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Organ burden and particle detection pattern in other organs after subacute exposure

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4		

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1. Description of task

Digestion and dilution of up to 1200 tissue samples in triplicate from lungs, olfactory bulbs, blood, lung-associated lymph nodes, spleen, liver, kidney, brain, heart, blood, feces for ICP-MS analysis starting with the high dose. The analyses will be targeted in a way that analyses will begin with the high dose in each organ to be studied. In case there are no GBP detectable, the lower dose will not be tested.

Preparation of inductively coupled plasma mass spectrometry (ICP-MS) equipment for analysis of up to 2600 organ tissue samples (installation of hydrofluoric acid kit). Running the analysis of organ samples. Maintenance of analytical equipment. The oral uptake will be quantified by feces, via feces analyses after 12 and 24 months (10 animals per dose and time, respectively). (BfR)

Preparation of up to 500 organ tissue samples that have given positive results with ICP-MS for structural analysis by time of flight secondary ion mass spectrometry (ToF-SIMS). These studies will be accompanied by Ion Beam Microscopy. Running the analysis of ca. 500 organ tissue samples. Maintenance of analytical equipment.

2. Description of work & main achievements

2.1 Summary

In a 28 day study, conducted according to OECD TG 412, CeO_2 nanoparticles (NM212, Ø 28 nm) were used as an exemplary substance to investigate potential low dose effects caused by chronic inhalation.

For this study, which was a range finding study for further examinations, focussing on potential chronic effects after long term low dose inhalation of CeO_2 (OECD TG 453), we examined organ burden of CeO_2 nanoparticles in the peripheral organs liver, kidney, blood, spleen, brain, heart and olfactory bulb on study days 28, 30/36, 62, 92 and 156.

The amount of cerium found in the examined organs is: liver > spleen > kidney > blood > heart, brain. The state of the art investigation showed particle retention depending on the dose group.

The 28 day study investigated three different dose groups: 0.5 mg/m³ (Low dose, LD), 5 mg/m³ (Mid dose, MD), and 25 mg/m³ (High dose, HD). The amount of CeO_2 deposited in the lung was already reported in deliverable 4.3.
These findings were taken as benchmark for the determination of the organ burden of the other secondary organs like liver and kidney.

Taking the administered amount of CeO_2 as percentage of the inhaled dose as a starting point, LD and MD showed a higher amount of inhaled CeO_2retained in liver and kidney compared to HD.
In contrast, for spleen at LD the percentage of inhaled dose of CeO_2 was lower than in MD and HD.

Looking at the absolute organ burden (ng / organ) a slightly different picture was found. The organ burden of CeO_2 in liver, spleen and kidney ranged as follows: HD > MD > LD. However, for spleen the organ burden was different.
Very low amounts of CeO_2 could be detected in blood which might be an indicator for the rapid distribution to peripheral organs after inhalative uptake and deposition in the lung. Additionally, low amounts of CeO_2 were detected in brain and heart. In contrary to the situation in the lung (please see deliverable 4.3), no linear correlation between dosage and CeO_2 burden was found in peripheral organs.

Based on these findings the following conclusions for the clearance mechanisms can be assumed. The process of clearance appears to be similar for all dose groups, when taking into account total peripheral organ burden (ng / organ) of CeO_2. For all three dose groups there was an increase of CeO_2 from the last day of inhalation till day 8 post exposure (MD and HD) and day 34 post exposure (LD), respectively. After that, the CeO_2 levels decreased till day 64 post exposure, before the CeO_2 levels increased again (day 128 post exposure). This suggests a continuous translocation of CeO_2 from lung to liver, spleen and kidney. Phagocytosis is assumed as main mechanism, responsible for clearance of particles.

Out of the investigation of tissue of the 28 day study we conclude that it is possible to determine the localization of CeO_2 particles in lung and liver tissue sections using imaging mass spectrometry (ToF-SIMS). Based on this finding, the ToF-SIMS technique will be used as a tool to study particle uptake and fate in organ tissues out of the 2-year inhalation study. The results achieved within ToF-SIMS investigations further demonstrate, that the technique is a suitable tool for identification of specific areas within the organ, where particle accumulation occurs. The ICP-MS results reported in D 4.3 and D 4.4 are in line with the ToF-SIMS results, a higher particle number concentration was detected in lung compared to liver.
The findings on organ burden and particle distribution confirm that liver, spleen and kidney are main target organs for CeO_2 following inhalative uptake. In blood, heart and brain lower concentrations in the ppb range were measured.

The comparison of organ burden (ICP-MS) and particle distribution pattern (ToF-SIMS and IBM), revealed that the CeO_2 found in secondary organs is mostly present in the form of particles. The pictures of particle distribution generated with ToF-SIMS and IBM suggest particle deposition in the lung followed by phagocytosis and translocation of agglomerates to peripheral organs.

Additionally, results of the 28 day study (deliverables 4.3 and 4.4) are serving as a basis for analysis of organ tissues out of the 2-year inhalation study. Preparations for the 28 day study was also used to develop and validate all necessary methods needed.
This will give the opportunity to compare organ burden, particle distribution and histopathological results to subsequent adverse effects as fibrosis, inflammation or formation of tumors.
Regarding the long-term inhalative exposure to low doses of CeO_2 (following deliverables), it might be possible to draw conclusions regarding conditions of overload and NOEAL for CeO_2 nano particles.

2.2 Description of the work carried out

2.2.1 Organ burden

2.2.1.A *Analytical Task*

The task within the deliverable was the determination of cerium in peripheral organs (liver, kidney, blood, spleen, brain, heart) and the particle distribution in organ samples, which gave positive results with the ICP-MS, after inhalation exposure in samples from the 28 day BASF CeO_2-study.

2.2.1.B *Method Development*

An appropriate analytical method was developed to determine the organ burden of the peripheral organs and blood. This method contains of three individual steps: a) freeze drying, b) wet chemical microwave assisted digestion and c) determination of cerium ions with ICP-MS.
The values of ^{140}Ce and ^{142}Ce isotopes are calculated as [ng CeO_2 / organ]. The analytical range of this method is 0.1 ppb - 20 ppb.

i. Freeze-drying

The roughly chopped organs were separated into samples of approximately 200 mg tissue. The individual tissue samples were homogenised, freeze-dried under vacuum and stored at -20 °C.

ii. Microwave wet-chemical digestion

The freeze-dried tissue samples were digested in separate digestion vessels each with an appropriate mixture of acid and oxidizing agent (2.5 mL H_2O, 2 mL HNO_3 (69 %), 1 mL H_2O_2 (30 %)). A suitable temperature and energy controlled digestion program was used to break down the tissue and transfer the CeO_2 nanoparticles into an ionisable form.
The gathered solutions were diluted further with water including the addition of the two internal standards indium (^{115}In) and lutetium (^{175}Lu). These standards served as bracketing standards at either side of the molecular mass of the two ^{140}Ce and ^{142}Ce isotopes.

iii. ICP-MS analysis

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

2.2.1.C *Validation and Transfer-Exercise*

The method above was in-house validated for all the six peripheral organs and blood. Therefore the tissue samples and blood were spiked with three differed concentrations (0.2 ppb, 2 ppb and 20 ppb).

i. Validation

The peripheral organs and blood 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 following criteria were tested - specificity / selectivity, linearity, stability, accuracy and precision, reproducibility, recovery - for all sample matrices, standards and media. Based on these data the limit of detection (LOD) the limit of quantification (LOQ) as well as the curve fitting and the range of the method was calculated according DIN 32645 “Chemical analysis - Decision limit, detection limit and determination limit under repeatability conditions - Terms, methods, evaluation”. Additionally appropriate system suitability criteria and a suitable certified reference material for cerium (BCR-667) have been selected to receive a high confidence and excellence in the gathered test values.

ii. Transfer-Exercise

In order to obtain values for the robustness of the method and the variability between different laboratories a transfer exercise was carried out between the Fraunhofer institute (ITEM) in Hannover and the BfR using spiked liver samples. The agreement of the two laboratories was 96.7%.

Thus the validation and the transfer-exercise were passed and the method was considered suitable for the purpose.

2.2.2 Particle distribution

2.2.2.A *Analytical Task*

The task according to the proposal was to identify particle clusters of CeO_2 in tissue slices of lungs and peripheral organs, which showed significant organ burden results. These clusters can consist of agglomerates or aggregates.
It was also aimed to detect the particle size of the clusters and to categorize the clusters in lung tissue slices. Additionally imaging mass spectrometry should reveal first information about the localization of the clusters. Liver tissue was analysed to get first information about the agglomeration state of the CeO_2 nano particles.
In a next step the particle detection patterns together with the chemical information of the ToF-SIMS analysis and the quantification results from the ion beam microscopy (IBM) will be combined to get all information from one distinct area of the tissue sections.

2.2.2.B *Sample preparation*

Paraffinated as well as deparaffinated lung slices were received from BASF. Deparaffinated samples were included in order to exclude the influence of paraffin onto ToF-SIMS investigations. Here is a brief description of the method used for sample preparation for ToF-SIMS and IBM samples: the tissue was fixed in formalin until microtome dissection. For microtome dissection the formalin fixed tissue was embedded in paraffin. Then 3 µm and 7 µm thick slices were cut. Half of the slices for the ToF-SIMS analysis were applied on gold wafers (3 µm) and subsequently deparaffinated and half of them were supplied as paraffinated samples. The lung slices for IBM (7 µm) were prepared on glass slides.

Frozen liver samples were cryo-sectioned into 3 µm slices on gold wafers, which were subsequently analysed using ToF-SIMS.

2.2.2.C *ToF-SIMS analysis*

All ToF-SIMS analysis was performed on a TOF-SIMS V instrument (ION-TOF GmbH). The TOF-SIMS V instrument was equipped with a Nano-Bismuth liquid metal cluster primary ion source. High resolution image data were recorded in burst alignment mode using Bi_3^+ primary ions. All images were recorded at 25keV acceleration voltage, using electron flooding for charge neutralization. The accumulated primary ion dose was kept below the static limit at 4 x 10^{12} ions/cm^2. Images were recorded using 512 x 512 pixels final resolution. All raw data has been collected and analyzed using the SurfaceSpec 6 software (ION-TOF GmbH). All cluster counting and categorization, where clusters are comprised of agglomerates or aggregates, was done using the freeware software programme DotCount v1.2 (MIT freeware programme).

2.2.2.D *IBM analysis*

The analysis was performed with particle-induced X-ray emission (proton-induced X-ray emission (PIXE)). This is a technique used to determine the elemental make-up of the tissue sections. When the tissue slice is exposed to an ion beam, atomic interactions occur that give off electro magnetic radiation of wavelengths in the x-ray part of the electromagnetic spectrum specific to an element. Thus, PIXE is considered a powerful yet non-destructive elemental analysis technique useful for the quantification of nano particles.

2.3 Results

The following results refer to the examined organs (lung and liver tissue sections, kidney, spleen, heart, brain, olfactory bulb and blood as whole organs). All organs mentioned, beside lung, were used for determination of organ burden of the 28 day inhalation study with CeO_2 nanoparticles. The results on lung burden of the 28 day study were already reported with deliverable 4.3.

Lung tissue samples (HD and control samples) were analysed using imaging mass spectrometry (ToF-SIMS) and IBM to get insights about the tissue distribution of the CeO_2 nano particle clusters and to quantify the CeO_2 clusters within lung tissue samples. Liver tissue slices were analysed using ToF-SIMS to get first insights into the CeO_2 distribution in the liver.

