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Organ burden, faeces analyses and particle detection pattern in other organs after chronic exposure

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1 Description of task

A two year inhalation study with cerium dioxide (NM-212 Ø 28 nm, source: JRC) has been performed according to OECD TG no. 453. Female Wistar rats were divided into five dose groups: 0 mg/m^3 (control group), 0.1 mg/m^3 (group 1), 0.3 mg/m^3 (group 2), 1 mg/m^3 (group 3) and 3 mg/m³ (group 4). After 3 and 12 months of exposure three animals per dose group were sacrificed. After 24 months of exposure another four animals per dose group (except control group: 2 animals) were sacrificed. The concentrations of CeO_2 in liver, spleen, kidney, brain, heart, lymph nodes, small intestine, bone marrow, blood and faeces were determined after 3, 12 and 24 months of exposure for groups 1, 2, 3 and 4. In femur (named “bone” in the following) the concentrations were determined for all dose groups after 24 months of exposure only. Control groups were also examined to check the background of the tissue matrices.

2 Description of work & main achievements

2.1 Summary

During this study we examined the concentrations of CeO_2 nanoparticles in liver, spleen, kidney, brain, heart, mesenteric and lung associated lymph nodes, small intestine, bone marrow, blood and faeces at termination time points of 3, 12 and 24 months. The CeO_2 concentrations in femur were determined after 24 months only.

The amounts of ceria found in each organ were dose group dependent. Lowest and highest concentrations of CeO_2 were found in groups 1 and 4, respectively.

The examination with inductively coupled plasma mass spectrometry (ICP-MS) revealed the following average CeO_2 distribution [CeO_2/organ] in the analysed matrices within each dose group, except the control group:

- after 3 months: lung** > lung associated lymph nodes (LALN) > liver > kidney > spleen > bone marrow* > small intestine* > mesenteric lymph nodes > heart > brain > olfactory bulb** > blood*
- after 12 months: lung associated lymph nodes > lung** > liver > kidney > spleen > bone marrow* > small intestine* > mesenteric lymph nodes > heart > brain > olfactory bulb** > blood*
- after 24 months: lung associated lymph nodes > lung** > liver > bone > kidney > spleen > bone marrow* > small intestine* > mesenteric lymph nodes > heart > brain > olfactory bulb** > blood*

* referred to [CeO_2/g]

** reported in deliverable 4.5

Two in-house validations of analytical methods for sample preparation and quantification of cerium in bone and faeces were performed. All specified parameters and analytical conditions were met. The validations were successful and the methods were used for examination of samples out of the 2-year inhalation study.

Studies on particle detection by time-of-flight secondary ion mass spectrometry (ToF-SIMS) and ion beam microscopy (IBM) were not performed so far, since the sample preparation of lungs by partner no.13 in cooperation with ITEM Hannover has required until February 10, 2016. ToF-SIMS and IBM analysis of these samples will be performed in the next months.

As a result of a the close cooperation with ITEM Hannover a further amendment with regard to the sample preparation of extrapulmonary organs out of the two year inhalation study (OECD TG no. 453) was completed. It was layed down, that for organs which were shown by ICP-MS analysis to contain significant concentrations of CeO_2, sample preparation for ToF-SIMS and IBM analysis will be performed by ITEM Hannover. Accordingly the examination of other organs than lung by ToF-SIMS and IBM will not start before March 2016.

2.2 Background of the task

In a 2 year inhalation study, conducted according to OECD TG no. 453, CeO_2 nanoparticles (NM-212, Ø 28 nm) were used as a representative of poorly soluble, respirable granular biodurable particles without known significant specific toxicity (GBP). The objective was to investigate potential low dose effects caused by chronic inhalation and also to compare the particle distribution in lung tissue by the use of imaging techniques. In order to correlate particle distribution with potential effects of CeO_2 nanoparticles, slices for ToF-SIMS and IBM studies are taken adjacent to those for histopathological investigations.

2.3 Description of the work carried out

2.3.1 Organ burden

2.3.1.1 Analytical task

The task within the deliverable was to determine the concentration of cerium in bone and faeces.

2.3.1.2 Method development

We have developed and validated two methods for sample preparation and analysis of cerium from CeO_2 nanoparticles (NM-212, Ø 28 nm) in i) bone and ii) faeces.

2.3.1.3 Freeze-drying

Always 100 mg of faeces were chopped, homogenized and freeze-dried under vacuum. Samples of bone were dried in a drying cabinet, chopped, homogenized and weighed as 100 mg subsamples. All samples were stored in 2-ml Eppendorf tubes at -80 °C.

2.3.1.4 Microwave digestion

The freeze-dried/dried samples were digested in separate vessels each with an appropriate mixture of acid and oxidizing agent (faeces: 2.5 ml H_2O, 2 ml HNO_3 (69 %), 1 ml H_2O_2 (30 %); bone: 2 ml HNO_3 (69%), 1 ml H_2O_2 (30%)). A separate suitable temperature and energy controlled digestion program was used each to break down the bone and faeces matrices and to dissolve the CeO_2 nanoparticles.

The gathered solutions were diluted further with MiliQ water including the addition of the two internal standards indium (^{115}In) and lutetium (^{175}Lu). These standards served as internal standards at either sides of the molecular mass of the two ^{140}Ce and ^{142}Ce isotopes.

2.3.1.5 ICP-MS analysis

Quantification was carried out with a quadrupole Thermo Fisher X Series II instrument. For analysis, ^{140}Ce and ^{142}Ce were selected out of the five naturally occurring cerium isotopes for quantification.

2.3.1.6 In-house validation

The two methods described above were in-house validated for bone and faeces. Therefore the samples were spiked with three different concentrations, appropriate to the concentrations expected according to the 2 year inhalation study: i) bone: 0.2 ppb, 2 ppb and 20 ppb, ii) faeces: 5 ppb, 10 ppb and 20 ppb.

Samples of bone and faeces were spiked with a certain CeO_2-concentration from a CeO_2-dispersion. The dispersion was prepared according to the NANOGENOTOX dispersion protocol. Nanoparticles NM-212 from the JRC and bovine serum albumin as medium were used.

2.3.1.7 Validation framework

The tissue samples were tested in line with DIN ISO/IEC 17025 “General requirements for the competence of testing and calibration laboratories” and the resulting SOP for the validation of analytical methods. The criteria specificity/selectivity, stability, accuracy and precision as well as recovery were tested for both. The uncertainty for ICP-MS measurements was determined. Additionally, appropriate system suitability criteria and a suitable certified reference material for cerium (BCR-670) were selected to receive a high confidence and excellence in the gathered test values. Thus the validation passed and the method was considered suitable for the purpose.

2.3.1.8 Quantification of cerium in tissues and faeces

Appropriate analytical methods for the quantification of cerium in bone and faeces have been developed.

Thus the determination of CeO_2 concentrations in both matrices and other organs was carried out with the methods previously established, comprising the steps of sampling, freeze drying, wet chemical microwave digestion and determination of cerium ions with ICP-MS.

The values of ^{140}Ce and ^{142}Ce isotopes are calculated as [CeO_2/organ] and [CeO_2/g], respectively. The analytical range of this method is 0.01 ppb - 20 ppb.

2.3.2 Particle distribution

The sample preparation of lungs and extrapulmonary organs for ToF-SIMS and IBM will be done by partner no.13 in cooperation with ITEM Hannover and is still in progress. Therefore no results on particle distribution can be reported so far.

2.4 Results

In the following, results of the method validations for the matrices specified above and for CeO_2 concentrations in several tissues and faeces are presented. All matrices were examined regarding their CeO_2 amounts during the 2 year inhalation study with CeO_2 nanoparticles (NM-212 from the JRC, Ø 28 nm).

The sample preparation of lungs and other organs for ToF-SIMS will be done by partner no.13 in cooperation with ITEM Hannover and is still in progress.

2.4.1 Organ burden

2.4.1.1 Validation

Two in-house validations for sample preparation and analysis of CeO_2 (NM-212) were performed using three independently prepared matrices of bone and faeces, respectively. Each sample was measured in triplicate by ICP-MS. Cerium amounts were calculated 2.3.1.3-2.3.1.5 using the internal standard $^{115}Indium$ (^{175}Lu showed comparable results). Recovery rates are reported in table 1. Results are given for faeces in figure 1 and for bone in figure 2. The uncertainty of ICP-MS was validated for four independently prepared liver samples spiked with 0.02, 0.2, 2 and 20 ppb CeO_2 (NM-212). Each sample concentration was measured ten times. Per sample 150 mg liver were weighed each and were prepared as described in *2.3.1.3 - 2.3.1.5*. Results are shown in table 2.

