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Working Party on Manufactured Nanomaterials

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Final Report

Investigation of nanoclay in standardized ecotoxicological tests

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Index of Content

1 Introduction

The intrinsic toxicity of nanoclay was tested in standard toxicity tests according to OECD test guidelines. The following tests were applied:

Test	Test guideline
Aquatic tests	
Fish, acute (static)	OECD 203
Daphnia magna, immobilization	OECD 202
Alga, growth	OECD 201
Sediment tests	
Sediment-dwelling organisms: *Chironomus riparius*, emergence (water application considered as environmentally relevant exposure)	OECD 219

Representative subsamples of the nanomaterial were used for the ecotoxicological tests. The samples were prepared according to GLP and ISO Guides 30-35 corresponding to the requirements defined in the OECD WPMN guidance document and in accordance with the requirements of the European Commission materials repository at the JRC.

2 Preliminary experiments

In preliminary experiments the behaviour of dispersed nanoparticles was investigated. Samples with the codes 0676, 0684 and 0691 were used.

Three concentrations in reconstituted tab water (100 mg/L, 10 mg/L, 1 mg/L) and two different dispersion procedures were compared. The water used for the preliminary experiments corresponded to the water used for the tests with daphnids, fish and chironomids.

For each measuring time and concentration a sample of 500 mL was individually prepared in glass bottles (500 mL). For the concentrations 100 mg/L and 10 mg/L, the required amounts of nanoclay were directly weighted into glass bottles. The concentration of 1 mg/L was achieved by a ten-fold dilution of the 10 mg/L stock dispersion.

According to the procedure established for TiO_2 (Hund-Rinke et al., 2010) the nanomaterial was suspended by stirring with magnetic fleas (1 min, 300 rpm) and ultrasonication in a bath sonicator filled to one third of the dispersion height in the bottles (Bandelin Sonorex RK 514 BH; 35 kHz; 215/860 W). Furthermore, an ultrasonication period of 30 min according to the procedure recommended by PROSPECT was applied. The particle size distribution was determined using a Malvern Zeta Sizer NanoZS. No specific refraction index is available for nanoclay. Therefore, the value for polystyrene latex, which is the value for the measuring device provided by the manufacturer, was used. Measuring times were as follows: test start (day 0), after 24 h, 48 h, 72 h and 96 h of incubation. To receive information on sedimentation the samples were not homogenized before measurement.

2.1 Results

The results are presented in Table 1 and Table 2. Measurements of 1 mg/L and 10 mg/L samples are at the limit of the measuring device. From peaks of the 100 mg/L samples (day 0 and day 1) it can be concluded that 30 min of ultrasonication resulted in slightly smaller

particles / agglomerates. At day 0 the diameters are 795 nm (3 min) and 695 nm (30 min). Furthermore sedimentation occurs during incubation.

Table 1: Behaviour (agglomeration, sedimentation) of nanoclay (NM-600) during 4 days of incubation

Day 0								
Concentration	Ultrasonication period [min]	Z-Average [nm] [1]	PDI [2]	Peak 1 [nm]	Peak 2 [nm]	Count Rate [3] [kcps]	Measurement Position [4]	Attenu-ation [5]
1 mg/L	3 (3 min) [7]	< detection limit						
1 mg/L	3 (30 min) [8]	< detection limit						
10 mg/L	3	< detection limit						
10 mg/L	30	1109	0.7	369	-	132	4.65	9
100 mg/L	3	1192	0.5	795	-	130	4.65	7
100 mg/L	30	827	0.4	695	-	162	4.65	7
Day 1 [6]								
10 mg/L	3	< detection limit						
10 mg/L	30	1221	0.4	852	-	181	4.65	9
100 mg/L	3	1002	0.4	791	-	173	4.65	9
100 mg/L	30	664	0.4	495	-	264	4.65	10
Day 2								
10 mg/L	3	1072	0.5	670	-	166	4.65	10
10 mg/L	30	960	0.5	639	-	283	4.65	10
100 mg/L	3	819	0.5	561	-	191	4.65	10
100 mg/L	30	719	0.5	486	-	122	4.65	10
Day 3								
10 mg/L	3	1295	0.7	638	-	128	4.65	10
10 mg/L	30	919	0.5	623	-	131	4.65	10
100 mg/L	3	890	0.5	610	-	132	4.65	10
100 mg/L	30	748	0.6	473	-	242	4.65	10
Day 4								
10 mg/L	3	1179	0.7	420	-	157	4.65	11
10 mg/L	30	1118	0.7	461	-	104	4.65	10
100 mg/L	3	1033	0.6	613	-	140	4.65	10
100 mg/L	30	724	0.6	460	-	87	4.65	10

[1] calculated value (cumulative mean); [2] increasing value indicates increasing polydispersity (maximum: 1); [3] best results with a count rate between 150 and 500 kilo counts per second (kcps); [4] measurement position in the middle of the measuring cell; [5] indicator for turbidity (high values indicate low turbidity; maximum: 11); [6] 1 mg/L samples below quantification limit; [7] prepared from 10 mg/L samples with 3 min of ultrasonic treatment; [8] prepared from 10 mg/L samples with 30 min of ultrasonic treatment

Table 2: Observations in the glass vessels containing the different concentrations of nanoclay (NM-600) during 4 days of incubation

	Ultrasonication period	1 mg/L	10 mg/L	100 mg/L
Day 0	3 min	Clear aqueous phase; no sedimentation	Clear aqueous phase; no sedimentation	Turbid aqueous phase
	30 min	Clear aqueous phase; no sedimentation	Clear aqueous phase; no sedimentation	Turbid aqueous phase
Day 1	3 min	Clear aqueous phase; no sedimentation	Clear aqueous phase; sedimented nanoclay	Clear aqueous phase with sedimented nanoclay
	30 min	Clear aqueous phase; no sedimentation	Clear aqueous phase; sedimented nanoclay	Slightly turbid aqueous phase; sedimented nanoclay
Day 2	3 min	No further consideration	Clear aqueous phase; sedimented nanoclay	Clear aqueous phase; sedimented nanoclay
	30 min	No further consideration	Clear aqueous phase; sedimented nanoclay	Clear aqueous phase; sedimented nanoclay
Day 3	3 min	No further consideration	Clear aqueous phase; sedimented nanoclay	Clear aqueous phase; sedimented nanoclay
	30 min	No further consideration	Clear aqueous phase; sedimented nanoclay	Clear aqueous phase; sedimented nanoclay
Day 4	3 min	No further consideration	Clear aqueous phase; sedimented nanoclay	Clear aqueous phase; sedimented nanoclay
	30 min	No further consideration	Clear aqueous phase; sedimented nanoclay	Clear aqueous phase; sedimented nanoclay

2.2 Conclusions

Differences between the ultrasonication periods of 3 min and 30 min are considered to be small. As many vessels have to be prepared for the experiments and an ultrasonication period of 30 min would increase the expenditure of time significantly, it was decided to perform the dispersion according to the procedure commonly applied for TiO_2 with the short period of ultrasonication:

1 min stirring and 3 min of ultrasonic treatment in an ultrasonic bath.

3 Tests with algae: OECD TG 201

3.1 Test principle

The purpose of this test is to determine the effects of a substance on the growth of freshwater microalgae and/or cyanobacteria. Exponentially growing test organisms are exposed to the test substance in batch cultures over a period of normally 72 hours. Despite the relatively brief test duration, effects over several generations can be assessed.

3.2 Materials and methods

3.2.1 Test guideline

The test was performed according to:

OECD 201 (23.06.2006): OECD guideline for testing of chemicals – Freshwater Alga and Cyanobacteria, Growth Inhibition Test.

3.2.2 GLP

The test was performed following the principles of GLP. In deviation to GLP the raw data have not been archived, and the quality assurance unit was not involved with respect to the inspection of the test, the raw data and the report. All laboratory equipment (e.g. balances, thermometer, pH-meter) was controlled and documented according to GLP.

3.2.3 Test substances

Nanoclay NM-600; vessel no. 0001

3.2.4 Analytical monitoring

Particle size distribution was determined at test start. Additionally, the modification of the particle size distribution was followed in separate vessels without algae during the test (day 1, 2, 3).

Zeta-potential and particle size distribution were measured using a Malvern Zeta-Sizer. The Zeta-potential was determined for the control and for the concentration of 11 mg/L. The particle size distribution was measured for every concentration.

At day 0, Si concentrations in the test suspensions were determined. For details of chemical analyses see chapter 7.

3.2.5 Details on test suspensions

Test concentrations were:

1.2 mg/L, 3.7 mg/L, 11.0 mg/L, 33.0 mg/L, 100.0 mg/L

The test suspensions (500 mL) were prepared with OECD medium (composition according to OECD TG 201 in 500 mL glass vessels (Schott)). Test concentrations of 11 - 100 mg/L were prepared individually. Accuracy of weighting increased the

demanded concentration by one position after the decimal point. The test concentration of 3.7 mg/L was prepared by dilution from the test concentration of 11 mg/L (168.182 mL adjusted to 500 mL), test concentration of 1.2 mg/L was prepared by dilution from the test concentration of 3.7 mg/L (162.162 mL adjusted to 500 mL

3.2.6 Test organism

The unicellular green alga *Pseudokirchneriella subcapitata* was chosen by experts as test organism representing freshwater primary producers.

Species: *Pseudokirchneriella subcapitata* (formerly *Selenastrum capricornutum*), Chlorophycea, Chlorophyta.

Origin: SAG, Culture Collection of Algae at Plant Physiological Institute of the University at Göttingen, Albrecht von Haller Institute, Untere Klarspüle 2, 37073 Göttingen, Germany, Catalogue No 61.81 SAG.

3.3 Study design

3.3.1 Study type

growth, static

3.3.2 Water medium type:

Algae test medium according to OECD TG 201

3.3.3 Total exposure duration

72 h: January 31st - February 3rd, 2011

No post-exposure observation period was performed.

3.3.4 Test conditions

Details on test conditions:

- Test temperature: 21.8 - 22.6 °C
- pH at test start: 7.38 - 7.51
  pH at test end: 7.66 - 8.13
- Light intensity: 3723 - 6945 lux

VEHICLE CONTROL PERFORMED: no

Reference substance: The sensitivity of the test organism is routinely checked using 3,5-dichlorophenol as primary standard following internal SOPs. The E_rC_{50} value of 5.18 mg/L is comparable with the result of an international ring test, namely 3.38 mg/L.

3.3.5 Any other information on materials and methods

Cultivation and pre-culture

The stock cultures were maintained fulfilling the criteria of the OECD guidelines. Three days prior to testing a pre-culture was established in OECD growth medium to obtain exponentially growing algae for the test.

Growth medium

A sterilized synthetic growth medium (OECD medium) according to OECD 201 was used for culture and preparation of the test medium.

Table 3: OECD medium: Algal medium according to OECD 201

	[mg/L]			[mg/L]
$NaHCO_3$	50		H_3BO_3	0.185
NH_4Cl	15		$MnCl_2$ x 4 H_2O	0.415
KH_2PO_4	1.6		$ZnCl_2$	0.003
$MgSO_4$ x 7 H_2O	15		$CoCl_2$ x 6 H_2O	0.0015
$MgCl_2$ x 6 H_2O	12		$CuCl_2$ x 2 H_2O	0.00001
$CaCl_2$ x 2 H_2O	18		Na_2MoO_4 x 2 H_2O	0.007
$FeCl_3$ x 6 H_2O	0.064		Na_2EDTA x 2 H_2O	0.1
			pH, at test start	approx. 7.5-8.0

Test vessels

Test vessels were 250 mL conical glass flasks covered with silicone-sponge caps. The vessels and caps were sterilized prior to use (autoclaving).

Temperature

During the exposure period, the incubation temperature was measured daily with a calibrated thermometer in an additionally prepared control vessel which was also incubated.

Light intensity

The light intensity was measured daily using an illuminance meter LI-189 (LI-COR, Lincoln, USA with radiation sensor) with a cosine (2π) receptor in lux on the level of the surface of the test media.

Determination of growth

Fluorescence measurements were performed instead of particle counts, since particle measurements cannot always distinguish between algae and nanomaterials / agglomerates. Algal biomass was determined after 0, 24, 48 and 72 h by recording the fluorescence intensity using a BioTek Synergy MX microtiter plate reader. 200 µL aliquots were transferred to microtiter plates prior to measurement.

Test performance

For preparing the test cultures for the growth test, every flask was filled with 100 mL of the respective test medium. 261 µL of the pre-culture (cell density 3.834 x 10^6 cells/mL) was added to the test vessels to achieve the initial cell concentration of 10,000 cells/mL. Three replicates were prepared for each concentration and six replicates for the controls.

At test start, the initial cell concentration was calculated based on the cell number of the pre-culture. Cell concentrations were measured using an electronic particle counter (CASY 1 Model TT, Schärfe System, Reutlingen, Germany). During the test, the cell concentrations were determined after 24, 48 and 72 h in samples taken directly from the test vessels.

The culture vessels were incubated at 22 ± 1 °C with a light intensity adjusted to approximately 4440-8880 Lux (60-120 µE m^{-2} s^{-1}) prior to the test and during the test. The cultures were oscillated by continuously stirring on a laboratory shaker with 100 rpm (Incubation Shaker Multitron ®, INFORS, Switzerland).

The pH values were measured in an additionally prepared replicate at test start and directly in the test vessels at the end of the test. During the exposure the incubation temperature was measured once a day in an additionally prepared control vessel, which was continuously incubated.

Data evaluation

- The evaluation of the concentration-effect-relationships and the calculation of the effect concentrations were based on the nominal concentrations of the test item.
- The fluorescence values were calculated in cell counts using a calibration curve.
- The mean value of the cell counts for each concentration plot was used for plotting growth curves.
- Calculation of the percent inhibition of growth rate [r] and yield [y] was performed according to the guideline and listed in a table.
- EC_{10} and EC_{50} values were determined together with 95% confidence intervals using Probit-analysis (Finney, 1984) assuming a log-normal distribution of the values by using ToxRat (ToxRat® Professional 2.09).
- The NOEC values for growth rate and yield were determined using the computer programme ToxRat (ToxRat® Professional 2.09). For growth rate and yield normal distribution was checked with the Shapiro-Wilk's Test (normal distribution shown), variance homogeneity was checked with Levene's Test (variance heterogeneity shown), NOEC was determined with Welch test for inhomogenous variances with Bonferroni-Holm adjustment.

3.4 Results

Zeta potential

Zeta potential and agglomeration behaviour of NM-600 are presented in Table 4 and Table 5. NM-600 has a negative Zeta potential in the test medium.

In contrast to the preliminary test the agglomeration behaviour showed two peaks which might be due to the different medium. Nevertheless, the peak indicating larger particles dominated. Higher concentrations resulted in larger particles. During incubation, the size of the peaks seems to decrease. This phenomenon may be due to shaking of the vessels during the incubation period. At present, the results cannot be interpreted properly. As we assume that knowledge concerning the measurement and interpretation of suspensions containing nanoparticles and their agglomerates will be increasing, it may be possible that the results obtained in this project can be interpreted retrospectively.

Table 4: Zeta potential

Sample	Zeta potential [mV]
NM 600 in mineral medium (application dispersion):	-25 mV
Mineral medium (OECD 201)	-9.8 mV

Table 5: Behaviour of the particles during the test

Concentration	Z-Average [nm] [1]	PDI [2]	Peak 1 [nm]	Peak 2 [nm]	Count Rate [3] [kcps]	Measurement Position [4]	Attenuation [5]
Day 0 (test start)							
11 mg/L	< detection limit						
33 mg/L	1920	1.000	565.2	[85.89] [6]	221.8	4.65	9
100 mg/L	1057	0.527	1013 (79 %)	214.4	133.6	4.65	7
Day 1 (24 h)							
11 mg/L	< detection limit						
33 mg/L	1161	0.689	913.3 (85 %)	194.7	153.9	4.65	8
100 mg/L	759.4	0.430	1039 (76 %)	256.8	130.6	4.65	7
Day 2 (48 h9							
11 mg/L	836.5	0.694	562.9 (89 %)	126.5	132.0	4.65	9
33 mg/L	888.6	0.608	841.0 (87 %)	174.1	156.9	4.65	8
100 mg/L	634.3	0.459	746.2 (90 %)	162.2	139.7	4.65	7
Day 3 (72 h)							
11 mg/L	677.3	0.622	363.1 (64 %)	705.5	137.5	4.65	9
33 mg/L	784.0	0.432	841.9 (82 %)	200.2	165.7	4.65	8
100 mg/L	591.4	0.372	718.1 (87 %)	176.3	134.6	4.65	7

[1] calculated value (cumulative mean); [2] increasing value indicates increasing polydispersity (maximum: 1); [3] best results with a count rate between 150 and 500 kilo counts per second (kcps); [4] measurement position in the middle of the measuring cell; [5] indicator for turbidity (high values indicate low turbidity; maximum: 11); 6 values in square brackets indicate that the peak is less than 10% of the main peak. 7 in case of two values, the values in round brackets indicate the percentage of the peak compared to the area of both peaks.

Test item concentrations

The results are presented in Table 6. A high silica concentration was detected in the control samples. This is due to the ubiquitous occurrence of silica (e.g. in dust). Because these aqueous media already show a high background concentration the calculated Si recoveries do not represent the recovery of the metal in nanoclay at all. For a better view and evaluation the mean of the controls per day was added to the nominal value for silica of the respective loading. This sum was set to 100%, and the recovery of the amount of silica in the sample in relation to this sum was determined. For a clear-cut result the amount of Si in the controls has to be less than 50% of the measured values in samples from test item loaded vessels.

The content of silica in nanoclay has to be estimated (see chapter 7). Nevertheless only in the highest test concentration a recovery above 80 % was calculated. Further determinations were performed (see chapter 3.6). Based on these results the use of

nominal concentrations for the calculation of the effects was considered to be suitable.

Table 6: Measured Si concentrations and recoveries for the test with algae

Sample	Mean Si [µg/L]	SD [µg/L]	Si nominal [µg/L]	mean Si control + Si nominal [µg/L]	Recovery of fortification in relation to measured Si [%]
control	3533	-	0	-	-
1.2 mg/L	1725	1371	362	3895	44.3
3.7 mg/L	1673	486	1115	4648	36.0
11 mg/L	3387	44.5	3315	6848	49.5
33 mg/L	6870	84.9	9946	13479	51.0
100 mg/L B	27319	705	30140	33673	81.1

Growth curves

The effect of the test item on the growth of *Pseudokirchneriella subcapitata* was tested with five graded concentrations. The fluorescence values were converted to cell counts using the calibration curve shown in Figure 1

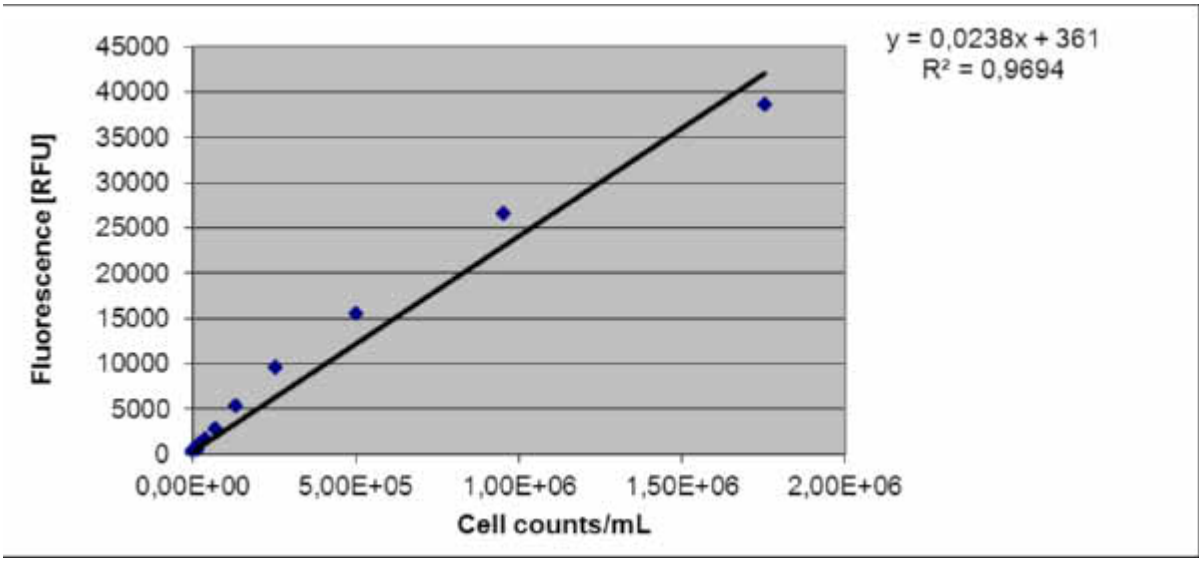

Figure 1: Calibration curve for the conversion of fluorescence in cell counts (fluorescence measured using a BioTek Synergy MX microtiter plate reader)

The cell counts dependent on the test item concentrations is listed below. The growth curves are shown in Figure 2

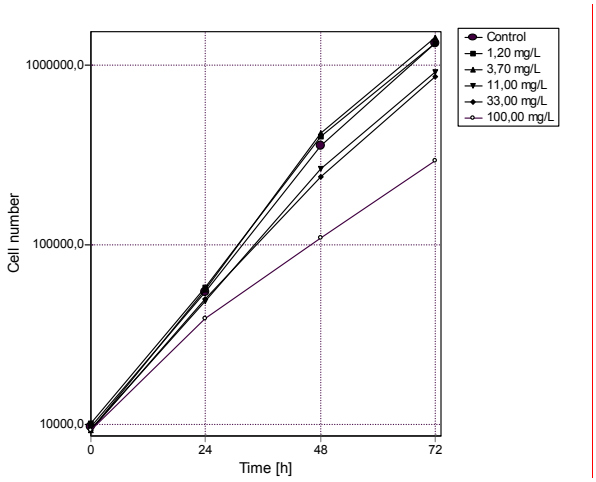

Figure 2: Cell counts of *Pseudokirchneriella subcapitata* dependent on nominal concentrations of the test item NM-600.

Effect concentrations

The percent inhibition of growth rate and yield depending on nominal test item concentrations are listed in (Table 7) and shown in Figure 3. Details on the test results are shown in chapter 3.10.2.

