

Comparison of Inhalation and Intratracheal Instillation as Testing Methods for Characterisation of Granular Biopersistent Particles (GBP)

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Intratracheal Instillation as Testing
Methods for Characterisation of Granular
Biopersistent Particles (GBP)**

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The responsibility for the contents of this publication lies with the authors.

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Comparison of Inhalation and Intratracheal Instillation as Testing Methods for Characterisation of Granular Biopersistent Particles (GBP)

Abstract

In this project F 2364 an in vivo validation study was conducted to evaluate the consistency of data with results obtained in the preceding intratracheal instillation study in project F 2336. The same test items were used and similar lung loads were achieved by calculating the target values after inhalation with the MPPD model. This approach served as a proof whether the instillation can be a reliable surrogate instead of the physiological inhalation route while assessing the GBP status of dust samples.

Comparison of bronchoalveolar lavage fluid (BALF) data

The comparison of the inflammatory potency of the 6 GBP candidates came out with a good accordance of the polymorphonuclear neutrophil (PMN) levels in the differential cell count for $\mu\text{-TiO}_2$. On day 3 post-treatment, PMN were not increased statistically significantly in the low and high dose groups, neither after instillation nor after inhalation. Thus, a very low inflammogenicity confirmed $\mu\text{-TiO}_2$ as GBP. $\mu\text{-BaSO}_4$ (low dose groups) showed a behaviour similar to $\mu\text{-TiO}_2$. However, for nano-TiO₂ P25 an evident difference was observed as a strong induction of PMN after instillation was not mirrored after inhalation. $\mu\text{-Eu}_2\text{O}_3$, $\mu\text{-ZrO}_2$ and nano-SiO₂ showed all a strong acute inflammation not meeting this GBP criterion.

Comparison of chemical analysis data of the lung burdens

Post-instillation, an average retention of approx. 70% of the total dose was analytically detected; therefore, the target lung loads for the inhalation study were calculated at this day 3 dose level using the MPPD model. The analysis revealed that these values were achieved satisfactorily in average. The clearance half-time showed a value close to the physiological rat lung clearance of approx. 60 days in both $\mu\text{-TiO}_2$ "Bayertitan T" dose groups and in the nano-TiO₂ P25 low dose group; in the latter high dose group $t_{1/2}=100$ days was only moderately increased (overload effect). In the $\mu\text{-BaSO}_4$ and amorphous silica groups, values similar to the instillation test were observed (25-30 days; low values indicating an additional dissolution effect). In the $\mu\text{-Eu}_2\text{O}_3$ and $\mu\text{-ZrO}_2$ groups increased half-times vs. the physiological value (= 60 days) indicated a clear surface chemistry-related toxicity and clearance retardation.

Conclusion

Considering the percentual PMN as well as the absolute PMN concentrations, the predominant observation is that inhalation induced a smaller PMN influx (with exception of biosoluble $\mu\text{-BaSO}_4$ and nano-SiO₂) at similar doses. This can be expected because of the physiological dust uptake and deposition by inhalation that is more gentle than intratracheal instillation (bolus effect!). Effects detected after instillation will not always allow a 'non inert' statement. The final setting of maximum tolerable clearance $t_{1/2}$ and PMN levels to define the GBP category should include inhalation. Doses at instillation testing of nanoparticles should not exceed volumetric values of 0.3 μl (using the correct agglomerate density).

Key words:

GBP, inhalation, PMN, retention, biosolubility

Vergleich von Inhalation und intratrachealer Instillation als Testmethoden zur Charakterisierung von granulären, biopersistenten Stäuben (GBS)

Kurzreferat

Im Vorhaben F 2364 wurde eine in vivo Validierungsstudie durchgeführt, um die Datenkonsistenz zu der vorhergehenden Intratrachealstudie F 2336 zu prüfen. Die gleichen Prüfsubstanzen und ähnliche Lungenbeladungen wurden sichergestellt (Berechnung der Zielbeladung mit dem MPPD-Modell). Dieser Ansatz diente als Nachweis, ob die Instillation eine zuverlässige Substitutionsmethode für die Inhalation sein kann, um den GBS-Status von Staubproben zu bewerten.

Vergleich der bronchoalveolären Lungenspülflüssigkeits- (BALF-) Daten

Der Vergleich der entzündlichen Reaktion der 6 GBS-Kandidaten ergab eine gute Übereinstimmung der Granulozyten-(PMN-)Konzentration im Differentialzellbild bei μ -TiO₂. An Tag 3 nach Behandlung waren die PMN in den Niedrig- und Hochdosisgruppen statistisch nicht signifikant erhöht, weder nach Instillation noch nach Inhalation. Der sehr schwache Entzündungseffekt bestätigte μ -TiO₂ als GBS. Ähnlich war es bei μ -BaSO₄ (Niedrigdosisgruppen). Bei nano-TiO₂ P25 trat jedoch insofern eine deutliche Differenz auf, als die starke PMN-Induktion nach Instillation nicht nach Inhalation beobachtet wurde. μ -Eu₂O₃, μ -ZrO₂ und nano-SiO₂ zeigten jeweils eine starke, akute Entzündungsreaktion und erfüllten das GBS-Kriterium somit nicht.

Vergleich der chemisch-analytischen Daten zur Lungenbeladung

An Tag 3 nach der Instillation wurde eine Lungenretention von ca. 70 % der Gesamtdosis analytisch nachgewiesen; daher wurden diese 70 %-Werte für die Inhalationsstudie als Ziel berechnet (MPPD-Modell). Die Analyse ergab, dass die Zielwerte ausreichend genau erreicht wurden. Die Clearancehalbwertszeiten lagen in beiden μ -TiO₂-Dosisgruppen und in den nano-TiO₂ P25-Niedrigdosisgruppen nahe dem physiologischen Wert (ca. 60 Tage); in den Hochdosisgruppen der letzteren war der t_{1/2}-Wert mit 100 Tagen nur mäßig erhöht (Überladungseffekt). In den μ -BaSO₄- und nano-SiO₂-Gruppen wurden ähnliche Werte wie im Instillationstest beobachtet (25-30 Tage; zusätzlich ist ein Löslichkeitseffekt wirksam). In den μ -Eu₂O₃- und μ -ZrO₂-Gruppen zeigten gegenüber dem physiologischen Wert (= ca. 60 Tage) erhöhte t_{1/2}-Werte deutlich eine Beteiligung der Oberflächenchemie und Clearanceverlangsamung.

Schlussfolgerung

Bei Betrachtung der prozentualen bzw. absoluten PMN-Werte wurde deutlich, dass diese nach Inhalation bei ähnlichen Dosen geringer ausfielen (mit Ausnahme der biolöslichen Stäube μ -BaSO₄ und nano-SiO₂). Erwartungsgemäß ist die physiologische Staubaufnahme und -deposition bei der Inhalation geringer in der Wirkung als der intratracheale Instillationstest (Boluseffekt!). Der IT-Test kann also nicht eindeutig ein 'nicht inert'-Urteil abgeben. Die Festsetzung maximal tolerabler t_{1/2}- und PMN-Werte für die Definition der GBS-Kategorie sollte auch auf Inhalation basiert sein. Dosen im IT-Test sollten volumetrische Werte von 0.3 μ l (Agglomeratdichte) nicht übersteigen.

Schlagwörter:

GBS, Inhalation, PMN, Retention, Biolöslichkeit

1 Information on the Study

Fraunhofer ITEM Study No:	02 N 15 535 (non-GLP)
Research Facility:	Fraunhofer ITEM
Fraunhofer ITEM Project Manager in vivo Toxicology:	Dr. Otto Creutzenberg
Aerosol Physics:	Prof. Dr. Wolfgang Koch
Clinical Chemistry:	Dr. Tanja Hansen
Chemical Analysis:	Dr. Sven Schuchardt
Sponsor's Study Manager:	Dr. Bruno Orthen
Projekt Initiation Date:	September 1, 2015
Project Completion Date:	July 31, 2017

2 Introduction

This inhalation study was conducted to validate the results of the preceding intratracheal instillation study (BAuA project F2336) under physiological conditions using the same test items, dosimetry and analytical endpoints, just the administration mode was varied. Therefore, details of the repeated experimental conditions can be found in the Final Report of BAuA project 2336. For calculation of the lung doses expected in 2-week exposure periods the Multi-Path Particle Dosimetry (MPPD) model was used (ANJILVEL & ASHGARIAN, 1995; RIVM, 2002).

3 Objectives

Using six granular dust candidates the solubility and non-inflammogenicity criteria for GBP dusts were experimentally determined. Two well-characterised inert dusts, i.e. $\mu\text{-TiO}_2$ Bayertitan T and $\mu\text{-BaSO}_4$ and four other candidates should be analysed in the rat model at the same volumetric lung burdens as used in the instillation study. The shortest exposure period to achieve this goal at tolerable aerosol concentrations ($<200 \text{ mg/m}^3$) were two weeks. Post-exposure the basic data defining "low solubility" and "non-adverse inflammation" of GBP dusts should be determined.

The experimental objectives were

- A. To perform 2-week inhalation studies with subsequent analysis of the BAL fluid and chemical analysis of the test item lung loads
 - o By measuring the inflammatory response in lung lavage fluid (BALF) at days 3 and 28;
 - o By determining the lung burdens at days 3, 28 and 90 and calculating clearance half-times.
- B. To check whether the results of the preceding instillation study could be conclusively validated in the in vivo study.

