	3	-	-	0	-	0
Gold 50%	1	32	-	48	-	-	24	-	10	-	9	-	-	4	-	1
	2	32	-	45.6	-	-	28	-	20	-	15	-	-	8	-	4
	3	32	-	45.6	-	-	28	-	12	-	6	-	-	2	-	1
	4	32	-	45.6	-	-	16	-	8	-	6	-	-	4	-	2
Vessels used for chemical analysis	1	32	-	48	-	-	28	-	26	-	36	-	-	14	-	6
	2	32	-	48	-	-	36	-	32	-	39	-	-	12	-	4
	3	32	-	40.8	-	-	26	-	14	-	12	-	-	6	-	3
	4	32	-	48	-	-	28	-	22	-	21	-	-	6	-	3
Date		7.12		9.12			12.12		14.12		16.12			19.12		21.12

Table 267: Oxygen concentration [%]: control, NM-330DIS (dispersant).

		O_2 %	O_2 %	O_2 %	O_2 %	O_2 %	O_2 %	O_2 %	O_2 %
Control	1	87.5				68.0			85.1
	2			90.3			83.4		
	3				84.2			83.4	
	4					77.0			90.5
Dispersant 0.1%	1	76.1					73.9		
	2			88.0				80.8	
	3				74.1				86.4
	4					71.4			
Dispersant 1.0%	1	78.5					55.8	74.6	
	2			80.0				74.9	
	3				77.8				89.8
	4					71.1			
Dispersant 10%	1	36.1	74.9				74.0		
	2		74.3	62.3				88.1	
	3		82.1		71.9				92.2
	4		82.3			73.5			
Dispersant 50%	1	49.4	83.6				1.6	*6.0	4.6
	2		85.9	80.9				*1.5	3.1
	3		74.8		45.8			*0.8	1.2
	4		87.4			27.1		*1.3	2.5
Date		29.11	30.11	2.12	6.12	9.12	12.12	16.12	20.12

* samples were aerated after the result of the low oxygen concentration was obtained, the samples were aerated; oxygen concentration was determined again, no improvement was achieved

Table 268: Oxygen concentration [%]: NM-330 (gold nanoparticles in dispersant).

		O_2 %	O_2 %	O_2 %	O_2 %	O_2 %	O_2 %	O_2 %	O_2 %
Gold 0.1%	1	76.5				74.1			
	2		88.0				85.8		
	3			70.9				76.0	
	4				75.1				
Gold 1.0%	1	82.3				72.3			
	2		93.1				82.9		
	3			69.1				84.2	
	4				74.6				
Gold 10%	1	84.0				67.7			
	2		95.6				85.6		
	3			77.9				81.5	
	4				79.0				
Gold 50%	1	84.9				70.0			
	2		90.4				48.8		
	3			71.6				87.0	
	4				73.7				
Vessels used for chemical analysis	1	78.3	87.5	67.3	60.7	4.0	52.6	83.8	
	2	71.3	90.9	67.0	61.6	66.2	71.1	83.8	
	3	85.2	91.8	65.5	72.0	65.0	77.6	83.9	
	4	80.6	80.6	69.7	62.1	65.6	78.1	82.5	
Date		29.11	2.12	6.12	9.12	13.12	16.12	20.12	

Table 269: Temperature (°C) and pH value: control, NM-330DIS (dispersant).

Date		2.12		9.12		16.12	
		Temp°C	pH	Temp°C	pH	Temp°C	pH
Control	1	20	7.93	20	7.08	20	8.50
	2		8.13		8.27		8.56
	3		8.14		8.32		8.43
	4		8.11		8.29		8.43
Dispersant 0.1%	1		8.06		8.32		8.46
	2		8.08		8.33		8.38
	3		8.10		8.34		8.44
	4		8.11		8.26		8.39
Dispersant 1.0%	1		8.25		8.48		8.53
	2		8.21		8.37		8.50
	3		8.25		8.53		8.58
	4		8.33		8.49		8.67
Dispersant 10%	1		8.66		9.19		9.30
	2		8.69		9.19		9.33
	3		8.71		9.18		9.28
	4		8.75		9.16		9.30
Dispersant 50%	1		6.70		8.97		9.29
	2		6.76		8.95		9.35
	3		6.72		8.21		9.28
	4		6.68		8.86		9.37

Table 270: Temperature (°C) and pH value: NM-330 (gold nanoparticles in dispersant).

Date		2.12		9.12		16.12	
		Temp°C	pH	Temp°C	pH	Temp°C	pH
Gold 0.1%	1	20	7.82	20	8.16	20	8.30
	2		7.82		8.23		8.36
	3		7.90		8.22		8.28
	4		7.93		8.30		8.34
Gold 1.0%	1		7.94		8.22		8.39
	2		7.98		8.17		8.37
	3		7.97		8.16		8.38
	4		8.03		8.19		8.30
Gold 10%	1		8.03		8.27		8.34
	2		8.06		8.30		8.37
	3		8.04		8.11		8.22
	4		8.03		8.30		8.39
Gold 50%	1		8.04		8.27		8.37
	2		8.04		8.19		8.23
	3		8.07		8.23		8.39
	4		8.07		8.18		8.43
Vessels used for chemical analysis	1		8.07		8.11		8.21
	2		8.04		8.11		8.34
	3		8.04		8.22		8.31
	4		8.04		8.10		8.39

Table 271: Number of hatched midges and sex: control, NM-330DIS (dispersant).

Date		8.12		9.12		10.12		11.12		12.12		13.12		14.12	
Day		14		15		16		17		18		19		20	
		♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂
Control	1	-	-	-	1	-	1	1	2	-	1	3	2	1	1
	2	-	2	-	1	4	2	3	1	2	-	-	-	1	2
	3	-	2	-	1	1	3	2	2	6	-	-	-	-	-
	4	-	-	-	3	-	2	2	4	2	1	2	-	1	-
Dispersant 0.1%	1	-	-	-	-	-	2	3	2	-	-	2	1	2	1
	2	-	1	-	-	-	-	1	1	-	1	-	2	1	1
	3	-	-	1	-	1	3	-	-	1	2	1	1	3	1
	4	-	-	-	-	-	-	1	2	-	-	1	2	1	1
Dispersant 1.0%	1	-	-	-	-	-	-	2	2	1	-	2	-	-	-
	2	-	-	-	2	-	1	2	1	-	-	1	1	-	-
	3	-	-	-	-	-	1	3	-	1	-	3	1	2	-
	4	-	1	-	1	1	4	-	2	1	2	-	2	-	1
Dispersant 10%	1	-	-	-	1	3	2	-	-	2	2	1	1	1	-
	2	*		-	-	2	1	2	3	2	-	1	1	2	-
	3	-	-	-	-	-	2	-	3	-	1	2	1	4	1
	4	-	1	-	2	2	3	-	2	-	1	2	3	-	1
Dispersant 50%	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	4	-	-	-		-	-	-	-	-	-	-	-	-	-

*One organism hatched (organism laid on the water surface), no identification of sex.

continued

Table 271: Number of hatched midges and sex: control, NM-330DIS (dispersant). (continued)

Date		15.12		16.12		17.12		18.12		19.12		20.12		21.12	
Day		21		22		23		24		25		26		27	
		♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂
Control	1	2	-	2	1	1	-	-	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	3	-	1	-	-	-	-	-	-	-	-	-	-	-	-
	4	3	-	-	-	-	-	-	-	-	-	-	-	-	-
Dispersant 0,1%	1	2	2	-	-	-	1	-	-	1	-	-	-	-	-
	2	3	-	3	*1	-	-	1	1	1	-	1	-	-	-
	3	-	2	-	-	-	-	1	-	-	-	1	-	-	-
	4	3	-	-	1	1	2	2	-	1	-	-	-	1	-
Dispersant 1,0%	1	-	-	1	1	-	-	2	-	1	-	3	1	-	-
	2	-	1	1	-	-	-	-	-	1	1	1	-	-	-
	3	2	-	1	1	2	-	-	-	-	1	1	-	-	-
	4	-	2	-	-	-	-	-	1	-	-	-	-	-	-
Dispersant 10%	1	1	-	-	-	-	-	1	1	1	-	-	-	-	-
	2	1	-	2	-	-	-	1	-	-	-	-	-	-	-
	3	1	-	-	1	-	-	-	-	-	-	-	1	1	-
	4	1	1	-	-	-	-	-	-	-	-	1	-	-	-
Dispersant 50%	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	4	-	-	-	-	-	-	-	-	-	-	-	-	-	-

*One ♂ dead (organism laid on the water surface), hatching half-finished

continued

Table 271: Number of hatched midges and sex: control, NM-330DIS (dispersant). (continued)

Date		22.12	
Day		28	
		♀	♂
Control	1	-	-
	2	-	-
	3	-	-
	4	-	-
Dispersant 0,1%	1	-	-
	2	-	-
	3	-	-
	4	-	-
Dispersant 1,0%	1	1	-
	2	1	-
	3	-	-
	4	-	-
Dispersant 10%	1	-	-
	2	-	-
	3	-	-
	4	-	-
Dispersant 50%	1	-	-
	2	-	-
	3	-	-
	4	-	-

Table 272: Number of hatched midges and sex: NM-330 (gold nanoparticles in dispersant).

Date		8.12		9.12		10.12		11.12		12.12		13.12		14.12	
Day		14		15		16		17		18		19		20	
		♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂
Gold 0,1%	1	-	-	-	1	-	-	2	1	1	3	2	1	-	2
	2	-	1	-	1	-	1	3	2	2	3	1	1	1	-
	3	-	-	-	-	-	1	-	1	1	1	-	1	2	1
	4	-	-	-	-	1	3	1	3	2	1	1	**	-	-
Gold 1,0%	1	-	-	-	-	-	6	1	1	1	3	2	-	2	1
	2	-	-	-	1	-	1	-	1	1	2	3	2	3	-
	3	-	-	-	-	-	-	-	2	1	3	-	2	1	1
	4	-	-	-	1	-	1	-	-	1	4	1	2	-	1
Gold 10%	1	-	-	-	1	-	-	-	2	-	1	-	1	2	4
	2	-	1	-	2	1	1	-	1	*		3	-	4	1
	3	-	-	-	-	-	-	-	2	-	2	-	-	2	1
	4	-	-	-	1	-	-	-	2	1	-	2	2	3	1
Gold 50%	1	-	-	-	-	1	2	-	1	1	3	1	1	2	3
	2	-	-	-	1	-	-	1	1	1	2	1	1	1	1
	3	-	-	-	1	-	1	1	1	1	1	-	1	3	4
	4	-	-	-	1	-	2	-	2	6	1	1	1	1	1
Vessels used for chemical analysis	1	-	-	-	-	-	-	-	1	1∞	2	-	-	-	1
	2	-	-	-	-	-	-	-	2	-	-	-	-	1	1
	3	-	-	-	3	1	1	-	-	2	-	2	1	1	1***
	4	-	-	-	-	-	2	-	2	-	2	-	-	2	1

continued

* One organism dead (organism laid on the water surface), not hatched, no identification of sex possible.

