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

Adaption of test guidelines

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

by

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15. Zusätzliche Angaben

Die verwendeten OECD-Tests sind geeignet, die Ökotoxizität ausgewählter Nanopartikel abzuschätzen, falls die Testmedien und damit der Organismen angemessen exponiert werden. Nanosilber repräsentiert eine untypische Situation, da es ein Ziel vieler Anwendungen ist, als ein Reservoir für die ständige Lösung von Silberionen zu dienen, die biozide Wirkung haben. Somit müssen die Partikelstabilität und die Verhinderung von Auflösungsvorgängen als experimentelles Ziel im Einzelfall diskutiert werden.

16. Zusammenfassung

Das nanopartikuläre Titandioxid NM-105 wurde mit dem Sedimentbewohner *Lumbriculus variegatus* in einem Wasser/Sediment-System in Anlehnung an OECD TG 225 getestet. NM-105-Dispersionen wurden mit über dem Sediment stehendem Wasser verdünnt, um Nominalkonz. von 15, 23, 39, 63, 100 mg NM-105/L zu erzielen. Diese wurden durch chemische Analytik der Ti-Konzentrationen bestätigt. Es wurden keine negativen Wirkungen auf Reproduktion oder Biomasse der Würmer beobachtet. Die NOEC ≥ 100 mg/L wurde in einem zweiten Test mit 100 mg NM-105/L bestätigt. Zu Testende waren die Ti-Konz. in den Würmern vergleichbar. Weiterhin wurde NM-105 in OECD TG 226-Tests mit der Raubmilbe *Hypoaspis aculeifer* untersucht. Die Testsubstrate wurden durch Mischung des Feststoffes mit künstlichem Boden (≥ 10mg/kg) oder Dosierung von NM-105-Dispersionen (≤ 10mg/kg) hergestellt. Bei 10 mg/kg wurde kein Einfluss der Dosiermethode auf die Testergebnisse beobachtet. Im ersten Haupttest verursachten 1, 10, 100 und 1000 mg NM-105/kg künstlicher Boden keine signifikante Wirkung. Im zweiten Test mit 1 und 1000 mg NM-105/kg zeigten beide Behandlungen im Vergleich zur Kontrolle signifikant niedrigere Zahlen juveniler Milben aufgrund der höheren statistischen Trennschärfe durch die verdoppelte Replikatzahl. Die Anwendung des Standarddesigns, das für Nanomaterialien geeignet war, ergab eine NOEC ≥ 1000 mg NM-105/kg. Nanopartikuläres Silber NM-300K wurde in zwei Fischtests mit *Danio rerio* nach OECD TG 210 in statischen Systemen mit 250 l untersucht. Die NM-300K-Dispersion (1:10) wurde leicht verdünnt, ultraschallbehandelt und direkt in die Testbecken dosiert. Die Wasserkörper wurden kontinuierlich durch je vier Pumpen bewegt, um eine homogene Verteilung zu erzeugen und Sedimentation zu minimieren Alle 7 Tage wurde das Testmedium gewechselt. Die Nominalkonz. waren 12.5, 25, 50, 100 und 200 µg Ag/L im ersten und 12.5, 50 und 100 µg/L im zweiten Haupttest. Messungen der Gesamt-Ag-Konz. ergaben 50-70 % der Nominalkonz. Der Anteil gelösten Silbers war ca. 3%. Der Schlupf war bis 136 µg/L (gemessene Konz.) nicht beeinträchtigt. Die Überlebensrate der Larven war ≥ 47 µg/L signifikant reduziert (NOEC= 23 µg/L). Den empfindlichsten Endpunkt stellte das Wachstum (NOEC = 5.9 µg/L) dar. Der Testansatz erwies für die Prüfung von Nanomaterialien als geeignet (Konstanz der Exposition, Empfindlichkeit, statistische Trennschärfe). Ag-Konz. in den Fischen waren mit steigender Ag-Konz. im Wasser erhöht und fanden sich vor allem im Darm.

17. Schlagwörter

$nTiO_2$; *Lumbriculus variegatus;* OECD 225*; Hypoaspis aculeifer;* OECD 226; nAg; Nanosilber; Fisch Early-Life Stage Test; OECD 210; *Danio rerio*; Chronische Toxizität; Dosiermethoden

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Report Cover Sheet

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

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15. Supplementary Notes
The applied guideline tests are suited for assessing ecotoxicity of selected nanoparticles, provided that exposure of the test media and subsequently of the test organisms is adequately performed. The situation with nanosilver is not a typical one, as the objective of using silver nanoparticles is to serve as reservoir for steady dissolution of free Ag ions that act as biocide. Thus, stability of the particles and prevention of dissolution have to be discussed in this case.

16. Abstract
The nanoparticulate titanium dioxide NM-105 was investigated with *Lumbriculus variegatus* in a sediment-water system according to OECD TG 225. Test media were prepared by dilution of NM-105-suspensions with the sediment-overlying water. Nominal test concentrations (confirmed by chemical analysis of Ti conc. in test media) were 15; 23; 39; 63 and 100 mg NM-105/L. No adverse effects on reproduction or biomass of the worms were observed. The NOEC ≥ 100 mg/L was confirmed in a second test with 100 mg NM-105/L. At test end, Ti conc. in worms were similar in all treatments. Additionally, NM-105 was investigated in OECD TG 226 tests with the predatory mite *Hypoaspis aculeifer*. Test substrates were prepared by mixing the solid powder into artificial soil (test conc. ≥ 10mg/kg) or application of NM-105-suspensions (≤ 10mg/kg). For 10 mg/kg, no influence of the application method on the endpoints was observed. No significant effect of the treatments 1; 10; 100; 1000 mg NM-105/kg artificial soil (dw) was detected in the 1st definitive test, the 2nd test at 1 and 1000 mg NM-105/kg showed significantly lower numbers of juvenile mites compared to the control in both treatments due to higher statistical power of the doubled number of replicates. For the standard design, which was proven to be applicable to the testing of nanomaterials, the NOEC was ≥ 1000 mg NM-105/kg.
The nanoparticulate silver NM-300 K was investigated in two fish early life stage toxicity tests (OECD TG 210) with *Danio rerio* in a 250 L static system. The NM-300 K dispersion (1:10) was slightly diluted, ultra-sonificated and directly applied to the test vessels. The water in all test aquaria was constantly moved by four pumps each for homogeneous distribution and minimized sedimentation. Every 7 days, the test medium was exchanged. The nominal test concentrations were 12.5; 25; 50; 100 and 200 µg Ag/L in the 1st test and 12.5; 50; 100 µg/L in the 2nd test. Chemical analysis of total Ag conc. in test media showed 50-70 % of nominal. The proportion of dissolved silver was approx. 3 %. Hatch was not affected up to 136 µg/L (mean measured conc.). Post-hatch survival was significantly reduced at conc. ≥ 47 µg/L, the NOEC was 23 µg/L. The most sensitive endpoint was growth with a NOEC of 5.9 µg/L. The test setup was suited for the testing of nanomaterials, proven by sensitive results and high statistical power. Total Ag conc. in the fish at test end significantly increased with increasing Ag conc. in the water. Most of the accumulated silver was located in the intestines.

17. Keywords
$nTiO_2$; *Lumbriculus variegatus;* OECD 225*; Hypoaspis aculeifer;* OECD 226;
nAg; nanosilver; Fish Early-Life Stage Test; OECD 210; *Danio rerio*;
chronic toxicity; application method

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List of abbreviations

CV:	Coefficient of variation
dw:	Dry weight
EC_x:	Effect concentration (x % effect size)
LOEC:	Lowest observed effect concentration
LOD:	Limit of detection
LOQ:	Limit of quantification
lx:	Lux
n.d.	Not determined due to mathematical reasons or inappropriate data
NOEC:	No observed effect concentration
rpm:	Rounds per minute
SD:	Standard deviation
TG:	Test guideline
WHC_{max}:	Maximum water holding capacity

1 Introduction

To date, limited data is available about behavior, persistence and effects of nanomaterial in the environment. At the moment, a comprehensive risk assessment of nanoparticles is not possible due to lack of data from field studies and experimental work [1–3].

One approach to reduce gaps of knowledge is initiated by the OECD. In frame of the "*Working-Party on Manufactured Nanomaterials*" OECD members and non-members function as sponsors and co-sponsors and become responsible for the safety testing of selected nanoparticles. Germany is a sponsor for nanoparticulate titanium dioxide and co-sponsor for nano silver and is responsible for the creation of the risk assessment for those particles with respect to potential effects on the environment and human health. Existing data are reviewed and areas of limited data are identified for further research.

Availability of data from chronic ecotoxicological studies with nano titanium dioxide and nano silver is scarce. For this reason, the German Federal Environment Agency supports studies to identify suitable technical procedures for testing of nanoparticles and ecotoxicological studies with terrestrial and aquatic test organisms. In the project "*Investigation of two widely used nanomaterials (TiO_2, Ag) in standardized ecotoxicological tests.*" (Support code 3709 65 416) several experiments with titanium dioxide and nano silver are performed according to OECD test guidelines [4]. To provide supplementary data from further experimental studies, the project presented in this report was initiated (support code 3709 65 418). The methods for application of particles into the test systems and determination of particle characteristics (i.e. zeta potential, size) developed in the previous project were applied in this project. The aim of the project presented here was the performance of the following studies:

- Sediment-water test with *Lumbriculus variegatus* according to OECD TG 225 [5] with the nano titanium dioxide NM-105 (Table 1),
- Reproduction test with *Hypoaspis aculeifer* in soil according to OECD TG 226 [6] with the nano titanium dioxide NM-105 (Table 1),
- Fish Early-life stage toxicity test with *Danio rerio* according to OECD TG 210 [7] with the nano silver NM-300K (Table 1).

Table 1: Properties of the applied nanomaterials.

TiO_2 nanomaterial	NM-105	Ag nanomaterial	NM-300K
Crystal structure	Rutile - Anatase	Condition	in dispersion NM-300K DIS
Purpose	active component for photo catalytic reactions	Primary particle size *	15 nm
Primary particle size *	21 nm		
Composition	TiO_2: > 99%	Data by the Joint Research Centre, European Commission	
BET	60 m^2/g		
Coating	none		
Condition	solid, powder		* according to Scherrer

2 Sediment-water test with *Lumbriculus variegatus* and NM-105

2.1 Test principle

The study was conducted in order to determine the potential impact of the test substance NM-105 on the survival, biomass and reproduction of the sediment-dwelling aquatic oligochaete *Lumbriculus variegatus*. To achieve this aim, adult worms of synchronised physiological state were exposed to a series of concentrations of the test item applied to the water phase of a sediment-water system. Artificial sediment and reconstituted water were used as media. Test vessels without the addition of the test substance served as controls. For measurement of physico-chemical parameters and for sampling for chemical analysis, 4 separate test vessels per treamtent and control were prepared and not included in evaluation of biological endpoints at test end. The test vessels were filled with sediment and 50% of the volume of the overlying water one day before beginning of exposure. The test organisms were introduced into the test vessels on the same day. On the following day, dispersions of the test substance were prepared and administerd into the test vessels to achieve the nominal concentrations in each test vessel. The test animals were exposed to the sediment-water systems for a period of 28 d. Prior to filling the test vessels, the sediment was amended with a mixture of dry, finely ground leaves of stinging nettle (*Urtica* sp.; urtica powder) and cellulose (i.e. α-cellulose powder) to a final amount of 0.5% of sediment dry weight to ensure that the worms survive, grow and reproduce under control conditions. The worms were not fed additionally during the exposure period.

Endpoints based on reproduction and growth – and if possible mortality – were assessed in comparison with the control. Parameters were the total number of surviving animals, and the dry weight of the surviving organisms.

The preferred endpoint of this study was the no observed effect concentration (NOEC) and the lowest observed effect concentration (LOEC) for reproduction and biomass reduction, respectively, compared to the control.

The studies were prepared at ECT (i.e. synchronising worms, preparation of artificial sediment, labelling of test vessels) and performed in the IME laboratory.

To verify the nominally applied concentrations and determine the behaviour and partitioning of the test item in the sediment-water system, samples were taken from the overlying water during the first definitive test. The samples were analytically measured for particles size and titanium concentrations at the IME. Additionally, worms were sampled after end of the second definitive test and uptake of titanium into the worms exposed to 100 mg/L NM-105 compared to controls was investigated.

2.2 Materials and methods

2.2.1 Test guideline

The test was performed according to:

OECD. 2007. OECD guideline for testing of chemicals. 225. Sediment-Water Lumbriculus Toxicity Test using spiked sediment. Organisation for Economic Co-operation and Development, Paris, France.

2.2.2 GLP

The test was not performed under GLP, but followed the principles. The use of any laboratory equipment was controlled and protocolled according to GLP. The quality assurance did not check any phase of the study, the raw data and study report.

2.2.3 Test substance

The test substance used in this study was nanoparticulate titanium dioxide NM-105.

2.3 Analytical monitoring

For determination of titanium concentration in overlying water, samples of control and test media were taken at beginning of exposure and 1, 7 and 28 d after beginning of exposure. For each sample subsamples of 5 mL were taken: one sample directly under the surface of the test medium, two samples by submerging the pipette to one and two third of the total height of the test medium, and one sample from approximately 1 cm over the sediment. The subsamples were pooled and transferred to the analytical laboratory where they were stored in a refrigerator until analysis.

Additionally, size of NM-105 particles in the test media was determined at beginning of exposure and 3 h, 1 d and 7 d after. Samples were taken from different depths of the respective test media applying the scheme described above. Measurement was performed using dynamic light scattering (DLS) in a Malvern Zeta-Sizer.

2.3.1 Details on sediment and water

Sediment was not included in analytical monitoring.

Chemical digestion of aqueous samples

Aqueous samples were vigorously shaken for approximately one minute before submitting them to chemical digestion. 4 mL sample was mixed with 1 mL of a mixture of hydrofluoric, nitric and hydrochloric acid (volume ratios 1:3:1). The mix was shaken and treated in an ultrasonic bath (Bandelin Sonorex RK 514 BH; 35 kHz; 215/860 W) for 30 minutes. To eliminate F^- before performing the chemical analysis, boric acid (4%) was added.

Analysis of titanium in digested sample

The chemical analysis of the digested water samples was performed with ICP-OES (inductively coupled plasma – optical emission spectrometry). Each sample was measured in triplicates. Calibrations were performed before measurements of samples with the following titanium concentrations: 0; 50; 100; 250; 500; 1000 µg/L. The mixture of hydrofluoric, nitric and hydrochloric acid was measured as a blank. Additionally, a TiO_2-positive control with 83.103 mg/L titanium was prepared, measured and recovery was determined.

2.3.2 Details on application

The test vessels were filled with sediment and 50% of the volume of the overlying water one day before beginning of exposure. The test organisms were introduced into the test vessels on the same day. On the following day, dispersions of the test substance were prepared and administerd into the test vessels to achieve the nominal concentrations in each test vessel. Immediately after application, test media were briefly stirred with a glass rod. In order to generate similar conditions as in the test vessels used for biological assessment, the vessels designated for analysis received a number of worms which provides a population density similar to the biological vessels. The test animals were exposed to the test item for a period of 28 d.

The dispersions were prepared by weighing the desired amount of powder NM-105 into a glass vessel and adding the respective volume of deionized water to gain the desired concentration. The dispersion was stirred for 60 seconds on a magnetic stirrer (900 rpm) and subsequently treated in an ultra-sonic water bath filled to one third of the dispersion height in the bottles (Bandelin Sonorex RK 514 BH; 35 kHz; 215/860 W) for three minutes (Hund-Rinke , [16]).

The application method of the test substance via the water phase is not described in the test guideline [5]. Application of test item via the water phase is described for the sediment dwelling dipteran larvae *Chironomus riparius* [8]. This method of administration was used in tests with *L. variegatus* since tests with *C. riparius* were performed at the IME (IME, 2012). This way, a higher degree of comparability of the results of the two test systems is achieved.

2.4 Test organisms

The test organism used in this study was the endobenthic oligochaete *Lumbriculus variegatus* (Müller). This species is tolerant to a wide range of sediment types, and is widely used for sediment toxicity and bioaccumulation testing. The species has been cultured at ECT Oekotoxikologie GmbH since January 1998. The animals were originally obtained from Fischfutter Etzbach (D-53894 Mechernich-Bergheim, Germany). The species identity of the cultured organisms was confirmed according to [9].

Lumbriculus variegatus is cultured at ECT in crystallising dishes containing quartz sand, and reconstituted water. The oligochaetes are held at 20 ± 2°C with a photo period of 16 h light (intensity up to 500 lx) and 8 h dark. In the culture, the worms are fed with fish food suspension (50 g/L TetraMin®).

10 days before the start of the test, the worms were artificially fragmented (synchronisation). This synchronisation was performed to avoid uncontrolled regeneration and subsequent high variation in test results. Adult worms, which did not show signs of recent morphallaxis, were used. These worms were placed onto a glass slide in a drop of culture water, and bisected in the median body region with a scalpel. The posterior ends were left to regenerate new heads in a culture vessel containing a 2 ± 1 cm layer of quartz sand and test medium. They were held at 20 ± 2°C until start of exposure. Feeding of the regenerated worms was done once on day seven after dissection, with fish food suspension. After regenerating, intact complete worms of similar size, which were actively swimming or crawling upon a gentle mechanical stimulus, were used for the test.

2.5 Study design

2.5.1 Study type

In the first definitive test, the nominal test substance concentrations were 15; 23; 39; 63 and 100 mg NM-105/L sediment overlying water. Additionally, worms were exposed under control conditions. The control and the highest test concentration were prepared with two different dilution media: medium used at ECT for culture and tests with *L. variegatus* and medium used in tests with *Chironomus riparius* at the IME. All other test concentrations were prepared only in dilution medium used at ECT. Four replicates were used for test substance concentration levels, and six replicates for the control. For measurement of physico-chemical parameters and for sampling for chemical analysis, 4 separate test vessels per treamtent and control were prepared and not included in evaluation of biological endpoints at test end.

The second definitive test was a limit test with a control and 100 mg NM-105/L. For control and treatment, 20 replicates were used. Again, 4 additional test vessels per treamtent and control were used for measurement of physico-chemical parameters.

The tests were prepared at ECT (i.e. synchronising worms, preparation of artificial sediment, labelling of test vessels) and performed in the IME laboratory.

2.5.2 Test duration type

The test period (exposure of the test organisms to the static spiked sediment-water system) was 28 days and is a long term study.

2.5.3 Water media type

Reconstituted fresh water was used as overlying water in the culture at ECT and in all tests. The composition and physical-chemical characteristics of the reconstituted water are according to OECD TG No. 203 [10]. The final concentrations of the salts in the reconstituted water were: $CaCl_2 \cdot 2\ H_2O$: 294.0 mg/L; $MgSO_4 \cdot 7\ H_2O$: 123.0 mg/L; $NaHCO_3$: 64.8 mg/L; KCl: 5.75 mg/L.

The required amount of reconstituted water was prepared within one month before use. During storage, the water was aerated. Before use the physical-chemical characteristics of the water were determined as required by the test guideline and checked for fulfilling the validity criteria (Table 2).

Table 2: Validity criteria for acceptance of suitable reconstituted water.

Parameter	Desired value
pH	7.5 – 8.0
Conductivity	550 – 650 µS/cm
Oxygen saturation	> 80%
Hardness	178 – 267 mg/L $CaCO_3$

Additionally, in the first test with *L. variegatus* the dilution medium usually used at the IME was used. It was purified drinking water, the purification including filtration with activated charcoal, passage through a limestone column and aeration. The following water quality data were obtained from regular measurements at the test facility during test performance: pH: 7.6 – 7.9; conductivity: 289 – 329 µS/cm; nitrate: 2.3 – 4.1 mg/L; nitrite: < 0.005 mg/L; ammonium (NH_4^+): < 0.01 mg/L; phosphate: 0.1 – 2.2 mg/L; calcium: 0.8 – 0.9 mmol/L; magnesium: 0.2 – 0.3 mmol/L; total hardness: 1.0 – 1.2 mmol/L; alkalinity: 2.1 – 2.9 mmol/L; DOC (NPOC): 0.8 – 3.8 mg/L; cadmium: < LOQ; chromium: < LOQ; copper: < LOQ – 7.8 µg/L; iron: < LOQ; manganese: < LOQ; zinc: <LOQ – 6.8 µg/L; lead: < LOQ.

2.5.4 Type of sediment

The composition of the artificial sediment was based on the recommendations in the test guideline and is shown in Table 3.

Table 3: Composition of the artificial sediment.

Constituent	Characteristics	% of sediment dry weight
Peat	*Sphagnum* moss peat, air dried, no visible plant remains, finely ground (particle size ≤ 0.5 mm)	5 ± 0.5
Quartz sand	Grain size: < 2 mm; > 50% of the particles should be in the range of 50–200 µm	75 – 76
Kaolinite clay	Kaolinite content ≥ 30%	20 ± 1
Urtica powder	*Folia urticae*; Caelo Caesar & Loretz GmbH, Hilden, in addition to dry sediment, finely ground (particle size ≤ 0.5 mm)	0.25%
Cellulose powder	α-Cellulose, in addition to dry sediment	0.25%
Organic carbon	Adjusted by addition of peat and sand	2 ± 0.5
Calcium carbonate	$CaCO_3$, pulverised, chemically pure, in addition to dry sediment	0.05 – 1[a]
Deionised Water	Conductivity ≤ 10 µS/cm , in addition to dry sediment	30 – 50

[a]: according to OECD TG 225 [5].

The peat was air dried and ground to a fine powder. A suspension of the required amounts of peat and deionised water was prepared using a high-performance homogenising device. The pH of this suspension was adjusted to 6.0 with $CaCO_3$. The suspension was conditioned for two days with gentle stirring at room temperature, to stabilise pH and establish a stable microbial component. The pH was measured again and was adjusted to 6.4. Then the peat suspension was mixed with the other constituents (quartz sand and kaolinite clay) to obtain a homogeneous sediment with a water content in the range of 30 to 50% of dry weight of the sediment. The pH of the sediment was measured directly in the substrate. Samples of the sediment were taken to determine the dry weight and the organic carbon content (TOC).

Prior to test start, the formulated sediment was conditioned for 7 d. For this purpose it was covered with reconstituted water (sediment-water volume ratio: 1:4 ± ≤ 0.5) and was incubated under the same conditions as in the subsequent test. Immediately before use in the test the supernatant was removed and the food (α-Cellulose and urtica powder) was mixed into the sediment.

2.5.5 Total exposure duration

The test period (exposure of the test organisms to the static sediment-water system) was 28 d.

2.5.6 Test conditions

The test was performed under the test conditions given in Table 4.

Table 4: Test conditions for tests with *Lumbriculus variegatus.*

Number of test organisms per test vessel at test start:	10 worms
Biological parameters:	number of worms, dry weight of worms per replicate
Observations:	at least 3 days per week
Test vessels:	glas vessels, 250 mL total volume with plastic lid
Sediment per test vessel	80 g wet weight
Height of sediment in test vessel:	ca. 1.5 cm
Volume of overlying water:	180 mL
Aeration of test vessels:	continuous aeration
Feeding during exposure:	food in sediment
Water change:	static; 3 days per week adjustment for evaporated test medium
Light regime:	16 light : 8 dark

Physico-chemical parameters were measured throughout the tests and are described in detail in Table 5 and Table 6.

Table 5: Physico-chemical parameters measured in the test media in the 1st test with *L. variegatus*.

Parameter	Measured at test begin	Measured during the test	Measured at test end
Water phase (minimum and maximum)			
Temperature [°C]	20.2 – 20.3	20.2 – 20.3	20.2 – 20.3
O_2 [mg/L]	7.84 – 8.11	5.65 – 8.21	7.65 – 8.18
pH	7.2 – 7.5	7.9 – 8.4	8.1 – 8.3
NH_4^+ [mg/L]	0.6 – 0.9	0.7 – 1.0	0.2 – 0.6
Hardness [mmol $CaCO_3$]	270 – 280 [A] 140 [B]	–	330 [A] 200 – 230 [B]
Light intensity [lx]	477 – 494	460 – 494	469 – 486
Sediment			
Total organic carbon [% dry weight]	2.32 ± 0.06 (Mean ± SD)	–	–
pH	6.8	–	–

[A]: measured in test vessels with ECT medium; [B]: measured in test vessels with IME medium.

Table 6: Physico-chemical parameters measured in the test media in the 2nd test with *L. variegatus*.

Parameter	Measured at test begin	Measured during the test	Measured at test end
Water phase (minimum and maximum)			
Temperature [°C]	20.2 – 20.3	20.2 – 20.3	20.2 – 20.3
O_2 [mg/L]	7.90 – 8.11	6.25 – 8.38	6.30 – 7.42
pH	7.5	8.3 – 8.7	8.5 – 8.7
NH_4^+ [mg/L]	0.6 – 0.7	0.7 – 1.5	1.2 – 1.4
Hardness [mmol $CaCO_3$]	270 – 280	–	410 – 450
Light intensity [lx]	527 – 545	523 – 546	544 – 569
Total organic carbon [% dry weight]	2.03 ± 0.09 (Mean ± SD)	–	–
pH	6.9	–	–

2.5.7 Any other method on materials and methods

Statistical evaluation of results

The total number of worms per replicate, and the total dry weight of the worms per replicate were assessed. In order to estimate mortalities, the numbers of worms that did not react to a gentle stimulus or showed signs of decomposition were considered to be dead.

For evaluation of effects of the test substance on total number of worms after 28 days of exposure, Fisher's Exact Binomial Test (multiple comparison, $p \leq 0.05$, 1-sided greater) was used to determine significant differences in the mean number of worms between test concentrations and the control. Treatment means

were compared by ANOVA followed by Dunnett's test (multiple comparison, 1-sided smaller; $p \leq 0.05$) and tested for statistically significant differences compared to the control. For evaluation of effects of NM-105 on the endpoints in the second test, the Student t test (pair-wise comparison, 1-sided smaller; $p \leq 0.05$) was used for comparison with controls. All statistical calculations were done based on the nominal concentrations.

The statistical software package ToxRat Professional 2.10 (ToxRat Solutions GmbH, Naheweg 15, D-52477 Alsdorf) was used for these calculations.

Chemical analysis of titanium concentration in worms

For the determination of titanium concentration in the worms from the second test, all worms were freeze dried, weighed and chemically digested with 5 mL nitric acid (69%) at 250°C. Subsequently, 500 µL fluoric acid (40%) was added to each sample and the samples were treated in an ultrasonic bath for 60 minutes. To eliminate F^- before performing the chemical analysis, boric acid (4%) was added to a final volume of 15 mL.

The chemical analysis of the digested samples was performed with ICP-OES (*inductively coupled plasma – optical emission spectrometry*). Each sample was measured in triplicates. Calibrations were performed before measurements of samples with the following titanium concentrations: 0; 50; 100; 250; 500; 1000 µg/L. A mixture of nitric and boric acid (volume ration 1:2) was measured as a blank.

2.6 Results

2.6.1 First definitive test with *Lumbriculus variegatus*

The particle sizes determined during exposure in the test media were between 402 and 1325 nm, declining over time. Details are in Table 7. Seven days after start of exposure the results from particle size measurement in test media showed no difference to particle sizes in controls, indicating sedimentation of NM-105. This result is supported by visual observation of increasing translucency of the water phase during the first week of exposure. Measurement of particle size was not continued after day 7 of the test.

Table 7: Measured particle sizes in test media from the 1st test with *L. variegatus.*

Concentration [mg NM-105/L sediment overlying medium]	Z-Average [d.nm]	PDI	Peak 1 [nm]	Peak 2 [nm]
Dispersions for application of NM-105 to water phase (begin of exposure)				
Control (ECT medium)	1618	1.0	237	413
15	2017	0.8	957	220
23	2431	0.7	938	1299
39	2478	0.5	1325	5494
63	4493	0.6	1227	792
100 (ECT medium)	4950	0.5	1172	–
100 (IME medium)	7696	0.6	959	80
3 hours after begin of exposure				
Control (ECT medium)	1618	1.0	237	413
15	2175	0.7	668	87
23	1573	0.7	721	334
39	2390	0.9	760	552
63	3340	0.9	1192	458
100 (ECT medium)	3991	0.8	932	–
100 (IME medium)	1991	0.6	1001	421
Day 1 after begin of exposure				
Control (ECT medium)	1561	1.0	289	–
15	771	0.7	402	–
23	1165	0.7	534	276
39	1854	0.9	540	236
63	1287	0.8	528	334
100 (ECT medium)	2964	0.9	540	16
100 (IME medium)	1594	0.7	746	158
Day 7 after begin of exposure				
Control (ECT medium)	1576	0.9	418	–
15	1214	0.8	405	525
23	1664	0.7	1123	202
39	1413	0.9	438	–
63	1252	0.8	463	–
100 (ECT medium)	1530	1.0	409	–
100 (IME medium)	1835	1.0	319	450

The results indicate no influence of the choice of dilution medium in controls on reproduction (Table 8) or dry weight (Table 9) of the worms. The nanomaterial NM-105 does not elicit a significant effect on the evaluated endpoints.

Table 8: No. of worms after 28 d exposure to control media and NM-105 (1st test with *L. variegatus*). Rep.: replicate of the respective treatment or control; SD: standard deviation.

Concentration [mg NM-105/L sediment overlying medium]	Number of worms at test end [n]						
	Rep. A	Rep. B	Rep. C	Rep. D	Rep. E	Rep. F	Mean ± SD
Control (ECT medium)	34	15	40	34	31	31	30.8 ± 8.4
Control (IME medium)	33	42	26	29	27	16	28.8 ± 8.6
15	31	26	27	7	-	-	22.8 ± 10.7
23	28	35	14	32	-	-	27.3 ± 9.3
39	29	21	18	34	-	-	25.5 ± 7.3
63	13	32	31	23	-	-	24.8 ± 8.8
100 (ECT medium)	39	35	24	13	-	-	27.8 ± 11.7
100 (IME medium)	34	30	34	10	-	-	28.5 ± 8.5

Table 9: Worm weight after 28 d exposure to control media and NM-105 (1st test with *L. variegatus*). Rep.: replicate of the respective treatment or control; SD: standard deviation.

Concentration [mg NM-105/L sediment overlying medium]	Total dry weight of worms [mg]						
	Rep. A	Rep. B	Rep. C	Rep. D	Rep. E	Rep. F	Mean ± SD
Control (ECT medium)	28.60	32.50	31.80	23.10	19.90	28.10	27.3 ± 4.9
Control (IME medium)	27.10	20.20	19.40	27.90	20.00	31.80	24.4 ± 5.2
15	22.30	28.60	22.50	18.20	-	-	22.9 ± 4.3
23	28.60	25.60	30.30	24.60	-	-	27.3 ± 2.6
39	26.70	27.20	33.30	22.80	-	-	27.5 ± 4.3
63	9.70	34.50	24.40	28.80	-	-	24.4 ± 10.6
100 (ECT medium)	25.20	22.80	28.10	25.50	-	-	25.4 ± 2.2
100 (IME medium)	32.30	22.20	24.80	31.40	-	-	27.8 ± 5.1

2.6.2 Second definitive test with *Lumbriculus variegatus*

In the second test, titanium concentrations in the test media were measured. Measured mean concentration in the treatment at begin of exposure is slightly above the nominal concentration of 100 mg NM-105/L sediment overlying medium and declines fast there after (Table 10). During the course of the test, titanium concentrations in the water phase of the treatment are increasing and reach approximately 1.5% of the initial titanium concentration at the end of the test.

As in the first test, the results of this test show no effect of NM-105 on the reproduction and the biomass of the worms (Table 11 and Table 12).

Table 10: Measured Ti concentrations in the test media (2nd test with L.variegatus).
The corresponding titanium concentration to 100 mg NM-105/L sediment overlying medium is 59 934.89 µg/L.

Sampling time after begin of exposure	Sample	Measured titanium concentration [µg/L]			Recovery, based on nominal concentrations [%]
		Measured value	Mean	SD	Mean ± SD
0 d	Control	1.606	1.61	–	–
	100 mg/L TiO_2	67430	66170	1781.9	110.4 ± 2.97
	100 mg/L TiO_2	64910			
1 d	Control	0.9948	0.995	–	–
	100 mg/L TiO_2	46.06	44.94	1.59	0.074 ± 0.0026
	100 mg/L TiO_2	43.81			
7 d	Control	91.2	85.49	8.08	–
	Control	79.77			
	100 mg/L TiO_2	595.2	599	5.37	0.999 ± 0.0090
	100 mg/L TiO_2	602.8			
28 d (Test end)	Control	134.5	135.55	1.48	–
	Control	136.6			
	100 mg/L TiO_2	945.3	930.6	20.78	1.55 ± 0.034
	100 mg/L TiO_2	915.9			

Table 11: No of worms after 28 d exposure to control medium and NM-105 (2nd test with *L. variegatus*).
SD: standard deviation.

Concentration [mg NM-105/ L sediment overlying medium]	Number of worms at test end [n]																				Mean ± SD
	Replicate																				
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	
Control	25	18	10	32	14	18	10	21	29	16	28	24	10	13	22	30	24	16	23	14	19.9 ± 7.9
100	13	27	30	23	25	30	27	19	21	9	25	24	18	15	0	25	19	10	0	29	21.6 ± 6.6

In this test, the biomass was determined as fresh weight, since the worms were sampled from the test vessels, pooled and left in dilution medium for gut purging over night. Subsequently the worms were killed with ethanol, weighed and transferred to the analytical laboratory, where they were freeze dried and stored at-20°C until chemical analysis.

Table 12: Worm weight after 28 d exposure to control medium and NM-105 (2nd test with *L. variegatus*).

Concentration [mg NM-105/L sediment overlying medium]	Total fresh weight of worms [mg]
Control	1472.1
100	1283.0

The results of the chemical analysis of the titanium concentrations in the worms as described in 2.5.7 are in Table 13. Comparing the titanium concentrations in worms exposed to NM-105 with worms exposed to control, no significant difference is detected.

Table 13: Ti concentrations measured in *L. variegatus* after 28 d exposure to control medium and NM-105.

Sample	Weight of freeze dried worms before chemical digestion [mg]	Dilution of sample by factor	Measured titanium concentration [µg/L]	Titanium concentration in worms [µg/g]	
				Value	Mean ± SD
Control	141.5	–	952	101	100 ± 1
Control	132.7	–	874	98.8	
100 mg/L	141.6	10	114	121	112 ± 12
100 mg/L	119.2	10	82	103	

2.7 Validity

In the two tests with *Lumbriculus variegatus* and the nanomaterial NM-105, all validity criteria were met (Table 14).

Table 14: Validity criteria according to OECD TG 225 [5] and values determined in the tests with *L. variegatus.*

Parameter	Recommended in guideline	Value determined in first test	Value determined in second test
Reproduction factor in controls	≥ 1.8	≥ 3.1 [A] ≥ 2.8 [B]	≥ 2.0
pH during test	6 – 9	7.2 – 8.4	7.5 – 8.7
Oxygen saturation	≥ 30%	> 62%	> 69%

[A]: Measured in test vessels with ECT medium; [B]: measured in test vessels with IME medium.

2.8 Additional experiments

No additional experiments were performed.

2.9 Conclusion

A NOEC ≥ 100 mg/L NM-105 was determined in the test.

2.10 Executive summary

The nanoparticulate titanium dioxide NM-105 was investigated in two tests with *Lumbriculus variegatus* in a sediment-water system [5]. The nominal test concentrations in the first test were 15; 23; 39; 63 and 100 mg NM-105/L sediment overlying water and 100 mg NM-105/L sediment overlying water in the second test. Chemical analysis of titanium concentrations in test media in the first test showed good agreement with nominal test concentrations.

In the investigated concentration range, NM-105 elicited no adverse effects in the worms in either test. A NOEC ≥ 100 mg NM-105/L sediment overlying water was determined.

2.11 Raw data

Table 15: Measured physical-chemical data during the 1st test with *Lumbriculus variegatus*.