Table 1: Recovery of CeO_2 NM-212 presented as ^{140}Ce in bone and faeces

	Recovery [%]		Relative Standard Deviation [%]	
Organ tissue spiked with CeO_2	**^{140}Ce**	**^{142}Ce**	**^{140}Ce**	**^{142}Ce**
bone 0.2 ppb	94.97	92.23	3.04	2.83
bone 2 ppb	94.60	94.90	0.44	1.41
bone 20 ppb	98.75	98.17	1.53	1.17
faeces 5 ppb	112.97	118.10	2.54	5.04
faeces 10 ppb	112.60	117.23	3.29	3.55
faeces 20 ppb	120.00	122.30	1.78	2.46

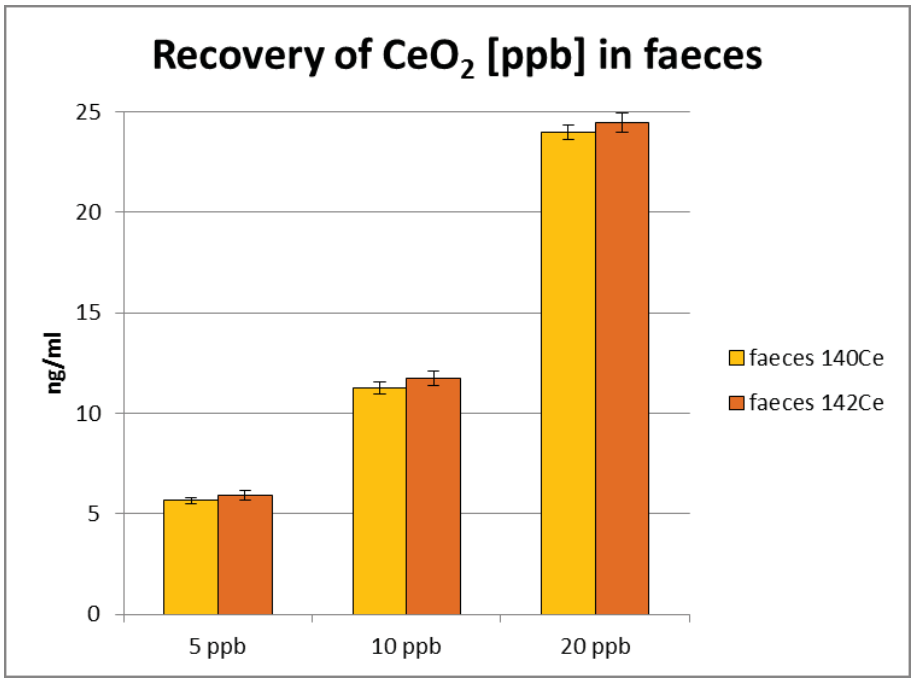

Figure 1: Recovery of CeO_2 NM-212 in faeces presented as ^{140}Ce and ^{142}Ce

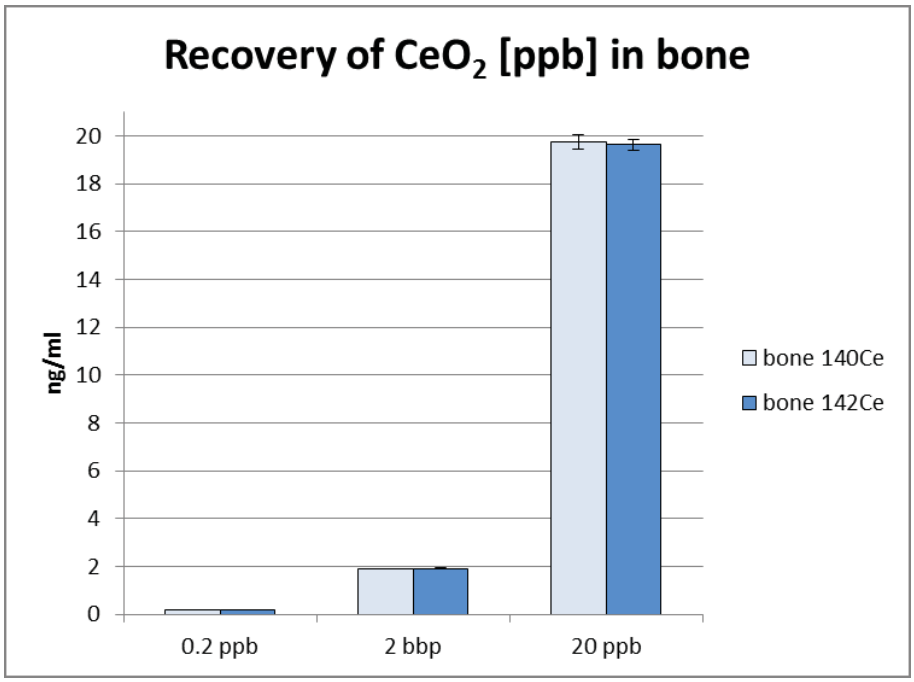

Figure 2: Recovery of CeO_2 NM-212 in bone presented as ^{140}Ce and ^{142}Ce

Experiments on recovery in three independently prepared samples (unspiked and spiked with 0.2 ppb, 2 ppb, 20 ppb CeO_2 for bone; unspiked and spiked with 5 ppb, 10 ppb, 20 ppb CeO_2 for faeces) revealed an average RSD < 2 % for bone and an average RSD < 3 % for faeces (both regarding ^{140}Ce and ^{115}In). Slight overpredictions were found for faeces because unspiked faeces samples already showed background concentrations of 1.7 ppb. These overpredictions were still in the range of minimal requirements.

Specificity of the analytical method for the isotopes ^{140}Ce, ^{142}Ce, ^{115}In and ^{175}Lu was demonstrated, linearity R was > 0.999. Sample stability was confirmed for up to 14 days for bone and 13 days for faeces samples.

The uncertainty of the ICP-MS measurements was calculated according to GUM and DIN V ENV 13005:1999-06 respectively. The determination of the uncertainty was performed using type A evaluation of standard uncertainty. Table 2 shows the measured Ce concentrations referred to internal standard ^{115}In. The uncertainty was equal when ^{175}Lu was used as internal standard.

The determined uncertainties for tissue samples and ICP-MS are:

- 14.4 % for 0.02 ppb
- 4.3 % for 0.2 ppb
- 2.1 % for 2 ppb
- 1.2 % for 20 ppb.

Table 2: Uncertainty of ICP-MS measurements

n=10 0.02 ppb	^{115}In ^{140}Ce	^{115}In ^{142}Ce	n=10 0.2 ppb	^{115}In ^{140}Ce	^{115}In ^{142}Ce
	0.015	0.012		0.152	0.150
	0.014	0.015		0.151	0.157
	0.015	0.015		0.150	0.160
	0.015	0.013		0.152	0.156
	0.014	0.015		0.151	0.157
	0.015	0.015		0.152	0.152
	0.014	0.014		0.153	0.157
	0.014	0.015		0.151	0.152
	0.014	0.015		0.149	0.148
	0.014	0.015		0.152	0.148
average	0.015	0.014	average	0.151	0.154
ASD	0.000	0.001	ASD	0.001	0.004
u [ng/ml]	**0.002**	**0.006**	**u [ng/ml]**	**0.006**	**0.027**
n=10 2 ppb	^{115}In ^{140}Ce	^{115}In ^{142}Ce	n=10 20 ppb	^{115}Indium ^{140}Ce	^{115}In ^{142}Ce
	1.405	1.427		14.446	14.534
	1.402	1.432		14.382	14.385
	1.398	1.446		14.377	14.482
	1.395	1.437		14.377	14.460
	1.396	1.422		14.375	14.428
	1.400	1.442		14.345	14.437
	1.398	1.421		14.379	14.518
	1.392	1.414		14.372	14.475
	1.395	1.419		14.357	14.485
	1.390	1.422		14.395	14.427
average	1.397	1.428	average	14.380	14.463
ASD	0.005	0.011	ASD	0.027	0.045
u [ng/ml]	**0.030**	**0.068**	**u [ng/ml]**	**0.170**	**0.286**

2.4.1.2 *Analytical task*

All concentrations measured with ICP-MS are mean values based on three (n = 3). For faeces three animals per dose group were examined after 3 and 12 months. After 24 months bone and faeces of four animals were examined each per dose group and two animals of the control groups. The mean values of three, four and two matrix samples each were again calculated as mean values for bone and faeces, respectively. The results for both are shown in table 3.