∞ Two organism dead (organism laid on the water surface), not hatched, no identification of sex possible.

** One ♂ dead (organism laid on the water surface), hatching half-finished.

*** One ♂ dead (organism laid on the water surface).

Table 272: Number of hatched midges and sex: NM-330 (gold nanoparticles in dispersant). (continued)

Date		15.12		16.12		17.12		18.12		19.12		20.12		21.12	
Day		21		22		23		24		25		26		27	
		♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂
Gold 0,1%	1	2	-	-	1	3	-	-	-	-	-	-	-	-	-
	2	-	1	1	-	1	-	-	-	1	-	-	-	-	-
	3	1*	-	3	1	1	-	2	-	1	-	-	-	1	-
	4	1	-	1	1	1	-	1	-	-	-	1	-	-	-
Gold 1,0%	1	2	-	-	-	-	-	1	-	-	-	-	-	-	-
	2	1	1	1	-	-	-	-	-	1	-	-	-	-	-
	3	5	-	1	-	1	-	-	-	-	1	-	-	-	-
	4	1**	3	1	-	1	1	-	-	-	1	-	-	-	-
Gold 10%	1	1+1 ***	1	-	-	1	1	1	-	2	2	2	-	-	-
	2	-	1	-	1	1	-	1	-	-	-	1	-	-	-
	3	2	-	1	-	1	-	-	1	-	-	-	-	1	-
	4	6	-	1	-	-	1	-	-	-	-	-	-	-	-
Gold 50%	1	2	-	-	-	-	1	-	-	-	-	1	-	-	-
	2	3	1	-	1	-	-	1	-	-	-	-	-	-	-
	3	1	3	-	-	-	-	1	-	-	-	-	-	-	-
	4	1	1	-	-	-	-	-	-	-	-	-	-	-	-
Vessels used for chemical analysis	1	-	1	-	-	3	-	1	1	-	-	1	-	-	-
	2	-	2	1	-	1	1	-	4	1	-	2	-	-	-
	3	-	2	1	-	-	-	1	-	-	-	-	-	-	-
	4	1	2	1	-	2	2	-	-	-	-	-	-	-	-

* One ♀ dead (organism laid on water surface), hatching half-finished

** One ♀ dead (organism laid on water surface), hatching half-finished

*** One organism dead (organism laid on water surface), not hatched, no identification of sex possible

continued

Table 272: Number of hatched midges and sex: NM-330 (gold nanoparticles in dispersant). (continued)

Date		22.12	
Day		28	
		♀	♂
Gold 0,1%	1	1	-
	2	-	-
	3	-	-
	4	-	-
Gold 1,0%	1	-	-
	2	-	-
	3	-	-
	4	-	-
Gold 10%	1	-	-
	2	-	-
	3	2	-
	4	-	-
Gold 50%	1	1	-
	2	-	-
	3	-	-
	4	-	-
Vessels used for chemical analysis	1	1	-
	2	-	-
	3	-	-
	4	-	-

21.8 Raw data – Reproduction test with daphnids (chapter 15)

21.8.1 P25 - first test

Table 273: P25 (1st test) – Test with daphnids: Ti concentrations.

Day 0, 7, 14: freshly prepared suspensions; day 2, 9, 16: supernatant after incubation of the suspensions for two days in the test vessels

	Nominal	Ti3372	Recovery	Ti3372	Recovery
	[µg/L]	[µg/L]	%	[µg/L]	%
		Day 0		**Day 2**	
Control 1	---	< LOD	---	< LOD	---
Control 2	---	< LOD	---	< LOD	---
0.05 mg/LTiO_2	30.0	20.7	69.1	7.20	24.0
0.05 mg/LTiO_2	30.0	20.1	67.2	3.44	11.5
0.1 mg/LTiO_2	59.9	---	---	8.91	14.9
0.1 mg/LTiO_2	59.9	38.1	63.6	9.54	15.9
0.5 mg/LTiO_2	300	193	64.4	41.1	13.7
0.5 mg/LTiO_2	300	196	65.4	46.4	15.5
1.0 mg/LTiO_2	599	450	75.1	89.4	14.9
1.0 mg/LTiO_2	599	420	70.1	117	19.5
5.0 mg/LTiO_2	2997	2570	85.8	75.3	12.6
5.0 mg/LTiO_2	2997	2487	83.0	91.7	15.3
20 mg/L stock suspension	11986	10728	89.5		
20 mg/L stock suspension	11986	10793	90.0		
Medium + TiO_2 85 mg/L	83103	90960	109	80140	96.4
Medium + TiO_2 85 mg/L	83103	91440	110	79380	95.5
Medium + TiO_2 85 mg/L	83103	88300	106	80720	97.1
Medium + TiO_2 85 mg/L	83103	90720	109	80160	96.5
Detection limit		5.64		1.67	
Quantification limit		18.8		5.58	

Continued

Table 273: P25 (1st test) – Test with daphnids: Ti concentrations. continued

	Nominal	Ti3372	Recovery	Ti3372	Recovery
	[µg/L]	[µg/L]	%	[µg/L]	%
		Day 7		**Day 9**	
Control 1	---	< LOD	---	< LOD	---
Control 2	---	< LOD	---	< LOD	---
0.05 mg/LTiO_2	30.0	19.2	64.2	1.02	3.40
0.05 mg/LTiO_2	30.0	19.1	63.8	0.86	2.86
0.1 mg/LTiO_2	59.9	36.0	60.0	2.70	4.51
0.1 mg/LTiO_2	59.9	38.2	63.7	1.99	3.33
0.5 mg/LTiO_2	300	212	70.7	7.7	2.56
0.5 mg/LTiO_2	300	189	63.0	9.3	3.11
1.0 mg/LTiO_2	599	434	72.4	18.4	3.07
1.0 mg/LTiO_2	599	434	72.4	23.3	3.89
5.0 mg/LTiO_2	2997	2285	76.3	130	4.34
5.0 mg/LTiO_2	2997	2308	77.0	136	4.55
20 mg/L stock suspension	11986	10045	83.8		
20 mg/L stock suspension	11986	10870	90.7		
Medium + TiO_2 85mg/L	83103	83040	99.9	77500	93.3
Medium + TiO_2 85mg/L	83103	82320	99.1	77240	92.9
Medium + TiO_2 85mg/L	83103	79960	96.2	78540	94.5
Medium + TiO_2 85mg/L	83103	81520	98.1	77240	92.9
Detection limit		4.74		2.06	
Quantification limit		15.8		6.88	

Continued

Table 273: P25 (1st test) – Test with daphnids: Ti concentrations. continued

	Nominal	**Ti3372**	**Recovery**	**Ti3372**	Recovery
	[µg/L]	**[µg/L]**	**%**	**[µg/L]**	%
		Day 14		**Day 16**	
Control 1	---	< LOD	---	< LOD	---
Control 2	---	< LOD	---	< LOD	---
0.05mg/L TiO_2	30.0	18.7	62.5	5.04	16.8
0.05mg/L TiO_2	30.0	20.4	68.1	5.39	18.0
0.1 mg/L TiO_2	59.9	42.2	70.4	9.21	15.4
0.1 mg/L TiO_2	59.9	40.9	68.3	9.11	15.2
0.5 mg/L TiO_2	300	196	65.5	41.7	13.9
0.5 mg/L TiO_2	300	163	54.4	42.3	14.1
1.0 mg/L TiO_2	599	445	74.3	94.7	15.8
1.0 mg/L TiO_2	599	457	76.3	80.1	13.4
5.0 mg/L TiO_2	2997	2575	85.9	547	18.2
5.0 mg/L TiO_2	2997	2577	86.0	535	17.8
20 mg/L stock suspension	11986	10275	85.7		
20 mg/L stock suspension	11986	10875	90.7		
Medium + TiO_2 85mg/L	83103	82840	99.7	83040	99.9
Medium + TiO_2 85mg/L	83103	82800	99.6	83220	100
Medium + TiO_2 85mg/L	83103	81840	98.5		
Medium + TiO_2 85mg/L	83103	82880	99.7		
Detection limit		1.76		0.941	
Quantification limit		5.86		3.14	

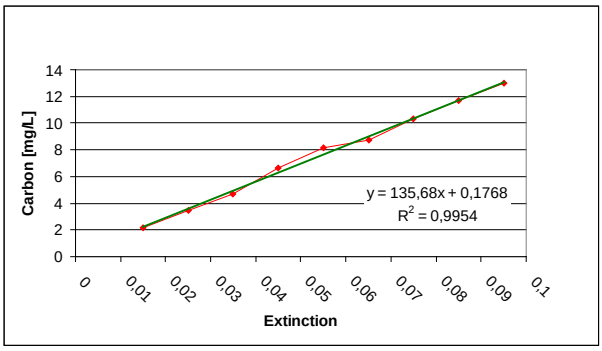


Figure 59: P25 - Calibration curve - extinction of algae density and organic carbon concentration.

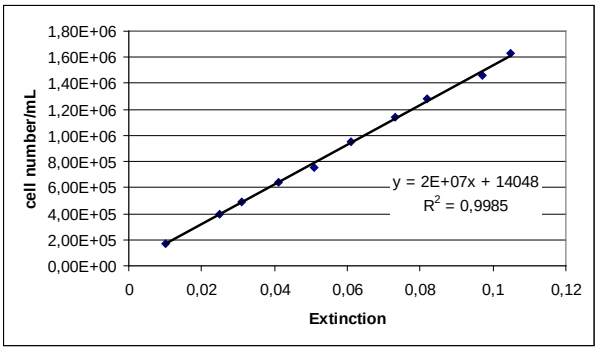


Figure 60: P25 - Calibration curve - extinction of algae density and cell number.