4 Selection and Basic Data of “6 Dusts Group”

Table 4.1 Selection of the 6 dusts to be used in the GBP project

Dust	Origin	Properties
1 μ-TiO₂ Bayertitan T	Bayer Produced in 1985	ρ : 4.3 98,17% TiO ₂ 99,5% Rutile BET: 1,9 m ² /g; EGME: 21,7 m ² /g μ -TiO ₂ ; MMGD: 1,8 μ m (GSD: 1,9) ζ -Potential: pH=4 -11,47 mV; pH=6 -35,08 mV; pH=8 -43,03 mV Toxicity profile: Prototype of an inert dust for in vivo tests at Fraunhofer ITEM (CREUTZENBERG et al., 2008)
2 nano-TiO₂ TiO₂ P25 Commercial sample, purchased and characterised by EU/JRC	Evonik	ρ : 4.3 - ρ_{Agg} : 1.6 (PAULUHN, 2011) BET: 60 m ² /g Anatase/Rutile 80%/20% Widely used „Standard titanium dioxide dust“ for in vivo toxicity testing Comm.: This TiO ₂ type (JRC code: NM-105), though consisting of an anatase/rutile 80%/20% mixture, exhibits a smaller toxic potential in lungs than the surface-modified pure rutile TiO ₂ types NM-103 and NM-104
3 μ-Eu₂O₃	American Elements	ρ : 7.4 A μ -sized dust containing a small part of nano-sized dust
4 μ-BaSO₄	Sigma-Riedel	ρ : 4.5 A microsized BaSO ₄ was selected CAS # 7727-43-7 Lot # SZBD0080V
5 μ-ZrO₂ Y-stabilised Zirconia (YSZ)	American Elements	ρ : 5.7 Stabilized zirconia or zirconium oxide Standard powder particle sizes average in the range of - 325 mesh, - 100 mesh, 10-50 microns and submicron (< 1 μ m) White high surface area particles available fully stabilized (8 mol%) or partially stabilized (3 mol%) or doped with yttria (yttrium oxide). Nanoscale yttria is typically 5 - 100 nanometers (nm) with specific surface area (SSA) of 25 - 50 m ² /g. Application: High temperature ceramics, technical ceramics, prostheses Solubility in water is given with 1 mg/l (20 °C).
6 nano-SiO₂ Amorphous SiO ₂ , “nano-structured”	JRC, Ispra	ρ : 2.2 (FRAUNHOFER IKTS, 2012) - ρ_{Agg} : 1.1 (AGS, 2015) JRC code: NM-200; precipitated amorphous SiO ₂ . The solubility of SiO ₂ in water is dependent on its modification or its grade of crystalline order. In case of quartz (crystalline SiO ₂) the solubility at 25 °C is approx. 10 mg SiO ₂ per litre water (solubility equilibrium may be reached very slowly only). In contrary, amorphous silica shows at 25 °C with approx. 120 mg/l water an evidently increased solubility. With increasing temperature solubility becomes higher (quartz: 60 mg/l water at 100 °C; amorphous silica: 1100 mg/l water at 75 °C. - Analysis by Fraunhofer IKTS: In water up to 14 days at a concentration of 5 g/l → approx. 5% SiO ₂ dissolves (also determined in RPMI w/ horse serum and FBS as well as in DMEM w/ FBS (up to 48h)) - Density: Raw (excl. voids) = 2.19 g/cm ³ (He-Pentapycnometer); bulk/tap = 0.12/0.16 g/cm ³ (Autotap, Quantachrome Instruments)

p: Raw densities; - ρ_{Agg} : Agglomerate densities

nano-structured: primary nanoparticles are agglomerated or aggregated/sintered due to aging

5 Conduct of Study

Staff

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Dipl.-Ing. Heiko Kock

Project Duration

Start of project:

September 1, 2015

Date of the Final Report:

July 31, 2017

6 Materials and Methods – 2-wk Inhalation Study

6.1 Nose-only Inhalation

Animal model

Male Wistar rats, strain Crl:WI(Han) were used in the study. Animals were exposed in two subsets each consisting of 210 rats. The total number amounted to 420 rats. At start of the exposure the rats were 9 weeks aged. The experimental design of the study is presented in Appendix 1.

Animal exposure

The particulate sample aerosols were generated by dispersing the dry powders. Dispersion was achieved by a feeding system and a high-pressure, high-velocity pressurised air dispersion nozzle developed by Fraunhofer ITEM (KOCH, 1998). For each nose-only exposure unit, the aerosol was generated by a high-pressure pneumatic disperser. The disperser was fed with the test substance under computerized control, i.e. with a feed back loop to the actual aerosol concentrations measured by an aerosol photometer (see Figure 6.1).

The photometer gives a scattering light signal which is proportional to the particle concentration, if the particle size distribution is constant. The ratio between photometer signal and concentration was determined throughout the study by comparing to gravimetric concentrations.

The aerosol was given to the rats by a flow-past nose-only inhalation exposure system which was used for previous particle and fiber inhalation studies at Fraunhofer ITEM. In this system, aerosols are supplied to each rat individually, and exhaled air is immediately exhausted. The airflow to each rat is approximately 1 l/min which is calculated to be laminar (rat minute volume: 0.2 l). Therefore measurement of the oxygen concentration is not necessary.

Prior to the 14-day exposure of rats, technical trials to adjust particle size distributions and exposure levels were conducted.

Monitoring and controlling the exposure atmospheres

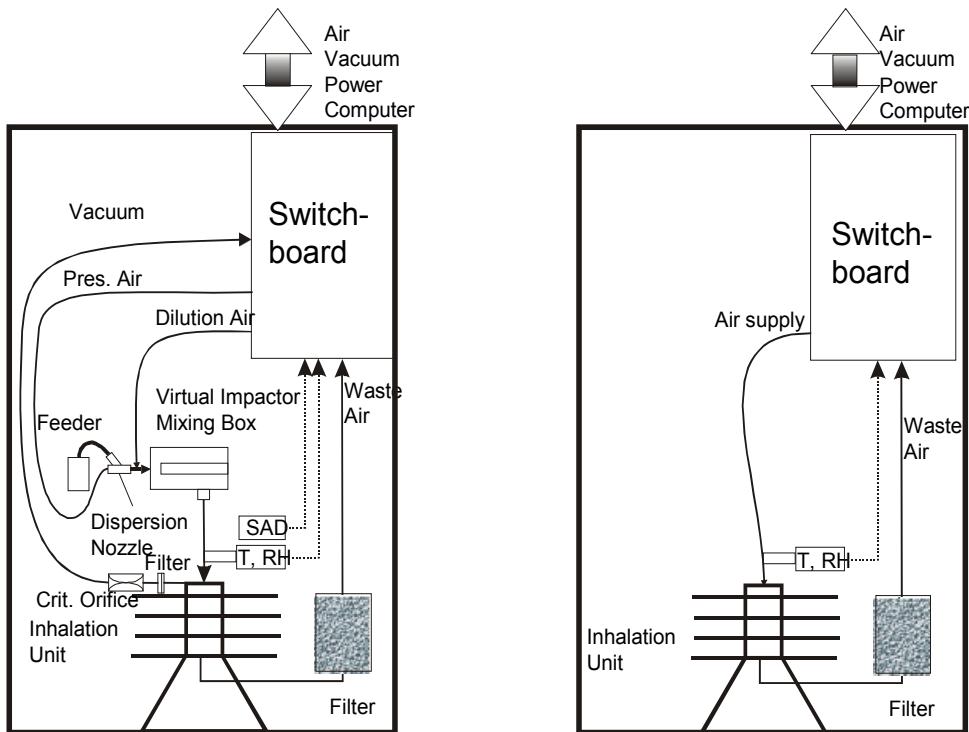
Air flow, temperature and relative humidity were measured continuously and recorded by 20-minute means. The limits were set at $22^\circ\text{C} \pm 2^\circ\text{C}$ for temperature and $55\% \pm 15\%$ for relative humidity. Animal room lighting was on a 12-hour light/dark cycle controlled by an automatic timing device.

Exposure of rats

For exposure to the test item the rats were restrained in acrylic tubes with adjustable backstops. The exposure tubes are arranged around a cylinder capable to take up 16 tubes per platform. The rat nose is located at the front end of a tube being connected to a cylinder delivering the aerosol. Through the thin pipes, the aerosol is supplied to each rat nose individually and exhaled air is drawn off immediately by a cylinder surrounding the aerosol delivering cylinder. The position of exposure tubes of rats at the cylinder is changed daily according to a rotation plan to minimize exposure differences due to geometry. The exposure units (1x clean air control, 6x treatment with dusts) were located each under a separate hood to prevent contamination among different dose groups.

Dust groups

Control Group



SAD: Scattering light aerosol detector - T: Temperature - RH: Relative humidity

Fig. 6.1 Aerosol generation set-up

For conversion of the dose volumes to dose masses raw densities were used. This is the regular approach for microscaled dusts because the void volume is negligible. For the nanoscaled dust TiO₂ P25 a measured agglomerate density of 1.6 g/cm³ (PAULUHN, 2011) was used. For nano-SiO₂ a raw density = 2.2 g/cm³ was determined by helium gas pycnometry (IKTS, 2012). According to the recommendation of the AGS (AGS, 2015) an agglomerate density of 2.2 * 50% = 1.1 g/cm³ was estimated for nano-SiO₂ to take into account the higher void volumes of nanomaterials.

Table 6.1 Raw and agglomerate densities of the test materials

Groups	Test substances	Raw density ρ	Agglomerate density ρ_{Agg}
2 + 3	μ -TiO ₂ - Bayertitan T	4.3	-
4 + 5	nano-TiO ₂ - TiO ₂ P25 EU/JRC	4.3	1.6 PAULUHN, 2011*
6 + 7	μ -Eu ₂ O ₃	7.4	-
8 + 9	μ -BaSO ₄	4.5	-
10 + 11	μ -ZrO ₂ - Y-stabilised	5.7	-
12 + 13	nano-SiO ₂ - NM-200	2.2 IKTS, 2012	1.1 AGS, 2015

* This value is very close to a value of 1.3 g/cm³ reported by DeLoid et al. (2014)

Table 6.2 Aerosol concentrations (recorded by aerosol photometer)

Group – Dust sample	Density * ρ (μ -dust) ρ_{Agg} (nano-dust)	Mean aerosol concentra- tion (mg/m ³)	SD	N	Target aerosol concentra- tion (mg/m ³) **	Calculated deposition efficiency (%); MPPD) and expected lung load (mg/ μ l per rat)
2 - μ -TiO ₂ Bayertitan T Low	4.3	27.56	4.96	13	36/24	9.3% 1.85/0.43
3 - μ -TiO ₂ Bayertitan T high		85.15	28.16	13	108/72	6.1% 3.74/0.87
4 - nano-TiO ₂ P25 Low	1.6	9.66	2.54	13	14/8	11.3% 0.79/0.49
5 - nano-TiO ₂ P25 high		29.12	7.74	13	42/24	11.0% 2.30/1.44
6 - μ -Eu ₂ O ₃ Low	7.4	31.85	2.26	13	31/31	4.9% 1.13/0.15
7 - μ -Eu ₂ O ₃ high		92.77	7.07	13	93/93	7.0% 4.68/0.63
8 - μ -BaSO ₄ Low	4.5	41.71	5.77	11	38/38	4.9% 1.47/0.33
9 - μ -BaSO ₄ high		135.83	23.95	12	114/143	4.2% 4.11/0.91
10 - μ -ZrO ₂ low	5.7	53.38	17.94	11	48/56	4.4% 1.69/0.30
11 - μ -ZrO ₂ high		163.95	10.84	12	144/167	4.6% 5.43/0.95
12 - nano- Amorphous SiO ₂ Low	1.1	11.01	2.05	12	9/11	4.7% 0.37/0.34
13 - nano- Amorphous SiO ₂ high		32.66	3.98	12	27/33	4.3% 1.01/0.92

SD Standard deviation

N Number of measurements

* Note: For nano-TiO₂ the $\rho_{\text{Agg}} = 1.6$ was measured (PAULUHN, 2011); for nano-SiO₂ 50% of the raw density excl. voids was used: $2.19 * 50\% = 1.1$ (IKTS, 2012; AGS, 2015)

** Aerosol concentrations were re-adjusted in the running study (see text) to optimise the final lung load

The duration of exposure was 6 hours/day, 5 days/week for 2 weeks. Animals were sacrificed 3 days after end of exposure. A second subset was sacrificed after 28 days to investigate recovery effects.

The MPPD model (ANJILVEL & ASHGARIAN, 1995; RIVM, 2002) was used to calculate the target lung doses after inhalation. The aerosol concentrations were adjusted to achieve in each group the volumetric dose as close as possible to those in the instillation study (i.e. 0.5 µl and 1.5 µl). As one third of the total dose is cleared very rapidly after intratracheal instillation, the target lung loads after inhalation were assigned with 0.3 µl and 0.9 µl per lung.