Note:

Dose group 0: 0 mg/m³ (control group)
Dose group 1: 0.1 mg/m³
Dose group 2: 0.3 mg/m³
Dose group 3: 1 mg/m³
Dose group 4: 3 mg/m³

Table 3: Overview of animals and tissues examined on CeO_2 concentration after chronic exposure

Animal no.	Dose group	Exposure time	Exposure concentration	Examined matrices
601	10	3 months	0 mg/m³	liver, spleen, kidney, brain, heart, lung associated lymph nodes, small intestine, bone marrow, faeces
602	10	3 months	0 mg/m³	liver, spleen, kidney, brain, heart, lung associated lymph nodes, small intestine, bone marrow, faeces
603	10	3 months	0 mg/m³	liver, spleen, kidney, brain, heart, lung associated lymph nodes, small intestine, bone marrow, faeces
611	11	3 months	0.1 mg/m³	liver, spleen, kidney, brain, heart, lung associated lymph nodes, small intestine, bone marrow, faeces
612	11	3 months	0.1 mg/m³	liver, spleen, kidney, brain, heart, lung associated lymph nodes, small intestine, bone marrow, faeces
613	11	3 months	0.1 mg/m³	liver, spleen, kidney, brain, heart, lung associated lymph nodes, small intestine, bone marrow, faeces
621	12	3 months	0.3 mg/m³	liver, spleen, kidney, brain, heart, lung associated lymph nodes, small intestine, bone marrow, faeces
622	12	3 months	0.3 mg/m³	liver, spleen, kidney, brain, heart, lung associated lymph nodes, small intestine, bone marrow, faeces
623	12	3 months	0.3 mg/m³	liver, spleen, kidney, brain, heart, lung associated lymph nodes, small intestine, bone marrow, faeces
631	13	3 months	1 mg/m³	liver, spleen, kidney, brain, heart, lung associated lymph nodes, small intestine, bone marrow, faeces
632	13	3 months	1 mg/m³	liver, spleen, kidney, brain, heart, lung associated lymph nodes, small intestine, bone marrow, faeces

Animal no.	Dose group	Exposure time	Exposure concentration	Examined matrices
633	13	3 months	1 mg/m³	liver, spleen, kidney, brain, heart, lung associated lymph nodes, small intestine, bone marrow, faeces
641	14	3 months	3 mg/m³	liver, spleen, kidney, brain, heart, lung associated lymph nodes, small intestine, bone marrow, faeces
642	14	3 months	3 mg/m³	liver, spleen, kidney, brain, heart, lung associated lymph nodes, small intestine, bone marrow, faeces
643	14	3 months	3 mg/m³	liver, spleen, kidney, brain, heart, lung associated lymph nodes, small intestine, bone marrow, faeces
604	20	12 months	0 mg/m³	liver, spleen, kidney, brain, heart, mesenteric and lung associated lymph nodes, small intestine, bone marrow, faeces
605	20	12 months	0 mg/m³	liver, spleen, kidney, brain, heart, mesenteric and lung associated lymph nodes, small intestine, bone marrow, faeces
606	20	12 months	0 mg/m³	liver, spleen, kidney, brain, heart, mesenteric and lung associated lymph nodes, small intestine, bone marrow, faeces
614	21	12 months	0.1 mg/m³	liver, spleen, kidney, brain, heart, mesenteric and lung associated lymph nodes, small intestine, bone marrow, faeces
615	21	12 months	0.1 mg/m³	liver, spleen, kidney, brain, heart, mesenteric and lung associated lymph nodes, small intestine, bone marrow, faeces
616	21	12 months	0.1 mg/m³	liver, spleen, kidney, brain, heart, mesenteric and lung associated lymph nodes, small intestine, bone marrow, faeces
624	22	12 months	0.3 mg/m³	liver, spleen, kidney, brain, heart, mesenteric and lung associated lymph nodes, small intestine, bone marrow, faeces
625	22	12 months	0.3 mg/m³	liver, spleen, kidney, brain, heart, mesenteric and lung associated lymph nodes, small intestine, bone marrow, faeces
626	22	12 months	0.3 mg/m³	liver, spleen, kidney, brain, heart, mesenteric and lung associated lymph nodes, small intestine, bone marrow, faeces

Animal no.	Dose group	Exposure time	Exposure concentration	Examined matrices
634	23	12 months	1 mg/m³	liver, spleen, kidney, brain, heart, mesenteric and lung associated lymph nodes, small intestine, bone marrow, faeces
635	23	12 months	1 mg/m³	liver, spleen, kidney, brain, heart, mesenteric and lung associated lymph nodes, small intestine, bone marrow, faeces
636	23	12 months	1 mg/m³	liver, spleen, kidney, brain, heart, mesenteric and lung associated lymph nodes, small intestine, bone marrow, faeces
644	24	12 months	3 mg/m³	liver, spleen, kidney, brain, heart, mesenteric and lung associated lymph nodes, small intestine, bone marrow, faeces
645	24	12 months	3 mg/m³	liver, spleen, kidney, brain, heart, mesenteric and lung associated lymph nodes, small intestine, bone marrow, faeces
646	24	12 months	3 mg/m³	liver, spleen, kidney, brain, heart, mesenteric and lung associated lymph nodes, small intestine, bone marrow, faeces
607	30	24 months	0 mg/m³	liver, spleen, kidney, brain, heart, mesenteric and lung associated lymph nodes, small intestine, bone marrow, bone, faeces
608	30	24 months	0 mg/m³	liver, spleen, kidney, brain, heart, mesenteric and lung associated lymph nodes, small intestine, bone marrow, bone, faeces
617	31	24 months	0.1 mg/m³	liver, spleen, kidney, brain, heart, mesenteric and lung associated lymph nodes, small intestine, bone marrow, bone, faeces
199	31	24 months	0.1 mg/m³	liver, spleen, kidney, brain, heart, mesenteric and lung associated lymph nodes, small intestine, bone marrow, bone, faeces
619	31	24 months	0.1 mg/m³	liver, spleen, kidney, brain, heart, mesenteric and lung associated lymph nodes, small intestine, bone marrow, bone, faeces
200	31	24 months	0.1 mg/m³	liver, spleen, kidney, brain, heart, mesenteric and lung associated lymph nodes, small intestine, bone marrow, bone, faeces

Animal no.	Dose group	Exposure time	Exposure concentration	Examined matrices
627	32	24 months	0.3 mg/m³	liver, spleen, kidney, brain, heart, mesenteric and lung associated lymph nodes, small intestine, bone marrow, bone, faeces
300	32	24 months	0.3 mg/m³	liver, spleen, kidney, brain, heart, mesenteric and lung associated lymph nodes, small intestine, bone marrow, bone, faeces
629	32	24 months	0.3 mg/m³	liver, spleen, kidney, brain, heart, mesenteric and lung associated lymph nodes, small intestine, bone marrow, bone, faeces
630	32	24 months	0.3 mg/m³	liver, spleen, kidney, brain, heart, mesenteric and lung associated lymph nodes, small intestine, bone marrow, bone, faeces
637	33	24 months	1 mg/m³	liver, spleen, kidney, brain, heart, mesenteric and lung associated lymph nodes, small intestine, bone marrow, bone, faeces
638	33	24 months	1 mg/m³	liver, spleen, kidney, brain, heart, mesenteric and lung associated lymph nodes, small intestine, bone marrow, bone, faeces
639	33	24 months	1 mg/m³	liver, spleen, kidney, brain, heart, mesenteric and lung associated lymph nodes, small intestine, bone marrow, bone, faeces
400	33	24 months	1 mg/m³	liver, spleen, kidney, brain, heart, mesenteric and lung associated lymph nodes, small intestine, bone marrow, bone, faeces
647	34	24 months	3 mg/m³	liver, spleen, kidney, brain, heart, mesenteric and lung associated lymph nodes, small intestine, bone marrow, bone, faeces
648	34	24 months	3 mg/m³	liver, spleen, kidney, brain, heart, mesenteric and lung associated lymph nodes, small intestine, bone marrow, bone, faeces
649	34	24 months	3 mg/m³	liver, spleen, kidney, brain, heart, mesenteric and lung associated lymph nodes, small intestine, bone marrow, bone, faeces
650	34	24 months	3 mg/m³	liver, spleen, kidney, brain, heart, mesenteric and lung associated lymph nodes, small intestine, bone marrow, bone, faeces

Statistical analysis with a one-way analysis of variances (ANOVA) for time and dose, DUNNETT's test and GRUBBs test were performed on the results of the matrices listed above in the frame of a combined chronic toxicity/carcinogenicity inhalation study (OECD TG no. 453). CeO_2 concentrations were not significant different from the control group for the following tissues:

- heart: group 1,2 after 24 months
- brain: group 1 after 3 months
- bone marrow: group 1-4 after 3 months
- blood: group 1-4 after 3, 12, 24 months

For all other tissues and dose groups DUNNETT's test revealed significant CeO_2 concentrations for the three termination time points (tables 3-14).