A concentration dependent inhibition could be observed. The NOEC values for effects on yield were 3.70 mg test item/L. Per expert judgement, the NOEC for the growth rate was determined to be 33.0 mg/L, as effects below 10% are considered not ecotoxicologically relevant.

The effect values for the 72 hour exposure period are compiled in Table 8. Details on the fluorescence values are shown in chapter 3.10.1.

The fluorescence of the algae is not influenced by nanoclay. In the presence of NM 600 (100 mg/L) algae resulted in a fluorescence comparable to algae in mineral medium (Table 9). Therefore, the observed inhibition is due to nanoclay and represents no false positive result for example by quenching of the signal.

Table 7: Percent inhibition of growth rate and yield by the test item compared to controls after 72 h

Test item, nominal [mg test item/L]	% Inhibition of growth rate	% Inhibition of yield
1.2	-1.3	-7.0
3.7	-4.5	-5.5
11.0	5.1	12.5
33.0	2.9	9.3
100.0	16.9	33.3

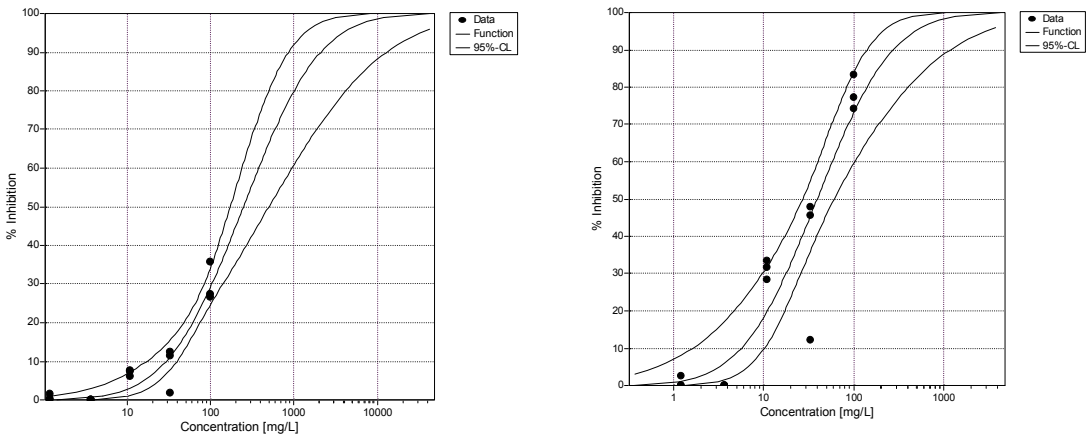

Figure 3: Concentration-effect curve showing the influence of the mean measured concentrations of NM-600 on %inhibition of growth rate (left) and yield (right) after 72 h.

Table 8: Effective concentrations of the test item based on nominal concentrations after exposure to *Pseudokirchneriella subspicata* for 72 hours

	Effective concentrations [mg/L] after 72 h (95% confidence interval [mg/L])			
		EC_{50}	EC_{10}	NOEC
Yield	Nanoclay NM-600	39.23 (27.04-60.64)	5.62 (1.62-10.15)	3.7
Growth rate	Nanoclay NM-600	248.48 [1] (172.64-502.62)	29.57 (17.26-39.58)	33.3

[1] exceeding the highest test concentration (100 mg/L)

Table 9: Fluorescence of algae, mineral medium, NM 600

Sample	Fluorescence
Mineral medium (OECD 201)	158 ± 2.7
NM 600 100 mg/L in mineral medium	157 ± 2.4
Algae (about 12 * 10^6 cells/mL; exposure condition at test end) in mineral medium	3621 ± 24.4
Algae in mineral medium + NM 600 (100 mg/L)	3640 ± 24.0

3.5 Validity

The alga growth inhibition test fulfils the validity criteria of OECD 201 (2006):

- The fluorescence in the control cultures increased by a factor of 137 within the test period of 72 h (validity criterion for biomass: > 16).
- Evaluation of the sectional growth rates of the controls:
  The mean of the replicate coefficients of variations in the section-by-section growth rate of controls was 17.7% (validity criterion ≤ 35%).
- The coefficient of variation of average specific growth rate in replicate control cultures during the whole test period was 1.2% (validity criterion ≤ 7%).

3.6 Further information

Due to the dissatisfactory recovery of the used concentrations (Table 6) a further, reduced test was performed. The same five test concentrations were prepared and analysed (Table 10). The growth test with algae was performed with two concentrations (11 mg/L; 33 mg/L). The results of the chemical analyses revealed a recovery of 90% and 94% resp. at the two highest test concentrations (100 mg/L, 33 mg/L). For the lower test concentrations recovery was above 100 %. The results of the growth test with algae are presented in Table 11. In Table 12 the percent effect of the two concentrations tested in the first and second test are presented. The concentration of 33 mg/L showed comparable effects in both tests, although the recovery was different (51 % in the first test; 94 % in the second test). The effect at the concentration of 11 mg/L was lower in the in the second test compared to the first test, also the recovery was much higher (49% in the first test; 186% in the second test). As the validity criteria were fulfilled in both tests, clear concentration-effect curves were obtained in the first test and comparable effects were obtained in the first and second test at 33 mg/L (the concentration with a clear effect) it was

assumed that the concentrations in the tests were correct. Therefore, the nominal test concentrations were used for the effect calculation.
Details of the analytical and effect data are presented in chapter 3.10 (second test).

Table 10: Measured Si concentrations and recoveries for the test with algae

Sample	Mean Si [µg/L]	SD [µg/L]	Si nominal [µg/L]	mean Si control + Si nominal [µg/L]	Recovery of fortification in relation to measured Si [%]
control	645	65	0	-	-
1.2 mg/L	3776	88	362	1007	375
3.7 mg/L	5560	69	1115	1760	316
11 mg/L	7378	39	3315	3960	186
33 mg/L	9929	40	9946	10591	94
100 mg/L B	27611	355	30140	30785	90

Table 11: Effect of two concentrations of nanoclay in the growth test with algae (incubation period: 72 h)

	Control	11 mg/L	33 mg/L
Number of replicates	6	3	3
Yield			
Mean value	1239502	1148893	772520
Std.Dev	112254	81760	73604
CV	9.1	7.1	9.5
Growth rate			
Mean value	1.655	1.622	1.511
Std.Dev	0.0337	0.0508	0.0273
CV	2.0	3.1	1.8

Table 12: Percent inhibition of the growth test with algae and percent recovery

	1st test		2nd test	
Test concentration	11 mg/L	33 mg/L	11 mg/L	33 mg/L
Yield				
Inhibition [%]	31.2	35.2	7.3	38
Growth rate				
Inhibition [%]	7.0	8.5	2.0	8.7
Chemical analyses				
Recovery [%]	49.5	51.0	186	94

3.7 Conclusion

Nanoclay NM-600 was tested in the growth test with algae according to OECD test guideline 201. A concentration dependent inhibition of growth rate and yield could be observed. The EC and NOEC values for growth rate and yield are summarised in the following table:

Table 13: Endpoint data in *Pseudokirchneriella subcapitata* exposed to nanoclay NM-600 for 72 hours based on nominal concentrations

	Effective concentrations [mg/L] after 72 h (95% confidence interval [mg/L])			
		EC_{50}	EC_{10}	NOEC
Yield	Nanoclay NM-600	39.23 (27.04-60.64)	5.62 (1.62-10.15)	3.7
Growth rate	Nanoclay NM-600	248.48 [1] (172.64-502.62)	29.57 (17.26-39.58)	33.3

[1] exceeding the highest test concentration (100 mg/L)

3.8 Executive summary

The toxicity of the test item **nanoclay NM-600** on the growth of the unicellular fresh water green alga *Pseudokirchneriella subcapitata* exposed for a test period of 72 hours under static conditions according to OECD 201 was determined.

The test item was suspended in sterilised growth medium. Three replicates for each concentration and six replicates for the controls (test medium only) were exposed to nominal 1.2, 3.7, 11.0, 33.0 and 100 mg test item/L.

A concentration dependent inhibition of growth rate and yield could be observed. The EC and NOEC values for growth rate and yield are summarised in the following table.

Table 14: Endpoint data in *Pseudokirchneriella subcapitata* exposed to nanoclay NM-600 for 72 hours based on nominal concentrations

	Effective concentrations [mg/L] after 72 h (95% confidence interval [mg/L])			
		EC_{50}	EC_{10}	NOEC
Yield	Nanoclay NM-600	39.23 (27.04-60.64)	5.62 (1.62-10.15)	3.7
Growth rate	Nanoclay NM-600	248.48 [1] (172.64-502.62)	29.57 (17.26-39.58)	33.3

[1] exceeding the highest test concentration (100 mg/L)

3.9 Interpretation of the effect data

Inhibition of algae growth was observed. With respect to the classification on labelling of chemical substances a 50 % inhibition > 100 mg/L (EC50; EC = effect concentration) results in no labelling. Usually the growth rate is used for the classification. Therefore, no labelling is necessary.

On the basis of ecotoxicological test data PNEC values (predicted no effect concentration) can be calculated by consideration of assessment factors. For a correct assessment following assessment factors applied (ECHA: Guidance on information requirements and chemical safety assessment; Chapter R.10: Characterisation of dose [concentration]-response for environment):

Available data	Assessment factor
At least one short-term L(E)C50 from each of three trophic levels (fish, invertebrates (preferred Daphnia) and algae)	1000
One long-term EC10 or NOEC (either fish or Daphnia)	100
Two long-term results (e.g. EC10 or NOECs) from species representing two trophic levels (fish and/or Daphnia and/or algae)	50
Long-term results (e.g. EC10 or NOECs) from at least three species (normally fish, Daphnia and algae) representing three trophic levels	10

For a correct assessment the test with fish is not available. For a first estimation the assessment was performed based on two tests (daphnids, algae). Due to their higher sensitivity, the algae are used for the assessment. Based on the NOEC (33.3 mg/L) a PNEC value of 0.6 mg/L is calculated (based on EC_{50}: 0.25 mg/L). Both values are comparable. Keeping in mind that it is a rough estimation (scientific standards not completely fulfilled), an environmental concentration of < ≈0.5 mg/L is considered to be tolerable.

3.10 Details on test data

3.10.1 Data on the ecotoxicological test

First test

Fluorescence of *Pseudokirchneriella subcapitata* as dependent on the concentration of the test item and time; Mean: arithmetic mean; Std.Dev.: standard deviation; n: number of replicates; CV: coefficient of variation (calculated from InputRawData)

Treatm. [mg/L]	Control	1.20	3.70	11.00	33.00	100.00
0 h	288.000	296.000	285.667	292.000	292.667	276.667
	286.667	300.333	295.667	287.333	291.333	280.000
	292.667	291.333	294.667	288.000	293.667	274.333
	295.333					
	294.667					
	297.000					
Mean:	292.389	295.889	292.000	289.111	292.556	277.000
Std.Dev.:	4.1762	4.5010	5.5076	2.5240	1.1706	2.8480
n:	6	3	3	3	3	3
CV:	1.4	1.5	1.9	0.9	0.4	1.0
24 h	819.333	852.333	854.333	718.000	840.667	673.333
	760.000	888.667	936.000	741.000	786.333	586.667
	831.000	818.000	799.667	756.667	735.667	617.667
	782.333					
	873.667					
	830.000					
Mean:	816.055	853.000	863.333	738.556	787.556	625.889
Std.Dev.:	40.0674	35.3381	68.6108	19.4489	52.5107	43.9145
n:	6	3	3	3	3	3
CV:	4.9	4.1	7.9	2.6	6.7	7.0
48 h	4599.667	4360.667	4913.667	3177.667	3284.667	1586.333
	3886.333	5541.667	6014.333	3169.667	3275.000	1258.333
	5250.000	4827.667	4805.333	3693.667	2662.000	1432.333
	4183.333					
	5064.667					
	4585.000					
Mean:	4594.833	4910.000	5244.445	3347.000	3073.889	1425.667
Std.Dev.:	514.0839	594.7893	668.9401	300.2488	356.7390	164.1016
n:	6	3	3	3	3	3
CV:	11.2	12.1	12.8	9.0	11.6	11.5
72 h	13298.667	13007.000	14234.667	10808.333	12748.667	4060.333
	13058.333	14153.000	15558.333	9976.667	8275.333	2717.333
	14464.000	13896.667	14256.333	10451.000	8692.333	3558.000
	13568.000					
	14550.000					
	13328.000					
Mean:	13711.167	13685.556	14683.110	10412.000	9905.444	3445.222
Std.Dev.:	637.8182	601.4607	758.0419	417.2024	2471.1147	678.5657
n:	6	3	3	3	3	3
CV:	4.7	4.4	5.2	4.0	24.9	19.7

Second test

Fluorescence in Pseudokirchneriella subcapitata as dependent on concentration of the test item and time; Mean: arithmetic mean; Std.Dev.: standard deviation; n: number of replicates; CV: coefficient of variation (calculated from InputRawData)

Treatm. [mg/L]	Control	11,00	33,00
0 h	220.000	247.333	248.333
	252.667	274.333	222.667
	237.667	223.667	233.667
	265.000		
	249.000		
	229.000		
Mean:	242.222	248.444	234.889
Std.Dev.:	16.5122	25.3516	12.8769
n:	6	3	3
CV:	6.8	10.2	5.5
24 h	1131.333	1278.667	1099.333
	1114.667	1066.000	1185.333
	1042.667	1201.333	998.333
	1169.667		
	1049.000		
	1070.667		
Mean:	1096.333	1182.000	1094.333
Std.Dev.:	50.4346	107.6434	93.6002
n:	6	3	3
CV:	4.6	9.1	8.6
48 h	7360.333	6594.667	6024.000
	7225.000	5049.000	4319.333
	6283.667	6990.333	5170.333
	6744.667		
	7213.000		
	7588.667		
Mean:	7069.222	6211.333	5171.223
Std.Dev.:	473.6964	1025.8666	852.3336
n:	6	3	3
CV:	6.7	16.5	16.5
72 h	31130.666	33392.332	21691.000
	35719.332	29023.000	18777.334
	29105.000	31045.666	22574.000
	33030.332		
	35804.668		
	36719.000		
Mean:	33584.832	31153.666	21014.111
Std.Dev.:	3023.4438	2186.6672	1986.7817
n:	6	3	3
CV:	9.0	7.0	9.5

3.10.2 Data on chemical analyses

First test

LOD/LOQ, correlation

The information about the LOD/LOQ and correlation coefficient are compiled in Table 15.

A representative calibration line is shown below.

The correlation factor (r) for respective calibration function was taken from ICP-OES instrument outputs.

The resulting values are reported in Table 57.

Table 15: LODs/LOQs, correlation

Measurement date, description	LOD [µg/L]	LOQ [µg/L]	Correlation factor r
May 19, 2011;	42	139*	0.9990

* internal LOQ calculation was performed with more digits.

Instrumental and analytical set-up of the ICP-OES:
Thermo IRIS Intrepid II
Thermo Electron Corporation, Germany
Analytical conditions
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
Wavelengths: 288.158 nm

Quality assurance measurements

According to the quality assurance requirement, the silica recovery in the recalibration standards was in the range of ± 15% of the certified value. However, regarding Si concentrations measured by ICP-OES, the mean recovery was 100 ± 4.6% (n = 5) for 250 µg/L.

Unfortunately, a reference water with a certified amount of Si is commercially not available.

Exact amounts of the nanoclay test item (104 mg/L and 55 mg/L, appropriately diluted to fit in the concentration range of samples) were introduced into ultrapure water.

Samples were taken, digested, appropriately diluted (dilution factor of 5 for 104 mg/L, 3 for 55 mg/L, respectively) to fit in the concentration range of the test samples and finally analysed. The amount of Si in nanoclay was calculated using an EDX analysis provided by NIA (NPL report). As energy dispersive X-ray spectroscopy only gives information about a specific surface area, the values have to be considered carefully. Therefore, the quality assurance requirement for the recovery of Si in nanoclay was set to 100 ± 30. The recovery of silica in nanoclay was determined to 119 ± 10% (n = 4) for 104 mg/L and 127 ± .20 for 55 (n = 4) mg/L.

Silica concentrations (34 – 70 µg/L) in digested ultrapure water blanks (in total n =5) were at least below LOQ (< 139 µg/L).

Reagent blanks (0.2% KOH, n = 18) were additionally analysed. The measured values were always below the LOD (42 µg/L).

Analytical results

In Table 16 the measurement results of nanoclay in aqueous samples from the algae test are compiled. A high silica concentration was detected in the controls. To calculate the recoveries the mean of the controls was added to the nominal value for silica of the respective loading. This sum was set to 100%, and the recovery of the amount of silica in the sample in relation to this sum was determined. For a clear-cut result the amount of Si in the controls have to be less than 50% of the measured values in samples from test item loaded vessels.

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Table 16: Measured Si concentrations and recoveries, test with algae (internal calculations were performed with more digits)

day 0									
Sample name	Measured Si [µg/L]	Dilution factor	Measured Si x dilution[µg/L]	Mean Si [µg/L]	SD [µg/L]	Si nominal [µg/L]	Si recovery [%]	Mean Si control + Si nominal [µg/L]	Recovery of fortification in relation to measured Si [%]
control d0 A	236	15	3533	1767	-	0	-	---	---
control d0 B	27	-	-						
1.2 mg/L d0 A	180	15	2694	1725	1371	362	476	3895	44.3
1.2 mg/L d0 B	50	15	755						
3.7 mg/L d0 A	134	15	2016	1673	486	1115	150	4648	36.0
3.7 mg/L d0 B	89	15	1329						
11 mg/L d0 A	224	15	3356	3387	44.5	3315	102	6848	49.5
11 mg/L d0 B	228	15	3419						
33 mg/L d0 A	231	30	6930	6870	84.9	9946	69.1	13479	51.0
33 mg/L d0 B	227	30	6810						
100 mg/L d0 A	358	75	26820	27319	705	30140	90.6	33673	81.1
100 mg/L d0 B	371	75	27818						

The light grey marked value is below LOD; the grey marked values are below LOQ.

Second test

Table 17: LODs/LOQs, correlation

Measurement date, description	LOD [µg/L]	LOQ [µg/L]	Correlation factor r
August 04, 2011;	18.1	60.2*	0.9990

* internal LOQ calculation was performed with more digits.

Table 18: Measured Si concentrations and recoveries, test with algae (internal calculations were performed with more digits)

day 0									
Sample name	Measured Si [µg/L]	Dilution factor	Measured Si x dilution[µg/L]	Mean Si [µg/L]	SD [µg/L]	Si nominal [µg/L]	Si recovery [%]	Mean Si control + Si nominal [µg/L]	Recovery of fortification in relation to measured Si [%]
control d0 A	39.9	15	599	345	-	0	-	---	---
control d0 B	46.0	-	691						
1.2 mg/L d0 A	256	15	3839	3776	65	362	1043	1007	375
1.2 mg/L d0 B	248	15	3714						
3.7 mg/L d0 A	374	15	5609	5560	88	1115	499	1760	316
3.7 mg/L d0 B	367	15	5511						
11 mg/L d0 A	494	15	7406	7378	69	3315	223	3960	186
11 mg/L d0 B	490	15	7350						
33 mg/L d0 A	332	30	9957	9929	39	9946	99.8	10591	93.7
33 mg/L d0 B	330	30	9900						
100 mg/L d0 A	365	75	27360	27611	40	30140	91.6	30785	89.7
100 mg/L d0 B	372	75	27863						

The grey marked values are below LOQ.

4 Tests with daphnids: OECD TG 202

4.1 Test principle

Specimens of the daphnids (*Daphnia magna*) were exposed to the test substance dissolved in water at a range of 5 concentrations for 48 hours. At the end of the test, the total number of mobile specimens was assessed.

4.2 Materials and methods

4.2.1 Test guideline

The test was performed according to:

OECD 202 (13.04.2004): OECD guideline for testing of chemicals – *Daphnia sp.* Acute Immobilisation Test

4.2.2 GLP

The test was performed following the principles of GLP. In deviation to GLP no raw data have been archived, and the quality assurance unit was not involved with respect to the inspection of the test, the raw data and the report. All laboratory equipment (e.g. balances. thermometer. pH-meter) were controlled and documented according to GLP.

4.2.3 Test substances

Nanoclay NM-600, vessel no. 0002

4.2.4 Analytical monitoring

Particle size distribution and zeta potential were determined at test start. Additionally the modification of the particle size distribution was determined at test end (day 2).

Zeta-potential and particle size distribution were measured using a Malvern Zeta-Sizer. The Zeta-potential was determined for the control and for the concentration of 11 mg/L. The particle size distribution was measured for every concentration.

For chemical analyses, at every sampling time (day 0, 1, 2) and test concentration two samples each with 20 mL were taken from vessels prepared just for these analyses but treated as the vessels used for the ecotoxicological determinations.

For details concerning the chemical analysis method see chapter 7.

4.2.5 Details on test suspensions

Test concentrations were:

1.2 mg/L. 3.7 mg/L. 11.0 mg/L. 33.0 mg/L. 100.0 mg/L

The test suspensions (500 mL) were prepared in purified tap water in 500 mL glass vessels (Schott)). Test concentrations of 11 - 100 mg/L were prepared individually. Accuracy of weighting increased the demanded concentration by one position after

the decimal point. The test concentration of 3.7 mg/L was prepared by dilution from the test concentration of 100 mg/L (18.5 mL adjusted to 500 mL). The test concentration of 1.2 mg/L was prepared by dilution from the test concentration of 33.0 mg/L (18.2 mL adjusted to 500 mL.

4.2.6 Test organism

The test organisms were young specimens of *Daphnia magna* (clone V), 4 - 24 hours old at test start.

Origin of the daphnids: German Federal Environment Agency (Umweltbundesamt), Institut für Wasser-. Boden- und Lufthygiene. Specimens used in the test were bred in the laboratory of the Fraunhofer IME.

Breeding conditions: Adult Daphnia at least 3 weeks old were separated from the stock population by sieving. Batches of 30 to 50 animals were held at room temperature in approx. 1.8 L dilution water for one week. During this week the daphnids were fed daily with an algal suspension (*Desmodesmus subspicatus*) and LiquizellR (HOBBY). Algae growing in the log-phase were centrifuged and the pellet was re-suspended in a few mL of medium. 30 mL of this suspension was given to 1 L Daphnia medium. The water was changed once per week. Newborn Daphnia were separated by sieving, the first generation was discarded.