In the first exposure week the concentrations were re-adjusted based on actual MMAD values measured in the running study.

Filter samples of the aerosols were taken daily to control the **aerosol concentrations** and to calibrate the aerosol photometers. These samples were collected at a port of the nose-only exposure unit, thus, under the same conditions the rats are inhaling the aerosol. The evaluation of filter samples was by gravimetical analysis. As a permanent control of the aerosol concentrations is guaranteed by photometers the scheduled filter sampling frequency is sufficient (in agreement with OECD guideline 412).

MMAD analysis (impactor measurements/TEM analysis of filter samples)

Impactor analysis was performed three times during the exposure period for each test item exposure unit (12 units) using a Marple cascade impactor. The mass median aerodynamic diameter (MMAD) was approx. 1 µm for µ-TiO₂ Bayertitan T, 0.6 µm for nano-TiO₂ P25 and 2-2.5 µm for the other dusts. Values are presented in Table 6.3.

Table 6.3 Results of MMAD determination (Marple impactor measurements)

Group	µ-TiO ₂ Bayertitan T low	µ-TiO ₂ Bayertitan T high	nano-TiO ₂ P25 low	nano-TiO ₂ P25 high	µ-Eu ₂ O ₃ low	µ-Eu ₂ O ₃ high
MMAD (µm) GSD (-)		1.23 (1.99)		0.51 (3.38)		1.68 (2.35)
	0.90 (2.52)	1.51 (2.13)	0.57 (3.98)	0.65 (3.67)	2.43 (2.46)	1.13 (3.65)
Mean MMAD (µm)	0.90	1.37	0.57	0.58	2.43	1.41
Mean GSD (-)	2.52	2.06	3.98	3.53	2.46	3.00
N	1	2	1	2	1	2

Group	µ-BaSO ₄ low	µ-BaSO ₄ high	µ-ZrO ₂ low	µ-ZrO ₂ high	nano- SiO ₂ low	nano- SiO ₂ high
MMAD (µm) GSD (-)						2.05 (3.50)
	1.85 (1.40)	2.23 (1.44)	2.59 (2.37)	2.78 (2.36)	1.97 (3.06)	2.45 (3.00)
	2.01 (1.37)	2.21 (1.49)	2.77 (2.55)	2.55 (1.69)	1.46 (3.85)	1.74 (3.31)
Mean MMAD (µm)	1.93	2.22	2.68	2.67	1.72	2.08
Mean GSD (-)	1.39	1.94	2.46	2.03	3.46	3.27
N	2	2	2	2	2	3

MMAD: Mass median aerodynamic diameter

GSD: geometric standard deviation (values given in brackets)

SD: standard deviation

Analysis of the bronchoalveolar lavage fluid (BALF)

Bronchoalveolar lavage was performed in 6 males per group after end of treatment (on days 3 and 28). The method of HENDERSON et al. (1987) was used with minor modifications.

Following preparation, the lungs were lavaged with saline using two lavages of 5 ml each. The lavage fluid was collected in calibrated tubes and the harvested volume was recorded. Until processing the BALF was kept on ice. Leukocyte concentrations of the lavagate were determined using a counting chamber and two cytosplots were prepared with a cytoseparator (Shandon Co., Frankfurt, Germany) for differential cell count (macrophages, neutrophils, lymphocytes).

Endpoints

After centrifugation of the BALF, biochemical indicators relevant for diagnosis of lung damage were determined in the supernatant (lactic dehydrogenase - LDH, β -glucuronidase, total protein). These parameters were analysed according to routine clinical chemistry protocols using a Cobas Fara device (Roche Co., Grenzach, Germany).

The justification of the parameters is given below:

Cytological parameters

- total cell count (recruitment of lung leukocytes)
- differential cell count (inflammatory (PMNs) or immunological (lymphocytes) reactions; a total of 400 leukocytes per rat were evaluated)

Biochemical parameters

- lactic dehydrogenase (LDH = cytosolic marker enzyme; increased permeability of membranes, cell damage and lysis)
- β -glucuronidase (measure of phagocytic activity of macrophages; lysis of macrophages)
- total protein (marker of transudation; damage of epithelial cells)

6.2 Chemical Analysis of Lung Loads after Inhalation

Retention analysis of “6-dust group” in lungs

After sacrifice the lungs were subjected to lyophilisation and subsequent low-temperature ashing and the test items retained in lung tissue were determined using ion-coupled plasma mass spectroscopy (ICP-MS). The soluble und insoluble moiety, the latter being the moiety gainable by filtration (0.2 μ m nucleopore filters; Whatman) were separately analysed.

7 Results – 2-wk Inhalation Study

7.1 Analysis of Bronchoalveolar Lavage Fluid (BALF)

In Figures 7.1 to 7.8 as well as in Table 7.1 results of differential cell count and biochemical analytes in the analysis of the bronchoalveolar lavage fluid (BALF) are presented.

To allow a comparison of the effects found in the differential cell count between the two administration modes, i.e. intratracheal instillation and inhalation, both illustrations are juxtaposed to each other. Mean data are presented in tables in Appendix 2. Lung weights are given in Appendix 3.

Differential cell count data following inhalation

- Polymorphonuclear (PMN) cell levels showed no increase in absolute numbers in the $\mu\text{-TiO}_2$ (rutile; PMN < 1%/ $< 2\%$) and a very slight increase in the $\mu\text{-BaSO}_4$ groups (PMN < 1%/ $< 6\%$) after 3 days. - After 28 days of recovery, PMN levels had returned fully to control levels.
- In the nano-TiO₂ P25 groups PMN levels of 4.3% (low dose; not statistically significantly increased) and 22.7% (high dose; ***) were detected. - After 28 days 5.3% and 17.9% (***)) were observed.
- Both, $\mu\text{-ZrO}_2$ and nano-SiO₂ induced a strong PMN inflammatory reaction, i.e. 28%/36% ($\mu\text{-ZrO}_2$) and 19%/50% (nano-SiO₂) in the low and high dose groups. – After 28 days, 6%/34% ($\mu\text{-ZrO}_2$) and 3.8%/3.4% (nano-SiO₂) were observed.
- $\mu\text{-Eu}_2\text{O}_3$ resulted in the low and high groups in a very strong inflammatory reaction that persisted during the 4-week recovery period. Cells were severely damaged and a conclusive evaluation of the cytospots was not possible.

Enzyme and protein analysis data

- The normalised data for lactic dehydrogenase (LDH), β -glucuronidase and total protein mirror the inflammatory effects observed with the PMN levels.
- $\mu\text{-TiO}_2$ (both doses), nano-TiO₂ P25 (low dose only) and $\mu\text{-BaSO}_4$ (both doses) behaved similar to the clean air controls. The nano-TiO₂ P25 high dose group showed a stronger response (with statistical relevance at day 3 and normalisation at day 28).
- $\mu\text{-ZrO}_2$ and nano-SiO₂ showed a strong inflammatory reaction at day 3 with partial recovery or full normalization at day 28, respectively.
- The rare earth $\mu\text{-Eu}_2\text{O}_3$ resulted in the low and high groups in a very strong inflammatory reaction that persisted during the 4-week recovery period.

Comparison inhalation versus intratracheal instillation

Comparing the two administration modes the predominant observation is that inhalation induced a smaller PMN influx (with exception of nano-SiO₂) amounting up to a decrease of approx. 70%. This can be expected because of the physiological dust uptake and deposition by inhalation that is more gentle than intratracheal instillation (bolus effect!). This is true also for nano-TiO₂ P25; here, the low dose group after instillation and the high dose group after inhalation have the same effective dose at day 3 as different assumptions of the agglomerate density have been used (see Tables 8.1 and 8.2).

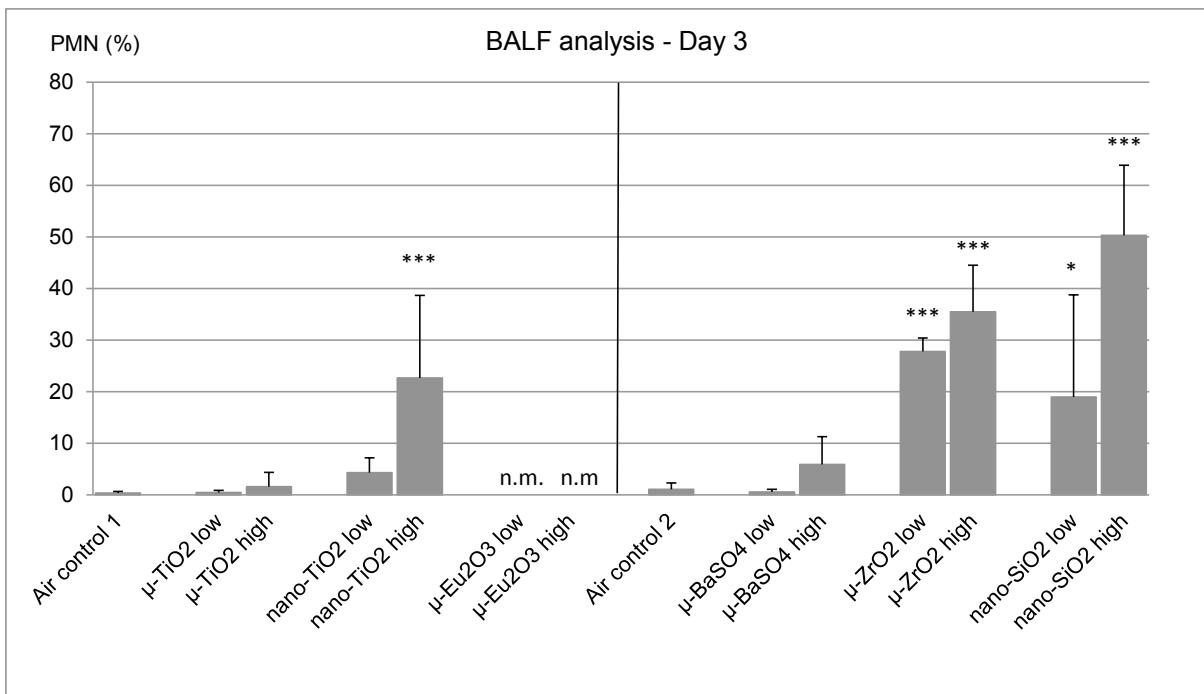


Fig. 7.1 Polymorphonuclear neutrophils (PMN) levels in BALF at day 3 following **inhalation**, percentual values

For comparison: Bar chart presenting data following intratracheal instillation (see F2336)