The one-way ANOVA revealed a significant effect of time concerning the CeO_2 concentration in lung, liver, spleen, kidney, LALN, mesenteric LN, bone marrow and faeces. For all other tissues no significant influence of time on the CeO_2 amounts was calculated.

A second one-way ANOVA revealed a significant effect of dose on the CeO_2 concentration in lung, liver, spleen, kidney, LALN, mesenteric LN, heart, olfactory bulb, bone and faeces. For all other tissues no significant effect of dose on CeO_2 concentration was observed.

GRUBBs test revealed no statistical outliers in matrices of kidney, brain, mesenteric LN, LALN, bone and faeces. Outliers were calculated for tissues of blood, bone marrow, heart, liver, small intestine and spleen.

Lung associated lymph nodes consist of mediastinal and tracheobronchial lymph nodes. Deficient values were measured with ICP-MS for samples 199, 630, 638, 648 in mediastinal lymph nodes. Thus the values of these LALN (mediastinal and tracheobronchial) were disregarded for mean value calculation (table 9)

Figures 4 to 15 and tables 3 and 14 show the calculated concentrations of CeO_2 after 3, 12 and 24 months of exposure according to table 2. The given amount of CeO_2 refers to the isotope ^{140}Ce, which was quantified based on the internal standard ^{115}In. Similar results were achieved using the isotope ^{142}Ce and the internal standard ^{175}Lu, respectively.

Table 4: CeO_2 liver burden [µg CeO_2/organ]

Cerium dioxide liver burden						
Exposure time	Value	control	0.1 mg/m³	0.3 mg/m³	1 mg/m³	3 mg/m³
3 months	animal 1	0.03	0.29	0.44	1.30	1.55
	animal 2	0.00	0.17	0.22	1.14	2.37
	animal 3	0.00	0.22	0.34	1.21	2.11
	mean	**0.00**	**0.23**	**0.33**	**1.22**	**2.01**
	± SD	0.00	0.06	0.11	0.08	0.42
12 months	animal 1	0.00	0.82	1.59	4.16	5.24
	animal 2	0.00	0.74	1.49	4.88	4.32
	animal 3	0.00	0.23	1.06	4.68	124.14[+]
	mean	**0.00**	**0.59**	**1.38**	**4.58**	**4.78**
	± SD	0.00	0.32	0.28	0.37	0.65
24 months	animal 1	0.00	0.21	2.61	3.82	8.21
	animal 2	0.00	1.18[+]	7.23[+]	4.11	10.17
	animal 3	*	0.37	2.25	2.19	12.34
	animal 4	*	0.32	3.02	2.10	16.10
	mean	**0.00**	**0.30**	**2.63**	**3.05**	**11.71**
	± SD	0.00	0.08	0.39	1.06	3.38

*: only two control animals were available
[+]: significant outlier; value is not considered for mean value calculations

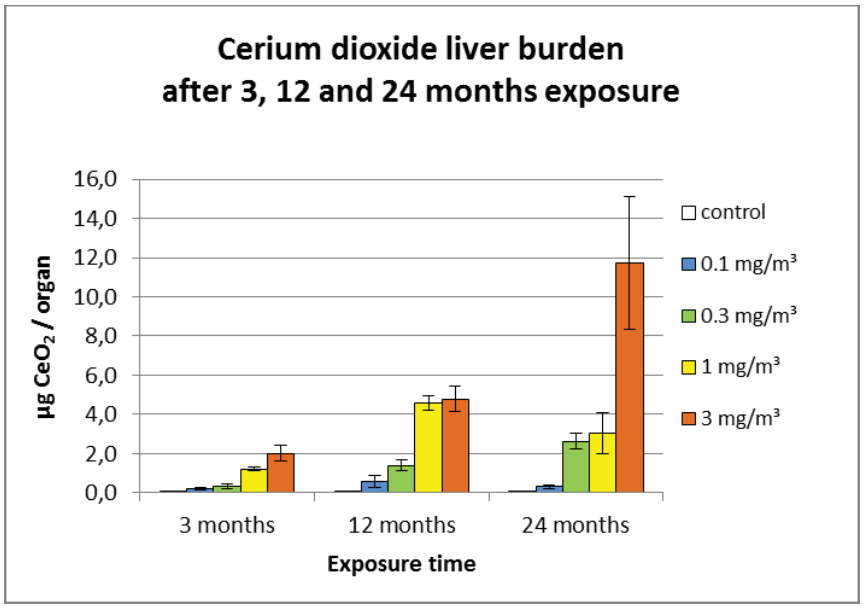

Figure 3: CeO_2 liver burden [µg CeO_2/organ]

The liver showed no clear CeO_2 distribution pattern (figure 3). On the one hand CeO_2 concentrations rose in group 2 and group 4 between month 3 and month 24. On the other hand in groups 1 and 3 CeO_2 concentrations increased between month 3 and month 12, and decreased between month 12 and 24. No

pattern for low doses (groups 1,2) or high doses (groups 3,4) can be deduced. The maximum average CeO_2 concentration of 11.71 µg/liver was detected after 24 months of inhalation in group 4 (table 4).

Table 5: CeO_2 spleen burden [ng CeO_2/organ]

Cerium dioxide spleen burden						
Exposure time	Value	control	0.1 mg/m³	0.3 mg/m³	1 mg/m³	3 mg/m³
3 months	animal 1	0.90	1.23	1.32	3.82	10.68
	animal 2	1.19	0.79	1.34	4.87	130.00
	animal 3	0.00	0.77	2.57	4.43	4.86
	mean	**0.70**	**0.93**	**1.75**	**4.37**	**48.51**
	± SD	0.62	0.35	0.72	2.25	4.11
12 months	animal 1	0.14	6.00	27.34	39.91	193.61
	animal 2	0.13	3.36	18.46	60.64	145.52
	animal 3	0.00	8.22	16.98	365.38	14635.48[+]
	mean	**0.09**	**5.86**	**20.93**	**155.31**	**169.57**
	± SD	0.08	5.44	9.33	34.26	14.25
24 months	animal 1	0.16	14.15	67.68	211.48	687.33
	animal 2	0.34	34.76	116.79	309.73	906.32
	animal 3	*	15.25	54.56	393.07	840.88
	animal 4	*	14.93	64.54	212.09	740.26
	mean	**0.25**	**19.77**	**75.89**	**281.59**	**793.70**
	± SD	0.13	4.18	42.59	113.19	115.20

*: only two control animals were available
[+]: significant outlier; value is not considered for mean value calculations

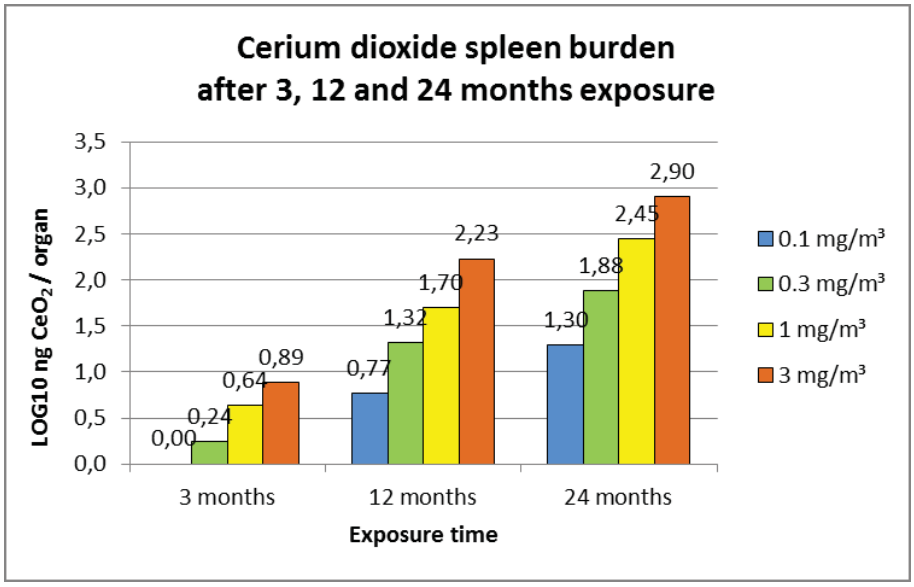

Figure 4: CeO_2 spleen burden [ng CeO_2/organ]

The spleen burden in figure 4 is displayed as LOG_{10} (ng CeO_2/organ) for better visibility (control groups and standard deviation are not shown due to the curve progression of the logarithm function). Average CeO_2 amounts in spleen ranged between 0.93 and 793.70 ng/organ in groups 1 and 4, respectively. As expected a clear increase of CeO_2 concentrations over time and with rising dose was observed (table 5 and figure 4).