Date	Day of exposure	Code / Test concentration [mg/L]	Temperature [°C]	Oxygen [mg/L]	pH [-]	Light intensity [lx]	Hardness [mmol CaCO3]	NH_4^+ [mg/L]
16. Nov 10	0	Control (ECT medium)	-	7.34	6.94	477	280	0.7
16. Nov 10	0	Control (IME medium)	-	7.29	7.21	-	140	0.6
16. Nov 10	0	15	-	6.64	7.36	-	-	0.8
16. Nov 10	0	23	-	5.68	7.46	-	-	0.8
16. Nov 10	0	39	-	7.42	7.46	-	-	0.9
16. Nov 10	0	63	-	6.38	7.47	-	-	0.7
16. Nov 10	0	100 (ECT medium)	-	6.73	7.53	-	270	0.9
16. Nov 10	0	100 (IME medium)	-	5.65	7.53	-	140	0.9
16. Nov 10	0	Water bath	20.2	-	-	494	-	-
19. Nov 10	3	Control (ECT medium)	-	-	-	-	-	0.8
19. Nov 10	3	Control (IME medium)	-	-	-	-	-	0.9
19. Nov 10	3	15	-	-	-	-	-	0.8
19. Nov 10	3	23	-	-	-	-	-	0.9
19. Nov 10	3	39	-	-	-	-	-	0.8
19. Nov 10	3	63	-	-	-	-	-	0.9
19. Nov 10	3	100 (ECT medium)	-	-	-	-	-	0.7
19. Nov 10	3	100 (IME medium)	-	-	-	-	-	1.0
19. Nov 10	3	Water bath	-	-	-	-	-	-
22. Nov 10	6	Control (ECT medium)	-	-	-	-	-	0.9
22. Nov 10	6	Control (IME medium)	-	-	-	-	-	0.9
22. Nov 10	6	15	-	-	-	-	-	1.0
22. Nov 10	6	23	-	-	-	-	-	0.9
22. Nov 10	6	39	-	-	-	-	-	0.8
22. Nov 10	6	63	-	-	-	-	-	0.8
22. Nov 10	6	100 (ECT medium)	-	-	-	-	-	0.9
22. Nov 10	6	100 (IME medium)	-	-	-	-	-	0.7
22. Nov 10	6	Water bath	-	-	-	-	-	-
23. Nov 10	7	Control (ECT medium)	-	7.52	8.12	479	-	0.8
23. Nov 10	7	Control (IME medium)	-	7.68	8.36	-	-	0.9
23. Nov 10	7	15	-	7.57	8.17	-	-	0.7
23. Nov 10	7	23	-	7.67	8.17	-	-	1.0
23. Nov 10	7	39	-	7.14	8.18	-	-	0.9

Date	Day of exposure	Code / Test concentration [mg/L]	Temperature [°C]	Oxygen [mg/L]	pH [-]	Light intensity [lx]	Hardness [mmol CaCO3]	NH_4^+ [mg/L]
23. Nov 10	7	63	-	7.95	8.23	-	-	1.0
23. Nov 10	7	100 (ECT medium)	-	7.65	8.22	-	-	1.0
23. Nov 10	7	100 (IME medium)	-	7.57	8.41	-	-	1.0
23. Nov 10	7	Water bath	20.3	-	-	494	-	-
24. Nov 10	8	Control (ECT medium)	-	-	-	-	-	0.9
24. Nov 10	8	Control (IME medium)	-	-	-	-	-	0.8
24. Nov 10	8	15	-	-	-	-	-	0.8
24. Nov 10	8	23	-	-	-	-	-	0.9
24. Nov 10	8	39	-	-	-	-	-	0.7
24. Nov 10	8	63	-	-	-	-	-	1.0
24. Nov 10	8	100 (ECT medium)	-	-	-	-	-	0.9
24. Nov 10	8	100 (IME medium)	-	-	-	-	-	0.9
24. Nov 10	8	Water bath	-	-	-	-	-	-
29. Nov 10	13	Control (ECT medium)	-	-	-	-	-	0.8
29. Nov 10	13	Control (IME medium)	-	-	-	-	-	0.7
29. Nov 10	13	15	-	-	-	-	-	0.8
29. Nov 10	13	23	-	-	-	-	-	0.7
29. Nov 10	13	39	-	-	-	-	-	0.7
29. Nov 10	13	63	-	-	-	-	-	0.7
29. Nov 10	13	100 (ECT medium)	-	-	-	-	-	0.8
29. Nov 10	13	100 (IME medium)	-	-	-	-	-	0.8
29. Nov 10	13	Water bath	-	-	-	-	-	-
30. Nov 10	14	Control (ECT medium)	-	6.89	7.85	465	-	0.7
30. Nov 10	14	Control (IME medium)	-	7.89	8.13	-	-	0.7
30. Nov 10	14	15	-	7.26	7.87	-	-	0.8
30. Nov 10	14	23	-	7.52	7.91	-	-	0.8
30. Nov 10	14	39	-	7.56	7.92	-	-	0.9
30. Nov 10	14	63	-	8.1	8.05	-	-	0.7
30. Nov 10	14	100 (ECT medium)	-	6.87	7.8	-	-	0.9
30. Nov 10	14	100 (IME medium)	-	7.6	8.1	-	-	0.8
30. Nov 10	14	Water bath	20.2	-	-	483	-	-
02. Dec 10	16	Control (ECT medium)	-	-	-	-	-	0.8
02. Dec 10	16	Control (IME medium)	-	-	-	-	-	0.7
02. Dec 10	16	15	-	-	-	-	-	0.8
02. Dec 10	16	23	-	-	-	-	-	0.8

Date	Day of exposure	Code / Test concentration [mg/L]	Temperature [°C]	Oxygen [mg/L]	pH [-]	Light intensity [lx]	Hardness [mmol CaCO3]	NH_4^+ [mg/L]
02. Dec 10	16	39	-	-	-	-	-	0.8
02. Dec 10	16	63	-	-	-	-	-	0.9
02. Dec 10	16	100 (ECT medium)	-	-	-	-	-	0.8
02. Dec 10	16	100 (IME medium)	-	-	-	-	-	0.8
02. Dec 10	16	Water bath	-	-	-	-	-	-
06. Dec 10	20	Control (ECT medium)	-	-	-	-	-	0.7
06. Dec 10	20	Control (IME medium)	-	-	-	-	-	0.7
06. Dec 10	20	15	-	-	-	-	-	0.8
06. Dec 10	20	23	-	-	-	-	-	0.8
06. Dec 10	20	39	-	-	-	-	-	0.8
06. Dec 10	20	63	-	-	-	-	-	0.7
06. Dec 10	20	100 (ECT medium)	-	-	-	-	-	0.8
06. Dec 10	20	100 (IME medium)	-	-	-	-	-	0.8
06. Dec 10	20	Water bath	-	-	-	-	-	-
07. Dec 10	21	Control (ECT medium)	-	7.3	7.9	460	-	0.8
07. Dec 10	21	Control (IME medium)	-	7.83	8.26	-	-	0.8
07. Dec 10	21	15	-	7.75	8.15	-	-	0.9
07. Dec 10	21	23	-	7.85	8.15	-	-	0.8
07. Dec 10	21	39	-	7.56	8.12	-	-	0.9
07. Dec 10	21	63	-	7.84	8.12	-	-	0.8
07. Dec 10	21	100 (ECT medium)	-	7.63	8.12	-	-	0.7
07. Dec 10	21	100 (IME medium)	-	7.98	8.26	-	-	0.8
07. Dec 10	21	Water bath	20.3	-	-	491	-	-
10. Dec 10	24	Control (ECT medium)	-	-	-	-	-	0.7
10. Dec 10	24	Control (IME medium)	-	-	-	-	-	0.8
10. Dec 10	24	15	-	-	-	-	-	7.0
10. Dec 10	24	23	-	-	-	-	-	0.7
10. Dec 10	24	39	-	-	-	-	-	0.7
10. Dec 10	24	63	-	-	-	-	-	0.8
10. Dec 10	24	100 (ECT medium)	-	-	-	-	-	0.9
10. Dec 10	24	100 (IME medium)	-	-	-	-	-	0.8
10. Dec 10	24	Water bath	-	-	-	-	-	-
13. Dec 10	27	Control (ECT medium)	-	-	-	-	-	0.8
13. Dec 10	27	Control (IME medium)	-	-	-	-	-	0.7
13. Dec 10	27	15	-	-	-	-	-	0.7

Date	Day of exposure	Code / Test concentration [mg/L]	Temperature [°C]	Oxygen [mg/L]	pH [-]	Light intensity [lx]	Hardness [mmol CaCO3]	NH_4^+ [mg/L]
13. Dec 10	27	23	-	-	-	-	-	0.7
13. Dec 10	27	39	-	-	-	-	-	0.8
13. Dec 10	27	63	-	-	-	-	-	0.7
13. Dec 10	27	100 (ECT medium)	-	-	-	-	-	0.7
13. Dec 10	27	100 (IME medium)	-	-	-	-	-	0.8
13. Dec 10	27	Water bath	-	-	-	-	-	-
14. Dec 10	28	Control (ECT medium)	-	7.92	8.16	469	330	0.6
14. Dec 10	28	Control (IME medium)	-	7.69	8.25	-	230	0.6
14. Dec 10	28	15	-	8.21	8.25	-	-	0.5
14. Dec 10	28	23	-	7.96	8.25	-	-	0.6
14. Dec 10	28	39	-	7.8	8.22	-	-	0.5
14. Dec 10	28	63	-	8.13	8.23	-	-	0.4
14. Dec 10	28	100 (ECT medium)	-	7.82	8.13	-	330	0.6
14. Dec 10	28	100 (IME medium)	-	8.04	8.33	-	200	0.2
14. Dec 10	28	Water bath	20.3	-	-	486	-	-

Table 16: Total organic carbon and pH measured in sediment used for the 1st test with *L. variegatus.*

Sample code	Total organic carbon [% dry weight]	pH
Sample 1	2.41	6.8
Sample 2	2.38	–
Sample 3	2.29	–
Sample 4	2.31	–
Mean ± SD	2.32 ± 0.06	–

Table 17: Measured physical-chemical data during the 2nd test with *L. variegatus*.

Date	Day of exposure	Code / Test concentration [mg/L]	Temperature [°C]	Oxygen [mg/L]	pH [-]	Light intensity [lx]	Hardness [mmol CaCO3]	NH_4^+ [mg/L]
24. Feb 11	0	Control	20.3	7.52	7.54	527	280	0.6
24. Feb 11	0	100	-	6.62	7.54	545	270	0.7
26. Feb 11	2	Control	-	-	-	-	-	0.8
26. Feb 11	2	100	-	-	-	-	-	0.9
01. March 11	5	Control	-	-	-	-	-	0.8
01. Mrz 11	5	100	-					0.9
03. March 11	7	Control	20.2	8.23	8.37	523	-	1
03. March 11	7	100	-	6.25	8.22	537	-	1.1
04. March 11	8	Control	-	-	-	-	-	1.1
04. March 11	8	100	-	-	-	-	-	1.1
08. March 11	12	Control	-	-	-	-	-	1.3
08. March 11	12	100	-	-	-	-	-	1.3
10. March 11	14	Control	20.3	7.7	8.46	529	-	1.2
10. March 11	14	100	-	7.02	8.32	546	-	1.3
11. March 11	15	Control	-	-	-	-	-	1.4
11. March 11	15	100	-	-	-	-	-	1.3
15. March 11	19	Control	-	-	-	-	-	1.3
15. March 11	19	100	-					1.5
17. March 11	21	Control	20.3	8.35	8.7	544	-	1.5
17. March 11	21	100	-	8.38	8.7	569	-	1.5
18. March 11	22	Control	-	-	-	-	-	1.5
18. March 11	22	100	-	-	-	-	-	1.4
22. March 11	26	Control	-	-	-	-	-	1.3
22. March 11	26	100	-					1.3
24. March 11	28	Control	20.3	6.3	8.5	544	450	1.2
24. March 11	28	100	-	7.4	8.67	569	410	1.4

Table 18: Total organic carbon and pH measured in sediment used for the 2nd test with *L. variegatus.*

Sample code	Total organic carbon [% dry weight]	pH
Sample 1	1.92	6.9
Sample 2	2.11	–
Sample 3	2.09	–
Sample 4	1.98	–
Mean ± SD	2.03 ± 0.09	–

Table 19: Validation of chemical analysis of Ti measurement in worm samples (2nd test with *L. variegatus*).

Sample code	Titanium [µg/L]	Ti3349 [cps]	Ti3361 [cps]	Ti3372 [cps]
Blank	0	-1.997	0.3912	0.8236
KalibStd-1	50	15.14	11.49	6.143
KalibStd-2	100	34.27	23.66	12.72
KalibStd-3	250	90.01	60.58	32.86
KalibStd-4	500	185.5	123.1	68.03
KalibStd-5	1000	352.2	231.9	125.3

Sample code	Date	Ti3349 [µg/L]	Ti3361 [µg/L]	Ti3372 [µg/L]
Blank HNO3/H3BO3 1:2	27 Jun 2011 14:15:21	-5.51	-4.468	-7.65
Blank HNO3/H3BO3 1:2	27 Jun 2011 14:17:33	-5.978	-4.41	-9.175
11KBW178	27 Jun 2011 14:19:46	-6.467	-3.574	-10.68
Ti 500 µg/L	27 Jun 2011 14:21:59	497	498.5	501.6
CPI 500 µg/L	27 Jun 2011 14:24:12	479.8	480.5	484
Blank HNO3/H3BO3 1:2	27 Jun 2011 14:26:26	-2.933	-5.81	-2.508
11KSA0402 a	27 Jun 2011 14:28:40	960	958.6	952.3
11KSA0402 b	27 Jun 2011 14:30:54	864.7	868.3	874.4
Blank HNO3/H3BO3 1:2	27 Jun 2011 14:33:08	-5.637	-4.614	-8.862
11KSA0403 a 1:10	27 Jun 2011 14:35:24	119.2	118.4	113.8
11KSA0403 b 1:10	27 Jun 2011 14:37:39	86.53	86.61	82.03
Blank HNO3/H3BO3 1:2	27 Jun 2011 14:39:54	-5.393	-5.169	-8.021
11KSA0403 a	27 Jun 2011 14:42:09	1324	1326	1307
11KSA0403 b	27 Jun 2011 14:44:21	1017	1020	1004
Blank HNO3/H3BO3 1:2	27 Jun 2011 14:46:33	-3.912	-4.969	-4.578
Blank HNO3/H3BO3 1:2	27 Jun 2011 14:48:47	-4.009	-5.713	-4.933
1:10Wurm110603+TiO_2c	27 Jun 2011 14:51:01	742.5	745.2	760.3
1:10Wurm110603+TiO_2d	27 Jun 2011 14:53:14	632.9	635.1	644.1
Blank HNO3/H3BO3 1:2	27 Jun 2011 14:55:28	-4.693	-4.745	-7.415
CPI 500 µg/L	27 Jun 2011 14:57:43	545.3	551.3	553.8
Blank HNO3/H3BO3 1:2	27 Jun 2011 14:59:58	-0.699	-9.327	8.627

Table 20: Validation of chemical analysis of Ti measurement in water samples (2nd test with *L. variegatus*).

Sample code	Titan [µg/L]	Ti3349 [cps]	Ti3361 [cps]	Ti3372 [cps]
Blank	0	-0.214	-1.345	1.164
KalibStd-1	50	13.58	7.671	6.096
KalibStd-2	100	28.46	17.27	11.19
KalibStd-3	250	72.77	45.76	26.6
KalibStd-4	500	143	92.14	51.42
KalibStd-5	1000	286.7	184.9	102.9

Sample code	Date	Ti3349 [µg/L]	Ti3361 [µg/L]	Ti3372 [µg/L]
Blank HFMix Borsäure	25 Feb 2011 13:32:23	-1.223	-1.233	1.431
Rec Ti 500 µg/L	25 Feb 2011 13:34:20	473.9	482.1	474
CPI 500 µg/L	25 Feb 2011 13:36:18	488.7	490.6	485.4
Blank HFMix Borsäure	25 Feb 2011 13:38:16	-1.223	-1.195	1.537
Kontrolle ECT d0	25 Feb 2011 13:40:15	-1.33	1.006	1.606
100mg/L ECT d0 1:200	25 Feb 2011 13:44:13	671.7	678.9	674.3
100mg/L ECT d0 1:200	25 Feb 2011 13:46:12	647.5	655.3	649.1
100 mg/L ECT d1	25 Feb 2011 13:48:12	44.12	46.51	46.06
100 mg/L ECT d1	25 Feb 2011 13:50:12	41.3	44.58	43.81
Blank HNO3 10 %	25 Feb 2011 13:52:13	0.2737	-3.794	6.707
Kontrolle ECT d1	25 Feb 2011 13:58:19	-1.396	1.191	0.9948

3 Reproduction test with *Hypoaspis aculeifer* and NM-105

3.1 Test principle

The purpose of the tests was to determine a NOEC/LOEC for the effects of the test substance on the reproduction of the mite *Hypoaspis aculeifer* by dermal, alimentary and respiratory uptake using a standardised artificial soil.

For this purpose, the test substance NM-105 was mixed with the substrate and filled into the test vessels. Into each test vessel, 10 adult mated female mites were put. The mites were taken from a synchronised culture between the 28th and 35th d after starting the respective culture and were thus of similar age (approx. 7 – 14 d after reaching the adult stage). At the beginning of the test and two times per weeks during the test, the mites were fed with prey mites (*Tyrophagus putrescentiae*). After 14 d of exposure, the soil including the mites of each test vessel were poured into extraction funnels and the mites were heat extracted by a modified infrared extractor according to Kempson et al. [11]. In the Kempson extractor, the vessels were placed between two horizontally separated compartments. The upper compartment was heated with infra-red light bulbs to the following temperatures for the respective time periods: 25°C/ 24 h; 35°C/20 h; 45°C/4 h. The lower compartment was cooled via a flow-through cooling unit to temperatures of 10-15°C. The resulting temperature gradient between upper and lower compartment of at least 10°C elicited a migration of the mites through the substrate towards the lower compartment, where the test organisms were collected in a vessel with plaster of paris. After end of extraction, the collected adult and juvenile mites were fixed in ethanol and counted per collection vessel separately.

Endpoints based on mortality and reproduction were assessed in comparison with the control. Parameters were the total number of surviving adult females and the total number of juvenile mites.

For each of the three tests with *H. aculeifer*, artificial soil was prepared at ECT and transferred to IME. Upon arrival, the artificial soil was treated with the test substance and transferred back to ECT, where it was stored over night at 20°C. The following day, the treated artificial soil was distributed to the test vessels and the test was started by introducing the test organisms.

To verify the nominally applied concentrations, samples were taken from the dispersions used for the application of the test substance to the artificial soil.

3.2 Materials and methods

3.2.1 Test guideline

The test was performed according to:

OECD. 2008. OECD guideline for testing of chemicals. 226. Predatory mite (*Hypoaspis* (Geolaelaps) *aculeifer*) reproduction test in soil. Organisation for Economic Co-operation and Development, Paris, France.

3.2.2 GLP

The test was not performed under GLP, but followed the principles. The use of any laboratory equipment was controlled and protocolled according to GLP. The quality assurance did not check any phase of the study, the raw data or the study report.

3.2.3 Test substance

The test substance used in this study was nanoparticulate titanium dioxide NM-105.

3.3 Analytical monitoring

Determination of titanium concentration in test substrates was not performed, since titanium concentrations in the untreated artificial soil were high and would impair accurate results in the investigated concentration levels.

Sizes of NM-105 particles in the dispersions used for application on the artificial soil with 1 and 10 mg NM-105/kg artificial soil (dw) were determined. Measurement was performed using dynamic light scattering (DLS) in a Malvern Zeta-Sizer.

3.3.1 Details on application

For each investigated test concentration, a vessel with 200 g artificial soil (dw) was used. The application of the test substance was performed with two different methods. The test concentrations 10, 100 and 1000 mg NM-105/kg artificial soil (dw) were prepared by application of NM-105 as powder, since the amount of powder was enough for homogenous distribution. The test concentrations 1 and 10 mg NM-105/kg artificial soil (dw) were prepared by application of NM-105 in dispersions. This way, a homogenous distribution of NM-105 was realized for otherwise very small amounts of NM-105 powder.

The test concentration of 10 mg NM-105/kg artificial soil (dw) was prepared with both methods of application, allowing a limited evaluation of the effect of the chosen method. The artificial soils used for each application method were prepared in order to reach 55% of the maximum water holding capacity after application of the powder or the dispersions, respectively. Finally, each test vessel was filled with an amount of test substrate corresponding to 20 g dw.

The dispersions used for application of the test substance with 1 and 10 mg/kg were prepared by weighing the desired amount of powder NM-105 into a glass vessel and adding the respective volume of deionized water to gain the desired concentration. Each dispersion was stirred for 60 seconds on a magnetic stirrer (900 rpm) and subsequently treated in an ultra-sonic water bath (Bandelin Sonorex RK 514 BH; 35 kHz; 215/860 W) for three minutes.

3.4 Test organisms

The culture of the gamasid mite *Hypoaspis* (Geolaelaps) *aculeifer* CANESTRINI (Acari: Laelapidae) used in this test is established at ECT since February 2002. The mites derive a culture of MITOX Laboratories (P.O. Box 92260, N-1090 AG Amsterdam, The Netherlands).

Synchronized adult mated female mites were used to start the test. For synchronized breeding, adult *H. aculeifer* were transferred to synchronisation units (180 females and 20 males per unit) 34 days before starting the test. Food (prey mites, *Tyrophagus putrescentiae*) was added. Two days later the mites were removed and only eggs were left in the synchronisation units. After three days the majority of the mites hatched, all remaining eggs were removed and food was added. 21 d after starting the respective culture, the mites reached the adult stage. Between 7 and 14 d later the mites were used for the tests.

3.5 Study design

3.5.1 Study type

A range finding test and two definitive tests were performed. In the range finding test and the first definitive test, the nominal test substance concentrations were 1; 10; 100 and 1000 mg NM-105/kg artificial soil (dw). Four replicates were used for each test substance concentration. The second definitive test was performed with 1 and 1000 mg NM-105/kg artificial soil (dw). Eight replicates were used for test substance concentrations. Additionally to the test substance treatments, the mites were exposed to untreated artificial soi as control treatment. For the controls eight replicates were investigated in each test.

The tests were prepared and performed at ECT. The application of the test substance NM-105 to the artificial soil was conducted in the IME laboratory.

3.5.2 Test duration

The test period (exposure of the test organisms to the treated artificial soil) was 14 days and is a long term study.

3.5.3 Type of substrate

The composition of the artificial soil was based on the test guideline and is shown in Table 21.

Table 21: Composition of the artificial substrate for the tests with *H. aculeifer*.

Constituent	Characteristics	% of substrate dry weight
Peat	*Sphagnum* moss peat, air dried, no visible plant remains, finely ground (particle size ≤0.5 mm)	5 ± 0.5
Quartz sand	Grain size: <2 mm; >50% of the particles should be in the range of 50–200 µm	74 – 75
Kaolin clay	Kaolinite content ≥30%	20 ± 1
Calcium carbonate	$CaCO_3$, pulverized, chemically pure, in addition to dry sediment	0.05 – 1

The peat was air dried and ground to a powder. All parts of the artificial soil were mixed until a homogeneous mixture was achieved. The pH value was adjusted to 6 ± 0.5 using calcium carbonate.

3.5.4 Total exposure duration

The test period (exposure of the test organisms to the spiked artificial soil) was 14 days.

3.5.5 Test conditions

The test was performed under conditions described in Table 22.

Table 22: Test conditions for tests with *H. aculeifer.*

Number of test organisms per test vessel at test start:	10 female, mated mites
Biological parameters assessed at test end:	number of adult female mites, number of juvenile mites
Test vessels:	glass vessels, 200 mL total volume, 5 cm diameter, covered tightly with perforated parafilm
Soil per test vessel	20 g dry weight
Feeding during exposure:	3, 7 and 10 days after test start with *Tyrophagus putrescentiae*
Light regime:	16 light : 8 dark; light intensity 400 – 800 lx

The water content of the control and test substrate was checked weekly by weighing the test vessels and comparing the weight with the initial weight. Losses of water > 2% were compensated by adding deionised water. Physico-chemical parameters were measured throughout the tests and are described in detail in Table 23.

Table 23: Physico-chemical parameters of the artificial soil in the tests with *H. aculeifer*.

Parameter	Recommended in test guideline	Measured values (min, max)		
		Range finding test	First definitive test	Second definitive test
pH	6.0 ± 0.5	6.4 – 6.5 (Test start) 6.6 – 6.7 (Test end)	6.2 – 6.5 (Test start) 6.2 – 6.3 (Test end)	5.5 – 5.6 (Test start) 5.5 – 5.6 (Test end)
Soil moisture [% WHCmax]	40 – 60	52.3 – 56.9 (Test start) 50.4 – 59.6 (Test end)	53.4 – 54.5 (Test start) 49.1 – 54.8 (Test end)	52.6 – 57.0 (Test start) 48.3 – 49.7 (Test end)
Temperature	20 ± 2°C	20.2 – 21.3°C	20.4 – 20.9 °C	20.2 – 20.9°C

3.5.6 Test concentrations

In the range finding test and the first definitive test, the nominal test substance concentrations were 1; 10; 100 and 1000 mg NM-105/kg artificial soil (dw). The second definitive test was performed with 1 and 1000 mg NM-105/kg artificial soil (dw).

3.5.7 Any other method on materials and methods

Statistical evaluation of results

Mortality of adult mites was assessed by evaluating mean number of dead or missing adult mites as absolute number and as percentage of the initial number at the start of the test for each concentration and for the control. Reproduction is represented by the mean number of juvenile mites at test end for each concentration and the control. Additional observations include any pathological or other symptoms or distinct changes in behaviour of the test organism during the course of the study.

For evaluation of effects of the test substance on mortality, Fisher's Exact Binomial Test (multiple comparison, $p \leq 0.05$, 1 -sided greater) was used to determine significant differences in the mean mortality of adult female mites after 14 days between test concentrations and the control. Effects on reproduction were assessed by checking data for normal distribution by R/s test procedure and for homogeneity by Cochran's test. Treatment means were compared by ANOVA followed by Dunnett's test (multiple comparison, 1-sided smaller; $p \leq 0.05$) and tested for statistically s ignificant differences compared to the control. For evaluation of effects of NM-105 on the endpoints in the second test, the Student t test (pair-wise comparison, 1-sided smaller; $p \leq 0.05$) was used for comparison with controls. All statistical calculations were done based on the nominal concentrations.

The statistical software package ToxRat® Professional 2.10 was used for these calculations.

3.6 Results

One range finding test and two definitive tests with *H. aculeifer* were conducted. Investigated test concentration and application methods varied between the tests and are shown in Table 24. As mentioned before (3.3.1), lower test concentrations were prepared by application of a dispersion while higher test concentrations were prepared by adding the powder NM-105 to the substrate. For the intermediate test concentration 10 mg/kg artificial soil (dw), both methods were applied and investigated. Usually, the application volumes of the dispersions were 20 mL. In the first definitive test, a third test substrate with 10 mg/kg artificial soil (dw) NM-105 was prepared with an application volume of 50 mL to provide easier mixing of NM-105 into the test substrate.

Table 24: Overview of tests conducted with *H. aculeifer* and NM-105.
Different methods of application of NM-105 to the respective test substrates are indicated (see 3.3.1 for details).

Test concentrations [mg NM-105/kg artificial soil (dw)]	Number of replicates	Method of application
Range finding test		
Control	8	–
1, 10	4	Dispersion (20 mL)
10, 100, 1000	4	Powder
First definitive test		
Control	8	–
1, 10	4	Dispersion (20 mL)
10	4	Dispersion (50 mL)
10, 100, 1000	4	Powder
Second definitive test		
Control	8	–
1	8	Dispersion
1000	8	Powder

3.6.1 Range finding test with *Hypoaspis aculeifer*

The range finding test was performed as a definitive test; results are shown in Table 25 and Table 26. After 14 days of exposure, a statistically significant (Dunnett's test, 1-sided smaller; $p \leq 0.05$) effect of 1000 mg NM-105/kg artificial soil (dw) on the number of juveniles was detected. Additionally, statistically significant effects on both assessed endpoints (survival of adult mites and number of juvenile mites) were detected at 1 mg NM-105/kg artificial soil (dw), but not in the test substrates with 10 and 100 mg NM-105/kg artificial soil (dw) (Figure 1).

Table 25: No of adult mites after 14 d of exposure to control substrate and NM-105 treated substrate in the range finding test with *H. aculeifer*.
Rep.: replicate of the respective treatment or control; SD: standard deviation. Susp.: Application of NM-105 via 20 mL dispersion; Powder: Application of NM-105 via powder.

Concentration [mg NM-105/kg artificial soil (dw)]	Number of adult mites at test end [n]								
	Rep. A	Rep. B	Rep. C	Rep. D	Rep. E	Rep. F	Rep. G	Rep. H	Mean ± SD
Control	9	9	10	10	9	10	8	10	9.4 ± 0.7
1	7	2	5	4	-	-	-	-	4.5 ± 2.1
10 - Susp.	7	7	9	10	-	-	-	-	8.3 ± 1.5
10 – Powder	10	9	9	10	-	-	-	-	9.5 ± 0.6
100	10	7	8	8	-	-	-	-	8.3 ± 1.3
1000	5	9	2	10	-	-	-	-	6.5 ± 3.7

Table 26: No of juvenile mites after 14 d of exposure to control substrate and NM-105 treated substrate in the range finding test with *H. aculeifer*.
Rep.: replicate of the respective treatment or control; SD: standard deviation; VarC: coefficient of variation. Susp.: Application of NM-105 via 20 mL dispersion; Powder: Application of NM-105 with powder.

Concentration [mg NM-105/kg artificial soil (dw)]	Number of juvenile mites at test end [n]									VarC [%]	Juveniles [% control]
	Rep. A	Rep. B	Rep. C	Rep. D	Rep. E	Rep. F	Rep. G	Rep. H	Mean ± SD		
Control	197	285	234	225	236	234	254	245	238.8 ± 25.1	10.5	–
1	178	45	63	143	-	-	-	-	107.3 ± 63.6	59.3	44.9
10 – Susp.	210	252	214	228	-	-	-	-	226.0 ± 19.0	8.4	94.7
10 - Powder	331	259	266	239	-	-	-	-	273.8 ± 39.8	14.6	114.7
100	202	259	237	172	-	-	-	-	217.5 ± 38.4	17.6	91.1
1000	190	209	15	48	-	-	-	-	115.5 ± 98.2	25.5	48.4

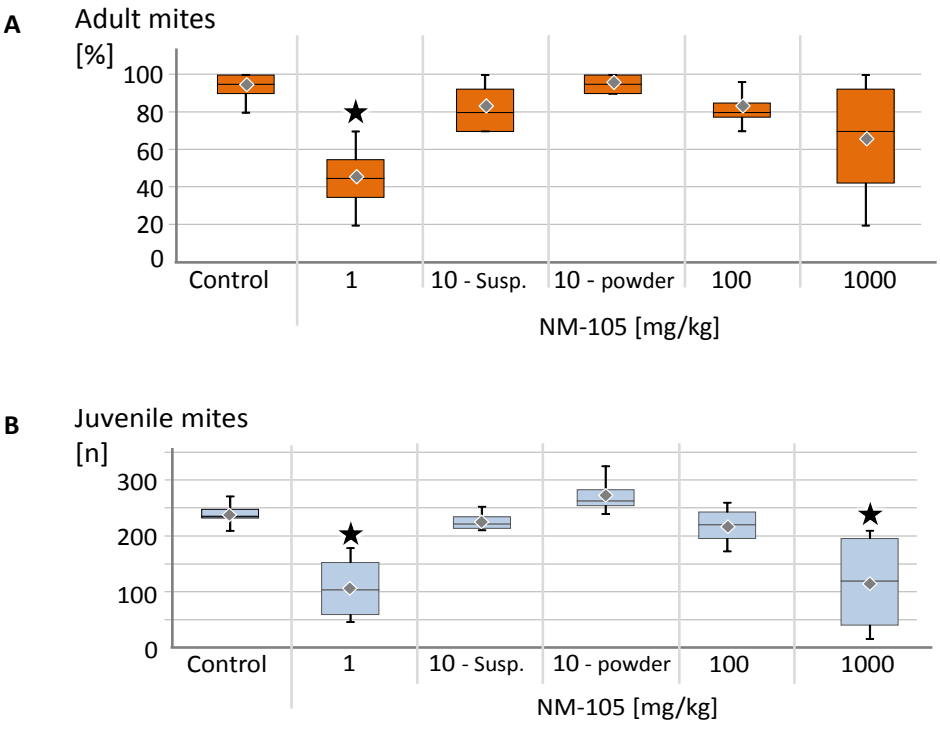

Figure 1: Results of the range finding test with *H. aculeifer*.
A:Survival of adult mites at test end in percentage to the number of mites at test start. **B**: Number of juveniles at test end. Boxes represent 2nd and 3rd quartiles of data, whiskers indicate minimum and maximum values. ♦: Mean; n=8 (controls); n=4 (treatments). ★: Statistically significant difference to control (Dunnett's test, 1-sided smaller; $p \leq 0.05$). Susp.: Application of NM-105 via 20 mL dispersion; powder: Application of NM-105 with powder.

3.6.2 First definitive test with *Hypoaspis aculeifer*

In the first definitive test, results from the range-finding test should be confirmed. Thus, the same concentration range was investigated.

The particle sizes determined in the dispersions used for preparation of test substrates were between 405 and 516 nm (Table 27). Because of the low volumes used for application, concentrations of NM-105 in dispersions were very high and resulted in high poly diversity in the measured samples, as indicated by poor PDI values.

Table 27: Measured particle sizes in dispersions used for preparation of test substrates for the 1st test with *H. aculeifer*.

Concentration [mg NM-105/kg artificial soil (dw)]	Z-Average [d.nm]	PDI	Peak 1 [nm]	Peak 2 [nm]
1	498	0.6	612	206
1 (repeated)	405	0.5	600	4276
10 (20 mL application volume)	516	0.6	226	726
10 (50 mL application volume)	479	0.5	571	194

At test end, no significant effects of NM-105 on the assessed endpoints were found (Table 28 and Table 29).

Table 28: No of adult mites after 14 d of exposure to control substrate and NM-105 treated substrate in the 1st definitive test with *H. aculeifer*.
Rep.: replicate of the respective treatment or control; SD: standard deviation. Susp.: Application of NM-105 via 20 mL and 50 mL dispersion; Powder: Application of NM-105 with powder.

Concentration [mg NM-105/kg artificial soil (dw)]	Number of adult mites at test end [n]								
	Rep. A	Rep. B	Rep. C	Rep. D	Rep. E	Rep. F	Rep. G	Rep. H	Mean ± SD
Control	9	10	6	10	9	10	9	10	9.1 ± 1.4
1	9	10	9	9	-	-	-	-	9.3 ± 0.5
10 – Susp.	10	7	10	4	-	-	-	-	7.8 ± 2.9
10 - Susp. 50	7	8	9	7	-	-	-	-	7.8 ± 1.0
10 - Powder	10	10	9	9	-	-	-	-	9.5 ± 0.6
100	10	6	9	9	-	-	-	-	8.5 ± 1.7
1000	9	10	9	9	-	-	-	-	9.3 ± 0.5

Table 29: No of juvenile mites after 14 d of exposure to control substrate and NM-105 treated substrate in the 1st definitive test with *H. aculeifer*.
Rep.: replicate of the respective treatment or control; SD: standard deviation; VarC: coefficient of variation. Susp.: Application of NM-105 via 20 mL and 50 mL dispersion; Powder: Application of NM-105 with powder.

Concentration [mg NM-105/kg artificial soil (dw)]	Number of juvenile mites at test end [n]									VarC [%]	Juveniles [% control]
	Rep. A	Rep. B	Rep. C	Rep. D	Rep. E	Rep. F	Rep. G	Rep. H	Mean ± SD		
Control	241	194	251	200	226	261	202	271	230.8	29.8	12.9	–
1	215	189	217	188	-	-	-	-	202.3	± 15.9	7.9	87.6
10 – Susp.	282	204	257	231	-	-	-	-	243.5	± 33.6	13.8	105.5
10 - Susp. 50	213	206	280	131	-	-	-	-	207.5	± 60.9	29.4	89.9
10 - Powder	287	261	280	228	-	-	-	-	264.0	± 26.4	10.0	114.4
100	128	185	170	238	-	-	-	-	180.3	± 45.4	25.2	78.1
1000	184	279	247	201	-	-	-	-	227.8	± 43.3	19.0	98.7

The survival of adult mites (Figure 2A) was for all treatments on the same level as in the control. Though the number of juvenile mites (Figure 2B) showed a higher variance between treatments and compared to the control, no significant effects were detected.