Beside lung and liver, no further organs were investigated using imaging techniques due to the low concentration of CeO_2 detected by ICP-MS.

2.3.1 Organ burden

All concentrations measured with ICP-MS are mean values based on three (n = 3) measurements with ICP-MS. For liver and kidney organs of three animals were examined on day 28. The mean values of three organs each were again calculated as mean value for liver and kidney respectively. The results of CeO_2 per organ where calculated according to the following table:

Organ	Sampling procedure	Number of samples	Mean value of CeO_2

liver	trim of were cut off; only inner part of left hepatic lobe was used	5	from average of 5 samples extrapolated to whole organ
kidney	whole kidneys were sampled	5 (almost always one kidney)	from average of 5 samples extrapolated to whole organ
spleen	whole spleen was sampled	3 or 4 (depending on total spleen weight)	summed up for whole organ
heart	whole heart was sampled	4	from average of 4 samples extrapolated to whole organ
brain	olfactory bulb was separated; whole organ except olf. bulb was sampled	4	from average of 4 samples extrapolated to whole organ
blood	whole blood was sampled	1 to 3	from total blood volume extrapolated to 20 mL blood per rat

Table 1: Overview – calculation of organ burden

The following table shows the organs per animal number examined for organ burden.

Organ	Day	Group 0 air (control) 0 mg/m^3	Group 1 CeO_2 (low dose) 0.5 mg/m^3	Group 2 CeO_2 (mid dose) 5 mg/m^3	Group 3 CeO_2 (high dose) 25 mg/m^3
Liver	d28	0/1; 0/2; 0/6	1/131;1/32; 1/36	2/61; 2/62; 2/66	3/91; 3/92; 3/96
	d30	-	-	-	-
	d36	-	1/43	2/73	3/103
	d62	0/20	1/49	2/79	3/110
	d92	0/25	1/55	2/85	3/115
	d156	0/26	1/56	2/86	3/116
Kidney	d28	0/1; 0/2; 0/6	1/131; 1/32;1/36	2/61; 2/62; 2/66	3/91; 3/92; 3/96
	d30	-	-	-	-
	d36	-	1/43	2/73	3/103
	d62	0/20	1/49	2/79	3/110
	d92	0/25	1/55	2/85	3/115
	d156	0/26	1/56	2/86	3/116
Spleen	d28	0/1	1/32	2/61	3/91
	d30	0/12	-	-	-
	d36	-	1/42	2/73	3/103
	d62	0/19	1/49	2/79	3/109
	d92	0/25	1/55	2/85	3/115
	d156	0/26	1/56	2/86	3/116
Brain	d28	0/6	1/36	2/66	3/96
	d30	0/12	-	-	-
	d36	-	1/43	2/73	3/103
	d62	0/24	1/54	2/84	3/114
	d92	0/25	1/55	2/85	3/115
	d156	0/26	1/56	2/86	3/116

Table 2: Overview of analysed organs per animal for each study group and time point (to be continued)

Organ	Day	Group 0 air (control) 0 mg/m^3	Group 1 CeO_2 (low dose) 0.5 mg/m^3	Group 2 CeO_2 (mid dose) 5 mg/m^3	*Group 3 CeO_2 (high dose) 25 mg/m^3*
Heart	d28	0/6	1/36	2/66	3/96
	d30	0/12	-	-	-

	d36	-	1/43	2/73	3/103
	d62	0/24	1/54	2/84	3/114
	d92	-	1/55	2/85	3/115
	d156	0/26	1/56	2/86	3/116
Blood	d28	0/6	1/36	2/66	3/96
	d30	0/12	-	-	-
	d36	-	1/43	2/73	3/103
	d62	-	1/54	2/84	3/114
	d92	0/25	1/55	2/85	3/115
	d156	0/26	1/56	2/86	3/116

Table 3: Overview of analysed organs per animal for each study group and time point (to be continued)

The following tables show the calculated amount of CeO_2 for a specific organ determined as explained above.
The stated amount of CeO_2 refers to isotope $^{140}CeO_2$, which was calculated based on internal standard ^{115}In. Results equal to the ones stated were obtained for isotope $^{142}CeO_2$ and internal standard ^{175}Lu respectively.

Note:

Group 0: Dose 0 mg/m³ (control)
Group 1: Dose 0.5 mg/m³ (low dose)
Group 2: Dose 5 mg/m³ (mid dose)
Group 3: Dose 25 mg/m³ (high dose)

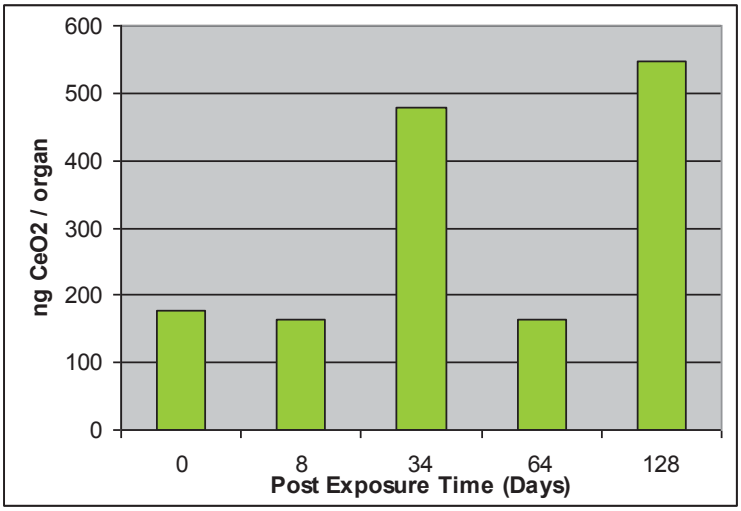
Organ	Day	Group	animal	CeO_2 [ng/organ]	absolute standard deviation [ng/organ]	relative standard deviation [%]
Liver	28	0	0/1	15.1	0.0	0.53
		0	0/2	15.8	0.0	0.22
		0	0/6	20.3	0.0	1.91
		0	**mean**	**17.1**	0.0	0.89
	62	0	0/20	5.3	0.001	0.93
	92	0	0/25	1.55	0.001	1.1
	156	0	0/26	3.2	0.001	0.39
Liver	28	1	1/31	190.9	0.0	0.55
		1	1/32	226.8	0.0	0.17
		1	1/36	116.7	0.0	0.22
		1	**mean**	**178.1**	0.0	0.31
	36	1	1/43	164.6	0.0	0.22
	62	1	1/49	477.6	0.0	0.26
	92	1	1/55	164.5	0.0	0.34
	156	1	1/56	546.2	0.0	0.35
Liver						

Table 4: Calculated amount of $^{140}CeO_2$ for liver; group 0 and group 1

Organ	Day	Group	animal	CeO2 [ng/organ]	absolute standard deviation [ng/organ]	relative standard deviation [%]
Liver	28	2	2/61	755.4	0.0	1.1

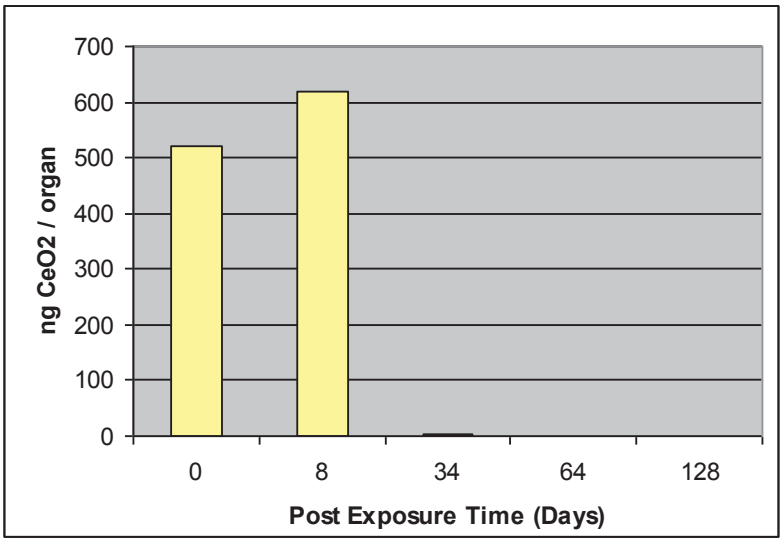
		2	2/62	378.7	0.0	0.43
		2	2/66	426.9	0.0	0.11
		2	**mean**	**520.4**	0.0	0.55
	36	2	2/73	620.2	0.0	0.14
	62	2	2/79	3.18	0.001	0.81
	92	2	2/85	0.55	0.001	1.1
	156	2	2/86	1.07	0.002	3.1
						
Liver	28	3	3/91	1166.4	0.0	1.17
		3	3/92	1132.2	0.0	0.18
		3	3/96	611.6	0.0	0.15
		3	**mean**	**970.1**	0.0	0.5
	30	3	3/102	1.26	0.0	0.26
	36	3	3/103	15.8	0.0	2.2
	62	3	3/110	3.91	0.0	0.0
	92	3	3/115	4.01	0.0	1.62
	156	3	3/116	1.26	0.0	1.85

Table 5: Calculated amount of $^{140}CeO_2$ for liver; group 2 and group 3 (to be continued)

Organ	Day	Group	animal	CeO2 [ng/organ]	absolute standard deviation [ng/organ]	relative standard deviation [%]

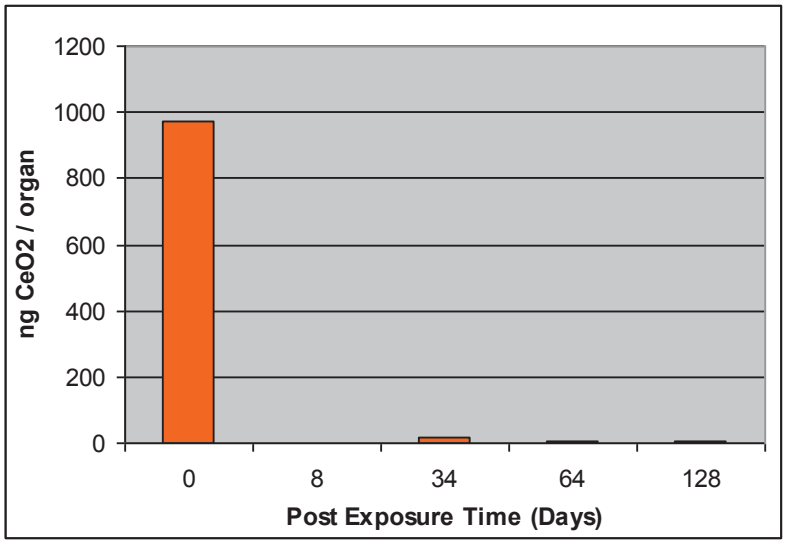
Liver	

Table 6: Calculated amount of $^{140}CeO_2$ for liver; group 3 (continued)