Table 6: CeO_2 kidney burden [ng CeO_2/organ]

	Cerium dioxide kidney burden					
Exposure time	Value	Control	0.1 mg/m³	0.3 mg/m³	1 mg/m³	3 mg/m³
3 months	animal 1	3.31	17.98	61.14	20.19	53.49
	animal 2	0.49	2.11	7.38	17.55	26.43
	animal 3	0.20	7.48	10.38	12.10	37.42
	mean	**1.34**	**9.19**	**26.30**	**16.62**	**39.11**
	± SD	0.21	3.80	2.12	3.86	7.77
12 months	animal 1	1.38	32.65	52.90	94.37	179.62
	animal 2	0.94	33.84	50.01	130.99	261.67
	animal 3	0.65	28.10	49.72	131.05	211.77
	mean	**0.99**	**31.53**	**50.87**	**118.80**	**217.69**
	± SD	0.37	3.03	1.76	21.17	41.34
24 months	animal 1	0.56	58.34	284.13	283.06	597.34
	animal 2	0.36	59.23	250.60	239.26	450.29
	animal 3	*	42.73	109.88	439.43	1171.86
	animal 4	*	55.40	202.78	309.62	1198.35
	mean	**0.46**	**53.93**	**211.85**	**317.84**	**854.46**
	± SD	0.14	7.64	75.73	86.10	386.64

*: only two control animals were available

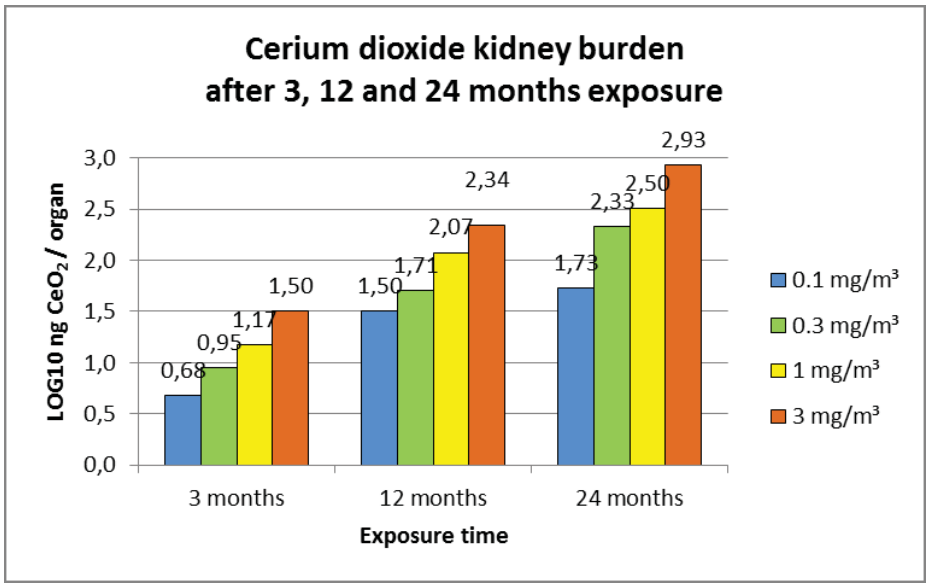

Figure 5: CeO_2 kidney burden [ng CeO_2/organ]

For reasons of visibility the kidney burden is also shown as LOG_{10} (ng CeO_2/organ; control groups and standard deviation is not shown due to the curve progression of the logarithm function). Average CeO_2 amounts in kidney ranged between 9.19 and 854.46 ng/organ in groups 1 and 4, respectively. Similarly, CeO_2 concentrations increased over time and with rising dose (table 6 and figure 5).

Table 7: CeO_2 brain burden [ng CeO_2/organ]

Cerium dioxide brain burden						
Exposure time	Value	control	0.1 mg/m³	0.3 mg/m³	1 mg/m³	3 mg/m³
3 months	animal 1	0.00	1.09	0.30	0.37	31.45
	animal 2	0.00	0.00	0.33	1.03	4.81
	animal 3	0.10	0.17	0.00	0.72	1.92
	mean	**0.03**	**0.42**	**0.21**	**0.71**	**3.37**
	± SD	0.06	0.59	0.18	0.33	2.04
12 months	animal 1	0.32	1.31	1.03	2.17	4.56
	animal 2	0.00	0.95	1.04	2.41	4.07
	animal 3	0.38	0.92	0.88	2.42	4.62
	mean	**0.23**	**1.06**	**0.98**	**2.33**	**4.42**
	± SD	0.20	0.22	0.09	0.14	0.30
24 months	animal 1	16.60	3.04	2.22	4.55	5.93
	animal 2	3.42	8.29	2.53	4.51	5.60
	animal 3	*	12.48	1.31	5.21	18.69
	animal 4	*	1.02	2.64	7.64	22.34
	mean	10.01	**6.21**	**2.17**	5.48	**13.14**
	± SD	**9.32**	5.18	0.60	1.48	8.65

*: only two control animals were available

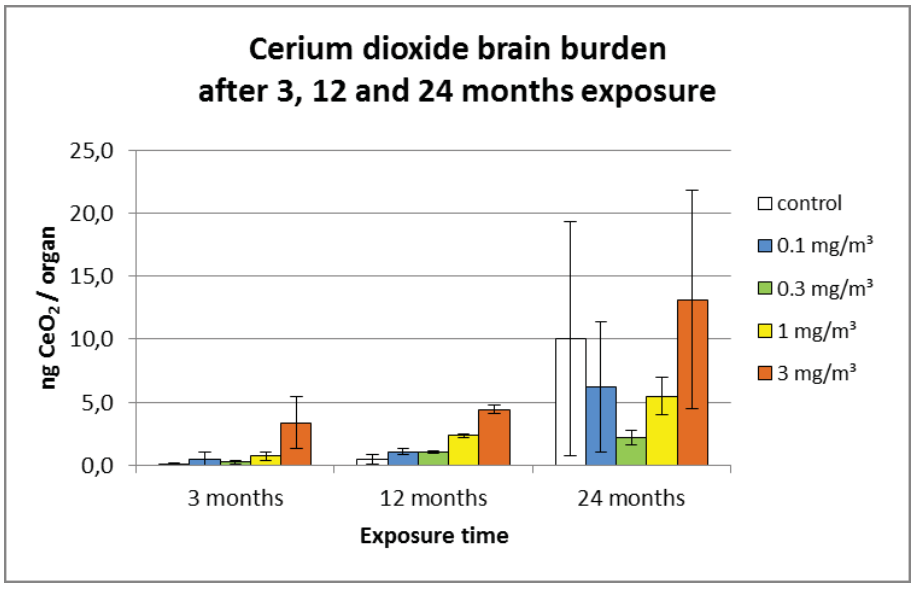

Figure 6: CeO_2 brain burden [ng CeO_2/organ]

Table 7 and figure 6 show a clear increase of CeO_2 concentrations in brain tissues in all dose groups and the control group until 12 months of exposure. After 24 months of exposure a high variability of CeO_2 concentrations was observed in general due to which no differentiation of dose groups and control is possible. The control levels after 24 months exposure were derived from 2 animals only.

Table 8: CeO_2 heart burden [ng CeO_2/organ]

Cerium dioxide heart burden						
Exposure time	Value	control	0.1 mg/m³	0.3 mg/m³	1 mg/m³	3 mg/m³
3 months	animal 1	0.12	0.29	0.78	1.71	2.71
	animal 2	0.05	0.79	0.61	1.19	5.01
	animal 3	0.08	0.81	0.76	1.89	4.98
	mean	**0.09**	**0.63**	**0.72**	**1.60**	**4.23**
	± SD	0.04	0.29	0.09	0.36	1.32
12 months	animal 1	0.49	1.85	4.33	6.67	7.88
	animal 2	0.47	1.85	3.35	7.18	13.27
	animal 3	0.40	1.55	3.24	8.26	14.45
	mean	**0.46**	**1.75**	**3.64**	**7.37**	**11.87**
	± SD	0.05	0.17	0.60	0.81	3.50
24 months	animal 1	66.62[+]	14.32	10.11	20.43	66.32
	animal 2	10.95	77.55[+]	12.33	16.96	37.99
	animal 3	*	7.13	7.74	56.42	75.61
	animal 4	*	4.59	14.14	28.33	52.97
	mean	**10.95**	**8.68**	**11.08**	**30.53**	**58.22**
	± SD	0.00	5.05	2.77	17.90	16.38

*: only two control animals were available
[+]: significant outlier; value is not considered for mean value calculations