Holding- and dilution-water: Purified drinking water was used as holding- and dilution water. The purification included filtration with activated charcoal passage through a limestone column and aeration. To avoid copper contamination plastic water pipes are used for the testing facilities. The following water chemistry data are recorded regularly in the testing facility and reported: pH, conductivity, dissolved oxygen content, content of nitrate, nitrite, ammonium (NH_4^+), phosphate, calcium, magnesium, total hardness, alkalinity, DOC content, content of metals (copper, iron, manganese and zinc).

Food: According to the OECD guideline 202, the daphnids were not fed during the test.

4.3 Study design

4.3.1 Study type

static

4.3.2 Water medium type

Freshwater

4.3.3 Total exposure duration

48 h: February 8th - February 10th, 2011

No post-exposure observation period was performed.

4.3.4 Test conditions

Total hardness:	1.1 mmol/L
Test temperature:	20.0 - 20.2 °C (permitted range: 20 ± 2 °C)
pH:	7.3 – 8.3 (permitted range: pH 6 – 9; variation less than 1.5)
Dissolved oxygen:	about 100% corresponding to about 8.6 mg/L (demanded threshold value: 3 mg/L)
Salinity:	289 µS/cm

Details on test conditions:

- Test vessel: glass beakers (60 mL) filled with 50 mL test suspension; covered with glass panes
- Aeration: no
- Renewal rate of test solution: no
- No. of organisms per vessel: 5
- No. of vessels per concentration (replicates): 4
- No. of vessels per control (replicates): 4
- Additional vessels per test concentration were prepared for chemical analyses.

Test medium / water parameters

Table 19: Specification of the applied purified tap water

Conductivity (µS/cm)	Alcalinity (mmol/l)	Tot. hardness (mmol/l)	Ca-hardness (mmol/l)	Mg-hardness (mmol/l)	NPOC (mg/L)	NO_3 (mg/L)
289	2.1	1.1	0.8	0.2	0.8055	4.1
NO_2 (mg/L)	NH_4 (mg/L)	PO_4 (mg/L)	Cl (mg/L)	Cd (µg/L)	Cr (µg/L)	Cu (µg/L)
<0.005	<0.01	0.19	< 0.02	< 0.852	<1.09	<4.56
Fe (µg/L)	Mn (µg/L)	Ni (µg/L)	Pb (µg/L)	Zn (µg/L)		
<8.42	<12.5	<2.11	<3.79	<5.83		

- Culture medium different from test medium: no
- Intervals of water quality measurement: once per month

Other test conditions

- Adjustment of pH: no
- Photoperiod: light/dark cycle 16/8 h
- Light intensity: 564 - 547 lux

Vehicle control: no

Reference substance: In order to confirm the sensitivity of the test species *Daphnia magna* (clone 5), acute immobilisation tests over 24 h with the reference substance (RS) $K_2Cr_2O_7$ were performed in regular intervals, as proposed by OECD 202.

Test performance

Young specimens of *D. magna* of similar age (4 – 24 h) were exposed to five concentrations of the test item under static conditions for a period of 48 h. After the test solution was distributed to the replicate beakers the test organisms were added immediately. The daphnids were exposed without aeration in 50 mL of test solution in numbered glass beakers of 50 mL nominal volume. Apparently dead animals were removed at the daily check.

In each medium pH value (pH-meter, e.g. WTW 535), oxygen concentration (e.g. WTW Digital-Sauerstoff-Messgerät Oxi Digi 550) and temperature (e.g. Digitalthermometer, Roth) were checked in the fresh and in the aged medium.

The numbers of dead animals were visually determined daily and dead specimens (if occurred) were removed. Any abnormalities in appearance and behaviour (if occurred) were recorded. Immobility was determined according to the OECD guideline 202. Specimens which were not able to swim within 15 seconds after gentle agitation of the test vessel were considered to be immobilized (even if they were still moving their antennae).

Data evaluation

- The evaluation of the concentration-effect-relationships and the calculations of effect concentrations were based on the nominal concentrations of the test item.
- All statistical calculations were performed using the computer programme ToxRat®.

4.4 Results

Zeta potential

The Zeta potential and the agglomeration behaviour of NM-600 are presented in Table 4 and Table 5. NM-600 has a negative Zeta potential in the test water. The negative charge in the test water was less pronounced compared to the charge in the mineral medium applied in the test with algae.

Particles / agglomerates could be determined at least at concentrations of 33 and 100 mg/L Even after an incubation period of 2 days particles were determined in the supernatant indicating that not all particles had sedimented. Higher concentrations resulted in larger particles at day 0 and at day 2 in the supernatant. At test end also particles in the concentration of 11 mg/L could be determined after shaking, which indicated the formation of larger particles during the incubation period which sedimented. At present, the results cannot be interpreted properly. As we assume that knowledge concerning the measurement and interpretation of suspensions containing nanoparticles and their agglomerates will be increasing, it may be possible that the results obtained in this project can be interpreted retrospectively.

Table 20: Zeta potential

Sample	Zeta potential [mV]
NM 600 in purified tap water, specification see Table 19	-18 mV
Purified tap water	-13 mV

Table 21: Behaviour of the particles during the test

Concentration	Z-Average [nm] [1]	PDI [2]	Peak 1 [nm]	Peak 2 [nm]	Count Rate [3] [kcps]	Measurement Position [4]	Attenuation [5]
Day 0 (test start)							
11 mg/L	< Detection limit						
33 mg/L	1524	0.767	848.0	[162.0] [6]	199.6	4.65	8
100 mg/L	1515	0.607	1089	[188.1]	150.6	4.65	7
Day 2 (48 h. test end) - supernatant							
11 mg/L	< Detection limit						
33 mg/L	1065	0.851	492.4	-	159.8	4.65	9
100 mg/L	1334	0.403	1001	-	285.7	4.65	8
Day 2 (48 h. test end) - after shaking of the test vessels and redispersion of the sedimented particles							
11 mg/L	1606	0.838	819.1	[140.6]	131.8	4.65	9
33 mg/L	1721	0.683	1068	[195.9]	208.4	4.65	8
100 mg/L	1802	0.595	873.3	-	156.3	4.65	7

[1] calculated value (cumulative mean); [2] increasing value indicates increasing polydispersity (maximum: 1); [3] best results with a count rate between 150 and 500 kilo counts per second (kcps); [4] measurement position in the middle of the measuring cell; [5] indicator for turbidity (high values indicate low turbidity; maximum: 11); [6] values in brackets indicate peaks with less than 10 % of the main peak.

Analytical concentrations

The results are presented in Table 22. Details are presented in Table 29 - Table 31.

A high silica concentration was detected in the controls. Because these aqueous media already show a high background concentration the calculated Si recoveries do not represent the recovery of the metal in nanoclay at all.

For a better view and evaluation the mean of the controls per day was added to the nominal value for silica of the respective loading. This sum was set to 100% and the recovery of the amount of silica in the sample in relation to this sum was determined. For a clear-cut result the amount of Si in the controls have to be less than 50% of the measured values in samples from test item loaded vessels.

The results show that at day 0 a sufficient recovery was obtained. During the incubation period sedimentation occurred. The lower recovery at day 1 compared to day 2 further indicates that sampling in the presence of sedimented nanoparticles is difficult. 20 mL were taken from a volume of 50 mL. Samples were taken from the overlaying water. Nevertheless, it cannot be excluded that sedimented particles were sucked resulting in an overestimation of the concentration in the overlaying water at least at day 2.

Table 22: Measured Si concentrations and recoveries

Concentration of nanoclay [mg/L]	Mean Si [µg/L]	SD [µg/L]	Si nominal [µg/L]	Mean Si control + Si nominal [µg/L]	Recovery of fortification in relation to measured Si [%]
Day 0					
control	4002	142	0	---	---
1.2	4317	865	368	4370	98.8
3.7	5081	277	1115	5117	99.3
11	7064	97	3315	7317	96.5
33	12708	522	9946	13948	91.1
100	29303	265	30139	34141	85.8
Day 1					
control	5913	653	0	---	---
1.2	6038	7	368	6281	96.1
3.7	5786	1116	1115	7028	82.3
11	6619	347	3315	9228	71.7
33	8670	611	9946	15859	54.7
100	8880	42	30139	36052	24.6
Day 2					
control	5390	702	0	---	---
1.2	5950	542	368	5758	103
3.7	6093	354	1115	6505	93.7
11	7259	104	3315	8705	83.4
33	7365	1379	9946	15336	48.0
100	25556	2615	30139	35529	71.9

Effects:

The mean values for immobilisation per treatment level are listed in Table 23 and Table 24. Only in the two highest test concentrations one daphnid died. Therefore, no EC_x-values could be determined. The observed immobilisation is not statistically significant and the NOEC is ≥100 mg/L.

Table 23: Mobile daphnids after 24 h and 48 h. Sum of all replicates [Ind.]. N = 20. Concentrations present nominal concentrations.

Incubation	Control	1.2 mg/LL	3.7 mg/L	11.0 mg/L	33.0 mg/L	100.0 mg/L
24 h	20	20	20	20	20	20
48 h	20	20	20	20	19	19

Table 24: Immobilisation after 24 h and 48 h. Mean values [%]. Concentrations given in nominal concentrations.

Incubation	Control	1.2 mg/LL	3.7 mg/L	11.0 mg/L	33.0 mg/L	100.0 mg/L
24 h	0	0	0	0	0	0
48 h	0	0	0	0	5	5

Physical/Pathological symptoms and changes in behaviour:

Neither significant signs of disease nor stress like discolouration or abnormal behaviour were observed in any replicate. All surviving specimens, up to the highest test item concentration, gave the impression of healthy condition.

Reference substance:

In order to confirm the sensitivity of the test species *Daphnia magna* (clone 5), acute immobilisation tests over 24 h with the reference substance (RS) $K_2Cr_2O_7$ are performed in regular intervals, as proposed by OECD 202. The results of the latest reference study (June 2010) are in agreement with historical 24 h EC_{50}-values obtained in this institute.

24 h EC_{50} value:

Immobilisation: 0.87 mg/L (95% CL: 0.77 – 0.99 mg/L)

4.5 Validity

The test is considered to be valid, since:

- Mortality in controls did not exceed 10%.
- The dissolved oxygen concentration at the end of the aging period (48 h) was ≥ 3 mg/L in control and test vessels.

4.6 Conclusion

Nanoclay NM-600 was tested in the acute test with *Daphnia magna*. Immobilisation was determined. Up to the highest test concentration of 100 mg/L no effect due to NM-600 was detected. The NOEC for immobilisation is ≥100 mg/L.

A high silica concentration was detected in the controls. The results show that at day 0 a sufficient recovery was obtained. During the incubation period sedimentation occurred.

4.7 Executive summary

The toxicity of the test item **nanoclay NM-600** on immobilisation of the species *Daphnia magna* was investigated. The daphnids were placed in water containing the test item in nominal concentrations of 1.2, 3.7, 11.0, 33.0 and 100 mg/L. The test was conducted for 48 h under static conditions. Effects on immobilisation were determined after 24 and 48 hours. Only in the two highest test concentrations one daphnid died. Therefore, no EC_x-values could be determined. The observed immobilisation is not statistically significant, and the NOEC is ≥100 mg/L.

A high silica concentration was detected in the controls. The results show that at day 0 a sufficient recovery was obtained. During the incubation period sedimentation occurred.

4.8 Details on test data

4.8.1 Data on the ecotoxicological test

Table 25: Oxygen concentration and pH-values in the daphnid test with nanoclay NM-600

Concentration [mg/L]	O_2 [mg/L] Test start	O_2 [mg/L] Test end	O_2 [mg/L] Test start	O_2 [mg/L] Test end	pH Test start	pH Test end
Control	8.9	9.1	102	103	7.3	8.2
1.2	8.6	9.0	101	101	7.7	8.2
3.7	8.7	9.2	101	102	7.7	8.3
11.0	8.4	8.8	98	99	7.8	8.3
33.0	8.3	8.7	97	98	7.8	8.3
100.0	8.0	8.7	93	98	8.0	8.3

Table 26: Mobile daphnids after 24 h and 48 h. Single values [Ind.] of the replicates. N = 5 per replicate. Concentrations given as nominal values.

	Replicate	Control	1.2 mg/L	3.7	11.0	33.0	100.0
24 h	1	5	5	5	5	5	5
	2	5	5	5	5	5	5
	3	5	5	5	5	5	5
	4	5	5	5	5	5	5
48 h	1	5	5	5	5	5	4
	2	5	5	5	5	5	5
	3	5	5	5	5	5	5
	4	5	5	5	5	4	5

Table 27: Immobilisation after 24 h and 48 h. Single values of the replicates [%]. Concentrations given as nominal values.

	Replicate	Control	1.2 mg/L	3.7	11.0	33.0	100.0
24 h	1	0	0	0	0	0	0
	2	0	0	0	0	0	0
	3	0	0	0	0	0	0
	4	0	0	0	0	0	0
48 h	1	0	0	0	0	0	0
	2	0	0	0	0	0	5
	3	0	0	0	0	0	0
	4	0	0	0	0	5	0

4.8.2 Data on chemical analyses

LODs/LOQs, correlation

The information about the LOD/LOQ and correlation coefficient are compiled in **Table 28**.

A representative calibration line is shown in the raw data 6.12.3.

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

Table 28: LODs/LOQs, correlation

Measurement date, description	LOD [µg/L]	LOQ [µg/L]	Correlation coefficient r
April 08, 2011; samples from day 0	22	73*	0.9990
April 28, 2011; samples from day 1, 2	15	49*	0.9997

* internal LOQ calculation was performed with more digits.

Instrumental and analytical set-up of the ICP-OES:
Thermo IRIS Intrepid II
Thermo Electron Corporation, Germany
Analytical conditions
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
Wavelengths: 288.158 nm

Quality assurance measurements

According to the quality assurance requirement, the silica recovery in recalibration standards was in the range of ± 15% of the certified value. However, regarding Si concentrations measured by ICP-OES, the mean recovery was 102 ± 1.9% (n = 2) for 100 µg/L and 100 ± 0.8% (n = 2) for 250 µg/L.

Unfortunately, a reference water with a certified amount of Si is not commercially available.

An exact amount of the nanoclay test item (104 mg/L, appropriately diluted to fit in the concentration range of samples) was introduced into ultrapure water. Samples were taken, digested, appropriately diluted (dilution factor 5) to fit in the concentration range of the test samples and finally analysed. The amount of Si in nanoclay was calculated using an EDX analysis provided by NIA (NPL report) As energy dispersive X-ray spectroscopy only gives information about a specific surface area the values have to be considered carefully. Therefore the quality assurance requirement for the recovery of Si in nanoclay was set to ± 30. The recovery of silica in nanoclay was determined to 106 ± 4%.

Silica concentrations in digested ultrapure water blanks were below LOQ (< 73 µg/L) for the measurement series from April 8, 2011. In the measurement series from April 28, 2011, elevated Si concentrations (72 – 127 µg/L) were found in digested ultrapure water blanks. However, these values were clearly below the amount of silica in method controls (media used for Daphnia test, Table 29 - Table 31). As silica is ubiquitous, a contamination cannot be completely excluded.

Digested reagent blanks (0.2% KOH) were additionally analysed. The measured values were always below the LOD of the respective measurement series (< 15 - < 22 µg/L).

Analytical results

In Table 29 - Table 31 the measurement results of nanoclay in aqueous samples from the *Daphnia* test are compiled. A high silica concentration was detected in the controls. To calculate the recovery the mean of the controls per day was added to the nominal value for silica of the respective loading. This sum was set to 100% and the

recovery of the amount of silica in the sample in relation to this sum was determined. For a clear-cut result the amount of Si in the controls have to be less than 50% of the measured values in samples from test item loaded vessels.

Table 29: Measured Si concentrations and recoveries, Daphnia test d0

Day 0									
Sample name	Measured Si [µg/L]	Dilution factor	Measured Si x dilution[µg/L]	Mean Si SD [µg/L]	SD [µg/L]	Si nominal [µg/L]	Si recovery [%]	Mean Si control + Si nominal [µg/L]	Recovery of fortification in relation to measured Si [%]
control d0 A	274	15	4103	4002	142	0	-		
control d0 B	260	15	3902						
1.2 mg/L d0 A	329	15	4929	4317	865	362	1193	4364	98.9
1.2 mg/L d0 B	247	15	3705						
3.7 mg/L d0 A	326	15	4886	5081	277	1115	456	5117	99.3
3.7 mg/L d0 B	352	15	5277						
11 mg/L d0 A	466	15	6996	7064	97	3315	213	7317	96.5
11 mg/L d0 B	476	15	7133						
33 mg/L d0 A	411	30	12339	12708	522	9946	128	13948	91.1
33 mg/L d0 B	436	30	13077						
100 mg/L d0 A	388	75	29115	29303	265	30140	97.2	34142	85.8
100 mg/L d0 B	393	75	29490						

Table 30: Measured Si concentrations and recoveries, Daphnia test d1

day 1									
Sample name	Measured Si [µg/L]	Dilution factor	Measured Si x dilution[µg/L]	Mean Si SD [µg/L]	SD [µg/L]	Si nominal [µg/L]	Si recovery [%]	Mean Si control + Si nominal [µg/L]	Recovery of fortification in relation to measured Si [%]
control d1 A	363	15	5451	5913	653	0			
control d1 B	425	15	6375						
1.2 mg/L d1 A	403	15	6044	6038	7	362	1668	6275	96.2
1.2 mg/L d1 B	402	15	6033						
3.7 mg/L d1 A	333	15	4997	5786	1116	1115	519	7028	82.3
3.7 mg/L d1 B	438	15	6575						
11 mg/L d1 A	425	15	6374	6619	347	3315	200	9228	71.7
11 mg/L d1 B	458	15	6864						
33 mg/L d1 A	275	30	8238	8670	611	9946	87.2	15859	54.7
33 mg/L d1B	303	30	9102						
100 mg/L d1 A	119	75	8910	8880	42	30140	29.5	36053	24.6
100 mg/L d1 B	118	75	8850						

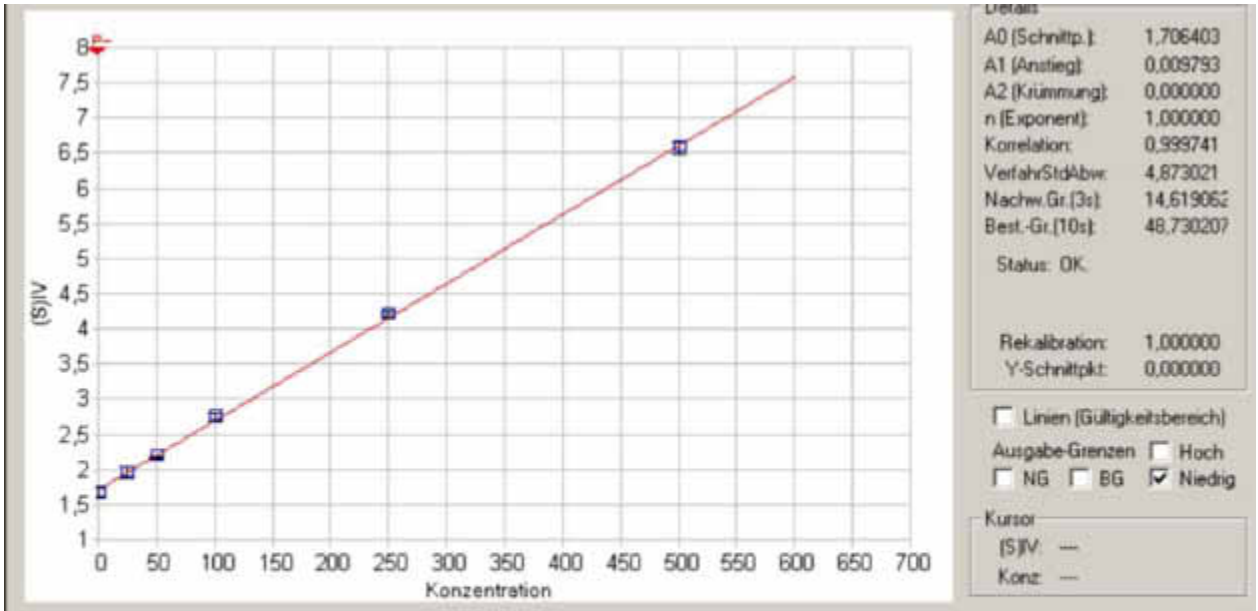
Table 31: Measured Si concentrations and recoveries, Daphnia test d2

day 2									
Sample name	Measured Si [µg/L]	Dilution factor	Measured Si x dilution[µg/L]	Mean Si SD [µg/L]	SD [µg/L]	Si nominal [µg/L]	Si recovery [%]	Measured Si corrected by background concentration [µg/L]	Recovery of Si [%]
control d1 A	326	15	4893	5390	702	0			
control d1 B	392	15	5886						
1.2 mg/L d1 A	371	15	5567	5950	542	362	1644	5752	103
1.2 mg/L d1 B	422	15	6333						
3.7 mg/L d1 A	390	15	5843	6093	354	1115	546	6505	93.7
3.7 mg/L d1 B	423	15	6344						
11 mg/L d1 A	489	15	7332	7259	104	3315	219	8705	83.4
11 mg/L d1 B	479	15	7185						
33 mg/L d1 A	213	30	6390	7365	1379	9946	74.0	15336	48.0
33 mg/L d1B	278	30	8340						
100 mg/L d1 A	316	75	23708	25556	2615	30140	84.8	35529	71.9
100 mg/L d1 B	365	75	27405						

Raw data examples

Example for ICP-OES calibration

Calibration data from the measurement performed on April 28, 2010 (Wavelength 288.158 nm)



	Standardname	Konzentration		Differenz		Signal		Gewicht
		Gegeben	Gefunden	Diff	%	(S)IV	StdAbw	
1	Blank	0,00000	-4,6009	-4,60	0,000	1,6613	0,070	1
2	KalibStd-1	25,000	24,741	-0,259	-1,03	1,9487	0,081	1
3	KalibStd-2	50,000	47,608	-2,39	-4,78	2,1726	0,018	1
4	KalibStd-3	100,00	105,62	5,62	5,62	2,7407	0,066	1
5	KalibStd-4	250,00	255,01	5,01	2,00	4,2036	0,034	1
6	KalibStd-5	500,00	496,63	-3,37	-0,675	6,5697	0,077	1