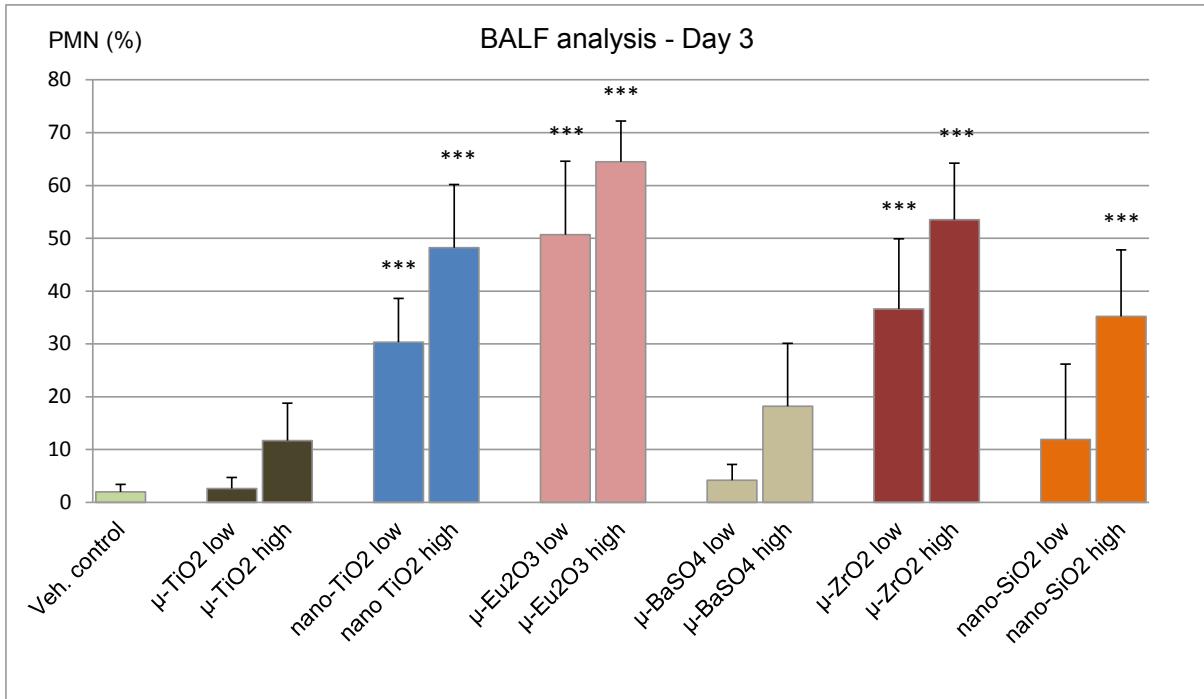


Fig. 7.2 Polymorphonuclear neutrophils (PMN) levels in BALF at day 3 following **intratracheal instillation**, percentual values

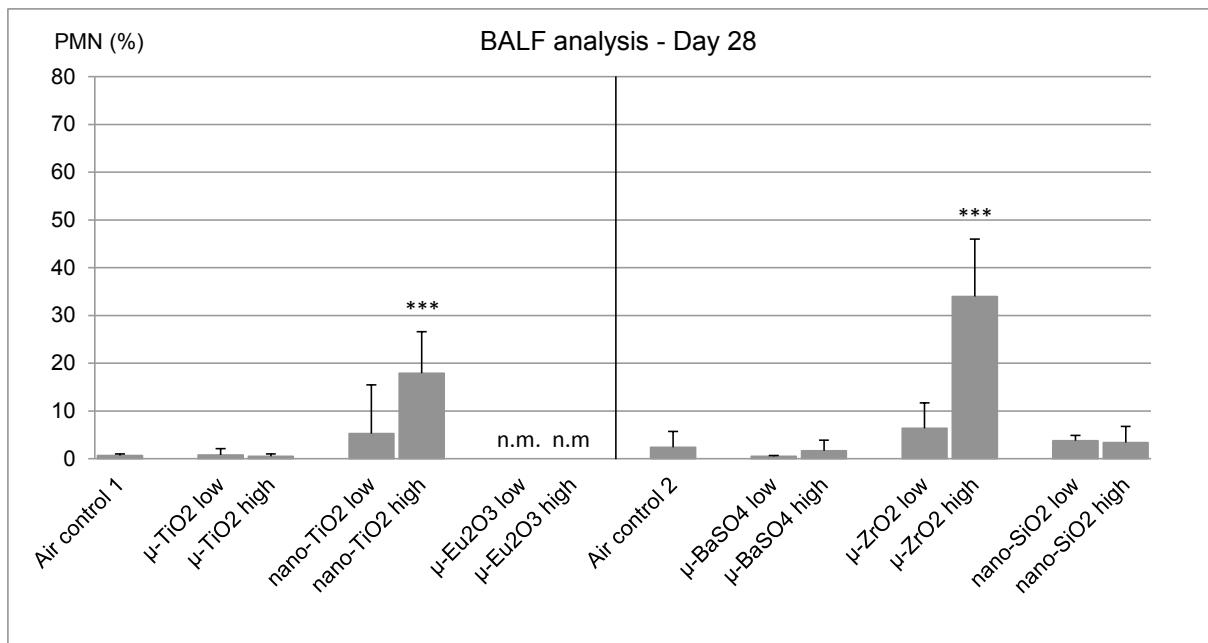


Fig. 7.3 Polymorphonuclear neutrophils (PMN) levels in BALF at day 28 following **inhalation**, percentual values

For comparison: Bar chart presenting data following intratracheal instillation (see F2336)

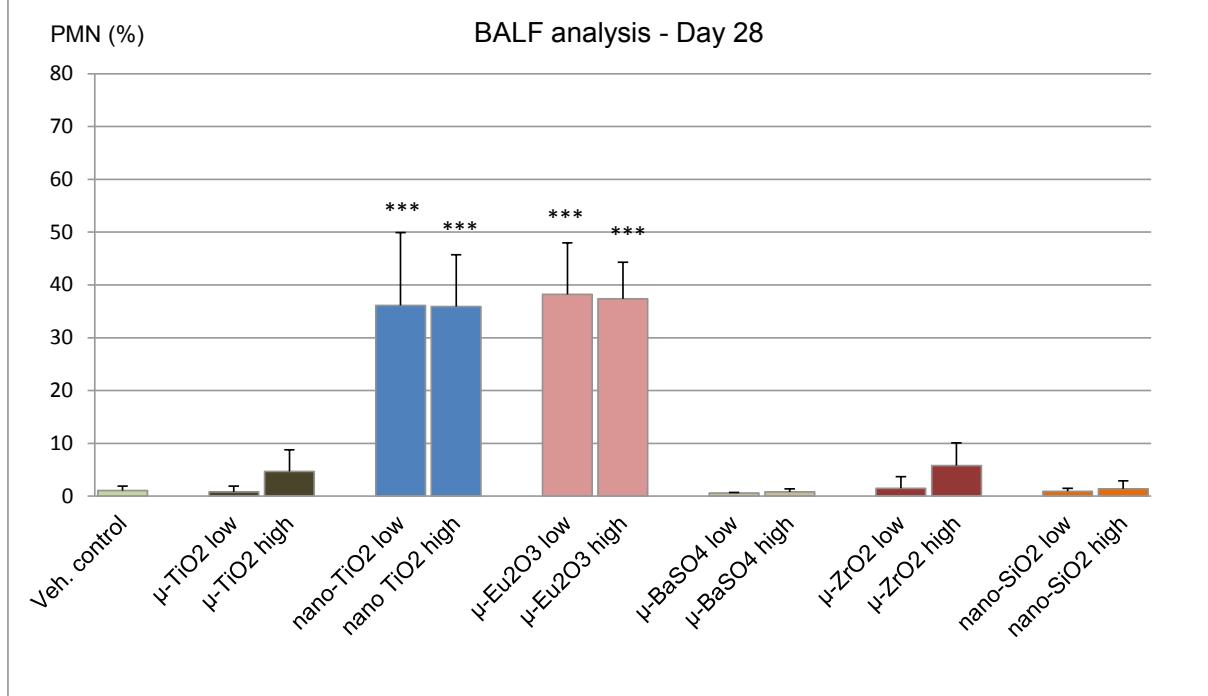


Fig. 7.4 Polymorphonuclear neutrophils (PMN) levels in BALF at day 28 following **intratracheal instillation**, percentual values

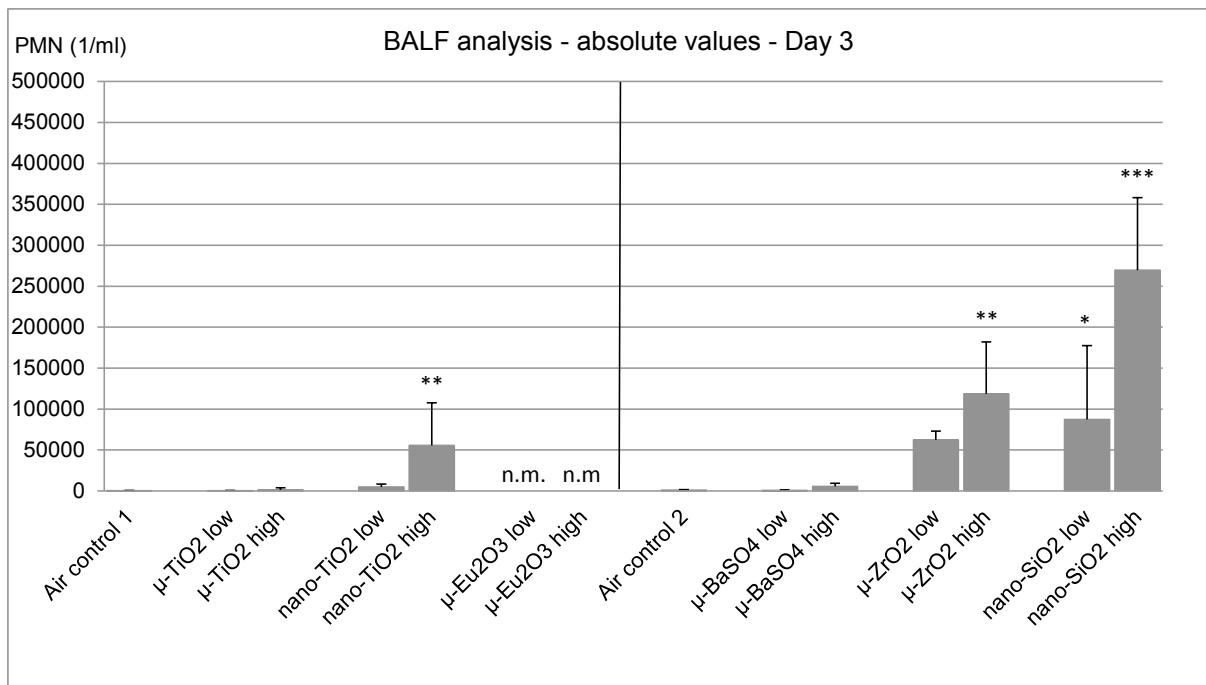


Fig. 7.5 Polymorphonuclear neutrophils (PMN) levels in BALF at day 3 following **inhalation**, absolute values

For comparison: Bar chart presenting data following intratracheal instillation (see F2336)