Table 274: P25 (1st test) – Test with daphnids: oxygen saturation of the overlaying water.

Values of the parallel test vessels throughout test duration [mg/L]: concentrations given as nominal concentrations of TiO_2

Date	Control		0.05 mg/L		0.1 mg/L		0.5 mg/L		1.0 mg/L		5.0 mg/L	
	aged	new	aged	new	aged	new	aged	new	aged	new	aged	new
Start		8.6		8.5		8.5		8.4		8.5		8.5
Day 2	8.7	8.0	8.5	7.8	8.4	7.8	8.1	7.8	8.2	8.0	8.1	8.1
Day 5	9.0	7.9	8.3	8.0	8.3	8.1	8.6	8.0	8.4	8.3	9.0	8.0
Day 7	8.7	8.1	8.7	8.0	8.5	8.1	8.6	8.1	8.6	8.1	8.9	8.1
Day 9	8.0	7.9	8.0	7.9	8.1	8.0	8.1	7.5	8.1	8.0	8.2	7.9
Day 12	7.8	8.4	7.8	8.3	7.8	8.3	7.9	8.3	7.8	8.2	7.9	8.1
Day 14	7.5	7.9	7.4	7.9	7.7	7.9	7.5	7.7	7.6	7.9	7.4	7.9
Day 16	8.1	8.4	7.6	8.2	7.3	8.0	7.6	7.9	7.4	7.9	8.0	8.1
Day 19	7.5	8.2	7.5	8.1	7.4	7.1	7.6	8.2	7.4	8.1	7.4	8.1
Day 21	7.8		7.6		7.7		7.6		7.8		7.4	

Table 275: P25 (1st test) – Test with daphnids: pH of the overlaying water.

Values of the parallel test vessels throughout test duration; concentrations given as nominal concentrations of TiO_2

Date	Control		0.05 mg/L		0.1 mg/L		0.5 mg/L		1.0 mg/L		5.0 mg/L	
	aged	new	aged	new	aged	new	aged	new	aged	new	aged	new
Start		8.5		8.5		8.4		8.5		8.5		8.5
Day 2	8.7	8.5	8.8	8.4	8.7	8.4	8.7	8.4	8.7	8.4	8.7	8.4
Day 5	8.8	8.5	8.8	8.6	8.8	8.6	8.8	8.6	8.8	8.5	8.8	8.5
Day 7	7.8	8.0	8.4	8.3	8.5	8.3	8.6	8.4	8.6	8.4	8.7	8.4
Day 9	8.2	8.0	8.3	8.0	8.3	8.0	8.4	8.0	8.3	8.0	8.4	8.0
Day 12	8.3	8.4	8.4	8.4	8.4	8.4	8.3	8.4	8.3	8.4	8.4	8.4
Day 14	8.2	8.3	8.2	8.3	8.2	8.3	8.3	8.3	8.3	8.3	8.3	8.3
Day 16	8.0	8.0	8.1	8.1	8.1	8.1	8.1	8.1	8.2	8.1	8.2	8.1
Day 19	8.2	8.3	8.3	8.5	8.3	8.5	8.3	8.5	8.3	8.5	8.3	8.5
Day 21	8.3		8.4		8.4		8.5		8.4		8.2	

Table 276: P25 (1st test) – Test with daphnids: temperature of the overlaying water [°C].

Measured in the climatic chamber

Day 0	Day 2	Day 5	Day 7	Day 9
20.7	21.0	21.0	21.0	21.1
Day 12	Day 14	Day 16	Day 19	Day 21
21.3	21.2	20.9	20.7	20.7

Table 277: P25 (1st test) – Test with daphnids: light intensity [lux].

Measured in the climatic chamber

Day 0	Day 2	Day 5	Day 7	Day 9
585	571	575	592	564
Day 12	Day 14	Day 16	Day 19	Day 21
585	579	563	587	591

Table 278: P25 (1st test) – Test with daphnids: extinction (585 nm) as measure for the density of algae used as feed.

Day 0	Day 2	Day 5	Day 7	Day 9
0.015	0.025	0.035	0.045	0.055
Day 12	Day 14	Day 16	Day 19	Day 21
0.065	0.075	0.090	0.100	---

Table 279: P25 (1st test) – Test with daphnids: offspring per replicate and day.

Concentrations given as nominal concentrations of TiO_2

Replicate	Control	0.05 mg/L	0.1 mg/L	0.5 mg/L	1.0 mg/L	5.0 mg/L
Day 6						
1	0	0	0	0	0	0
2	0	0	0	0	0	0
3	0	0	0	0	0	0
4	0	0	0	0	0	0
5	0	0	0	0	0	0
6	0	0	0	0	0	0
7	0	0	0	0	0	0
8	0	0	0	0	0	0
9	0	0	0	0	0	0
10	0	0	0	0	0	0
Day 7						
1	0	0	0	0	0	0
2	0	0	0	0	0	0
3	0	0	0	0	0	0
4	0	0	0	0	0	0
5	0	0	0	0	0	0
6	0	0	0	0	0	0
7	0	0	0	0	0	0
8	0	0	0	0	0	0
9	0	0	0	0	0	0
10	0	0	0	0	0	0

Continued

Table 279: P25 (1st test) – Test with daphnids: offspring per replicate and day. continued

Replicate	Control	0.05 mg/L	0.1 mg/L	0.5 mg/L	1.0 mg/L	5.0 mg/L
Day 8						
1	0	0	0	0	0	0
2	0	0	0	0	0	0
3	0	0	0	0	0	0
4	0	0	8	0	0	0
5	0	0	0	0	0	0
6	0	0	0	0	0	0
7	0	0	0	0	0	0
8	0	0	0	0	0	0
9	0	0	0	0	0	0
10	0	0	13	0	0	0
Day 9						
1	0	3	10	2	0	0
2	0	0	0	4	0	0
3	15	0	0	4	0	0
4	0	0	0	0	5	0
5	0	2	0	9	0	0
6	12	0	0	0	0	0
7	0	9	0	0	10	0
8	9	0	0	7	14	0
9	0	9	2	11	0	0
10	0	0	2	5	4	0
Day 10						
1	0	0	0	0	12	0
2	0	5	0	0	0	10
3	0	0	15	0	0	12
4	19	0	0	17	0	0
5	19	0	0	0	9	12
6	0	0	13	14	17	12
7	18	0	12	0	0	18
8	0	20	0	0	0	17
9	16	0	0	0	0	8
10	0	0	0	0	0	0

Continued

Table 279: P25 (1st test) – Test with daphnids: offspring per replicate and day. continued

Replicate	Control	0.05 mg/L	0.1 mg/L	0.5 mg/L	1.0 mg/L	5.0 mg/L
Day 12						
1	12	14	18	23	0	12
2	0	0	11	0	12	0
3	19	9	0	17	23	0
4	0	17	20	0	19	15
5	0	17	13	17	7	0
6	24	13	1	0	0	0
7	0	20	0	16	16	0
8	21	0	17	25	27	0
9	0	16	17	17	13	0
10	14	18	12	17	20	12
Day 14						
1	22	0	0	0	15	19
2	17	20	22	24	29	21
3	0	19	27	0	0	23
4	27	0	0	27	0	22
5	25	0	26	0	21	26
6	0	0	22	21	24	19
7	24	0	25	23	0	26
8	0	32	0	0	0	19
9	30	0	0	0	26	13
10	20	0	0	0	0	19
Day 16						
1	0	23	25	27	0	0
2	21	27	0	22	0	0
3	29	0	33	30	24	0
4	0	28	30	0	27	0
5	0	33	0	24	0	0
6	24	25	0	0	0	0
7	0	31	0	0	26	0
8	30	0	27	29	25	0
9	0	24	24	20	0	0
10	0	29	27	26	23	0

Continued

Table 279: P25 (1st test) – Test with daphnids: offspring per replicate and day. continued

Replicate	Control	0.05 mg/L	0.1 mg/L	0.5 mg/L	1.0 mg/L	5.0 mg/L
Day 19						
1	23	32	27	22	27	22
2	0	19	29	0	25	25
3	30	21	0	30	32	26
4	28	27	31	27	30	21
5	30	33	20	27	26	28
6	28	26	22	27	23	20
7	29	31	25	19	26	25
8	31	34	21	26	27	27
9	33	29	28	27	19	16
10	21	27	32	29	27	18
Day 21						
1	1	0	0	3	28	23
2	25	0	26	18	30	30
3	0	25	31	0	0	33
4	32	0	20	27	0	19
5	34	0	28	0	23	29
6	0	0	31	3	24	26
7	27	0	26	29	0	33
8	0	35	0	0	0	28
9	34	0	0	0	25	24
10	31	0	0	0	0	23

21.8.2 P25 - second test

Table 280: P25 (2nd test) - Test with daphnids: Ti concentration.