Example for ICP-OES raw data printout

Example printout from the measurement performed on April 28, 2010.
The measurement values used for evaluation are marked

Nanoclay SiO2_Daphnie_d1 d2_110428 Hanskne

45	Pro: 33,0mg/L d2SiO2 D 1:2 04/28/2011 13:46:55 KONZ Custom ID1: Custom ID2: Custom ID3:					
	Al1670	Al3092	Si2124	Si2216	Si2516	Si2881
Einheit	µg/L	µg/L	µg/L	µg/L	µg/L	µg/L
Mittel	12,65	73,63	279,2	282,8	278,5	278,0
StdAbw	32,56	3,39	0,9	1,0	4,0	9,9
% RSD	257,4	4,598	0,3062	0,3544	1,450	3,578
Mess.#1	-24,90	73,77	278,2	281,7	278,3	285,4
Mess.#2	29,80	70,18	279,5	283,6	282,7	282,0
Mess.#3	33,04	76,95	279,9	283,1	274,6	266,7
46	Pro: 100mg/L d2SiO2 C 1:5 04/28/2011 13:50:08 KONZ Custom ID1: Custom ID2: Custom ID3:					
	Al1670	Al3092	Si2124	Si2216	Si2516	Si2881
Einheit	µg/L	µg/L	µg/L	µg/L	µg/L	µg/L
Mittel	72,71	104,0	306,7	313,0	308,8	315,1
StdAbw	43,20	1,6	0,8	2,0	2,2	1,9
% RSD	59,41	1,508	0,2665	0,6292	0,6996	0,5869
Mess.#1	90,91	105,6	306,4	313,6	307,7	317,7
Mess.#2	103,8	103,9	307,6	310,8	311,2	314,1
Mess.#3	23,39	102,5	306,1	314,6	307,3	316,5
47	Pro: 100mg/L d2SiO2 D 1:5 04/28/2011 13:53:20 KONZ Custom ID1: Custom ID2: Custom ID3:					
	Al1670	Al3092	Si2124	Si2216	Si2516	Si2881
Einheit	µg/L	µg/L	µg/L	µg/L	µg/L	µg/L
Mittel	93,11	96,48	362,0	363,6	365,9	363,4
StdAbw	35,32	3,00	2,3	2,6	2,5	6,9
% RSD	37,93	3,108	0,6252	0,7239	0,6714	1,881
Mess.#1	81,31	93,10	362,3	366,3	368,7	372,8
Mess.#2	132,8	97,49	359,6	363,4	364,1	364,3
Mess.#3	65,21	98,84	364,1	361,1	364,9	359,2
48	Pro: Blank 0,2 % KOH 04/28/2011 13:56:31 KONZ Custom ID1: Custom ID2: Custom ID3:					
	Al1670	Al3092	Si2124	Si2216	Si2516	Si2881
Einheit	µg/L	µg/L	µg/L	µg/L	µg/L	µg/L
Mittel	-5,572	44,42	-2,737	-3,922	2,447	-0,2513
StdAbw	48,59	1,30	0,844	2,219	6,037	2,299
% RSD	872,0	2,917	30,83	56,57	246,7	914,7
Mess.#1	45,91	43,85	-3,658	-3,916	-2,705	0,0642
Mess.#2	-50,62	45,91	-2,001	-1,707	0,9567	-2,692
Mess.#3	-12,01	43,51	-2,553	-6,145	9,089	1,873
49	Pro: Blank 0,2 % KOH 04/28/2011 13:59:43 KONZ Custom ID1: Custom ID2: Custom ID3:					
	Al1670	Al3092	Si2124	Si2216	Si2516	Si2881
Einheit	µg/L	µg/L	µg/L	µg/L	µg/L	µg/L
Mittel	-19,52	45,25	-4,380	-5,779	-5,409	-2,513
StdAbw	42,72	4,06	0,509	1,855	3,782	6,797
% RSD	218,8	8,974	11,63	32,11	69,92	337,6
Mess.#1	-44,19	40,60	-3,950	-3,922	-8,600	4,188
Mess.#2	-44,18	48,06	-4,942	-5,781	-1,232	-9,280

5 Tests with fish: OECD TG 203

5.1 Test principle

The objective of the study was to assess the effects of a 96 h exposure to the test item of zebra fish (*Danio rerio*), survival and signs of intoxication, according to the OECD test guideline 203. Seven zebra fish each were exposed to three concentrations of the test item and an untreated dilution water control under static conditions.

5.2 Materials and methods

5.2.1 Test guideline

The test was performed according to:

OECD 203 (17.07.1992): OECD guideline for testing of chemicals – Fish, acute toxicity test.

5.2.2 GLP

The test was performed following the principles of GLP. In deviation to GLP the raw data have not been archived and the quality assurance unit was not involved with respect to the inspection of the test, the raw data and the report. All laboratory equipment (e.g. balances, thermometers, pH-meter) were controlled and documented according to GLP.

5.2.3 Test substances

Nanoclay NM-600, vessels no. 0006, 0676, 0691

5.2.4 Analytical monitoring

The particle size distribution was determined at test start and at day 1, day 2 and day 4 (test end).

For sampling a 20 mL volumetric pipette was used. During sampling the pipette was moved through the whole water body. The sample was frozen in glass vessels until the analyses were performed.

The particle size distribution was measured using a Malvern Zeta-Sizer. For the zeta-potential the value of the test with daphnids was used. In both tests the same aqueous medium (purified drinking water) was applied.

Test concentrations were determined at test start and at day 1, day 2 and day 4 (test end). For sampling a 20 mL volumetric pipette was used. During sampling the pipette was moved through the whole water body. The sample was frozen in glass vessels until analyses were performed. For details of chemical analyses see chapter 7.

5.2.5 Details on test suspensions

Test concentrations were:

1.0 mg/L, 10.0 mg/L, 100.0 mg/L

The test suspensions were prepared by adding 10 mg, 100 mg and 1000 mg in purified tap water (5 L). Glass vessels were used. The dispersions were stirred (1 min) and treated with ultrasound in an ultrasonic bath (3 min). The suspensions were transferred in full glass aquaria (12 L) and 5 L of purified drinking water added.

5.2.6 Test organism

Justification for use: *Danio re*rio is listed in the OECD TG 203 as test organism representing aquatic vertebrates.

Specification: *Danio rerio* (Teleostei, Cyprinidae)

Length: 2 cm ± 1 cm

Source: in house culture

Origin of the strain: West Aquarium GmbH
37431 Bad Lauterberg, Germany.

Holding: The fish were reared in water of the same quality as used in the test (purified drinking water) for at least three months until the start of exposure.

Health: The criteria of the test guideline 203 were followed. Only healthy fish without visible diseases and abnormalities were used in the study.

Holding- and dilution-water: Purified drinking water was used as holding- and dilution water. The purification included filtration with activated charcoal, passage through a limestone column, and aeration. To avoid copper contamination, plastic water pipes are used. The following water chemistry data are recorded regularly in the testing facility and are reported: pH, conductivity, dissolved oxygen content, content of nitrate, nitrite, ammonium (NH4+), phosphate, calcium, magnesium, total hardness, alkalinity, DOC content, content of metals (copper, iron, manganese and zinc).

5.3 Study design

5.3.1 Study type

static

5.3.2 Water medium type

Freshwater

5.3.3 Total exposure duration

96 h: February 28th - March 04th, 2011

No post-exposure observation period was performed.

5.3.4 Test conditions

Total hardness:	1.1 mmol/L
Test temperature:	22.4 - 22.8 °C (recommended range: 21 - 25 °C)
pH:	8.1 – 8.7 (recommended range: pH 6.0 – 8.5)
Dissolved oxygen:	about 100% corresponding to about 8.6 mg/L (demanded threshold value: ≥ 60%)
Salinity:	289 µS/cm

Details on test conditions:

- Test vessel: glass beakers (12 L) filled with 10 L test suspension
- Aeration: yes
- Stirring: the aquaria were placed on magnetic stirrers. Stirring was performed with magnetic bars. To avoid damage of the fish the magnetic bars were covered with a wired cage.
- Photoperiod: yes (12/12 h)
- Renewal rate of test solution: no
- No. of organisms per vessel: 7
- No. of vessels per concentration (replicates): 1
- No. of vessels per control (replicates): 1

Temperature, pH, and oxygen concentration of the water were measured in each vessel directly before adding the fish and afterwards daily.

Test medium / water parameters

Purified drinking water was used. The purification included filtration with activated charcoal, passage through a lime-stone column and aeration until oxygen saturation. Based on periodical measurements the dilution water was characterised as follows:

Table 32: Specification of the applied purified tap water

Conductivity (µS/cm)	Alcalinity (mmol/l)	Tot. hardness (mmol/l)	Ca-hardness (mmol/l)	Mg-hardness (mmol/l)	NPOC (mg/L)	NO_3 (mg/L)
289	2.1	1.1	0.8	0.2	0.8055	4.1
NO_2 (mg/L)	NH_4 (mg/L)	PO_4 (mg/L)	Cl (mg/L)	Cd (µg/L)	Cr (µg/L)	Cu (µg/L)
<0.005	<0.01	0.19	< 0.02	< 0.852	<1.09	<4.56
Fe (µg/L)	Mn (µg/L)	Ni (µg/L)	Pb (µg/L)	Zn (µg/L)		
<8.42	<12.5	<2.11	<3.79	<5.83		

- Culture medium different from test medium: no
- Intervals of water quality measurement: once per months

Vehicle control: no

Reference substance: In order to confirm the sensitivity of the test species *Danio rerio* mortality tests over 96 h with the reference substance $CuSO_4$ are performed in regular intervals. The 96 h LC_{50}-values obtained in this institute are within the range of 0.06 - 0.11 mg/L.

.

Test performance

With respect to animal protection the test was performed with a reduced number of test concentrations and test animals.

The test vessels were full glass aquaria of 12 L with approx. 10 litres of test solution. Seven fish each were exposed to three nominal concentrations and a control for a period of 96 h. In Figure 4 the test design is presented. To achieve distribution of the nanomaterials in the test vessels stirring with magnetic bars was performed during the test. To avoid damage of the fish, the magnetic bar was protected by a mesh.

Figure 4: Test design of the fish test

Control | NM-600: 1 mg/L | NM-600: 1 mg/L | Stirring with magnetic bar (protected)

No feeding occurred throughout the test. The tanks were subjected to a light/dark cycle of 12/12 hours. Water temperature was adjusted to 23 ± 2 °C in the water bath holding all test vessels. All fish were measured for length and weight before the

beginning of the study and observed daily for mortality and any other abnormalities in appearance and behaviour.

Data evaluation

- The evaluation of the concentration-effect-relationships and the calculations of effect concentrations were based on the nominal concentrations of the test item.
- No effect occurred, and therefore no calculations were performed.

5.4 Results

Zeta potential

The Zeta potential of NM-600 is presented in Table 33. NM-600 has a negative Zeta potential in the test water. The negative charge in the test water was less pronounced compared to the charge in the mineral medium applied in the test with algae.

Table 33: Zeta potential

Sample	Zeta potential [mV]
NM 600 in purified tap water, specification see Table 19 (application dispersion):	-18 mV
Purified tap water	-13 mV

Particle size distribution

At day 0, day 1, day 2 and day 4 particle size distributions were determined with the device Malvern Nano ZS at the two higher test concentrations (10 and 100 mg/L). Differences in peak positions between the different measuring days were small. It is assumed that due to the stirring no sedimentation of the particles took place. The hydrodynamic diameter (Z-average) which is a calculated value, increased. At present, the results cannot be interpreted properly. As we assume that knowledge concerning the measurement and interpretation of suspensions containing nanoparticles and their agglomerates will be increasing, it may be possible that the results obtained in this project can be interpreted retrospectively. In Table 34 measuring results and applied measuring parameter are presented.

Table 34: Particle size distribution in the reproduction test with daphnids with nanoclay

Concentration [mg/L]	Z-Average nm] [1]	PDI [2]	Peak 1 [nm] [6]	Peak 2 [nm] [6]	Count Rate [3] [kcps]	Measurement Position [4]	Attenu-ation [5]
Day 0							
10 mg/L	1608	0.8	743	-	86	4.65	9
100 mg/L	1673	0.4	1036	-	153	4.65	7
Day 1							
11 mg/L	1732	0.8	754	-	134	4.65	9
100 mg/L	1751	0.4	1122	-	159	4.65	7
Day 2							
10 mg/L	2538	0.9	792	-	75	4.65	9
100 mg/L	1804	0.5	1252	-	164	4.65	7
Day 4							
10 mg/L	2629	1	770	-	258	4.65	10
100 mg/L	1815	0.4	1182	-	178	4.65	7

[1] calculated value (cumulative mean); [2] increasing value indicates increasing polydispersity (maximum: 1); [3] best results with a count rate between 150 and 500 kilo counts per second (kcps); [4] measurement position in the middle of the measuring cell; [5] indicator for turbidity (high values indicate low turbidity; maximum: 11); [6] 1 mg/L samples below quantification limit; [7] prepared from 10 mg/L samples with 3 min of ultrasonic treatment; [8] prepared from 10 mg/L samples with 30 min of ultrasonic treatment

[6] In the case of more than two peaks, value in brackets gives percentage of the single peak compared to all peaks (prerequisite, the peak exceeds 10%)

Analytical concentrations

The results are presented in Table 35. Details are presented in Table 42 - Table 45. A high silica concentration was detected in the control samples. This is due to the ubiquitous occurrence of silica (e.g. in dust). A high silica concentration was detected in the controls. Because these aqueous media already show a high background concentration the calculated Si recoveries do not represent the recovery of the metal in nanoclay. For a better view and evaluation the mean of the controls per day was added to the nominal value for silica of the respective loading. This sum was set to 100%, and the recovery of the amount of silica in the sample in relation to this sum was determined. For a clear-cut result the amount of Si in the controls have to be less than 50% of the measured values in samples from test item loaded vessels.

In the fish test the suspensions in the test vessels were determined. Due to the high background concentration, the lowest concentration (1 mg/L) could not be determined with sufficient accuracy. For the higher concentrations recovery was higher. During the incubation period of 96 h recovery in the individual test vessels of the two higher test concentrations (10, 100 mg/L) increased indicating an improved distribution of the nanomaterials in the large test vessels due to stirring. Recoveries above 80% were determined after 1 day (10 mg/L) and after 2 days (100 mg/L). Due to the recoveries of 87 and 88% at the end of the test the nominal test concentrations were used for the calculation of NOEC and LOEC values.

Table 35: Measured Si concentrations and recoveries

Concentration of nanoclay [mg/L]	Mean Si [µg/L]	SD [µg/L]	Si nominal [µg/L]	Mean Si control + Si nominal [µg/L]	Recovery of fortification in relation to measured Si [%]
Day 0					
control	4260	246	0	---	---
1	4317	221	301	4424	92.7
10	6842	35	3014	7137	88.8
100	28714	599	30140	34263	77.2
Day 1					
control	4528	203	0	---	---
1	4630	340	301	4405	96.7
10	7143	458	3014	7118	94.4
100	31050	350	30140	34244	79.6
Day 2					
control	4260	246	0	---	---
1	4317	221	301	4561	94.6
10	6842	35	3014	7274	94.1
100	28714	599	30140	34400	83.5
Day 4					
control	4528	203	0		
1	4630	340	301	4829	95.9
10	7143	458	3014	7542	94.7
100	31050	350	30140	34668	89.6

Effects:

No mortality and no effect on the behaviour of the test fish were observed (Table 36, Table 37). Therefore, no EC_x-values could be determined. The NOEC is ≥100 mg/L.

Table 36: Clinical signs during the test period of 96 h (number of fish with symptoms), 7 fish per concentration were introduced. Concentrations given as nominal concentrations

Incubation	3 h	24 h	48 h	72 h	96 h
Control	0	0	0	0	0
1 mg/L	0	0	0	0	0
10 mg/L	0	0	0	0	0
100 mg/L	0	0	0	0	0

Table 37: Cumulative mortality during the test period of 96 h (number of dead fish), 7 fish per concentration were introduced. Concentrations given as nominal concentrations

Incubation	3 h	24 h	48 h	72 h	96 h
Control	0	0	0	0	0
1 mg/L	0	0	0	0	0
10 mg/L	0	0	0	0	0
100 mg/L	0	0	0	0	0

Reference substance:

In order to confirm the sensitivity of the test species *Danio rerio* mortality tests over 96 h with the reference substance $CuSO_4$ are performed in regular intervals. The 96 h LC_{50}-values obtained in this institute are within the range of 0.06 - 0.11 mg/L.

5.5 Validity

The test is considered to be valid since:

- Mortality in controls did not exceed 10%.
- Constant conditions were maintained throughout the test.
- The dissolved oxygen concentration was ≥ 60% throughout the test.

5.6 Conclusion

Nanoclay NM-600 was tested for mortality of the species *Danio rerio*. A static test with nominal concentrations of 1.0, 10.0, and 100 mg/L was performed. During the incubation the test dispersions were stirred. To avoid damaging of the fish the magnetic bar used for stirring was protected by a mesh. Effects on mortality and behaviour were determined after 96 hours. No fish died or showed abnormal behaviour. Therefore, no LC_x-values could be determined. The NOEC is ≥ 100 mg/L.

A high silica concentration was detected in the control samples. This is due to the ubiquitous occurrence of silica (e.g. in dust). In the fish test the suspensions in the test vessels were determined. Due to the high background concentration, the lowest concentration (1 mg/L) could not be determined with sufficient accuracy. During the incubation period of 96 h recovery in the individual test vessels of the two higher test concentrations (10, 100 mg/L) increased indicating an improved distribution of the nanomaterials in the large test vessels due to stirring. Recoveries above 80% were determined after 1 day (10 mg/L) and after 2 days (100 mg/L). Due to the recoveries of 87 and 88% at the end of the test the nominal test concentrations were used for the calculation of NOEC and LOEC values.

5.7 Executive summary

The toxicity of the test item **nanoclay NM-600** on the mortality of the species *Danio rerio* was investigated. The fish were placed in water containing the test item in nominal concentrations of 1.0, 10.0, and 100 mg/L. The test was conducted for 96 h

under static conditions. Effects on mortality and behaviour were determined after 96 hours. No fish died or showed abnormal behaviour. Therefore, no LC_x-values could be determined. The NOEC is ≥ 100 mg/L.

A high silica concentration was detected in the control samples. This is due to the ubiquitous occurrence of silica (e.g. in dust). In the fish test the suspensions in the test vessels were determined. Due to the high background concentration, the lowest concentration (1 mg/L) could not be determined with sufficient accuracy. For the higher concentrations recovery was higher. During the incubation period of 96 h recovery in the individual test vessels of the two higher test concentrations (10, 100 mg/L) increased indicating an improved distribution of the nanomaterials in the large test vessels due to stirring. Recoveries above 80% were determined after 1 day (10 mg/L) and after 2 days (100 mg/L). Due to the recoveries of 87 and 88% at the end of the test the nominal test concentrations were used for the calculation of NOEC and LOEC values.

5.8 Details on test data

5.8.1 Data on ecotoxicological tests

Table 38: Fish weights (g) at test start, n= 28

Concentration [mg/L]	Control	1 mg/L	10 mg/L	100 mg/L
Min	0.100	0.130	0.130	0.170
Max	0.170	0.210	0.210	0.210
Mean	0.137	0.171	0.157	0.191
Standard deviation	0.024	0.030	0.036	0.012

Table 39: Oxygen saturation [%] of the test suspensions throughout the test

Nominal concentration [mg/L]	0 h	24 h	48 h	72 h	96 h	Mean	Min	Max
Control	97	97	97	100	98	98	97	100
1.0	95	98	95	99	98	97	95	99
10.0	92	96	97	97	95	95	92	97
100.0	91	96	97	95	93	94	91	97

Table 40: pH values of the test suspensions throughout the test

Nominal concentration [mg/L]	0 h	24 h	48 h	72 h	96 h	Mean	Min	Max
Control	8.1	8.4	8.4	8.6	8.6	8.4	8.1	8.6
1.0	8.1	8.4	8.4	8.6	8.6	8.4	8.1	8.6
10.0	8.2	8.4	8.4	8.6	8.6	8.4	8.2	8.6
100.0	8.3	8.5	8.4	8.6	8.7	8.5	8.3	8.7

5.8.2 Data on chemical analyses

LOD/LOQ, correlation

The information about the LOD/LOQ and correlation coefficient are compiled in Table 41.

A representative calibration line is shown below.

The correlation factor (r) for respective calibration function was taken from ICP-OES instrument outputs.

The resulting values are reported in Table 41.

Table 41: LODs/LOQs, correlation

Measurement date, description	LOD [µg/L]	LOQ [µg/L]	Correlation factor r
August 04, 2011;	48	160*	0.9992

* Internal LOQ calculation was performed with more digits.

Instrumental and analytical set-up of the ICP-OES:
Thermo IRIS Intrepid II
Thermo Electron Corporation, Germany
Analytical conditions
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
Wavelengths: 288.158 nm

Quality assurance measurements

According to the quality assurance requirement, the silica recovery in recalibration standards was in the range of ± 15% of the certified value. However, regarding Si concentrations measured by ICP-OES, the mean recovery was 99.3 ± 1.0% (n = 3) for 250 µg/L.

Unfortunately, a reference water with a certified amount of Si is not commercially available.

Exact amounts of the nanoclay test item (104 mg/L and 55 mg/L, appropriately diluted to fit in the concentration range of the samples) were introduced into ultrapure water. Samples were taken, digested, appropriately diluted (dilution factor of 5 for 104 mg/L, 3 for 55 mg/L, respectively) to fit in the concentration range of the test samples and finally analysed. The amount of Si in nanoclay was calculated using an EDX analysis provided by NIA (NPL report). As energy dispersive X-ray spectroscopy only gives information about a specific surface area, the values have to be considered carefully. Therefore, the quality assurance requirement for the recovery of Si in nanoclay was set to 100 ± 30. The recovery of silica in nanoclay was determined to 111 ± 8% (n = 2) for 104 mg/L and 96.5 ± .0.7% (n = 2) for 55 mg/L.