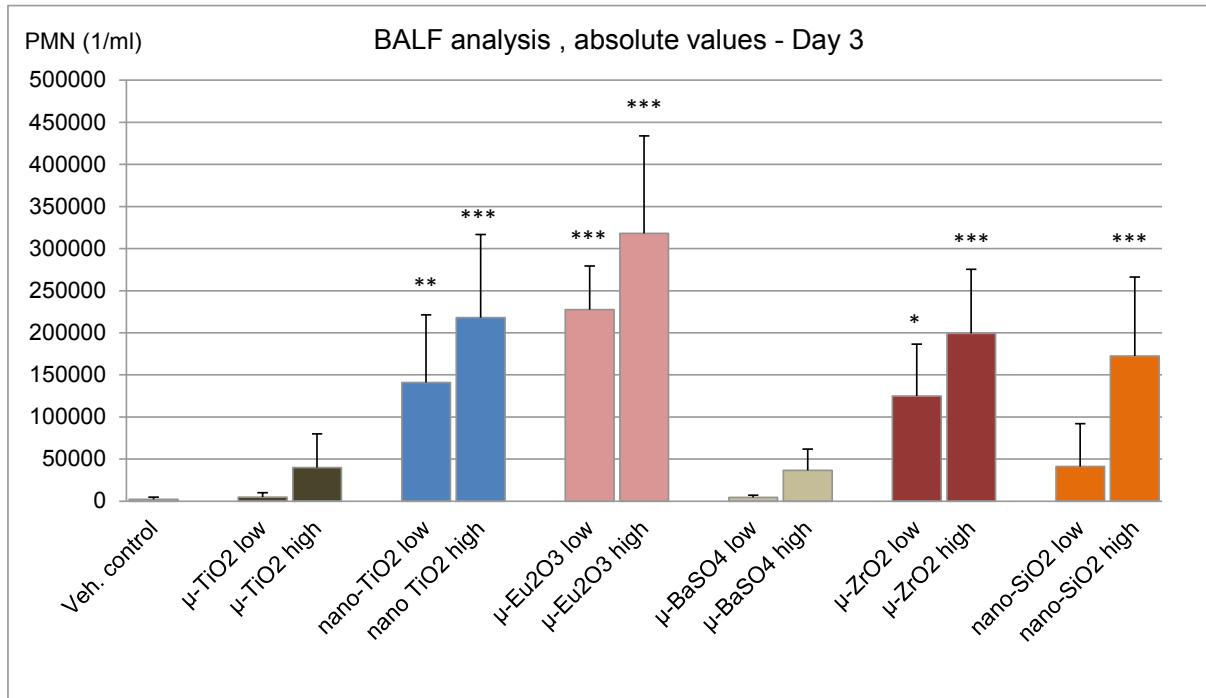


Fig. 7.6 Polymorphonuclear neutrophils (PMN) levels in BALF at day 3 following **intratracheal instillation**, absolute values

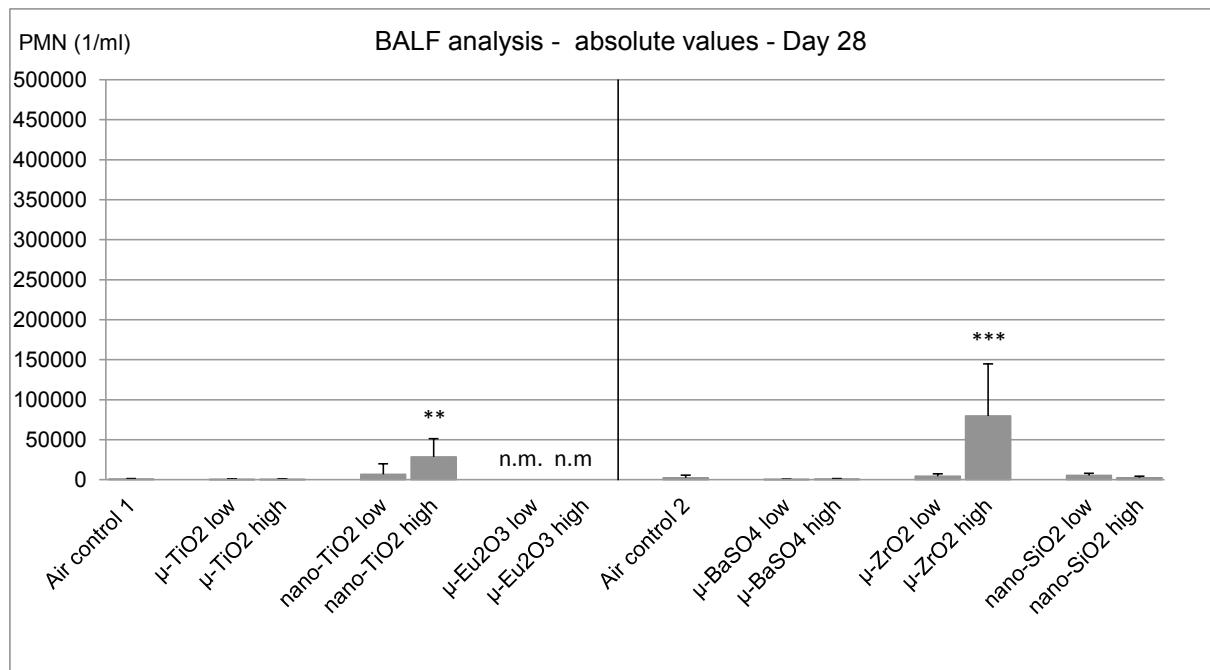


Fig. 7.7 Polymorphonuclear neutrophils (PMN) levels in BALF at day 28 following **inhalation**, absolute values

For comparison: Bar chart presenting data following intratracheal instillation (see F2336)

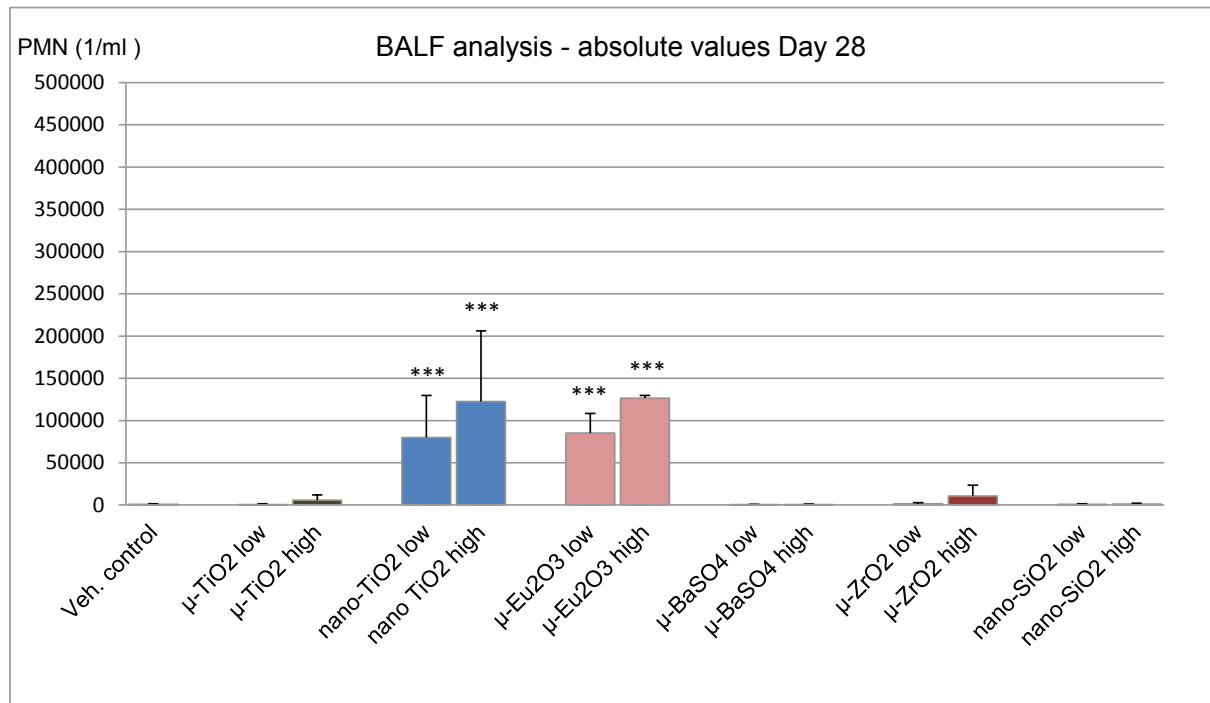


Fig. 7.8 Polymorphonuclear neutrophils (PMN) levels in BALF at day 28 following **intratracheal instillation**, absolute values

Table 7.1 Normalised data of analytes in the BALF**Day 3** (normalized data: vehicle control = 100%) – Two subsets → two vehicle controls

Males		LDH U/L	GLU U/L	TP mg/L	
1m	Mean	100	100	100	1 st control
2m	Mean	104	60	100	
3m	Mean	110	77	103	
4m	Mean	144	93	122	
5m	Mean	223*	123	164	
6m	Mean	623**	5060**	1139**	
7m	Mean	680**	5257**	1220**	
1m	Mean	100	100	100	2 nd control
8m	Mean	115	122	114	
9m	Mean	110	100	108	
10m	Mean	243**	278	233**	
11m	Mean	413**	683**	470**	
12m	Mean	333**	261	217**	
13m	Mean	519**	500**	319**	

Day 28

Males		LDH U/L	GLU U/L	TP mg/L	
1m	Mean	100	100	100	
2m	Mean	95	82	99	
3m	Mean	106	107	112	
4m	Mean	109	118	107	
5m	Mean	155	143	127	
6m	Mean	716**	5689**	1478**	
7m	Mean	896**	7089**	1912**	
1m	Mean	100	100	100	2 nd control
8m	Mean	86	96	88	
9m	Mean	85	96	96	
10m	Mean	105	96	100	
11m	Mean	193**	239	179*	
12m	Mean	88	87	94	
13m	Mean	91	87	89	

Statistics Test: Dunnett Test: * - 5% significance level; ** - 1% significance level

Group 1 - Vehicle Control	Group 2 - μ -TiO ₂ low	Group 3 - μ -TiO ₂ high
Group 4 - nano-TiO ₂ low	Group 5 - nano-TiO ₂ high	Group 6 - μ -Eu ₂ O ₃ low
Group 7 - μ -Eu ₂ O ₃ high	Group 8 - μ -BaSO ₄ low	Group 9 - μ -BaSO ₄ high
Group 10 - μ -ZrO ₂ low	Group 11 - μ -ZrO ₂ high	Group 12 - nano-SiO ₂ low
Group 13 - nano-SiO ₂ high		

LDH Lactic dehydrogenase - GLU β -Glucuronidase - TP Total protein

7.2 Retention Analysis of the Test Items in Lungs

The chemical analysis resulted in the following lung loads retained after 3, 28 and 90 days after end of exposure. Individual data are shown in Appendix 4. The mean data are illustrated in bar charts given in Appendix 5. - For comparison, the calculated $t_{1/2}$ of the preceding study using intratracheal instillation as administration mode are juxtaposed to the inhalation-derived $t_{1/2}$ (see table 7.8); calculation of $t_{1/2}$: Appendix 6.