(day 0, 7, 14: freshly prepared suspensions; day 2, 9, 16: supernatant after incubation of the suspensions for two days in the test vessels

	Nominal	Ti3372	Recovery	Ti3372	Recovery	Ti3372	Recovery
	[µg/L]	[µg/L]	%	[µg/L]	%	[µg/L]	%
		Day 0		Day 1 (daily renewal of medium)		Day 2 (renewal of medium three times a week)	
Control 1	---	< LOD	---	< LOD	---	< LOD	---
Control 2	---	< LOD	---	< LOD	---	< LOD	---
1.0 mg/LTiO_2	599	564	94.0	163	27.2	65.6	10.9
1.0 mg/LTiO_2	599	574	95.7	202	33.7	63.3	10.6
5.0 mg/LTiO_2	2997	2540	84.8	673	22.4	260	8.67
5.0 mg/LTiO_2	2997	2353	78.5	683	22.8	258	8.62
20 mg/L stock suspension	11986	10100	84.3				
20 mg/L stock suspension	11986	10170	84.8				
Medium + TiO_2 85 mg/L	83103	82840	99.7	82840	99.7	82840	99.7
Medium + TiO_2 85 mg/L	83103	82800	99.6	82800	99.6	82800	99.6
Detection limit		5.99		5.99		5.99	
Quantification limit		20.0		20.0		20.0	

Continued

Table 280: P25 (2nd test) - Test with daphnids: Ti concentration, continued

	Nominal	Ti3372	recovery	Ti3372	recovery	Ti3372	recovery
	[µg/L]	[µg/L]	%	[µg/L]	%	[µg/L]	%
		Day 7		Day 8 (daily renewal of medium)		Day 9 (renewal of medium three times a week)	
Control 1	---	< LOD	---	< LOD	---	< LOD	---
Control 2	---	< LOD	---	< LOD	---	< LOD	---
1.0 mg/LTiO_2	599	513	85.6	254	42.4	230	38.4
1.0 mg/LTiO_2	599	509	84.9	254	42.3	230	38.4
5.0 mg/LTiO_2	2997	2675	89.3	1124	37.5	826	27.6
5.0 mg/LTiO_2	2997	2665	88.9	1131	37.7	828	27.6
20 mg/L stock suspension	11986	10930	91.2				
20 mg/L stock suspension	11986	11233	93.7				
Medium + TiO_2 85mg/L; 1 : 200	83103	85340	103	85340	103	85340	103
Medium + TiO_2 85mg/L; 1 : 200	83103	83380	100	83380	100	83380	100
Medium + TiO_2 85mg/L; 1 : 200	83103	85200	103	85200	103	85200	103
Medium + TiO_2 85mg/L; 1 : 2000	83103	83720	101	83720	101	83720	101
Medium + TiO_2 85mg/L; 1 : 2000	83103	83740	101	83740	101	83740	101
Medium + TiO_2 85mg/L; 1 : 2000	83103	81660	98.3	81660	98.3	81660	98.3
TiO_2 250 µg/L	250	257	103	257	103	257	103
TiO_2 250 µg/L	250	257	103	257	103	257	103
TiO_2 250 µg/L	250	254	102	254	102	254	102
CPI 100 µg/L	100	101	101	101	101	101	101
CPI 100 µg/L	100	101	101	101	101	101	101
Detection limit		8.09		8.09		8.09	
Quantification limit		27.0		27.0		27.0	

Continued

Table 280: P25 (2nd test) - Test with daphnids: Ti concentration. continued

	Nominal	Ti3372	recovery	Ti3372	recovery	Ti3372	recovery
	[µg/L]	[µg/L]	%	[µg/L]	%	[µg/L]	%
		Day 14		**Day 15 (daily renewal of medium)**		**Day 16 (renewal of medium three times a week)**	
Control 1	---	< LOD	---	< LOD	---	< LOD	---
Control 2	---	< LOD	---	< LOD	---	< LOD	---
1.0 mg/LTiO_2	599	616	103	295	49.2	464	77.4
1.0 mg/LTiO_2	599	620	103	297	49.5	464	77.4
5.0 mg/LTiO_2	2997	2978	99.4	1268	42.3	685	22.8
5.0 mg/LTiO_2	2997	2961	98.8	1274	42.5	696	23.2
20 mg/L stock suspension	11986	12178	102				
20 mg/L stock suspension	11986	12108	101				
Medium + TiO_2 85mg/L; 1 : 200	83103	85340	103	85340	103	85340	103
Medium + TiO_2 85mg/L; 1 : 200	83103	83380	100	83380	100	83380	100
Medium + TiO_2 85mg/L; 1 : 200	83103	85200	103	85200	103	85200	103
Medium + TiO_2 85mg/L; 1 : 2000	83103	83720	101	83720	101	83720	101
Medium + TiO_2 85mg/L; 1 : 2000	83103	83740	101	83740	101	83740	101
Medium + TiO_2 85mg/L; 1 : 2000	83103	81660	98.3	81660	98.3	81660	98.3
TiO_2 250 µg/L	250	257	103	257	103	257	103
TiO_2 250 µg/L	250	257	103	257	103	257	103
TiO_2 250 µg/L	250	254	102	254	102	254	102
CPI 100 µg/L	100	101	101	101	101	101	101
CPI 100 µg/L	100	101	101	101	101	101	101
Detection limit		8.09		8.09		8.09	
Quantification limit		27.0		27.0		27.0	

Table 281: P25 (2nd test) – Test with daphnids: oxygen saturation of the overlaying water.

Values of the parallel test vessels throughout test duration [mg/L]; concentrations given as nominal concentrations of TiO_2

Date	Control		1 mg/L		5 mg/L		Control		1.0 mg/L		5.0 mg/L	
	Medium renewal three times a week						Daily medium renewal					
	aged	new	aged	new	aged	new	aged	new	aged	new	aged	new
Start		9.0		8.6				9.0				
Day 1							8.3	8.5	8.3	8.2	8.3	8.4
Day 2	8.7	8.9	8.4	8.5	8.3	8.6	8.7	8.9	8.5	8.3	8.6	8.4
Day 3							8.9	8.3	8.5	8.5	8.7	8.5
Day 4							8.2	8.2	7.5	8.2	8.2	8.2
Day 5	8.6	8.4	8.3	8.5	8.5	8.3	8.7	8.4	8.5	8.4	8.6	8.6
Day 6							9.0	8.8	8.7	8.4	8.5	8.3
Day 7	8.1	8.2	8.0	8.1	8.3	8.3	8.4	8.2	8.6	8.0	8.1	8.0
Day 8							8.1	8.1	8.2	7.8	8.1	8.1
Day 9	8.6	7.6	8.3	8.0	8.4	8.2	8.6	7.6	8.8	8.3	8.6	8.0
Day 10							9.1	8.0	8.4	8.3	8.8	8.5
Day 11							8.6	8.3	8.7	8.2	8.6	8.0
Day 12	8.5	8.3	7.9	7.6	7.9	7.7	8.3	8.3	8.3	8.3	8.3	7.7
Day 13							8.6	8.1	8.5	8.1	8.4	8.3
Day 14	8.3	8.3	8.0	8.2	8.0	8.3	8.4	8.3	8.5	7.9	8.3	8.3
Day 15							9.6	8.5	9.4	8.4	9.4	8.3
Day 16	7.9	7.9	8.6	8.1	8.5	8.3	8.6	7.9	8.8	7.7	8.3	8.3
Day 17							8.6	8.0	8.7	8.2	8.7	8.1
Day 18							8.1	7.8	8.3	8.3	8.4	8.0
Day 19	7.8	8.6	8.2	8.9	7.8	8.7	8.4	8.6	8.9	8.3	8.3	8.8
Day 20							8.9	8.3	9.5	8.2	9.3	8.6
Day 21	9.4		9.3		8.9		10.0		9.6		10.2	

Table 282: P25 (2nd test) – Test with daphnids: pH of the overlaying water.

Values of the parallel test vessels throughout test duration; concentrations given as nominal concentrations of TiO_2

Date	Control		1 mg/L		5 mg/L		Control		1.0 mg/L		5.0 mg/L	
	Medium renewal three times a week						Daily medium renewal					
	aged	new	aged	new	aged	new	aged	new	aged	new	aged	new
Start		8.3		8.3		8.3		8.3		8.3		8.3
Day 1							8.4	8.3	8.4	8.3	8.4	8.3
Day 2	8.5	7.9	8.6	8.4	8.6	8.2	8.6	7.9	8.6	8.4	8.4	8.3
Day 3							8.5	8.2	8.6	8.1	8.6	8.1
Day 4							8.4	8.2	8.5	8.2	8.5	8.2
Day 5	8.7	8.0	8.6	8.0	8.6	8.0	8.5	8.0	8.5	8.0	8.5	8.0
Day 6							8.4	8.2	8.5	8.3	8.5	8.2
Day 7	8.7	8.4	8.6	8.4	8.6	8.4	8.6	8.4	8.7	8.4	8.6	8.4
Day 8							8.7	8.2	8.8	8.2	8.7	8.2
Day 9	8.3	8.4	8.7	8.3	8.7	8.3	8.7	8.4	8.8	8.4	8.7	8.4
Day 10							8.8	8.2	8.9	8.2	8.8	8.2
Day 11							8.3	8.1	8.5	8.1	8.5	8.1
Day 12	8.7	8.3	8.5	8.3	8.5	8.3	8.7	8.3	8.7	8.3	8.6	8.3
Day 13							8.5	8.3	8.6	8.3	8.6	8.1
Day 14	8.7	8.5	8.6	8.4	8.7	8.4	8.7	8.5	8.8	8.5	8.7	8.4
Day 15							8.9	8.2	8.9	8.3	8.9	8.3
Day 16	8.7	8.1	8.7	8.3	8.6	8.2	8.4	8.1	8.6	8.1	8.6	8.3
Day 17							8.9	8.5	8.9	8.4	8.9	8.4
Day 18							8.4	8.2	8.6	8.2	8.6	8.2
Day 19	8.6	8.0	8.5	8.2	8.5	8.3	8.7	8.0	9.0	8.2	8.9	8.3
Day 20							8.9	8.3	9.0	8.3	9.0	8.3
Day 21	8.9		8.9		8.8		9.1		8.9		8.1	

Table 283: P25 (2nd test) – Test with daphnids: temperature of the overlaying water [°C] in the climatic chamber.

Day 0	Day 2	Day 5	Day 7	Day 9
20.1	19.9	20.6	20.5	20.5
Day 12	Day 14	Day 16	Day 19	Day 21
20.4	20.5	20.3	20.1	20.5

Table 284: P25 (2nd test) – Test with daphnids: light intensity [lux] in the climatic chamber.

Day 0	Day 2	Day 5	Day 7	Day 9
562	581	607	592	560
Day 12	Day 14	Day 16	Day 19	Day 21
572	567	580	567	573

Table 285: P25 (2nd test) – Test with daphnids: extinction (585 nm) as measure for the density of algae used as feed.

Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
0.015	0.020	0.025	0.030	0.030	0.035	0.040
Day 7	Day 8	Day 9	Day 10	Day 11	Day 12	Day 13
0.045	0.050	0.055	0.0.060	0.060	0.065	0.070
Day 14	Day 15	Day 16	Day 17	Day 18	Day 19	Day 20
0.075	0.080	0.090	0.095	0.095	0.100	0.100

Table 286: P25 (2nd test) – Test with daphnids: offspring per replicate and day.

Concentrations given as nominal concentrations of TiO_2

Date	Control	1 mg/L	5 mg/L	Control	1.0 mg/L	5.0 mg/L
	Medium renewal three times a week			Daily medium renewal		
Day 6						
1	0	0	0	0	0	0
2	*	0	0	0	0	0
3	0	0	0	0	0	0
4	0	0	0	0	0	0
5	0	0	0	0	0	0
6	0	0	0	0	0	0
7	0	0	0	0	0	0
8	0	0	0	0	0	0
9	0	0	0	0	0	0
10	0	0	0	0	0	0

* Brood pouch of daphnid contained algae; no reproduction; organism was not considered for the calculation of reproduction

Date	Control	1 mg/L	5 mg/L	Control	1.0 mg/L	5.0 mg/L
	Medium renewal three times a week			Daily medium renewal		
Day 7						
1	0	0	0	0	0	0
2	*	0	0	0	0	0
3	0	0	0	0	0	0
4	0	0	0	0	0	0
5	0	0	0	0	0	0
6	0	0	0	0	0	0
7	0	0	0	0	0	0
8	0	0	0	0	0	0
9	0	0	0	0	0	0
10	0	0	0	0	0	0

* Brood pouch of daphnid contained algae; no reproduction; organism was not considered for the calculation of reproduction

Continued

Table 286: P25 (2nd test) – Test with daphnids: offspring per replicate and day, continued

Date	Control	1 mg/L	5 mg/L	Control	1.0 mg/L	5.0 mg/L
	Medium renewal three times a week			Daily medium renewal		
Day 8						
1	0	0	0	0	0	0
2	*	0	0	0	0	0
3	0	0	0	0	0	0
4	0	0	0	0	0	0
5	0	0	0	0	0	0
6	0	0	0	0	0	0
7	0	0	0	0	0	0
8	0	0	0	0	0	0
9	0	0	0	0	0	0
10	0	0	0	0	0	0

* Brood pouch of daphnid contained algae; no reproduction; organism was not considered for the calculation of reproduction

Date	Control	1 mg/L	5 mg/L	Control	1.0 mg/L	5.0 mg/L
	Medium renewal three times a week			Daily medium renewal		
Day 9						
1	0	0	0	0	5	0
2	*	0	11	0	0	3
3	0	7	0	0	0	0
4	0	0	0	0	0	0
5	1	0	0	0	0	0
6	0	0	0	7	0	12
7	0	0	0	0	0	2
8	0	0	0	7	6	0
9	0	0	0	0	Organism dead	6
10	0	0	0	0	0	0

* Brood pouch of daphnid contained algae; no reproduction; organism was not considered for the calculation of reproduction

Continued

Table 286: P25 (2nd test) – Test with daphnids: offspring per replicate and day, continued

Date	Control	1 mg/L	5 mg/L	Control	1.0 mg/L	5.0 mg/L
	Medium renewal three times a week			Daily medium renewal		
Day 10						
1	0	0	0	0	0	16
2	*	0	0	2	organism dead	0
3	0	0	0	14	0	0
4	0	10	8	16	5	13
5	0	7	0	0	organism dead	13
6	0	0	15	0	13	0
7	9	7	0	7	0	0
8	0	0	0	0	0	16
9	0	14	8	19	-	0
10	0	15	0	4	0	0

* Brood pouch of daphnid contained algae; no reproduction; organism was not considered for the calculation of reproduction

Date	Control	1 mg/L	5 mg/L	Control	1.0 mg/L	5.0 mg/L
	Medium renewal three times a week			Daily medium renewal		
Day 11						
1				8	0	0
2				0	-	0
3				0	0	12
4				0	0	0
5				0	-	0
6				0	0	0
7				0	10	0
8				0	0	0
9				0	-	0
10				0	0	11

Continued

Table 286: P25 (2nd test) – Test with daphnids: offspring per replicate and day. continued

Date	Control	1 mg/L	5 mg/L	Control	1.0 mg/L	5.0 mg/L
	Medium renewal three times a week			Daily medium renewal		
Day 12						
1	14	4	0	0	24	0
2	*	6	13	0	-	7
3	17	20	15	0	0	0
4	11	0	0	0	0	0
5	15	0	16	0	-	0
6	21	15	0	20	0	2
7	0	0	3	0	0	0
8	3	11	6	18	12	0
9	13	0	0	0	-	16
10	19	0	13	0	9	0

* Brood pouch of daphnid contained algae; no reproduction; organism was not considered for the calculation of reproduction

Date	Control	1 mg/L	5 mg/L	Control	1.0 mg/L	5.0 mg/L
	Medium renewal three times a week			Daily medium renewal		
Day 13						
1				0	0	19
2				14	-	5
3				27	6	0
4				1	23	27
5				7	-	15
6				0	22	14
7				16	0	0
8				0	0	26
9				17	-	0
10				8	0	0

Continued

Table 286: P25 (2nd test) – Test with daphnids: offspring per replicate and day. continued

Date	Control	1 mg/L	5 mg/L	Control	1.0 mg/L	5.0 mg/L
	Medium renewal three times a week			Daily medium renewal		
Day 14						
1	4	22	15	0	0	10
2	*	0	0	0	-	0
3	3	1	24	0	0	20
4	8	2	21	20	0	0
5	21	28	0	0	-	10
6	5	26	26	0	0	0
7	1	15	7	0	17	0
8	25	23	0	0	0	0
9	23	20	24	9	-	0
10	9	31	0	0	0	23

* Brood pouch of daphnid contained algae; no reproduction; organism was not considered for the calculation of reproduction

Date	Control	1 mg/L	5 mg/L	Control	1.0 mg/L	5.0 mg/L
	Medium renewal three times a week			Daily medium renewal		
Day 15						
1				9	0	0
2				0	-	1
3				0	0	0
4				0	0	0
5				1	-	0
6				25	0	0
7				0	0	28
8				20	18	0
9				0	-	0
10				9	11	0

Continued

Table 286: P25 (2nd test) – Test with daphnids: offspring per replicate and day. continued

Date	Control	1 mg/L	5 mg/L	Control	1.0 mg/L	5.0 mg/L
	Medium renewal three times a week			Daily medium renewal		
Day 16						
1	0	0	0	0	21	0
2	*	10	31	17	-	24
3	9	32	0	0	0	0
4	22	0	0	0	1	24
5	30	24	28	12	-	0
6	25	0	0	0	10	17
7	0	22	0	27	0	0
8	0	0	16	0	0	25
9	2	6	0	0	-	0
10	0	0	25	0	0	0

* Brood pouch of daphnid contained algae; no reproduction; organism was not considered for the calculation of reproduction

Date	Control	1 mg/L	5 mg/L	Control	1.0 mg/L	5.0 mg/L
	Medium renewal three times a week			Daily medium renewal		
Day 17						
1				0	0	19
2				0	-	0
3				0	0	23
4				20	22	0
5				0	-	23
6				0	18	0
7				0	0	0
8				0	0	0
9				21	-	0
10				0	0	0

Continued

Table 286: P25 (2nd test) – Test with daphnids: offspring per replicate and day. continued

Date	Control	1 mg/L	5 mg/L	Control	1.0 mg/L	5.0 mg/L
	Medium renewal three times a week			Daily medium renewal		
Day 18						
1				21	0	0
2				0	-	0
3				0	0	0
4				0	0	0
5				0	-	6
6				17	0	0
7				0	3	26
8				20	19	0
9				0	-	13
10				0	0	22

Date	Control	1 mg/L	5 mg/L	Control	1.0 mg/L	5.0 mg/L
	Medium renewal three times a week			Daily medium renewal		
Day 19						
1	18	21	16	0	5	0
2	*	14	30	0	-	24
3	11	22	25	0	0	0
4	28	0	30	0	0	35
5	23	0	31	20	-	0
6	16	28	23	0	0	24
7	11	23	15	0	0	0
8	29	23	18	12	0	0
9	26	0	24	0	-	0
10	2	27	28	17	0	0

* Brood pouch of daphnid contained algae; no reproduction; organism was not considered for the calculation of reproduction

Continued

Table 286: P25 (2nd test) – Test with daphnids: offspring per replicate and day. continued

Date	Control	1 mg/L	5 mg/L	Control	1.0 mg/L	5.0 mg/L
	Medium renewal three times a week			Daily medium renewal		
Day 20						
1				0	23	0
2				17	-	0
3				29	5	2
4				0	0	0
5				0	-	0
6				0	0	0
7				30	0	0
8				0	0	4
9				26	-	0
10				0	0	0

Date	Control	1 mg/L	5 mg/L	Control	1.0 mg/L	5.0 mg/L
	Medium renewal three times a week			Daily medium renewal		
Day 21						
1	23	38	23	0	0	23
2	*	0	0	0	-	0
3	14	0	32	0	0	26
4	3	0	22	26	0	0
5	7	29	0	0	-	0
6	20	23	38	7	0	0
7	22	0	25	0	27	0
8	36	32	0	0	0	0
9	27	19	27	0	-	0
10	4	30	0	0	0	27

* Brood pouch of daphnid contained algae; no reproduction; organism was not considered for the calculation of reproduction

21.8.3 P25 – third test

Table 287: P25 (3rd test) – Test with daphnids: oxygen saturation of the overlaying water.