Three ultrapure water blanks were digested along with the test samples. Unfortunately, in two of them the Silica concentrations were above the LOQ (324 µg/L and 228 µg/L). However, they were in the range of silica concentrations determined in controls from the fish test. Only in one of the digested blanks the analysed amount of silica was below LOD (< 48 µg/L). This may be due to a potential contamination with dust.

Reagent blanks (0.2% KOH, n = 9) were additionally analysed. The measured values were always below the LOD (< 48 µg/L).

Analytical results

In Table 42 - Table 45 the measurement results of nanoclay in aqueous samples from the fish test are compiled. A high silica concentration was detected in the controls. Because these aqueous media already show a high background concentration, the calculated Si recoveries do not represent the recovery of the metal in nanoclay. For a better view and evaluation the mean of the controls per day was added to the nominal value for silica of the respective loading. This sum was set to 100%, and the recovery of the amount of silica in the sample in relation to this sum was determined. For a clear-cut result the amount of Si in the controls have to be less than 50% of the measured values in samples from test item loaded vessels.

Table 42: Measured Si concentrations and recoveries, fish test 3 h (internal calculations were performed with more digits)

3h									
Sample name	Measured Si [µg/L]	Dilution factor	Measured Si x dilution[µg/L]	Mean Si [µg/L]	SD [µg/L]	Si nominal [µg/L]	Si recovery [%]	Mean Si control + Si nominal [µg/L]	Recovery of fortification in relation to measured Si [%]
control d0 A	279	0	4182	4123	84	0	-	-	-
control d0 B	271	0	4064						
1 mg/L d0 A	269	15	4031	4103	103	301	1361	4424	92.7
1 mg/L d0 B	278	15	4176						
10 mg/L d0 A	419	15	6281	6335	77	3014	210	7137	88.8
10 mg/L d0 B	426	15	6390						
100 mg/L d0 A	350	75	26243	26438	276	30140	87.7	34263	77.2
100 mg/L d0 B	355	75	26633						

Table 43: Measured Si concentrations and recoveries, fish test 24 h (internal calculations were performed with more digits)

24h									
Sample name	Measured Si [µg/L]	Dilution factor	Measured Si x dilution[µg/L]	Mean Si [µg/L]	SD [µg/L]	Si nominal [µg/L]	Si recovery [%]	Mean Si control + Si nominal [µg/L]	Recovery of fortification in relation to measured Si [%]
control d0 A	274	0	4103	4104	2	0	-	-	-
control d0 B	274	0	4106						
1 mg/L d0 A	289	15	4329	4260	98	301	1413	4405	96.7
1 mg/L d0 B	279	15	4191						
10 mg/L d0 A	448	15	6713	6721	12	3014	223	7118	94.4
10 mg/L d0 B	449	15	6729						
100 mg/L d0 A	368	75	27630	27270	509	30140	90.5	34244	79.6
100 mg/L d0 B	359	75	26910						

Table 44: Measured Si concentrations and recoveries, fish test 48 h (internal calculations were performed with more digits)

48h									
Sample name	Measured Si [µg/L]	Dilution factor	Measured Si x dilution[µg/L]	Mean Si [µg/L]	SD [µg/L]	Si nominal [µg/L]	Si recovery [%]	Mean Si control + Si nominal [µg/L]	Recovery of fortification in relation to measured Si [%]
control d0 A	296	0	4434	4260	246	0	-	-	-
control d0 B	272	0	4086						
1 mg/L d0 A	298	15	4473	4317	221	301	1432	4561	94.6
1 mg/L d0 B	277	15	4161						
10 mg/L d0 A	458	15	6867	6842	35	3014	227	7274	94.1
10 mg/L d0 B	455	15	6818						
100 mg/L d0 A	377	75	28290	28714	599	30140	95.3	34400	83.5
100 mg/L d0 B	389	75	29138						

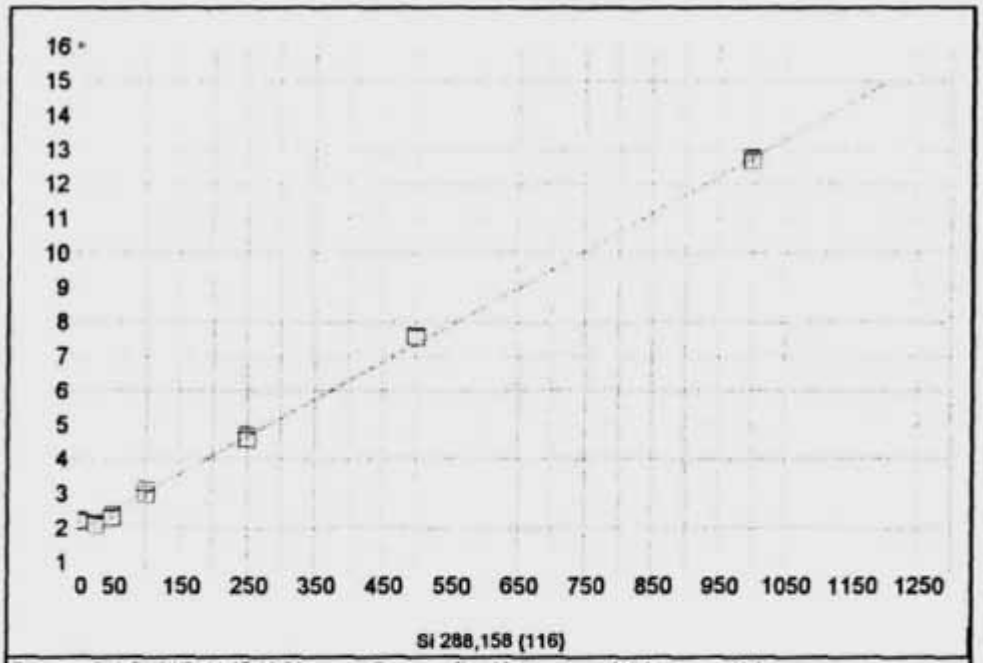
Table 45: Measured Si concentrations and recoveries, fish test 96 h (internal calculations were performed with more digits)

96h									
Sample name	Measured Si [µg/L]	Dilution factor	Measured Si x dilution[µg/L]	Mean Si [µg/L]	SD [µg/L]	Si nominal [µg/L]	Si recovery [%]	Mean Si control + Si nominal [µg/L]	Recovery of fortification in relation to measured Si [%]
control d0 A	292	0	4385	4528	203	0	-	-	-
control d0 B	311	0	4671						
1 mg/L d0 A	293	15	4389	4630	340	301	1536	4829	95.9
1 mg/L d0 B	325	15	4871						
10 mg/L d0 A	455	15	6819	7143	458	3014	237	7542	94.7
10 mg/L d0 B	498	15	7467						
100 mg/L d0 A	411	75	30803	31050	350	30140	103	34668	89.6
100 mg/L d0 B	417	75	31298						

Raw data examples

Example for ICP-OES calibration

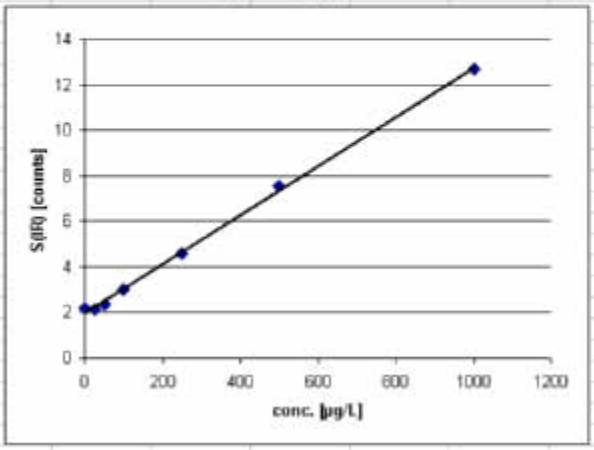
Calibration data from the measurement performed on August 4, 2011 (Wavelength 288.158 nm).



Regress.-Dat: 08/04/2011 15:19:21		Regress.-Typ Linear		Wichtung:	Keine
A0 (Schnittp.):	1,950886		Rekalibration	1,000000	
A1 (Anstieg):	0,010807		Y-Schnittp:	0,000000	
A2 (Krümmung):	0,000000				
n (Exponent):	1,000000				
Korrelation:	0,999194		Status:	OK.	
Verfahr.-StdAbw:	16,031328				
NG:	48,093983				
BG:	160,313278				

Std.-Name	Gesetzte Konz.	Gefund. Konz.	Differenz	% Diff.	(S)IR	Std-Abw	Gewicht
Blank	0,00000	22,048	22,0	0,000	2,1891	0,047	1
KalibStd-1	25,000	14,337	-10,7	-42,7	2,1058	0,046	1
KalibStd-2	50,000	35,209	-14,8	-29,6	2,3314	0,060	1
KalibStd-3	100,00	96,463	-3,54	-3,54	2,9933	0,127	1
KalibStd-4	250,00	244,53	-5,47	-2,19	4,5934	0,070	1
KalibStd-5	500,00	519,38	19,4	3,88	7,5637	0,022	1
KalibStd-6	1000,0	993,04	-6,96	-0,696	12,682	0,060	1

conc. [µg/L]	S(IR) [counts]
0	2.1891
25	2.1058
50	2.3314
100	2.9933
250	4.5934
500	7.5637
1000	12.682



Example for ICP-OES raw data printout

% RSD	0,3593	0,1832	1,261	0,7169
Mess.#1	256,8	260,3	261,9	273,1
Mess.#2	257,7	259,7	258,0	270,2
Mess.#3	255,9	260,7	264,6	269,5
21	Pro: 3h 1mg/L A 08/04/2011 11:54:49 KONZ Custom ID1: Custom ID2: Custom ID3:			
	Si2124	Si2216	Si2516	Si2881
Einheit	µg/L	µg/L	µg/L	µg/L
Mittel	254,6	256,7	259,4	268,7
StdAbw	1,6	0,8	1,3	3,6
% RSD	0,6473	0,3097	0,5169	1,345
Mess.#1	252,7	255,9	257,9	270,8
Mess.#2	255,8	257,5	259,8	264,6
Mess.#3	255,3	256,8	260,5	270,8
22	Pro: 3h 1mg/L B 08/04/2011 11:57:58 KONZ Custom ID1: Custom ID2: Custom ID3:			
	Si2124	Si2216	Si2516	Si2881
Einheit	µg/L	µg/L	µg/L	µg/L
Mittel	255,6	262,0	263,0	278,4
StdAbw	1,1	1,5	6,5	10,9
% RSD	0,4299	0,5711	2,481	3,913
Mess.#1	254,8	261,3	256,1	287,3
Mess.#2	255,3	263,7	263,9	281,7
Mess.#3	256,9	261,0	269,1	266,2
23	Pro: 3h 10mg/L A 08/04/2011 12:01:07 KONZ Custom ID1: Custom ID2: Custom ID3:			
	Si2124	Si2216	Si2516	Si2881
Einheit	µg/L	µg/L	µg/L	µg/L
Mittel	400,5	402,8	398,4	418,7
StdAbw	2,3	1,5	2,6	4,4
% RSD	0,5764	0,3831	0,6418	1,047
Mess.#1	401,0	402,3	401,2	414,6
Mess.#2	402,6	404,5	397,7	418,1
Mess.#3	398,0	401,6	396,2	423,3
24	Pro: 3h 10mg/L B 08/04/2011 12:04:17 KONZ Custom ID1: Custom ID2: Custom ID3:			
	Si2124	Si2216	Si2516	Si2881
Einheit	µg/L	µg/L	µg/L	µg/L
Mittel	418,8	419,9	414,1	426,0
StdAbw	2,0	1,0	8,4	2,4
% RSD	0,4869	0,2376	2,028	0,5621
Mess.#1	420,5	420,4	423,7	428,7
Mess.#2	416,5	420,5	409,9	424,0
Mess.#3	419,2	418,7	408,5	425,5
25	Pro: 3h 100mg/L A 1:5 08/04/2011 12:07:28 KONZ Custom ID1: Custom ID2: Custom ID3:			
	Si2124	Si2216	Si2516	Si2881
Einheit	µg/L	µg/L	µg/L	µg/L
Mittel	338,4	343,3	338,6	349,9

6 Test with Chironomids

6.1 Test principle

The sediment-dwelling larvae (first instar) of the freshwater dipteran *Chironomus riparius* were placed in a sediment-water test system with defined artificial sediment. The overlaying water was spiked with the test item at a range of concentrations. The test item was applied once. Chironomid emergence was measured at the end of the test after 28 days of incubation as the endpoint. The test was performed according to the guideline OECD 219. Using appropriate statistical methods it was analysed whether there was a statistically significant difference in emergence rate, development time and rate, and sensitivity of the sexes between the treatment and the control.

6.2 Materials and methods

6.2.1 Test guideline

The test was performed according to:

OECD Guidelines for the Testing of Chemicals Test No. 219: Sediment-Water Chironomid Toxicity Using Spiked Water (2004)

6.2.2 GLP

The test was performed following the principles of GLP. In deviation to GLP the raw data have not been archived, and the quality assurance unit was not involved with respect to the inspection of the test, the raw data and the report. All laboratory equipment (e.g. balances, thermometers, pH-meters) were controlled and documented according to GLP.

6.3 Test substances

- NM-600 (Vessel No 0017 and 0007)

6.4 Analytical monitoring

For the control and for each concentration one additional vessel only used for analytical measurements was applied. These additional vessels were treated as the control vessels and the test vessels used for the assessment of the nanomaterials.

At several times aqueous samples (50 mL) were taken at four different depths (about 2.0 cm; 4.0 cm; 5.5 cm; 6.5 cm). The samples were combined. About 20 mL were used for the analyses. The remaining amount was carefully returned into the test vessels without disturbing the sediment.

Zeta-potential and particle size distribution were measured using a Malvern Zeta-Sizer.

Temperature and pH were measured in the overlaying water at test start / end and during the study in all vessels once a week. Dissolved oxygen was measured at test start and twice a week in one representative vessel per treatment; at test end it was measured in all test vessels. Hardness and ammonia were measured in the controls and in one test vessel at the highest concentration at the start and the end of the study.

Test item concentrations in the overlaying water were determined 3 h after adding the test substance, at day 1, 2, 7, 14 and 28. For details of chemical analyses see chapter 7.

6.4.1 Details on sediment and water

Artificial sediment components

Sphagnum peat, air-dried, finely ground	5%
Kaolinite, air-dried	20%
Industrial quartz sand, air-dried	75%

The test substrate was wetted with deionised water to reach a water content of about 25% to 30%. According to the Guideline a water content of 30% to 50% is recommended. Our experience shows that a lower water content results in a more homogenous distribution of the sediment in the individual vessels. Pulverized calcium carbonate of chemically pure quality ($CaCO_3$) was added to adjust the pH of the final mixture of the sediment to 7.0 ± 0.5. The organic carbon content of the final mixture was 2.2%, which was in the demanded range of 2% ± 0.5%.

Water

Purified tap water was used as test water.

6.4.2 Details on application

The nominal concentrations in the test containers with the test item were 15, 23, 39, 63, and 100 mg test item/L. Four replicates per concentration were conducted.

For each vessel a 500 mL stock dispersion of the nanomaterials in tap water was prepared. For the double concentrated dispersion of the final test concentration the respective amount of nanomaterial was weighted in brown glass vessels using a suitable balance. 500 mL of tap water was added, and the mixture was stirred (magnetic stirrer, 300 rpm) and ultrasonic treatment in a water bath (3 min, 500 W). The stock dispersion was added thoroughly to the water column in the test vessels 24 h after addition of the test specimens. Due to the large amount of stock dispersion, mixing occurred during addition. There was no further mixing to avoid disturbance of the sediment.

6.5 Test organism

The test organisms were the first instar larvae from the dipteran *Chironomus riparius*.

Origin of the midges: Bayer Crop Science AG, 40789 Monheim, Germany. Specimens used in the test were bred in the laboratory of the Fraunhofer IME.

Breeding conditions: On a layer of diatomaceous earth purified tap water was used. The dipterans were fed daily with powder of TetraMin® Hauptfutter (Tetra Werke, Melle, Germany).

Pretreatment: Four to five days before adding the test organisms to the test vessels, egg masses were taken from the cultures and placed in small aerated vessels with test water at about 20 °C. First instar larvae (1 days post hatching) were used in the test. As the larvae were added one day before spiking the age of the larvae was about 2 days at day 0 (day 0 = day of spiking the water phase).

6.6 Study design

6.6.1 Study type

Reproduction, static

6.6.2 Study type

Laboratory study

6.6.3 Test type

Static

6.6.4 Test duration type

Long-term

6.6.5 Water media type

Freshwater

6.6.6 Type of sediment

Artificial sediment

6.6.7 Total exposure duration

The exposure period was 28 days.

- March 07th - April 4th 2011

No post-exposure observation period was performed.

6.6.8 Test conditions

Hardness:	Test start: 130 – 140 mg/L as $CaCO_3$ equivalents (demanded threshold value of 400 mg/L as $CaCO_3$ equivalents) and 130 mg/L as $CaCO_3$ equivalents in one representative replicate of the highest test concentration. Test end: 120 – 130 mg/L as $CaCO_3$ equivalents in the controls and 120 mg/L as $CaCO_3$ equivalents in one representative replicate of the highest test concentration.
Test temperature:	20.3 °C (permitted range: 20 ± 2 °C)
pH:	8.3 – 8.5 (permitted range: pH 6 – 9)
Dissolved oxygen:	About 100% at test start and test end (demanded threshold value: 60%)
Ammonia:	Test start: 1.2 - 1.4 (control); 1.2 (highest test concentration) Test end: 4.0 - 6.0 (control); 0.4 (highest test concentration)
Nominal concentrations:	The nominal concentrations in the test containers with TiO_2 were 1.2, 3.7, 11, 33, and 100 mg test item/L.

Details on test conditions:

The light intensity was measured using an illuminance meter (MINOLTA) with photometric sensor in Lux. With 593 – 679 lx the permitted range of about 500 – 1000 lx was kept.

Reference substance:

According to the guideline a test with a reference substance is not compulsory necessary.
2-Chloracetamid was tested in a sediment-water-chironomid toxicity test using spiked sediment (OECD 218).

6.6.9 Any other information on materials and methods

Control treatment

The control consists of sediment, tap water and chironomids. Four replicates per control were conducted.

Statistical method

Data evaluation:

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

Statistical calculations:

Calculations were performed with the computer software ToxRat Professional version 2.10.4.1 by ToxRat® Solutions GmbH.

Food

Powder of TetraMin® Hauptfutter was used for feeding the larva. According to the guideline, the food ratios for the first 10 days were 0.25 – 0.5 mg TetraMin® / larvae/day, from day 10 on the food ratio was increased up to 0.5 – 1.0 mg TetraMin® /larvae/day.

Test container

Round glass beakers (3L) were used as test vessels. The vessels were filled up to 2 cm height with wet artificial sediment (corresponding to 370 g dry mass). The overlaying water was 8 cm high (ratio sediment : water about 1:4). The containers were covered with glass plates. After 10 days, emergence traps were placed on the test vessels, the glass plates remained on the emergence traps to avoid evaporation. Aeration of overlaying water was provided through a glass pipette fixed 2-3 cm above the sediment layer (at least 1 bubble /second).

Test procedure

Sediment was filled in the test vessels. 400 mL of tap water was added, and the sediment-water system was gently aerated for several days prior to addition of the test organisms. Batches of twenty larvae were placed into each vessel.

After an incubation of 24 h, 500 mL of the freshly prepared stock dispersion of the nanomaterials was added. Further 100 mL of tap water were used to rinse the vessels containing the stock dispersions. To avoid separation of sediment ingredients during the addition of test water and stock dispersion, the surface of the water column was covered with a stainless steel disc while water was poured onto it. The disc was removed immediately afterwards. Due to the large amount of stock dispersion, mixing

occurred during addition. There was no further mixing to avoid disturbance of the sediment.

The test was carried out at 20 °C ± 2 °C and at 16 hours photoperiod (500 –1000 lux). The exposure duration was 28 days. The development time and total number of fully emerged male and female midges were determined. Test vessels were observed daily for visual assessment of abnormal behaviour. Emergence was counted daily. After identification the midges were removed from the test vessel. At test end, the test vessels were observed for visible pupae that had failed to emerge.

6.7 Results

Zeta potential

The Zeta potential and the agglomeration behaviour of NM-600 are presented in Table 46. NM-600 has a negative Zeta potential in the test water.

Table 46: Zeta potential

Sample	Zeta potential [mV]
NM 600 in purified tap water, specification see Table 19 (application dispersion):	-18 mV
Purified tap water	-13 mV

Particle size distribution

At day 1, day 2, day 7 and day 14 particle size distributions were determined with the device Malvern Nano ZS. At day 1 and day 2 differences in the peak position of control and higher test concentrations were observed. At day 7 and day 14, no differences were detected indicating sedimentation of the applied nanoclay. At present, the results cannot be interpreted properly. As we assume that knowledge concerning the measurement and interpretation of suspensions containing nanoparticles and their agglomerates will be increasing, it may be possible that the results obtained in this project can be interpreted retrospectively. In Table 47 measuring results and applied measuring parameters are presented.