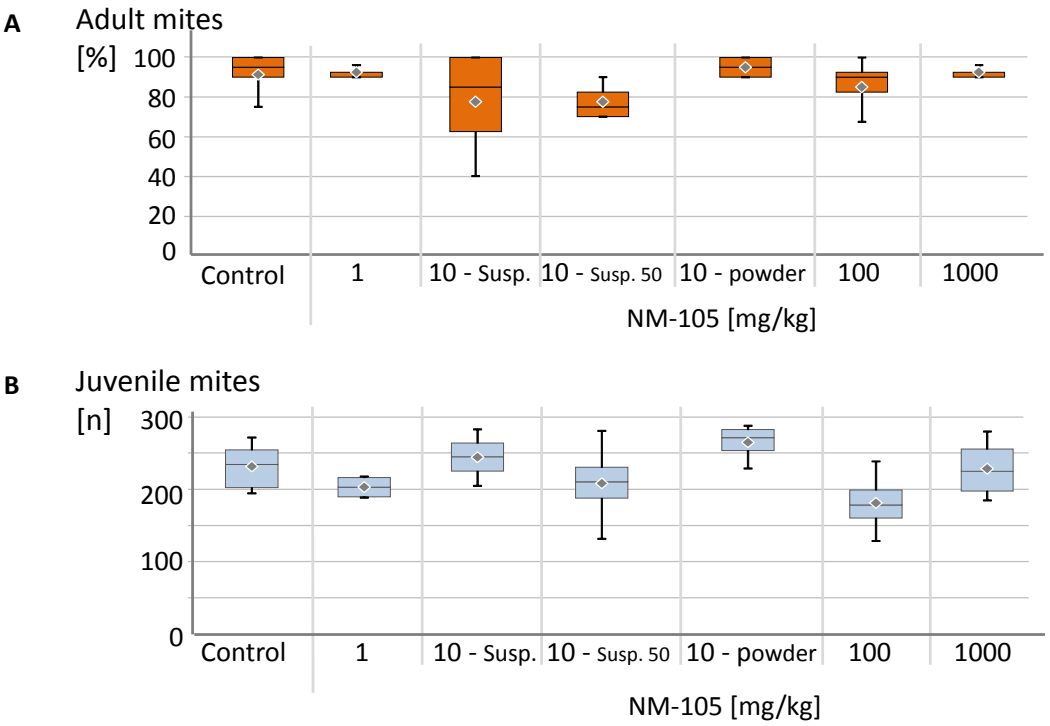

Figure 2: Results of the 1st definitive test with *H. aculeifer*.
A: Survival of adult mites at test end in percentage to the number of mites at test start.
B: Number of juveniles at test end.
Boxes represent 2nd and 3rd quartiles of data, whiskers indicate minimum and maximum values. ♦: Mean; n=8 (controls); n=4 (treatments). No significant difference to control.

3.6.3 Second definitive test with *Hypoaspis aculeifer*

The second definitive test was performed as a limit test to confirm the findings of the previous, first definitive test. Since in the range finding test statistically significant effects were found in the highest and the lowest test concentrations 1000 and 1 mg NM-105/kg artificial soil (dw), these two concentrations were chosen for this test. The results show no statistically significant effects of NM-105 on the survival of adult mites (Table 30 and Figure 3A). The number of juvenile mites at test end is approximately 16 and 20% lower in the treatments as in the control (Table 31 and Figure 3B). Though the decline is not as strong as in the range finding test, the effect is statistically significant (Student t test, 1-sided smaller; $p \leq 0.05$).

Table 30: No of adult mites after 14 d of exposure to control substrate and NM-105 treated substrate in the 2nd definitive test with *H. aculeifer*.
Rep.: replicate of the respective treatment or control; SD: standard deviation.

Test concentration [mg/kg]	Number of adult mites at test end [n]								
	Rep. A	Rep. B	Rep. C	Rep. D	Rep. E	Rep. F	Rep. G	Rep. H	Mean ± SD
Control	10	10	9	9	10	10	9	9	9.5 ± 0.5
1	9	9	9	8	10	9	8	9	8.9 ± 0.6
1000	9	10	10	10	10	9	7	10	9.4 ± 1.1

Table 31: No of juvenile mites after 14 d of exposure to control substrate and NM-105 treated substrate in the 2nd definitive test with *H. aculeifer*.
Rep.: replicate of the respective treatment or control; SD: standard deviation; VarC: coefficient of variation.

Test concentration [mg/kg]	Number of juvenile mites at test end [n]										
	Rep. A	Rep. B	Rep. C	Rep. D	Rep. E	Rep. F	Rep. G	Rep. H	Mean ± SD	VarC [%]	Juveniles [% control]
Control	298	348	286	317	288	254	288	188	283.4 ± 47.1	16.6	–
1	208	233	164	226	278	271	231	284	236.9 ± 40.3	17.0	83.6
1000	200	247	233	194	285	218	240	200	227.1 ± 30.7	12.9	80.1

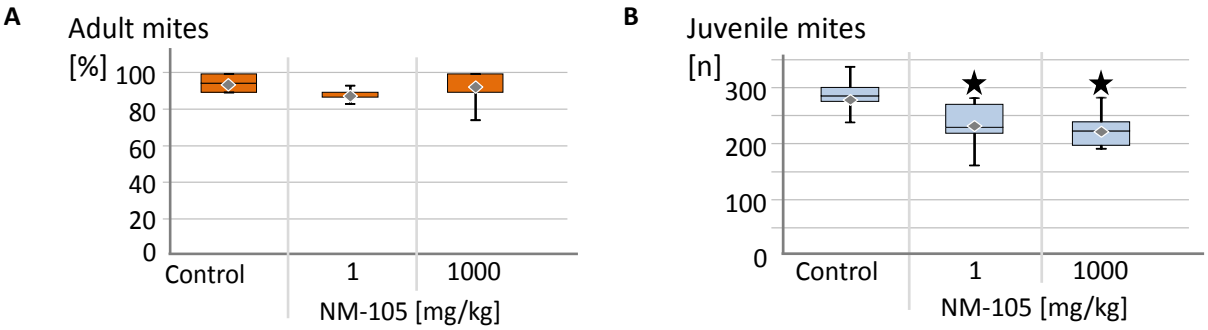

Figure 3: Results of the 2nd definitive test with *H. aculeifer*.
A: Survival of adult mites at test end in percentage to the number of mites at test start.
B: Number of juveniles at test end.
Boxes represent 2nd and 3rd quartiles of data, whiskers indicate minimum and maximum values. ♦: Mean; n=8 (controls); n=4 (treatments). ★: Significant difference to control (Student t test; $p \leq 0.05$).

3.7 Validity

In the three tests performed with NM-105 and the predatory mite *Hypoaspis aculeifer*, all validitiy criteria described in the test guideline were met (Table 32).

Table 32: Validity criteria according to OECD TG 226 [6] and values determined in the controls of the tests with *H. aculeifer.*

Parameter	Required in guideline	Range finding test	First definitive test	Second definitive test
Mean adult female mortality [% of introduced mites]	≤ 20%	0 – 20% (Min. Max) 6.25 ± 7.4% (Mean ± SD)	0 – 40% (Min. Max) 8.8 ± 13.6% (Mean ± SD)	0 – 10% (Min. Max) 5.0 ± 5.3% (Mean ± SD)
Mean number of juveniles per replicate	≥ 50	197 – 285 (Min. Max) 238.8 ± 25.1 (Mean ± SD)	194 – 271. (Min. Max) 230.8 ± 29.8 (Mean ± SD)	188 – 348 (Min. Max) 283.4 ± 47.1 (Mean ± SD)
Coefficient of variation for number of juveniles [%]	≤ 30	10.5	12.9	16.6

3.8 Additional experiments

3.8.1 Reference Test

A reference test was performed according to the test guideline.

Date of work: March 11, – April 05, 2011.

Results:

Mortality: Three percent mortality was observed in the control and 0% to 15% mortality was observed at all concentrations of boric acid tested.

Reproduction: The EC_{50} value was calculated by Probit analysis using Linear Max. Likelihood Regression as 134.0 mg boric acid/kg soil (dry weight) (95% confidence limits = 123.8 – 144.9 mg boric acid/kg soil (dry weight).

Comments: The observed effect is within the range expected from the guideline (EC_{50} in the range between 100 and 500 mg boric acid/kg soil (dry weight) and hence acceptable sensitivity of the test system is assured.

3.9 Conclusion

A NOEC could not be determined, since the definitive tests with NM-105 showed differing results, the second one exhibiting an effect at the lowest test concentration. Though statistically significant results differ, a lower number of offspring at 1mg NM-105/kg artificial soil (dw) compared to the control is noticeable in both tests, the effect size being nearly the same. The likely reason for the lack of significance

in the first definitive test is based on the test design. In the first test, four replicates were used per treatment, as recommended in the test guideline [6]. Since the second test was performed with only two treatments, the number of replicates was increased to eight per treatment. The higher number of replicates increased the statistical power of the test. This is not related to the testing of nanomaterials but true for all test items. When applying the standard design with four replicates per treatment and considering the high quality of the tests concerning the validity criteria, the NOEC can be set to ≥ 1000 mg NM-105 /L.

3.10 Executive summary

NM-105, a nanoparticulate titanium dioxide, was investigated in a range finding test and two definitive tests with the predatory mite *Hypoaspis aculeifer* as described in the test guideline [6]. The following treatments were investigated in the tests:

Range finding test:	Control; 1; 10; 10; 100; 1000 mg NM-105/kg artificial soil (dw)
First definitive test:	Control; 1; 10; 10; 10; 100; 1000 mg NM-105/kg artificial soil (dw)
Second definitive test:	Control; 1; 1000 mg NM-105/kg artificial soil (dw)

For preparation of test substrate with 1 and 10 mg NM-105/kg artificial soil (dw), NM-105 was applied via a dispersion. In all tests, application volume was 20 mL. In the first definitive test, the test concentration 10 mg NM-105/kg artificial soil (dw) was prepared additionally by application of 50 mL dispersion. Test concentrations with 10, 100 and 1000 mg NM-105/kg artificial soil (dw) were prepared by mixing the powder NM-105 into the substrate. This way, 10 mg NM-105/kg artificial soil (dw) was prepared by mixing powder NM-105 into the substrate and application of dispersion.

In the range finding test, a significant lower number of juvenile mites was found at the lowest and highest investigated test concentration (1 and 1000 mg NM-105/kg artificial soil (dw)), but not in the intermediate test concentrations. At 1 mg NM-105/kg artificial soil (dw) the survival of adult mites was lower than in the controls and any of the treatments. It was considered that the mites exposed to 1 mg NM-105/kg artificial soil (dw) might have been damaged during transfer into the test vessels and died before onset of reproduction. Since the reasons for the low number of juvenile mites at 1 mg NM-105/kg artificial soil (dw) could not be ascertained, the first definitive test was conducted at the same test concentrations. After 14 d of exposure to NM-105, no statistically significant difference between treatments and control was detected. The second definitive test was conducted as a limit-test with doubled number of replicates for the treatments. Usually, in a limit test one treatment is used. Since reasons for the result from the range finding tests were not clearly determined and to prove whether results from the first definitive test are reproducible, 1 and 1000 mg NM-105/kg artificial soil (dw) were investigated. When evaluating the number of juvenile mites after the exposure period, total differences in comparison with the control were less pronounced than in the range finding test, but nevertheless significantly lower due to enhanced

statistical power. For the standard design, which was proven to be applicable to the testing of nanomaterials, the NOEC was ≥ 1000 mg NM-105/L.

3.11 Raw data

Table 33: Determination of maximum water holding capacity (WHCmax) in artificial soil used for range finding test and 1st definitive test with *H. aculeifer*.

Replicate	Tara + frit		Sample + tara		Sample - tara		Water content	WHCmax
	dry	wet	wet	dry	dry	wet		
	[g]	[g]	[g]	[g]	[g]	[g]	[g]	[% dry weight]
1	29.8	30.5	78.8	63.1	33.3	48.3	15.0	45.0
2	29.9	30.3	78.2	62.6	32.7	47.9	15.2	46.5
3	30.1	30.7	78.1	62.7	32.6	47.4	14.8	45.4

Table 34: Determination of maximum water holding capacity (WHCmax) in artificial soil used for 2nd definitive test with *H. aculeifer*.

Replicate	Tara + frit		Sample + tara		Sample - tara		Water content	WHCmax
	dry	wet	Wet	dry	dry	wet		
	[g]	[g]	[g]	[g]	[g]	[g]	[g]	[% dry weight]
1	29.8	30.4	72.7	58.2	28.4	42.3	13.9	48.9
2	29.8	30.2	72.2	57.9	28.1	42.0	13.9	49.5
3	29.5	30.1	73.7	58.7	29.2	43.6	14.4	49.3

Table 35: pH at test start and end in test substrates of the range finding test with *H. aculeifer*.

Code / Test concentration	pH at start of exposure	pH at end of exposure
Control	6.5	6.7
1	6.5	6.6
10 - Susp.	6.4	6.7
10 – Powder	6.4	6.7
100	6.5	6.7
1000	6.5	6.7

Table 36: Soil moisture at start and end of exposure of the range finding test with *H. aculeifer*. WHCmax: maxiumum water holding capacity [% dry weight].

Code / Test concentration	Tara [g]	Soil + Tara [g]	Soil + tara, after drying [g]	Soil dry weight [g]	Water loss [g]	Soil moisture [% dry weight]	Soil moisture [% WHCmax]
Day 0 (Test start)							
C0	45.40	55.60	53.50	8.10	2.10	25.93	56.86
1	55.80	65.83	63.90	8.10	1.93	23.83	52.25
10 - Susp.	54.90	64.90	62.90	8.00	2.00	25.00	54.82
10 - Powder	55.00	65.00	63.00	8.00	2.00	25.00	54.82
100	55.60	65.60	63.60	8.00	2.00	25.00	54.82
1000	59.00	69.00	67.00	8.00	2.00	25.00	54.82
Day 14 (Test end)							
C0	58.11	60.88	60.32	2.21	0.56	25.34	55.57
1	43.92	45.09	44.85	0.93	0.24	25.81	56.59
10 - Susp.	45.80	46.57	46.42	0.62	0.15	24.19	53.06
10 - Powder	40.23	41.14	40.97	0.74	0.17	22.97	50.38
100	85.61	86.64	86.42	0.81	0.22	27.16	59.56
1000	56.34	57.59	57.33	0.99	0.26	26.26	57.59

Table 37: pH at test start and test end in test substrates of the 1st definitive test with *H. aculeifer*.

Code / Test concentration	pH at start of exposure	pH at end of exposure
Control	6.49	6.32
1	6.18	6.19
10 - Susp.	6.25	6.22
10 - Susp. - 50	6.33	6.28
10 – Powder	6.34	6.23
100	6.35	6.23
1000	6.30	6.18

Table 38: Soil moisture at start and end of exposure of the 1st definitive test with *H. aculeifer*. WHCmax: maxiumum water holding capacity [% dry weight].

Code / Test concentration	Tara [g]	Soil + Tara [g]	Soil + tara, after drying [g]	Soil dry weight [g]	Water loss [g]	Soil moisture [% dry weight]	Soil moisture [% WHCmax]
Day 0 (Test start)							
C0	54.88	64.17	62.32	7.44	1.85	24.87	54.53
1	46.29	56.57	54.55	8.26	2.02	24.46	53.63
10 - Susp.	42.29	52.35	50.37	8.08	1.98	24.50	53.74
10 - Susp. - 50	45.49	55.49	53.50	8.01	1.99	24.84	54.48
10 - Powder	54.88	64.12	62.31	7.43	1.81	24.36	53.42
100	55.99	65.97	64.00	8.01	1.97	24.59	53.93
1000	40.53	50.98	48.90	8.37	2.08	24.85	54.50
Day 14 (Test end)							
C0	44.20	53.90	52.10	7.90	1.80	22.78	49.97
1	85.60	95.40	93.50	7.90	1.90	24.05	52.74
10 - Susp.	42.40	52.20	50.30	7.90	1.90	24.05	52.74
10 - Susp. - 50	42.30	51.80	49.90	7.60	1.90	25.00	54.82
10 - Powder	59.00	68.30	66.60	7.60	1.70	22.37	49.05
100	54.90	65.00	63.00	8.10	2.00	24.69	54.15
1000	55.20	64.30	62.60	7.40	1.70	22.97	50.38

Table 39: pH measured at test start and end in test substrates of the 2nd definitive test with *H. aculeifer*.

Code / Test concentration	pH at start of exposure	pH at end of exposure
Control	5.52	5.61
1	5.50	5.49
1000	5.61	5.48

Table 40: Determination of soil moisture at start and end of exposure of the 2nd definitive test with *H. aculeifer*.
WHCmax: maxiumum water holding capacity [% dry weight].

Code / Test concentration	Tara [g]	Soil + Tara [g]	Soil + tara, after drying [g]	Soil dry weight [g]	Water loss [g]	Soil moisture [% dry weight]	Soil moisture [% WHCmax]
Day 0 (Test start)							
C0	45.90	56.20	54.00	8.10	2.20	27.16	55.20
1	56.40	66.90	64.60	8.20	2.30	28.05	57.01
1000	58.10	68.80	66.60	8.50	2.20	25.88	52.61
Day 14 (Test end)							
C0	55.60	65.80	63.80	8.20	2.00	24.39	49.57
1	41.90	52.40	50.40	8.50	2.00	23.53	47.82
1000	52.50	62.70	60.80	8.30	1.90	22.89	46.53

4 Fish early life stage toxicity test with *Danio rerio* and nanosilver

4.1 Test principle

The study was conducted in order to determine the potential chronic impact of the test item nanosilver on fish. As the fish early life stage test (OECD TG 210) comprises different life stages and performances of fish, it is generally accepted as chronic toxicity test. The endpoints to be observed are hatch, stage-specific survival and growth. Fertilized eggs of the zebrafish *Danio rerio* were exposed to a series of concentrations of the test item for 35 days. Besides generation of data for hazard assessment for the most sensitive life stages of fish, the objective was to adapt the OECD TG 210 test protocol to the testing of nanomaterials. Thus, we tried to achieve a particle concentration being as homogeneous and constant as possible, even when in case of nanosilver the effects are assumed to be caused by dissolved silver ions. As dosing of nanoparticles in a flow through-system via dosing pumps can be difficult depending on the dispersing, adsorptive and/or electrostatic properties, and due to the relative persistence of nanoparticles, we decided to use semi-static exposure conditions. To minimize stress to the early life stages, exchange of test media was scheduled after 7, 14, 21 and 28 days by tranferring the fish to freshly prepared test aquaria.

To verify the nominally applied concentrations and determine the losses of the test item in water body, due to sedimentation, samples were taken from the centre of the water body two times a week, one day after media renewal and one before the next renewal and measured for total silver concentrations. During an orientation study, samples were taken on days 1, 2, 5 and 7 at different locations of the water body and analysed for particle size distribution and total silver concentrations to determine homogeneity of the dispersion. In an additional test, fish were exposed for 28 days (weekly renewal of test dispersion) and analysed for uptake and partitioning of total silver.

4.2 Materials and methods

4.2.1 Test guideline

The test was performed according to:

OECD. 1993. OECD guideline for testing of chemicals. 210. Fish, Early Life Stage Toxicity Test. Organisation for Economic Co-operation and Development, Paris, France.

4.2.2 GLP

The test was not performed under GLP, but followed the principles. The use of any laboratory equipment was controlled and protocolled according to GLP. The quality assurance did not check any phase of the study, the raw data and study report.

4.2.3 Test vessels

260 L glass aquaria for each concentration and the control with dimensions of 0.98 x 0.48 x 0.55 m (l x w x h) were filled to a depth of 51 cm, corresponding to 240 L of test dispersion. To achieve homogeneous distribution of nanoparticles, the water column was agitated by four flow pumps (WP 300, Tetra GmbH, Melle, Germany), placed in the bottom corners of each vessel. Pseudo-replicate test cages were placed at the water surface of the aquaria, each containing an individual test group of 20 fertilized eggs. As test cages, sieves of stainless steel (ISO 3310-1) were used with a diameter 10 cm and a brim height of 4.5 cm, the sieve net at the bottom at a mesh width of 355 µm. Due to the agitation of the water body and the moving water surface, no additional aeration was necessary. The aquaria were placed on shelves containing light units for each aquarium (two neon lamps per vessel, light intensity approximately 1500 lux, measured 5 cm above the water surface in the middle of the test vessel), with the light/dark cycle adjusted to 14 hours on/10 hours off.

In the first test, one vessel per treatment was equipped with four ELS cages of stainless steel wires close to the water surface (pseudo-replicates), each stocked with 20 fertilized eggs at test start. A one-week pre-test showed the concentration of total silver and the particle size distribution in the mid of the vessel and the four ELS cages to be sufficiently constant. Thus, every 7 days the ELS cages were transferred in cleaned vessels with freshly prepared test dispersion. The NOEC und LOEC of this test were verified in a second test with two vessels and six ELS cages per concentration. At test end, surviving fish were measured for the content of total silver. As range finder for the test concentrations we performed a fish - embryo test, exhibiting 48 h NOECs of 200 µg/L for survival and 100 µg/L for heart beat frequency, respectively. Thus, we dosed 200, 100, 50, 25 und 12.5 µg total silver/L, verified by ICP-MS before and after each medium exchange.

4.2.4 Test substance

The test substance used in this study was dispersed (1:10) nanoparticulate silver NM-300 K.

4.2.5 Analytical monitoring

For determination of the silver concentration in the test dispersion, samples of control and test media were taken in the centre of the water body one day after setup and each renewal of the test dispersion as well as shortly before the following renewal and test end, respectively (definitive tests). In the initial orientation tests, samples were taken from the centre of the water body and from the four pseudo-replicate fish cages. Samples were stored in a refrigerator until analysis for total silver. In the definitive tests, samples were taken from the centre of the water body and analyszed for total silver. In the additional uptake test, samples were analyzed for total as well as for dissolved silver.

Additionally, size of silver particles in the test dispersion was determined during the orientation test at 400 µg Ag/L, representing a 7 day interval between test media exchanges, at beginning of exposure (1 h)

and after 1, 2, 5 and 7 days. Samples were taken from the centre of the water body. Measurement was performed using dynamic light scattering (DLS) in a Malvern Zeta-Sizer.

Reagents for silver analysis

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

Commercially available multielement ICP-standard containing 1000 mg/L Ag in nitric acid 2-3 % (CertiPUR®, ICP Multi Element Standard Solution IV, CertiPUR®, Merck, Darmstadt, Germany) and single element ICP-standard containing 1000 mg/L Ag in nitric acid 2-3 % (CertiPUR®, ICP Single Element Standard Solution Ag, CertiPUR®, Merck, Darmstadt, Germany) were used to prepare appropriate stock solutions and respective calibration solutions. All prepared standard solutions had a final HNO3 concentration of 10 %.

(Certified) reference materials and verifying of 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 certified with 10.9 µg/L Ag and TM-DWS.2 certified with 9.9 µg/L).

For the determination of Ag in zebrafish and prepared tissues certified reference materials (NIST 2977 mussel tissue, purchased from NIST, USA and DOLT-4 dogfish liver, purchased from Environment Canada) were digested along with actual samples and the recoveries for Ag were determined.

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-ionizer "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). They were purchased from Gilson (Abimed, Langenfeld, Germany) and Eppendorf (Wesseling, Germany).

ICP-OES

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. Before measurement calibration was performed using concentrations to fit in the optimal working range of the samples. The calibration formula was calculated using the linear regression algorithm of the ICP-OES instrument software. Correlation coefficient (r) was at least 0.999238. 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 LOD/LOQ were determined to 5.1 µg/L and 17 µg/L, respectively.

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

- Thermo IRIS Intrepid II, Thermo Electron Corporation, Germany

Analytical conditions:

- Nebulizer: Concentric glass nebulizer, Thermo Electron Corporation, Dreieich, Germany
- Spray chamber: Glass cyclonic spray chamber, Thermo Electron Corporation, Dreieich, Germany
- Nebulizer gas flow: 0.68 L/min
- Make-up gas flow: 0.5 L/min
- RF power: 1150 W
- Wavelength: 328.068 nm

ICP-MS

Silver concentrations of aqueous samples were measured using an Agilent 7500ce ICP-MS (Agilent Technologies, Waldbronn, Germany). Isotopes 107Ag or 109Ag were applied for evaluation. Before measurement calibrations were performed using concentrations to fit in the optimal working range of the samples. The calibration formula was calculated using the linear regression algorithm of the ICP-MS instrument software. Correlation coefficients (r) were at least 0.9981. 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: 9 * method standard deviation from calibration line.

Coefficients of determination (r) for respective calibration functions were taken from ICP-MS instrument outputs. Typical LOD and LOQ were between 0.005 – 0.012 µg/L and 0.015 – 0.036 µg/L, respectively.

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

- Agilent 7500ce, Agilent Technologies, Waldbronn, Germany

Analytical conditions:

- Nebulizer: Micromist, Agilent Technologies, Waldbronn, Germany
- Spray chamber: Scott type spray chamber, Agilent Technologies, Waldbronn, Germany
- Carrier gas flow: 0.90 L/min
- Make-up gas flow: 0.14 L/min
- RF power: 1500 W
- Isotopes: 103Rh (standard for quality assurance) 107Ag, 109Ag

Digestion of aqueous samples

After thoroughly shaking the samples (vortexer) 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 UltraPure water were added. The subsequent digestion was performed using an Ultra Clave II microwave (MLS GmbH, Leutkirch im Allgaeu, 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 analyzed by ICP-MS for its amount of silver.

Digestion of Zebrafish

The obtained Zebrafish were cooled in liquid nitrogen and then grinded by a cooled mortar and pistil. Subsequently, the samples were transferred into a Christ Alpha lyophilisation device. The samples freeze-dried until constant was reached. Afterwards, approx. 200 mg were weighed into quartz vessels, 5 mL of conc. nitric acid were added and finally digested in the microwave using the same program as mentioned above.

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 analyzed by ICP-OES for its amount of silver.

Digestion of fish tissues

The complete tissues were directly weighed into quartz vessels, 5 mL of conc. nitric acid were added and finally digested in the microwave using the same program as mentioned above.

Thereafter, the digested samples were poured into volumetric flasks and filled up with ultrapure water to an exact volume of 10 mL, due to lower inweights and expected silver concentrations. This final solution was analyzed by ICP-MS for its amount of silver.

Centrifugal Filtration method

In order to separate silver ions from nano silver the centrifugal filtration method was applied. Therefore, 5 mL of the test solution were transferred into centrifugal filtration tubes (vivaspin 6, 3 kDa, Sartorius Stedim, Germany). These tubes were directly centrifuged at 3600 g for 60 min at 20 °C. Due to the filtration membrane nearly all particles remain as residue, whereas the filtrate contains the silver ions.

To compare aqueous samples containing nano silver with the Ag+-ions in the filtrates after centrifugation the same digestion procedure (see above) was applied for the filtrate with minor modifications: Instead of 5 mL of sample volume, only 4 mL were available after filtration. After filling up the digested filtrate to 15 mL, the solution was analysed by ICP-MS.

4.2.6 Quality assurance measurements

The certified reference materials (CRMs) TMDA-70 (certified with 10.9 µg/L Ag) and TM-DWS.2 (certified with 9.9 µg/L Ag) were analysed as quality assurance sample with solution samples from the test. CRMs were diluted if necessary to fit in the concentration range of aqueous test samples. According to the quality assurance requirement, the silver recovery was in the range of 100 ± 15% of the certified value. However, regarding Ag concentrations measured by ICP-OES and ICP-MS, the mean recovery (accuracy) and precision over all series for non-digested TMDA-70 were 107 ± 5 % (n = 2), 98.6 ± 5.0 (n = 22, dilution factor 5), 101 ± 5 (n = 18, dilution factor 10) and 98.9 ± 6.4 (n = 12, dilution factor 20).

Mean recovery (accuracy) and precision of ICP-MS measurements over all series for non-digested TM-DWS.2 were 96.5 ± 3.3 % (n = 24, dilution factor 5) and 101 ± 5 % (n = 15, dilution factor 10).

To verify the digestion procedure the CRMs NIST 2977 mussel tissue (certified with 4.58 µg/g Ag) and DOLT-4 dogfish liver (certified with 0.93 µg/g Ag) were digested and analyzed along with zebrafish and tissue samples from the tests. According to the quality assurance requirements, the silver recovery was in the range of 100 ± 20 %. Mean recovery (accuracy) and precision for ICP-OES measurement of digested NIST 2977 material was 96.1 ± 6.5 % (n =3) and for ICP-MS quantification of DOLT-4 material was 83.2 ± 1.9 % (n = 9).

4.2.7 Details on application

The test vessels were filled with test water and temperated before beginning of exposure. On the starting day of exposure, dispersions of the test substance were prepared and administerd into the test vessels to achieve the nominal concentrations in each test vessel. 2 mL containing bottles of NM-300 K were filled up to 10 mL with aqua dest., ultra-sonificated for 15 min (Bandelin Sonorex RK 514 BH; 35 kHz; 215/860 W) and directly applied to the test vessels. Example: For the 100 µg/L-treatment, 1.25 mL of the application dispersion (25 mg Ag) were added to a 250 L vessel.

The test organisms were introduced into the test vessels on the same day. The test animals were exposed to the test item for a period of 35 d.

4.2.8 Test organisms

The test organism used in this study was the cyprinid teleost fish *Danio rerio*. This species is one of the recommended species by the test guideline and standard test fish within European chemicals regulation. The used strain was originally obtained from the no longer existing West Aquarium GmbH, 37431 Bad Lauterberg, Germany, and is cultured at Fraunhofer IME under inbreeding conditions since more than 20 years. It is comparably close to the wild type , closely following embryonic and sexual development as described by Hisaoka et co-authors [12, 13] and Takahashi [14].

Parental fish for the production of fertilized eggs for the study were held in aquaria with a total volume of 150 L. Holding water was of the same quality as that used in the test (i.e., purified drinking water, see below).

Holding temperature was 26 °C ± 1 °C. 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 TetraMin® Hauptfutter (Tetra Werke, Melle, Germany) and brine shrimp nauplii (*Artemia salina*). The brood stock was visually checked every 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. Beforehand, fertilisation rate was checked to be at least 70% before accepting the batch as parental fish for the production of fish used in the early life stage test.

The introduction of lighting induced mating and spawning of fish. Eggs were collected after settling in a spawning-tray (all glass), which was placed at the bottom of the holding vessels. The spawning tray was covered with a lattice (stainless steel) to prevent adults from feeding on the eggs, and artificial plant substrate (modified method according to Nagel [15]) to stimulate spawning onto the tray. Collected eggs were transferred from the spawning-tray onto a sieve, rinsed with clean water to remove faeces and residues of food, and then placed in glass dishes. Fertilised eggs (confirmed by microscopic determination of early cleavage stages) were then pipetted (using a widened and de-burred pipette tip) into the test chambers.

4.3 Study design

4.3.1 Study type

For range finding purposes, fish embryo tests were performed, resulting in a NOEC (lethal effects) of 200 µg/L and a LOEC (15% mortality) of 400 µg/L after 48 h. The most sensitive effect was a significant reduction of the heart beat frequency at 200 µg/L (NOEC: 100 µg/L).

In an orientation test under definitive test conditions, a nominal test item concentration of 400 µg/L was applied to investigate the homogeneity and stability of the test dispersion in terms of total silver concentration as well as of particle size for 7 days, the scheduled interval of test medium exchange. At the same time, the threshold concentration for lethal effects derived from the fish embryo test was investigated under definitive test conditions.

The first definitive test was performed with one vessel per treatment, equipped with four larvae cages of stainless steel wires close to the water surface (pseudo-replicates), each stocked with 20 fertilized eggs at test start. The nominal test concentrations were 200, 100, 50, 25 und 12.5 µg total silver/L. A test medium control and a dispergent control without silver nanoparticles were run in parallel. Every 7 days, a second set of aquaria was prepared and dosed, and the larvae cages were transferred.

The NOEC und LOEC of this test were verified in a second definitive test with two vessels per concentration and test medium control, containing six larvae cages, each. As in the first test some vessels showed inconsistent results due to a contamination of the tap water pipe with chlorine, the second test was performed at 100, 50 und 12.5 µg/L. At test end, surviving fish were measured for the content of total silver.

In an additional test (see 4.6), juvenile fish were exposed in the test system to nominal 25 and 100 µg Ag/L for 21 days (renewal of test dispersion after 7 and 14 d) to investigate main uptake routes and silver distribution in different fish tissues as well as the concentration of total and dissolved silver at start and end of the renewal period in the test aquaria. At the end of the study, fish were dissected in 1) head and skin (including gills), 2) intestines and 3) filet. The different parts of the fish were pooled and analyzed for total silver.

4.3.2 Test duration type

As the test duration comprises the early life stages and performances of fish, the test can be considered a chronic study. In any case, the duration type is a long-term study.

4.3.3 Water media type

The test medium was purified drinking water, the purification including filtration with activated charcoal, passage through a limestone column and aeration.

The following water quality data were obtained from regular measurements at the test facility during the performance of the definitive tests: pH: 7.5 – 8.0; conductivity: 271 – 328 µS/cm; nitrate: 1.9 – 3.1 mg/L; nitrite: < 0.005 – 0.027 mg/L; ammonium (NH_4^+): ≤ 0.01 mg/L; phosphate: < 0.1 – 0.6 mg/L; calcium: 0.8 – 0.9 mmol/L; magnesium: 0.1 – 0.5 mmol/L; total hardness: 1.0 – 1.3 mmol/L; alkalinity: 2.0 – 2.4 mmol/L; DOC (NPOC): 0.5 – 1.2 mg/L; cadmium: < LOQ – 0.27 µg/L; chromium: < LOQ – 0.33 µg/L; copper: < LOQ – 4.2 µg/L; iron: < LOQ – 12.5 µg/L; manganese: < LOQ – 0.24 µg/L; zinc: <LOQ – 7.4 µg/L; lead: < LOQ – 2.0 µg/L.

4.3.4 Total exposure duration

Total exposure duration to the weekly renewed test dispersion was 35 days.

4.3.5 Test conditions

The test was performed under the test conditions given in Table 41. Physico-chemical parameters were measured throughout the tests and are described in detail in Table 42,Table 43 and Table 44.