Organ	Day	Group	animal	CeO_2 [ng/organ]	absolute standard deviation [ng/organ]	relative standard deviation [%]
Kidney	28	0	0/1	2.38	0.0	5.9
		0	0/2	1.63	0.0	11.1
		0	0/6	0.27	0.0	6.8
		0	**mean**	**1.55**	0.0	7.9
	62	0	0/20	1.16	0.0	8.6
	92	0	0/25	0.89	0.0	6.4
	156	0	0/26	0.85	0.0	6.3
Kidney	28	1	1/31	6.41	0.0	6.6
		1	1/32	4.7	0.0	3.4
		1	1/36	2.8	0.0	2.7
		1	**mean**	**4.64**	0.0	4.2
	36	1	1/43	4.44	0.0	2.1
	62	1	1/49	19.58	0.001	4.1
	92	1	1/55	6.06	0.0	1.9
	156	1	1/56	9.31	0.0	1.2

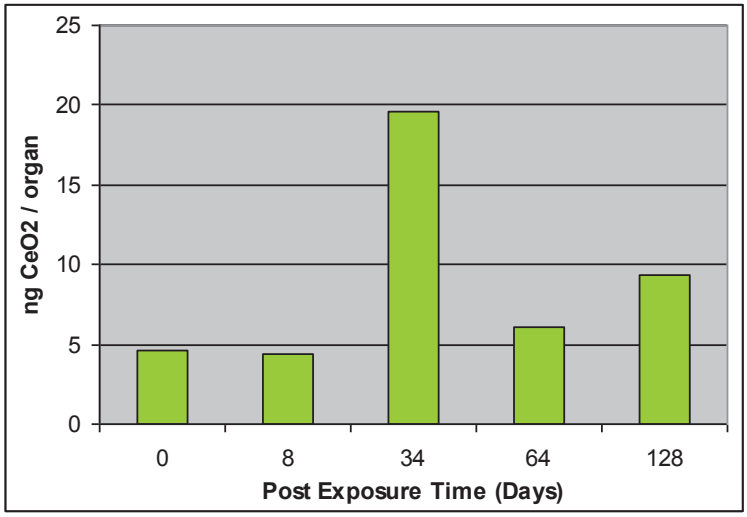


Table 7: Calculated amount of $^{140}CeO_2$ for kidney; group 0 and group 1

Organ	Day	Group	animal	CeO_2 [ng/organ]	absolute standard deviation [ng/organ]	relative standard deviation [%]

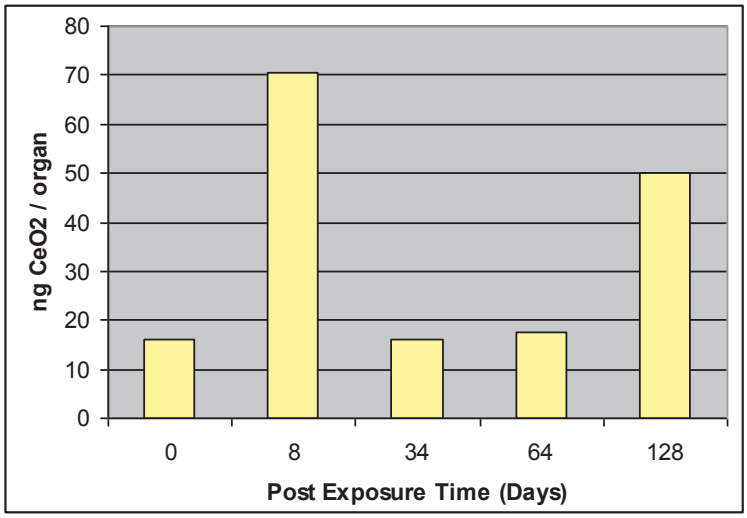
Kidney	28	2	2/61	16.3	0.0	3.1
		2	2/62	18.9	0.0	5.2
		2	2/66	12.9	0.0	2.0
		2	**mean**	**16.0**	0.0	3.4
	36	2	2/73	70.4	0.0	3.3
	62	2	2/79	16.0	0.0	1.0
	92	2	2/85	17.6	0.0	2.0
	156	2	2/86	50.2	0.0	1.5
						
Kidney	28	3	3/91	28.0	0.0	1.7
		3	3/92	36.8	0.0	8.2
		3	3/96	17.7	0.0	2.1
		3	**mean**	**27.5**	0.0	4.0
	36	3	3/103	29.5	0.0	1.8
	62	3	3/110	73.8	0.0	4.5
	92	3	3/115	55.8	0.0	1.1
	156	3	3/116	102.9	0.0	1.3

Table 8: Calculated amount of $^{140}CeO_2$ for kidney; group 2 and group 3 (to be continued)

Organ	Day	Group	animal	CeO_2 [ng/organ]

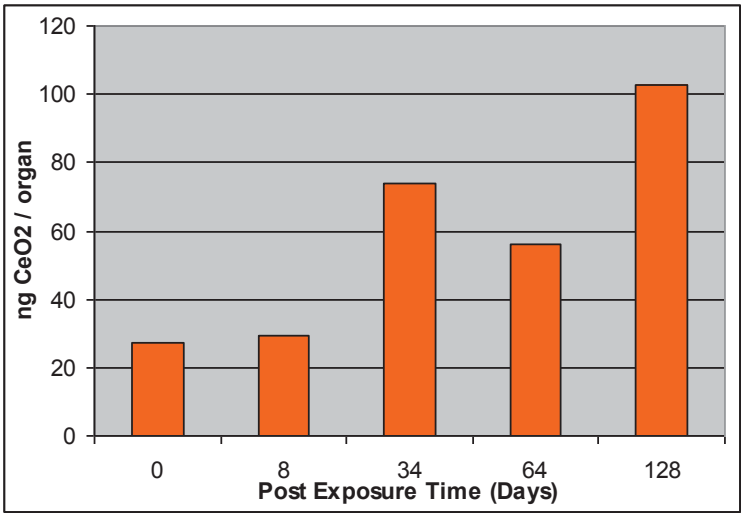


Table 9: Calculated amount of $^{140}CeO_2$ for kidney; group 3 (continued)

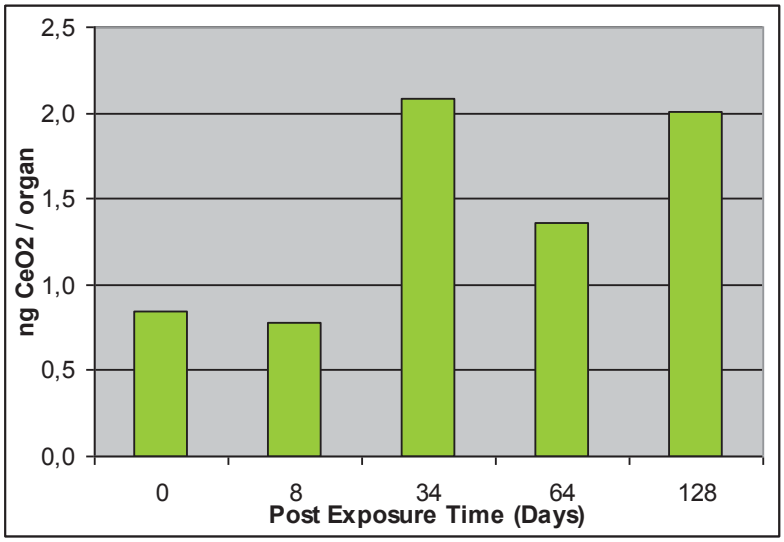
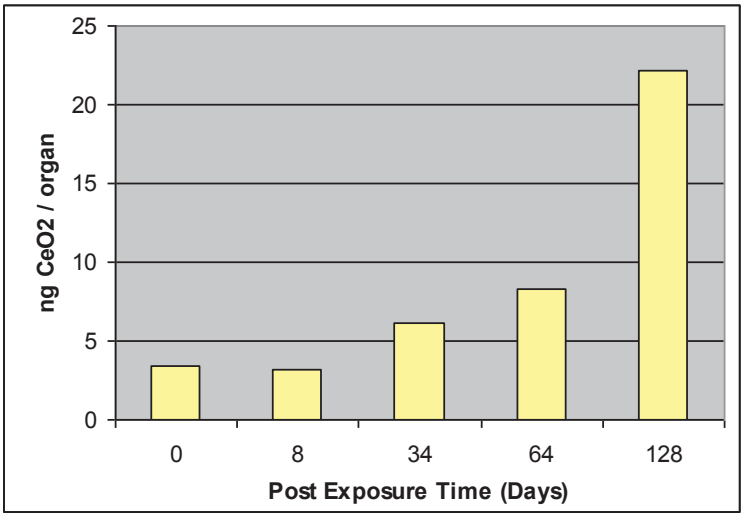
Organ	Day	Group	animal	CeO_2 [ng/organ]
Spleen	28	0	0/1	0.52
	30	0	0/12	0.13
	62	0	0/19	0.26
	92	0	0/25	0.05
	156	0	0/26	0.11
Spleen	28	1	1/32	0.84
	36	1	1/42	0.78
	62	1	1/49	2.08
	92	1	1/55	1.36
	156	1	1/56	2.00
				
Spleen	28	2	2/61	3.43
	36	2	2/73	3.22
	62	2	2/79	6.10
	92	2	2/85	8.26
	156	2	2/86	22.17

Table 10: Calculated amount of $^{140}CeO_2$ for spleen; group 0, group 1 and group 2 (to be continued)

Organ	Day	Group	animal	CeO_2 [ng/organ]



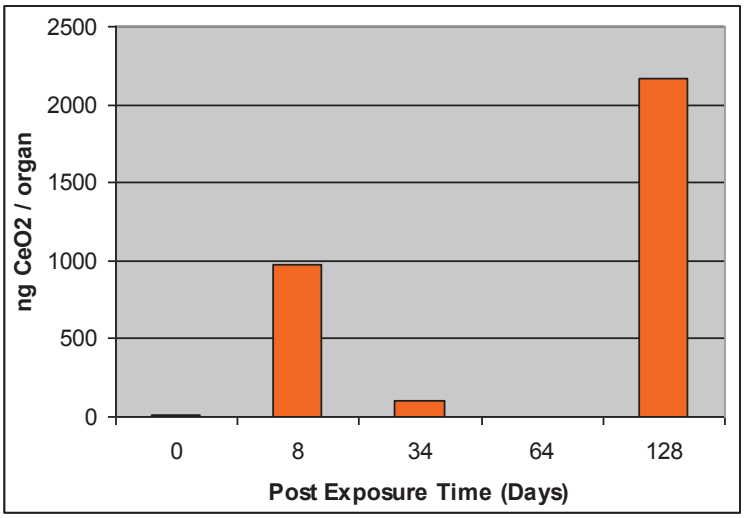
	28	3	3/91	9.07
	36	3	3/103	979.0
	62	3	3/114	106.7
	92	3	3/115	0.06
	156	3	3/116	2165.8
Spleen				

Table 11: Calculated amount of $^{140}CeO_2$ for spleen; group 2 and group 3 (continued)

Organ	Day	Group	animal	CeO_2 [ng/organ]	absolute standard deviation [ng/organ]	relative standard deviation [%]
Brain	28	0	0/6	0.77	0.03	3.9
	30	0	0/12	0.67	0.02	2.2
	62	0	0/24	1.19	0.07	5.8
	92	0	0/25	0.93	0.03	3.5
	156	0	0/26	0.76	0.02	3.1
Brain	28	1	1/36	0.54	0.008	1.5
	36	1	1/43	0.90	0.05	5.0
	62	1	1/54	0.70	0.02	2.7
	92	1	1/55	1.29	0.08	6.3
	156	1	1/56	1.20	0.03	2.3
Brain	28	2	2/66	2.5	0.09	3.5
	36	2	2/73	1.27	0.04	2.9
	62	2	2/84	1.17	0.03	2.2
	92	2	2/85	1.37	0.09	6.3
	156	2	2/86	1.31	0.04	2.8