Table 47: Particle size distribution in the reproduction test with daphnids with nanoclay

Concentration [mg/L]	Z-Average nm] [1]	PDI [2]	Peak 1 [nm] [6]	Peak 2 [nm] [6]	Count Rate [3] [kcps]	Measurement Position [4]	Attenu-ation [5]
Day 1							
Control	1541	1	244	-			
1.2 mg/L	2703	1	260	-	463	4.65	11
3.7 mg/L	1778	1	491	-	50	4.65	8
11 mg/L	2096	1	429	-	239	4.65	8
33 mg/L	1769	1	568	-	117	4.65	8
100 mg/L	1365	0.6	861	-	189	4.65	8
Day 2							
Control	879	0.8	274 [87%]	66 [13%]	88	4.65	10
11 mg/L	1828	1	453	92	71	4.65	8
100 mg/L	1410	0.6	799	-	146	4.65	7
Day 7							
Control	1673	1	465	-	149	4.65	7
11 mg/L	1177	0.8	455	-	153	4.65	7
100 mg/L	1678	1	422	-	115	4.65	7
Day 14							
Control	1038	0.6	704	-	191	4.65	6
11 mg/L	963	0.6	570	-	161	4.65	6
100 mg/L	999	0.6	658	-	261	4.65	6

[1] calculated value (cumulative mean); [2] increasing value indicates increasing polydispersity (maximum: 1); [3] best results with a count rate between 150 and 500 kilo counts per second (kcps); [4] measurement position in the middle of the measuring cell; [5] indicator for turbidity (high values indicate low turbidity; maximum: 11); [6] 1 mg/L samples below quantification limit; [7] prepared from 10 mg/L samples with 3 min of ultrasonic treatment; [8] prepared from 10 mg/L samples with 30 min of ultrasonic treatment

[6] In the case of more than two peaks, value in brackets gives percentage of the single peak compared to all peaks (prerequisite, the peak exceeds 10%)

Test item concentrations:

In Table 48 the measurement results of nanoclay in aqueous samples from the chironomid test are compiled. A high silica concentration was detected in the controls. This is due to the ubiquitous occurrence of silica (e.g. in dust) but it is also due to the silica sand used for the preparation of the sediment. A high silica concentration was detected in controls. Because these aqueous media already show a high background concentration, the calculated Si recoveries do not represent the recovery of the metal in nanoclay. For a better view and evaluation the mean of the controls per day was added to the nominal value for silica of the respective loading. This sum was set to 100%, and the recovery of the amount of silica in the sample in relation to this sum was determined. For a clear-cut result the amount of Si in the controls have to be less than 50% of the measured values in samples from test item loaded vessels.

For the assessment of the recoveries it has to be taken into account that small differences in control and treated samples result in a high inaccuracy of the results. At day 0, the recovery in the stock dispersions of 77 - 125% was determined. As the content of silica in nanoclay has to be estimated (see chapter 7) and as the difference of control and treated samples was small at least in the two lowest concentrations,

the recovery was considered to be sufficient to use the nominal concentrations for the calculation of the effect concentrations.

During the incubation period sedimentation was observed at least for the higher test concentrations. In the last two measurements (day 14, day 28) for some concentrations increased silica concentrations were determined in the overlaying water. It is assumed that the increase is due to the silica concentration in the sediment which is also distributed into the overlaying water by diffusion and by moving activity of the midges. The difference between the control and the individual concentrations became smaller during the incubation period and followingly the accuracy of the recovery concentration decreases.

Table 48: Measured Si concentrations and recoveries, test with chironomids

Concentration of nanoclay [mg/L]	Mean Si [µg/L]	SD [µg/L]	Si nominal [µg/L]	Mean Si control + Si nominal [µg/L]	Recovery of fortification in relation to measured Si [%]
Day 0 (double concentrated stock suspension)					
control	4631	78	0	---	---
2.4	5535	67	723	5354	103
7.4	6680	93	2230	6861	97.3
22	10754	172	6631	11262	95.5
66	22658	223	19892	24523	92.3
200	51039	1826	60279	64910	78.6
Day 1					
control	6223	160	0	---	---
1.2	7427	510	362	6585	113
3.7	9002	410	1115	7338	123
11	9824	1255	3315	9538	103
33	11015	2471	9946	16169	68.1
100	22215	308	30140	36363	61.1
Day 2					
control	8813	566	0	---	---
1.2	8047	864	362	9175	87.7
3.7	8568	787	1115	9928	86.3
11	7703	474	3315	12126	63.5
33	9275	163	9946	18759	49.4
100	18011	5	30140	38953	46.2
Day 7					
control	10926	223	0	---	---
1.2	12334	372	362	11288	109
3.7	11926	1475	1115	12041	99.0
11	12232	1471	3315	14241	85.9
33	10167	2613	9946	20872	48.7
100	13065	4476	30140	41066	31.8
Day 14					
control	34140	382	0	---	---
1.2	24771	119	362	34502	71.8
3.7	23655	547	1115	35255	67.1
11	20031	246	3315	37455	53.5
33	22193	202	9946	44086	50.3
100	29573	583	30140	64280	46.0
Day 28					
control	11179	249	0	---	---
1.2	10143	212	362	11541	87.9
3.7	12200	735	1115	12294	99.2
11	11342	767	3315	14494	78.3
33	13001	1140	9946	21125	61.5
100	12267	38	30140	41319	29.7

Effects:

Summarized results are presented in Table 49 - Table 51.

No significant effect on emergence and development rate were observed. Statistical differences were observed for the development time, and NOEC and LOEC values could be determined. However, the differences showed no concentration-effect relationship. The highest concentration showed lower effects than the concentration of 33 mg/L. This might be due to higher agglomeration and lower bioavailability in higher test concentrations.

Table 49: NOEC, LOEC and EC_{10} values for the test with chironomids

	NOEC	LOEC	EC_{10}
Emergence rate of males and females	≥ 100	> 100	n.d.
Development rate of males and females	≥ 100	> 100	n.d.
Development rate of males	≥ 100	> 100	n.d.
Development rate of females	≥ 100	> 100	n.d.
Development time of males and females	≥ 100	> 100	n.d.
Development time of males	≥ 100	> 100	n.d.
Development time of females	3.7	11	n.d.

The NOEC was ≥ 100 mg/L and $EC_{10,}$ $EC_{20,}$ and EC_{50} values of the biological endpoints were > 100 mg/L.

Physical/pathological symptoms and changes in behaviour:

Neither physical nor pathological symptoms were obtained. All specimens gave the impression of healthy condition. There was only one dead emerged animal in the second concentration (23 mg/L) at day 18.

Emergence rate:

The results of emergence are presented in Table 50.

No concentration/effect dependency on emergence rate due to nanoclay was detected. The NOEC (no observed effect concentration) for the tested species *Chironomus riparius* was found to be > 100 mg/L for males, females and the combined sexes.

Table 50: Emergence at test end. Emerged midges [Ind.] and emergence rate [% of introduced larvae]; concentrations given as nominal values.

Control	1.2 mg/L	3.7 mg/L [1]	11 mg/L	33 mg/L	100 mg/L
Emerged midges [Ind.]					
73	78	78	74	77	79
Emerged midges [%]					
91.3	97.5	96.2	92.5	96.3	98.8
Emerged midges [males]					
32	42	41	36	41	40
Emergence rate males [%]					
40.0	52.5	50.6	45.0	51.3	50.0
Emerged midges [females]					
41	36	37	38	36	39
Emergence rate females [%]					
51.3	45.0	45.7	47.5	45.0	48.8

[1] 81 (instead of 80) midges introduced in the four vessels

Development time and rate:

The results of development time and rate are presented as mean values (Table 51). No influence on development rate due to nanoclay was detected. Statistical differences were observed for the development time; however, the differences showed no concentration-effect relationship. The highest concentration showed lower effects than the concentration of 33 mg/L. This might be due to higher agglomeration and lower bioavailability at higher test concentrations. But also biological variance as reason for this observation is not completely excluded.

Table 51: Development time and rate. Development time [d] and rate [1/d] of midges; concentrations given as nominal values. * Significant deviation when compared with control (Williams Multiple Sequential t-test, p < 0.05; one-sided).

Control	1.2 mg/L	3.7 mg/L	11 mg/L	33 mg/L	100 mg/L
Development time midges					
18.3	18.0	17.7	17.5	17.0 *	17.3
Development rate midges					
0.056	0.056	0.058	0.058	0.059	0.058
Development time males					
16.5	17.1	16.3	16.2	15.9	16.0
Development rate males					
0.061	0.059	0.062	0.062	0.063	0.063
Development time females					
19.7	19.1	19.2	18.7 *	18.2 *	18.7 *
Development rate females					
0.052	0.053	0.053	0.054	0.055	0.054

6.8 Validity

The test is considered valid since

- the mean emergence in controls was 92% (70% mentioned in the guideline) at test end.
- *C. riparius* development time of most adults in controls was between 16 – 23 days after their insertion into the test vessels, which is between the demanded range of 12 – 23 days. However, one further animal developed at day 24, 25 and 26.
- at the end of the test the dissolved oxygen concentration was at least 60% of the air saturation level at the temperature used, and the pH of overlaying water was in a range of 6 – 9 in all test vessels.
- the water temperature differed not more than ± 1 °C between vessels and was maintained within the temperature range of 20 ± 2°C.

6.9 Conclusion

Nanoclay up to a concentration of 100 mg/L resulted in no negative impact on the emergence of larvae in a spiked water sediment test with chironomids. The NOEC is ≥ 100 mg/L.

6.10 Conclusion

Nanoclay (1.2, 3.7, 11, 33, and 100 mg test item/L) was tested in the chironomid test with spiked water (OECD 219).

Nanoclay up to a concentration of 100 mg/L was considered to result in no negative impact on the emergence of larvae in a spiked water sediment test with chironomids. The NOEC is ≥ 100 mg/L.

6.11 Executive summary

Nanoclay was tested in the chironomid test with spiked water (OECD 219). The nominal concentrations in the test containers were 1.2, 3.7, 11, 33, and 100 mg test item/L.

There was sedimentation of nanclay resulting in low nanoclay concentrations in the overlaying water.

Nanoclay up to a concentration of 100 mg/L was considered to result in no negative impact on the emergence of larvae in a spiked water sediment test with chironomids. The NOEC is ≥ 100 mg/L.

6.12 Details on test data

6.12.1 Physico-chemical test parameters

Table 52: Physico-chemical test parameters

		Test start						Test end					
		O_2 %	Temp °C	pH	TH mmol/l	NH_4 mg/L	Light lux	O_2 %	Temp °C	pH	TH mmol/l	NH_4 mg/L	Light lux
Control	1	98.0	20.3	8.32	1.4	1.4	593 - 609	99.8	20.3	8.48	1.2	6.0	643 - 679
	2	97.0		8.29	1.4	1.3		90.5		8.38	1.3	6.0	
	3	97.1		8.28	1.4	1.5		92.3		8.31	1.3	6.0	
	4	98.5		8.41	1.4	1.4		90.9		8.42	1.2	6.0	
1.2 mg/L	1	99.8		8.44				97.1		8.46			
	2	99.8		8.48				99.6		8.49			
	3	98.0		8.38				99.8		8.56			
	4	99.7		8.45				95.4		8.50			
3.7 mg/L	1	99.9		8.45				99.0		8.56			
	2	99.7		8.44				96.7		8.42			
	3	99.7		8.48				98.0		8.35			
	4	99.9		8.47				99.1		8.51			
11 mg/L	1	99.1		8.48				95.7		8.37			
	2	99.2		8.44				95.8		8.40			
	3	99.7		8.45				90.2		8.30			
	4	99.9		8.45				98.8		8.37			
33 mg/L	1	98.0		8.43				91.3		8.28			
	2	98.1		8.40				98.4		8.37			
	3	99.2		8.40				93.3		8.33			
	4	97.3		8.36				97.5		8.37			
100 mg/L	1	98.2		8.36				93.8		8.36			
	2	98.8		8.38				87.5		8.18			
	3	96.8		8.48	1.3	1.2		91.7		8.36	1.2	0.4	
	4	98.8		8.48				96.3		8.33			
Vessels used for chemical analysis	K	94.0		8.11	1.4	1.4		95.7		8.48	1.2	7.0	
	1	99.2		8.38				99.3		8.43			
	2	98.3		8.36				99.2		8.40			
	3	99.1		8.44				96.7		8.26			
	4	97.5		8.35				92.7		8.25			
	5	99.3		8.52	1.4	0.9		99.4		8.32	1.2	0.8	

Water hardness (TH): 1 mmol corresponds to 100 mg $CaCo_3$ equivalent.

6.12.2 Data concerning test performance and test organism

Table 53: Addition of Food (TetraMin grinded)

		Day -1	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11	Day 12
		mg	mg	mg	mg	mg	mg	mg	mg	mg	mg	mg	mg	mg	mg
Control	1	10	-	5	16	-	24	-	-	20	-	26	-	48	-
	2	10	-	5	16	-	24	-	-	20	-	26	-	48	-
	3	10	-	5	16	-	24	-	-	20	-	26	-	48	-
	4	10	-	5	16	-	24	-	-	20	-	26	-	48	-
1.2 mg/L	1	10	-	5	16	-	24	-	-	20	-	26	-	48	-
	2	10	-	5	16	-	24	-	-	20	-	26	-	48	-
	3	10	-	5	16	-	24	-	-	20	-	26	-	48	-
	4	10	-	5	16	-	24	-	-	20	-	26	-	48	-
3.7 mg/L	1	10	-	5	16	-	24	-	-	20	-	26	-	48	-
	2	10	-	5	16	-	24	-	-	20	-	26	-	48	-
	3	10	-	5	16	-	24	-	-	20	-	26	-	48	-
	4	10	-	5	16	-	24	-	-	20	-	26	-	48	-
11 mg/L	1	10	-	5	16	-	24	-	-	20	-	26	-	48	-
	2	10	-	5	16	-	24	-	-	20	-	26	-	48	-
	3	10	-	5	16	-	24	-	-	20	-	26	-	48	-
	4	10	-	5	16	-	24	-	-	20	-	26	-	48	-
33 mg/L	1	10	-	5	16	-	24	-	-	20	-	26	-	48	-
	2	10	-	5	16	-	24	-	-	20	-	26	-	48	-
	3	10	-	5	16	-	24	-	-	20	-	26	-	48	-
	4	10	-	5	16	-	24	-	-	20	-	26	-	48	-
100 mg/L	1	10	-	5	16	-	24	-	-	20	-	26	-	48	-
	2	10	-	5	16	-	24	-	-	20	-	26	-	48	-
	3	10	-	5	16	-	24	-	-	20	-	26	-	48	-
	4	10	-	5	16	-	24	-	-	20	-	26	-	48	-
Vessels used for chemical analysis	K	10	-	5	16	-	24	-	-	20	-	26	-	48	-
	1	10	-	5	16	-	24	-	-	20	-	26	-	48	-
	2	10	-	5	16	-	24	-	-	20	-	26	-	48	-
	3	10	-	5	16	-	24	-	-	20	-	26	-	48	-
	4	10	-	5	16	-	24	-	-	20	-	26	-	48	-
	5	10	-	5	16	-	24	-	-	20	-	26	-	48	-
Date		6.3.		08.3	09.3		11.3			14.3		16.3		18.3	

Table 53: Addition of Food (TetraMin grinded)

		Day 13	Day 14	Day 15	Day 16	Day 17	Day 18	Day 19	Day 20	Day 21	Day 22	Day 23	Day 24	Day 25	Day 26
		mg	mg	mg	mg	mg	mg	mg	mg	mg	mg	mg	mg	mg	mg
Control	1	-	32	-	25.6	-	30	-	-	10	-	0	-	0	-
	2	-	32	-	25.6	-	30	-	-	4	-	4	-	3	-
	3	-	32	-	28.8	-	30	-	-	10	-	8	-	12	-
	4	-	32	-	24	-	33	-	-	14	-	8	-	9	-
1.2 mg/L	1	-	32	-	27.2	-	33	-	-	6	-	2	-	3	-
	2	-	32	-	24	-	21	-	-	2	-	0	-	0	-
	3	-	32	-	25.6	-	15	-	-	0	-	0	-	0	-
	4	-	32	-	28.8	-	42	-	-	14	-	2	-	3	-
3.7 mg/L	1	-	32	-	24	-	24	-	-	2	-	0	-	0	-
	2	-	32	-	24	-	27	-	-	4	-	2	-	3	-
	3	-	32	-	25.6	-	30	-	-	10	-	2	-	3	-
	4	-	32	-	16	-	21	-	-	4	-	0	-	0	-
11 mg/L	1	-	32	-	27.2	-	27	-	-	8	-	6	-	6	-
	2	-	32	-	24	-	24	-	-	6	-	2	-	3	-
	3	-	32	-	25.6	-	18	-	-	2	-	2	-	3	-
	4	-	32	-	19.2	-	27	-	-	4	-	4	-	6	-
33 mg/L	1	-	32	-	16	-	18	-	-	2	-	0	-	0	-
	2	-	32	-	22.4	-	15	-	-	2	-	0	-	0	-
	3	-	32	-	20.8	-	21	-	-	6	-	6	-	9	-
	4	-	32	-	25.6	-	12	-	-	2	-	0	-	0	-
100 mg/L	1	-	32	-	25.6	-	24	-	-	0	-	0	-	0	-
	2	-	32	-	19.2	-	15	-	-	2	-	2	-	3	-
	3	-	32	-	20.8	-	21	-	-	4	-	2	-	3	-
	4	-	32	-	25.6	-	27	-	-	0	-	0	-	0	-
Vessels used for chemical analysis	K	-	32	-	27.2	-	27	-	-	2	-	0	-	0	-
	1	-	32	-	25.6	-	33	-	-	8	-	0	-	0	-
	2	-	32	-	24	-	21	-	-	4	-	0	-	0	-
	3	-	32	-	25.6	-	27	-	-	12	-	10	-	12	-
	4	-	32	-	27.2	-	39	-	-	2	-	0	-	0	-
	5	-	32	-	27.2	-	27	-	-	4	-	2	-	3	-
Date			21.3		23.3		25.3			28.3		30.3		1.4.	

Table 53: Addition of Food (TetraMin grinded)

		Day 27	Day 28
		mg	mg
Control	1	-	-
	2	4	-
	3	4	-
	4	4	-
1.2 mg/L	1	6	-
	2	-	-
	3	12	-
	4	2	-
3.7 mg/L	1	-	-
	2	8	-
	3	-	-
	4	2	-
11 mg/L	1	6	-
	2	6	-
	3	10	-
	4	20	-
33 mg/L	1	16	-
	2	8	-
	3	8	-
	4	2	-
100 mg/L	1	2	-
	2	2	-
	3	6	-
	4	2	-
Vessels used for chemical analysis	K	8	-
	1	4	-
	2	12	-
	3	10	-
	4	4	-
	5	12	-
Date		23.6	

Table 54: Oxygen concentration [%]

		O_2 %	O_2 %	O_2 %	O_2 %	O_2 %	O_2 %	O_2 %	O_2 %
Control	1					86.8			
	2		94.7					91.1	
	3			90.0					
	4				93.9				
1.2 mg/L	1					80.4			
	2		98.2				97.9		
	3			95.5				98.7	
	4				93.1				95.5
3.7 mg/L	1		97.0				93.4		
	2			92.7				97.2	
	3				96.4				98.7
	4					91.9			
11 mg/L	1				97.3				97.3
	2					90.1			
	3		97.6				93.3		
	4			94.8				98.3	
33 mg/L	1					86.8			
	2		97.3				96.5		
	3			94.0				94.9	
	4				94.1				99.3
100 mg/L	1		95.7				89.9		
	2			92.6				95.3	
	3				95.9				98.2
	4					85.0			
Vessels used for chemical analysis	1		96.8	92.2	91.2	83.9	88.0	95.2	98.6
	2		97.5	95.1	97.8	93.0	97.4	97.1	100.4
	3		97.4	95.6	96.6	87.8	96.4	93.6	98.6
	4		98.7	95.9	98.0	94.2	97.4	96.9	99.0
	5		96.2	93.0	95.7	87.8	93.4	96.0	97.5
Date		8.03.	11.3.	15.3.	18.3.	22.3.	25.3.	29.3.	1.4.

Table 55: Temperature (°C) und pH value

Date		14.03.2011		21.03.2011		28.03.2011	
		Temp °C	pH	Temp °C	pH	Temp °C	pH
Control	1	20.3	8.38	20.3	8.26	20.3	8.14
	2		8.43		8.24		8.26
	3		8.39		8.30		8.03
	4		8.41		8.31		8.03
1.2 mg/L	1		8.39		8.25		8.14
	2		8.48		8.37		8.37
	3		8.49		8.37		8.40
	4		8.41		8.28		8.22
3.7 mg/L	1		8.46		8.34		8.35
	2		8.44		8.33		8.37
	3		8.43		8.28		7.89
	4		8.44		8.34		8.21
11 mg/L	1		8.45		8.32		8.33
	2		8.41		8.32		8.32
	3		8.37		8.30		8.11
	4		8.45		8.34		8.37
33 mg/L	1		8.41		8.30		8.22
	2		8.44		8.33		8.25
	3		8.43		8.33		8.09
	4		8.44		8.24		8.30
100 mg/L	1		8.38		8.27		8.30
	2		8.39		8.27		8.14
	3		8.42		8.31		8.36
	4		8.45		8.32		8.36
Vessels for chemical analysis	1		8.27		8.20		8.22
	2		8.41		8.35		8.45
	3		8.40		8.34		8.44
	4		8.41		8.32		8.37
	5		8.38		8.28		8.40

Table 56: Number of hatched midges and sex

Date		22.3.11		23.3.11		24.3.11		25.3.11		26.3.11		27.3.11		28.3.11	
Day		15		16		17		18		19		20		21	
		♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂
Control	1	-	1	-	3	-	3	1	2	1	1	1	-	2	-
	2	-	2	1	1	-	1	2	3	1	-	1	1	4	1
	3	-	2	-	-	1	3	3	1	3	1	1	-	-	-
	4	-	1	1	3	-	1	3	-	1	-	1	1	1	-
1.2 mg/L	1	-	1	-	2	2	1	1	2	1	1	3	2	1	-
	2	-	1	-	4	2	4	-	2	2	-	2	-	2	-
	3	-	-	-	4	2	5	1	3	2	2	1	-	-	-
	4	-	-	-	2	-	1	2	1	2	-	1	2	1	1
3.7 mg/L	1	-	1	-	4	2	2	-	3	1	1	2	-	2	1
	2	-	3	1	1	-	2	2	2	3	-	3	-	1	-
	3	-	1	-	3	1	2	2	1	2	2	-	-	1	-
	4	-	2	-	8	1	-	2	-	1	1	1	1	1	-
11 mg/L	1	-	1	-	2	2	-	6	-	2	1	1	-	1	-
	2	-	1	-	4	1	5	-	1	1	1	3	-	-	-
	3	-	1	-	3	1	4	4	1	1	-	3	1	-	-
	4	-	1	-	7	1	1	1	-	3	-	3	-	1*	-
33 mg/L	1	-	1	-	9	1	2	1	-	2	-	1	-	1	1
	2	-	2	-	4	-	4	5	-	3	-	1	-	-	-
	3	-	2	-	5	1	1	4	-	3	-	1	-	-	-
	4	-	1	-	3	2	3	5	2	2	-	1	-	-	-
100 mg/L	1	-	2	-	2	-	4	4	-	4	-	1	-	3	-
	2	-	2	-	6	-	4	2	1	1	-	3	-	-	-
	3	-	1	-	6	2	2	2	-	1	-	1	1	1	1
	4	-	-	-	4	1	3	2	1	7	-	1	-	1	-
Vessels for chemical analysis	K	-	3	-	-	-	4	4	-	4	-	2	-	2	-
	1	-	4	-	-	-	2	3	-	6	-	-	-	1	-
	2	-	1	-	4	3	2	1	2	1	2	-	-	2	-
	3	-	1	-	3	1	4	2	-	1	-	2	-	-	-
	4	-	1	-	2	-	3	1	-	10	-	1	-	1	-
	5	-	2	-	1	-	3	5	-	3	1	-	-	3	-

Table 56: Number of hatched midges and sex

Date		29.3.11		30.3.11		31.3.11		1.4.11		2.4.11		3.4.11		4.4.11	
		22		23		24		25		26		27		28	
		♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂
Control	1	3	-	2	-	-	-	-	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	1	-	-	-	-	-	-	-
	3	-	-	1	-	-	-	-	-	-	-	-	-	-	-
	4	3	-	-	-	1	-	-	-	1	-	-	-	-	-
1.2 mg/L	1	-	-	2	-	-	-	-	-	-	-	-	-	-	-
	2	1	-	-	-	-	-	-	-	-	-	-	-	-	-
	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	4	4	-	1	1	-	-	-	-	-	-	-	-	-	-
3.7 mg/L	1	1	-	1	-	-	-	-	-	-	-	-	-	-	-
	2	1	-	-	-	-	-	-	-	-	-	-	-	-	-
	3	3*	-	-	-	-	-	-	-	-	-	-	-	-	-
	4	1	-	1	-	-	-	-	-	-	-	-	-	-	-
11 mg/L	1	1	-	-	-	1	-	-	-	-	-	-	-	-	-
	2	1	-	-	1	-	-	-	-	-	-	-	-	-	-
	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	4	-	-	-	-	-	-	-	-	-	-	-	-	-	-
33 mg/L	1	1	-	-	-	-	-	-	-	-	-	-	-	-	-
	2	-	1	-	-	-	-	-	-	-	-	-	-	-	-
	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	4	1	-	-	-	-	-	-	-	-	-	-	-	-	-
100 mg/L	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	3	1	-	-	-	1	-	-	-	-	-	-	-	-	-
	4	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Vessels for chemical analysis	K	1	-	-	-	-	-	-	-	-	-	-	-	-	-
	1	2	-	2	-	-	-	-	-	-	-	-	-	-	-
	2	-	-	2	-	-	-	-	-	-	-	-	-	-	-
	3	1	-	-	-	1	-	-	-	-	-	-	-	-	-
	4	1	-	-	-	-	-	-	-	-	-	-	-	-	-
	5	1	-	-	-	-	-	-	-	-	-	-	-	-	-

6.12.3 Data on chemical analyses

LODs/LOQs, correlation

The information about the LOD/LOQ and correlation coefficient are compiled in Table 57.