Table 7.2 Total burden – approx. dose 0.3 µl

Dust #			(µg/lung)			(% of total dose)		
			Day 3	Day 28	Day 90			
1	$\mu\text{-TiO}_2$	Mean	996	593	318	100	59,5	31,9
		SD	259	160	96			
2	nano-TiO_2	Mean	438	242	113	100	55,3	25,8
		SD	155	89	41			
3	$\mu\text{-Eu}_2\text{O}_3$	Mean	1223	1274	1194	100	104,2	97,6
		SD	232	236	156			
4	$\mu\text{-BaSO}_4$	Mean	931	223	20	100	24,0	2,1
		SD	170	93	6,0			
5	$\mu\text{-ZrO}_2$	Mean	1364	1219	732	100	89,4	53,7
		SD	263	273	73			
6	nano-SiO_2	Mean	196	95	24	100	48,5	12,2
		SD	33	19	7,7			

Table 7.3 Total burden – approx. dose 0.9 µl

Dust #			(µg/lung)			(% of total dose)		
			Day 3	Day 28	Day 90			
1	$\mu\text{-TiO}_2$	Mean	3351	1606	881	100	47,9	26,3
		SD	738	268	110			
2	nano-TiO_2	Mean	1491	1270	818	100	85,2	54,9
		SD	198	272	123			
3	$\mu\text{-Eu}_2\text{O}_3$	Mean	3604	3368	2790	100	93,5	77,4
		SD	644	378	661			
4	$\mu\text{-BaSO}_4$	Mean	1282	478	113	100	37,3	8,8
		SD	358	100	115			
5	$\mu\text{-ZrO}_2$	Mean	3788	3723	2724	100	98,3	71,9
		SD	482	382	581			
6	nano-SiO_2	Mean	330	127	39	100	38,5	11,8
		SD	49	37	9,3			

Total dose: The lung load analysed at day 3 was set = 100%

Table 7.4 Particulate burden – Dose 0.3 µl

Dust #		(µg/lung)			(% of total dose)		
		Day 3	Day 28	Day 90			
1	µ-TiO ₂	Mean	993	588	317	99,7	59,0
		SD	258	158	96		
2	nano-TiO ₂	Mean	436	241	113	99,5	55,0
		SD	155	89	41		
3	µ-Eu ₂ O ₃	Mean	1117	954	842	91,3	78,0
		SD	240	106	140		
4	µ-BaSO ₄	Mean	925	221	20	99,4	23,7
		SD	170	93	5,8		
5	µ-ZrO ₂	Mean	1356	1213	729	99,4	88,9
		SD	262	273	73		
6	nano-SiO ₂	Mean	191	91	21	97,4	46,4
		SD	33	19	7,7		

Table 7.5 Particulate burden – Dose 0.9 µl

Dust #		(µg/lung)			(% of total dose)		
		Day 3	Day 28	Day 90			
1	µ-TiO ₂	Mean	3348	1602	879	100	47,8
		SD	739	268	110		
2	nano-TiO ₂	Mean	1488	1267	815	99,8	85,0
		SD	198	270	123		
3	µ-Eu ₂ O ₃	Mean	3499	2995	2106	97,1	83,1
		SD	693	415	671		
4	µ-BaSO ₄	Mean	1271	475	113	99,1	37,1
		SD	357	100	114		
5	µ-ZrO ₂	Mean	3767	3712	2718	99,4	98,0
		SD	479	381	580		
6	nano-SiO ₂	Mean	323	120	34	97,9	36,4
		SD	49	41	7,1		

Total dose: The lung load analysed at day 3 was set = 100%

Table 7.6 Ionic burden – Dose 0.3 µl

Dust #		(µg/lung)			(% of total dose)		
		Day 3	Day 28	Day 90			
1	µ-TiO ₂	Mean	1.2	0.7	0.7	0,12	0,07
		SD	0.5	0.3	0.3		
2	nano-TiO ₂	Mean	2.1	1.5	0.6	0,48	0,34
		SD	0,8	0,8	0.1		
3	µ-Eu ₂ O ₃	Mean	106	320	353	8,7	26,2
		SD	56	333	233		28,9
4	µ-BaSO ₄	Mean	6,0	2,1	0,4	0,64	0,23
		SD	1,4	1,0	0.3		0,04
5	µ-ZrO ₂	Mean	8,5	6,3	3,2	0,62	0,46
		SD	1,8	0,5	0.9		0,23
6	nano-SiO ₂	Mean	5,4	4,4	3,0	2,8	2,2
		SD	1,9	1,2	0.3		1,5

Table 7.7 Ionic burden – Dose 0.9 µl

Dust #		(µg/lung)			(% of total dose)		
		Day 3	Day 28	Day 90			
1	µ-TiO ₂	Mean	2,3	1,6	1,5	0,07	0,05
		SD	0,5	0,7	0.1		
2	nano-TiO ₂	Mean	3,1	3,3	3,7	0,21	0,22
		SD	1,7	1,8	0,9		
3	µ-Eu ₂ O ₃	Mean	105	372	684	2,9	10,3
		SD	68	159	720		19,0
4	µ-BaSO ₄	Mean	10,3	3,3	0,8	0,80	0,26
		SD	3,8	0,7	0,4		0,06
5	µ-ZrO ₂	Mean	20	12	5,6	0,53	0,32
		SD	4,8	1,9	2,0		0,15
6	nano-SiO ₂	Mean	7,3	4,4	3,2	2,2	1,3
		SD	1,7	1,0	1,1		0,97

Total dose: The lung load analysed at day 3 was set = 100%

Biokinetics of the test items following inhalation (in comparison to instillation)

The physiological rat lung clearance has been precisely investigated using low soluble radioactively tagged particles, e.g. ^{85}Sr -polystyrene particles resulting in half-times of 50-70 days; therefore, a mean of approx. 60 days is a well-documented experimental value (MORROW, 1988; MUHLE et al., 1990; BELLMANN et al., 1991).

Using intratracheal instillation, there is the possibility that the injected material can partially be either "coughed up in those species with such capability, especially if recovery from anesthesia is too rapid or if the volume of instillate is too large, or could be quickly cleared from the trachea" (DRISCOLL et al., 2000). To exclude the cleared mass underlying rapid clearance mechanisms from half-time calculation for the deep lung, only the retained masses (determined by chemical analysis) at day 3, 28 and 90 (not any calculated doses at day 0) were included into this calculation in the preceding instillation study; see report on F 2336.

In the inhalation study (F 2364), half-times were also calculated using the retained masses at 3, 28 and 90 days, thus, excluding the rapid clearance effects. Comparison of half-times:

A. The clearance half-times after instillation (see Table 7.8; values *in italics*) showed a value close to the physiological rat lung clearance of approx. 60 days in the $\mu\text{-TiO}_2$ "**Bayertitan T**" low dose group. In the high dose group a 2- to 3-fold increased half-time (141 days) was observed (overload effect). In the **nano-TiO₂ P25** group a half-time close to 60 days was calculated in the low dose group (very slight clearance retardation).

In the $\mu\text{-BaSO}_4$ and **nano-SiO₂** groups (either the low and high dose groups), smaller values in the range of 25-40 days were calculated indicating an additional dissolution effect.

In the $\mu\text{-Eu}_2\text{O}_3$ and $\mu\text{-ZrO}_2$ groups increased half-times, i.e. 4- to 5-fold (low and high dose) and 2- to 4-fold, respectively, were calculated indicating a clear surface-chemistry-related contribution to the toxicity and increase of the clearance half-times. Dissolution of particles: $\mu\text{-TiO}_2$ "Bayertitan T" and nano-TiO₂ P25 showed very low ionic moieties regarding the total lung burden. Levels in lungs at all 3 time-points did not exceed the 0.1% percentage except TiO₂ P25 in the high dose $\rightarrow \geq 0.2\%$.

B. Following inhalation, the $\mu\text{-TiO}_2$ "**Bayertitan T**" low and high dose groups as well as the **nano-TiO₂ P25** low dose group resulted in half-times within the physiological range, even the **nano-TiO₂ P25** high dose showed a significantly lower value of 100 days (as compared to 870 days after instillation). For the other groups principally the same half-times as observed after instillation were detected (see Table 7.8). The calculation of the clearance half-times is presented in Appendix 6.

Dissolution of particles: $\mu\text{-Eu}_2\text{O}_3$ resulted in the highest ionic percentages of all 6 dusts amounting to a range of 9-29% of the total mass in lungs.

$\mu\text{-BaSO}_4$ showed low ionic moieties of 0.8% or lower regarding the total lung burden (at all 3 time-points). $\mu\text{-ZrO}_2$ showed low ionic moieties regarding the total lung burden. Levels in lungs at all 3 time-points did not exceed the 0.65% percentage.

Nano-SiO₂ showed low ionic moieties of 2.2% or lower regarding the total lung burden (at all 3 time-points).

Comparison $\mu\text{-Eu}_2\text{O}_3$ vs. **nano-SiO₂**: Both show high solubility, however, the ionic moiety of Eu₂O₃ is eliminated from lungs more slowly than that of amorphous SiO₂. The difference may be caused by different transport mechanisms of the ions.

To reveal the mechanistic reason for the very specific behavior of BaSO₄ a comprehensive toxicokinetic investigation was conducted by KONDURU et al., 2014. A high biosolubility was found for a nano-BaSO₄ after lung instillation. The same was observed in this BAuA project using a microscaled type of BaSO₄ particles.

Table 7.8 Calculated half-time values (absolute values and percentual values vs. 60 days of physiological rat lung clearance)

	Clearance half-time (days)		Clearance half-time (in % vs. the physiological half-time in rat lungs; 60 days = 100%)	
	Inhalation	<i>Instillation</i>	Inhalation	<i>Instillation</i>
$\mu\text{-TiO}_2$	55	47	92	78
	49	89	82	148
nano-TiO_2	47	141	78	235
	100	866	167	1443
$\mu\text{-Eu}_2\text{O}_3$	2310	277	3850	462
	224	347	373	578
$\mu\text{-BaSO}_4$	23	26	38	43
	23	39	38	65
$\mu\text{-ZrO}_2$	96	133	160	222
	165	257	275	428
nano-SiO_2	29	25	49	42
	29	27	48	45

The selection of an appropriate density value is not simply done as reliable, experimentally determined values are regularly scarce, in particularly for nanoparticle agglomerates. For example, the raw density of TiO₂ is 4.3 g/cm³; in the GBP experiment with intratracheal instillation (F 2336), 3.8 g/cm³ was chosen as agglomerate density for nano-TiO₂. For comparison, a density of 4.3 x ½ = 2.2 g/cm³ was the 50%-value recommended by the AGS (AGS, 2015) to estimate unknown agglomerate densities. PAULUHN (2011) determined an agglomerate density of 1.6 g/cm³ applying gas pycnometry. Using the most realistic value of Pauluhn the analysed dose of 440/1490 µg in the nano-TiO₂ P25 low and high dose groups of the inhalation study would correspond to 275/930 nl administered volume, respectively, in the deep lung after 3 days. Overall, in the low dose group no lung overload was existing, independently on the chosen agglomeration density.

In the $\mu\text{-ZrO}_2$ low dose groups the half-time was increased (96 days). Lower half-times than 60 days observed in the $\mu\text{-BaSO}_4$ and nano-SiO₂ groups indicate an additive half-time effect due to an evident biosolubility. Correspondingly, in the high dose groups increased values were detected indicating overload effects in the nano-TiO₂ P25 and $\mu\text{-ZrO}_2$ high dose groups.