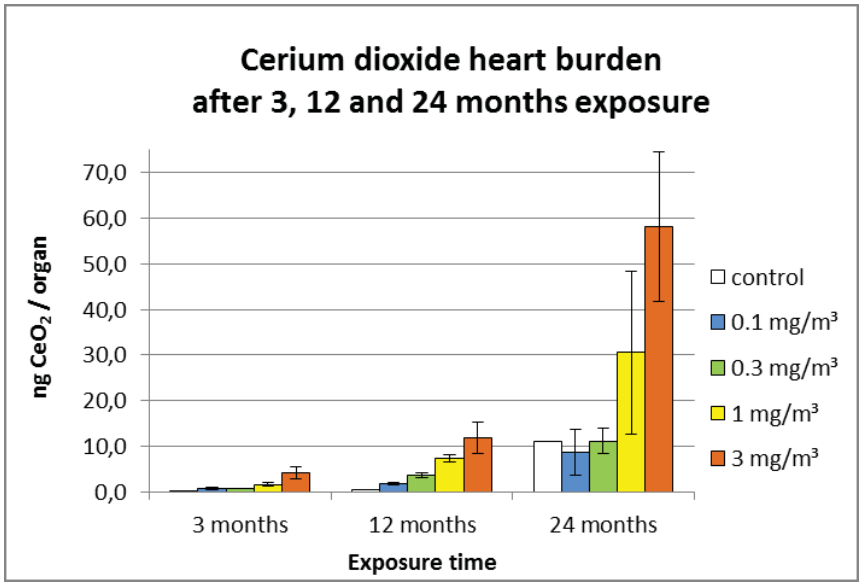

Figure 7: CeO_2 heart burden [ng CeO_2/organ]

The CeO_2 concentrations detected in heart tissue show an increasing tendency between month 3 and month 24. However, the maximum levels of dose and control groups in heart are with 75.61 respectively 66.62 ng/organ very close and no statistic differentiation is possible (table 8).

Table 9: CeO_2 mesenteric lymph node burden [ng CeO_2/organ]

Cerium dioxide mesenteric LN burden						
Exposure time	Value	control	0.1 mg/m³	0.3 mg/m³	1 mg/m³	3 mg/m³
12 months	animal 1	0.36	2.28	5.36	10.62	36.33
	animal 2	0.51	2.09	4.36	24.49	53.67
	animal 3	0.16	2.14	2.37	13.66	55.08
	mean	**0.35**	**2.17**	**4.03**	**16.26**	**48.36**
	± SD	0.18	0.10	1.52	7.29	10.44
24 months	animal 1	0.57	1.80	3.90	17.66	85.20
	animal 2	0.28	2.92	13.94	21.09	43.23
	animal 3	*	1.60	4.64	65.43	79.38
	animal 4	*	1.91	9.02	52.86	83.90
	mean	**0.42**	**2.06**	**7.88**	**39.26**	**72.93**
	± SD	0.20	0.59	4.63	23.57	19.96

*: only two control animals were available

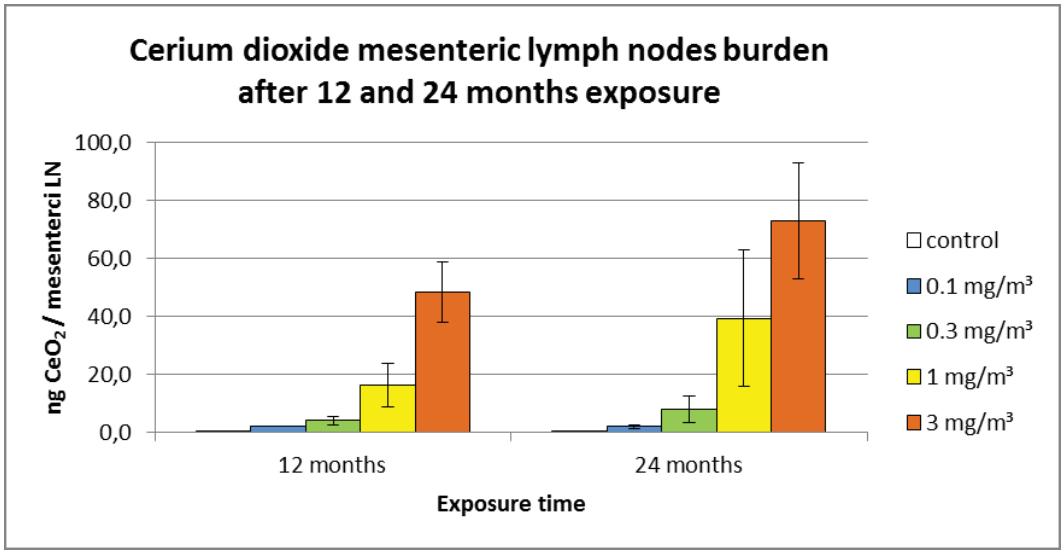

Figure 8: CeO_2 mesenteric LN burden [ng CeO_2/organ]

The mesenteric lymph node burden (table 9, figure 8) rose between month 12 and month 24. CeO_2 levels were in the range of those found in heart. Average amounts varied between 2.06 and 72.93 ng/mesenteric LN.

Table 10: CeO_2 LALN burden [µg CeO_2/organ]

Cerium dioxide LALN burden						
Exposure time	Value	control	0.1 mg/m³	0.3 mg/m³	1 mg/m³	3 mg/m³
3 months	animal 1	0.00	0.02	n.m.	1.87	n.m.
	animal 2	0.00	0.01	0.10	1.76	30.39
	animal 3	0.00	2.85+	0.12	0.35	n.m.
	mean	**0.00**	**0.01**	**0.11**	**1.33**	**30.39**
	± SD	0.00	0.01	0.01	0.85	0.00
12 months	animal 1	0.00	0.64	20.33	149.22	595.44
	animal 2	0.00	0.09	23.39	216.72	534.84
	animal 3	0.00	1.10	11.94	131.12	809.74
	mean	**0.00**	**0.61**	**18.55**	**165.68**	**646.67**
	± SD	0.00	0.50	5.93	45.11	144.43
24 months	animal 1	0.00	0.19	9.71	611.67	1901.30
	animal 2	0.00	0.52+	21.26	174.59+	678.21+
	animal 3	*	0.52	6.79	416.29	3004.81
	animal 4	*	0.29	1.46+	689.38	2210.35
	mean	**0.00**	**0.33**	**12.59**	**572.45**	**2372.15**
	± SD	0.00	0.17	7.65	140.71	569.27

*: only two control animals were available
+: significant outlier; value is not considered for mean value calculations
n.m.: not measurable; sample got lost

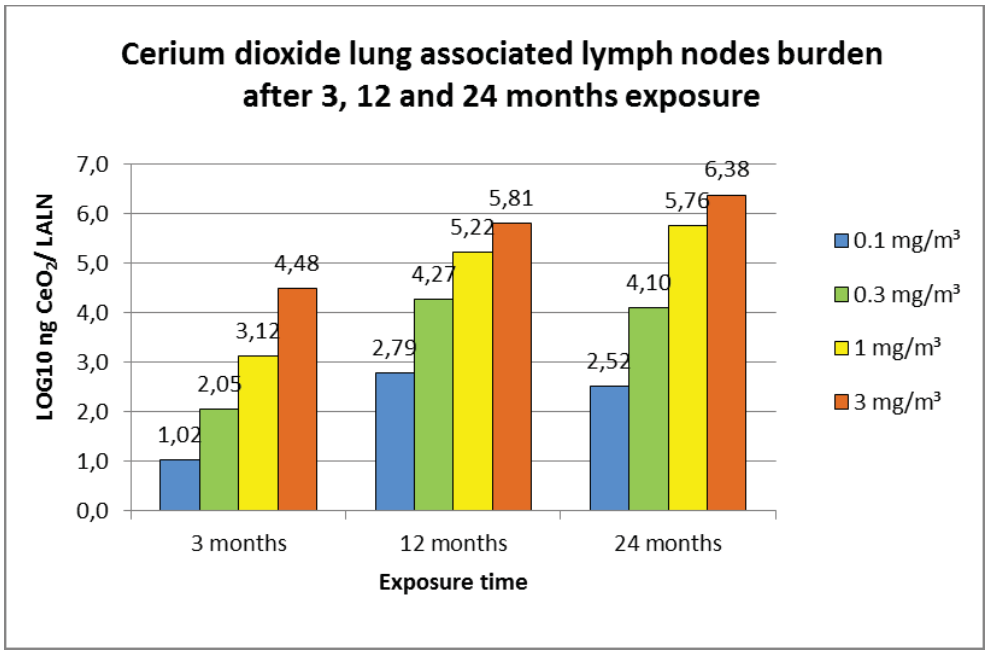

Figure 9: CeO_2 LALN burden [µg CeO_2/organ]

For reasons of visibility the LALN burden is shown as LOG_{10} (µg CeO_2/LALN; control groups and standard deviation is not shown due to the curve progression of the logarithm function). Average CeO_2 amounts in LALN ranged between 0.01 and 2372.15 µg/LALN in groups 1 and 4, respectively. In the low-dose groups 1 and 2, CeO_2 concentrations increased between month 3 and month 12 and slightly decreased between month 12 and month 24. This progression may indicate a declining clearance process. On the contrary, CeO_2 concentrations in high-dose groups 3 and 4 increased over time (table 10 and figure 9). The translocation from lung to lymph nodes seems to be still ongoing subsequently to long-term exposure.