A representative calibration line is shown below.

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

The resulting values are reported in Table 57.

Table 57: LODs/LOQs, correlation

Measurement date, description	LOD [µg/L]	LOQ [µg/L]	Correlation coefficient r
May 19, 2011; samples from day 1, 2, 7	42	139*	0.9993
May 24, 2011; samples from day 0, 14, 28	24	79*	0.9993

* Internal LOQ calculation was performed with more digits.

Instrumental and analytical set-up of the ICP-OES:
Thermo IRIS Intrepid II
Thermo Electron Corporation, Germany
Analytical conditions
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
Wavelengths: 288.158 nm

Quality assurance measurements

According to the quality assurance requirement, the silica recovery in recalibration standards was in the range of ± 15% of the certified value. However, regarding Si concentrations measured by ICP-OES, the mean recovery was 93.9 ± 9.4% (n = 8) for 250 µg/L.

Unfortunately, a reference water with a certified amount of Si is not commercially available.

Exact amounts of the nanoclay test item (104 mg/L and 55 mg/L, appropriately diluted to fit in the concentration range of the samples) was introduced into ultrapure water.

Samples were taken, digested, appropriately diluted (dilution factor of 5 for 104 mg/L and 3 for 55 mg/L, respectively) to fit in the concentration range of the test samples and finally analysed. The amount of Si in nanoclay was calculated using an EDX analysis provided by NIA (NPL report). As energy dispersive X-ray spectroscopy only gives information about a specific surface area the values have to be considered carefully. Therefore, the quality assurance requirement for the recovery of Si in nanoclay was set to 100 ± 30. The recovery of silica in nanoclay was determined to 122 ± 15% for 104 mg/L and 104 ± .44 for 55 mg/L.

Silica concentrations (3.9 – 70 µg/L) in digested ultrapure water blanks (in total n =13) were below LOQ (< 79 – 139 µg/L), except for two of these which exhibited measured silica concentrations of 544 µg/L and 567 µg/L. This may be due to a potential contamination with dust.

Reagent blanks (0.2% KOH) were additionally analysed. The measured values were always below the LOD of the respective measurement series (< 24 - < 42 µg/L).

Analytical results

A high silica concentration was detected in controls. To calculate the recoveries the background concentrations were subtracted for the concentrations measured in the treated samples.

Table 58: Measured Si concentrations and recoveries, chironomid test d0

Day 0								
Sample name	Measured Si [µg/L]	Dilution factor	Measured Si. * dilution [µg/L]	Mean Si [µg/L]	SD [µg/L]	Si nominal [µg/L]	Mean Si control + Si nominal [µg/L]	Recovery of fortification in relation to measured Si [%]
control d0 A	312	15	4686	4631	78	0	-	-
control d0 B	305	15	4575					
2.4 mg/L d0 A	366	15	5489	5535	67	723	5354	103
2.4 mg/L d0 B	372	15	5582					
7.4 mg/L d0 A	450	15	6746	6680	93	2230	6861	97.3
7.4 mg/L d0 B	441	15	6614					
22 mg/L d0 A	725	15	10875	10754	172	6631	11262	95.5
22 mg/L d0 B	709	15	10632					
66 mg/L d0 A	304	75	22815	22658	223	19892	24523	92.3
66 mg/L d0 B	300	75	22500					
200 mg/L d0 A	634	82.5	52330	51039	1826	60279	64910	78.6
200 mg/L d0 B	603	82.5	49748					

Table 59: Measured Si concentrations and recoveries, chironomid test d1

Day 1								
Sample name	Measured Si [µg/L]	Dilution factor	Measured Si. * dilution [µg/L]	Mean Si [µg/L]	SD [µg/L]	Si nominal [µg/L]	Measured Si corrected by background concentration [µg/L]	Recovery of Si [%]
control d1 A	407	15	6110	6223	160	0	-	-
control d1 B	422	15	6336					
1.2 mg/L d1 A	471	15	7067	7427	510	362	6585	113
1.2 mg/L d1 B	519	15	7788					
3.7 mg/L d1 A	581	15	8712	9002	410	1115	7338	123
3.7 mg/L d1 B	620	15	9293					
11 mg/L d1 A	714	15	10712	9824	1255	3315	9538	103
11 mg/L d1 B	596	15	8937					
33 mg/L d1 A	425	30	12762	11015	2471	9946	16169	68.1
33 mg/L d1 B	309	30	9267					
100 mg/L d1 A	299	75	22433	22215	308	30140	36363	61.1
100 mg/L d1 B	293	75	21998					

Table 60: Measured Si concentrations and recoveries, chironomid test d2

Day 2								
Sample name	Measured Si [µg/L]	Dilution factor	Measured Si. * dilution [µg/L]	Mean Si [µg/L]	SD [µg/L]	Si nominal [µg/L]	Measured Si corrected by background concentration [µg/L]	Recovery of Si [%]
control d2 A	561	15	8412	8813	566	0	-	-
control d2 B	614	15	9213					
1.2 mg/L d2 A	577	15	8658	8047	864	362	9175	87.7
1.2 mg/L d2 B	496	15	7436					
3.7 mg/L d2 A	608	15	9125	8568	787	1115	9928	86.3
3.7 mg/L d2 B	534	15	8012					
11 mg/L d2 A	491	15	7368	7703	474	3315	12126	63.5
11 mg/L d2 B	536	15	8039					
33 mg/L d2 A	313	30	9390	9275	163	9946	18759	49.4
33 mg/L d2 B	305	30	9159					
100 mg/L d2 A	240	75	18015	18011	5	30140	38953	46.2
100 mg/L d2 B	240	75	18008					

Table 61: Measured Si concentrations and recoveries, chironomid test d7

Day 7									
Sample name	Measured Si [µg/L]	Dilution factor	Measured Si. * dilution [µg/L]	Mean Si [µg/L]	SD [µg/L]	Si nominal [µg/L]	Si recovery [%]	Measured Si corrected by background concentration [µg/L]	Recovery of Si [%]
control d7 A	718	15	10769	10926	223	0	-	-	-
control d7 B	739	15	11084						
1.2 mg/L d7 A	805	15	12071	12334	372	362	3407	11288	109
1.2 mg/L d7 B	840	15	12597						
3.7 mg/L d7 A	726	15	10883	11926	1475	1115	1070	12041	99.0
3.7 mg/L d7 B	865	15	12969						
11 mg/L d7 A	746	15	11192	12232	1471	3315	369	14241	85.9
11 mg/L d7 B	885	15	13272						
33 mg/L d7 A	277	30	8319	10167	2613	9946	102	20872	48.7
33 mg/L d7 B	401	30	12015						
100 mg/L d7 A	216	75	16230	13065	4476	30140	43.3	41066	31.8
100 mg/L d7 B	132	75	9900						

Table 62: Measured Si concentrations and recoveries, chironomid test d14

Day 14									
Sample name	Measured Si [µg/L]	Dilution factor	Measured Si. * dilution [µg/L]	Mean Si [µg/L]	SD [µg/L]	Si nominal [µg/L]	Si recovery [%]	Measured Si corrected by background concentration [µg/L]	Recovery of Si [%]
control d14 A	1129	30	33870	34140	382	0	-	-	-
control d14 B	1147	30	34410						
1.2 mg/L d14 A	829	30	24855	24771	119	362	6843	34502	71.8
1.2 mg/L d14 B	823	30	24687						
3.7 mg/L d14 A	801	30	24042	23655	547	1115	2122	35255	67.1
3.7 mg/L d14 B	776	30	23268						
11 mg/L d14 A	662	30	19857	20031	246	3315	604	37455	53.5
11 mg/L d14 B	674	30	20205						
33 mg/L d14 A	298	75	22335	22193	202	9946	223	44086	50.3
33 mg/L d14 B	294	75	22050						
100 mg/L d14 A	194	150	29160	29573	583	30140	98.1	64280	46.0
100 mg/L d14 B	200	150	29985						

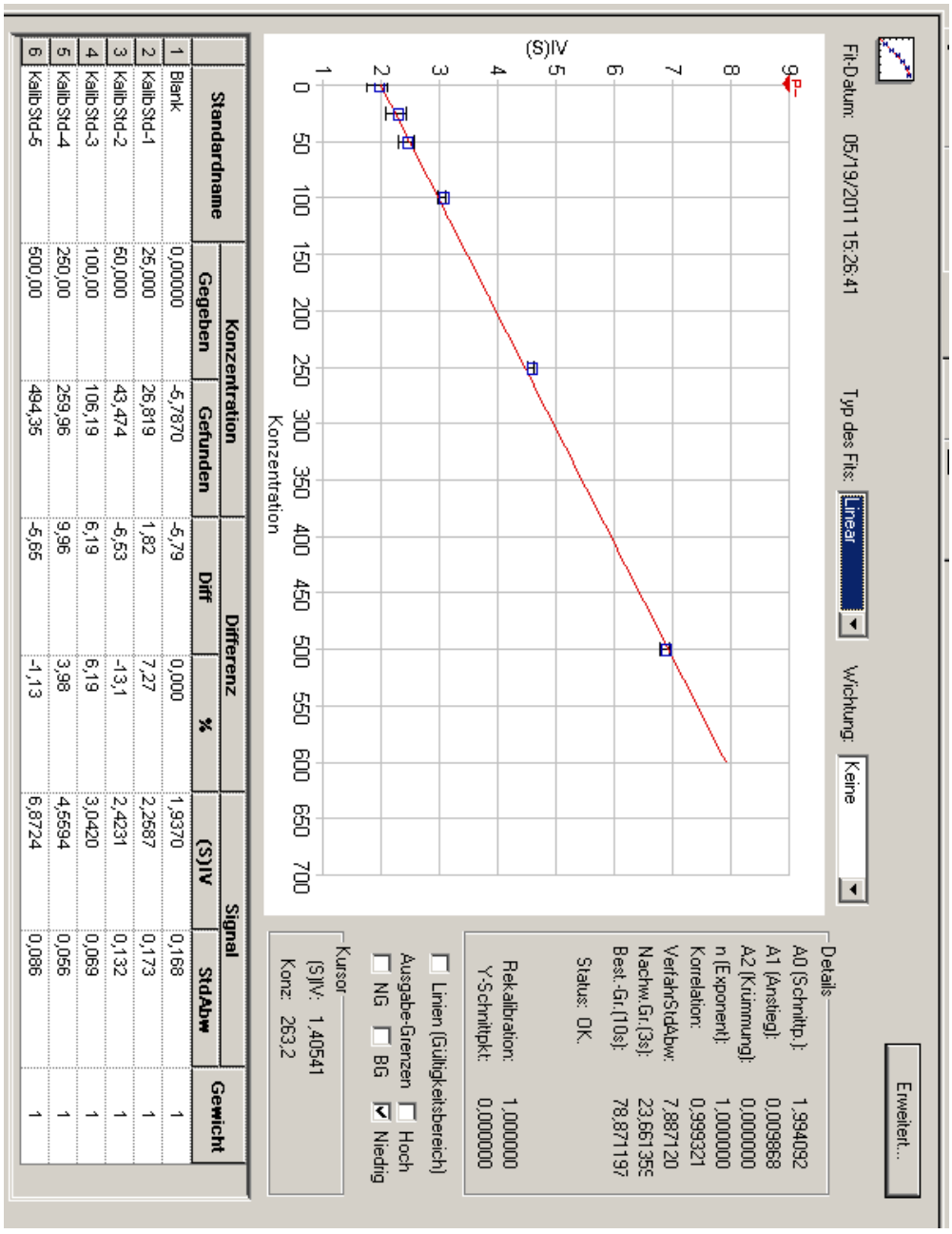
Table 63: Measured Si concentrations and recoveries, chironomid test d28

Day 28									
Sample name	Measured Si [µg/L]	Dilution factor	Measured Si. * dilution [µg/L]	Mean Si [µg/L]	SD [µg/L]	Si nominal [µg/L]	Si recovery [%]	Measured Si corrected by background concentration [µg/L]	Recovery of Si [%]
control d28 A	734	15	11003	11179	249	0	-	-	-
control d28 B	757	15	11355						
1.2 mg/L d28 A	686	15	10293	10143	212	362	2802	11541	87.9
1.2 mg/L d28 B	666	15	9993						
3.7 mg/L d28 A	848	15	12720	12200	735	1115	1094	12294	99.2
3.7 mg/L d28 B	779	15	11681						
11 mg/L d28 A	792	15	11885	11342	767	3315	342	14494	78.3
11 mg/L d28 B	720	15	10800						
33 mg/L d28 A	184	75	13808	13001	1140	9946	131	21125	61.5
33 mg/L d28 B	163	75	12195						
100 mg/L d28 A	82	150	12294	12267	38	30140	40.7	41319	29.7
100 mg/L d28 B	82	150	12240						

Raw data examples

Example for ICP-OES calibration

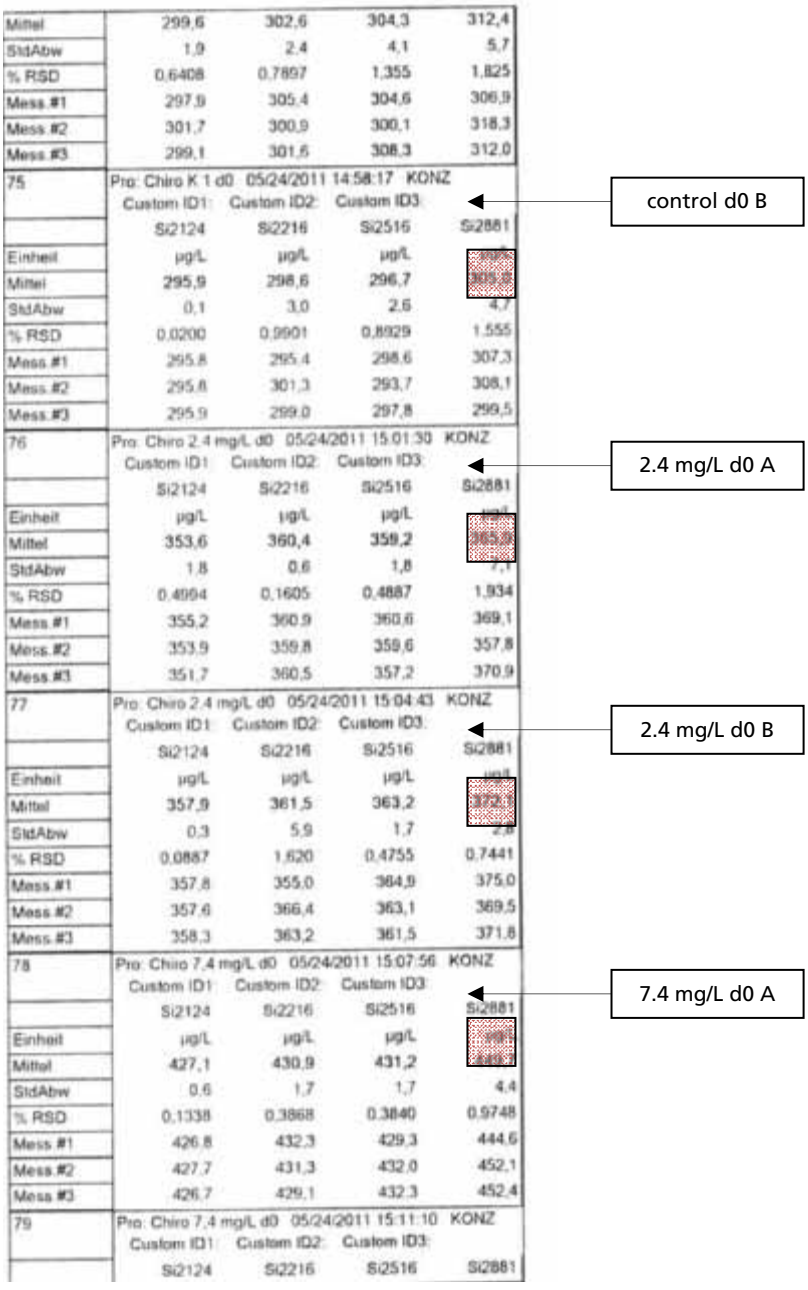
Calibration data from the measurement performed on May 24, 2011 (Wavelength 288.158 nm).



	Standardname	Konzentration		Differenz		Signal		Gewicht
		Gegeben	Gefunden	Diff	%	(S)IV	StdAbw	
1	Blank	0,00000	-5,7870	-5,79	0,000	1,9370	0,168	1
2	KalibStd-1	25,000	26,819	1,82	7,27	2,2587	0,173	1
3	KalibStd-2	50,000	43,474	-6,53	-13,1	2,4231	0,132	1
4	KalibStd-3	100,00	106,19	6,19	6,19	3,0420	0,069	1
5	KalibStd-4	250,00	259,96	9,96	3,98	4,5594	0,056	1
6	KalibStd-5	500,00	494,35	-5,65	-1,13	6,8724	0,086	1

Example for ICP-OES raw data printout

Example printout from the measurement performed on May 24, 2011. The measurement values used for evaluation are marked.



Mittel	299,6	302,6	304,3	312,4
StdAbw	1,9	2,4	4,1	5,7
% RSD	0,6408	0,7897	1,355	1,825
Mess.#1	297,9	305,4	304,6	306,9
Mess.#2	301,7	300,9	300,1	318,3
Mess.#3	299,1	301,6	308,3	312,0
75	Pro: Chiro K 1 d0 05/24/2011 14:58:17 KONZ			
	Custom ID1:	Custom ID2:	Custom ID3:	
	Si2124	Si2216	Si2516	Si2881
Einheit	µg/L	µg/L	µg/L	
Mittel	295,9	298,6	296,7	
StdAbw	0,1	3,0	2,6	4,7
% RSD	0,0200	0,9901	0,8929	1,555
Mess.#1	295,8	295,4	298,6	307,3
Mess.#2	295,8	301,3	293,7	308,1
Mess.#3	295,9	299,0	297,8	299,5
76	Pro: Chiro 2,4 mg/L d0 05/24/2011 15:01:30 KONZ			
	Custom ID1:	Custom ID2:	Custom ID3:	
	Si2124	Si2216	Si2516	Si2881
Einheit	µg/L	µg/L	µg/L	
Mittel	353,6	360,4	359,2	
StdAbw	1,8	0,6	1,8	7,1
% RSD	0,4994	0,1605	0,4887	1,934
Mess.#1	355,2	360,9	360,6	369,1
Mess.#2	353,9	359,8	359,6	357,8
Mess.#3	351,7	360,5	357,2	370,9
77	Pro: Chiro 2,4 mg/L d0 05/24/2011 15:04:43 KONZ			
	Custom ID1:	Custom ID2:	Custom ID3:	
	Si2124	Si2216	Si2516	Si2881
Einheit	µg/L	µg/L	µg/L	
Mittel	357,9	361,5	363,2	
StdAbw	0,3	5,9	1,7	2,9
% RSD	0,0887	1,620	0,4755	0,7441
Mess.#1	357,8	355,0	364,9	375,0
Mess.#2	357,6	366,4	363,1	369,5
Mess.#3	358,3	363,2	361,5	371,8
78	Pro: Chiro 7,4 mg/L d0 05/24/2011 15:07:56 KONZ			
	Custom ID1:	Custom ID2:	Custom ID3:	
	Si2124	Si2216	Si2516	Si2881
Einheit	µg/L	µg/L	µg/L	
Mittel	427,1	430,9	431,2	
StdAbw	0,6	1,7	1,7	4,4
% RSD	0,1338	0,3868	0,3840	0,9748
Mess.#1	426,8	432,3	429,3	444,6
Mess.#2	427,7	431,3	432,0	452,1
Mess.#3	426,7	429,1	432,3	452,4
79	Pro: Chiro 7,4 mg/L d0 05/24/2011 15:11:10 KONZ			
	Custom ID1:	Custom ID2:	Custom ID3:	
	Si2124	Si2216	Si2516	Si2881

control d0 B

2.4 mg/L d0 A

2.4 mg/L d0 B

7.4 mg/L d0 A

7 Details on chemical analyses

Digestion and quantification of nano SiO_2 (nanoclay) in aqueous samples

7.1 Abbreviations and definitions

CoA	Certificate of Analysis
conc.	Concentration
CRM	certified reference material
d	days(s)
ICP-OES	inductively coupled plasma-optical emission spectrometry
LOD	limit of detection
LOQ	limit of quantification
EDX	energy dispersive X-ray spectroscopy

Limit of detection (LOD): The limit of detection is the lowest quantity of a substance in a sample that can be detected reliably. It can be calculated using the method standard deviation determined from the calibration line (LOD = 3 * method standard deviation). Measurements in this range are only qualitative as the uncertainty is too high to quantify concentrations.