Values of the parallel test vessels throughout test duration [mg/L]; concentrations given as nominal concentrations of TiO_2

Date	Control		1 mg/L		5 mg/L		Control		1.0 mg/L		5.0 mg/L	
	Ultrasonication period: 3 min						Ultrasonication period: 30 min					
	aged	new	aged	new	aged	new	aged	new	aged	new	aged	new
Start		8.1		8.1		8.0		8.1		8.2		7.9
Day 2	10.5	9.8	9.6	8.2	8.9	8.4	10.5	9.8	8.5	8.0	8.6	7.7
Day 5	8.9	8.3	9.2	8.1	9.4	7.8	8.9	8.3	8.6	7.7	7.9	7.0
Day 7	8.4	7.3	8.4	7.2	7.9	7.2	8.4	7.3	7.8	6.3	7.6	6.1
Day 9	8.8	7.6	8.9	8.0	8.9	8.0	8.8	7.6	8.6	7.6	9.2	7.5
Day 12	8.1	7.6	7.9	7.6	8.1	7.6	8.1	7.6	7.9	7.2	8.2	7.0
Day 14	8.5	8.3	9.1	8.2	8.7	8.0	8.5	8.3	8.7	7.5	8.7	7.5
Day 16	8.6	8.2	8.2	7.9	8.5	8.0	8.6	8.2	8.2	7.8	8.4	7.9
Day 19	8.8	8.1	8.9	8.1	9.0	8.1	8.8	8.1	9.2	7.5	9.1	7.5
Day 21	9.6		9.3		9.8		9.6		10.4		9.8	

Table 288: P25 (3rd test) – Test with daphnids: pH of the overlaying water.

Values of the parallel test vessels throughout test duration; concentrations given as nominal concentrations of TiO_2

Date	Control		1 mg/L		5 mg/L		Control		1.0 mg/L		5.0 mg/L	
	Ultrasonication period: 3 min						Ultrasonication period: 30 min					
	aged	new	aged	new	aged	new	aged	new	aged	new	aged	new
Start		8.1		8.1		8.1		8.1		8.0		8.1
Day 2	8.6	8.3	8.6	8.3	8.6	8.3	8.6	8.3	8.6	8.4	8.6	8.4
Day 5	8.7	8.4	8.8	8.4	8.9	8.4	8.7	8.4	8.8	8.4	8.8	8.4
Day 7	8.7	7.7	8.6	7.9	8.6	8.0	8.7	7.7	8.6	8.0	8.7	8.0
Day 9	8.6	8.2	8.6	8.2	8.6	8.2	8.6	8.2	8.5	8.3	8.7	8.2
Day 12	8.4	8.2	8.4	8.4	8.3	8.4	8.4	8.2	8.4	8.1	8.5	8.2
Day 14	8.5	8.4	8.6	8.3	8.5	8.3	8.5	8.4	8.6	8.3	8.6	8.3
Day 16	8.4	8.4	8.4	8.1	8.5	8.1	8.4	8.4	8.4	8.1	8.4	8.2
Day 19	8.3	8.4	8.3	8.5	8.3	8.5	8.3	8.4	8.5	8.4	8.5	8.5
Day 21	8.6		8.7		8.6		8.6		8.8		8.6	

Table 289: P25 (3rd test) – Test with daphnids: temperature of the overlaying water [°C] in the climatic chamber.

Day 0	Day 2	Day 5	Day 7	Day 9
21.2	20.5	20.9	21.0	20.7
Day 12	Day 14	Day 16	Day 19	Day 21
21.3	20.8	20.9	20.9	21.0

Table 290: P25 (3rd test) – Test with daphnids: light intensity [lux] in the climatic chamber.

Day 0	Day 2	Day 5	Day 7	Day 9
886	867	891	885	861
Day 12	Day 14	Day 16	Day 19	Day 21
842	850	834	847	811

Table 291: P25 (3rd test) – Test with daphnids: extinction (585 nm) as measure for the density of algae used as feed.

Day 0	Day 2	Day 5	Day 7	Day 9
0.015	0.025	0.035	0.045	0.55
Day 12	Day 14	Day 16	Day 19	
0.065	0.075	0.090	0.100	

Table 292: P25 (3rd test) – Test with daphnids: offspring per replicate and day.

Concentrations given as nominal concentrations of TiO_2

Date	Control	1 mg/L	5 mg/L	Control	1.0 mg/L	5.0 mg/L
	Ultrasonication period: 3 min			Ultrasonication period: 30 min		
Day 6						
1	0	0	0	0	0	0
2	0	0	0	0	0	0
3	0	0	0	0	0	0
4	0	0	0	0	0	0
5	0	0	0	0	0	0
6	0	0	0	0	0	0
7	0	0	0	0	0	0
8	0	0	0	0	0	0
9	0	0	0	0	0	0
10	0	0	0	0	0	0

Date	Control	1 mg/L	5 mg/L	Control	1.0 mg/L	5.0 mg/L
	Ultrasonication period: 3 min			Ultrasonication period: 30 min		
Day 7						
1	0	0	0	0	0	0
2	0	0	0	0	0	0
3	0	0	0	0	0	0
4	0	0	0	0	0	0
5	0	0	0	0	0	0
6	0	0	0	0	0	0
7	0	0	0	0	0	0
8	0	0	0	0	0	0
9	0	0	0	0	0	0
10	0	0	0	0	0	0

Continued

Table 292: P25 (3rd test) – Test with daphnids: offspring per replicate and day. continued

Date	Control	1 mg/L	5 mg/L	Control	1.0 mg/L	5.0 mg/L
	Ultrasonication period: 3 min			Ultrasonication period: 30 min		
Day 8						
1	0	0	0	0	0	0
2	0	0	11	0	0	0
3	0	0	0	0	0	0
4	0	0	0	0	0	0
5	0	0	0	0	0	0
6	0	0	0	0	0	0
7	0	0	0	0	0	0
8	0	0	0	0	0	0
9	0	0	0	0	0	0
10	0	0	0	0	0	0

Date	Control	1 mg/L	5 mg/L	Control	1.0 mg/L	5.0 mg/L
	Ultrasonication period: 3 min			Ultrasonication period: 30 min		
Day 9						
1	6	0	2	6	11	9
2	8	8	4	8	9	3
3	12	11	0	12	12	10
4	9	5	10	9	8	5
5	3	8	9	3	9	8
6	0	10	0	0	0	11
7	9	6	4	9	16	0
8	4	3	8	4	12	6
9	8	7	6	8	6	0
10	2	9	0	2	5	5

Continued

Table 292: P25 (3rd test) – Test with daphnids: offspring per replicate and day. continued

Date	Control	1 mg/L	5 mg/L	Control	1.0 mg/L	5.0 mg/L
	Ultrasonication period: 3 min			Ultrasonication period: 30 min		
Day 10						
1	0	10	0	0	0	0
2	0	organism dead	0	0	0	0
3	0	0	16	0	0	0
4	0	0	0	0	0	0
5	0	2	0	0	0	0
6	0	0	0	0	0	0
7	0	0	organism dead	0	0	16
8	0	0	0	0	0	0
9	0	1	0	0	0	0
10	0	0	0	0	0	0

Date	Control	1 mg/L	5 mg/L	Control	1.0 mg/L	5.0 mg/L
	Ultrasonication period: 3 min			Ultrasonication period: 30 min		
Day 12						
1	25	0	13	25	22	20
2	19	-	22	19	19	15
3	22	24	0	22	20	16
4	16	21	17	16	22	17
5	19	23	18	19	14	14
6	15	17	20	15	19	15
7	15	17	-	15	21	0
8	15	19	18	15	16	16
9	20	18	16	20	13	9
10	17	20	16	17	12	13

Continued

Table 292: P25 (3rd test) – Test with daphnids: offspring per replicate and day. continued

Date	Control	1 mg/L	5 mg/L	Control	1.0 mg/L	5.0 mg/L
	Ultrasonication period: 3 min			Ultrasonication period: 30 min		
Day 14						
1	0	23	29	0	0	0
2	0	-	0	0	0	0
3	0	0	30	0	0	0
4	0	0	0	0	0	0
5	0	0	0	0	0	0
6	0	0	0	0	0	0
7	0	0	-	0	0	18
8	0	0	0	0	0	0
9	0	0	0	0	0	17
10	0	0	24	0	0	12

Date	Control	1 mg/L	5 mg/L	Control	1.0 mg/L	5.0 mg/L
	Ultrasonication period: 3 min			Ultrasonication period: 30 min		
Day 16						
1	27	0	20	27	27	18
2	31	-	23	31	21	21
3	27	26	5	27	27	25
4	31	31	22	31	26	23
5	36	27	25	36	29	21
6	30	25	30	30	28	20
7	30	21	-	30	32	18
8	30	24	18	30	27	0
9	34	28	19	34	17	0
10	28	32	0	28	22	8

Continued

Table 292: P25 (3rd test) – Test with daphnids: offspring per replicate and day. continued

Date	Control	1 mg/L	5 mg/L	Control	1.0 mg/L	5.0 mg/L
	Ultrasonication period: 3 min			Ultrasonication period: 30 min		
Day 19						
1	0	20	1	0	25	0
2	30	-	10	30	33	23
3	31	31	13	31	33	19
4	33	15	26	33	27	24
5	34	25	26	34	24	19
6	28	Organism dead	27	28	31	20
7	27	2	-	27	27	25
8	27	21	25	27	27	21
9	31	26	25	31	27	21
10	29	31	20	29	26	24

Date	Control	1 mg/L	5 mg/L	Control	1.0 mg/L	5.0 mg/L
	Ultrasonication period: 3 min			Ultrasonication period: 30 min		
Day 21						
1	32	27	0	32	0	23
2	0	-	12	0	0	0
3	0	0	19	0	0	0
4	0	0	4	0	0	0
5	0	0	0	0	0	0
6	0	-	0	0	0	0
7	0	23	-	0	0	32
8	0	0	0	0	0	0
9	0	0	0	0	0	23
10	0	1	29	0	17	29

21.9 Raw data – Immobilisation test with daphnids (chapter 16)

21.9.1 NM-330 – pre-test (range finder)

Table 293: NM-330 – Acute test with daphnids: number of immobilized daphnids in the pre-test (range finder).