Table 11: CeO_2 small intestine burden [ng CeO_2/g tissue]

Cerium dioxide small intestine burden						
Exposure time	Value	control	0.1 mg/m³	0.3 mg/m³	1 mg/m³	3 mg/m³
3 months	animal 1	16.39	27.68	17.72	64.81	195.28
	animal 2	12.16	14.69	63.85	74.75	182.79
	animal 3	29.23	23.40	28.90	63.62	85.38
	mean	**19.26**	**19.43**	**36.82**	**67.73**	**154.48**
	± SD	8.89	7.85	24.06	6.11	60.17
12 months	animal 1	4.60	9.91	22.79	18.52	379.08
	animal 2	2.75	8.38	3.23[+]	32.29	117.99
	animal 3	3.01	28.90[+]	21.81	35.70	25.14
	mean	**3.45**	**9.15**	**22.30**	**28.84**	**174.07**
	± SD	1.00	1.08	0.69	9.09	183.51
24 months	animal 1	0.79	2.21	12.32	36.45	269.15
	animal 2	1.31	6.27	20.56	53.93	58.93
	animal 3	*	2.98	6.74	45.08	305.75
	animal 4	*	4.03	7.94	20.77	34.76
	mean	**1.05**	**3.87**	**11.89**	**39.06**	**167.15**
	± SD	0.37	1.76	6.26	14.12	123.62

*: only two control animals were available
[+]: significant outlier; value is not considered for mean value calculations

Despite the high standard deviations observed for samples of small intestine (table 11), CeO_2 concentrations of low-dose groups 1 and 2 decreased between months 3 and 24. Concentrations in group 3 decreased between month 3 and month 12 and increased between months 12 and 24. Contrastingly the CeO_2 levels in group 4 increased between months 3 and 12 and decreased between months 12 and 24. No clear pattern for low-/high-dose groups can be stated. Faeces adhering to samples of small intestine could be an explanation for high standard deviations and the biased distribution pattern.

Table 12: CeO_2 bone marrow burden [ng CeO_2/g tissue]

Cerium dioxide bone marrow burden						
Exposure time	Value	control	0.1 mg/m³	0.3 mg/m³	1 mg/m³	3 mg/m³
3 months	animal 1	15.89	8.93	5.90	2.83	1.63[+]
	animal 2	60.06	13.46	3.32	7.11	39.42
	animal 3	2.55	5.41	8.64	14.08	23.14
	mean	**26.17**	**11.19**	**5.95**	**8.01**	**31.28**
	± SD	30.10	4.04	2.66	5.68	11.51
12 months	animal 1	1.77	31.48	25.16	48.28	231.62
	animal 2	11.76	13.69	42.47	149.86	195.18
	animal 3	28.23	42.92	23.20	99.12	3439.18[+]
	mean	**13.92**	**29.36**	**30.28**	**99.08**	**213.40**
	± SD	13.36	14.73	10.61	50.79	25.77
24 months	animal 1	3.07	11.41	59.05	152.22	330.42
	animal 2	2.98	46.29	88.21	211.84	492.06
	animal 3	*	19.81	76.59	382.20	229.37
	animal 4	*	28.33	57.98	167.49	345.33
	mean	**3.02**	**26.46**	**70.46**	**228.44**	**349.30**
	± SD	**0.07**	14.91	14.59	105.58	108.22

*: only two control animals were available
[+]: significant outlier; value is not considered for mean value calculations

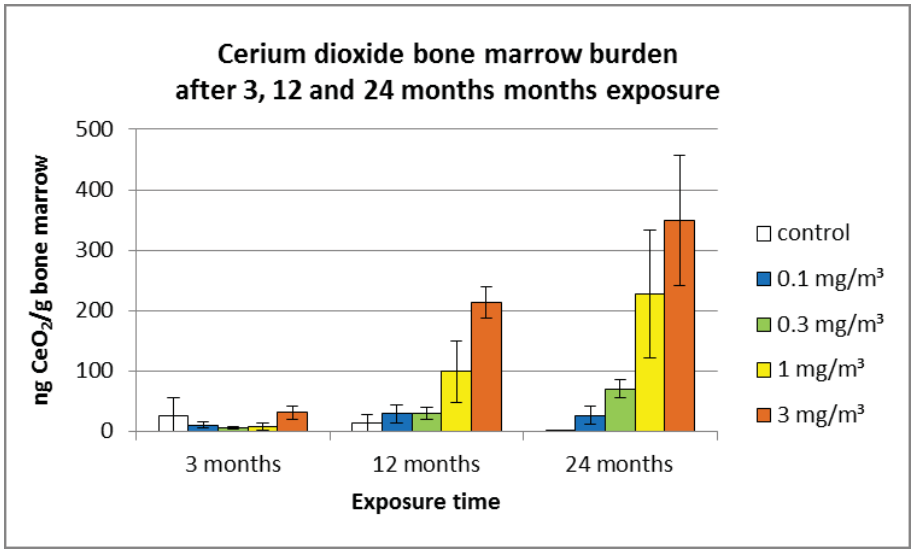

Figure 11: CeO_2 bone marrow burden [ng CeO_2/g tissue]

The CeO_2 concentrations in groups 2-4 clearly rose between months 3 and 24. In group 1 the CeO_2 levels in bone marrow seem to be constant (table 12, figure 11). Average concentrations ranged between 5.95 and 349.30 ng/g bone marrow in groups 2 and 4, respectively. However after 3 months of inhalation, CeO_2 concentrations in control groups were on the same level as in all dose groups (figure 11). The complete bone marrow is estimated to account for 3.2 % of rat bodyweight. That would mean average CeO_2 concentrations

between 47.62 and 4470.99 ng/whole bone marrow in groups 2 and 4, respectively. These calculations refer to average body weights of 250 g, 337 g and 400 g for rats after 3, 12 and 24 months inhalation.

Table 13: CeO_2 bone (femur) burden [µg CeO_2/skeleton]

	Cerium dioxide skeleton burden					
Exposure time		control	0.1 mg/m³	0.3 mg/m³	1 mg/m³	3 mg/m³
24 months	animal 1	0.07	4.33	9.63	25.62	45.05
	animal 2	0.10	2.22	12.30	18.43	33.39
	animal 3	*	4.87	7.19	22.61	54.00
	animal 4	*	3.91	9.95	30.22	47.11
	mean	**0.09**	**3.83**	**9.77**	**24.22**	**44.89**
	± SD	0.02	1.15	2.09	4.97	8.57

*: only two control animals were available

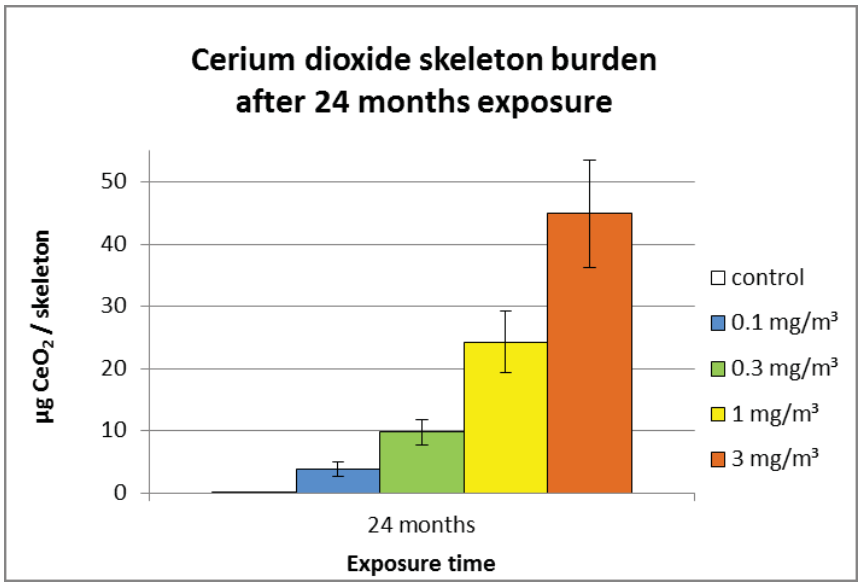

Figure 12: CeO_2 skeleton burden [µg CeO_2/skeleton]