8 Summary

14-day inhalation study

The duration of exposure was 6 hours/day, 5 days/week for 2 weeks. The Multipath Particle Deposition (MPPD) model (RIVM, 2002) was used to calculate the target lung doses after inhalation. The aerosol concentrations were adjusted to achieve in each group the same volumetric dose as administered in the instillation study (i.e. 0.5 µl and 1.5 µl; actually approx. 0.33 µl and 1 µl). In the first exposure week the concentrations were re-adjusted based on actual MMAD values measured in the running study.

Animals were sacrificed 3 days after end of exposure; a second subset was sacrificed after 28 days to investigate recovery effects in bronchoalveolar lavage fluid (BALF). For the chemical analysis of retained masses in lungs sacrifices were scheduled at days 3, 28 and 90 after end of exposure.

Analysis of the bronchoalveolar lavage fluid (BALF)

Differential cell count data

- Polymorphonuclear (PMN) cell levels showed no increase in absolute numbers after treatment in the µ-TiO₂ (rutile; PMN < 1%/ $< 2\%$) and a very slight increase in the µ-BaSO₄ groups (PMN < 1%/ $< 6\%$) 3 days upon cessation of exposure. - After 28 days of recovery, PMN levels had returned fully to control levels.
- In the nano-TiO₂ P25 groups PMN levels of 4.3% (low dose; not statistically significantly increased) and 22.7% (high dose; ***) were detected. - After 28 days 5.3% and 17.9% (***)) were observed.
- Both, µ-ZrO₂ and nano-SiO₂ induced strong PMN inflammatory reactions (20%-50%) in the low and high dose groups. – After 28 days, PMN levels of 6%/34% (µ-ZrO₂) and 3.8%/3.4% (nano-SiO₂) were observed.
- µ-Eu₂O₃ resulted in the low and high groups in a very strong inflammatory reaction that persisted during the 4-week recovery period. Cells were severely damaged and a conclusive evaluation of the cytospot slides was not possible.

Enzyme and protein analysis data

- The normalised data for lactic dehydrogenase (LDH), β-glucuronidase and total protein mirror the inflammatory effects observed with the PMN levels.
- µ-TiO₂ (both doses), nano-TiO₂ P25 (low dose only) and µ-BaSO₄ (both doses) behaved similar to the clean air controls.
- µ-ZrO₂ and nano-SiO₂ showed a strong inflammatory reaction at day 3 with partial recovery or full normalization at day 28, respectively.
- The rare earth µ-Eu₂O₃ resulted in the low and high groups in a very strong inflammatory reaction that persisted during the 4-week recovery period.

Chemical analysis of the lung burdens

- The target volumetric lung doses were satisfactorily achieved.

Conclusion: Comparison of inhalation versus intratracheal instillation

Considering the percentual PMN as well as the absolute PMN concentrations, the predominant observation is that inhalation induced a smaller PMN influx (with exception of soluble µ-BaSO₄ and nano-SiO₂) at similar doses (65% lower at maximum). This can be expected because of the physiological dust uptake and deposition by

inhalation that is more gentle than intratracheal instillation (bolus effect!). This is true also for nano-TiO₂ P25; for this dust, the low dose group after inhalation and the high dose group after inhalation should be compared as they have the same effective dose at day 3 (different assumptions of the agglomerate density had been used; see Tables 8.1 and 8.2).

Table 8.1 Inhalation study: Clearance half-time and results of BAL analysis

Dust	Dose (μ l/rat) predicted	Effective dose day 3 (μ l/rat)	Clearance half-time (d) ³	BAL PMN (%) day 3	BAL PMN (%) day 28	BAL PMN (cells/ml) day 3	BAL PMN (cells/ml) day 28
Vehicle control I	0	0	approx. 60	0.4	0.7	317	862
μ -TiO ₂ Bayertitan T	0.3	0.23	55	0.5	0.8	331	450
	0.9 ¹	0.78	49	1.6	0.5	1398	465
nano-TiO ₂ P25	0.3	0.27	47	4.3	5.3	4821	6636
	0.9	0.93	100	***22.7	***17.9	**55731	**28448
μ -Eu ₂ O ₃	0.3	0.17	2310	n.m.	n.m.	n.m.	n.m.
	0.9	0.49	224	n.m.	n.m.	n.m.	n.m.
Vehicle control II	0	0	approx. 60	1.1	2.4	919	2267
μ -BaSO ₄	0.3	0.21	23	0.6	0.5	652	516
	0.9	0.28	23	5.9	1.7	5333	698
μ -ZrO ₂	0.3	0.24	96	***27.8	6.4	62459	4266
	0.9	0.66	165	***35.5	***34.0	**118624	***79762
nano-SiO ₂ NM-200	0.3	0.18	29	*19.0	3.8	*87152	5327
	0.9	0.30	29	***50.3	3.4	***269609	2258

Statistics Test: Dunnett's Test: * - 5%; ** - 1%; *** -0.1% significance level

¹ 0.9 μ l/rat is considered as overload dose; the lower dose as compared to the instillation study (1.5 μ l) was chosen to achieve a better comparability of the actual deep lung burdens in the two studies

Table 8.2 For comparison: Instillation study - Clearance half-time and results of BAL analysis

Dust	Dose (μ l/rat)	Effective dose day 3 (μ l/rat)	Clearance half-time (d) ³	BAL PMN (%) day 3	BAL PMN (%) day 28	BAL PMN (cells/ml) day 3	BAL PMN (cells/ml) day 28
Vehicle control	0	0	ca. 60	2.0	1.1	2233	853
μ -TiO ₂ Bayertitan T	0.5	0.37 (74%) ²	47	2.6	0.8	4982	571
	1.5 ¹	1.32 (88%)	141	11.7	4.7	40005	5740
nano-TiO ₂ P25	1.19	0.85 (72%)	89	***30.3	***36.1	**141098	***79986
	3.56	2.40 (67%)	866	***48.2	***35.9	***217983	***122517
μ -Eu ₂ O ₃	0.5	0.38 (76%)	277	***50.7	***38.2	***227489	***84921
	1.5	1.17 (78%)	347	***64.5	***37.4	***318245	***126353
μ -BaSO ₄	0.5	0.29 (58%)	26	4.2	0.6	4653	648
	1.5	0.99 (66%)	39	18.2	0.8	36738	623
μ -ZrO ₂	0.5	0.26 (52%)	133	***36.6	1.5	*124975	1329
	1.5	0.92 (61%)	257	***53.5	5.8	***199373	10603
nano-SiO ₂ NM-200	1.0	0.19 (19%)	25	11.9	0.9	41147	901
	3.0	0.49 (16%)	27	***35.2	1.4	***172360	1140

Statistics Test: Dunnett's Test: * - 5%; ** - 1%; *** -0.1% significance level

¹ 1.5 μ l/rat is considered as overload dose

² (74%) = about 1/3 is not reaching or quickly removed from the lower respiratory tract; an average retention of approx. 70% as compared to the administered total dose was detected on day 3

³ Based on total burden (particulate + ionic); standard value rat: 60 days

Clearance half-times and PMN levels with evident differences (inhalation vs. instillation) are juxtaposed vertically with same colour.

9 Literature

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Appendix 1 Experimental Design of the 2-wk Inhalation Study

Male Wistar rats, Crl:WI(Han) - Inhalation study

BAL: at day 3 and 28; chemical analysis at day 3, 28 and 90 after end of exposure

Part 1: Nov. 9 - 24, 2015 - 2-wk inhalation test → BAL → Chemical Analysis
 Wistar rats, strain: Crl:WI(Han), males only Day 3 und 28 Day 3, 28 and 90 of recovery

Group	Aerosol	Dose day 3* (µl per lung) target (actual)	Aerosol conc.* (µl/m ³)	Aerosol conc.* (mg/m ³)	Animal number per group		
					Day 3 Rec	Day 28 Rec	Day 90 Rec
1	Clean air	0	0	0	6/6	6/6	6
2	TiO ₂ Bayertitan T μ-TiO ₂ ρ = 4.3	ca. 0.3 (0.23)	6.4	27.6	6/6	6/6	6
		ca. 0.9 (0.78)	19.8	85.2	6/6	6/6	6
4	nano-TiO ₂ TiO ₂ P25 EU/JRC ρ = 1.6 Aggl	ca. 0.3 (0.27)	4.4	9.7	6/6	6/6	6
		ca. 0.9 (0.93)	13.2	29.1	6/6	6/6	6
6	μ-Eu ₂ O ₃ ρ = 7.4	ca. 0.3 (0.17)	8.6	31.9	6/6	6/6	6
		ca. 0.9 (0.49)	25.1	92.8	6/6	6/6	6
		Total of rats: 210			84	84	42

Part 2: Jan. 25 - Feb. 9, 2016 - 2-wk inhalation test → BAL → Chemical Analysis

Group	Aerosol	Dose* (µl per lung) target (actual)	Aerosol conc.* (µl/m ³)	Aerosol conc.* (mg/m ³)	Animal number per group		
					Day 3 Rec	Day 28 Rec	Day 90 Rec
1	Clean air	0	0	0	6/6	6/6	6
8	μ-BaSO ₄ ρ = 4.5	ca. 0.3 (0.21)	9.3	41.7	6/6	6/6	6
		ca. 0.9 (0.28) ¹	30.2	135.8	6/6	6/6	6
10	Y-stabilised ZrO ₂ μ-ZrO ₂ ρ = 5.7	ca. 0.3 (0.24)	9.4	53.4	6/6	6/6	6
		ca. 0.9 (0.66)	28.8	164.0	6/6	6/6	6
12	Amorphous SiO ₂ NM-200 ρ = 1.1 Aggl	ca. 0.3 (0.18)	10.0	11.0	6/6	6/6	6
		ca. 0.9 (0.30)	29.7	32.7	6/6	6/6	6
		Total of rats: 210			84	84	42

¹ 0.9 µl/rat is considered as overload dose; compared to the instillation study (there 1.5 µl) a lower dose was selected to achieve a better comparability of both administration modes in terms of the actual burdens in deep lungs

Appendix 2 Analysis of Bronchoalveolar Lavage Fluid (BALF)

Sacrifice at day 3 following inhalation

Differential cell count – means

Wistar Han Males		Leukocyte concentration (cell/ml)	Macrophages (%)	PMN (%)	Lymphocytes (%)
Group					
1 Control Group	Mean	82292	99.0	0.4	0.5
	Std	46573	0.5	0.3	0.2
	N	6	6	6	6
2 $\mu\text{-TiO}_2$ low	Mean	70833	99.2	0.5	0.3
	Std	27894	0.4	0.4	0.2
	N	6	6	6	6
3 $\mu\text{-TiO}_2$ high	Mean	82917	98.0	1.6	0.3
	Std	35064	2.7	2.8	0.3
	N	6	6	6	6
4 nano-TiO ₂ P25 low	Mean	109063	95.1	4.3	0.7
	Std	29290	3.0	2.9	0.3
	N	6	6	6	6
5 nano-TiO ₂ P25 high	Mean	214583	*** 76.0	*** 22.7	1.3
	Std	74666	16.7	16.0	1.0
	N	6	6	6	6
6 $\mu\text{-Eu}_2\text{O}_3$ low	Mean	405000	n.m	n.m	n.m
	Std	96644	n.m	n.m	n.m
	N	6	6	6	6
7 $\mu\text{-Eu}_2\text{O}_3$ high	Mean	463333	n.m	n.m	n.m
	Std	338728	n.m	n.m	n.m
	N	6	6	6	6