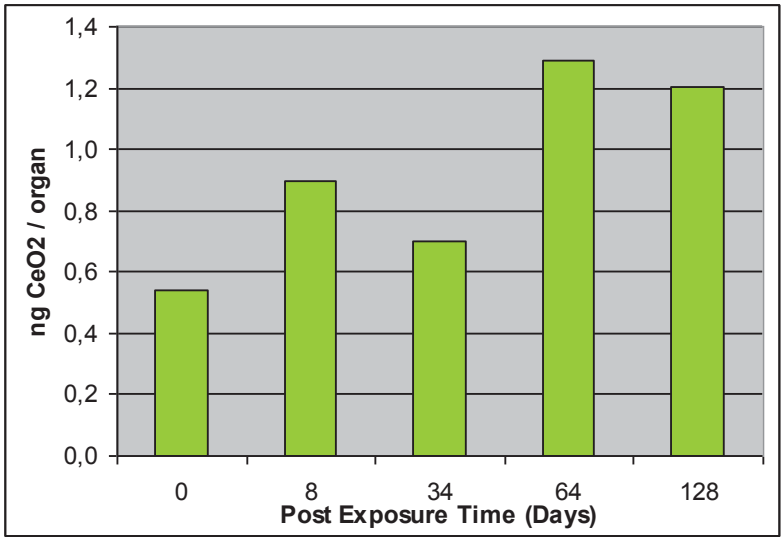


Table 12: Calculated amount of $^{140}CeO_2$ for brain; group 0, group 1 and group 2 (to be continued)

Organ	Day	Group	animal	CeO_2 [ng/organ]	absolute standard deviation	relative standard deviation

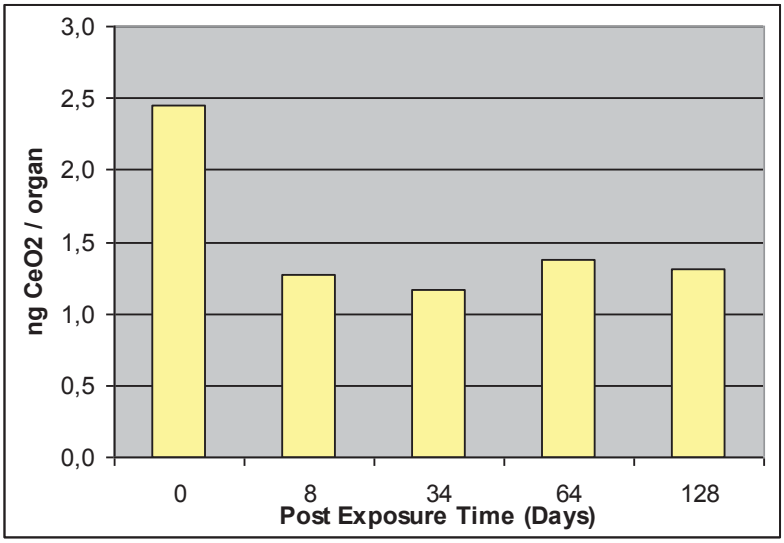
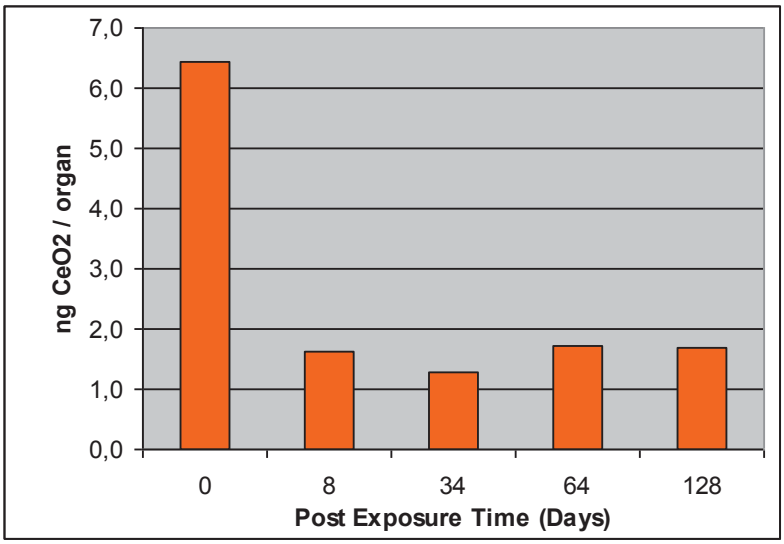
					[ng/organ]	[%]
Brain						
Brain	28	3	3/96	6.45	0.67	10.4
	36	3	3/103	1.61	0.05	3.3
	62	3	3/114	1.29	0.07	5.1
	92	3	3/115	1.71	0.11	6.7
	156	3	3/116	1.69	0.07	4.0
						

Table 13: Calculated amount of $^{140}CeO_2$ for spleen; group 2 and group 3 (continued)

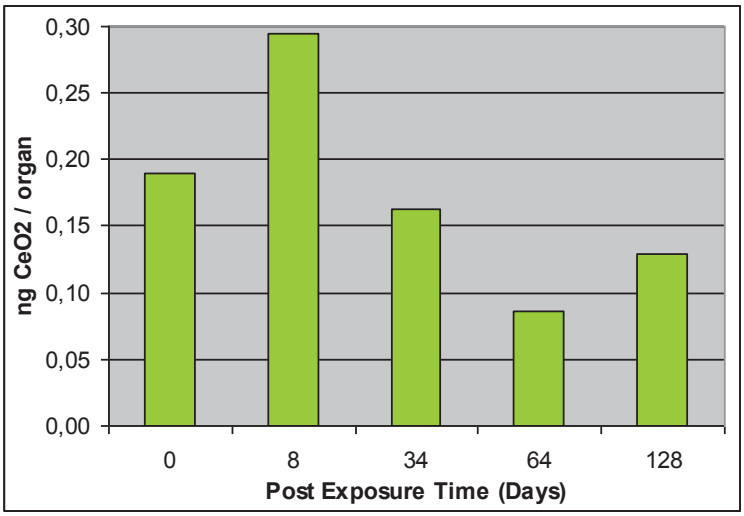
Organ	Day	Group	animal	CeO_2 [ng/organ]
Olfactory bulb	28	0	0/6	0.02
	30	0	0/12	0.04
	62	0	0/24	0.06
	156	0	0/26	0.02
Olfactory bulb	28	1	1/36	0.19
	36	1	1/43	0.30
	62	1	1/54	0.16
	92	1	1/55	0.09
	156	1	1/56	0.13
				
Olfactory bulb	28	2	2/66	0.25
	36	2	2/73	0.49
	62	2	2/84	0.40
	92	2	2/85	0.16
	156	2	2/86	0.20

Table 14: Calculated amount of $^{140}CeO_2$ for olfactory bulb; group 0, group 1 and group 2 (to be continued)

Organ	Day	Group	animal	CeO_2 [ng/organ]

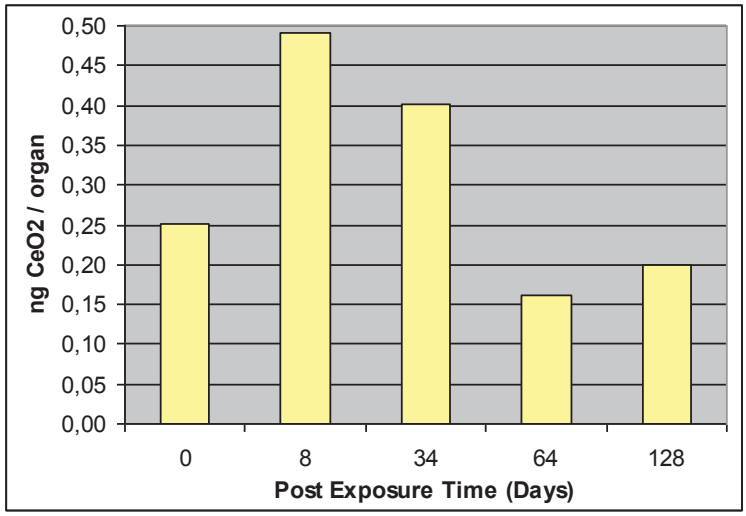
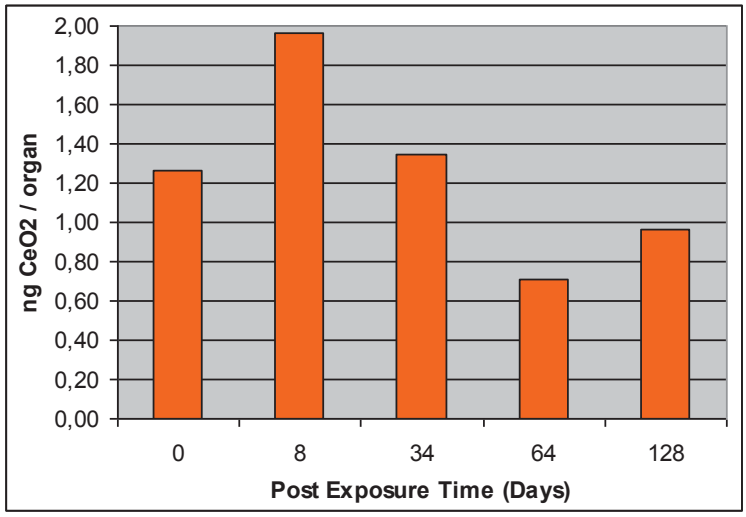
Olfactory bulb				
	28	3	3/96	1.26
	36	3	3/103	1.97
	62	3	3/114	1.34
	92	3	3/115	0.71
	156	3	3/116	0.97
Olfactory bulb				

Table 15: Calculated amount of $^{140}CeO_2$ for olfactory bulb; group 2 and group 3 (continued)

Organ	Day	Group	animal	CeO_2 [ng/organ]
Heart	28	0	0/6	0.19
	30	0	0/12	0.57
	62	0	0/24	0.19
	156	0	0/26	0.21
Heart	28	1	1/36	0.48
	36	1	1/43	0.14
	62	1	1/54	0.51
	92	1	1/55	0.52
	156	1	1/56	0.63
Heart	28	2	2/66	0.87
	36	2	2/73	2.70
	62	2	2/84	1.38
	92	2	2/85	1.24
	156	2	2/86	4.69

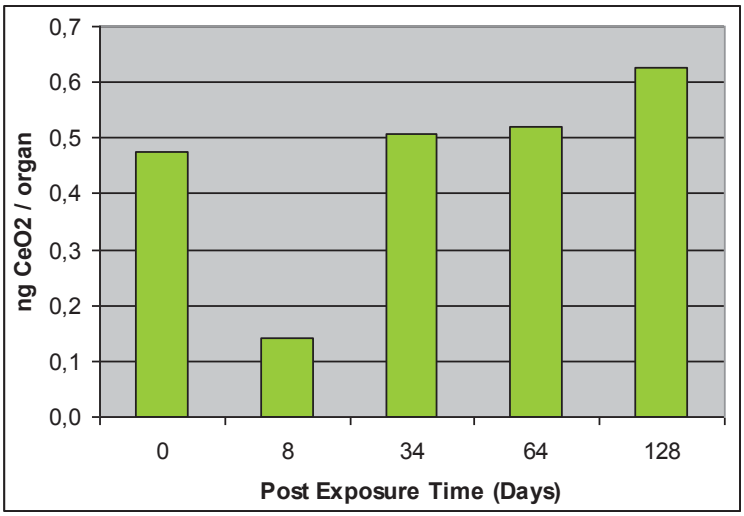


Table 16: Calculated amount of $^{140}CeO_2$ for heart; group 0, group 1 and group 2 (to be continued)