Table 41: Test conditions for tests with *Danio rerio.*

Number of test organisms per pseudo-replicate larvae cage at test start:	20 fertilized eggs
Biological parameters:	hatch, survival rate, size at test end (length, group weight)
Observations:	Daily
Test vessels:	glas vessels, 240 L test dispersion
Water movement:	4 pumps (each corner of the bottom of each aquarium)
Aeration of test vessels:	None
Feeding during exposure:	From day 6: breeding food (Tetra, AZ 000) twice daily ad libitum From day 16 : ground TetraMin flake food twice daily ad libitum From day 9 on: addition of brine shrimp nauplii (*Artemia salina*)
Water change:	Every 7 days transfer of larvae cages in freshly prepared aquaria
Light regime:	14 light : 10 dark

Table 42: Physico-chemical parameters measured in the test media in the orientation test with *Danio rerio.*

	pH		Temperature (°C)		O2 (mg/l)		O2 (%)	
test day	control	400 µg/L	control	400 µg/L	control	400 µg/L	control	400 µg/L
0	7.9	8.2	26.3	25.7	8.6	7.3	94	105
1	8.1	8.3	25.5	26.0	7.9	7.2	92	100
2	8.1	8.3	25.2	25.9	7.8	7.4	94	98
3	8.2	8.4	24.9	25.6	8.0	7.5	96	100
4	8.2	8.4	25.0	25.7	7.7	7.5	92	96
5	8.3	8.4	25.1	25.6	8.9	8.9	100	100
6	8.4	8.4	25.5	26.2	8.1	8.0	97	98
7	8.4	8.5	25.8	26.3	7.7	7.8	95	94
median/mean	8.2	8.4	25.4	25.9	8.1	7.7	95	99
Sd			0.5	0.3	0.4	0.6	3	3

Table 43: Physico-chemical parameters measured in the test media in the 1st test with *Danio rerio.*
Only minima and maxima of the fresh and aged medium shown (5 measurements weekly)

Parameter	Week 1		Week2		Week 3		Week 4		Week 5	
	new	Old	new	old	new	old	new	Old	new	old
Temperature [°C]	25.5-25.8	25.8-26.1	25.0-25.5	26.1-26.4	25.0-25.8	25.6-26.1	25.2-25.6	26.0-26.5	25.1-25.5	26.1-26.5
O_2 [mg/L]	7.3-8.1	6.9-7.0	7.2-7.4	6.6-7.1	7.4-8.1	6.9-7.1	6.9-7.0	6.4-7.0	7.1-7.6	6.4-7.2
O_2 [%]	92-101	87-89	92-94	87-92	94-99	88-92	90-93	83-91	91-97	83-91
pH	8.0-8.1	8.4	8.1-8.2	8.5-8.6	8.2	8.4-8.5	8.1	8.4-8.5	8.2	8.4-8.5

Table 44: Physico-chemical parameters measured in the test media in the 2nd test with *Danio rerio*.
Only minima and maxima of the fresh and aged medium shown (5 measurements weekly)

Parameter	Week 1		Week2		Week 3		Week 4		Week 5	
	new	Old	new	old	new	old	new	Old	new	Old
Temperature [°C]	26.3-27.3	25.9-26.6	25.5-26.3	26.3-26.9	25.0-26.0	25.9-26.6	25.5-26.1	26.4-26.8	25.1-26.1	25.7-26.5
O_2 [mg/L]	7.4-7.8	6.8-7.2	7.2-8.1	6.9-7.1	7.4-7.5	6.9-7.2	7.3-7.8	6.4-7.0	7.3-7.5	6.0-6.8
O_2 [%]	96-100	88-94	94-98	90-92	93-96	89-92	95-100	84-87	92-96	76-87
pH	8.5-8.7	8.6-8.7	8.4-8.6	8.6-8.7	8.4-8.7	8.5-8.6	8.5-8.7	8.5	8.6-8.7	8.2-8.5

4.3.6 Any other method on materials and methods

Endpoint observation and statistical analysis

The mean number of hatched fish, the mean number of surviving fish (post-hatch success), mean total length of fish and the dry weight of the pooled fish group (1st definitive test) or mean individual wet weight (2nd definitive test) were determined per replicate. Observations on hatching and survival as well as on abnormal appearance of behaviour were made daily. Dead embryos, larvae and juvenile fish were removed as soon as observed. After 21, 28 and 35 days larvae/juvenile fish were photographed (digital camera: Olympus C 1400 L) and the survival rates as well as the lengths of the fish were determined using digital image processing (UTHSCSA ImageTool Version 3.0; University of Texas Health Science Center at San Antonio).

The statistical software package ToxRat Professional 2.10 (ToxRat Solutions GmbH, Naheweg 15, D-52477 Alsdorf) was used for regression analysis and hypothesis testing. The statistical reports are attached as annex 1.

4.4 Results

4.4.1 Orientation study: Stability/homogeneity of test dispersion; hatch/survival after 7 d

To investigate stability and homogeneity of the test dispersion under definitive test conditions, a nominal test item concentration of 400 µg/L was applied to investigate particle size distributions (Table 45) and total silver concentration in different parts of the water body (Table 46) for 7 days, the scheduled interval of test medium exchange. The tested concentration caused 15 % mortality in the range finding fish embryo test (LOEC) and represented the lower limit for the determination of particle sizes.

Table 45: Measured particle sizes in the orientation test with *Danio rerio.*

	Peak 1		Peak 2	
Time	nm	%	nm	%
1 h	54	79	14	21
1 d	approx. 50	approx. 70	approx. 100	21
2 d	56	75	438	13
5 d	56	70	217	30
7 d	152	56	44	44

The main peak of the particle size distribution (70-80 % of the particles) was constantly measured at a particle size of 50-60 nm for the first five days (Table 45). The size of the remaining particles (20-30 %) highly varied between 14 and 440 nm. At the end of the investigated interval, the particle size of the less dominant main peak (56 %) was increased. At the tested concentration, the measurements were highly uncertain, but indicated an acceptable size stability of the main particle fraction under the test system conditions for at least five days. Concerning the concentration of total silver in the water body, concentrations varied by a factor of 2-3 between different times and locations. Due to the visible water movement, no location was observed to be priviledged: The time weighted average concentrations were calculated to be between 82 and 114 % of nominal at all sampling sites. There was no time-dependent decrease of the total silver concentration due to sedimentation. Thus, a media exchange interval of one week was considered appropriate. Under definitive test conditions, the mortality of fish larvae until and after hatch was between 75 % and 100 % in the four pseudo-replicate cages, resulting in a mean total success of only 11 % (Table 47). Thus, in the first definitive test concentrations of 200, 100, 50, 25, and 12.5 µg total Ag/L were applied.

Table 46: Total Ag concentrations (µg/L) in different parts of the water body in the orientation test with *Danio rerio.*
Values are means of 4 (1h), 3 (1d) and 2 (2-7d) replicate measurements, respectively.

Time	Control	Cage 1	Cage 2	Cage 3	Cage 4	Centre
1 h	3.5	305	280	285	269	317
1 d	4.7	319	258	201	219	267
2 d	5.0	sample lost	555	391	377	540
5 d	1.7	397	487	442	363	542
7 d	8.8	232	286	548	293	328
Time weighted Average	4.2	339	430	397	330	455
% of nominal	1.1	85	108	99	82	114

Table 47: Hatch and survival of *Danio rerio* larvae in the orientation test.
Numbers at test start = introduced fertilized eggs. Numbers on days 1-7 = hatched alive larvae.
Decreasing numbers with time indicate mortality of hatched larvae.

		Control				400 µg/L			
		Cage 1	Cage 2	Cage 3	Cage 4	Cage 1	Cage 2	Cage 3	Cage 4
start	13.04.2011	20	20	20	20	20	20	20	20
day 1	14.04.2011	0	0	0	0	0	0	0	0
day 2	15.04.2011	0	0	0	0	0	0	0	0
day 3	16.04.2011	0	3	7	1	2	2	0	1
day 4	17.04.2011	11	11	15	9	0	2	0	1
day 5	18.04.2011	20	18	18	17	3	4	0	2
day 6	19.04.2011	20	18	18	17	3	4	2	2
day 7	20.04.2011	20	19	19	19	4	5	0	0
	%	100	95	95	95	20	25	0	0
	Mean			96.3				11.3	
	Standard deviation			2.5				13.1	

4.4.2 First definitive test with *Danio rerio*

In the 1st definitive test, total silver concentrations in the test media were measured in a range between 53 % and 98 % of nominal concentrations with mean measured concentrations of 70 % ± 2 % for all treatments (Table 48). During the intervals of media exchange, a trend of decreasing concentrations was observed, except the value at the lowest concentration on day 6.

In correspondence with the NOEC for lethal effects of the fish embryo test performed as range finding test, hatch was complete in all pseudo-replicate cages of all test concentrations between 25 and 200 µg/L (nominal concentration, Table 49). The only treatments with clearly reduced hatch were the vehicle control run with the dispersant only and the lowest test concentration.

Table 48: Total Ag concentrations measured in the 1st test with *Danio rerio*.

		Total Ag concentration (% of nominal)						
Date	**Day**	**Control**	**Vehicle**	**12.5 µg/L**	**25 µg/L**	**50 µg/L**	**100 µg/L**	**200 µg/L**
17.05.2011	0							
19.05.2011	2	0.0	0.0	79.2	72.1	68.5	69.5	70.0
23.05.2011	6	0.0	0.0	98.4	70.5	53.8	55.2	68.2
26.05.2011	9	0.0	0.0	59.3	74.6	69.0	67.8	69.0
30.05.2011	13	0.0	0.0	59.1	63.3	58.6	66.3	65.7
01.06.2011	15	0.0	0.0	80.4	85.5	80.2	80.2	
06.06.2011	20	0.0	0.0	73.9	71.8	64.7	70.5	
09.06.2011	23	0.0	0.0	82.6	90.3	85.8	84.5	
14.06.2011	28	0.0	0.0	56.8	65.0	63.4	63.1	
16.06.2011	30	0.0	0.0	73.2	73.4	79.1	72.6	
20.06.2011	34	0.0	0.0	53.3	58.3	63.4	63.0	
21.06.2011	35							
Mean				71.6	72.5	68.6	69.2	68.2
Sd				14.3	9.7	10.2	8.5	1.8
CV %				20.0	13.3	14.8	12.3	2.7
Total Ag concentration (µg/L)				8.95	18.1	34.3	69.2	136
Max concentration (µg/L)				10.1	21.4	40.1	80.2	138

Table 49: No of hatched *Danio rerio* larvae in the first nine days of the 1st definitive test.

D	**Control**				**Vehicle**				**12.5 µg/L**				**25 µg/L**				**50 µg/L**				**100 µg/L**				**200 µg/L**			
	1	**2**	**3**	**4**	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**
1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3	8	9	8	12	2	3	2	0	2	0	0	0	2	0	3	3	0	0	2	1	3	1	4	1	0	0	0	0
4	11	12	12	15	4	6	3	3	5	0	3	3	4	8	4	11	10	8	8	9	10	13	9	7	3	2	4	6
5	17	19	18	18	7	10	5	4	10	1	3	3	11	11	8	15	15	15	16	17	18	17	15	16	17	16	16	14
6	18	19	18	18	9	10	7	5	10	1	3	6	11	13	9	15	18	18	17	19	18	17	17	17	17	16	16	15
7	19	20	19	19	10	10	8	7	10	3	6	8	16	18	14	19	20	20	20	20	20	20	19	20	19	20	19	20
8	20	20	20	20	11	11	10	9	11	5	8	8	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20
9	20	20	20	20	13	11	10	9	12	7	8	8	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20

Between 31st of May and 9th of June, we ran an additional control vessel and vessels at treatments with nominal 12.5 and 6.25 µg/L without test media exchange (Table 50), to check whether the reduced hatch was due to a kind of low-dose effect or due to non-dose related problems in the unicate test vessels. The control had to be run a further time between 14th and 23rd of June. Until day 9 of the (restarted) vessels, all introduced fertilized eggs hatched. The reason for the failure of hatch in three of 11 test vessels a problem at the water supply station could be identified later. During modification of the cleaning process, temporary chlorine overdosing obviously had resulted in enhanced concentrations of chloroalkanes which could still be measured in rarely used water tubes two weeks later. The failing two vessels of the 1st definitive test were the first ones filled with water before dosing the test item. This explanation does not

fit to the additional control vessel. However, it is obvious that also in this vessel a water quality problem occurred, as all pseudo-replicates were affected.

Table 50: No of hatched *Danio rerio* larvae in the first nine days of the 1st definitive test, additional vessels.

D	Control				Control restarted				6.25 µg/L				12.5 µg/L			
	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0	0	6	0	0	2	8	5	9
4	1	0	0	2	3	0	0	0	2	20	6	10	6	12	14	16
5	2	3	4	4	6	9	2	2	11	20	8	16	16	20	16	18
6	5	5	4	5	12	11	5	7	15	20	10	20	19	20	17	20
7	6	5	4	6	16	19	15	15	18	20	17	20	20	20	20	20
8	6	7	7	8	20	20	18	17	18	20	20	20	20	20	20	20
9	6	7	7	8	20	20	20	20	20	20	20	20	20	20	20	20

Table 51: Cumulative number of dead *Danio rerio* during the 1st definitive test.
Only treatments with successful hatch included.
Days 7-18: daily counts of dead larvae/fish. Days 21, 28, 35: Calculated from photographies for length measurements.

D	control				25 µg/L				50 µg/L				100 µg/L				200 µg/L			
	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
7																				
8															1		17	20	14	17
9										2					1	1	19	20	15	19
10	1	1	1		1					2					1	1	20	20	20	19
11	4	3	3	2	1					2					1	1	20	20	20	19
12	4	3	5	3	1					2					1	2				
13	4	4	6	3	1					2					1	2				
14	6	5	7	3	1	1			5	4	5	6	10	7	8	10				
15	6	5	7	3	1	2	4	1	5	5	5	7	12	9	9	11				
16	6	5	7	3	2	3	5	1	5	5	5	7	13	10	10	11				
17	7	6	9	3	2	3	5	1	5	5	6	8	13	10	10	11				
18	7	6	9	3	2	3	5	1	5	5	6	8	13	10	10	11				
21	7	6	9	3	3	5	5	1	5	5	8	9	13	10	10	11				
28	7	8	9	7	4	5	5	2	5	5	9	10	13	10	10	11				
35	7	8	9	7	4	5	5	4	5	5	9	10	13	10	10	11				

In the further course of the 1st definitive test, mortality of hatched larvae was recorded (Table 51). Post-hatch success in the control was 61%, which is below the quality criterion of 70% given by the guideline (7). For the remaining test concentrations (vessels with low hatch excluded), which all showed 100% hatch, a concentration-response relationship was observed regarding post-hatch success (Table 52). In the highest test concentration (200 µg/L), all larvae died after being transferred to fresh test dispersion on day 7. At 100 µg Ag/L, unusual mortality started on day 14 and resulted in a survival rate of 45% at test end. At 50 µg/L, post hatch success was similar compared to the control (NOEC), but also below the quality criterion. It cannot be excluded that weak control success masked effects at 50 µg/L. For growth, measured as total length (mean of measured individuals) and group weight (total dry weight of all fish in

the pseudo-replicate), a clear reduction was observed already at 25 µg/L. The few surviving individuals at 12.5 µg/L were comparable to the control. However, as growth is clearly density-dependent, comparable sizes at lower densities may also indicate effects.

Due to the water quality issues which became obvious in some test vessels, the first definitive test results are loaded with uncertainties. These should be reduced in the confirming second definitive test with the NOEC and LOEC of the first test, tested in true replicate vessels with more pseudo-replicates. However, the NOEC and LOEC were questionable. The 12.5 µg/L treatment could not be properly evaluated, but was hypothesized as absolute NOEC. 100 µg/L showed clear significant and population-relevant effects and were identified as clear effect concentration. Due to limited space in the facility, only one further test concentration was possible. We decided to test 50 µg/L, as this treatment represented the NOEC for post-hatch success.

Table 52: Effect overview table of the 1st definitive test with *Danio rerio*.
Lengths are means of individual lengths per pseudo-replicate. Weights are pooled group weights (dry weight) of all fish in a pseudo-replicate. Mean and SD (standard deviation) refer to treatment (pseudo-)replicates. Concentrations are given as nominal concentrations.

D	**control**				**25 µg/L**				**50 µg/L**				**100 µg/L**				**200 µg/L**			
	1	**2**	**3**	**4**	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**
Hatch (%)	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
Post-hatch (%)	65	60	55	65	80	75	75	80	75	75	55	50	35	50	50	45	0	0	0	0
Mean	61.3				77.5				63.8				45.0*				0*			
SD	4.8				2.9				13.1				7.1				0			
Length (cm)	1.30	1.33	1.23	1.16	1.00	1.08	1.01	0.98	1.09	0.91	1.11	1.01	1.27	0.97	1.10	1.01	-	-	-	-
Mean	1.26				1.02*				1.03*				1.08*				-			
SD	0.08				0.05				0.09				0.13				-			
Group weight (mg dw)	62	61	44	45	32	51	35	35	47	35	25	20	29	24	27	19	-	-	-	-
Mean	53				38*				32*				25*				-			
SD	10				9				12				4				-			

*: statistically significant deviation compared to control, $p<0.05$, one-sided smaller (see annex 1)

4.4.3 Second definitive test with *Danio rerio*

In the second definitive test, total silver concentrations were measured in the test media. After test end, the surviving fish were analyzed for silver to learn about silver bioaccumulation. To enhance the fish biomass for analyses, 6 pseudo-replicate cages with 20 fertilized eggs each were placed in each of two replicate aquaria per treatment.

Total silver concentrations in the test media were measured in a range between 10 % and 81 % of nominal concentrations with mean measured concentrations of 47 % ± 6 % for all treatments (Table 53).

During the intervals of media exchange, concentrations clearly decreased, indicating sedimentation, Obviously, in the 2nd test the pump performance had decreased.

Table 53: Total Ag concentrations measured in the 2nd test with *Danio rerio*.

Date	Day	Total Ag concentration (% of nominal)							
		Control		12.5 µg/L		50 µg/L		100 µg/L	
		Rep 1	Rep 2	Rep 1	Rep 2	Rep 1	Rep 2	Rep 1	Rep 2
02.08.2011	0								
04.08.2011	2	0.0	0.0	34.6	38.7	60.4	49.2	59.9	59.3
08.08.2011	6	0.0	0.0	9.9	16.0	42.3	17.2	43.9	51.5
11.08.2011	9	0.0	0.0	60.6	81.3	63.2	63.0	54.5	68.2
16.08.2011	13	0.0	0.0	11.6	65.7	29.1	32.1	33.0	40.5
18.08.2011	16	0.0	0.0	63.4	64.7	56.3	55.6	52.9	61.7
22.08.2011	20	0.0	0.0	37.2	54.5	37.0	31.1	30.9	38.3
25.08.2011	23	0.0	0.0	61.8	61.0	55.1	61.2	51.6	55.8
29.08.2011	27	0.0	0.0	21.0	37.7	17.0	24.7	19.8	23.1
01.09.2011	30	0.0	0.0	72.8	65.8	62.5	67.4	59.7	70.5
05.09.2011	34	0.0	0.0	42.8	42.6	46.8	53.8	37.6	34.2
06.09.2011	35								
Mean				41.5	52.8	46.9	45.5	44.4	50.3
Sd				22.6	19.0	15.5	17.8	13.6	15.7
CV %				54.5	36.0	33.1	39.0	30.6	31.2
Total Ag concentration (µg/L)				5.2	6.6	23.5	22.8	44.4	50.3
Max concentration (µg/L)				9.1	10.2	31.6	31.5	54.5	61.7

As in the 1st definitive test, hatch was complete in all pseudo-replicate cages of all test concentrations (Table 54) (except control (I), cage 5 and 50 µg/L (I), cage I: 95 % hatch). At 12.5 µg/L, there was no indication of an effect on hatch, confirming the conclusion from the 1st test that reduced hatch was due to water quality problems rather than due to low dose effects.

Table 54: No of hatched *Danio rerio* larvae in the first eight days of the 2nd definitive test.

	day	0	1	2	3	4	5	6	7	8
Treatment	Cage									
control (I)	1				10	17	19	20	20	
	2				9	11	20	20	20	
	3				9	16	19	20	20	
	4				5	13	20	20	20	
	5				3	13	19	19	19	
	6				6	15	20	20	20	
control (II)	1				7	9	12	15	19	20
	2				6	10	16	18	19	20
	3				8	13	16	20	20	
	4				6	15	17	20	20	
	5				7	12	15	18	19	20
	6				8	14	17	20	20	
12.5 µg/L (I)	1				1	4	8	16	19	20
	2				2	5	14	18	18	20
	3				5	11	17	19	20	
	4				8	14	18	20	20	
	5				8	11	15	20	20	
	6				6	9	15	19	20	
12.5 µg/L (II)	1				3	10	17	19	20	
	2				5	14	18	18	19	20
	3				4	9	18	19	20	
	4				2	12	20	20	20	
	5				6	17	20	20	20	
	6				8	13	20	20	20	
50 µg/L (I)	1				1	8	19	19	19	
	2				2	10	20	20	20	
	3				12	20	20	20	20	
	4				5	10	19	20	20	
	5				4	15	20	20	20	
	6				2	15	20	20	20	
50 µg/L (II)	1				5	12	20	20	20	
	2				1	14	20	20	20	
	3				6	11	20	20	20	
	4				3	13	20	20	20	
	5				4	10	20	20	20	
	6				7	15	20	20	20	
100 µg/L (I)	1				2	13	20	20	20	
	2				0	9	20	20	20	
	3				9	15	20	20	20	
	4				0	10	20	20	20	
	5				1	11	20	20	20	
	6				0	11	20	20	20	
100 µg/L (II)	1				2	9	20	20	20	
	2				2	9	18	20	20	
	3				1	7	19	19	20	
	4				5	12	20	20	20	
	5				2	15	20	20	20	
	6				4	8	18	19	20	

Table 55: Cumulative number of dead *Danio rerio* during the 2nd definitive test.
Days 6-18: daily counts of dead larvae/fish. Days 21, 28, 35: Calculated from photographies for length measurements.

	day	6	7	8	9	10	11	12	13	14	15	16	17	18	21	28	35
Treatment	Cage																
control (I)	1										1	1	2	2	2	2	2
	2			2	3	3	4	4	4	4	4	4	4	4	3	4	4
	3														0	0	0
	4								1	1	1	2	3	3	4	4	4
	5		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	6								1	1	1	1	2	2	2	2	2
control (II)	1														2	2	2
	2														0	0	0
	3	1	1	1	1	1	1	1	1	1	1	1	1	1	3	3	3
	4											1	2	2	3	3	3
	5											1	1	1	4	5	5
	6						2	2	2	2	2	2	2	2	3	3	3
12.5 µg/L	1									1	1	1	1	1	2	1	2
(I)	2							1	1	1	1	1	1	1	3	1	3
	3						1	1	1	1	1	1	1	1	2	1	2
	4														0	0	0
	5														1	1	1
	6						1	1	1	1	1	1	1	1	4	4	4
12.5 µg/L	1														1	1	1
(II)	2								1	1	1	1	1	1	3	3	3
	3														0	0	1
	4								1	1	1	1	1	1	4	4	4
	5							1	1	1	1	1	1	1	1	1	1
	6														0	1	1
50 µg/L (I)	1		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	2														0	0	0
	3		1	1	1	1	1	1	2	2	2	2	2	2	2	2	2
	4														0	0	0
	5														0	0	0
	6														0	0	0
50 µg/L (II)	1					1	1	1	1	1	1	1	1	1	2	2	2
	2		1	1	1	1	1	2	2	2	2	2	3	3	4	4	4
	3														0	0	0
	4														0	0	0
	5														1	2	2
	6														1	1	1
100 µg/L (I)	1			15	15	15	15	15	15	15	15	15	15	15	15	15	15
	2			7	7	7	7	7	7	7	7	7	7	7	6	6	7
	3								1	1	1	1	1	1	1	1	1
	4				1	1	1	1	1	1	1	1	1	1	2	2	2
	5			9	9	9	9	9	9	9	9	9	9	9	9	9	9
	6			12	12	12	12	12	12	12	12	13	13	13	14	14	14
100 µg/L (II)	1										1	1	1	1	1	1	1
	2				1	2	2	3	3	4	4	5	5	5	5	6	6
	3					1	1	1	1	1	2	2	3	3	5	5	5
	4									2	2	2	2	2	3	4	4
	5		1	1	1	1	1	1	1	1	3	3	3	3	4	4	4
	6			1	1	1	1	1	1	1	2	2	2	2	2	2	2

Table 56: Effect overview table of the 2nd definitive test with *Danio rerio*.
Lengths: mean total lengths per fish and cage. Weights: mean wet weights per fish and cage. SD: standard deviation. Treatments: nominal concentrations. * statistically significant effect (p<0.05).

		Hatch (%)	Post-hatch success (%)		Length (cm)			Weight (mg)		
			mean	SD		mean	SD		mean	SD
Treatment	Cage									
control (I)	1	100	90		1.43			29.6		
	2	100	80		1.48			29.3		
	3	100	100		1.46			27.4		
	4	100	80		1.39			27.0		
	5	95	100		1.42			28.2		
	6	100	90	88.3	1.43	1.42	0.04	27.6	27.3	2.0
control (II)	1	100	90		1.35			23.4		
	2	100	100		1.38			24.4		
	3	100	85		1.49			28.1		
	4	100	85		1.44			28.9		
	5	100	75		1.40			28.2		
	6	100	85		1.40			25.3		
12.5 µg/L (I)	1	100	90		1.45			27.7		
	2	100	85		1.39			23.8		
	3	100	90		1.41			25.8		
	4	100	100		1.31			19.6		
	5	100	95		1.42			24.9		
	6	100	80	90.4	1.45	1.39	0.06	25.3	25.5	3.2
12.5 µg/L (II)	1	100	95		1.37			26.4		
	2	100	85		1.36			28.5		
	3	100	95		1.40			26.5		
	4	100	80		1.44			30.4		
	5	100	95		1.25			20.0		
	6	100	95		1.42			27.4		
50 µg/L (I)	1	95	100		1.42			25.6		
	2	100	100		1.36			21.6		
	3	100	90		1.36			22.2		
	4	100	100		1.28			19.7		
	5	100	100		1.29			21.4		
	6	100	100	95.4	1.27	1.31*	0.05	19.0	20.3 *	2.1
50 µg/L (II)	1	100	90		1.28			20.2		
	2	100	80		1.33			18.9		
	3	100	100		1.31			18.1		
	4	100	100		1.30			19.3		
	5	100	90		1.27			18.2		
	6	100	95		1.28			19.7		
100 µg/L (I)	1	100	25		1.52			28.5		
	2	100	65		1.41			25.5		
	3	100	95		1.18			14.2		
	4	100	90		1.22			15.4		
	5	100	55		1.38			23.0		
	6	100	30	70.8*	1.48	1.30*	0.12	29.8	19.8 *	5.6
100 µg/L (II)	1	100	95		1.24			16.7		
	2	100	70		1.28			19.5		
	3	100	75		1.29			18.3		
	4	100	80		1.14			14.5		
	5	100	80		1.26			18.1		
	6	100	90		1.18			14.0		

In the further course of the 2nd definitive test, mortality of hatched larvae was recorded. Post-hatch success in all pseudo-replicates of the control and of all treatments except the highest test concentration was 75 – 100 % (Table 55). At 100 µg/L, mortality of 35 - 75 % occurred in four cages of aquarium 1 after larvae transfer to fresh test dispersion on day 7. The other eight cages at the highest concentration exhibited a post-hatch success comparable to the other treatments (70 – 95 %), resulting in a pseudo-replicate mean of 70.8 % (Table 56). Due to the very high post-hatch success in the controls (88 %) and the low variability, the deviation was statistically significant. For growth, measured as total length (mean of measured individuals per cage, pseudo-replicate statistics), significant reduction was found at 50 and 100 µg/L.

As additional information, fish surviving after 35 days were analyzed for total silver concentrations. Increased total silver concentrations in water resulted in increased concentrations in fish (Table 57). As for most metals, the relative accumulation decreases with increasing concentration.

Table 57: Ag concentrations in pooled fish of the 2nd definitive test with *Danio rerio*.

Treatment	Cage	µg Ag/kg fish	mean	SD	µg Ag/L water	BCF
control (I)	1-3	-4.4	70	60	0.1	700
	4-6	109				
control (II)	1-3	49.2				
	4-6	127				
12.5 µg/L (I)	1-3	3974	3530	427	6	590
	4-6	3619				
12.5 µg/L (II)	1-3	3581				
	4-6	2947				
50 µg/L (I)	1-3	9665	9755	514	23	420
	4-6	10507				
50 µg/L (II)	1-3	9422				
	4-6	9426				
100 µg/L (I)	1-6	18653	16477	3077	47	350
100 µg/L (II)	1-6	14301				

Statistical evaluation of the endpoints was performed for the two definitive studies using mean measured concentrations. The obviously enhanced stress in the 1st test resulted in worse control performance (below validity criterion), but at the same time in more pronounced effects (length). The overall results are coincident with the 2nd test. The statistical power for all endpoints was sufficient to identify deviations from controls of more than 7% as significant effects.

Table 58: Summary effect table of the fish early life stage tests with *Danio rerio*. Calculations are based on mean measured concentrations during the test (µg Ag/L)

	LOEC (effect)	NOEC (effect)	EC_{50}	95%-CI	EC_{10}
Hatch					
Orientation	**400** (80%)	n.d.	n.d.	n.d.	n.d.
1st test	n.d.	**136** (0%)	n.d.	n.d.	n.d.
2nd test	n.d.	> 47 (0%)	n.d.	n.d.	n.d.
Post-hatch success					
1st test	69 (27%)	34 (0%)	72	67-77	53
2nd test	**47** (20%)	**23** (0%)	62	56-68	41
Length*					
1st test	18 (19%)	n.d.	n.d.	n.d.	n.d.
2nd test	**23** (8%)	**5.9** (2%)	n.d.	n.d.	n.d.
Weight*					
1st test	18 (23%)	n.d.	n.d.	n.d.	n.d.
2nd test	**23** (26%)	**5.9** (7%)	n.d.	n.d.	n.d.

4.5 Validity

In the definitive tests with *Danio rerio* and nanosilver, all validity criteria were met only by the 2nd test (Table 59). However, the 1st test is providing valuable information and confirming evidence.

Table 59: Validity criteria according to the revision of OECD TG 210 [5] and values determined in the two definitve tests with *Danio rerio*.

Parameter	Recommended in guideline	Value determined in first test	Value determined in second test
Hatching success	90 %	100 %	99 %
Post-hatch success	≥ 75 %	61 %	88 %
Water temperature within the range recommended for the test species	26 ± 1 °C	25 -27°C	25 - 27°C
Oxygen saturation	≥ 60 %	> 83 %	> 76 %

4.6 Additional experiments

4.6.1 Uptake and distribution experiment

To investigate the main uptake route and distribution of nanosilver in fish, an additional test was set up in the test systems. As the fish at the end of the early life stage test were too small for a differential analysis, we exposed 15 bigger juvenile fish each to 25 und 100 µg Ag/L for 21 d to differentiate total Ag residues in the different tissues 1) head, gills and skin, 2) intestines (stomach, guts), and 3) filet and rest (organs, bones) (Table 61). The results (3 fish pooled) were compared between the different tissues and related to the water concentrations (Table 60).

Table 60: Additional uptake test with *Danio rerio:* Total and dissolved Ag concentrations in the water (µg/L).

		Control		25 µg/L		100 µg/L	
Date	**Day**	**total**	**centrifuged**	**total**	**centrifuged**	**total**	**centrifuged**
29.11.2011	0	0.51	0.23	17.7	0.34	66.6	3.15
05.12.2011	6	0.87	0.19	14.5	0.63	60.5	0.92
06.12.2011	7	0.36	0.09	17.3	0.41	72.2	2.67
12.12.2011	13	0.31	0.12	14.4	0.38	72.9	2.79
13.12.2011	14	0.25	0.09	18.8	0.65	76.0	3.20
19.12.2011	20	0.34	0.18	9.3	0.55	55.6	1.00
Mean		0.44	0.15	15.3	0.50	67.3	2.29
SD		0.05	0.05	4.7	0.13	11.0	1.17
CV %		11	32	31	27	16	51
% of nominal				61.4	2.0	67.3	2.3
Dissolved (% of total)			34		3.3		3.4

Table 61: Additional uptake test with *Danio rerio:* Total Ag concentrations in different tissues (µg/kg).

Fish	**Control**			**25 µg/L**			**100 µg/L**		
	Head & skin	**Stomach & guts**	**Inner fish**	**Head & skin**	**Stomach & guts**	**Inner fish**	**Head & skin**	**Stomach & guts**	**Inner fish**
1	20.3	43.4	17.2	115	734°	54.9	667	6370	228
2	23.8	96.6	9.0	1046°	3899	105	548	13504	310
3	*	77.6	8.2	172	5040	48.4	471	36444	364
4	15.2	57.8	9.0	139	4395	327	573	27486	451
5	15.7	42.9	12.9	109	6676	45.2	535	17867	723
Mean	18.7	63.7	11.3	134	5003	116	559	20334	415
SD	4.0	23.2	3.8	28.4	1210	120	71.2	11816	190
CV %	21.6	36.4	33.6	21.2	24.2	104	12.7	58.1	45.8

* weighing error, sample excluded

° value regarded as outlier and excluded from statistical evaluation

For the tissue portions „head and skin“ and “inner fish” including filet and inner organs, similar total silbver concentrations were determined within each treatment. Stomach and guts exhibit approximately four times higher total silver concentrations than the other tissues in control fish, and approximately 45 times higher concentrations compared to other tissues in fish treated with nanosilver. Thus, total silver concentrations in fish as measured in the 2nd definitive test consist of a small part due to uptake as dissolved silver and a major part taken up as (or associated with) particles via ingestion. The latter fraction mainly remains in the guts without entering the inner fish but may contribute to the slight silver accumulation by providing dissolved silver ions.

4.7 Conclusion

A NOEC of 5.9 µg/L of nanosilver was determined in the valid 2nd test, based on an effect on growth, measured as total lengths. The threshold concentration for acute mortality of sensitive yolk sac larvae, most probably resulting from exposure to dissolved silver directly after renewal of the test dispersions (low complexation by organic carbon from feed and faeces), was determined in the 100 µg/L-treatment, total silver measured to be approximately 60 µg/L. The fish embryo test used as range finding test was able to predict the sensitivity of hatch, but not that of following life stages.

A comparison with literature data on fish early life stage or juvenile sensitivity to silver nanoparticles (e.g. no significant effects on growth at 6.2 µg/L in Sheepshead minnow [17]) indicates a high sensitivity of the described setup (static test with moving water, 7d – renewal of test dispersion, zebrafish as test species) concerning endpoints relevant for population dynamics and regulation.

Measurement of total silver concentrations in the fish after the exposure period showed significantly increasing concentrations with increasing Ag concentration in the test medium. In an additional experiment it was shown that most of the accumulated silver is located in the guts.

Pseudo-replicate cages located in one vessel per test concentration only may run into the risk of a bias by uneven conditions (1st test)). Thus, true replicates (containing pseudo-replicate chambers) are preferred, which can be statistically compared and combined if they do not differ significantly (2nd test).

4.8 Executive summary

Nanoparticulate silver was investigated in two fish early life stage toxicity tests [7] with *Danio rerio* in a large static system (250 L), in which the test dispersion was permanently mixed by pumps. The nominal test concentrations in the first test were 12.5; 25; 50; 100 and 200 µg Ag/L water and 12.5; 50 and 100 µg Ag/L water in the second test. In the 1st test one aquarium per treatment was used, containing four fish cages (pseudo-replicates). In the 2nd test, two aquaria per treatment contained six fish cages each. Chemical analysis of total silver concentrations in test media showed approximately 70 % of nominal concentrations during the 1st test and 50 % during the 2nd test. The proportion of dissolved silver was approximately 3 %. Hatch was not affected up to 136 µg/L (mean measured). Post-hatch survival was significantly reduced at concentrations ≥ 47 µg/L, the NOEC was determined to be 23 µg/L. The most sensitive endpoint was growth, measured as total individual length and wet weight with a NOEC of 5.9 µg/L.

The test setup was demonstrated to be suited for the testing of nanomaterials, proven by sensitive results and high statistical power.

4.9 Raw data

Table 62: Measured physical-chemical data during the 1st definitive test with *Danio rerio*.