Limit of quantification (LOQ): The limit of quantification is the quantity of a substance in a sample that can be measured reliably. It can be calculated using the method standard deviation determined from the calibration line (LOQ = 10 * method standard deviation). At concentrations that are below the limit of quantification (and above the limit of detection), the presence of a substance can be detected but not quantified.

Remarks on data processing: Measurements and results were reported with 2 - 3 digits. However, calculations with the data were performed with more digits (e.g. in Microsoft Excel-files).

7.2 Procedure

7.2.1 Digestion of aqueous samples

The samples were deep-frozen until digestion. Prior to digestion the samples were thoroughly shaken (vortexer). The digestion procedure of nanoclay containing samples was developed and finally potassium hydroxide was used: 0.1 mL of 30% KOH solution was added to 1 mL of the sample, and the mixture was transferred into microwave tubes consisting of Teflon® to avoid potential dissolving of silica from quartz glass tubes. After digesting by microwave irradiation (Ultra Clave II, MLS, Leutkirch, Germany) the samples were brought to a volume of 15 mL with Ultra Pure Water (Pure Lab Ultra water purification system, purified water resistivity >18 MΩ·cm) and the silica concentration was determined by ICP-OES with a matrix adjusted calibration.

The following microwave digestion method was applied:

Step 1: 25 min heating to 220 °C
Step 2: 220 °C remained for 30 min

7.3 Analytical measurement

7.3.1 Reagents for silica analysis

Potassium hydroxide was of “Emsure” quality (supplied by VWR, Darmstadt). The water used was purified using a Pure Lab Ultra water purification system (purified water resistivity >18 MΩ·cm).

A commercially available silica ICP-standard containing 1000 mg/L Si in sodium hydroxide solution 2% (lot no. HC074649) was used to prepare appropriate stock solutions and respective calibration solutions. All prepared standard solutions had a final base concentration of 2%.

7.3.2 Materials verifying the method

An exact amount of the nanoclay test item was introduced into ultrapure water, and samples were taken for digestion and analysis. The obtained recoveries in the measurement series verified the method.

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

The amounts of silica in digested reagent blanks were additionally determined.

7.3.3 Laboratory equipment

All materials used for sample treatment were suitable for analyses of silica. It is inevitable not to use glassware due to potential dissolving of silica from glass. Therefore, polypropylene and Teflon® tubes were applied. The pipettes used (disposable polypropylene tips) were adjustable to variable volumes (50 - 250 µL, 200 - 1000 µL, 1000 - 5000 µL) and were purchased from Gilson (Abimed, Langenfeld, Germany) and Eppendorf (Wesseling, Germany).

7.3.4 ICP-OES

Silicium concentrations of aqueous samples were measured using an IRIS Intrepid II ICP-OES (Thermo Electron, Dreieich, Germany). Silicium was detected at the wavelength of 288.158 nm. Calibrations were performed before each measurement. Depending on concentration range in samples the following calibration solutions were used: blank, 25 µg/L, 50 µg/L, 100 µg/L, 250 µg/L, and 500 µg/L. The calibration formulas were calculated using the linear regression algorithm of the ICP-OES instrument software. Correlation coefficients (r) were at least 0.9990. For each sample, at least three internal measurements were performed, and the mean was calculated and printed by the instrument software. Two measurement series were performed.

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

7.3.5 Certificate of silica ICP standard

Eingang 26.03.11/14

MERCK

Certificate of Analysis
CertiPUR® Reference Material

Silicon ICP Standard 1000 mg/l Si CertiPUR®

1.70365.0100 **Lot No.: HC074649**

This Certificate of Analysis is based on the data from the Merck Calibration Laboratory for ICP-OES, according to DIN EN ISO / IEC 17025.
Accredited by the DKD (Deutscher Kalibrierdienst).

DAR Reg.-No.: DKD-K-14302
Ref. Calibration Certificate: 422/DKD-K-14302/10-04

Composition: Silicon oxide in sodium hydroxide solution® 2%

Assay: **979 mg/kg** **Analysis: ICP-OES**
1000 mg/l (calculated)

Measurement Uncertainty: **± 5 mg/kg (± 0.5%)**
This value represents the expanded uncertainty (*U*) for a coverage probability of 95%. Refer to page 2 for further details.

Traceability: This ICP Standard has been measured applying high precision ICP-OES in comparison to the corresponding **NIST SRM® 3150, lot 071204**

Trace impurities µg/ml:

Ag	<0.02	Cr	<0.05	In	<0.02	Ni	<0.02	Sb	<0.02	Tl	<0.02
Al	<0.05	Cu	<0.02	Ir	<0.02	Os	<0.20	Sc	<0.05	Tm	<0.02
As	<0.20	Dy	<0.02	K	**<2.50**	P	<0.20	Se	<0.20	U	<0.02
Au	<0.02	Er	<0.02	La	<0.02	Pb	<0.05	Si	+	V	<0.02
B	<0.05	Eu	<0.02	Li	<0.02	Pd	<0.02	Sm	<0.02	W	<0.05
Ba	<0.05	Fe	<0.10	Lu	<0.02	Pr	<0.02	Sn	<0.02	Y	<0.02
Be	<0.02	Ga	<0.02	Mg	<0.02	Pt	<0.02	Sr	<0.10	Yb	<0.02
Bi	<0.20	Gd	<0.02	Mn	<0.02	Rb	<0.02	Ta	<0.05	Zn	<0.02
Ca	<0.05	Ge	<0.02	Mo	<0.02	Re	<0.02	Tb	<0.02	Zr	<0.02
Cd	<0.02	Hf	<0.02	Na	**Matrix**	Rh	<0.02	Te	<0.20		
Ce	<0.02	Hg	<0.02	Nb	<0.05	Ru	<0.02	Th	<0.02		
Co	<0.02	Ho	<0.02	Nd	<0.02	S	<0.20	Ti	<0.05		

Date of release: 2010-04-08

Minimum shelf life: 2013-04-30

A. Yildirim

Dipl.-Ing Ayfer Yildirim
(responsible laboratory manager quality control)

7.3.6 Results from EDX analysis and calculation sheet for the amount of Si

Table 1. **Elemental composition (atomic%) according to EDX analysis of NM-600 (0153) Bentonite by stoichiometry (oxygen) of sample that was: a) applied as a thin layer on to an SEM stub b) packed into a specially adapted SEM stub. Table shows the average value (and corresponding 1 SD) from 12 replicate spectra.**

a)

	Composition / atomic%								
	Na	Mg	Al	Si	S	Ca	Fe	O	Sum
Average	2.85	1.29	8.61	22.25	0.27	0.29	1.61	62.84	100.00
SD	0.38	0.10	0.22	0.37	0.21	0.10	0.31	0.15	

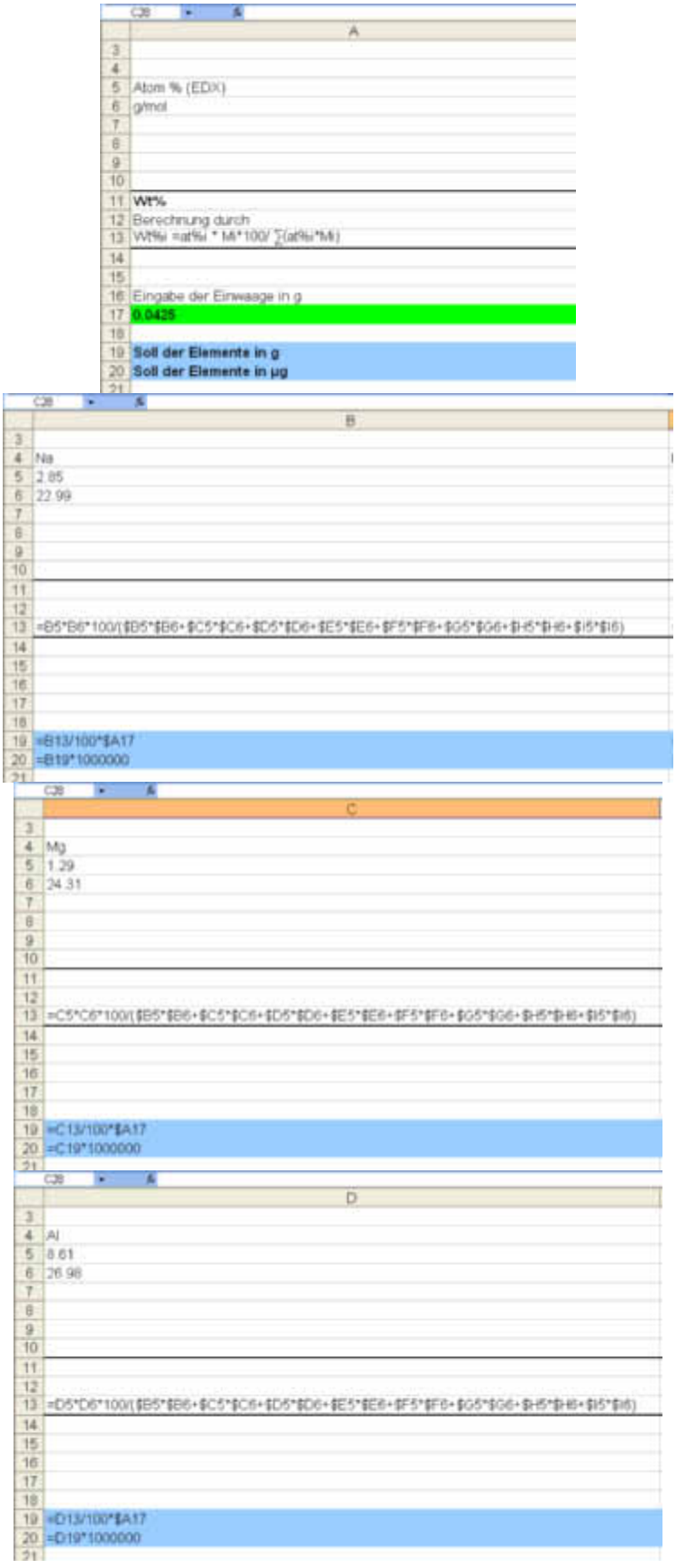
b)

	Composition / atomic%								
	Na	Mg	Al	Si	S	Ca	Fe	O	Sum
Average	2.79	1.30	8.51	22.16	0.39	0.37	1.57	62.90	100.00
SD	0.51	0.11	0.40	0.70	0.39	0.20	0.42	0.21	

Reference: Contract NN148 Version 2 Page 6 of 30
Checked by: S. J. S.

	A	B	C	D	E	F	G	H	I	J	K
1											
2	**Berechnung anhand EDX Analy:**	**Vorsicht, da nur semiquantitativ**			**Grünes Feld ausfüllen**						
3											
4		Na	Mg	Al	Si	S	Ca	Fe	O	**Prüfsumme**	
5	Atom % (EDX)	2.82	1.295	8.56	22.205	0.33	0.33	1.59	62.87	**100**	***richtig***
6	g/mol	22.99	24.31	26.98	28.09	32.07	40.08	55.85	15.9994		
7											
8											
9											
10											
11	**Wt%**										
12	Berechnung durch										
13	Wt%i =at%i * Mi*100/ ∑(at%i*Mi)	3.13273713	1.52121501	11.159676	30.139863	0.51138593	0.63911282	4.2909769	48.6052333	**100**	***richtig***
14											
15											
16	Eingabe der Einwaage in g										
17	**0.0425**										
18											
19	**Soll der Elemente in g**	0.00133141	0.00064652	0.00474286	0.01280936	0.00021734	0.00027162	0.00182367	0.02065722	**0.0425**	***richtig***
20	**Soll der Elemente in µg**	1331.41328	646.51638	4742.86229	12809.3568	217.33902	271.622948	1823.66518	20657.2241	**42500**	***richtig***

Values taken from EDX printout

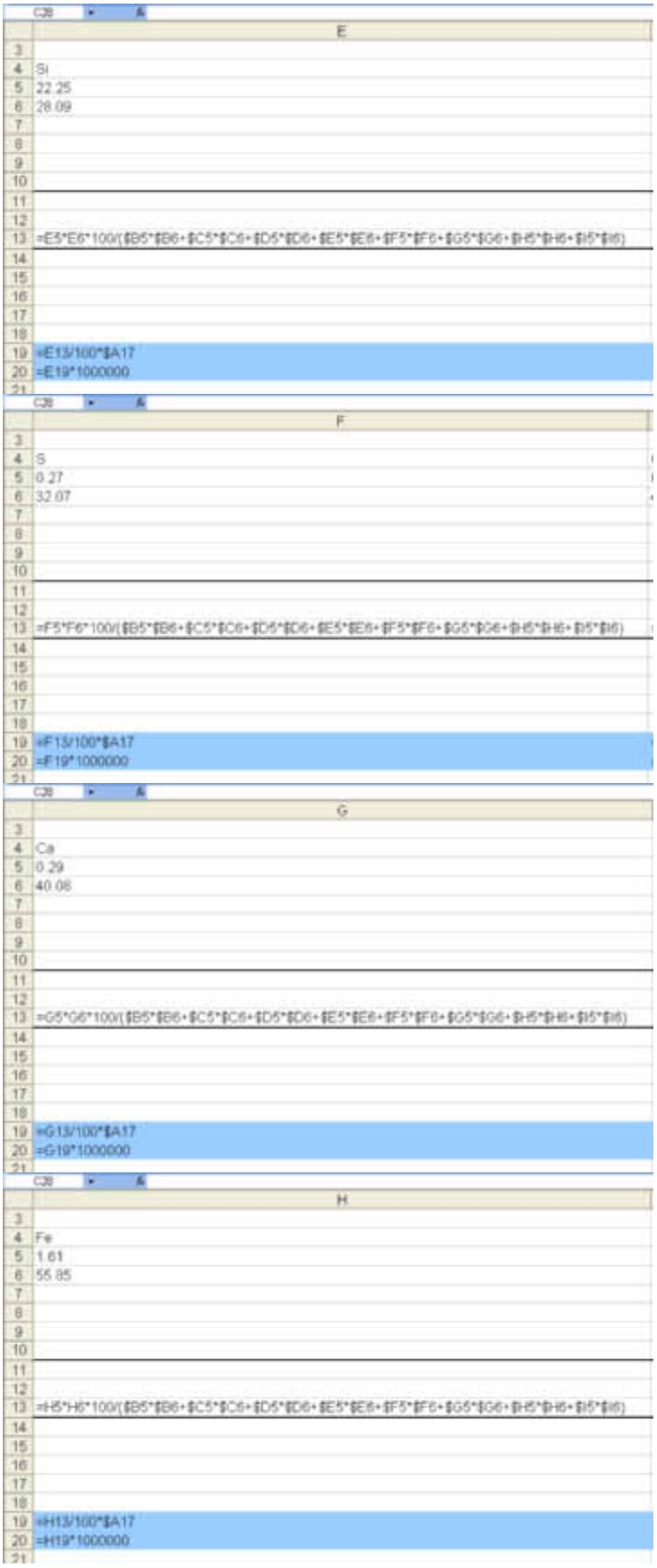


	A
3	
4	
5	Atom % (EDX)
6	g/mol
7	
8	
9	
10	
11	**Wt%**
12	Berechnung durch
13	Wt% =at% * M*100/ $\sum$(at%i*Mi)
14	
15	
16	Eingabe der Einwaage in g
17	**0.0425**
18	
19	**Soll der Elemente in g**
20	**Soll der Elemente in µg**
21	

	B
3	
4	Na
5	2.85
6	22.99
7	
8	
9	
10	
11	
12	
13	=B5*B6*100/($B5*$B6+$C5*$C6+$D5*$D6+$E5*$E6+$F5*$F6+$G5*$G6+$H5*$H6+$I5*$I6)
14	
15	
16	
17	
18	
19	=B13/100*$A17
20	=B19*1000000
21	

	C
3	
4	Mg
5	1.29
6	24.31
7	
8	
9	
10	
11	
12	
13	=C5*C6*100/($B5*$B6+$C5*$C6+$D5*$D6+$E5*$E6+$F5*$F6+$G5*$G6+$H5*$H6+$I5*$I6)
14	
15	
16	
17	
18	
19	=C13/100*$A17
20	=C19*1000000
21	

	D
3	
4	Al
5	8.61
6	26.98
7	
8	
9	
10	
11	
12	
13	=D5*D6*100/($B5*$B6+$C5*$C6+$D5*$D6+$E5*$E6+$F5*$F6+$G5*$G6+$H5*$H6+$I5*$I6)
14	
15	
16	
17	
18	
19	=D13/100*$A17
20	=D19*1000000
21	



C28 A

E

3	
4	Si
5	22.25
6	28.09
7	
8	
9	
10	
11	
12	
13	=E5*E6*100/($B5*$B6+$C5*$C6+$D5*$D6+$E5*$E6+$F5*$F6+$G5*$G6+$H5*$H6+$I5*$I6)
14	
15	
16	
17	
18	
19	=E13/100*$A17
20	=E19*1000000
21	

C28 A

F

3	
4	S
5	0.27
6	32.07
7	
8	
9	
10	
11	
12	
13	=F5*F6*100/($B5*$B6+$C5*$C6+$D5*$D6+$E5*$E6+$F5*$F6+$G5*$G6+$H5*$H6+$I5*$I6)
14	
15	
16	
17	
18	
19	=F13/100*$A17
20	=F19*1000000
21	

C28 A

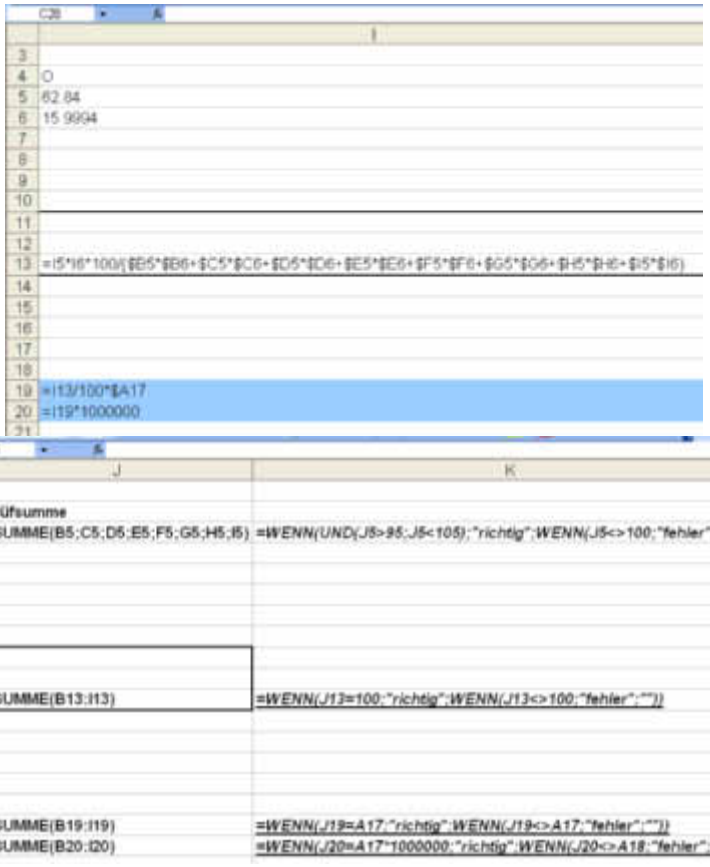
G

3	
4	Ca
5	0.29
6	40.08
7	
8	
9	
10	
11	
12	
13	=G5*G6*100/($B5*$B6+$C5*$C6+$D5*$D6+$E5*$E6+$F5*$F6+$G5*$G6+$H5*$H6+$I5*$I6)
14	
15	
16	
17	
18	
19	=G13/100*$A17
20	=G19*1000000
21	

C28 A

H

3	
4	Fe
5	1.61
6	55.85
7	
8	
9	
10	
11	
12	
13	=H5*H6*100/($B5*$B6+$C5*$C6+$D5*$D6+$E5*$E6+$F5*$F6+$G5*$G6+$H5*$H6+$I5*$I6)
14	
15	
16	
17	
18	
19	=H13/100*$A17
20	=H19*1000000
21	



	I
3	
4	0
5	62.84
6	15.9994
7	
8	
9	
10	
11	
12	
13	=I5*I6*100/($E5*$E6+$C5*$C6+$D5*$D6+$E5*$E6+$F5*$F6+$G5*$G6+$H5*$H6+$I5*$I6)
14	
15	
16	
17	
18	
19	=I13/100*$A17
20	=I19*1000000
21	

	J	K
3		
4	**Prüfsumme**	
5	=SUMME(B5;C5;D5;E5;F5;G5;H5;I5)	*=WENN(UND(J5>95;J5<105);"richtig";WENN(J5<>100;"fehler";""))*
6		
7		
8		
9		
10		
11		
12		
13	=SUMME(B13:I13)	*=WENN(J13=100;"richtig";WENN(J13<>100;"fehler";""))*
14		
15		
16		
17		
18		
19	=SUMME(B19:I19)	*=WENN(J19=A17;"richtig";WENN(J19<>A17;"fehler";""))*
20	=SUMME(B20:I20)	*=WENN(J20=A17*1000000;"richtig";WENN(J20<>A18;"fehler";""))*

8 References

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