Organ	Day	Group	animal	CeO_2 [ng/organ]

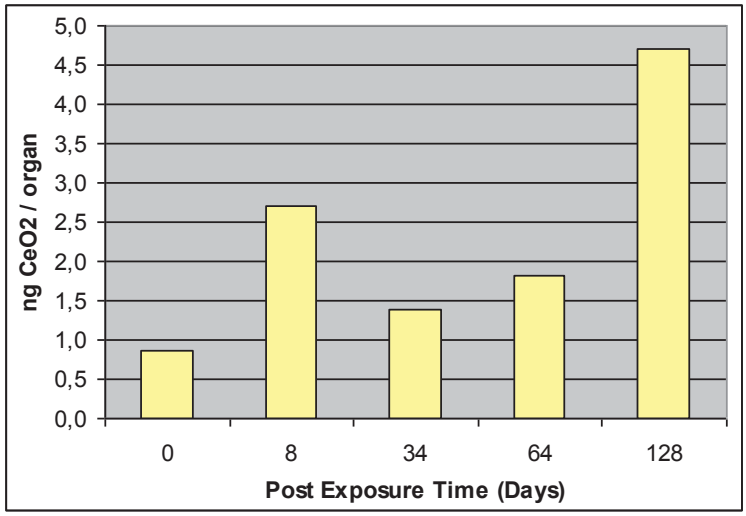
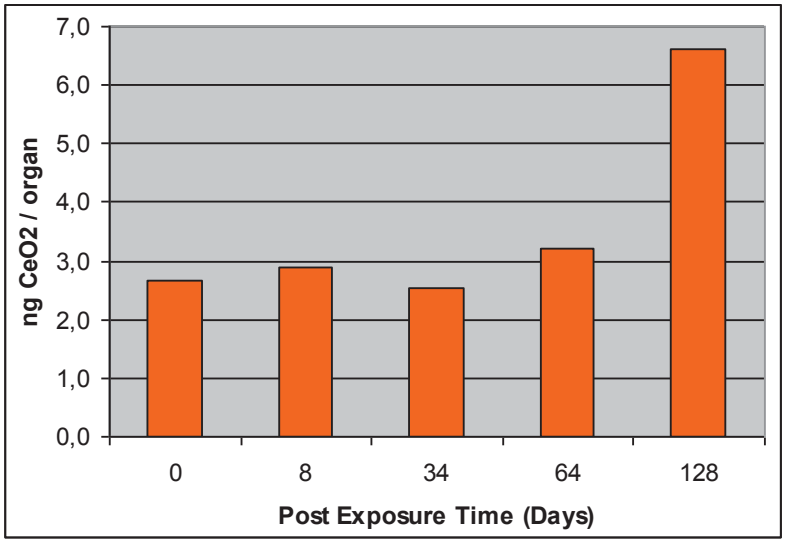
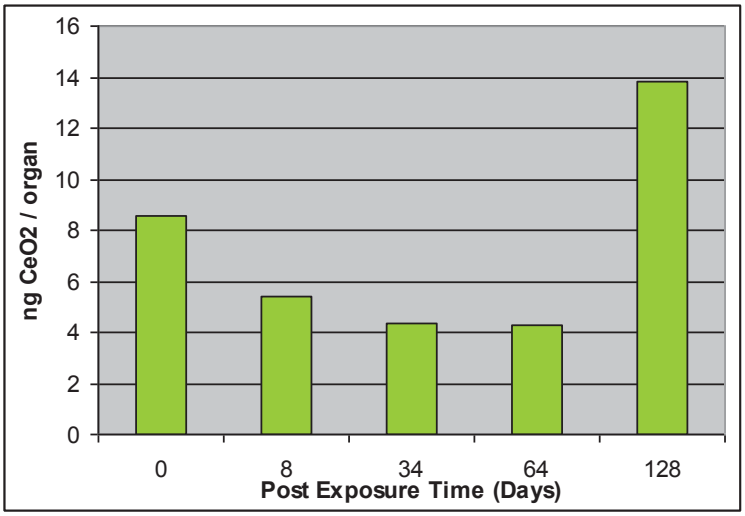
Heart				
	28	3	3/96	2.68
	36	3	3/103	2.87
	62	3	3/114	2.52
	92	3	3/115	3.21
	156	3	3/116	6.62
Heart				

Table 17: Calculated amount of $^{140}CeO_2$ for heart; group 2 and group 3 (continued)

Organ	Day	Group	animal	CeO_2 [ng/20ml]	absolute standard deviation [ng/20 ml]	relative standard deviation [%]
Blood	28	0	0/6	8.98	0.00	0.68
	30	0	0/12	26.4	0.00	1.33
	92	0	0/25	7.3	0.00	0.25
	156	0	0/26	4.03	0.00	0.21
Blood	28	1	1/36	8.55	0.00	0.78
	36	1	1/43	5.42	0.00	0.97
	62	1	1/54	4.35	0.00	0.62
	92	1	1/55	4.28	0.00	1.3
	156	1	1/56	13.85	0.00	0.0



Organ	Day	Group	animal	CeO_2 [ng/20ml]]	absolute standard deviation [ng/20 ml]	relative standard deviation [%]
Blood	28	2	2/66	44.4	0.00	0.71
	36	2	2/73	4.07	0.00	0.58
	62	2	2/84	5.94	0.00	0.67
	92	2	2/85	2.61	0.00	0.73
	156	2	2/86	6.65	0.00	0.45

Table 18: Calculated amount of $^{140}CeO_2$ for blood; group 0, group 1 and group 2 (to be continued)

Organ	Day	Group	animal	CeO_2 [ng/20ml]	absolute standard	relative standard

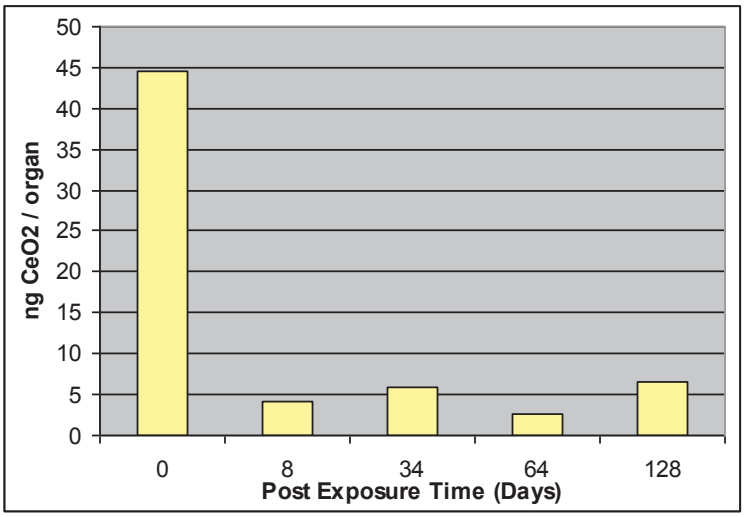
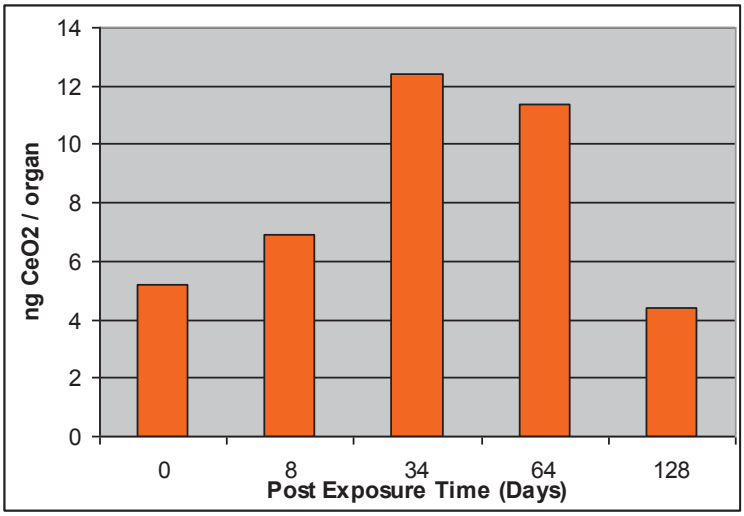
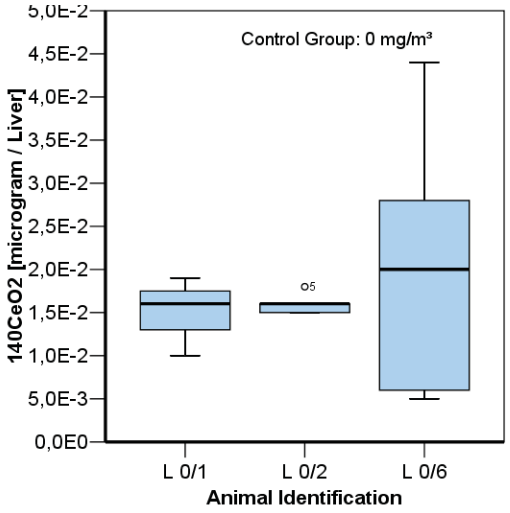
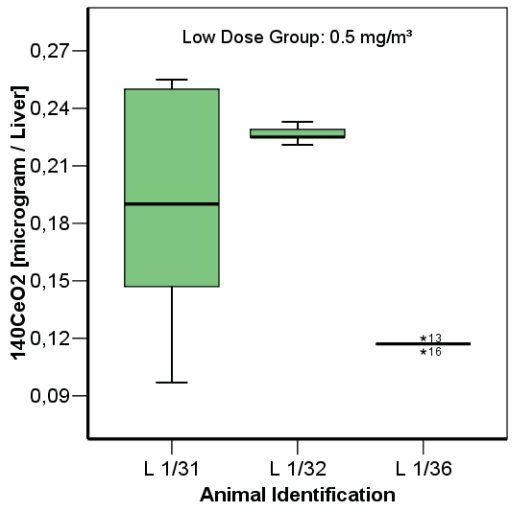
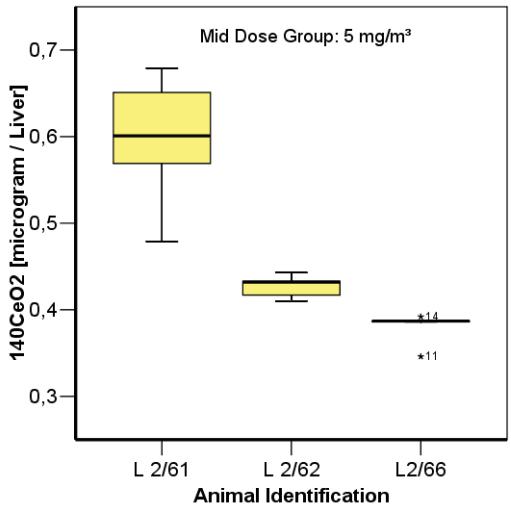
					deviation [ng/20ml]	deviation [%]
Blood						
	28	3	3/96	5.20	0.00	0.91
	36	3	3/103	6.89	0.00	0.51
	62	3	3/114	12.41	0.00	1.47
	92	3	3/115	11.35	0.00	0.47
	156	3	3/116	4.38	0.00	0.52
Blood						

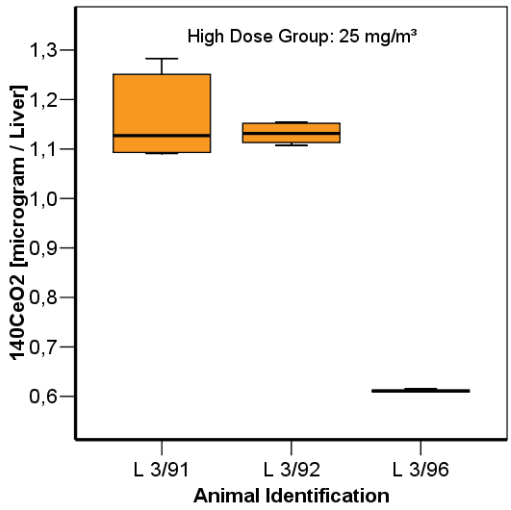
Table 19: Calculated amount of $^{140}CeO_2$ for blood; group 2 and group 3 (continued)

The following tables give an overview of the variability of one dose group for liver and kidney based on three animals each. The animals used for this calculation are from time point day 28.