Applied charge of NM-300 K	Date	Day of exposure	Treatmen t [µg/L]	Temperature [°C]	Oxygen [mg/L]	Oxygen saturation [%]	pH [-]
Charge no. 06107	17.05.2011	0	control	25.5	8.1	101	8.08
		0	dispersant	25.8	7.4	95	8.04
		0	12.5	25.6	7.6	96	8.07
		0	25	25.5	7.4	94	8.10
		0	50	25.5	7.5	95	8.08
		0	100	25.6	7.3	92	8.08
		0	200	25.8	7.4	95	8.10
	18.05.2011	1	control	25.3	8.1	98	8.20
		1	dispersant	25.5	8.2	98	8.18
		1	12.5	25.2	8.1	97	8.24
		1	25	25.2	8.1	96	8.22
		1	50	25.2	8.2	98	8.24
		1	100	25.3	8.2	98	8.20
		1	200	25.4	8.2	98	8.25
	19.05.2011	2	control	24.9	7.4	94	8.24
		2	dispersant	25.2	7.1	90	8.33
		2	12.5	24.9	7.2	91	8.36
		2	25	25.0	7.0	89	8.42
		2	50	25.0	7.1	90	8.38
		2	100	24.7	7.2	91	8.41
		2	200	24.9	7.1	89	8.41
	20.05.2011	3	control	26.0	7.2	93	8.23
		3	dispersant	26.3	7.1	92	8.27
		3	12.5	25.9	7.1	92	8.33
		3	25	25.7	7.1	92	8.33
		3	50	26.4	7.1	91	8.33
		3	100	26.0	7.1	91	8.33
		3	200	26.4	7.1	92	8.33
	23.05.2011	6	control	26.0	7.0	88	8.38
		6	dispersant	25.9	6.9	88	8.43
		6	12.5	25.6	6.9	87	8.41
		6	25	26.0	7.0	89	8.43
		6	50	25.9	6.9	88	8.43
		6	100	25.8	7.0	89	8.41
		6	200	26.1	7.0	89	8.41
Charge no. 06092	24.05.2011	7	control	25.3	7.3	93	8.12
		7	dispersant	25.0	7.3	93	8.16
		7	12.5	25.2	7.4	93	8.12
		7	25	25.0	7.4	94	8.15
		7	50	25.4	7.2	92	8.17
		7	100	25.5	7.3	93	8.14
		7	200	25.0	7.3	94	8.15
	25.05.2011	8	control	25.3	7.5	94	8.24
		8	dispersant	25.0	7.4	93	8.26
		8	12.5	25.2	7.3	92	8.23
		8	25	25.2	7.2	92	8.23
		8	50	25.2	7.4	93	8.23
		8	100	25.1	7.4	93	8.26
		8	200	24.7	7.4	93	8.25
	26.05.2011	9	control	25.3	7.3	92	8.28
		9	dispersant	24.9	7.1	92	8.32
		9	12.5	24.8	7.0	90	8.30
		9	25	25.1	7.1	91	8.32
		9	50	24.9	7.1	91	8.34
		9	100	24.9	7.2	91	8.32
		9	200	24.6	7.3	92	8.32

Applied charge of NM-300 K	Date	Day of exposure	Treatmen t [µg/L]	Temperature [°C]	Oxygen [mg/L]	Oxygen saturation [%]	pH [-]
	27.05.2011	10	control	25.8	7.1	90	8.36
		10	dispersant	25.1	6.9	87	8.35
		10	12.5	24.6	7.2	89	8.34
		10	25	24.8	7.2	90	8.38
		10	50	24.9	7.0	89	8.36
		10	100	24.9	7.1	90	8.35
		10	200	24.7	7.1	90	8.37
Charge no. 0387	30.05.2011	13	control	26.4	6.6	87	8.46
		13	dispersant	26.3	6.9	90	8.46
		13	12.5	26.1	7.3	92	8.49
		13	25	26.1	6.9	90	8.54
		13	50	26.1	6.7	88	8.51
		13	100	26.1	7.1	91	8.52
		13	200	26.4	7.1	92	8.56
	31.05.2011	14	control	25.0	7.5	95	8.22
		14	dispersant	25.8	8.1	99	8.21
		14	12.5	25.2	7.7	96	8.20
		14	25	25.5	7.5	95	8.20
		14	50	25.5	7.4	94	8.20
		14	100	25.2	7.5	94	8.22
		14	add. control	25.6	7.1	92	8.46
		14	6.25	26.8	7.4	94	8.48
		14	12.5	25.6	7.7	93	8.05
	01.06.2011	15	control	25.4	7.1	89	8.33
		15	dispersant	25.4	7.2	89	8.33
		15	12.5	25.4	7.2	89	8.41
		15	25	25.2	7.3	91	8.36
		15	50	25.4	7.2	90	8.38
		15	100	25.2	7.3	90	8.41
		15	add. control	25.8	7.3	91	8.61
		15	6.25	26.4	7.1	88	8.61
		15	12.5	25.6	7.2	89	8.44
	03.06.2011	17	control	25.5	6.7	84	8.39
		17	dispersant	25.9	6.9	89	8.39
		17	12.5	25.2	7.2	89	8.41
		17	25	25.5	7.1	89	8.42
		17	50	25.6	6.8	86	8.41
		17	100	25.4	7.0	88	8.40
		17	add. control	25.9	6.9	87	8.53
		17	6.25	26.0	7.0	88	8.57
		17	12.5	25.9	7.0	87	8.48
Charge no. 06111	06.06.2011	20	control	25.8	7.1	90	8.36
		20	dispersant	26.1	7.1	92	8.41
		20	12.5	25.6	6.9	90	8.39
		20	25	25.7	6.9	88	8.45
		20	50	25.8	6.9	88	8.41
		20	100	25.9	7.0	91	8.39
		20	add. control	26.4	7.0	91	8.52
		20	6.25	26.1	7.2	92	8.54
		20	12.5	26.1	6.9	90	8.47
	07.06.2011	21	control	25.3	7.0	91	8.11
		21	dispersant	25.6	6.9	93	8.09
		21	12.5	25.2	7.0	90	8.13
		21	25	25.5	7.0	90	8.10
		21	50	25.5	7.0	91	8.09
		21	100	25.6	7.0	90	8.09
		21	add. control	25.6	6.7	88	8.47
		21	6.25	25.7	7.0	90	8.14
		21	12.5	26.0	6.7	87	8.44

Applied charge of NM-300 K	Date	Day of exposure	Treatmen t [µg/L]	Temperature [°C]	Oxygen [mg/L]	Oxygen saturation [%]	pH [-]
	08.06.2011	22	control	26.1	6.9	91	8.24
		22	dispersant	26.2	7.0	92	8.24
		22	12.5	25.7	7.0	94	8.31
		22	25	25.8	7.1	93	8.24
		22	50	25.6	7.2	94	8.24
		22	100	25.9	7.0	92	8.24
		22	add. control	26.3	7.1	93	8.54
		22	6.25	26.7	6.9	90	8.37
		22	12.5	26.1	6.8	87	8.50
	09.06.2011	23	control	25.9	6.6	85	8.31
		23	dispersant	25.6	6.9	89	8.39
		23	12.5	25.2	7.0	89	8.41
		23	25	24.9	7.2	91	8.41
		23	50	24.9	7.1	90	8.40
		23	100	24.5	7.1	90	8.39
		23	add. control	26.0	7.0	91	8.54
		23	6.25	25.6	7.1	90	8.47
		23	12.5	25.7	7.1	89	8.51
	10.06.2011	24	control	25.5	6.8	86	8.36
		24	dispersant	25.6	6.9	88	8.38
		24	12.5	24.7	7.2	91	8.36
		24	25	24.7	7.3	91	8.33
		24	50	24.8	7.2	91	8.37
		24	100	24.6	7.3	91	8.35
		24	add. control	25.9	6.9	88	8.49
		24	6.25	25.3	7.3	91	8.45
		24	12.5	25.6	7.2	90	8.45
	14.06.2011	28	control	26.5	6.4	83	8.38
		28	dispersant	26.1	6.9	89	8.48
		28	12.5	26.0	7.0	91	8.49
		28	25	26.1	6.9	90	8.44
		28	50	26.1	6.7	87	8.43
		28	100	26.3	6.8	88	8.43
Charge no. 06126	14.06.2011	28	control	25.2	7.4	95	8.20
		28	dispersant	25.5	7.2	92	8.20
		28	12.5	25.2	7.1	91	8.22
		28	25	25.5	7.3	93	8.20
		28	50	25.5	7.5	96	8.20
		28	100	25.1	7.6	97	8.21
		28	add. control	26.4	7.0	91	8.69
		28	6.25	25.8	7.4	95	8.26
		28	12.5	26.3	7.3	92	8.50
	15.06.2011	29	control	25.4	7.2	92	8.22
		29	dispersant	25.7	7.3	94	8.25
		29	12.5	25.2	7.4	94	8.29
		29	25	25.5	7.3	93	8.26
		29	50	25.5	7.2	92	8.26
		29	100	25.4	7.4	94	8.27
		29	add. control	26.0	7.3	93	8.65
		29	6.25	26.1	7.4	94	8.36
		29	12.5	25.7	7.2	92	8.46
	16.06.2011	30	control	25.2	6.9	88	8.29
		30	dispersant	26.1	7.0	89	8.30
		30	12.5	25.7	6.9	89	8.34
		30	25	25.8	7.2	91	8.32
		30	50	25.8	7.1	90	8.32
		30	100	25.4	7.2	92	8.32
		30	add. control	25.6	7.3	92	8.65
		30	6.25	26.4	7.1	92	8.44
		30	12.5	25.9	7.0	90	8.41

Applied charge of NM-300 K	Date	Day of exposure	Treatmen t [µg/L]	Temperature [°C]	Oxygen [mg/L]	Oxygen saturation [%]	pH [-]
	17.06.2011	31	control	26.1	6.8	87	8.36
		31	dispersant	26.4	6.8	88	8.38
		31	12.5	26.1	7.0	91	8.43
		31	25	26.4	7.0	90	8.43
		31	50	26.4	6.7	85	8.40
		31	100	26.3	6.9	88	8.42
		31	add. control	26.1	7.4	93	8.68
		31	6.25	26.8	7.0	90	8.50
		31	12.5	26.4	7.0	88	8.46
	20.06.2011	34	control	26.4	6.7	86	8.46
		34	dispersant	26.5	6.8	87	8.45
		34	12.5	26.3	6.7	85	8.47
		34	25	26.6	6.7	86	8.45
		34	50	26.6	6.6	86	8.56
		34	100	26.4	6.7	86	8.48
		34	add. control	26.3	7.1	91	8.70
		34	6.25	27.0	6.9	90	8.54
		34	12.5	26.4	7.0	88	8.48
	21.06.2011	35	control	26.1	6.4	83	8.44
		35	dispersant	26.4	6.8	89	8.42
		35	12.5	26.3	7.2	91	8.45
		35	25	26.5	7.0	91	8.45
		35	50	26.5	6.8	89	8.45
		35	100	26.4	6.8	89	8.45
		35	add. control	26.3	7.1	92	8.69
		35	6.25	26.9	6.9	90	8.54
		35	12.5	26.4	7.0	89	8.49

Table 63: Measured physical-chemical data during the 2nd definitive test with *Danio rerio*.

Date	Day of exposure	Conc. [µg/L]	Temp. [°C]	O_2 [mg/L]	O_2 saturat. [%]	pH
02.08.2011	0	control (I)	26.5	7.6	98	8.55
	0	control (II)	26.6	7.4	96	8.45
	0	12.5 (I)	26.4	7.8	98	8.60
	0	12.5 (II)	26.3	7.8	100	8.62
	0	50 (I)	26.3	7.6	98	8.57
	0	50 (II)	26.3	7.6	98	8.64
	0	100 (I)	27.3	7.7	99	8.66
	0	100 (II)	26.4	7.6	98	8.66
03.08.2011	1	control (I)	26.6	7.3	93	8.62
	1	control (II)	26.4	7.0	90	8.57
	1	12.5 (I)	26.4	6.9	89	8.62
	1	12.5 (II)	26.6	6.9	89	8.64
	1	50 (I)	26.5	7.1	89	8.64
	1	50 (II)	26.5	7.0	90	8.64
	1	100 (I)	27.4	7.1	90	8.71
	1	100 (II)	26.5	6.9	90	8.67
04.08.2011	2	control (I)	26.3	7.0	90	8.60
	2	control (II)	26.5	7.2	93	8.59
	2	12.5 (I)	26.3	7.2	92	8.64
	2	12.5 (II)	26.3	7.3	93	8.65
	2	50 (I)	26.4	6.9	87	8.66
	2	50 (II)	26.4	7.0	89	8.62
	2	100 (I)	27.3	6.9	89	8.67
	2	100 (II)	26.4	7.0	89	8.65
05.08.2011	3	control (I)	25.8	7.3	95	8.59
	3	control (II)	25.2	7.3	94	8.59
	3	12.5 (I)	24.3	7.2	92	8.64
	3	12.5 (II)	25.6	7.2	92	8.63
	3	50 (I)	24.6	7.3	93	8.63
	3	50 (II)	24.6	7.2	92	8.62

Date	Day of exposure	Conc. [µg/L]	Temp. [°C]	O_2 [mg/L]	O_2 saturat. [%]	pH
	3	100 (I)	25.0	7.4	94	8.63
	3	100 (II)	24.5	7.3	93	8.63
08.08.2011	6	control (I)	26.6	7.2	94	8.65
	6	control (II)	26.3	7.1	93	8.62
	6	12.5 (I)	25.9	7.0	92	8.62
	6	12.5 (II)	26.6	7.0	91	8.63
	6	50 (I)	26.1	7.0	91	8.62
	6	50 (II)	26.1	6.8	88	8.60
	6	100 (I)	26.5	7.0	90	8.62
	6	100 (II)	26.1	6.9	90	8.63
09.08.2011	7	control (I)	25.5	8.1	98	8.61
	7	control (II)	25.5	7.7	97	8.44
	7	12.5 (I)	25.5	7.5	96	8.58
	7	12.5 (II)	26.3	7.2	95	8.62
	7	50 (I)	25.5	7.5	95	8.61
	7	50 (II)	25.7	7.4	95	8.51
	7	100 (I)	25.6	7.6	95	8.61
	7	100 (II)	25.8	7.6	94	8.48
10.08.2011	8	control (I)	25.3	7.1	89	8.59
	8	control (II)	26.4	7.1	91	8.43
	8	12.5 (I)	25.5	7.3	92	8.64
	8	12.5 (II)	25.5	7.2	90	8.67
	8	50 (I)	25.7	7.2	91	8.67
	8	50 (II)	25.6	7.3	92	8.59
	8	100 (I)	25.6	7.1	90	8.64
	8	100 (II)	25.7	7.3	91	8.58
11.08.2011	9	control (I)	25.5	7.3	92	8.65
	9	control (II)	26.0	7.2	93	8.55
	9	12.5 (I)	25.1	7.4	93	8.68
	9	12.5 (II)	26.3	7.2	92	8.68
	9	50 (I)	25.8	7.2	93	8.70
	9	50 (II)	25.9	7.4	94	8.64
	9	100 (I)	25.9	7.3	93	8.70
	9	100 (II)	25.8	7.2	92	8.64
12.08.2011	10	control (I)	26.1	7.2	93	8.59
	10	Control (II)	26.4	7.2	94	8.56
	10	12.5 (I)	25.8	7.2	93	8.67
	10	12.5 (II)	26.4	7.1	92	8.65
	10	50 (I)	25.9	7.2	93	8.70
	10	50 (II)	25.7	7.2	93	8.67
	10	100 (I)	25.6	7.3	93	8.70
	10	100 (II)	25.6	7.2	93	8.65
15.08.2011	13	Control (I)	26.5	6.9	90	8.60
	13	Control (II)	26.7	6.9	91	8.61
	13	12.5 (I)	26.3	7.0	91	8.64
	13	12.5 (II)	26.9	6.9	90	8.62
	13	50 (I)	26.4	7.1	91	8.65
	13	50 (II)	26.6	7.0	90	8.65
	13	100 (I)	26.5	7.1	92	8.66
	13	100 (II)	26.6	7.0	90	8.66
16.08.2011	14	control (I)	25.3	7.5	95	8.65
	14	control (II)	25.2	7.5	95	8.49
	14	12.5 (I)	25.2	7.5	96	8.43
	14	12.5 (II)	26.0	7.5	93	8.68
	14	50 (I)	25.4	7.4	94	8.45
	14	50 (II)	25.4	7.5	94	8.43
	14	100 (I)	25.4	7.4	94	8.70
	14	100 (II)	25.0	7.5	94	8.68
17.08.2011	15	control (I)	26.0	7.3	92	8.61
	15	control (II)	25.8	7.3	93	8.52

Date	Day of exposure	Conc. [µg/L]	Temp. [°C]	O_2 [mg/L]	O_2 saturat. [%]	pH
	15	12.5 (I)	25.6	7.5	93	8.48
	15	12.5 (II)	26.0	7.4	94	8.61
	15	50 (I)	25.7	7.4	93	8.49
	15	50 (II)	25.8	7.3	93	8.48
	15	100 (I)	26.4	7.4	93	8.64
	15	100 (II)	25.7	7.3	93	8.61
18.08.2011	16	control (I)	25.7	7.1	91	8.62
	16	control (II)	24.9	7.1	91	8.53
	16	12.5 (I)	24.3	7.2	91	8.52
	16	12.5 (II)	25.6	7.3	93	8.62
	16	50 (I)	24.6	7.2	90	8.53
	16	50 (II)	24.4	7.2	90	8.53
	16	100 (I)	24.8	7.3	92	8.66
	16	100 (II)	24.3	7.3	92	8.63
19.08.2011	17	control (I)	26.6	7.2	92	8.59
	17	control (II)	26.3	7.1	93	8.58
	17	12.5 (I)	25.7	7.1	92	8.50
	17	12.5 (II)	26.6	7.1	92	8.61
	17	50 (I)	25.8	7.2	92	8.53
	17	50 (II)	25.7	6.9	90	8.53
	17	100 (I)	26.4	7.1	92	8.62
	17	100 (II)	25.6	7.3	93	8.60
22.08.2011	20	control (I)	26.6	7.0	90	8.52
	20	control (II)	26.3	6.9	89	8.52
	20	12.5 (I)	25.9	6.9	89	8.49
	20	12.5 (II)	26.5	7.0	91	8.54
	20	50 (I)	26.3	7.0	89	8.58
	20	50 (II)	26.1	7.0	89	8.52
	20	100 (I)	26.6	7.0	90	8.60
	20	100 (II)	26.1	7.2	92	8.60
23.08.2011	21	control (I)	25.9	7.5	95	8.66
	21	control (II)	25.6	7.8	100	8.49
	21	12.5 (I)	25.5	7.6	97	8.66
	21	12.5 (II)	26.1	7.7	95	8.74
	21	50 (I)	25.7	7.8	96	8.66
	21	50 (II)	25.8	7.6	97	8.62
	21	100 (I)	25.6	7.4	96	8.64
	21	100 (II)	25.9	7.3	95	8.64
24.08.2011	22	Control (I)	26.5	7.4	95	8.65
	22	Control (II)	26.4	7.3	96	8.47
	22	12.5 (I)	26.4	7.2	93	8.62
	22	12.5 (II)	26.6	7.5	93	8.65
	22	50 (I)	26.4	7.4	95	8.60
	22	50 (II)	26.4	7.4	94	8.55
	22	100 (I)	26.4	7.6	95	8.62
	22	100 (II)	26.1	7.2	94	8.62
25.08.2011	23	control (I)	26.1	6.9	89	8.52
	23	control (II)	26.5	6.8	88	8.40
	23	12.5 (I)	25.4	7.0	89	8.53
	23	12.5 (II)	26.4	6.8	88	8.55
	23	50 (I)	25.5	7.1	89	8.56
	23	50 (II)	25.5	6.9	89	8.52
	23	100 (I)	25.2	7.1	91	8.56
	23	100 (II)	25.2	7.0	89	8.56
26.08.2011	24	control (I)	26.8	6.5	86	8.48
	24	control (II)	27.0	6.6	87	8.44
	24	12.5 (I)	26.4	6.6	85	8.51
	24	12.5 (II)	27.0	6.7	87	8.54
	24	50 (I)	26.4	6.7	88	8.53
	24	50 (II)	26.4	6.6	87	8.48

Date	Day of exposure	Conc. [µg/L]	Temp. [°C]	O_2 [mg/L]	O_2 saturat. [%]	pH
	24	100 (I)	26.4	6.9	90	8.56
	24	100 (II)	26.4	6.7	89	8.54
29.08.2011	27	control (I)	26.8	6.6	86	8.47
	27	control (II)	26.6	6.5	85	8.46
	27	12.5 (I)	26.4	6.6	86	8.47
	27	12.5 (II)	26.5	6.5	84	8.48
	27	50 (I)	26.6	7.0	87	8.54
	27	50 (II)	26.5	6.4	84	8.48
	27	100 (I)	26.5	6.6	86	8.54
	27	100 (II)	26.6	6.7	86	8.54
30.08.2011	28	control (I)	25.4	7.5	94	8.62
	28	control (II)	25.5	7.5	96	8.56
	28	12.5 (I)	25.1	7.3	93	8.72
	28	12.5 (II)	26.1	7.3	92	8.69
	28	50 (I)	25.4	7.4	94	8.74
	28	50 (II)	25.5	7.4	93	8.70
	28	100 (I)	25.6	7.4	94	8.71
	28	100 (II)	25.5	7.3	92	8.69
31.08.2011	29	control (I)	25.7	7.0	89	8.67
	29	control (II)	25.5	7.1	91	8.52
	29	12.5 (I)	25.5	7.0	89	8.64
	29	12.5 (II)	25.5	7.1	89	8.58
	29	50 (I)	25.5	7.1	89	8.59
	29	50 (II)	25.5	7.1	91	8.58
	29	100 (I)	26.0	6.9	88	8.64
	29	100 (II)	25.5	7.1	90	8.59
01.09.2011	30	control (I)	25.7	6.7	85	8.54
	30	control (II)	25.8	6.7	85	8.44
	30	12.5 (I)	25.4	6.7	85	8.54
	30	12.5 (II)	25.2	6.5	82	8.43
	30	50 (I)	25.6	6.6	85	8.54
	30	50 (II)	25.6	6.7	85	8.54
	30	100 (I)	25.6	7.0	88	8.58
	30	100 (II)	25.4	6.9	86	8.57
02.09.2011	31	control (I)	26.1	6.6	83	8.41
	31	control (II)	26.3	6.5	84	8.35
	31	12.5 (I)	26.0	6.8	87	8.45
	31	12.5 (II)	25.6	6.6	84	8.40
	31	50 (I)	26.3	6.7	85	8.49
	31	50 (II)	26.3	6.7	86	8.49
	31	100 (I)	26.1	6.8	88	8.51
	31	100 (II)	26.1	6.5	85	8.51
05.09.2011	34	control (I)	26.5	6.2	80	8.31
	34	control (II)	26.5	6.0	77	8.26
	34	12.5 (I)	26.3	6.2	80	8.32
	34	12.5 (II)	25.8	6.6	85	8.41
	34	50 (I)	26.6	6.4	83	8.42
	34	50 (II)	26.5	6.4	82	8.41
	34	100 (I)	26.8	6.8	88	8.47
	34	100 (II)	26.6	6.5	83	8.41
06.09.2011	35	control (I)	26.2	6.2	79	8.26
	35	control (II)	26.4	6.0	76	8.24
	35	12.5 (I)	26.0	6.7	85	8.41
	35	12.5 (II)	25.7	6.5	82	8.31
	35	50 (I)	26.4	6.7	84	8.40
	35	50 (II)	26.3	6.5	84	8.41
	35	100 (I)	26.5	6.8	87	8.45
	35	100 (II)	26.3	6.6	84	8.40

Table 64: 1st definitive test with *Danio rerio*: Raw data of water analyses.
ICP-OES at 20.05.2011 (samples: 19.05), and 23.05.2011 (samples: 23.05.). Ag3280 used for analysis.

Date	Day	sample	Ag3280 µg/L Probe	Ag3382 µg/L Probe	Ag3280 µg/L Testlsg.	Ag3382 µg/L Testlsg.	Recovery %
19.05.2011	2	Ag d2 Vehicle 3ml A	-0.05	-0.59	-0.25	-2.94	
	2	Ag d2 Vehicle 3ml B	-0.08	-0.85	-0.42	-4.27	
	2	Ag d2 Control 3ml A	-0.36	0.05	-1.8	0.26	
	2	Ag d2 Control 3ml B	0.06	0.04	0.29	0.2	
	2	Ag d2 12.5µg/L 3ml A	1.72	2.94	8.59	14.7	68.7
	2	Ag d2 12.5µg/L 3ml B	2.24	3.21	11.2	16.1	89.6
	2	Ag d2 25 µg/L 3ml A	3.69	3.52	18.5	17.6	73.8
	2	Ag d2 25 µg/L 3ml B	3.52	4.16	17.6	20.8	70.3
	2	Ag d2 50 µg/L 3ml A	7.23	9.34	36.2	46.7	72.3
	2	Ag d2 50 µg/L 3ml B	6.46	7.06	32.3	35.3	64.6
	2	Ag d2 100 µg/L 3ml A	13.6	13.3	68	66.3	68
	2	Ag d2 100 µg/L 3ml B	14.2	15.4	70.9	77	70.9
	2	Ag d2 200 µg/L 3ml A	27.6	26.7	138	134	69.1
	2	Ag d2 200 µg/L 3ml B	28.3	25.8	142	129	70.8
	2	TM-DWS.2	9.93	10.9			100
	2	TM-DWS.2	9.75	11.4			98
	2	TM-DWS.2	9.31	11.6			93.7
	2	TM-DWS.2	9.66	10.9			97.2
	2	TMDA 70	10.7	11.2			98
	2	TMDA 70	10.8	10			98.7
	2	TMDA 70	11.2	10.7			103
	2	TMDA 70	11.1	11.3			102
	2	Ag 25 µg/L	23.5	24			94.1
	2	Ag 25 µg/L	24.3	24.5			97.4
	2	MerkIV 50 µg/L	48.1	49.6			96.2
	2	MerkIV 50 µg/L	48.3	45.9			96.6
23.05.2011	6	Ag d6 Vehicle 3ml A	1.02	2.68	5.12	13.38	
	6	Ag d6 Vehicle 3ml B	0.03	0.15	0.16	0.76	
	6	Ag d6 Control 3ml A	0.65	0.2	3.25	1.02	
	6	Ag d6 Control 3ml B	0.31	-1.84	1.54	-9.2	
	6	Ag d6 12.5µg/L 3ml A	2.94	3.29	14.72	16.4	117.7
	6	Ag d6 12.5µg/L 3ml B	1.98	2.96	9.9	14.8	79
	6	Ag d6 25 µg/L 3ml A	3.39	2.96	16.9	14.8	67.7
	6	Ag d6 25 µg/L 3ml B	3.66	2.96	18.3	14.8	73.2
	6	Ag d6 50 µg/L 3ml A	5.53	3.18	27.7	15.9	55.3
	6	Ag d6 50 µg/L 3ml B	5.22	7.26	26.1	36.3	52.2
	6	Ag d6 100 µg/L 3ml A	11.6	10.4	57.9	52.1	57.9
	6	Ag d6 100 µg/L 3ml B	10.5	12	52.4	60.1	52.4
	6	Ag d6 200 µg/L 3ml A	27.2	26.9	136	135	67.9
	6	Ag d6 200 µg/L 3ml B	27.4	25	137	125	68.4
	6	MerkIV 3ml 200µg/L A	42.1	42	210	210	105
	6	MerkIV 3ml 200µg/L B	42.1	42.3	211	212	105
	6	MerkIV 50 µg/L	50.4	49.5			101
	6	TM-DWS.2	10.42	14			105
	6	TM-DWS.2	10.28	11.6			103
	6	TM-DWS.2	10.18	11.1			102
	6	TM-DWS.2	10.65	12.2			107
	6	TMDA 70	11.3	12.6			104
	6	TMDA 70	10.1	11.4			92.2
	6	TMDA 70	12	11.2			110
	6	TMDA 70	11.2	12.3			102
	6	Ag 25 µg/L	25.1	24.5			100
	6	Ag 25 µg/L	24.6	26			98.3
26.05.2011	9	Ag d6 Vehicle 3ml A	0.3442	-0.2927	1.72	-1.46	
	9	Ag d6 Vehicle 3ml B	-0.5324	2.228	-2.66	11.14	
	9	Ag d6 Control 3ml A	0.5368	0.9692	2.68	4.85	
	9	Ag d6 Control 3ml B	0.1618	-0.7881	0.81	-3.94	
	9	Ag d6 12.5µg/L 3ml A	1.084	5.254	5.42	26.3	43.4

Table 64-2: ICP-OES at 27.05.2011 (samples: 26.05), and 31.05.2011 (samples: 30.05.). Ag3280 used for analysis.

Date	Day	sample	Ag3280 µg/L Probe	Ag3382 µg/L Probe	Ag3280 µg/L Testlsg.	Ag3382 µg/L Testlsg.	Recovery %
26.05.2011	9	Ag d6 12.5µg/L 3ml B	1.879	6.295	9.4	31.5	75.2
	9	Ag d6 25 µg/L 3ml A	4.03	4.703	20.2	23.5	80.6
	9	Ag d6 25 µg/L 3ml B	3.431	7.897	17.2	39.5	68.6
	9	Ag d6 50 µg/L 3ml A	7.007	8.392	35.0	42.0	70.1
	9	Ag d6 50 µg/L 3ml B	6.779	7.557	33.9	37.8	67.8
	9	Ag d6 100 µg/L 3ml A	12.75	11.68	63.8	58.4	63.8
	9	Ag d6 100 µg/L 3ml B	14.33	15.12	71.7	75.6	71.7
	9	Ag d6 200 µg/L 3ml A	27.84	29.46	139	147	69.6
	9	Ag d6 200 µg/L 3ml B	27.35	26.78	137	134	68.4
	9	MerkIV 3ml 200µg/L A	40.77	40.77	204	204	102
	9	MerkIV 3ml 200µg/L B	40.43	41.3	202	207	101
	9	TM-DWS.2	11.78	12.89			119
	9	TM-DWS.2	10.76	10.69			108
	9	TM-DWS.2	11.75	11.9			118
	9	TM-DWS.2	11.32	13.82			114
	9	TMDA 70	12.34	9.862			113
	9	TMDA 70	11.6	11.95			106
	9	TMDA 70	12.01	11.78			110
	9	TMDA 70	12.66	11.57			116
	9	Ag 25 µg/L	24.7	24.14			98.8
	9	Ag 25 µg/L	24.95	25.74			99.8
	9	MerkIV 50 µg/L	52	51.59			104
	9	MerkIV 50 µg/L	52.11	50.91			104
30.05.2011	13	Ag d2 Vehicle 3ml A	-0.37	-1.75	-1.84	-8.73	
	13	Ag d2 Vehicle 3ml B	-0.12	-1.20	-0.60	-6.01	
	13	Ag d2 Vehicle 5ml C	-0.09	0.00	-0.28	-0.01	
	13	Ag d2 Control 3ml A	-0.65	-0.82	-3.24	-4.10	
	13	Ag d2 Control 3ml B	0.32	0.11	1.60	0.53	
	13	Ag d2 Control 5ml C	0.04	-0.93	0.12	-2.79	
	13	Ag d2 12.5µg/L 3ml A	1.26	1.69	6.31	8.4	50.5
	13	Ag d2 12.5µg/L 3ml B	1.09	1.09	5.5	5.4	43.7
	13	Ag d2 12.5µg/L 5ml C	1.21	3.05	3.64	9.15	29.1
	13	Ag d2 25 µg/L 3ml A	2.96	2.56	14.8	12.8	59.1
	13	Ag d2 25 µg/L 3ml B	3.44	2.18	17.2	10.9	68.8
	13	Ag d2 25 µg/L 5ml C	4.72	5.71	14.2	17.1	56.7
	13	Ag d2 50 µg/L 3ml A	5.74	4.90	28.7	24.5	57.4
	13	Ag d2 50 µg/L 3ml B	5.85	5.39	29.2	27.0	58.5
	13	Ag d2 50 µg/L 5ml C	10.13	10.73	30.4	32.2	60.8
	13	Ag d2 100 µg/L 3ml A	12.3	12.3	61.3	61.3	61.3
	13	Ag d2 100 µg/L 3ml B	12.8	12.1	64.0	60.7	64.0
	13	Ag d2 100 µg/L 5ml C	20.5	21.3	61.4	64.0	61.4
	13	Ag d2 200 µg/L 3ml A	26.7	26.9	133	135	66.7
	13	Ag d2 200 µg/L 3ml B	24.8	24.7	124	123	62.1
	13	Ag d2 200 µg/L 5ml C	42.5	44.8	128	134	63.8
	13	MerkIV 3ml 200µg/L A	40.5	41.1	202	205	101
	13	MerkIV 3ml 200µg/L B	42.2	42.0	211	210	105
	13	TM-DWS.2	10.28	8.6			103
	13	TM-DWS.2	9.68	11.1			97.4
	13	TM-DWS.2	9.82	12.0			98.8
	13	TM-DWS.2	8.30	11.7			83.5
	13	TMDA 70	10.4	12.4			95.8
	13	TMDA 70	11.5	9.6			105
	13	TMDA 70	12.0	13.4			110
	13	TMDA 70	12.7	10.2			116
	13	Ag 25 µg/L	24.0	22.1			96.2
	13	Ag 25 µg/L	25.4	24.7			101
	13	MerkIV 50 µg/L	50.7	53.6			101
	13	MerkIV 50 µg/L	52.5	49.9			105

Table 64-3: ICPMS at 01.06.2011 and 06.06.2011 (based on Ag/107 (#1)).

Date	Day	sample	Ag / 107 [#1]	Ag / 107 [#2]	Ag / 109 [#1]	Ag / 109 [#2]	Recov. %
01.06.2011	15	blank01	0.006371	0.00516	0.007009	0.01151	
	15	UHQ 1:3 a	0.001859	0.003716	0.001564	0.001093	
	15	UHQ 1:3 b	0.00343	0.002413	0.003413	0.005177	
	15	Ag Control A d15 1:3	0.01569	0.006872	0.01515	0.009302	
	15	Ag Control B d15 1:3	0.006015	0.00634	0.01686	0.006613	
	15	Ag Control A d15 1:3 neu	1.357	1.26	1.354	1.254	
	15	Ag Control B d15 1:3 neu	1.355	1.365	1.33	1.295	
	15	Ag Vehicle A d15 1:3	0.01267	0.01387	0.01161	0.01043	
	15	Ag Vehicle B d15 1:3	0.01161	0.01912	0.01004	0.01459	
	15	TMDWS 1:5a	2.086	1.908	2.099	1.906	105.3
	15	TMDWS 1:5b	2.025	1.873	2.03	1.858	101.8
	15	Merck IV 50 µg/L 1:3	14.98	21.55	14.91	21.47	89.4
	15	blank02	0.01147	0.01548	0.01133	0.02003	
	15	Ag 6,25 µg/L d15 A 1:3	4.338	3.903	4.316	3.888	207.2
	15	Ag 6,25 µg/L d15 B 1:3	3.296	3.688	3.275	3.718	157.2
	15	Ag 12,5 µg/L d15 A 1:3	3.25	3.904	3.242	3.892	77.8
	15	Ag 12,5 µg/L d15 B 1:3	3.461	3.397	3.458	3.374	83.0
	15	blank03	0.002569	0.001442	0.002603	0.004236	
	15	Ag 12,5 µg/L d15 A 1:3 neu	3.156	3.049	3.162	3.055	75.9
	15	Ag 12,5 µg/L d15 B 1:3 neu	2.857	3.283	2.84	3.288	68.2
	15	Ag 25 µg/L d15 A 1:3	7.247	5.518	7.158	5.491	85.9
	15	Ag 25 µg/L d15 B 1:3	7.114	7.777	7.089	7.728	85.1
	15	blank03	0.001493	0.001378	0.001768	0.001098	
	15	Ag 50 µg/L d15 A 1:3	12.47	11.57	12.38	11.54	74.3
	15	Ag 50 µg/L d15 B 1:3	14.43	13.59	14.34	13.7	86.0
	15	Ag 100 µg/L d15 A 1:3	24.75	24.26	24.56	24.04	73.7
	15	Ag 100 µg/L d15 B 1:3	27.15	28.44	26.17	28.22	78.5
	15	blank04	0.01118	0.02084	0.01025	0.02187	
	15	TMDA70c 1:10	1.151	1.31	1.147	1.295	105.2
	15	TMDA70d 1:10	1.196	1.026	1.166	1.055	107.0
	15	blank05	0.001246	0.001393	0.0007734	0.0009956	
06.06.2011	20	blank01	0.008758	0.005623	0.00776	0.01068	
	20	UHQ 1:3 a	0.01035	0.006662	0.01042	0.01084	
	20	UHQ 1:3 b	0.003734	0.002048	0.004011	0.005205	
	20	Ag Control A d20 1:3	0.03692	0.02964	0.04151	0.03939	
	20	Ag Control B d20 1:3	0.02452	0.02193	0.02345	0.02082	
	20	Ag Control A d20 1:3 neu	1.336	1.29	1.31	1.372	
	20	Ag Control B d20 1:3 neu	1.625	1.392	1.597	1.38	
	20	Ag Vehicle A d20 1:3	0.006953	0.006732	0.006076	0.004634	
	20	Ag Vehicle B d20 1:3	0.003478	0.001731	0.004443	0.003744	
	20	TMDWS 1:5a	2.26	2.154	2.181	2.087	109.4
	20	TMDWS 1:5b	2.042	1.834	2.001	1.856	100.4
	20	Merck IV 25 µg/L 1:3	8.726	7.632	8.603	7.569	103.2
	20	blank02	0.003106	0.002944	0.002712	0.006937	
	20	Ag 6,25 µg/L d20 A 1:3	4.887	4.443	4.806	4.437	230.7
	20	Ag 6,25 µg/L d20 B 1:3	4.531	4.425	4.511	4.457	216.5
	20	Ag 12,5 µg/L d20 A 1:3	3.013	3.182	2.973	3.134	71.4
	20	Ag 12,5 µg/L d20 B 1:3	3.198	2.958	3.182	3.072	76.4
	20	blank03	0.003395	0.000382	0.002475	0.001966	
	20	Ag 12,5 µg/L d20 A 1:3 neu	2.576	2.38	2.536	2.399	60.9
	20	Ag 12,5 µg/L d20 B 1:3 neu	2.596	2.534	2.581	2.649	61.9
	20	Ag 25 µg/L d20 A 1:3	6.252	5.641	6.198	5.64	74.4
	20	Ag 25 µg/L d20 B 1:3	5.825	5.995	5.76	5.94	69.1
	20	blank04	0.002508	0.001018	0.003049	0.004699	
	20	Ag 50 µg/L d20 A 1:3	9.963	11.06	9.854	11.1	59.1
	20	Ag 50 µg/L d20 B 1:3	11.87	9.453	11.72	9.565	70.3
	20	Ag 100 µg/L d20 A 1:3	24.19	19.03	23.98	19.31	71.9
	20	Ag 100 µg/L d20 B 1:3	23.21	26.25	23	26.55	69.0
	20	blank05	0.01181	0.01759	0.01308	0.01841	
	20	TMDA70c 1:10	1.151	1.04	1.148	1.016	105.3
	20	TMDA70d 1:10	1.158	0.9936	1.134	1.035	104.0

	20	blank06	0.0001039	-0.0003325	0.0007573	0.001656	
	20	Stammlsg.A20g/L 1:30	69.82	57.78	69.43	58.47	104.1
	20	Stammlsg.B20g/L 1:30	67.15	61.76	66.81	62.08	100.2
	20	blank07	0.01591	0.01382	0.01569	0.01594	

Table 64.4: ICPMS at 09.06.2011 (based on Ag/107 (#1)) and 14.06.2011 (based on Ag/107 (#2)).