The CeO_2 concentrations clearly increased with increasing inhalation dose. Unfortunately, bone samples were only available for the termination time point 24 month. CeO_2 concentrations are given in µg/whole skeleton. As whole bone amount of the body weight in rats, 6 % and a homogenous distribution of CeO_2 in bone are anticipated. This resulted in average CeO_2 concentrations between 3.83 and 44.89 µg/whole skeleton in groups 1 and 4, respectively. These calculations refer to average body weights of 250 g, 337 g and 400 g for rats after 3, 12 and 24 month inhalation.
In bone, more CeO_2 was found in comparison to bone marrow (table 12, table 13)

Table 14: CeO_2 blood burden [ng CeO_2/ml]

		Cerium dioxide blood burden				
Exposure time	Value	control	0.1 mg/m³	0.3 mg/m³	1 mg/m³	3 mg/m³
3 months	animal 1	0.31	0.30	1.16	1.38	0.67
	animal 2	4.13	0.00	0.10	0.20	0.10
	animal 3	0.00	0.00	0.22	0.13	0.20
	mean	**1.48**	**0.10**	**0.49**	**0.57**	**0.32**
	± SD	2.30	0.17	0.58	0.70	0.07
12 months	animal 1	0.00	0.00	2.26	0.29	0.76
	animal 2	0.00	0.09	3.74	0.44	0.68
	animal 3	0.09	0.96	1.71	0.61	0.71
	mean	**0.03**	**0.35**	**2.57**	**0.52**	**0.72**
	± SD	0.05	0.53	1.05	0.12	0.04
24 months	animal 1	1.00	1.01	0.66	1.55	0.99
	animal 2	1.03	2.11	1.17	0.81	1.52
	animal 3	*	24.57[+]	0.74	0.50	1.53
	animal 4	*	0.40	0.29	1.38	2.43
	mean	**1.01**	**1.17**	**0.71**	**1.06**	**1.62**
	± SD	0.02	0.86	0.36	0.49	0.60

*: only two control animals were available
[+]: significant outlier; value is not considered for mean value calculations

Blood burden was very low in general. CeO_2 concentrations ranged between 0.10 and 2.57 ng/ml. No clear CeO_2 distribution pattern over time and between dose groups could be derived.

Table 15: CeO_2 concentrations in faeces [µg CeO_2/g faeces]

		Cerium dioxide concentrations in faeces				
Exposure time	Value	control	0.1 mg/m³	0.3 mg/m³	1 mg/m³	3 mg/m³
3 months	animal 1	0.29	1.34	0.89	6.98	28.52
	animal 2	0.34	0.24	0.76	4.38	28.37
	animal 3	0.67	0.47	0.87	6.58	70.70
	mean	**0.44**	**0.68**	**0.84**	**5.98**	**28.44**
	± SD	0.20	0.58	0.07	1.40	0.10
12 months	animal 1	0.25	3.32	5.73	15.67	58.35
	animal 2	0.22	1.58	4.65	9.57	48.24
	animal 3	0.25	1.92	6.19	28.96	30.39
	mean	**0.24**	**2.27**	**5.52**	**18.07**	**45.66**
	± SD	0.02	0.92	0.79	9.92	14.16
24 months	animal 1	0.06	0.92	2.57	5.57	25.30
	animal 2	0.03	0.82	3.26	10.52	16.93
	animal 3	*	0.95	2.22	16.18	14.63
	animal 4	*	0.77	2.46	17.30	7.82
	mean	**0.04**	**0.86**	**2.63**	**12.39**	**16.17**
	± SD	0.02	0.09	0.45	5.43	7.21

*: only two control animals were available

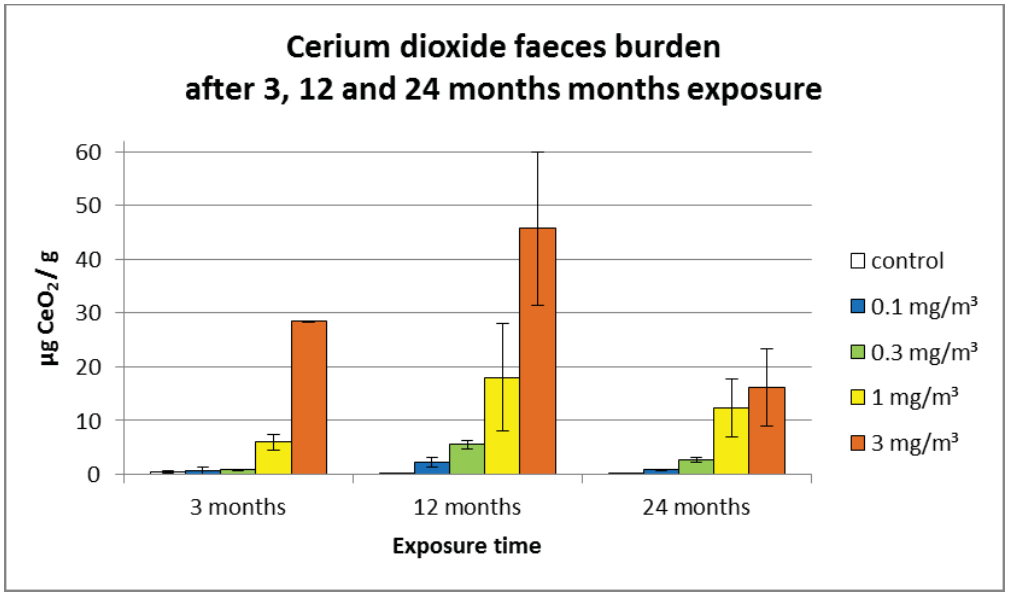

Figure 14: CeO_2 concentrations in faeces [µg CeO_2/g faeces]

CeO_2 concentrations in faeces (figure 14) showed a clear progress in all dose groups between month 3 and month 12, while a decrease was observed between months 12 and 24. This may indicate a slowed clearance through faecal excretion. Average CeO_2 amounts ranged between 0.68 and 45.66 µg/g (table 15). Thus faecal excretion could be in the range of some hundred µg/day.

2.4.2 Particle distribution

The sample preparation of lungs and other organs out of the 2 year inhalation study for ToF-SIMS is performed by partner no. 13 in cooperation with ITEM Hannover. ToF-SIMS and IBM analysis will be performed during the next months.

2.5 Evaluation and conclusions

The examination of CeO_2 concentrations in organs and faeces out of the 2 year inhalation study with CeO_2 nanoparticles (NM-212) according to OECD TG no. 453 has been completed successfully. Method validations and quantification of cerium dioxide nanoparticles in tissues of lung, liver, spleen, kidney, brain, olfactory bulb, heart, mesenteric and lung associated lymph nodes, small intestine, bone marrow, bone, blood and faeces were performed in groups of 1,2,3,4 and the control groups after inhalation for 3, 12 and 24 months.

While studies on organ burden quantification (Task 4.3) progressed according to the project planning, there is a delay of the results on particle distribution pattern (Task 4.4) due to a delayed availability of samples. Further attention needs to be given to the close cooperation with histopathological and histochemical tasks in order to identify molecular changes that are relevant for toxic or carcinogenic effects.

2.5.1 Organ burden

In this deliverable (4.6) we reported the CeO_2 amounts in liver, spleen, kidney, brain, heart, mesenteric and lung associated lymph nodes, small intestine, bone marrow, bone, blood and faeces. There is a significant correlation between inhaled aerosol concentration and organ burden and faecal concentration, respectively. In all tissues despite blood, significant amounts of CeO_2 could be detected. Among all examined matrices, concentrations of ceria were highest in LALN, faeces, bone and bone marrow and also liver. Tissues of blood, brain, heart and mesenteric lymph nodes showed low and very low concentrations for all dose groups 1-4. Generally, CeO_2 concentrations increased with exposure time and with increasing dose. However, compared to the progression of lung burden between months 3 and 24 (deliverable 4.5), no linear increase of CeO_2 burden could be observed for any other organs or faeces.

Open questions address the storage of CeO_2 in bone and the dissolution of ceria under circumstances of low pH-values like in the liver. Examinations by ToF-SIMS may give hints about particle distribution and the chemical status of CeO_2 in bone and liver.

2.5.2 Particle distribution

The sample preparation of lungs for ToF-SIMS will be done by partner no. 13 in cooperation with ITEM Hannover and is still in progress.

2.6 Data management

The methods and endpoints addressed within this deliverable 4.6 (ICP-MS analysis and ToF-SIMS analysis) were reported to CIRABC and are available as ISA-TAB-Nano templates online.

3 Deviations from the work plan

There is a delay of the results on particle distribution pattern (Task 4.4) due to a delayed availability of samples. Further attention needs to be given to the close cooperation with histopathological and histochemical tasks in order to identify molecular changes that are relevant for toxic or carcinogenic effects.

4 References / Selected sources of information (optional)

No

5 List of abbreviations (optional)

No

Annexes (optional)

No