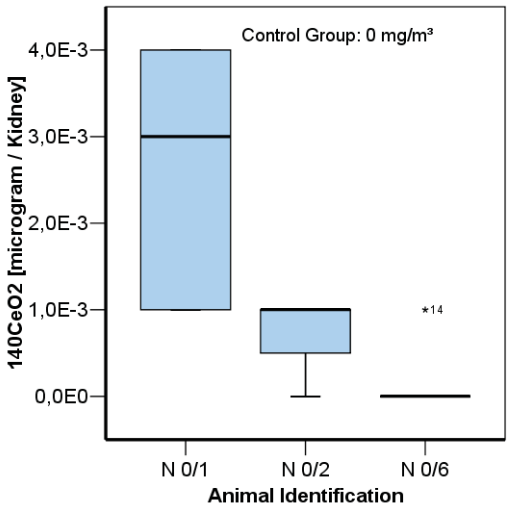
Organ	Day	Group	animal	CeO_2 [µg/organ]	absolute standard deviation [µg/organ]	relative standard deviation [%]
Liver	28	0	0/1	0.015		
		0	0/2	0.016		
		0	0/6	0.020		
		0	**mean**	**0.017**	**0.003**	**15.57**
						

Organ	Day	Group	animal	CeO_2 [µg/organ]	absolute standard deviation [µg/organ]	relative standard deviation [%]
Liver	28	1	1/31	0.19		
		1	1/32	0.23		
		1	1/36	0.12		
		1	**mean**	**0.18**	**0.056**	**30.93**
						

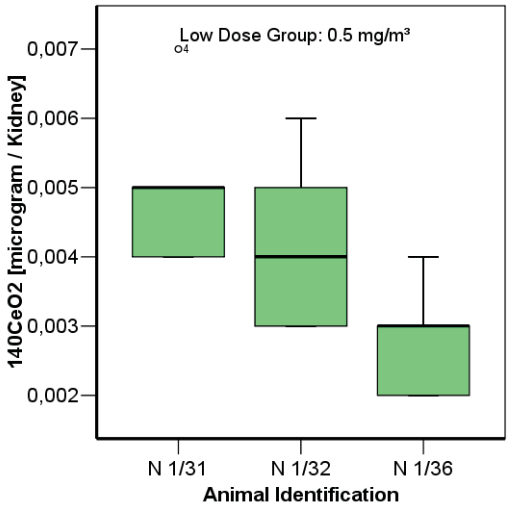
Organ	Day	Group	animal	CeO_2 [µg/organ]	absolute standard deviation [µg/organ]	relative standard deviation [%]
Liver	28	2	2/61	0.76		
		2	2/62	0.43		
		2	2/66	0.38		
		2	**mean**	**0.52**	**0.21**	**39.71**
						

Organ	Day	Group	animal	CeO_2 [µg/organ]	absolute standard deviation [µg/organ]	relative standard deviation [%]
Liver	28	3	3/91	1.17		
		3	3/92	1.13		
		3	3/96	0.61		
		3	**mean**	**0.97**	**0.31**	**32.21**
						

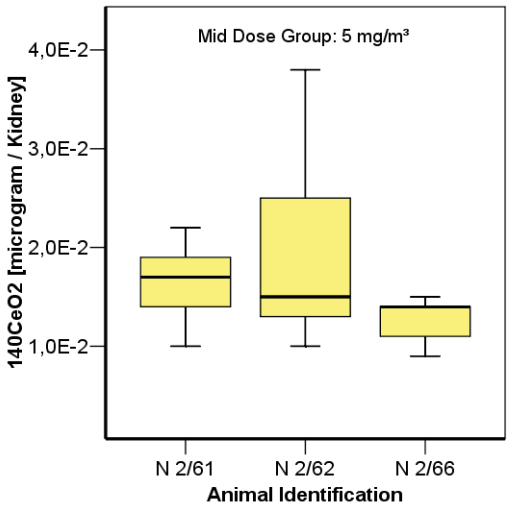
Organ	Day	Group	Animal Identification	CeO_2 [µg/organ]	absolute standard deviation [µg/organ]	relative standard deviation [%]
Kidney	28	0	0/1	0.002		
		0	0/2	0.002		
		0	0/6	0.0		
		0	**mean**	**0.001**	**0.001**	**115.5**



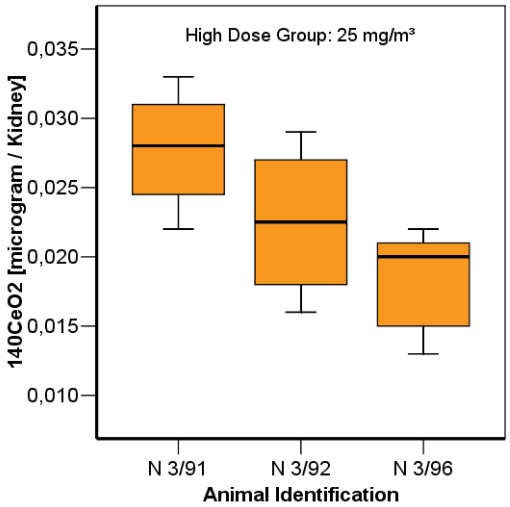
Organ	Day	Group	Animal Identification	CeO_2 [µg/organ]	absolute standard deviation [µg/organ]	relative standard deviation [%]
Kidney	28	1	1/31	0.006		
		1	1/32	0.005		
		1	1/36	0.003		
		1	**mean**	**0.005**	**0.003**	**64.3**



Organ	Day	Group	Animal Identification	CeO_2 [µg/organ]	absolute standard deviation [µg/organ]	relative standard deviation [%]
Kidney	28	2	2/61	0.016		
		2	2/62	0.019		
		2	2/66	0.003		
		2	**mean**	**0.005**	**0.03**	**18.75**



Organ	Day	Group	Animal Identification	CeO_2 [µg/organ]	absolute standard deviation [µg/organ]	relative standard deviation [%]
Kidney	28	3	3/91	0.028		
		3	3/92	0.037		
		3	3/96	0.018		
		3	**mean**	**0.028**	**0.01**	**33.95**



Particle distribution

The aim of this study was to identify particle clusters of CeO_2 in tissue slices of rat lungs and peripheral organs. Organs for which a high burden of CeO_2 has been confirmed (lung and liver) by ICP-MS, were investigated first (see chapter 2.3.1, page 6). Thus, the following examinations focus on the detection of CeO_2 nano particle clusters in these organs.

2.3.1.A *Lung tissue sections – ToF-SIMS*

Preliminary experiments showed that the deparaffinated lung tissue sections were better suited for ToF-SIMS analysis with subsequent particle detection than the paraffinated ones. The following sections show therefore only the results for the deparaffinated lung tissue sections.

For the particle distribution 6 times 6 pictures (500 µm x 500 µm) were acquired from each of the analysed lung tissue sections. One deparaffinated lung tissue section from three CeO_2 treated animals (25 mg/m^3) each were analyzed (10I151-100-2 II/4; 10I151-100-2 III/2 and 10I151-100-2 III/4) and 6 times 6 pictures were taken from a control deparaffinated lung tissue section from an untreated control animal (10I151-16-2 II/1). The stepper mode in the SurfaceLab 6 software was used to get large area images (see Figure 1 and Figure 2).

The CeO^+ clusters from the acquired ToF-SIMS pictures were analysed for total cluster counting and cluster size determination using the freeware software programme DotCount v1.2 (MIT freeware programme). All clusters were categorized into three groups: Group 1: 1 to 1.4 µm^2 cluster size, Group 2: 1.5 to 2.4 µm^2, Group 3: 2.5 to 3.5 µm^2 , Group 4: 3.5 to 4.5 µm^2 and Group 5 > 4.5 µm^2. An overview of the observed cluster number is shown in Figure 6. A total area of 9 mm^2 was analysed from sample 10I151-100-2 III/4. The largest observed CeO_2 cluster size was 5.8 µm^2.

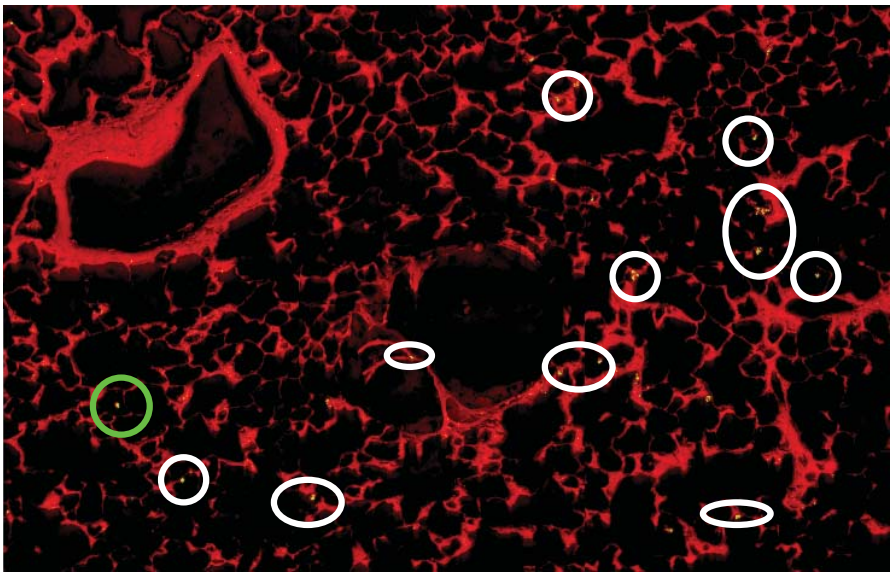


Figure 1: ToF-SIMS overlay picture (1.5 mm x 1 mm) of total ions acquired (red colour) and CeO^+ signal (yellow colour) from CeO_2 nano particle agglomerates. The bright red structures result from the analyzed lung tissue section. The green circle shows an area with high CeO^+ signal density. From that area a 50 µm x 50 µm picture was acquired (see Figure 3). The white circles show areas with high CeO^+ signal density.