Date	Day	sample	Ag / 107 [#1]	Ag / 107 [#2]	Ag / 109 [#1]	Ag / 109 [#2]	Recov. %
09.06.2011	23	UHQ 1:3 a	0.004861	0.008174	0.005414	0.004569	
	23	UHQ 1:3 b	0.003236	0.0006185	0.002461	0.001233	
	23	Ag Control A d23 1:3	0.00487	0.005189	0.006827	0.003865	
	23	Ag Control B d23 1:3	0.005199	0.004077	0.006748	0.004878	
	23	Ag Vehicle A d23 1:3	0.01645	0.01159	0.01451	0.009037	
	23	Ag Vehicle B d23 1:3	0.007603	0.003162	0.005515	0.002059	
	23	TMDWS 1:5a	2.192	2.081	2.158	2.059	109.9
	23	TMDWS 1:5b	2.365	1.951	2.391	1.977	118.6
	23	Merck IV 25 µg/L 1:3	8.196	9.725	8.098	9.609	98.4
	23	blank02	0.009239	0.0094	0.009428	0.006536	
	23	Ag 6.25µg/L d23 A 1:3 new	3.864	3.426	3.838	3.451	185.5
	23	Ag 6.25µg/L d23 B 1:3 new	3.542	3.134	3.525	3.16	170.0
	23	Ag 12.5µg/L d23 A 1:3	3.796	2.692	3.771	2.672	91.1
	23	Ag 12.5µg/L d23 B 1:3	3.088	3.808	3.089	3.708	74.1
	23	Ag 25µg/L d23 A 1:3	7.344	7.114	7.322	7.028	88.1
	23	Ag 25µg/L d23 B 1:3	7.706	6.511	7.718	6.55	92.5
	23	blank04	0.0009423	0.00203	0.001117	0.002	
	23	Ag 50µg/L d23 A 1:3	15.15	13.18	15.2	13.01	90.9
	23	Ag 50µg/L d23 B 1:3	13.43	13.82	13.42	13.93	80.6
	23	Ag 100µg/L d23 A 1:3	28.69	23.6	28.34	23.47	86.1
	23	Ag 100µg/L d23 B 1:3	27.64	23.81	27.53	23.94	82.9
	23	blank05	0.006571	0.006749	0.009623	0.008663	
	23	TMDA70c 1:10	1.209	1.018	1.232	1	110.9
	23	TMDA70d 1:10	1.253	0.8958	1.249	0.9537	115.0
	23	blank06	-0.0001443	0.001148	0.0006694	0.0004038	
14.06.2011	28	Blank01	0.0295	0.04178	0.03076	0.0447	
	28	UHQ 1:3 a	0.00466	0.003369	0.003752	0.002098	
	28	UHQ 1:3 b	0.002261	0.002629	0.001987	0.001261	
	28	Ag Control A d28 1:3	0.006372	0.004513	0.0067	0.003146	
	28	Ag Control B d28 1:3	0.009569	0.004429	0.008672	0.004686	
	28	Ag Vehicle A d28 1:3	0.002195	0.001168	0.002483	0.002498	
	28	Ag Vehicle B d28 1:3	0.004368	0.001872	0.005754	0.002741	
	28	TMDWS 1:5a	1.955	1.908	1.957	1.942	95.7
	28	TMDWS 1:5b	2.213	1.994	2.207	1.949	100.0
	28	Merck IV 25µg/L 1:3	7.714	8.163	7.627	8.303	98.0
	28	blank02	0.003873	0.005368	0.00483	0.00444	
	28	Ag 6.25µg/L d28 A 1:3 new	3.913	3.82	3.865	3.804	183.4
	28	Ag 6.25µg/L d28 B 1:3 new	5.196	4.439	5.172	4.418	213.1
	28	Ag 12.5µg/L d28 A 1:3	2.588	2.411	2.553	2.449	57.9
	28	Ag 12.5µg/L d28 B 1:3	2.582	2.316	2.539	2.348	55.6
	28	blank03	0.0001255	0.001388	1.54E-05	0.0005702	
	28	Ag 25µg/L d28 A 1:3	5.511	5.899	5.47	5.72	70.8
	28	Ag 25µg/L d28 B 1:3	6.133	4.937	6.083	5.018	59.2
	28	Ag 50µg/L d28 A 1:3	10.38	10.32	10.28	10.33	61.9
	28	Ag 50µg/L d28 B 1:3	11.14	10.8	11.08	10.81	64.8
	28	blank04	0.001804	0.002729	0.002765	0.001451	
	28	Ag 100µg/L d28 A 1:3	22.84	22.3	22.69	22.09	66.9
	28	Ag 100µg/L d28 B 1:3	21.28	19.74	21.03	19.84	59.2
	28	blank05	0.008929	0.0137	0.01103	0.01703	
	28	TMDA70c 1:10	1.053	1.077	1.038	1.03	98.8
	28	TMDA70d 1:10	1.118	1.005	1.1	1.079	92.2
	28	blank06	-0.0003472	0.0004995	7.02E-05	-0.0001136	

Table 64.5: ICPMS at 16.06.2011 (samples: 16.06.) and 14.06.2011 (samples: 14.06.), Ag/107 (#2) used for analysis.

Date	Day	sample	Ag / 107 [#1]	Ag / 107 [#2]	Ag / 109 [#1]	Ag / 109 [#2]	Recovery

							%
16.06.2011	30	blank01	0.1018	0.1095	0.1019	0.1177	
	30	UHQ 1:3 a	0.007149	0.007653	0.007099	0.008628	
	30	UHQ 1:3 b	0.002724	0.005404	0.0026	0.003989	
	30	Ag Control A d30 1:3	0.00588	0.003865	0.004808	0.004603	
	30	Ag Control B d30 1:3	0.006236	0.007431	0.006837	0.005693	
	30	Ag Vehicle A d30 1:3	0.003355	0.004608	0.004	0.005027	
	30	Ag Vehicle B d30 1:3	0.002187	0.003616	0.002123	0.002174	
	30	TMDWS 1:5a	1.804	1.911	1.812	1.871	95.8
	30	TMDWS 1:5b	1.875	1.989	1.882	2.014	99.7
	30	Merck IV 25µg/L 1:3	8.411	8.348	8.427	8.201	100.2
	30	blank02	0.002775	0.01022	0.003936	0.00605	
	30	Ag 6.25 µg/L d30 A 1:3 neu	2.992	2.883	3.035	2.851	138.4
	30	Ag 6.25 µg/L d30 B 1:3 neu	2.847	3.107	2.858	3.058	149.1
	30	Ag 12.5 µg/L d30 A 1:3	3.274	2.98	3.303	2.964	71.5
	30	Ag 12.5 µg/L d30 B 1:3	3.188	3.12	3.204	3.054	74.9
	30	blank03	0.0009206	0.004721	0.00255	0.003815	
	30	Ag 25 µg/L d30 A 1:3	5.979	6.347	6.001	6.418	76.2
	30	Ag 25 µg/L d30 B 1:3	5.33	5.876	5.316	5.915	70.5
	30	Ag 50 µg/L d30 A 1:3	11.43	13.34	11.44	13	80.0
	30	Ag 50 µg/L d30 B 1:3	11.66	13.04	11.68	13.04	78.2
	30	blank04	0.0099	0.01287	0.008816	0.01018	
	30	Ag 100 µg/L d30 A 1:3	23.74	25.27	23.88	25.26	75.8
	30	Ag 100 µg/L d30 B 1:3	23.25	23.14	23.31	22.69	69.4
	30	blank05	0.02655	0.03224	0.02808	0.03589	
	30	TMDA70c 1:10	1.063	1.071	1.058	1.084	98.3
	30	TMDA70d 1:10	1.048	1.039	1.048	0.9846	95.3
	30	blank06	0.0006351	0.002529	0.002442	0.004998	
20.06.2011	34	blank01	0.05691	0.1128	0.05239	0.1021	
	34	UHQ 1:3 a	0.01551	0.01312	0.01627	0.01506	
	34	Ag Control A d34 1:3	0.004286	0.004598	0.004794	-0.002455	
	34	Ag Control B d34 1:3	0.00475	0.00435	0.006068	-0.0004092	
	34	Ag Vehicle A d34 1:3	0.00575	0.004199	0.004574	0.001447	
	34	Ag Vehicle B d34 1:3	0.003518	0.004241	0.002863	0.001606	
	34	TMDWS 1:5a	1.618	1.952	1.642	1.996	97.9
	34	TMDWS 1:5b	1.907	1.971	1.898	1.956	98.8
	34	Merck IV 25 µg /L 1:3	8.438	8.609	8.429	8.674	103.3
	34	blank02	0.0122	0.01512	0.01182	0.01212	
	34	Ag 6.25µg/L A 1:3 new	2.525	2.477	2.52	2.46	118.9
	34	Ag 6.25µg/L B 1:3 new	1.954	2.978	1.922	3.013	142.9
	34	Ag 12.5µg/L d34 A 1:3	2.266	2.245	2.257	2.352	53.9
	34	Ag 12.5µg/L d34 B 1:3	2.15	2.191	2.17	2.247	52.6
	34	blank03	0.001806	0.003521	0.002064	-0.003853	
	34	Ag 25µg/L d34 A 1:3	4.52	4.549	4.519	4.781	54.6
	34	Ag 25µg/L d34 B 1:3	4.92	5.168	4.92	5.028	62.0
	34	Ag 50µg/L d34 A 1:3	10.58	10.63	10.49	10.65	63.8
	34	Ag 50µg/L d34 B 1:3	9.765	10.5	9.776	10.61	63.0
	34	blank04	0.008723	0.01214	0.0462	0.007938	
	34	Ag 100µg/L d34 A 1:3	20.87	20.89	20.92	20.95	62.7
	34	Ag 100µg/L d34 B 1:3	20.14	21.07	20.22	20.85	63.2
	34	blank05	0.02443	0.03634	0.02413	0.02543	
	34	TMDA70c 1:10	1.014	1.043	1.021	1.034	97.5
	34	TMDA70d 1:10	0.9876	1.107	0.9975	1.118	103.5
	34	blank06	-0.0003427	0.002368	0.000158	-0.004047	

Table 65: 2nd definitive test with *Danio rerio*: Raw data of water analyses. Analyses based on Ag / 107 (#2). ICPMS on 08.08.2011 (samples from 04.08) and on 16.08 (samples from 08.08.) Calibration: MERCK -Ag single element in HNO3 10%. Samples digested in ULTRACLAVE (5mL sample filled up to 15mL).

Date	Day	sample	Ag / 107 [#1]	Ag / 107 [#2]	Ag / 109 [#1]	Ag / 109 [#2]	Recovery %
04.08.2011	2	blank	1.60E-11	3.10E-11	-1.33E-11	-3.81E-11	
	2	0.1 µg/L	0.1031	0.09367	0.09943	0.07575	
	2	0.25 µg/L	0.2612	0.2677	0.256	0.2464	
	2	0.5 µg/L	0.4425	0.5404	0.4302	0.5449	
	2	1.0 µg/L	0.9836	0.8646	0.9838	0.907	
	2	2.5 µg/L	2.453	2.379	2.477	2.418	
	2	5.0 µg/L	4.959	5	4.996	4.907	
	2	10 µg/L	9.832	9.459	9.831	9.551	
	2	25 µg/L	25.08	24.49	25.07	24.93	
	2	50 µg/L	54.1	50.37	54.02	50.14	
	2	blank01	0.01339	0.01965	0.01318	0.02541	
	2	UHQ 1:3 a	0.00494	0.003927	0.005039	0.006307	
	2	UHQ 1:3 b	0.007251	0.005961	0.007879	0.005021	
	2	Ag Control.I A d2 1:3	0.02506	0.01877	0.023	0.03137	
	2	Ag Control.I Bd2 1:3	0.04112	0.03875	0.04351	0.05091	
	2	Ag Control.II A d2 1:3	0.01304	0.0128	0.01161	0.00792	
	2	Ag Control.II B d2 1:3	0.01653	0.01397	0.01594	0.01941	
	2	TMDWS 1:5a	1.972	1.995	1.998	1.948	100.1
	2	TMDWS 1:5b	1.902	1.913	1.907	1.91	95.9
	2	TMDA70a 1:20	0.5663	0.4969	0.5586	0.5201	91.2
	2	TMDA70b 1:20	0.5402	0.4659	0.5563	0.4965	85.5
	2	blank02	0.0004433	-0.002468	0.0008499	-5.63E-05	
	2	12.5 µg/L I A 1:3	1.598	1.418	1.556	1.442	34.0
	2	12.5 µg/L I B1:3	1.468	1.465	1.466	1.384	35.2
	2	12.5 µg/L II A 1:3	1.756	1.646	1.777	1.794	39.5
	2	12.5 µg/L II B1:3	1.805	1.576	1.811	1.652	37.8
	2	blank03	0.001093	-0.0008967	0.001702	-0.0008232	
	2	50 µg/L I A 1:3	10.04	10.08	10.02	10.22	60.5
	2	50 µg/L I B1:3	10.1	10.05	10.1	9.9	60.3
	2	50 µg/L II A 1:3	8.544	8.279	8.591	8.31	49.7
	2	50 µg/L II B1:3	8.499	8.123	8.526	8.232	48.7
	2	blank04	0.005997	0.02106	0.008553	0.01123	
	2	100 µg/L I A 1:3	21.61	21.48	21.78	21.64	64.4
	2	100 µg/L I B1:3	18.29	18.42	18.42	18.55	55.3
	2	100 µg/L II A 1:3	19.64	19.48	19.66	19.75	58.4
	2	100 µg/L II B1:3	19.88	20.04	20	19.93	60.1
	2	blank05	0.01615	0.0194	0.0151	0.02248	
	2	TMDWS 1:5c	1.975	1.92	1.937	1.93	96.3
	2	TMDWS 1:5d	1.989	1.952	1.992	1.966	97.9
	2	TMDA70c 1:20	0.6333	0.5452	0.6245	0.6406	100.0
	2	TMDA70d 1:20	0.6604	0.5548	0.6407	0.6213	101.8
	2	blank07	0.05658	0.05725	0.05699	0.08476	
08.08.2011	6	blank	4.20E-11	3.67E-12	3.80E-11	1.79E-10	
	6	0.1 µg/L	0.1014	0.09724	0.1016	0.1113	
	6	0.25 µg/L	0.2582	0.245	0.2626	0.2283	
	6	0.5 µg/L	0.525	0.4675	0.5231	0.4969	
	6	1.0 µg/L	1.046	0.9361	1.06	0.9819	
	6	2.5 µg/L	2.654	2.306	2.669	2.313	
	6	5.0 µg/L	5.271	5.297	5.262	5.253	
	6	10 µg/L	11.44	10.21	11.46	10.3	
	6	25 µg/L	24.31	22.9	24.45	22.85	
	6	50 µg/L	50.02	50.99	49.94	51	
	6	blank01	0.01783	0.03298	0.01795	0.03085	
	6	UHQ 1:3 a	0.001222	0.00413	0.002612	-0.00116	
	6	UHQ 1:3 b	0.001436	0.004878	0.001853	0.0003238	

Table 65-2: ICPMS on 16.08.2011 (samples from 08.08.and 11.08) and on 22.08.2011 (samples from 15.08.). Analyses based on Ag / 107 (#2).

Date	Day	Sample	Ag / 107 [#1]	Ag / 107 [#2]	Ag / 109 [#1]	Ag / 109 [#2]	Recovery %
08.08.2011	6	Ag Control I A d6 1:3	0 .0198	0.02488	0.01962	0.01202	
	6	Ag Control I B d6 1:3	0.01717	0.01841	0.02013	0.01982	
	6	Ag Control II A d6 1:3	0.01468	0.01491	0.01174	0.008283	
	6	Ag Control II B d6 1:3	0.01109	0.01319	0.01061	0.01708	
	6	TMDWS a 1:5	1.982	1.812	2.008	1.83	90.9
	6	TMDWS b 1:5	2.059	1.916	2.044	1.895	96.1
	6	TMDA70a 1:10	1.173	1.079	1.203	0.8792	99.0
	6	TMDA70b 1:10	1.127	1.107	1.096	1.063	101.6
	6	Blank02	-0.001053	-5.40E-05	-0.0003498	-0.001991	
	6	Ag 12.5 µg/L IA d6 1:3	0.4642	0.4047	0.4637	0.4027	9.7
	6	Ag 12.5 µg/L IB d6 1:3	0.462	0.4225	0.4593	0.3711	10.1
	6	Ag 12.5 µg/L IIA d6 1:3	0.7805	0.5991	0.783	0.6328	14.4
	6	Ag 12.5 µg/L IIB d6 1:3	0.7803	0.7352	0.7659	0.6862	17.6
	6	Blank03	0.0003632	-0.0008029	0.001305	-0.002193	
	6	Ag 50 µg/L IA d61:3	7.552	6.916	7.6	6.79	41.5
	6	Ag 50 µg/L IB d6 1:3	7.741	7.19	7.781	6.978	43.1
	6	Ag 50 µg/L IIA d6 1:3	3.099	2.812	3.073	2.833	16.9
	6	Ag 50 µg/L IIB d6 1:3	2.918	2.913	2.932	2.825	17.5
	6	Blank04	1.25E-05	0.002301	0.001142	0.002046	
	6	Ag 100 µg/L IA d6 1:3	14.82	14.42	14.88	14.32	43.3
	6	Ag 100 µg/L IB d6 1:3	15.16	14.84	15.24	14.7	44.5
	6	Ag 100 µg/L IIA d6 1:3	18.78	17.15	18.64	17.18	51.5
	6	Ag 100 µg/L IIB d6 1:3	18.8	17.13	18.81	17.17	51.4
	6	Blank05	0.002405	0.007174	0.002921	0.006741	
	6	TMDA70c 1:10	1.112	1.113	1.084	1.063	102.1
	6	TMDA70d 1:10	1.211	1.148	1.198	1.052	105.3
	6	Blank06	-0.0003688	-0.0008029	0.0006136	-0.00137	
11.08.2011	9	Ag Control I A d9 1:3	0.03222	0.01931	0.02649	0.02027	
	9	Ag Control.I B d9 1:3	0.02708	0.02279	0.03262	0.02782	
	9	Ag Control II A d9 1:3	0.02709	0.0239	0.02575	0.02308	
	9	Ag Control II B d9 1:3	0.02508	0.02162	0.02566	0.01894	
	9	Blank07	0.001009	0.0002211	0.001718	-0.0001897	
	9	Ag 12.5 µg/L IA d9 1:3	2.728	2.573	2.744	2.482	61.8
	9	Ag 12.5 µg/L IB d9 1:3	2.862	2.471	2.882	2.524	59.3
	9	Ag 12.5 µg/L IIA d9 1:3	4.181	3.457	4.216	3.482	83.0
	9	Ag 12.5 µg/L IIB d9 1:3	3.691	3.318	3.656	3.269	79.6
	9	Blank08	0.06746	0.05877	0.06981	0.0594	
	9	Ag 50 µg/L IA d9 1:3	11.72	10.47	11.73	10.34	62.8
	9	Ag 50 µg/L IB d9 1:3	10.78	10.58	10.73	10.52	63.5
	9	Ag 50 µg/L IIA d9 1:3	11.28	10.92	11.16	10.92	65.5
	9	Ag 50 µg/L IIB d9 1:3	11.9	10.08	11.96	10.07	60.5
	9	Blank09	0.0416	0.03947	0.03495	0.03359	
	9	Ag 100 µg/L IA d9 1:3	19.77	17.87	19.95	18.44	53.6
	9	Ag 100 µg/L IB d9 1:3	19.5	18.45	19.68	18.33	55.4
	9	Ag 100 µg/L IIA d9 1:3	22.84	22.56	22.93	22.9	67.7
	9	Ag 100 µg/L IIB d9 1:3	24.9	22.91	24.99	22.37	68.7
	9	Blank10	0.01393	0.01755	0.01186	0.01511	
	9	TMDWS a 1:5	2.182	1.955	2.146	1.996	98.0
	9	TMDWS b 1:5	2.121	1.97	2.153	1.936	98.8
	9	TMDA70a 1:10	1.223	1.094	1.235	1.126	100.4
	9	TMDA70b 1:10	1.162	1.069	1.188	1.046	98.1
	9	Blank11	0.001518	0.004681	0.002341	0.002278	
15.08.2011	13	Blank	1.45E-11	---	4.19E-12	---	
	13	0.1 µg/L	0.1	0.08421	0.09729	0.09124	
	13	0.25 µg/L	0.2218	0.2187	0.2206	0.2574	
	13	0.5 µg/L	0.464	0.4936	0.4795	0.4864	
	13	1.0 µg/L	1.019	.9417	1.005	1.02	
	13	2.5 µg/L	2.446	2.489	2.454	2.431	

Table 65-3: ICPMS on 22.08.2011 (samples from 15.08. and 18.08.). Analyses based on Ag / 107 (#2).

Date	Day	sample	Ag / 107 [#1]	Ag / 107 [#2]	Ag / 109 [#1]	Ag / 109 [#2]	Recovery %
15.08.2011	13	5.0 µg/L	4.987	5.058	4.99	5.143	
	13	10 µg/L	10.11	9.845	10.04	9.874	
	13	25 µg/L	23.49	25.64	23.57	25.8	
	13	50 µg/L	50.74	49.71	50.71	49.61	
	13	blank01	0.004856	0.01201	0.006577	0.01198	
	13	UHQ 1:3 a	0.00368	0.00279	0.003068	0.005609	
	13	UHQ 1:3 b	0.001632	0.007669	0.001496	0.003846	
	13	Ag Control I A d13 1:3	0.01953	0.01412	0.01803	0.02181	
	13	Ag Control I B d13 1:3	0.0204	0.01375	0.01709	0.017	
	13	Ag Control II A d13 1:3	0.02502	0.02735	0.02581	0.023	
	13	Ag Control II B d13 1:3	0.02657	0.02388	0.02531	0.03482	
	13	TMDWS 1:5a	1.859	1.861	1.859	1.892	93.3
	13	TMDWS 1:5b	1.674	1.874	1.666	1.954	94.0
	13	TMDA70a 1:5	2.197	1.978	2.195	1.95	90.7
	13	TMDA70b 1:5	2.025	2.121	2.035	2.103	97.3
	13	blank02	0.0002727	0.001316	0.0001272	0.0006596	
	13	Ag 12.5 µg/L I A d13 1:3	.4952	0.4814	0.4948	0.4931	11.6
	13	Ag 12.5 µg/L I B d13 1:3	0.5196	0.4851	0.5308	0.471	11.6
	13	Ag 12.5 µg/L II A d13 1:3	2.317	2.838	2.305	2.976	68.1
	13	Ag 12.5 µg/L II B d13 1:3	2.495	2.639	2.512	2.703	63.3
	13	blank03	0.0006036	0.0007364	0.0005476	0.0007303	
	13	Ag 50 µg/L I A d13 1:3	4.874	4.888	4.867	5.075	29.3
	13	Ag 50 µg/L I B d13 1:3	4.743	4.798	4.781	4.953	28.8
	13	Ag 50 µg/L II A d13 1:3	5.297	5.292	5.311	5.423	31.8
	13	Ag 50 µg/L II B d13 1:3	5.394	5.397	5.371	5.527	32.4
	13	blank04	0.002981	0.006159	0.004319	0.007703	
	13	Ag 100 µg/L I A d13 1:3	11.19	10.9	11.14	11.27	32.7
	13	Ag 100 µg/L I B d13 1:3	11.15	11.09	11.16	11.42	33.3
	13	Ag 100 µg/L II A d13 1:3	13.33	13.07	13.4	13.28	39.2
	13	Ag 100 µg/L II B d13 1:3	13.26	13.9	13.33	13.9	41.7
	13	blank05	0.003133	0.01192	0.004876	0.006418	
	13	TMDA70c 1:5	2.05	2.072	2.055	2.153	95.0
	13	TMDA70d 1:5	2.075	2.08	2.101	2.059	95.4
18.08.2011	16	blank06	1.15E-05	0.001375	-8.00E-05	---	
	16	Ag Control I A d16 1:3	0.05082	0.05178	0.04689	0.05278	
	16	Ag Control I B d16 1:3	0.04946	0.05012	0.04547	0.04873	
	16	Ag Control II A d16 1:3	0.03402	0.02893	0.03328	0.0403	
	16	Ag Control II B d16 1:3	0.03809	0.03628	0.03607	0.04954	
	16	blank07	-0.0002439	0.0007413	0.0008502	---	
	16	Ag 12.5 µg/L I A d16 1:3	3.028	2.587	3.041	2.673	62.1
	16	Ag 12.5 µg/L I B d16 1:3	2.562	2.69	2.553	2.82	64.6
	16	Ag 12.5 µg/L II A d16 1:3	2.691	2.667	2.683	2.707	64.0
	16	Ag 12.5 µg/L II B d16 1:3	2.693	2.723	2.674	2.777	65.4
	16	blank08	0.001269	0.003002	0.001015	0.004425	
	16	Ag 50 µg/L I A d16 1:3	10.61	9.35	10.63	9.322	56.1
	16	Ag 50 µg/L I B d16 1:3	9.483	9.475	9.47	9.641	56.9
	16	Ag 50 µg/L II A d16 1:3	9.467	8.979	9.462	9.085	53.9
	16	Ag 50 µg/L II B d16 1:3	9.852	9.537	9.754	9.718	57.2
	16	blank09	0.0001852	0.002179	0.000477	0.002178	
	16	Ag 100 µg/L I A d16 1:3	18.94	18	19.03	18.41	54.0
	16	Ag 100 µg/L I B d16 1:3	17.34	17.23	17.4	17.68	51.7
	16	Ag 100 µg/L II A d16 1:3	20.82	20.94	20.72	21.32	62.8
	16	Ag 100 µg/L II B d16 1:3	20.14	20.2	20.41	20.58	60.6
	16	blank10	0.004135	0.01071	0.00443	0.009033	
	16	TMDWS 1:5c	1.863	1.902	1.876	1.886	95.4
	16	TMDWS 1:5d	1.752	1.831	1.743	1.795	91.8
	16	TMDA70e 1:5	2.073	1.994	2.086	2.092	91.5
	16	TMDA70f 1:5	2.022	2.014	2.006	1.997	92.4
	16	blank11	0.0003917	0.001592	-0.0001707	0.001551	

Table 65-4: ICPMS on 29.08.2011 (samples from 22.08. and 24.08.). Analyses based on Ag / 107 (#2).

Date	Day	sample	Ag / 107 [#1]	Ag / 107 [#2]	Ag / 109 [#1]	Ag / 109 [#2]	Recovery %
22.08.2011	20	blank	4.08E-11	-3.55E-11	5.05E-11	8.20E-11	
	20	0.1 µg/L	0.09765	0.08739	0.09656	0.1026	
	20	0.25 µg/L	0.2462	0.2119	0.2486	0.2337	
	20	0.5 µg/L	0.4667	0.5414	0.4604	0.5484	
	20	1.0 µg/L	1.032	1.036	1.067	1.038	
	20	10 µg/L	9.526	9.985	9.498	10.04	
	20	2.5 µg/L	2.447	2.487	2.492	2.481	
	20	25 µg/L	23.39	24.75	23.42	24.76	
	20	5.0 µg/L	5.021	4.936	5.042	4.965	
	20	50 µg/L	50.9	50.13	50.88	50.11	
	20	blank01	0.004726	0.006327	0.005296	0.008841	
	20	UHQ 1:3 a	0.003831	0.005637	0.002827	0.001572	
	20	UHQ 1:3 b	0.0006562	-0.001251	0.0001006	-0.0005389	
	20	Ag Control I A d20 1:3	0.05426	0.05168	0.05416	0.04534	
	20	Ag Control I B d20 1:3	0.04669	0.04655	0.04456	0.05196	
	20	Ag Control II A d20 1:3	0.04338	0.04703	0.04479	0.03499	
	20	Ag Control II B d20 1:3	0.04211	0.03892	0.04106	0.0533	
	20	TMDWS 1:5a	1.861	1.896	1.891	1.92	95.1
	20	TMDWS 1:5b	1.808	2.049	1.805	2.037	103
	20	TMDA70a 1:5	1.081	1.195	1.106	1.152	110
	20	TMDA70b 1:5	1.063	1.044	1.078	1.035	95.8
	20	blank02	-0.0003135	-0.001251	0.0003839	-0.0005724	
	20	Ag 12.5 µg/L I A d20 1:3	1.536	1.535	1.536	1.523	36.8
	20	Ag 12.5 µg/L I B d20 1:3	1.61	1.568	1.613	1.582	37.6
	20	Ag 12.5 µg/L II A d20 1:3	2.317	2.273	2.308	2.276	54.6
	20	Ag 12.5 µg/L II B d20 1:3	2.261	2.264	2.285	2.254	54.3
	20	blank03	-0.001153	-0.0006331	-0.000733	-0.001863	
	20	Ag 50 µg/L I A d20 1:3	6.009	6.099	6.053	6.064	36.6
	20	Ag 50 µg/L I B d20 1:3	5.756	6.23	5.853	6.118	37.4
	20	Ag 50 µg/L II A d20 1:3	5.215	5.298	5.177	5.267	31.8
	20	Ag 50 µg/L II B d20 1:3	5.369	5.053	5.408	5.056	30.3
	20	blank04	-0.0009781	-0.0006056	0.0001996	-0.0005619	
	20	Ag 100 µg/L I A d20 1:3	10.24	10.55	10.31	10.77	31.7
	20	Ag 100 µg/L I B d20 1:3	10.29	10.02	10.36	9.994	30.1
	20	Ag 100 µg/L II A d20 1:3	13.29	12.65	13.35	13	38.0
	20	Ag 100 µg/L II B d20 1:3	12.13	12.86	12.24	13.08	38.6
	20	blank05	0.0002805	8.59E-05	0.001377	-0.001863	
	20	TMDA70c 1:5	1.024	1.058	1.027	1.03	97.1
	20	TMDA70d 1:5	1.035	1.03	1.067	1.053	94.5
	20	blank06	-0.0005924	0.001294	-0.0001641	0.00253	
25.08.2011	23	Ag Control I A d23 1:3	0.03685	0.04749	0.03749	0.03929	
	23	Ag Control I B d23 1:3	0.03373	0.03095	0.03304	0.03141	
	23	Ag Control II A d23 1:3	0.02324	0.02461	0.02329	0.02242	
	23	Ag Control II B d23 1:3	0.03318	0.03182	0.03685	0.03529	
	23	blank07	0.0007558	-0.0006117	0.0004508	-0.001228	
	23	Ag 12.5 µg/L I A d23 1:3	2.289	2.479	2.304	2.528	59.5
	23	Ag 12.5 µg/L I B d23 1:3	2.559	2.666	2.555	2.74	64.0
	23	Ag 12.5 µg/L II A d23 1:3	2.426	2.584	2.448	2.657	62.0
	23	Ag 12.5 µg/L II B d23 1:3	2.418	2.499	2.444	2.541	60.0
	23	blank08	-0.0003534	0.001334	-0.0006247	-0.001863	
	23	Ag 50 µg/L I A d23 1:3	9.18	9.304	9.303	9.392	55.8
	23	Ag 50 µg/L I B d23 1:3	9.864	9.044	9.848	9.041	54.3
	23	Ag 50 µg/L II A d23 1:3	10.1	10.33	10.21	10.14	62.0
	23	Ag 50 µg/L II B d23 1:3	9.705	10.06	9.725	10.09	60.4
	23	blank09	-0.0002034	0.002785	0.0003745	-0.001188	
	23	Ag 100 µg/L I A d23 1:3	16.79	17.14	16.9	17.2	51.4
	23	Ag 100 µg/L I B d23 1:3	17.33	17.24	17.46	17.28	51.7
	23	Ag 100 µg/L II A d23 1:3	19.26	18.62	19.5	18.78	55.9
	23	Ag 100 µg/L II B d23 1:3	19.05	18.55	19.24	18.48	55.7
Date	Day	sample	Ag / 107 [#1]	Ag / 107 [#2]	Ag / 109 [#1]	Ag / 109 [#2]	Recov. %

	23	blank10	0.00463	0.007622	0.005805	0.006722	
	23	TMDWS 1:5c	1.856	1.844	1.894	1.872	92.5
	23	TMDWS 1:5d	1.707	1.871	1.767	1.864	93.8
	23	TMDA70e 1:5	0.997	1.093	1.032	1.045	100
	23	TMDA70f 1:5	1.036	1.05	1.046	1.106	96.3
	23	blank11	-0.0009475	-0.001251	0.0000193	0.001132	

Table 65-5: ICPMS on 02.09.2011 (samples from 29.08, repeated and 01.09.). Analyses based on Ag / 107 (#2).