Concentrations given as percentage of NM-330 and NM-330DIS in the test medium

	Control	NM-330 (gold nanoparticles in dispersant)			NM-330DIS (dispersant of gold nanoparticles)		
		1 %	10 %	50 %	1 %	10 %	50 %
Incubation period: 24 h							
Replicate 1	0	0	0	0	0	3	5
Replicate 2	0	0	0	0	0	4	5
Replicate 3	0	---	---	---	---	---	---
Replicate 4	0	---	---	---	---	---	---
Immobile daphnids (total)	0	0	0	0	0	7	10
Immobile daphnids (%)	0	0	0	0	0	70	100
Incubation period: 48 h							
Replicate 1		0	0	0	0	5	5
Replicate 2		0	0	0	1	5	5
Replicate 3		---	---	---	---	---	---
Replicate 4		---	---	---	---	---	---
Immobile daphnids (total)	0	0	0	0	1	10	10
Immobile daphnids (%)	0	0	0	0	10	100	100

21.9.2 NM-330 - main test

Table 294: NM-330 – Acut test with daphnids: chemical analysis.

	dilution	Measured value considering the different wave length for determination			measured value * dilution		
		Au1978 [3] µg/L	Au2082 µg/L	Au2427 µg/L	Au1978 [3] µg/L	Au2082 µg/L	Au2427 µg/L
Test start: 0 h							
Control	10	0.423 [1]	2.76 [1]	-2.31 [1]	4.23 [1]	27.6 [1]	-23.1 [1]
NM-330 5%	20	134	136	143	2680	2712	2866
NM-330 10%	50	100	101	106	4985	5045	5310
Test end: 48 h							
Control	10	0.670 [1]	3.30 [2]	-2.64 [1]	6.70 [1]	33.0 [2]	-26.4 [1]
NM-330 5%	10	42.7	45.4	37.4	427	454	374
NM-330 10%	10	53.8	56.8	51.5	538	568	515

[1]Limit of detection; [2]Limit of determination; [3]used for calculation of concentration

Table 295: NM-330 – Acut test with daphnids: calibration.

			Measured value considering the different wave length for determination			measured value * dilution			Recovery		
	dilu-tion	nomi-nal	Au1978 [3] µg/L	Au2082 µg/L	Au2427 µg/L	Au1978 [3] µg/L	Au2082 µg/L	Au2427 µg/L	Au1978 [3] rec %	Au2082 rec %	Au2427 rec %
nano Gold reference material (value not certified)											
NIST 8011 A mg/L	250	51.56	201	203	203	50325	50625	50675	97.6	98.2	98.3
NIST 8011 B mg/L	250	51.56	200	202	198	49950	50450	49600	96.9	97.8	96.2
NIST 8011 A mg/L	250	51.56	207	205	204	51725	51200	50900	100	99.3	98.7
NIST 8011 B mg/L	250	51.56	202	202	203	50425	50550	50650	97.8	98.0	98.2
recalibration standard											
Standard Au 50 µg/L		50	48.1	51.0	49.2				96.2	102	98.4
Standard Au 50 µg/L		50	47.0	49.3	51.8				94.0	98.6	104
Limit of detec-tion			4.29	3.21	7.50	< NG					
Limit of deter-mination			14.3	10.7	25.0	< BG					

[3]used for calculation of concentration

Ecotoxicological test

Table 296: NM-330 – Acute test with daphnids: number of immobilized daphnids in the main test.

Concentrations given as percentage of NM-330 and NM-330DIS in the test medium

		NM-330 (gold nanoparticles in dispersant)		NM-330DISs (dispersant of gold nanoparticles)				
	Control	5 %	10 %	0.625 %	1.25 %	2.5 %	5 %	10 %
Incubation period: 24 h								
Replicate 1	0	0	0	0	0	0	0	5
Replicate 2	0	0	0	0	0	0	4	4
Replicate 3	0	0	0	0	0	0	1	3
Replicate 4	0	0	0	0	0	0	2	4
Immobile daphnids (total)	0	0	0	0	0	0	7	16
Immobile daphnids (%)	0	0	0	0	0	0	35	80
Incubation period: 48 h								
Replicate 1	0	0	0	0	1	1	3	5
Replicate 2	1	0	0	0	0	1	5	5
Replicate 3	0	1	1	0	3	1	2	5
Replicate 4	0	0	0	0	2	0	3	5
Immobile daphnids (total)	1	1	1	0	6	3	13	20
Immobile daphnids (%)	5	5	5	0	30	15	65	100

21.10 Raw data – growth test with algae – Au (chapter 17):

21.10.1 NM-330 – first test

Table 297: NM-330 – Test with algae: number of algae (test concentrations achieved by dilution with ultrapure water).

Time		Treatment [%]								
0 h	Replicate	Control	0.63	1.25	2.5	5.00	10	20	40	80
	1	19552	20350	17395	16793	14426	12423	6289	3277	0
	2	19972	18796	19650	17941	14174	11148	7535	1387	0
	3	18880	19244	17661	17325	13880	10812	7087	1779	0
	4	19944								
	5	18613								
	6	20756								
	Mean	19619.5	19463.1	18235.3	17352.9	14159.7	11461.3	6970.1	2147.5	0.0
	Std.Dev	785.6	800.2	1232.3	574.7	273.4	849.7	631.4	997.9	0.0
	CV%	4.0	4.1	6.8	3.3	1.9	7.4	9.1	46.5	
24 h	Replicate									
	1	139986	118445	142577	127241	119706	129300	92381	144958	80336
	2	135602	135210	143725	122101	130560	115406	81261	144482	68207
	3	133137	138627	138487	114342	128964	117465	118796	128417	69790
	4	117283								
	5	122171								
	6	127843								
	Mean	129337.1	130761.0	141596.6	121227.8	126409.9	120723.6	97479.0	139285.7	72777.8
	Std.Dev	8555.5	10801.6	2753.2	6493.7	5860.5	7498.1	19279.8	9415.3	6593.4
	CV%	6.6	8.3	1.9	5.4	4.6	6.2	19.8	6.8	9.1
48 h	Replicate									
	1	740938	567269	670658	597927	607801	579468	390490	293403	60476.2
	2	671331	681246	632311	541751	607535	508739	304244	313403	43809.5
	3	649888	655882	614398	549538	614874	509342	485294	273011	46302.5
	4	547871								
	5	569314								
	6	562955								
	Mean	623716.2	634799.3	639122.3	563071.9	610070.0	532516.3	393342.7	293272.6	50196.1
	Std.Dev	76285.2	59842.3	28742.1	30435.6	4162.4	40662.3	90558.9	20196.4	8989.7
	CV%	12.2	9.4	4.5	5.4	0.7	7.6	23.0	6.9	17.9
72 h	Replicate									
	1	1974216	1430462	1347619	973473	894468	801120	482255	304286	59019.6
	2	1759398	1541793	1196765	887661	899748	657339	353782	316275	43095.2
	3	1603193	1365196	1132619	1067045	939076	636919	498403	279300	44313.7
	4	1339272								
	5	1361695								
	6	1356050								
	Mean	1565637.3	1445817.0	1225667.6	976059.8	911097.1	698459.4	444813.3	299953.3	48809.5
	Std.Dev	261782.5	89294.0	110375.6	89719.8	24373.5	89491.4	79248.2	18864.3	8863.2
	CV%	16.7	6.2	9.0	9.2	2.7	12.8	17.8	6.3	18.2

Table 298: NM-330 – Test with algae: number of algae (test concentrations achieved by dilution with dispersant).

Time		Treatment [%]								
0 h	Replicate	Control	0.63	1.25	2.5	5.00	10	20	40	80
	1	21905	13291	15854	13207	9818	6359	1653	0	0
	2	[1])	16765	14062	12101	11373	7087	2311	0	0
	3	19146	12465	13754	13039	10490	6513	1373	0	0
	4	19440								
	5	19804								
	6	20252								
	Mean	20109.2	14173.7	14556.5	12782.4	10560.2	6652.7	1778.7	0.0	0.0
	Std.Dev	1085.6	2281.6	1134.5	596.2	779.7	383.8	481.7	0.0	0.0
	CV%	5.4	16.1	7.8	4.7	7.4	5.8	27.1		
24 h	Replicate									
	1	174216	30392	47185	31891	29846	22171	11092	5728	35770
	2	[1])	32423	31961	28319	28039	20742	13880	5700	46359
	3	155490	34986	30924	34272	27843	19706	12241	4916	43810
	4	149720								
	5	155266								
	6	147353								
	Mean	156409.0	32600.4	36690.0	31493.9	28576.1	20873.0	12404.3	5448.2	41979.5
	Std.Dev	10559.2	2302.1	9103.6	2996.0	1104.1	1237.7	1400.7	461.1	5526.3
	CV%	6.8	7.1	24.8	9.5	3.9	5.9	11.3	8.5	13.2
48 h	Replicate									
	1	884832	33964	46947	31429	26092	22311	11289	6723	118782
	2	[1])	31667	31639	30560	25644	20854	14258	7199	149230
	3	823782	34398	30168	37003	26485	19398	11989	6737	133109
	4	778473								
	5	812479								
	6	788543								
	Mean	817621.8	33342.7	36251.2	32997.2	26073.8	20854.3	12511.7	6886.1	133706.8
	Std.Dev	41712.5	1467.6	9291.8	3496.0	420.5	1456.6	1552.1	271.0	15232.9
	CV%	5.1	4.4	25.6	10.6	1.6	7.0	12.4	3.9	11.4
72 h	Replicate									
	1	2155406	31387	40448	28193	25504	18754	9202	5924	101414.57
	2	[1])	28361	31008	26793	23389	17003	11345	5896	118025.21
	3	1963992	31176	28347	31317	23950	16443	11499	5210	109691.88
	4	1715406								
	5	1780966								
	6	1767605								
	Mean	1876675.1	30308.1	33268.0	28767.5	24281.0	17399.6	10681.6	5676.9	109710.6
	Std.Dev	181919.8	1689.2	6359.0	2315.9	1095.7	1205.5	1284.0	404.5	8305.3
	CV%	9.7	5.6	19.1	8.1	4.5	6.9	12.0	7.1	7.6

[1]) Sample defect

Table 299: NM-330DIS – Test with algae: number of algae.