Figure 2 shows a second section, analysed with ToF-SIMS for particle localization. Both green circles, numbered 1 and 2, show regions, where pictures of smaller area were acquired for exact CeO_2 nano particle cluster localization. Again, these two pictures could locate larger clusters within macrophages, indicating that predominantly macrophages are used to clear nano particle burden.

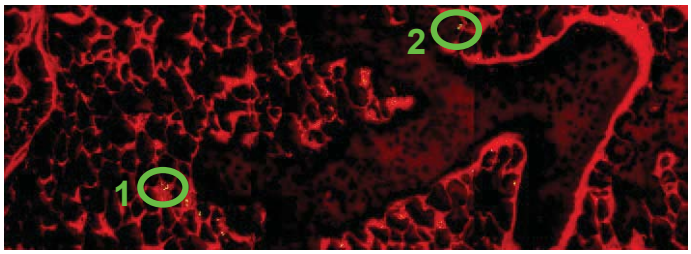


Figure 2: ToF-SIMS overlay picture (1.5 mm x 500 µm) of total ions acquired (red colour) and CeO^+ signal (yellow colour) from CeO_2 nano particle agglomerates. The bright red structures result from the analyzed lung tissue section. The green circle (depicted as 1 and 2 in the picture) show areas with high CeO^+ signal density (see Figure 4 and Figure 5).

Additionally 16 pictures (either 50 µm x 50 µm or 75 µm x 75 µm) were taken from areas with high CeO_2 agglomerate densities in the 500 µm x 500 µm lung tissue areas analyzed to get better insights in the exact location of the CeO_2 nano particle agglomerates (see Figure 3, Figure 4 and Figure 5). All acquired pictures indicate the localization of CeO_2 nano particle clusters within alveolar macrophages (see Figure 3, Figure 4 and Figure 5).

a) b)

Figure 3:

a) ToF-SIMS overlay picture (50 µm x 50 µm) of total ions acquired (red colour) and CeO^+ signal (yellow colour) from CeO_2 nano particle clusters within a macrophage. The bright red structures result from the analyzed lung tissue section. The green circle shows the accumulation of CeO_2 nano particle an area with high CeO^+ signal density.

b) Detailed picture of nano particle clusters, depicted as red areas within a macrophage cell, reconstructed from CeO^+ signal using the SurfaceLab 6 ION-TOF software. Visible are four larger CeO_2 nano particle clusters and 11 smaller nano particle clusters.

Figure 4 (see Figure 4, region 2) again shows the accumulation of CeO_2 nano particle clusters within a single alveolar macrophage.

a) b)

Figure 4:

a) ToF-SIMS overlay picture (50 µm x 50 µm) of total ions acquired (red colour) and CeO^+ signal (green-yellow colour) from CeO_2 nano particle clusters within a macrophage. The bright red structures result from the analyzed lung tissue section. The green circle shows the accumulation of CeO_2 nano particle clusters within a single alveolar macrophage.

b) Detailed picture of nano particle clusters, depicted as red areas within a macrophage cell, reconstructed from CeO^+ signal using the SurfaceLab 6 ION-ToF software. Visible are one larger, nine medium sized and 17 smaller CeO_2 nano particle clusters.

Figure 5 shows an area, where alveolar macrophages (ca. five) accumulate. Also in this area, CeO_2 clusters are only found within alveolar macrophages.

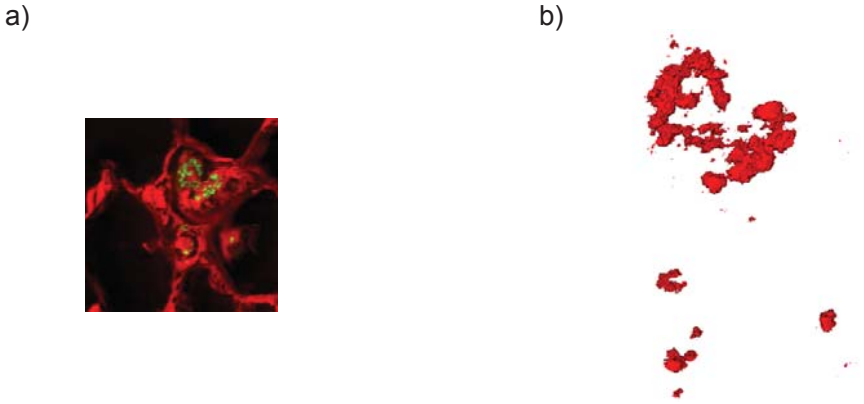


Figure 5

a) ToF-SIMS overlay picture (75 µm x 75 µm) of total ions acquired (red colour) and CeO^+ signal (green-yellow colour) from CeO_2 nano particle clusters within an area, where alveolar macrophages accumulate. The bright red structures result from the analyzed lung tissue section.

b) Detailed picture of nano particle clusters, depicted as red areas, within alveolar macrophages, reconstructed from CeO^+ signal using the SurfaceLab 6 ION-TOF software.

An example for CeO_2 nano particle cluster counting and categorization was performed on a total area of 9 mm^2 from sample 10I151-100-2 III/4. A total of 583 CeO_2 nano particle clusters were recorded in that area. The largest recorded CeO_2 nano particle cluster had an area of 5.8 μm^2. An overview of the cluster size distribution gives Figure 6.

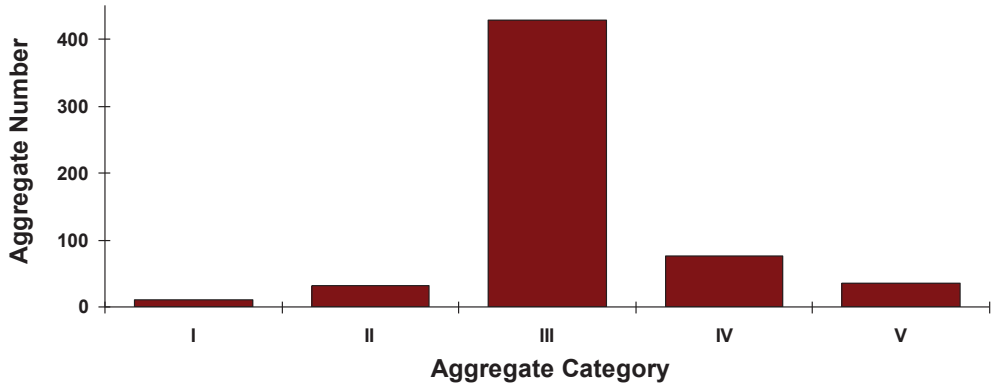


Figure 6: CeO_2 nano particle cluster categorization from a total area of 9 mm^2 of sample 10I151-100-2 III/4. 10 clusters were recorded in group 1, 32 clusters in group 2, 428 in group 3, 77 in group 4 and 36 in group 5. Group

1: 1 to 1.4 µm^2 cluster size, Group 2: 1.5 to 2.4 µm^2, Group 3: 2.5 to 3.5 µm^2 , Group 4: 3.5 to 4.5 µm^2 and Group 5 > 4.5µm^2.

For the 36 pictures acquired from the control deparaffinated lung tissue section (10I151-16-2 II/1) no CeO_2 nano particle clusters could be observed.

2.3.1.B *Liver tissue sections – ToF-SIMS*

For the particle detection with ToF-SIMS 6 times 6 pictures (500 µm x 500 µm) were acquired from one treated liver cryo-section (L2/72; Group 2, CeO_2 (MD) 5 mg/m^3) and one untreated liver cryo-section (L0/12; Group 0, CeO_2 (control dose) 0 mg/m^3). The results showed only a very weak signal increase for the CeO_2 nano particle dimer signal in treated samples (see Figure 7). The signal is missing in the control spectra. Therefore, preliminary results suggests, that the cerium detected with ICP-MS may result from CeO_2 nano particle dimers in the liver.

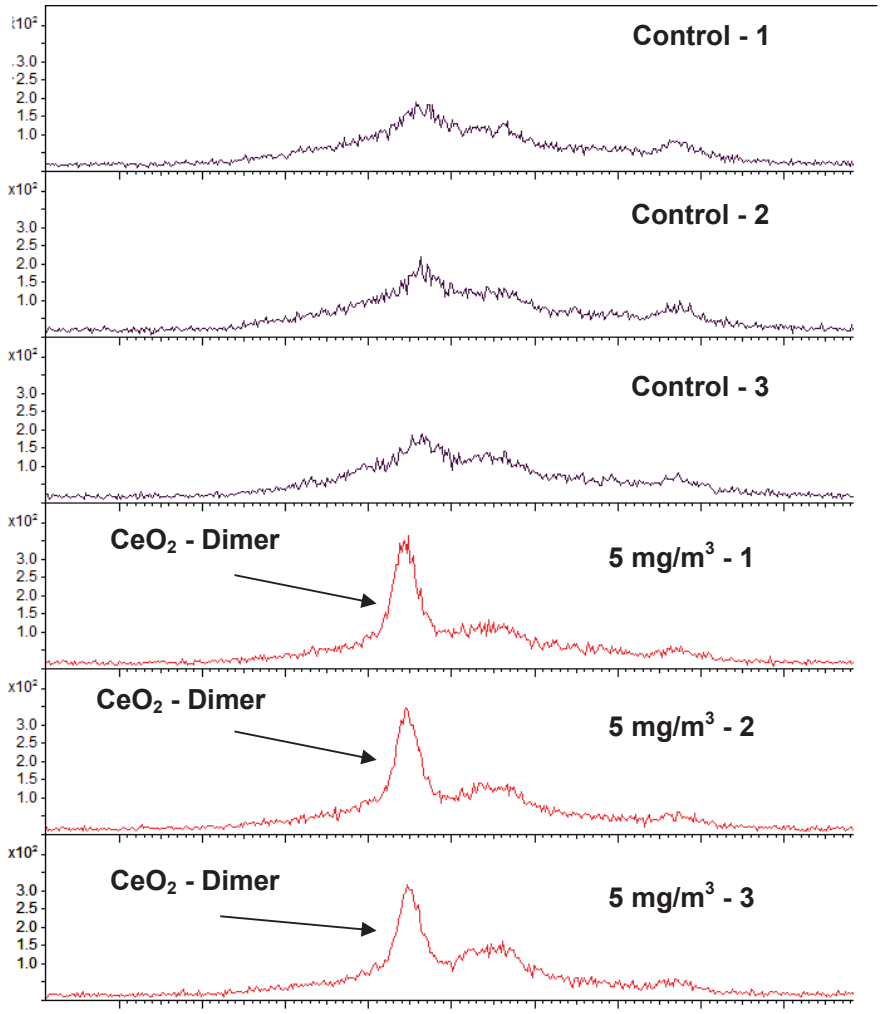


Figure 7: ToF-SIMS spectra from a control liver tissue section (Control 1 to Control 3) and of three spectra from a liver section of a 5 mg/m^3 CeO_2 exposed animal (5mg/m^3 – 1 to 5 mg/m^3 – 3). The controls lack a distinct peak at the CeO_2 dimer position, whilst in the treated samples the CeO_2 dimer peak is clearly visible.

Thus, the range finding experiments are considered satisfying and liver sections from the test group 3, CeO_2 (HD) 25 mg/m^3 could be analysed successfully. Therefore, it is concluded, that the limit concentration per organ for a particle localisation by ToF-SIMS is around 500 ng CeO_2 (see also chapter 2.3.1 organ burden, page 6).