Date	Day	sample	Ag / 107 [#1]	Ag / 107 [#2]	Ag / 109 [#1]	Ag / 109 [#2]	Recovery %
29.08.2011	27	0.25 µg/L	0.2487	0.2746	0.238	0.2769	
	27	0.5 µg/L	0.4965	0.4881	0.5227	0.4779	
	27	1.0 µg/L	1.02	1.032	0.9973	0.987	
	27	2.5 µg/L	2.626	2.36	2.587	2.332	
	27	5.0 µg/L	4.993	5.578	5.027	5.431	
	27	10 µg/L	10.12	9.782	10.08	9.936	
	27	25 µg/L	24.8	25.63	24.8	25.77	
	27	50 µg/L	51.8	50.71	51.74	50.93	
	27	Blank01	0.01274	0.007448	0.0138	0.01368	
	27	UHQ 1:3 a	0.002264	0.003788	0.002571	0.004396	
	27	UHQ 1:3 b	0.001511	0.002253	0.001746	0.002164	
	27	Ag Control I A d27 1:3	0.02325	0.02272	0.02652	0.02752	
	27	Ag Control I B d27 1:3	0.03186	0.02863	0.0324	0.02792	
	27	Ag Control II A d27 1:3	0.03441	0.02866	0.03306	0.03556	
	27	Ag Control II B d27 1:3	0.06521	0.06057	0.05942	0.05874	
	27	TMDWS 1:5a	1.853	1.905	1.831	1.943	97.5
	27	TMDWS 1:5b	1.898	1.859	1.929	1.943	94.8
	27	TMDA70a 1:5	2.092	2.056	2.104	2.035	96.7
	27	TMDA70b 1:5	2.056	2.102	2.074	2.116	91.2
	27	blank02	-0.0001701	0.0003063	-0.0004907	0.0008151	
	27	Ag 12.5 µg/L I A d27 1:3	0.7707	0.8979	0.7581	0.8632	41.6
	27	Ag 12.5µg/L I B d27 1:3	0.9238	0.8544	0.9256	0.8919	43.9
	27	Ag 12.5 µg/L II A d27 1:3	1.493	1.526	1.485	1.529	41.6
	27	Ag 12.5 µg/L II B d27 1:3	1.603	1.614	1.608	1.623	43.5
	27	blank03	-0.0006329	0.003862	-0.0009654	0.001674	
	27	Ag 50 µ/L I A d27 1:3	2.691	2.814	2.688	2.716	43.8
	27	Ag 50 µg/L I B d27 1:3	2.72	2.843	2.714	2.789	49.7
	27	Ag 50µg/L II A d27 1:3	3.945	4.055	3.993	4.153	52.2
	27	Ag 50 µg/L II B d27 1:3	4.111	4.164	4.089	4.102	55.4
	27	blank04	-0.0007646	0.00124	-0.0001195	0.002548	
	27	Ag 100 µg/L I A d27 1:3	6.205	6.093	6.249	6.27	37.4
	27	Ag 100 µg/L I B d27 1:3	6.839	7.091	6.784	7.044	37.8
	27	Ag 100 µg/L II A d27 1:3	6.609	6.948	6.634	6.911	36.1
	27	Ag 100 µg/L II B d27 1:3	8.382	8.448	8.449	8.321	32.3
	27	Blank05	0.001162	0.004443	0.00226	0.003803	
	27	TMDA70c 1:5	2.099	2.177	2.171	2.207	99.9
	27	TMDA70d 1:5	2.216	2.238	2.262	2.231	103
	27	blank06	0.0001725	0.001656	0.0001014	---	
01.09.2011	30	Ag Control I A d30 1:3	0.03858	0.02874	0.03237	0.05008	
	30	Ag Control I B d30 1:3	0.03974	0.03244	0.03677	0.03846	
	30	Ag Control II A d30 1:3	0.01911	0.02598	0.01932	0.01388	
	30	Ag Control II B d30 1:3	0.02485	0.02942	0.03002	0.02924	
	30	Blank07	-0.0001812	-0.001343	-9.58E-06	0.001986	
	30	Ag 12.5 µg/L I A d30 1:3	2.94	2.834	3.001	2.846	68.0
	30	Ag 12.5 µg/L I B d30 1:3	2.964	3.23	2.939	3.378	77.5
	30	Ag 12.5 µg/L II A d30 1:3	2.783	2.672	2.76	2.759	64.1
	30	Ag 12.5 µg/L II B d30 1:3	2.788	2.81	2.781	2.781	67.4
	30	blank08	-0.0002675	-0.0002944	-0.0002219	0.005482	
	30	Ag 50 µg/L I A d30 1:3	10.58	10.56	10.65	10.65	63.4
	30	Ag 50 µg/L I B d30 1:3	10.38	10.26	10.4	10.34	61.6
Date	Day	sample	Ag / 107 [#1]	Ag / 107 [#2]	Ag / 109 [#1]	Ag / 109 [#2]	Recov. %

	30	Ag 50 µg/L II A d30 1:3	11.37	10.74	11.38	10.77	64.4
	30	Ag 50 µg/L II B d30 1:3	11.32	11.71	11.42	11.87	70.3
	30	blank09	0.002329	0.004138	0.00105	0.002148	
	30	Ag 100 µg/L I A d30 1:3	19.36	19.72	19.52	19.61	59.2
	30	Ag 100 µg/L I B d30 1:3	19.47	20.02	19.54	19.98	60.1
	30	Ag 100 µg/L II A d30 1:3	23.09	23.3	23.24	22.96	69.9
	30	Ag 100 µg/L II B d30 1:3	25.64	23.66	25.56	23.25	71.0
	30	blank10	0.004871	0.009066	0.00763	0.01281	
	30	TMDWS 1:5c	1.943	1.996	1.985	2.045	100
	30	TMDWS 1:5d	1.97	2.064	1.933	2.034	104
	30	TMDA70e 1:5	2.236	2.167	2.24	2.21	99.4
	30	TMDA70f 1:5	2.227	2.364	2.269	2.281	108
	30	blank11	0.000842	0.001208	0.0001018	0.002473	

Table 65-6: ICPMS on 05.09.2011 (samples from 05.09.). Analyses based on Ag / 107 (#2).

Date	Day	sample	Ag / 107 [#1]	Ag / 107 [#2]	Ag / 109 [#1]	Ag / 109 [#2]	Recovery %
05.09.2011	34	0.25 µg/L	0.2766	0.2899	0.305	0.2339	
	34	0.5 µg/L	0.4524	0.5013	0.4722	0.4929	
	34	1.0 µg/L	1.025	0.9582	1.011	1.021	
	34	2.5 µg/L	2.614	2.501	2.606	2.667	
	34	5.0 µg/L	4.789	4.88	4.843	5.031	
	34	10 µg/L	10.12	9.697	10.15	9.492	
	34	25 µg/L	28.48	24.08	28.52	24.22	
	34	50 µg/L	47.19	55.61	47.18	54.68	
	34	blank01	0.006989	0.01142	0.005511	0.01494	
	34	UHQ 1:3 a	0.01364	0.01153	0.01115	0.01021	
	34	UHQ 1:3 b	0.01902	0.0142	0.02009	0.01887	
	34	Ag Control I A d34 1:3	0.08801	0.09481	0.08799	0.09578	
	34	Ag Control I B d34 1:3	0.05713	0.06371	0.06445	0.06285	
	34	Ag Control II A d34 1:3	0.06393	0.06085	0.05731	0.06213	
	34	Ag Control II B d34 1:3	0.07418	0.06408	0.0798	0.09629	
	34	TMDWS 1:5a	1.836	1.945	1.835	2.038	97.5
	34	TMDWS 1:5b	1.83	1.89	1.817	1.978	94.8
	34	TMDA70a 1:5	2.053	2.108	2.04	2.171	96.7
	34	TMDA70b 1:5	2.178	1.988	2.187	2.061	91.2
	34	blank02	0.0002324	0.00675	0.001941	-0.0009932	
	34	Ag 12.5 µg/L I A d34 1:3	1.72	1.732	1.693	1.753	41.6
	34	Ag 12.5 µg/L I B d34 1:3	1.734	1.831	1.686	1.778	43.9
	34	Ag 12.5 µg/L II A d34 1:3	1.542	1.735	1.54	1.651	41.6
	34	Ag 12.5 µg/L II B d34 1:3	1.679	1.813	1.69	1.901	43.5
	34	blank03	0.002539	0.007157	0.001726	0.0001489	
	34	Ag 50 µg/L I A d34 1:3	7.122	7.301	7.091	7.431	43.8
	34	Ag 50 µg/L I B d34 1:3	7.992	8.29	8.106	8.377	49.7
	34	Ag 50 µg/L II A d34 1:3	8.296	8.693	8.275	8.673	52.2
	34	Ag 50 µg/L II B d34 1:3	8.044	9.237	8.073	9.236	55.4
	34	blank04	0.002308	0.0128	0.005343	0.01016	
	34	Ag 100 µg/L I A d34 1:3	11.88	12.46	11.97	12.62	37.4
	34	Ag 100 µg/L I B d34 1:3	11.04	12.59	11.07	12.66	37.8
	34	Ag 100 µg/L II A d34 1:3	11.24	12.02	11.23	11.89	36.1
	34	Ag 100 µg/L II B d34 1:3	11.41	10.78	11.49	10.84	32.3
	34	blank05	0.005519	0.01088	0.004958	0.008055	
	34	TMDWS 1:5c	1.904	1.938	1.919	1.904	97.2
	34	TMDWS 1:5d	1.881	2.012	1.84	1.98	101
	34	TMDA70e 1:5	2.114	2.261	2.108	2.16	104
	34	TMDA70f 1:5	2.123	2.25	2.143	2.23	103
	34	blank06	0.001004	0.001812	0.002228	0.004995	

Table 66: Additional uptake test with *Danio rerio*: Raw data of water analyses.
ICPMS at 09.12.2011 (samples from 29.11, 5.12, 6.12) and 21.12.2011 (samples from 12.12, 13.12. 19.12.), Calibration: MERCK -Ag single element in HNO3 10%. Samples digested in ULTRACLAVE (5 mL sample filled up to 15mL, dilution factor 3; measurement on 21.12.2011, centrifuged : 4 mL sample filled up to 15 mL, df 3.75)

File:	Sample:	Cu/63 [#1]	Cu/63 [#2]	Ag/107 [#1]	Ag/107 [#2]	Ag/109 [#1]	Ag/109 [#2]
001_STD.D#	blank	3.70E-07	-3.57E-07	3.09E-11	-2.39E-11	9.71E-12	4.53E-11
002_STD.D#	0.1	142.7	131.3	0.09359	0.1065	0.09219	0.1211
003_STD.D#	0.25	43.86	43.06	0.2379	0.251	0.242	0.2443
004_STD.D#	0.5	36.66	34.63	0.4839	0.4994	0.4672	0.4695
005_STD.D#	1	22.68	19.52	1.001	0.9935	1.02	0.9651
006_STD.D#	2.5	143.7	152.8	2.263	2.519	2.293	2.423
007_STD.D#	5	21.53	19.23	5.077	4.845	5.112	4.7
008_STD.D#	10	31.46	33.17	10.16	10.32	10.18	10.24
009_STD.D#	25	77.41	78.88	24.86	25.39	24.91	25.23
010_STD.D#	50	9.492	8.302	50.04	49.76	50.01	49.87
SMPL001.D#	blank01	-5.824	-3.858	0.03755	0.07122	0.03683	0.0765
SMPL002.D#	TMDWS.2 a 1:10	3957	3826	0.9914	1.033	0.9945	1.049
SMPL003.D#	TMDWS.2 b 1:10	3799	3778	0.9591	1.054	0.9454	1.008
SMPL004.D#	TMDA70a 1:5	19610	17790	2.204	2.247	2.231	2.257
SMPL005.D#	TMDA70b 1:5	18590	17120	2.135	2.185	2.132	2.186
SMPL006.D#	blank02	-0.303	3.301	0.001108	0.0004203	0.0005941	0.0004421
SMPL007.D#	UHQ A	166.3	171.6	0.05102	0.05703	0.0451	0.04844
SMPL008.D#	UHQ B	189.8	193.1	0.06763	0.0672	0.06306	0.07172
SMPL009.D#	d0 control centrif. 2911 I	236	226	0.0754	0.07982	0.07485	0.06509
SMPL010.D#	d0 control centrif. 2911 II	292.3	268.7	0.04933	0.04795	0.05294	0.05569
SMPL011.D#	d0 25µg/L centrif. 2911 I	166.2	156.6	0.08783	0.09846	0.08912	0.09762
SMPL012.D#	d0 25µg/L centrif. 2911 II	227.1	222.1	0.09462	0.09219	0.09826	0.1044
SMPL013.D#	d0 100µg/L centrif. 2911 I	149.8	136.3	0.9717	0.9755	0.9628	1.019
SMPL014.D#	d0 100µg/L centrif. 2911 II	161.2	148	0.706	0.7571	0.6961	0.7478
SMPL015.D#	blank03	-6.754	-4.07	-0.0003497	-0.0004303	-0.0001688	-0.001223
SMPL016.D#	d0 control A 2911	249.6	259.5	0.1673	0.188	0.1677	0.1892
SMPL017.D#	d0 control B 2911	245.4	231.6	0.173	0.1842	0.1791	0.1712
SMPL018.D#	d0 25µg/L A 2911	318.9	296.1	5.917	6.085	5.863	6.073
SMPL019.D#	d0 25µg/L B 2911 II	197.3	182.5	5.896	6.035	5.873	6.066
SMPL020.D#	d0 100µg/L A 2911 I	200.8	184.8	22.44	23.2	22.4	23.22
SMPL021.D#	d0 100µg/L B 2911 II	220.9	214.6	21.96	23.36	21.98	23.13
SMPL022.D#	blank04	-6.833	-4.946	0.003271	0.005542	0.003093	0.005936
SMPL023.D#	d6 control centrif. 0512 I	205.3	180.7	0.05606	0.04404	0.05301	0.04475
SMPL024.D#	d6 control centrif. 0512 II	282.2	277.5	0.04623	0.04073	0.04963	0.04664
SMPL025.D#	d6 25µg/L centrif. 0512 I	292.2	274.7	0.08012	0.08897	0.07808	0.07581
SMPL026.D#	d6 25µg/L centrif. 0512 II	294.4	288.1	0.2578	0.2751	0.2579	0.2685
SMPL027.D#	d6 100µg/L centrif. 0512 I	188.1	185.9	0.1516	0.1606	0.1536	0.1649
SMPL028.D#	d6 100µg/L centrif. 0512 II	244.3	231	0.3416	0.3467	0.3433	0.3606
SMPL029.D#	blank05	-6.92	-5.421	0.0006056	-0.0004157	-0.0004403	3.28E-05
SMPL030.D#	TMDA70c 1:5	18210	17320	2.333	2.276	2.366	2.253
SMPL031.D#	TMDA70d 1:5	16940	17750	2.181	2.312	2.167	2.368
SMPL032.D#	blank06	-5.454	-2.763	0.0003078	1.61E-05	0.0001545	-0.0003772
SMPL033.D#	d6 control A 0512 I	231.1	230.2	0.2809	0.3034	0.2855	0.3144
SMPL034.D#	d6 control B 0512 II	280.3	269.2	0.2983	0.3235	0.2967	0.3256
SMPL035.D#	d6 25µg/L A 0512 I	233.8	223.7	4.803	5.048	4.766	5.024
SMPL036.D#	d6 25µg/L B 0512 II	216	212.4	4.851	5.178	4.902	5.228
SMPL037.D#	d6 100µg/L A 0512 I	215.8	196.9	20.24	20.54	20.32	20.48
SMPL038.D#	d6 100µg/L B 0512 II	253.3	265.1	20.08	22.36	20.22	22.37
SMPL039.D#	blank07	-6.565	-4.212	0.01604	0.02349	0.01432	0.02633
SMPL040.D#	d0 control centrif. 0612 I	288.4	281.6	0.03058	0.03811	0.03505	0.03055
SMPL041.D#	d0 control centrif. 0612 II	203.3	218.8	0.0163	0.01616	0.01567	0.01312
SMPL042.D#	d0 25µg/L centrif. 0612 I	156.5	143.5	0.08575	0.08664	0.0813	0.08085
SMPL043.D#	d0 25µg/L centrif. 0612 II	193.3	187.6	0.1343	0.1304	0.124	0.1321
SMPL044.D#	d0 100µg/L centrif. 0612 I	199.2	191.7	0.638	0.6703	0.6557	0.6831
SMPL045.D#	d0 100µg/L centrif. 061 II	292.2	296.4	0.7862	0.8426	0.7926	0.8749

File:	Sample:	Cu/63 [#1]	Cu/63 [#2]	Ag/107 [#1]	Ag/107 [#2]	Ag/109 [#1]	Ag/109 [#2]
SMPL046.D#	blank08	-7.124	-5.229	0.0001299	0.001226	0.0007182	0.0004076
SMPL047.D#	d0 control A 0612	470.1	455.4	0.1199	0.1185	0.1228	0.1327
SMPL048.D#	d0 control B 0612	161.2	144.3	0.1206	0.1205	0.1092	0.1255
SMPL049.D#	d0 25µg/L A 0612	113.8	109	5.684	6.273	5.728	6.176
SMPL050.D#	d0 25µg/L B 0612	116.8	103.1	5.879	6.173	5.886	6.162
SMPL051.D#	d0 100µg/L A 0612	140.9	125.4	23.76	24.85	23.71	24.43
SMPL052.D#	d0 100µg/L B 0612	141.8	122.8	24.35	24.93	24.34	24.87
SMPL053.D#	blank09	-6.977	-5.452	0.01951	0.03449	0.02072	0.03029
SMPL054.D#	TMDWS.2 c 1:10	3608	3757	1.035	1.065	0.993	1.071
SMPL055.D#							

Sequence error! Restart; Sample "055" lost.

File:	Sample:	Cu/63 [#1]	Cu/63 [#2]	Ag/107 [#1]	Ag/107 [#2]	Ag/109 [#1]	Ag/109 [#2]
SMPL056.D#	TMDA70e 1:5	17470	17610	2.236	2.305	2.216	2.324
SMPL057.D#	TMDA70f 1:5	17070	17320	2.237	2.249	2.231	2.313
SMPL058.D#	blank10	-5.94E+00	-1.74E+00	6.30E-04	-8.52E-04	3.58E-04	-3.96E-04
001_STD.D#	blank	2.55E-08	-4.02E-07	2.45E-11	-2.93E-12	2.65E-11	-2.18E-12
002_STD.D#	0.1	270.1	206.8	0.1282	0.1012	0.1278	0.09006
003_STD.D#	0.25	27.28	21.81	0.2783	0.2408	0.274	0.2353
004_STD.D#	0.5	36.83	35.11	0.555	0.4765	0.5429	0.4988
005_STD.D#	1	25.98	19.6	1.17	1.005	1.167	0.9905
006_STD.D#	2.5	49.96	42.78	3.069	2.645	3.058	2.584
007_STD.D#	5	41.81	39.11	5.438	5.266	5.443	5.222
008_STD.D#	10	10.42	12.37	11.24	11.68	11.29	11.55
009_STD.D#	25	70.55	74.03	24.35	24.26	24.33	24.33
010_STD.D#	50	19.55	18.35	62.39	56.63	62.24	56.12
SMPL001.D#	blank01	27.02	22.61	0.00597	0.02278	0.006035	0.01442
SMPL002.D#	TMDWS.2 a 1:10	4950	4099	1.138	0.9956	1.156	0.9793
SMPL003.D#	TMDWS.2 b 1:10	4537	4178	1.081	0.9707	1.066	1
SMPL004.D#	TMDA70a 1:10	11690	10220	1.264	1.16	1.289	1.126
SMPL005.D#	TMDA70b 1:10	11650	10260	1.272	1.113	1.276	1.12
SMPL006.D#	blank02	31.64	28.61	-0.0005315	0.00155	0.0003147	0.0003507
SMPL007.D#	UHQ A	337.4	293.8	0.07207	0.0522	0.06535	0.05014
SMPL008.D#	UHQ B	601.2	418.5	0.118	0.08536	0.1188	0.09197
SMPL009.D#	d6 control centrif. 12.12 I	302.1	261.2	0.03501	0.03018	0.03381	0.03174
SMPL010.D#	d6 control centrif. 12.12 II	207.9	217.9	0.02697	0.03239	0.02755	0.02995
SMPL011.D#	d6 25µg/L centif. 12.12 I	357.5	293	0.2039	0.1862	0.2045	0.1812
SMPL012.D#	d6 25µg/L centif. 12.12 II	249	181.4	0.02387	0.01887	0.01861	0.01682
SMPL013.D#	d6 100µg/L centif. 12.12 I	361.1	318.4	0.3109	0.2889	0.3077	0.2784
SMPL014.D#	d6 100µg/L centif. 1212 II	222.8	194.7	1.319	1.2	1.304	1.187
SMPL015.D#	Blank03	15.75	14.17	0.0003525	0.0007094	0.001059	0.001822
SMPL016.D#	d6 control A 12.12	344.4	296.6	0.1257	0.1109	0.1214	0.1119
SMPL017.D#	d6 control B 12.12	198.5	185	0.09824	0.09726	0.09431	0.08971
SMPL018.D#	d6 25µg/L A 12.12	172	143.3	5.365	4.758	5.399	4.694
SMPL019.D#	d6 25µg/L B 12.12	219.5	179.1	5.593	4.828	5.647	4.879
SMPL020.D#	d6 100µg/L A 12.12	306.3	306.5	26.1	26.57	26.2	26.32
SMPL021.D#	d6 100µg/L B 12.12	242.7	175.7	27.35	22	27.37	21.83
SMPL022.D#	Blank04	7.31	8.296	0.03185	0.02865	0.02734	0.03231
SMPL023.D#	d0 control centrif. 13.12 I	306.6	239.1	0.02013	0.01538	0.02293	0.01338
SMPL024.D#	d0 control centrif. 13.12 II	184	145.2	0.04244	0.03016	0.04266	0.0382
SMPL025.D#	d0 25µg/L centif. 13.12 I	228.6	178.5	0.2022	0.1678	0.2138	0.169
SMPL026.D#	d0 25µg/L centif. 13.12 II	266.1	231.2	0.2187	0.1789	0.2116	0.1843
SMPL027.D#	d0 100µg/L centif. 13.12 I	179.5	157.4	1.064	0.9451	1.07	0.9194
SMPL028.D#	d0 100µg/L centif. 13.12 II	206.8	175.7	0.8162	0.7636	0.8304	0.7236
SMPL029.D#	blank05	15.9	14.01	5.41E-05	-3.16E-05	0.0001368	0.0003457
SMPL030.D#	TMDA70c 1:10	10400	10100	1.206	1.147	1.214	1.153
SMPL031.D#	TMDA70d 1:10	12570	9146	1.411	0.9987	1.468	1.074
SMPL032.D#	blank06	21.82	21.93	0.0003001	0.002218	-0.0001634	-0.0003405
SMPL033.D#	d0 control A 13.12	191.4	150.9	0.07278	0.06827	0.07064	0.07308
SMPL034.D#	d0 control B 13.12	162.7	150.1	0.1012	0.09607	0.1045	0.1044
SMPL035.D#	d0 25µg/L A 13.12	196	153.3	7.701	6.234	7.651	6.123
SMPL036.D#	d0 25µg/L B 13.12	239.5	205	7.155	6.314	7.154	6.393

File:	Sample:	Cu/63 [#1]	Cu/63 [#2]	Ag/107 [#1]	Ag/107 [#2]	Ag/109 [#1]	Ag/109 [#2]
SMPL037.D#	d0 100µg/L A 13.12	165.9	141.6	26.84	25.11	26.94	25
SMPL038.D#	d0 100µg/L B 13.12	199.5	156.4	30.24	25.54	30.16	25.36
SMPL039.D#	blank07	5.202	7.368	0.01815	0.02466	0.01983	0.02148
SMPL040.D#	d6 control centrif. 19.12 I	259.9	268.1	0.0228	0.03047	0.02356	0.02709
SMPL041.D#	d6 control centrif. 19.12 II	353.7	314.2	0.06893	0.06569	0.06132	0.05158
SMPL042.D#	d6 25µg/L centif. 19.12 I	293.7	267.9	0.1626	0.1681	0.1656	0.1677
SMPL043.D#	d6 25µg/L centif. 19.12 II	311.3	261.4	0.1537	0.1251	0.1412	0.1205
SMPL044.D#	d6 100µg/L centif. 19.12 I	280.9	241.9	0.1533	0.1174	0.1511	0.1299
SMPL045.D#	d6 100µg/L centif. 19.12 II	188.5	153.2	0.4763	0.4162	0.4765	0.4081
SMPL046.D#	Blank08	7.878	9.29	-0.0003574	-4.88E-05	-0.0004072	-2.59E-05
SMPL047.D#	d6 control A 19.12	141.6	120.6	0.1625	0.1467	0.1617	0.1524
SMPL048.D#	d6 control B 19.12	127.2	109.8	0.0911	0.08149	0.08893	0.08898
SMPL049.D#	d6 25µg/L A 19.12	154.2	127.1	3.68	3.309	3.661	3.305
SMPL050.D#	d6 25µg/L B 19.12	201.8	180.8	3.186	2.924	3.213	2.947
SMPL051.D#	d6 100µg/L A 19.12	204.4	172.9	20.13	18.84	20.29	18.76
SMPL052.D#	d6 100µg/L B 19.12	190.6	162.3	20.43	18.21	20.46	18.16
SMPL053.D#	Blank09	7.21	7.13	0.001864	0.004698	0.000334	0.002468
SMPL054.D#	TMDWS.2 c 1:10	4759	4196	1.148	1.005	1.167	1.042
SMPL055.D#	TMDWS.2 d 1:10	4598	4156	1.126	0.9994	1.163	1.017
SMPL056.D#	TMDA70e 1:10	10600	9871	1.235	1.125	1.237	1.12
SMPL057.D#	TMDA70f 1:10	10650	10210	1.236	1.114	1.254	1.16
SMPL058.D#	blank10	19.11	17.01	-9.29E-05	0.001085	0.0006351	0.0003204

Table 67: Additional uptake test with *Danio rerio*: Raw data of tissue analyses.
ICPMS at 11.01. and 12.01.2012; Calibration: MERCK -Ag single element in HNO3 10%. Samples digested in ULTRACLAVE and filled up to 10mL)

File:	Sample:	Cu/63 [#1]	Cu/63 [#2]	Ag/107 [#1]	Ag/107 [#2]	Ag/109 [#1]	Ag/109 [#2]	mg fresh weight
001_STD.D#	blank	-1.61E-07	-1.35E-07	-1.53E-11	-1.51E-11	-3.14E-11	-3.31E-11	
002_STD.D#	0.1	713.2	639.7	0.09738	0.08704	0.09753	0.0911	
003_STD.D#	0.25	206.7	206.8	0.232	0.2393	0.2345	0.2692	
004_STD.D#	0.5	120.3	119.1	0.4795	0.4607	0.4665	0.4766	
005_STD.D#	1	13.36	15.04	0.9622	1.056	0.9806	1.035	
006_STD.D#	2.5	18.06	17	2.411	2.53	2.42	2.672	
007_STD.D#	5	96.26	94.2	4.751	4.997	4.741	5.126	
008_STD.D#	10	21.44	20.46	9.354	9.56	9.3	9.66	
009_STD.D#	25	71.53	78.29	24.01	28.82	24.01	28.82	
010_STD.D#	50	10.64	7.84	50.65	48.17	50.66	48.13	
SMPL001.D#	blank01	-0.03576	-3.085	0.04516	0.05796	0.04755	0.06009	
SMPL002.D#	BW297	136.4	105.2	0.1329	0.144	0.1346	0.1352	
SMPL003.D#	BW298	76.65	71.99	0.4766	0.5112	0.4648	0.5229	
SMPL004.D#	BW299	231.2	208.3	0.06115	0.07466	0.06502	0.07003	
SMPL005.D#	BW300	79.72	76.39	0.06233	0.06848	0.05452	0.05695	
SMPL006.D#	BW301	142.7	143.7	0.4798	0.5448	0.4678	0.5606	
SMPL007.D#	BW302	121.8	95.26	0.5121	0.5066	0.5128	0.5366	
SMPL008.D#	TMDWS.2 a 1:10	4341	4216	0.9766	1.027	0.9828	1.079	
SMPL009.D#	TMDWS.2 b 1:10	4413	4270	0.9982	1.037	0.9813	1.031	
SMPL010.D#	TMDA70a 1:20	5331	5131	0.5599	0.5858	0.5399	0.5513	
SMPL011.D#	TMDA70b 1:20	5117	5045	0.5224	0.5386	0.5242	0.5601	
SMPL012.D#	blank02	2.487	2.129	0.0004197	0.0009143	0.0003132	0.0002816	
SMPL013.D#	guts control I	3283	3125	0.3376	0.3458	0.3429	0.3651	0.079
SMPL014.D#	guts control II	4464	4260	0.8783	0.8932	0.8793	0.9303	0.091
SMPL015.D#	guts control III	4672	4661	0.9339	1.011	0.9151	0.9841	0.118
SMPL016.D#	guts control IV	6538	6861	0.7605	0.8317	0.7573	0.8893	0.131
SMPL017.D#	guts control V	5917	5708	0.5436	0.5529	0.5454	0.5797	0.127
SMPL018.D#	blank03	9.627	8.468	0.001304	0.001681	-0.0001279	0.0001576	
SMPL019.D#	head control I	7941	7682	0.5122	0.5289	0.5128	0.5066	0.253
SMPL020.D#	head control II	7523	7648	0.6143	0.6186	0.6249	0.6118	0.263
SMPL021.D#	head control III	7139	7190	0.4094	0.3926	0.4068	0.4295	

File:	Sample:	Cu/63 [#1]	Cu/63 [#2]	Ag/107 [#1]	Ag/107 [#2]	Ag/109 [#1]	Ag/109 [#2]	mg fresh weight
SMPL022.D#	head control IV	8657	8332	0.4627	0.4409	0.4573	0.4366	0.300
SMPL023.D#	head control V	5961	5868	0.3195	0.3341	0.3173	0.3035	0.202
SMPL024.D#	blank04	8.1	6.604	-0.0001355	0.000321	-0.0001608	5.07E-05	
SMPL025.D#	filet control I	5525	5596	0.3731	0.3915	0.3782	0.4104	0.220
SMPL026.D#	filet control II	7599	7851	0.3434	0.3586	0.3446	0.3846	0.383
SMPL027.D#	filet control III	5797	5896	0.2446	0.2604	0.234	0.2363	0.285
SMPL028.D#	filet control IV	7824	8328	0.307	0.3372	0.3002	0.346	0.332
SMPL029.D#	filet control V	4089	4056	0.2853	0.3026	0.2794	0.2874	0.216
SMPL030.D#	blank05	6.542	6.695	-0.0001743	0.001183	-0.0008315	0.0000658	
SMPL031.D#	08DL076	147400	151400	15.07	15.35	14.95	15.41	
SMPL032.D#	08DL077	147000	148100	14.89	15.06	14.78	15.19	
SMPL033.D#	08DL078	154000	152500	15.64	15.37	15.58	15.57	
SMPL034.D#	blank06	120.6	149.3	0.01439	0.0173	0.01821	0.02325	
SMPL035.D#	guts 25µg/L I	4529	4515	9.449	9.75	9.395	9.754	0.128
SMPL036.D#	guts 25µg/L II	7133	6967	67.1	65.76	67.85	65.97	0.174
SMPL037.D#	guts 25µg/L III	3792	3424	37.83	35.07	37.8	35.28	0.075
SMPL038.D#	guts 25µg/L IV	5243	5196	50.06	51.38	49.66	51.55	0.113
SMPL039.D#	guts 25µg/L V	4492	4417	68.37	68.69	68.1	68.85	0.102
SMPL040.D#	blank07	8.886	12.13	0.1027	0.1361	0.09616	0.1176	
SMPL041.D#	head 25µg/L I	5650	5579	2.589	2.731	2.615	2.728	0.227
SMPL042.D#	head 25µg/L II	4134	4081	19.02	19.65	19.03	19.62	0.182
SMPL043.D#	head 25µg/L III	6917	7082	4.875	5.025	4.738	5.009	0.276
SMPL044.D#	head 25µg/L IV	7485	7533	3.338	3.431	3.36	3.514	0.242
SMPL045.D#	head 25µg/L V	7458	7684	2.577	2.695	2.629	2.742	0.241
SMPL046.D#	blank08	9.021	11.45	0.003319	0.00253	0.005219	0.006577	
SMPL047.D#	08DL079	142400	145700	14.53	14.84	14.53	15.05	
SMPL048.D#	08DL080	137300	145000	14.1	14.74	13.98	15.06	
SMPL049.D#	08DL081	145300	152800	15.07	15.57	14.97	15.63	
SMPL050.D#	blank09	94.64	135.5	0.01243	0.0163	0.01431	0.0191	
SMPL051.D#	TMDWS.2 c 1:10	3802	4064	0.9429	1.002	0.9623	1.023	
SMPL052.D#	TMDWS.2 d 1:10	3860	4035	0.9458	1.006	0.9643	1.029	
SMPL053.D#	TMDA70c 1:20	4634	4841	0.5111	0.5686	0.5269	0.5174	
SMPL054.D#	TMDA70d 1:20	4950	5243	0.5314	0.56	0.5251	0.5494	
SMPL055.D#	blank09	2.457	0.8361	0.0005598	0.00132	0.0000125	0.003446	

File:	Sample:	Cu/ 63 [#1]	Cu/ 63 [#2]	Ag/107 [#1]	Ag/107 [#2]	Ag/109 [#1]	Ag/109 [#2]	mg fresh weight
001_STD.D#	blank	5.02E-08	-4.19E-07	-2.91E-12	1.72E-11	1.95E-12	1.67E-11	
002_STD.D#	0.1	59.81	60.8	0.0965	0.1055	0.0986	0.09792	
003_STD.D#	0.25	669.8	678.6	0.241	0.2465	0.2339	0.2497	
004_STD.D#	0.5	129.4	133.6	0.4894	0.4747	0.492	0.517	
005_STD.D#	1	16.61	17.47	0.9703	0.995	0.9826	0.9821	
006_STD.D#	2.5	53.72	57.51	2.476	2.495	2.456	2.49	
007_STD.D#	5	151.8	171.7	4.97	5.549	4.899	5.584	
008_STD.D#	10	26.32	25.17	9.935	9.932	9.958	9.928	
009_STD.D#	25	76.69	77.26	24.81	25.14	24.66	25.55	
010_STD.D#	50	15.2	12.11	50.99	50.26	50.65	49.92	
011_STD.D#	100	91.69	92.06	99.56	99.81	99.77	99.88	
SMPL001.D#	blank01	3.336	5.823	0.05757	0.1032	0.05256	0.1052	
SMPL002.D#	BW303	287.5	220.4	0.01345	0.01162	0.01317	0.01395	
SMPL003.D#	BW304	28.29	27.02	0.03034	0.03749	0.02972	0.03317	
SMPL004.D#	BW305	231.7	211	0.009199	0.008771	0.01111	0.01015	
SMPL005.D#	BW306	178.5	173.6	0.03204	0.02462	0.029	0.03286	
SMPL006.D#	BW307	22.53	19.35	0.1949	0.2035	0.1926	0.2076	
SMPL007.D#	BW308	81.11	61.8	0.07242	0.07979	0.07287	0.08072	
SMPL008.D#	TMDWS.2 a 1:10	4518	4338	1.018	1.057	1.032	0.9976	
SMPL009.D#	TMDWS.2 b 1:10	4470	4439	1.026	1.022	1.019	1.015	
SMPL010.D#	TMDA70a 1:20	6579	6458	0.563	0.5856	0.5708	0.5756	
SMPL011.D#	TMDA70b 1:20	6175	6350	0.5625	0.556	0.5485	0.5928	
SMPL012.D#	blank02	2.21	2.456	-0.0008134	0.0004152	0.0000678	-0.0000673	
SMPL013.D#	filet 25µg/L I	8101	7583	1.836	1.881	1.823	1.813	0.332

File:	Sample:	Cu/63 [#1]	Cu/63 [#2]	Ag/107 [#1]	Ag/107 [#2]	Ag/109 [#1]	Ag/109 [#2]	mg fresh weight
SMPL014.D#	filet 25µg/L II	6934	6580	3.701	3.739	3.63	3.736	0.345
SMPL015.D#	filet 25µg/L III	8913	8250	1.732	1.66	1.678	1.741	0.347
SMPL016.D#	filet 25µg/L IV	8400	8107	11.36	11.65	11.27	11.42	0.345
SMPL017.D#	filet 25µg/L V	8975	8623	1.511	1.534	1.506	1.518	0.333
SMPL018.D#	blank03	10.42	13.27	0.002115	0.001436	0.002932	0.003574	
SMPL019.D#	guts 100µg/L I	9838	9259	88.04	86.34	87.9	86.37	0.138
SMPL020.D#	guts 100µg/L II	8853	8233	176.9	176.6	176.9	175.7	0.131
SMPL021.D#	guts 100µg/L III	4671	4288	199.5	197.5	196.8	197.4	0.054
SMPL022.D#	guts 100µg/L IV	7363	6702	289.8	303.7	288.6	302.3	0.105
SMPL023.D#	guts 100µg/L V	5202	4814	147.2	149	148.3	148.6	0.083
SMPL024.D#	blank04	4.954	8.216	0.874	0.9775	0.8574	0.9492	
SMPL025.D#	08DL082	173700	171000	16.09	16.45	15.95	16.33	
SMPL026.D#	08DL083	165800	162900	15.46	15.6	15.18	15.52	
SMPL027.D#	08DL084	167200	170000	15.68	16.07	15.53	16.1	
SMPL028.D#	08DL085	169200	171000	16.44	16.61	16.15	16.59	
SMPL029.D#	blank05	146.4	193.3	0.4736	0.4899	0.4693	0.5363	
SMPL030.D#	head 100µg/L I	6121	5872	16.33	16.42	16.27	16.41	0.244
SMPL031.D#	head 100µg/L II	5508	5368	11.28	11.56	11.07	11.44	0.202
SMPL032.D#	head 100µg/L III	5760	5604	9.037	9.045	8.947	9.104	0.190
SMPL033.D#	head 100µg/L IV	5171	4880	10.11	10.06	10.02	10.1	0.175
SMPL034.D#	head 100µg/L V	5300	5176	10.17	10.33	10.06	10.33	0.188
SMPL035.D#	blank06	7.231	9.441	0.2365	0.2663	0.2479	0.2579	
SMPL036.D#	Filet 100µg/L I	5787	5435	6.316	6.224	6.185	6.342	0.271
SMPL037.D#	filet 100µg/L II	7946	7780	10.42	10.89	10.22	10.79	0.330
SMPL038.D#	filet 100µg/L III	9711	9214	12.35	12.4	12.24	12.22	0.336
SMPL039.D#	filet 100µg/L IV	8859	8312	16.56	16.48	16.31	16.39	0.362
SMPL040.D#	filet 100µg/L V	7518	7171	25.82	25.44	25.3	25.55	0.350
SMPL041.D#	blank07	5.78	9.151	0.09184	0.1093	0.09122	0.116	
SMPL042.D#	TMDWS.2 c 1:10	4885	4530	1.147	1.11	1.123	1.101	
SMPL043.D#	TMDWS.2 d 1:10	4715	4428	1.113	1.111	1.081	1.107	
SMPL044.D#	TMDA70c 1:20	5273	5339	0.572	0.6176	0.5727	0.6164	
SMPL045.D#	TMDA70d 1:20	5482	5268	0.5916	0.5747	0.5939	0.5807	
SMPL046.D#	blank08	4.975	5.56	0.02728	0.02956	0.02864	0.03197	

5 Summary

5.1 Results from ecotoxicological studies

5.1.1 Titanium dioxide

The nanoparticulate titanium dioxide NM-105 was investigated in two tests with *Lumbriculus variegatus* in a sediment-water system according to the OECD TG 225 [5]. The nominal test concentrations in the first test were 15; 23; 39; 63 and 100 mg/L NM-105 and 100 mg/L NM-105 in the second test. Chemical analysis of titanium concentrations in test media in the first test showed good agreement with nominal test concentrations.