Time		Treatment [%]								
0 h	Replicate	Control	0.63	1.25	2.5	5.00	10	20	40	80
	1	14664	20098	21078	18291	18908	17913	16190	15420	14132
	2	16050	21373	19776	18950	18221	16975	16611	16204	15924
	3	16008	20602	18824	19608	18697	19314	16961	15840	15098
	4	16681								
	5	14300								
	6	17059								
	Mean	15793.7	20690.9	19892.6	18949.6	18608.8	18067.2	16587.3	15821.7	15051.4
	Std.Dev	1096.2	641.9	1132.0	658.3	351.6	1177.1	385.7	392.5	897.3
	CV%	6.9	3.1	5.7	3.5	1.9	6.5	2.3	2.5	6.0
24 h	Replicate									
	1	171569	121779	113207	78683	48880	33838	31204	29650	31289
	2	165756	168641	124090	79188	51653	34090	28627	24426	31541
	3	160924	179748	110658	69412	44678	32353	29692	23782	29986
	4	166569								
	5	157059								
	6	157731								
	Mean	163268.0	156722.7	115985.1	75761.0	48403.4	33426.7	29841.3	25952.4	30938.4
	Std.Dev	5669.0	30767.7	7133.5	5504.4	3511.7	938.4	1295.0	3218.3	834.4
	CV%	3.5	19.6	6.2	7.3	7.3	2.8	4.3	12.4	2.7
48 h	Replicate									
	1	995980	723515	509356	191989	85574	47787	30042	22927	30196
	2	950238	856289	594454	191751	89790	51176	28711	19958	30210
	3	923361	897549	493711	163291	78964	47745	26639	20014	29888
	4	979762								
	5	920378								
	6	933543								
	Mean	950543.9	825784.3	532507.0	182343.6	84775.9	48902.9	28464.1	20966.4	30098.0
	Std.Dev	31164.5	90938.5	54214.7	16500.2	5457.1	1969.1	1715.1	1698.3	182.1
	CV%	3.3	11.0	10.2	9.0	6.4	4.0	6.0	8.1	0.6
72 h	Replicate									
	1	2835938	1061975	567297	227521	98768	60420	38347	21331	29342
	2	2725196	1154188	614958	209244	108109	62353	33515	19202	28613
	3	2647535	1204230	487703	218263	93992	59482	33291	18936	27647
	4	2776078								
	5	2663838								
	6	2623389								
	Mean	2711995.8	1140130.7	556652.7	218342.7	100289.4	60751.6	35051.4	19822.6	28534.1
	Std.Dev	82423.4	72161.7	64291.7	9138.9	7180.8	1464.0	2856.6	1312.7	850.1
	CV%	3.0	6.3	11.5	4.2	7.2	2.4	8.1	6.6	3.0

21.10.2 NM-330 – second test

Table 300: NM-330 – Test with algae: number of algae (test concentrations achieved by dilution with ultrapure water).

Time		Treatment [%]								
0 h	Replicate	Control	0.63	1.25	2.5	5.00	10	20	40	80
	1	14062	12115	12017	8880	9594	4832	854	0	0
	2	13487	15196	11863	10084	8557	3585	2003	0	0
	3	12689	12549	11625	10854	8725	4776	1261	0	0
	4	13431								
	5	12619								
	6	12087								
	Mean	13062.6	13286.6	11834.7	9939.3	8958.9	4397.8	1372.5	0.0	0.0
	Std.Dev	721.5	1667.8	197.6	995.3	556.2	704.1	582.4	0.0	0.0
	CV%	5.5	12.6	1.7	10.0	6.2	16.0	42.4		
24 h	Replicate									
	1	119986	83137	109874	97647	95868	85252	65476	63179	27073
	2	121779	115322	105812	92465	102661	79160	51471	69678	29146
	3	100322	105546	104132	88221	106653	81947	64790	51359	27381
	4	114202								
	5	110952								
	6	100966								
	Mean	111367.9	101335.2	106606.0	92777.8	101727.4	82119.5	60578.9	61405.2	27866.5
	Std.Dev	9176.7	16500.5	2952.3	4720.7	5452.5	3049.9	7895.5	9287.6	1118.5
	CV%	8.2	16.3	2.8	5.1	5.4	3.7	13.0	15.1	4.0
48 h	Replicate									
	1	582577	421863	536092	460266	461204	370980	273585.4	111638.7	21681
	2	579104	571919	508711	418333	446232	351232	201498.6	125182.1	20840
	3	465840	544118	501218	399790	462283	361821	231092.4	109495.8	17311
	4	494300								
	5	508501								
	6	447577								
	Mean	512983.2	512633.1	515340.8	426129.8	456573.3	361344.5	235392.2	115438.8	19944.0
	Std.Dev	56718.2	79829.0	18357.8	30982.8	8971.6	9882.6	36235.3	8505.6	2318.7
	CV%	11.1	15.6	3.6	7.3	2.0	2.7	15.4	7.4	11.6
72 h	Replicate									
	1	1350742	1098824	954272	761120	782983	697577	595952.4	186610.6	27073
	2	1307493	1182045	941933	648739	680854	670126	426092.4	205616.2	29146
	3	1022577	1261148	929160	639216	711947	706821	512773.1	177437	27381
	4	970770								
	5	1092353								
	6	943109								
	Mean	1114507.5	1180672.3	941788.0	683025.2	725261.4	691507.9	511606.0	189888.0	27866.5
	Std.Dev	174373.2	81171.2	12556.6	67799.9	52350.1	19085.3	84936.0	14372.7	1118.5
	CV%	15.6	6.9	1.3	9.9	7.2	2.8	16.6	7.6	4.0

Table 301: NM-330DIS – Test with algae: number of algae.

Time		Treatment [%]								
0 h	Replicate	Control	0.63	1.25	2.5	5.00	10	20	40	80
	1	11863	7997	9006	9230	9300	10182	8824	13235	11092
	2	12521	8543	9230	7185	10588	10224	9216	11667	12955
	3	12479	8852	9188	8053	8319	9314	10322	10546	12577
	4	12577								
	5	11933								
	6	13711								
	Mean	12514.0	8464.1	9141.0	8155.9	9402.4	9906.6	9453.8	11816.1	12208.2
	Std.Dev	663.5	432.7	119.1	1026.3	1137.9	513.9	777.2	1350.7	984.6
	CV%	5.3	5.1	1.3	12.6	12.1	5.2	8.2	11.4	8.1
24 h	Replicate									
	1	115294	15238	15294	17045	15938	16905	40966	82339	66443
	2	121863	14202	16148	14104	17185	19356	38291	64692	80462
	3	119356	16821	16975	9314	15560	21261	36891	60308	80560
	4	113669								
	5	108067								
	6	111737								
	Mean	114997.7	15420.2	16139.1	13487.4	16227.8	19173.7	38716.2	69113.0	75821.7
	Std.Dev	5032.7	1319.0	840.4	3902.2	850.1	2183.6	2070.8	11661.9	8122.7
	CV%	4.4	8.6	5.2	28.9	5.2	11.4	5.3	16.9	10.7
48 h	Replicate									
	1	578796	27087	11232	17087	24188	28571	101036	340910.4	400630.25
	2	611709	28039	13277	14510	26022	37563	94397.8	253263.3	460028.01
	3	595546	33403	13319	10770	24034	40392	86582.6	247479	484397.76
	4	567983								
	5	561765								
	6	574048								
	Mean	581641.0	29509.8	12609.7	14122.3	24747.9	35508.9	94005.6	280550.9	448352.0
	Std.Dev	18680.4	3405.4	1192.9	3176.0	1106.4	6172.3	7234.9	52352.8	43087.1
	CV%	3.2	11.5	9.5	22.5	4.5	17.4	7.7	18.7	9.6
72 h	Replicate									
	1	1416289	23529	13543	25798	38894	46751	161036	682549	1146414.6
	2	1521779	23908	14594	22129	40812	57185	143431	494355.7	1117675.1
	3	1370518	28375	13880	17759	37829	61877	137633	501064.4	1042268.9
	4	1362017								
	5	1365070								
	6	1405280								
	Mean	1406825.4	25270.8	14005.6	21895.4	39178.3	55270.8	147366.9	559323.1	1102119.5
	Std.Dev	60623.0	2695.3	536.4	4024.7	1511.8	7742.6	12187.9	106769.5	53787.2
	CV%	4.3	10.7	3.8	18.4	3.9	14.0	8.3	19.1	4.9

21.10.3 NM-330 – third test

Table 302: NM-330 and NM-330DIS – Test with algae: number of algae (test concentrations achieved by dilution with ultrapure water).

Time		Treatment [%]					
		NM-330			NM-330DIS		
0 h	Replicate	Control	40,00	80,00	Control	40,00	80,00
	1	9119	6813	6143	9119	8071	8616
	2	9539	8218	5430	9539	7442	7904
	3	8491	8029	5786	8491	7631	8050
	4	9706			9706		
	5	9350			9350		
	6	9078			9078		
	Mean	9213.8	7686.9	5786.2	9213.8	7714.9	8190.1
	Std.Dev	428.4	762.3	356.4	428.4	322.7	376.4
	CV%	4.6	9.9	6.2	4.6	4.2	4.6
24 h	Replicate						
	1	52872	44927	34654	52872	11866	11656
	2	44990	45472	34927	44990	9413	12537
	3	37715	44130	32327	37715	10084	13543
	4	32222			32222		
	5	48491			48491		
	6	45073			45073		
	Mean	43560.4	44842.8	33969.3	43560.4	10454.2	12578.6
	Std.Dev	7457.5	674.8	1428.7	7457.5	1267.7	944.1
	CV%	17.1	1.5	4.2	17.1	12.1	7.5
48 h	Replicate						
	1	311321	230881	157757	311321	9497	12621
	2	275115	227379	158407	275115	9832	14969
	3	221908	225723	171090	221908	8721	14654
	4	210566			210566		
	5	298155			298155		
	6	258470			258470		
	Mean	262589.1	227994.4	162417.9	262589.1	9350.1	14081.1
	Std.Dev	40435.0	2633.0	7517.4	40435.0	569.9	1274.6
	CV%	15.4	1.2	4.6	15.4	6.1	9.1
72 h	Replicate						
	1	1789602	444130	108281	1789602	9665	13816
	2	1676143	431447	113229	1676143	8574	11740
	3	1321803	386122	113166	1321803	5136	9560
	4	1405157			1405157		
	5	1789832			1789832		
	6	1654298			1654298		
	Mean	1606139.1	7791.8	11705.1	1606139.1	7791.8	11705.1
	Std.Dev	197920.3	2363.4	2128.1	197920.3	2363.4	2128.1
	CV%	12.3	30.3	18.2	12.3	30.3	18.2