2.3.1.C *Kidney tissue sections – ToF-SIMS*

The organ burden results for kidney in the given three test groups are significantly below the current limit concentration of 500 ng CeO_2 per organ for a successful localisation. The highest

detected CeO_2 concentrations are around 0.1 µg CeO_2 (average) for test group 3 (HD) . Hence, additional method development has to be done in order to push current detection limits even further.

2.3.1.D *Lung tissue sections – IBM*

The distributions of CeO_2 nanoparticles in rat tissues from the 25 mg/m^3 group (HD) are shown as an overview in large lung sections (400 µm x 400 µm) and also in single alveoli at higher resolution (Figure 10). The lung tissue represents a loose structure containing alveoli and bronchiole. Mucines being highly glycosylated negatively charged proteins are the main component of airway mucus. Part of their negative charge is provided by sulphate groups. The linings of the bronchiole are correspondingly recognized by their high sulphur content in the sulphur elemental images. In both experiments the nanoparticles could be detected and show a nonhomogeneous cerium distribution in lung alveoli and pneumocytes as marked by green contours.

The average CeO_2-concentration in single pneumocytes (Figure 8) was found to be about 1780 ppm, which is comparable with the P or S cellular element concentration. The average alveolar nano particle concentration can be considered as the truly effective doses at cellular level. These findings are fundamental for comparing *in vitro* with *in vivo* data on quantitative grounds.

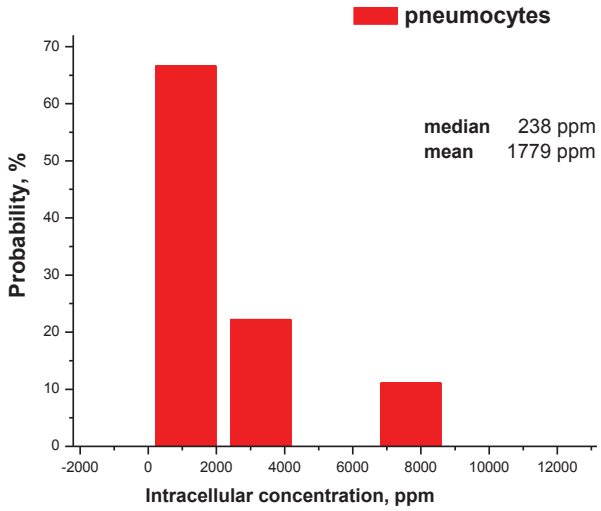


Figure 9: Histogram of nano particle content in pneumocytes in the HD group

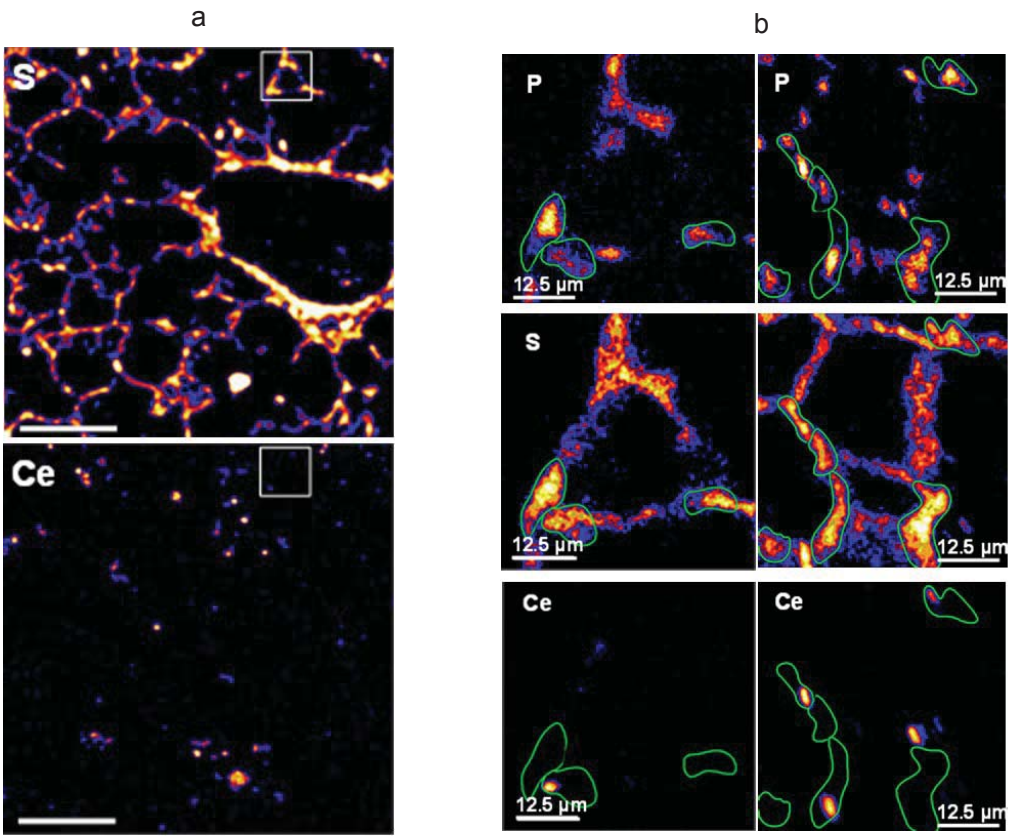


Figure 10: Proton Induces X-ray Emission (PIXE) images of lung tissue slices from rats which had been exposed to CeO_2 aerosol (25 mg/m^3) for 28 days.

a: Sulphur and cerium distribution in lung tissue. The bar is 100 µm.

b: A specific region containing single alveoli was selected and inspected at higher resolution. The pneumocytes are marked by green contours. The average CeO_2-concentration in single pneumocytes was found to be about 1780 ppm, which is comparable with the P or S cellular element concentration. The bar is 12.5 µm.

2.4 Evaluation of the results

The work on organ burden quantification (Task 4.3), estimation of the particle distribution pattern (Task 4.4) in samples out of the 28 day and 2 year inhalation studies is progressing according to the project planning. 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.

2.4.1 Organ burden

Within D 4.3, Lung burden of rats after 28 days of inhalative exposure against CeO_2 nanoparticles (NM 212) was reported with 33.7, 517 and 2268 µg for low (0.5 mg/m³), medium (5 mg/m³) and high (25 mg/m³) dose group respectively. A linear correlation between dosage and CeO_2 burden of the lung was observed. At both, MD and HD, lung clearance during a 128 day post exposition period was impaired, indicating particle overload at doses above LD. A translocation of CeO_2 from lung to lung associated lymph nodes was more pronounced at MD and HD. (see Title of D4.3 according to (amended) DoW: Lung burden and particle detection and quantification in olfactory bulbs, blood - subacute exposure). In comparison to the results reported for lung in D 4.3, we here report concentrations of CeO_2 in liver of animals out of the same study and at the same day of 178.1, 520.4 and 970.1 ng / organ for low, medium and high dose group respectively. In kidney 4.6, 16.0 and 27.5 ng / organ were detected for LD, MD and HD.Contrastingly to lung, no linear correlation between dosage and organ burden was found for liver and kidney. At lower dosages a higher percentage of inhaled CeO_2 was detected in both organs.

Furthermore, CeO_2 was analyzed in blood, brain, heart, olfactory bulbs and spleen of rats out of the 28 day study: 5.2, 10.4, 2.68, 1.26 and 9.7 ng were detected after the end of exposition in these organs out of animals of the HD respectively.

The CeO_2 concentrations measured in peripheral organs confirm that a translocation of the substance into organs behind the respiratory tract occurs following inhalative uptake. Measured concentrations are orders of magnitude below the amount of CeO_2 analyzed in lung. However, contrastingly to the situation there, a rather continuous clearance of CeO_2 via peripheral organs than a clear decrease of secondary organ burden over the post exposure period seems to be the case. The results for liver, spleen and lymph nodes (deliverable 4.3) though might be an indication of clearance by the mononuclear phagocyte system. Thus, possible routes of CeO_2 translocation and the excretion via feces remain to be investigated.

The difference in organ burden of liver and kidney between different animals of the same dose group (except control) was found to be at a maximum of 32.7 % for liver of LD. Based on this, we conclude, that there is a significant difference of CeO_2 organ burden of liver, kidney and also other secondary organs depending on the individual characteristics of each rat. Also first examinations of organ burden out of the long term inhalation study revealed that variability between animals of the same dose group.

The results are currently used for preparation of appropriate techniques of sample preparation for the long term low dose inhalation study. Main target is the identification of a potential relation between particle agglomeration, distribution and histochemical findings as e.g. inflammation or tumour proliferation. Furthermore, it is the aim to be able to precisely identify overload and no observed adverse effective levels for CeO_2. This could make a large contribution to the human health risk assessment, occupational precautions as well as to the animal welfare.

2.4.2 Particle distribution

Unimpaired clearance in the rat lung is described by elimination half-lifes of 60-90 days. When determining the elimination half-lifes from the MD and HD it became evident that 60-90 days are strongly exceeded.

The results show that in deparaffinated lung tissue sections CeO_2 nano particle agglomerates could be detected which also be confirmed by ICP analysis. The detected clusters were not evenly

distributed within the lung tissue. In an area of 9 mm^2 583 nano particle clusters could be detected. The majority of the nano particle clusters detected have a size range from 2.5 to 3.5 μm^2. ToF-SIMS pictures taken from smaller areas indicate a high CeO_2 nano particle load. The overview pictures (500 µm x 500 µm) point towards the location of the nano particle clusters within alveolar macrophages. The pictures also show alveolar macrophage accumulation in specific lung tissue areas. Particle accumulation was also confirmed by Ion Beam Microscopy (IBM) measurements, showing an enhanced concentration of approximately 1700 ppm cerium dioxide in single pneumocytes.

In addition the localisation technique could be further improved to determine limit concentrations of CeO_2 nano particles per organ of up to 500 ng. So it was possible to assess the particle load of liver sections not only for the HD, but also for the MD.

These results demonstrate that the ToF-SIMS technique can be used successfully for investigation of deparrafinized samples and thus that the technique can be directly linked with histochemical/histopatholical investigations of tissue slices out of the same paraffin block. This is important in order to link histochemical/histopathological findings and chemical analysis.

3 Deviations from the workplan

Initially it was planned to use cryo embedded tissue samples for investigation of particle distribution pattern by Time-of-Flight Secondary Ion Mass Spectrometry (ToF-SIMS). However, histopathological investigation of tissues out of the 28 day and 2 year inhalation studies with CeO_2 is using paraffin embedded samples. In order to correlate mass spectrometric analysis to the histopathological findings, it was decided to use paraffin embedded samples. Paraffin embedded samples have to be deparaffinised prior to ToF-SIMS analysis in order to avoid a matrix influence on the analysis. This step is requiring additional time and has delayed generation of results. Furthermore, the maximum concentrations of CeO_2 detected in liver and kidney were in the range of 1 µg and 0.1 µg per kg tissue respectively, while in lung a maximum of 2789 µg per kg tissue was found. Considering the low concentrations in other organs, ToF-SIMS investigations were started with lung and liver samples out of the 28 day study. The total number of 500 ToF-SIMS slices mentioned in the document of work is not realistic due to the complexity of the samples. One picture takes one day. Therefore we choose to make 6 pictures per tissue that gave positive results with ICP-MS and probably can be detected by ToF-SIMS.

4 Conclusions

The deliverable could be completed satisfactory.