In the investigated concentration range, NM-105 elicited no adverse effects on reproduction or biomass of the worms in either test. A NOEC ≥ 100 mg/L was determined. Measurement of titanium concentrations in the worms at test end showed no significant difference between control worms and worms exposed to 100 mg NM-105/L.

Additionally, NM-105 was investigated in tests with the predatory mite *Hypoaspis aculeifer* as described in the OECD TG 226 [6]. In a range finding test and the first definitive test, 1; 10; 100; 1000 mg NM-105/kg artificial soil (dw) were used to investigate effects. The second definitive test was performed with 1; 1000 mg NM-105/kg artificial soil (dw).

In the range finding test, a significantly lower number of juvenile mites was found at the lowest and highest investigated test concentration (1 and 1000 mg NM-105/kg artificial soil (dw)), but not in the intermediate test concentrations. At 1 mg NM-105/kg artificial soil (dw), the survival of adult mites was lower than in the controls and any of the treatments. It was considered that the mites exposed to 1 mg NM-105/kg artificial soil (dw) could have been damaged during transfer into the test vessels and died before onset of reproduction. However, it is not clear why this should have happened exclusively to mites used for the lowest test concentration, since the transfer of mites into the test vessels was conducted randomized over all test concentrations. In the first definitive test, no significant difference between treatments and control was detected. The results of the second definitive test show a significantly lower number of juvenile mites at 1 and 1000 mg NM-105/kg artificial soil (dw) than in the control. Total differences compared to the control were comparable to the first definitive test, but significant due to higher statistical power of the doubled number of replicates. For the standard design, which was proven to be applicable to the testing of nanomaterials, the NOEC was ≥ 1000 mg NM-105/kg.

5.1.2 Nano silver

The nanoparticulate silver NM-300 K was investigated in two fish early life stage toxicity tests with *Danio rerio* in a 250 L static system according to the OECD TG 210 [7]. The nominal test concentrations in the

first test were 12.5; 25; 50; 100 and 200 µg Ag/L and 12.5, 50 and 100 µg Ag/L in the second test. Chemical analysis of total silver concentrations in test media showed approximately 70 % of nominal concentrations during the 1st test and 50 % during the 2nd test. The proportion of dissolved silver was approximately 3 %. Hatch was not affected up to 136 µg/L (mean measured). Post-hatch survival was significantly reduced at concentrations ≥ 47 µg/L, the NOEC was determined to be 23 µg/L. The most sensitive endpoint was growth, measured as total individual length with a NOEC of 5.9 µg/L.

The test setup was demonstrated to be suited for the testing of nanomaterials, proven by sensitive results and high statistical power.

Measurement of total silver concentrations in the fish after the exposure period showed significantly increasing concentrations with increasing Ag concentration in the test medium. In an additional experiment it was shown that most of the accumulated silver is located in the intestines.

5.2 Suitability of application methods

5.2.1 Titanium dioxide

The application methods used for preparation of test media with NM-105 were developed by Hund-Rinke and colleagues [4], [16].

For the tests with *L. variegatus*, test media were prepared by dilution of NM-105-dispersions with the sediment-overlying water. Volumes used for application were high compared to final volumes and homogenous distribution of the test item in the test media was easily achieved by stirring test media with a glass rod.

In the tests with *H. aculeifer*, the test substrates were prepared by mixing the solid powder into the artificial soil (for test concentrations ≥ 10mg/kg) or application of NM-105-dispersions (test concentrations ≤ 10mg/kg). Distributing very small amounts of solid NM-105 in the test substrates would have been very difficult; by using dispersions a homogenous distribution was readily performed. For 10 mg/kg, no influence of selected application method on the biological endpoints was observed.

5.2.2 Nano silver

The application method used for preparation of test media with NM-300 K was developed by Hund-Rinke and colleagues [4]. The NM-300 K dispersion (1:10) was slightly diluted with aqua dest., ultra-sonificated for 15 min (Bandelin Sonorex RK 514 BH; 35 kHz; 215/860 W) and directly applied to the test vessels. The water in the test aquaria was constantly moved by four pumps per vessel, enabling homogeneous distribution and minimizing sedimentation. Every 7 days, the test medium was exchanged entirely by transferring the test organisms in freshly prepared aquaria. This caused enhanced mortality of yolk sac larvae, most probably resulting from exposure to dissolved silver directly after renewal of the test dispersions (low complexation by organic carbon from feed and faeces). However, as the overall NOEC is

resulting from growth effects being more sensitive by one order magnitude, acute effects by dissolved silver do not drive the test results.

Pseudo-replicate cages located in one vessel per test concentration only may run into the risk of a bias by uneven conditions (1st test)). Thus, true replicates (containing pseudo-replicate chambers) are preferred, which can be statistically compared and combined if they do not differ significantly (2nd test).

5.3 Suitability of guideline tests for assessing ecotoxicity of selected nanoparticles

The applied guideline tests are suited for assessing ecotoxicity of selected nanoparticles, provided that exposure of the test media and subsequently of the test organisms is adequately performed. The situation with nanosilver is not a typical one, as the objective of using silver nanoparticles is to serve as reservoir for steady dissolution of free Ag ions that act as biocide. Thus, stability of the particles and prevention of dissolution have to be discussed in this case. For nanomaterials aimed to be stable and with e.g. electrostatic properties, dosing aquatic tests via flow-through systems is neither necessary nor feasible in certain cases. For these materials, the proposed static system, preferably with two replicate aquaria per treatment, is a good alternative.

6 Literature

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ANNEX 1: Statistical evaluation of endpoints

A 1.1 Fish Early Life Stage Toxicity test: First study

A 1.1.1 Post hatch survival, day 35

Statistical Characteristics of the Sample

Tab. 1: Statistical characteristics: Mean: arithmetic mean (X); Med: median; Min: minimum value, Max: maximum value; n: sample size; s: standard deviation; s%: coefficient of variation; s(X): standard error; %s(X): %standard error; 95%l, 95%u: lower, upper 95%-confidence limits.

Treatm. [µg/L]	Mean	Med	Min	Max	n	s	%s	s(X)	%s(X)	95%l	95%u
Control	0.9	0.9	0.8	0.9	4	0.05	5.4	0.02	2.7	0.8	1.0
25	1.1	1.1	1.0	1.1	4	0.03	3.2	0.02	1.6	1.0	1.1
50	0.9	0.9	0.8	1.0	4	0.14	14.9	0.07	7.4	0.7	1.2
100	0.7	0.8	0.6	0.8	4	0.07	9.8	0.04	4.9	0.6	0.9
200	0.0	0.0	0.0	0.0	4	0.00	n.d.	0.00	n.d.	0.0	0.0

One-way Analysis of Variance

Tab. 2: One-way Analysis of Variance: Source: source of variance; SS: sum of squares; df: degrees of freedom; MSS: mean sum of squares; F: test statistic: p: probability

Source	SS	df	MSS	F	p(F)
Treatment	2.89	4	0.72	129.508	< 0.001
Residuals	0.08	15	0.01		
Total	2.97	19			

p(F) is smaller than or equal to the selected significance level of 0.05; therefore, treatments are significantly different.

The first analysis revealed significant results: Pretesting is continued.

Shapiro-Wilk´s Test on Normal Distribution

Tab. 3: Shapiro-Wilk´s Test on Normal Distribution; Mean: arithmetic mean; n: sample size; p(ShapiroWilk´s W): probability of the W statistic. In case p(ShapiroWilk´s W) is greater than the chosen significance level, the normality hypothesis(Ho) is accepted.

Treatm. [µg/L]	Mean	s	n
Control	0.9	0.05	4
25	1.1	0.03	4
50	0.9	0.14	4
100	0.7	0.07	4
200	0.0	0.00	4

Results:

Number of residues = 11; Shapiro-Wilk´s W = 0.983; p(W) = 0.819; p(W) is greater than the selected significance level of 0.05; therefore, treatment data do not significantly deviate from normal distribution.

Normality check was passed (p > 0.05).

Levene´s Test on Variance Homogeneity (with Residuals)

Tab. 4: Levene´s Test on Variance Homogeneity (with Residuals): Source: source of variance; SS: sum of squares; df: degrees of freedom; MSS: mean sum of squares; F: test statistic: p: probability

Source	SS	df	MSS	F	p(F)
Treatment	0.00	4	0.00	14.771	< 0.001
Residuals	0.00	15	0.00		
Total	0.00	19			

Based on the pre-selected significance level of 0.05, the Levene test indicates variance heterogeneity!

Variance homogeneity check was not passed

Welch-t test for Inhomogeneous Variances with Bonferroni-Holm Adjustment

Tab. 5: Multiple sequentially rejective comparisons after Welch of treatments with "Control" by the t test procedure. Significance was Alpha = 0.05, one-sided smaller; Mean: arithmetic mean; n: sample size; s^2: variance; %MDD: minimum detectable difference to Control (in percent of Control); t: sample t; p(t): probability of sample t for Ho: $\mu 1 = \mu 2$; Alpha(i): adjusted significance levels; the differences are significant in case p(i) <= Alpha(i) ; dfm: modified degrees of freedom due to heteroscedasticity.(Control(c) and treatment(t) variance was applied: $s^2(c)/nc + s^2(t)/nt$, each).

Treatm. [µg/L]	Mean	s^2	df	%MDD	t	p(t)	Alpha(i)	Sign.
Control	0.9	0.0						
25	1.1	0.0	5	-6.7	5.93	0.999	0.050	-
50	0.9	0.0	3	-25.9	0.40	0.643	0.025	-
100	0.7	0.0	5	-14.1	-3.78	0.006	0.017	+
200	0.0	0.0	0	n.d.	n.d.	n.d.	n.d.	*

+: significant; -: non-significant; *: test could not be performed; n.d.: not determined

Based on the results of the Welch t-test, a NOEC of 50 µg/L is suggested.

A 1.1.2 Length, day 35

Statistical Characteristics of the Sample

Tab. 1: Statistical characteristics: Mean: arithmetic mean (X); Med: median; Min: minimum value, Max: maximum value; n: sample size; s: standard deviation; s%: coefficient of variation; s(X): standard error; %s(X): %standard error; 95%l, 95%u: lower, upper 95%-confidence limits.

Treatm. [µg/L]	Mean	Med	Min	Max	n	s	%s	s(X)	%s(X)	95%l	95%u
Control	1.3	1.3	1.2	1.3	4	0.08	6.1	0.04	3.0	1.1	1.4
25	1.0	1.0	1.0	1.1	4	0.04	4.3	0.02	2.1	0.9	1.1
50	1.0	1.1	0.9	1.1	4	0.09	8.8	0.05	4.4	0.9	1.2
100	1.1	1.1	1.0	1.3	4	0.13	12.3	0.07	6.1	0.9	1.3

One-way Analysis of Variance

Tab. 2: One-way Analysis of Variance: Source: source of variance; SS: sum of squares; df: degrees of freedom; MSS: mean sum of squares; F: test statistic: p: probability

Source	SS	df	MSS	F	p(F)
Treatment	0.14	3	0.05	5.678	0.012
Residuals	0.10	12	0.01		
Total	0.24	15			

p(F) is smaller than or equal to the selected significance level of 0.05; therefore, treatments are significantly different.

Shapiro-Wilk´s Test on Normal Distribution

Tab. 3: Shapiro-Wilk´s Test on Normal Distribution; Mean: arithmetic mean; n: sample size; p(ShapiroWilk´s W): probability of the W statistic. In case p(ShapiroWilk´s W) is greater than the chosen significance level, the normality hypothesis(Ho) is accepted.

Treatm. [µg/L]	Mean	s	n
Control	1.3	0.08	4
25	1.0	0.04	4
50	1.0	0.09	4
100	1.1	0.13	4

Results:

Number of residues = 16; Shapiro-Wilk´s W = 0.958; p(W) = 0.632; p(W) is greater than the selected significance level of 0.05; therefore, treatment data do not significantly deviate from normal distribution.

Normality check was passed (p > 0.05).

Levene´s Test on Variance Homogeneity (with Residuals)

Tab. 4: Levene´s Test on Variance Homogeneity (with Residuals): Source: source of variance; SS: sum of squares; df: degrees of freedom; MSS: mean sum of squares; F: test statistic: p: probability

Source	SS	df	MSS	F	p(F)
Treatment	0.00	3	0.00	1.567	0.249
Residuals	0.00	12	0.00		
Total	0.00	15			

Based on the pre-selected significance level of 0.05, the Levene test indicates variance homogeneity!

Variance homogeneity check was passed.
Normal distribution and variance homogeneity requirements are fulfilled.

Williams Multiple Sequential t-test Procedure

Tab. 5: Comparison of treatments with "Control" by the t test procedure after Williams. Significance was Alpha = 0.05, one-sided smaller; Mean: arithmetic mean; n: sample size; s: standard deviation; LhM: max. likelihood mean; %MDD: minimum detectable difference to Control (in percent of Control); t: sample t; t*: critical t for Ho: µ1 = µ2 = ... = µk; the differences are significant in case |t| > |t*| (The residual variance of an ANOVA was applied; df = N - k; N: sum of treatment replicates n(i); k: number of treatments).

Treatm. [µg/L]	Mean	s	df	LhM	%MDD	t	t*	Sign.
Control	1.3	0.092						
25	1.0	0.092	12	1.0	-9.2	-3.24	-1.78	+
50	1.0	0.092	12	1.0	-9.7	-3.24	-1.87	+
100	1.1	0.092	12	1.0	-9.8	-3.24	-1.90	+

+: significant; -: non-significant

The NOEC is lower than 25 µg/L.

A 1.1.3 Group dry weight, day 35

Statistical Characteristics of the Sample

Tab. 1: Statistical characteristics: Mean: arithmetic mean (X); Med: median; Min: minimum value, Max: maximum value; n: sample size; s: standard deviation; s%: coefficient of variation; s(X): standard error; %s(X): %standard error; 95%l, 95%u: lower, upper 95%-confidence limits.

Treatm. [µg/L]	Mean	Med	Min	Max	n	s	%s	s(X)	%s(X)	95%l	95%u
Control	53.0	53.0	44.0	62.0	4	9.83	18.6	4.92	9.3	37.2	68.8
25	38.3	35.0	32.0	51.0	4	8.62	22.5	4.31	11.3	24.4	52.1
50	31.8	30.0	20.0	47.0	4	11.93	37.6	5.96	18.8	12.5	51.0
100	24.8	25.5	19.0	29.0	4	4.35	17.6	2.17	8.8	17.7	31.8

One-way Analysis of Variance

Tab. 2: One-way Analysis of Variance: Source: source of variance; SS: sum of squares; df: degrees of freedom; MSS: mean sum of squares; F: test statistic: p: probability

Source	SS	df	MSS	F	p(F)
Treatment	1740.69	3	580.23	6.989	0.006
Residuals	996.25	12	83.02		
Total	2736.94	15			

p(F) is smaller than or equal to the selected significance level of 0.05; therefore, treatments are significantly different.

The first analysis revealed significant results: Pretesting is continued.

Shapiro-Wilk´s Test on Normal Distribution

Tab. 3: Shapiro-Wilk´s Test on Normal Distribution; Mean: arithmetic mean; n: sample size; p(ShapiroWilk´s W): probability of the W statistic. In case p(ShapiroWilk´s W) is greater than the chosen significance level, the normality hypothesis(Ho) is accepted.

Treatm. [µg/L]	Mean	s	n
Control	53.0	9.83	4
25	38.3	8.62	4
50	31.8	11.93	4
100	24.8	4.35	4

Results:

Number of residues = 15; Shapiro-Wilk´s W = 0.948; p(W) = 0.496; p(W) is greater than the selected significance level of 0.05; therefore, treatment data do not significantly deviate from normal distribution.

Normality check was passed (p > 0.05).

Levene´s Test on Variance Homogeneity (with Residuals)

Tab. 4: Levene´s Test on Variance Homogeneity (with Residuals): Source: source of variance; SS: sum of squares; df: degrees of freedom; MSS: mean sum of squares; F: test statistic: p: probability

Source	SS	df	MSS	F	p(F)
Treatment	17731.29	3	5910.43	1.525	0.258
Residuals	46505.56	12	3875.46		
Total	64236.86	15			

Based on the pre-selected significance level of 0.05, the Levene test indicates variance homogeneity!

Variance homogeneity check was passed.
Normal distribution and variance homogeneity requirements are fulfilled.

Williams Multiple Sequential t-test Procedure

Tab. 5: Comparison of treatments with "Control" by the t test procedure after Williams. Significance was Alpha = 0.05, one-sided smaller; Mean: arithmetic mean; n: sample size; s: standard deviation; LhM: max. likelihood mean; %MDD: minimum detectable difference to Control (in percent of Control); t: sample t; t*: critical t for Ho: μ1 = μ2 = ... = μk; the differences are significant in case |t| > |t*| (The residual variance of an ANOVA was applied; df = N - k; N: sum of treatment replicates n(i); k: number of treatments).

Treatm. [µg/L]	Mean	s	df	LhM	%MDD	t	t*	Sign.
Control	53.0	9.112						
25	38.3	9.112	12	38.3	-21.7	-2.29	-1.78	+
50	31.8	9.112	12	31.8	-22.8	-3.30	-1.87	+
100	24.8	9.112	12	24.8	-23.1	-4.38	-1.90	+

+: significant; -: non-significant

The NOEC is lower than 25 µg/L.

A 1.2 Fish Early Life Stage Toxicity tests: Second study

A 1.2.1 Post hatch survival, day 35

Statistical Characteristics of the Sample

Tab. 1: Statistical characteristics: Mean: arithmetic mean (X); Med: median; Min: minimum value, Max: maximum value; n: sample size; s: standard deviation; s%: coefficient of variation; s(X): standard error; %s(X): %standard error; 95%l, 95%u: lower, upper 95%-confidence limits.

Treatm. [µg/L]	Mean	Med	Min	Max	n	s	%s	s(X)	%s(X)	95%l	95%u
Control	1.3	1.2	1.0	1.6	12	0.19	15.1	0.06	4.4	1.1	1.4
12.5	1.3	1.3	1.1	1.6	12	0.13	10.3	0.04	3.0	1.2	1.4
50	1.4	1.6	1.1	1.6	12	0.18	12.4	0.05	3.6	1.3	1.5
100	1.0	1.1	0.5	1.3	12	0.27	26.5	0.08	7.6	0.9	1.2

One-way Analysis of Variance

Tab. 2: One-way Analysis of Variance: Source: source of variance; SS: sum of squares; df: degrees of freedom; MSS: mean sum of squares; F: test statistic: p: probability

Source	SS	df	MSS	F	p(F)
Treatment	1.02	3	0.34	8.486	< 0.001
Residuals	1.76	44	0.04		
Total	2.77	47			

p(F) is smaller than or equal to the selected significance level of 0.05; therefore, treatments are significantly different.

The first analysis revealed significant results: Pretesting is continued.

Shapiro-Wilk´s Test on Normal Distribution

Tab. 3: Shapiro-Wilk´s Test on Normal Distribution; Mean: arithmetic mean; n: sample size; p(ShapiroWilk´s W): probability of the W statistic. In case p(ShapiroWilk´s W) is greater than the chosen significance level, the normality hypothesis(Ho) is accepted.

Treatm. [µg/L]	Mean	s	n
Control	1.3	0.19	12
12.5	1.3	0.13	12
50	1.4	0.18	12
100	1.0	0.27	12

Results:

Number of residues = 23; Shapiro-Wilk´s W = 0.967; p(W) = 0.629; p(W) is greater than the selected significance level of 0.05; therefore, treatment data do not significantly deviate from normal distribution.

Normality check was passed (p > 0.05).

Levene´s Test on Variance Homogeneity (with Residuals)

Tab. 4: Levene´s Test on Variance Homogeneity (with Residuals): Source: source of variance; SS: sum of squares; df: degrees of freedom; MSS: mean sum of squares; F: test statistic: p: probability

Source	SS	df	MSS	F	p(F)
Treatment	0.02	3	0.01	2.484	0.073
Residuals	0.10	44	0.00		
Total	0.12	47			

Based on the pre-selected significance level of 0.05, the Levene test indicates variance homogeneity!

Variance homogeneity check was passed.
Normal distribution and variance homogeneity requirements are fulfilled.

Williams Multiple Sequential t-test Procedure

Tab. 5: Comparison of treatments with "Control" by the t test procedure after Williams. Significance was Alpha = 0.05, one-sided smaller; Mean: arithmetic mean; n: sample size; s: standard deviation; LhM: max. likelihood mean; %MDD: minimum detectable difference to Control (in percent of Control); t: sample t; t*: critical t for Ho: µ1 = µ2 = ... = µk; the differences are significant in case |t| > |t*| (The residual variance of an ANOVA was applied; df = N - k; N: sum of treatment replicates n(i); k: number of treatments).

Treatm. [µg/L]	Mean	s	df	LhM	%MDD	t	t*	Sign.
Control	1.3	0.200						
12.5	1.3	0.200	44	1.4	-10.8	1.06	-1.68	-
50	1.4	0.200	44	1.4	-11.3	1.06	-1.76	-
100	1.0	0.200	44	1.0	-11.4	-2.99	-1.78	+

+: significant; -: non-significant

Based on the results of the Williams test, a NOEC of 50 µg/L is suggested.

A 1.2.2 Length, day 35

Statistical Characteristics of the Sample

Tab. 1: Statistical characteristics: Mean: arithmetic mean (X); Med: median; Min: minimum value, Max: maximum value; n: sample size; s: standard deviation; s%: coefficient of variation; s(X): standard error; %s(X): %standard error; 95%l, 95%u: lower, upper 95%-confidence limits.

Treatm. [µg/L]	Mean	Med	Min	Max	n	s	%s	s(X)	%s(X)	95%l	95%u
Control	1.4	1.4	1.4	1.5	12	0.04	2.9	0.01	0.8	1.4	1.4
12.5	1.4	1.4	1.3	1.5	12	0.06	4.3	0.02	1.2	1.4	1.4
50	1.3	1.3	1.3	1.4	12	0.05	3.5	0.01	1.0	1.3	1.3
100	1.3	1.3	1.1	1.5	12	0.12	9.5	0.04	2.7	1.2	1.4

One-way Analysis of Variance

Tab. 2: One-way Analysis of Variance: Source: source of variance; SS: sum of squares; df: degrees of freedom; MSS: mean sum of squares; F: test statistic: p: probability

Source	SS	df	MSS	F	p(F)
Treatment	0.13	3	0.04	7.630	< 0.001
Residuals	0.25	44	0.01		
Total	0.38	47			

p(F) is smaller than or equal to the selected significance level of 0.05; therefore, treatments are significantly different.

The first analysis revealed significant results: Pretesting is continued.

Shapiro-Wilk´s Test on Normal Distribution

Tab. 3: Shapiro-Wilk´s Test on Normal Distribution; Mean: arithmetic mean; n: sample size; p(ShapiroWilk´s W): probability of the W statistic. In case p(ShapiroWilk´s W) is greater than the chosen significance level, the normality hypothesis(Ho) is accepted.

Treatm. [µg/L]	Mean	s	n
Control	1.4	0.04	12
12.5	1.4	0.06	12
50	1.3	0.05	12
100	1.3	0.12	12

Results:

Number of residues = 34; Shapiro-Wilk´s W = 0.975; p(W) = 0.625; p(W) is greater than the selected significance level of 0.05; therefore, treatment data do not significantly deviate from normal distribution.

Normality check was passed (p > 0.05).

Levene´s Test on Variance Homogeneity (with Residuals)

Tab. 4: Levene´s Test on Variance Homogeneity (with Residuals): Source: source of variance; SS: sum of squares; df: degrees of freedom; MSS: mean sum of squares; F: test statistic: p: probability

Source	SS	df	MSS	F	p(F)
Treatment	0.00	3	0.00	6.047	0.002
Residuals	0.00	44	0.00		
Total	0.00	47			

Based on the pre-selected significance level of 0.05, the Levene test indicates variance heterogeneity!

Variance homogeneity check was not passed

Welch-t test for Inhomogeneous Variances with Bonferroni-Holm Adjustment

Tab. 5: Multiple sequentially rejective comparisons after Welch of treatments with "Control" by the t test procedure. Significance was Alpha = 0.05, one-sided smaller; Mean: arithmetic mean; n: sample size; s^2: variance; %MDD: minimum detectable difference to Control (in percent of Control); t: sample t; p(t): probability of sample t for Ho: μ1 = μ2; Alpha(i): adjusted significance levels; the differences are significant in case p(i) <= Alpha(i) ; dfm: modified degrees of freedom due to heteroscedascity.(Control(c) and treatment(t) variance was applied: $s^2(c)/nc + s^2(t)/nt$, each).

Treatm. [µg/L]	Mean	s^2	df	%MDD	t	p(t)	Alpha(i)	Sign.
Control	1.4	0.0						
12.5	1.4	0.0	19	-2.5	-1.59	0.064	0.050	-
50	1.3	0.0	21	-2.9	-6.12	< 0.001	0.017	+
100	1.3	0.0	13	-5.7	-3.32	0.003	0.025	+

+: significant; -: non-significant; n.d.: not determined

Based on the results of the Welch t-test, a NOEC of 12.5 µg/L is suggested.

A 1.2.3 Group wet weight, day 35

Statistical Characteristics of the Sample

Tab. 1: Statistical characteristics: Mean: arithmetic mean (X); Med: median; Min: minimum value, Max: maximum value; n: sample size; s: standard deviation; s%: coefficient of variation; s(X): standard error; %s(X): %standard error; 95%l, 95%u: lower, upper 95%-confidence limits.

Treatm. [µg/L]	Mean	Med	Min	Max	n	s	%s	s(X)	%s(X)	95%l	95%u
Control	0.5	0.5	0.4	0.5	12	0.04	9.9	0.01	2.9	0.4	0.5
12.5	0.4	0.5	0.4	0.5	12	0.05	10.9	0.01	3.2	0.4	0.5
50	0.4	0.4	0.3	0.5	12	0.05	12.8	0.01	3.7	0.3	0.4
100	0.2	0.3	0.1	0.3	12	0.06	23.6	0.02	6.8	0.2	0.3

One-way Analysis of Variance

Tab. 2: One-way Analysis of Variance: Source: source of variance; SS: sum of squares; df: degrees of freedom; MSS: mean sum of squares; F: test statistic: p: probability

Source	SS	df	MSS	F	p(F)
Treatment	0.34	3	0.11	47.061	< 0.001
Residuals	0.11	44	0.00		
Total	0.44	47			

p(F) is smaller than or equal to the selected significance level of 0.05; therefore, treatments are significantly different.

The first analysis revealed significant results: Pretesting is continued.

Shapiro-Wilk´s Test on Normal Distribution

Tab. 3: Shapiro-Wilk´s Test on Normal Distribution; Mean: arithmetic mean; n: sample size; p(ShapiroWilk´s W): probability of the W statistic. In case p(ShapiroWilk´s W) is greater than the chosen significance level, the normality hypothesis(Ho) is accepted.

Treatm. [µg/L]	Mean	s	n
Control	0.5	0.04	12
12.5	0.4	0.05	12
50	0.4	0.05	12
100	0.2	0.06	12

Results:

Number of residues = 44; Shapiro-Wilk´s W = 0.971; p(W) = 0.322; p(W) is greater than the selected significance level of 0.05; therefore, treatment data do not significantly deviate from normal distribution.

Normality check was passed (p > 0.05).

Levene´s Test on Variance Homogeneity (with Residuals)

Tab. 4: Levene´s Test on Variance Homogeneity (with Residuals): Source: source of variance; SS: sum of squares; df: degrees of freedom; MSS: mean sum of squares; F: test statistic: p: probability

Source	SS	df	MSS	F	p(F)
Treatment	0.00	3	0.00	0.308	0.819
Residuals	0.00	44	0.00		
Total	0.00	47			

Based on the pre-selected significance level of 0.05, the Levene test indicates variance homogeneity !

Variance homogeneity check was passed.
Normal distribution and variance homogeneity requirements are fulfilled.

Williams Multiple Sequential t-test Procedure

Tab. 5: Comparison of treatments with "Control" by the t test procedure after Williams. Significance was Alpha = 0.05, one-sided smaller; Mean: arithmetic mean; n: sample size; s: standard deviation; LhM: max. likelihood mean; %MDD: minimum detectable difference to Control (in percent of Control); t: sample t; t*: critical t for Ho: µ1 = µ2 = ... = µk; the differences are significant in case |t| > |t*| (The residual variance of an ANOVA was applied; df = N - k; N: sum of treatment replicates n(i); k: number of treatments).

Treatm. [µg/L]	Mean	s	df	LhM	%MDD	t	t*	Sign.
Control	0.5	0.049						
12.5	0.4	0.049	44	0.4	-7.4	-0.86	-1.68	-
50	0.4	0.049	44	0.4	-7.8	-4.28	-1.76	+
100	0.2	0.049	44	0.2	-7.9	-10.68	-1.78	+

+: significant; -: non-significant

Based on the results of the Williams test, a NOEC of 12.5 µg/L is suggested.

A 1.2.4 Single wet weight, day 35

Statistical Characteristics of the Sample

Tab. 1: Statistical characteristics: Mean: arithmetic mean (X); Med: median; Min: minimum value, Max: maximum value; n: sample size; s: standard deviation; s%: coefficient of variation; s(X): standard error; %s(X): %standard error; 95%l, 95%u: lower, upper 95%-confidence limits.

Treatm. [µg/L]	Mean	Med	Min	Max	n	s	%s	s(X)	%s(X)	95%l	95%u
Control	27.3	27.9	23.4	29.6	12	1.97	7.2	0.57	2.1	26.0	28.5
12.5	25.5	26.1	19.6	30.4	12	3.19	12.5	0.92	3.6	23.5	27.6
50	20.3	19.7	18.1	25.6	12	2.12	10.4	0.61	3.0	19.0	21.7
100	19.8	18.2	14.0	29.8	12	5.61	28.3	1.62	8.2	16.2	23.4

One-way Analysis of Variance

Tab. 2: One-way Analysis of Variance: Source: source of variance; SS: sum of squares; df: degrees of freedom; MSS: mean sum of squares; F: test statistic: p: probability

Source	SS	df	MSS	F	p(F)
Treatment	504.01	3	168.00	13.434	< 0.001
Residuals	550.27	44	12.51		
Total	1054.27	47			

p(F) is smaller than or equal to the selected significance level of 0.05; therefore, treatments are significantly different.

The first analysis revealed significant results: Pretesting is continued.

Shapiro-Wilk´s Test on Normal Distribution

Tab. 3: Shapiro-Wilk´s Test on Normal Distribution; Mean: arithmetic mean; n: sample size; p(ShapiroWilk´s W): probability of the W statistic. In case p(ShapiroWilk´s W) is greater than the chosen significance level, the normality hypothesis(Ho) is accepted.

Treatm. [µg/L]	Mean	s	n
Control	27.3	1.97	12
25.0	25.5	3.19	12
50.0	20.3	2.12	12
100.0	19.8	5.61	12

Results:

Number of residues = 48; Shapiro-Wilk´s W = 0.956; p(W) = 0.071; p(W) is greater than the selected significance level of 0.05; therefore, treatment data do not significantly deviate from normal distribution.

Normality check was passed (p > 0.05).

Levene´s Test on Variance Homogeneity (with Residuals)

Tab. 4: Levene´s Test on Variance Homogeneity (with Residuals): Source: source of variance; SS: sum of squares; df: degrees of freedom; MSS: mean sum of squares; F: test statistic: p: probability

Source	SS	df	MSS	F	p(F)
Treatment	5069.74	3	1689.91	5.701	0.002
Residuals	13042.87	44	296.43		
Total	18112.61	47			

Based on the pre-selected significance level of 0.05, the Levene test indicates variance heterogeneity!

Variance homogeneity check was not passed

Welch-t test for Inhomogeneous Variances with Bonferroni-Holm Adjustment

Tab. 5: Multiple sequentially rejective comparisons after Welch of treatments with "Control" by the t test procedure. Significance was Alpha = 0.05, one-sided smaller; Mean: arithmetic mean; n: sample size; s^2: variance; %MDD: minimum detectable difference to Control (in percent of Control); t: sample t; p(t): probability of sample t for Ho: µ1 = µ2; Alpha(i): adjusted significance levels; the differences are significant in case p(i) <= Alpha(i) ; dfm: modified degrees of freedom due to heteroscedascity.(Control(c) and treatment(t) variance was applied: $s^2(c)/nc + s^2(t)/nt$, each).

Treatm. [µg/L]	Mean	s^2	df	%MDD	t	p(t)	Alpha(i)	Sign.
Control	27.3	3.9						
25	25.5	10.2	18	-6.9	-1.60	0.063	0.050	-
50	20.3	4.5	21	-7.0	-8.33	< 0.001	0.017	+
100	19.8	31.5	13	-13.6	-4.36	< 0.001	0.025	+

+: significant; -: non-significant; n.d.: not determined

Based on the results of the Welch t-test, a NOEC of 12.5 µg/L is suggested by the program.