Statistics Test: Dunnett Test: * - 5%; ** - 1%; *** - 0.1% significance level

n.m. Not measured/evaluated because cells were too severely damaged

Group 1 – Control	Group 2 - Bayertitan low	Group 3 - Bayertitan high
Group 4 - TiO ₂ P ₂₅ low	Group 5 - TiO ₂ P25 high	Group 6 - Eu ₂ O ₃ low
Group 7 - Eu ₂ O ₃ high	Group 8 - BaSO ₄ low	Group 9 - BaSO ₄ high
Group 10 - ZrO ₂ low	Group 11 - ZrO ₂ high	Group 12 - SiO ₂ low
Group 13 - SiO ₂ high		

MPh – Macrophages; PMN – Polymorphonuclear cells; Lympho – Lymphocytes

Analysis of Bronchoalveolar Lavage Fluid (BALF) – cont'd
 Sacrifice at day 3 following inhalation
 Differential cell count – means

Wistar Han Males		Leukocyte concentration (cell/ml)	Macrophages (%)	PMN (%)	Lymphocytes (%)
Group					
1 Control Group	Mean	99375	98.2	1.1	0.7
	Std	42342	1.7	1.2	0.5
	N	6	6	6	6
8 $\mu\text{-BaSO}_4$ low	Mean	103542	98.6	0.6	0.8
	Std	32855	1.1	0.5	1.1
	N	6	6	6	6
9 $\mu\text{-BaSO}_4$ high	Mean	99479	93.9	5.9	0.3
	Std	22501	5.4	5.4	0.2
	N	6	6	6	6
10 $\mu\text{-ZrO}_2$ low	Mean	224063	*** 71.3	*** 27.8	0.9
	Std	25865	2.9	2.6	0.4
	N	6	6	6	6
11 $\mu\text{-ZrO}_2$ high	Mean	320313	*** 61.5	*** 35.5	*** 3.0
	Std	89622	8.8	9.0	1.2
	N	6	6	6	6
12 SiO_2 NM-200 low	Mean	415417	* 80.5	* 19.0	0.6
	Std	108446	20.3	19.8	0.8
	N	6	6	6	6
13 SiO_2 NM-200 high	Mean	532083	*** 48.4	*** 50.3	1.3
	Std	75658	13.5	13.6	1.0
	N	6	6	6	6

Statistics Test: Dunnett Test: * - 5%; ** - 1%; *** - 0.1% significance level

Group 1 – Control	Group 2 - Bayertitan low	Group 3 - Bayertitan high
Group 4 - TiO_2 P ₂₅ low	Group 5 - TiO_2 P25 high	Group 6 - Eu_2O_3 low
Group 7 - Eu_2O_3 high	Group 8 - BaSO_4 low	Group 9 - BaSO_4 high
Group 10 - ZrO_2 low	Group 11 - ZrO_2 high	Group 12 - SiO_2 low
Group 13 - SiO_2 high		

MPh – Macrophages; PMN – Polymorphonuclear cells; Lympho – Lymphocytes

Analysis of Bronchoalveolar Lavage Fluid (BALF) – cont'd
 Sacrifice at day 3 following inhalation
 Differential cell count – means, absolute

Wistar Han Males		Leucocyte conc.(1/ml)	MPh (1/ml)	PMN (1/ml)	Lympho (1/ml)
Group					
1 Control Group	Mean	82292	81558	317	417
	Std	46573	46183	255	228
	N	6	6	6	6
2 μ -TiO ₂ low	Mean	70833	70281	331	221
	Std	27894	27652	226	227
	N	6	6	6	6
3 μ -TiO ₂ high	Mean	82917	81265	1398	254
	Std	35064	34758	2549	264
	N	6	6	6	6
4 nano-TiO ₂ P25 low	Mean	109063	103463	4821	779
	Std	29290	27395	3647	490
	N	6	6	6	6
5 nano-TiO ₂ P25 high	Mean	214583	**155791	**55713	**3079
	Std	74666	41191	51883	2846
	N	6	6	6	6
6 μ -Eu ₂ O ₃ low	Mean	**405000	n.m.	n.m.	n.m.
	Std	96644	n.m.	n.m.	n.m.
	N	6	6	6	6
7 μ -Eu ₂ O ₃ high	Mean	***463333	n.m.	n.m.	n.m.
	Std	338728	n.m.	n.m.	n.m.
	N	6	6	6	6

Statistics Test: Dunnett Test: * - 5%; ** - 1%; *** - 0.1% significance level

Analysis of Bronchoalveolar Lavage Fluid (BALF) – cont'd
 Sacrifice at day 3 following inhalation
 Differential cell count – means, absolute

Wistar Han Males		Leucocyte conc.(1/ml)	MPh (1/ml)	PMN (1/ml)	Lympho (1/ml)
Group					
1 Control Group	Mean	99375	97790	919	667
	Std	42342	42193	640	400
	N	6	6	6	6
8 μ -BaSO ₄ low	Mean	103542	102127	652	763
	Std	32855	32739	651	895
	N	6	6	6	6
9 μ -BaSO ₄ high	Mean	99479	93895	5333	252
	Std	22501	22995	4046	196
	N	6	6	6	6
10 μ -ZrO ₂ low	Mean	*224063	159627	62459	1976
	Std	25865	17683	10581	998
	N	6	6	6	6
11 μ -ZrO ₂ high	Mean	***320313	*192572	**118624	***9116
	Std	89622	41600	63287	2949
	N	6	6	6	6
12 SiO ₂ NM-200 low	Mean	***415417	***325754	*87152	2511
	Std	108446	86446	90285	3365
	N	6	6	6	6
13 SiO ₂ NM-200 high	Mean	***532083	***255882	***269609	***6593
	Std	75658	78429	88564	4410
	N	6	6	6	6

Statistics Test: Dunnett Test: * - 5%; ** - 1%; *** - 0.1% significance level

Group 1 - Control

Group 2 - μ -TiO₂ low

Group 3 - μ -TiO₂ high

Group 4 - nano-TiO₂ low

Group 5 - nano-TiO₂ high

Group 6 - μ -Eu₂O₃ low

Group 7 - μ -Eu₂O₃ high

Group 8 - μ -BaSO₄ low

Group 9 - μ -BaSO₄ high

Group 10 - μ -ZrO₂ low

Group 11 - μ -ZrO₂ high

Group 12 - nano-SiO₂ low

Group 13 - nano-SiO₂ high

MPh – Macrophages; PMN – Polymorphonuclear cells; Lympho – Lymphocytes

Analysis of Bronchoalveolar Lavage Fluid (BALF) – cont'd
 Sacrifice at day 28 following inhalation
 Differential cell count – means

Wistar Han Males		Leukocyte concentration (cell/ml)	Macrophages (%)	PMN (%)	Lymphocytes (%)
Group					
1 Control Group	Mean	130938	99.1	0.7	0.2
	Std	29221	0.5	0.3	0.2
	N	6	6	6	6
2 $\mu\text{-TiO}_2$ low	Mean	76146	99.1	0.8	0.1
	Std	37653	1.3	1.3	0.2
	N	6	6	6	6
3 $\mu\text{-TiO}_2$ high	Mean	99792	99.5	0.5	0.0
	Std	64654	0.4	0.5	0.1
	N	6	6	6	6
4 nano-TiO ₂ P25 low	Mean	95938	94.5	5.3	0.2
	Std	44968	10.2	10.2	0.2
	N	6	6	6	6
5 nano-TiO ₂ P25 high	Mean	140000	*** 81.8	*** 17.9	0.3
	Std	54440	8.6	8.7	0.4
	N	6	6	6	6
6 $\mu\text{-Eu}_2\text{O}_3$ low	Mean	1549167	n.m.	n.m.	n.m.
	Std	1726744	n.m.	n.m.	n.m.
	N	6	6	6	6
7 $\mu\text{-Eu}_2\text{O}_3$ high	Mean	1148750	n.m.	n.m.	n.m.
	Std	845638	n.m.	n.m.	n.m.
	N	6	6	6	6

Statistics Test: Dunnett Test: * - 5%; ** - 1%; *** - 0.1% significance level

n.m. Not measured/evaluated because cells were too severely damaged

Group 1 – Control

Group 2 - Bayertitan low

Group 3 - Bayertitan high

Group 4 - TiO₂ P₂₅ low

Group 5 - TiO₂ P25 high

Group 6 - Eu₂O₃ low

Group 7 - Eu₂O₃ high

Group 8 - BaSO₄ low

Group 9 - BaSO₄ high

Group 10 - ZrO₂ low

Group 11 - ZrO₂ high

Group 12 - SiO₂ low

Group 13 - SiO₂ high

MPh – Macrophages; PMN – Polymorphonuclear cells; Lympho - Lymphocytes

Analysis of Bronchoalveolar Lavage Fluid (BALF) – cont'd
 Sacrifice at day 28 following inhalation
 Differential cell count – means

Wistar Han Males		Leukocyte concentration (cell/ml)	Macrophages (%)	PMN (%)	Lymphocytes (%)
Group					
1 Control Group	Mean	98125	97.0	2.4	0.7
	Std	38452	3.7	3.3	0.9
	N	6	6	6	6
8 $\mu\text{-BaSO}_4$ low	Mean	98646	99.3	0.5	0.2
	Std	25834	0.3	0.2	0.3
	N	6	6	6	6
9 $\mu\text{-BaSO}_4$ high	Mean	63229	98.0	1.7	0.3
	Std	23892	2.4	2.2	0.4
	N	6	6	6	6
10 $\mu\text{-ZrO}_2$ low	Mean	79375	93.2	6.4	0.4
	Std	23164	5.5	5.3	0.4
	N	6	6	6	6
11 $\mu\text{-ZrO}_2$ high	Mean	227188	*** 65.4	*** 34.0	0.7
	Std	174349	12.0	12.0	0.1
	N	6	6	6	6
12 SiO_2 NM-200 Low	Mean	133333	95.5	3.8	0.7
	Std	52813	1.0	1.1	0.7
	N	6	6	6	6
13 SiO_2 NM-200 High	Mean	72292	96.1	3.4	0.5
	Std	25065	3.7	3.4	0.4
	N	6	6	6	6

Statistics Test: Dunnett Test: * - 5%; ** - 1%; *** - 0.1% significance level

Group 1 – Control	Group 2 - Bayertitan low	Group 3 - Bayertitan high
Group 4 - TiO_2 P ₂₅ low	Group 5 - TiO_2 P25 high	Group 6 - Eu_2O_3 low
Group 7 - Eu_2O_3 high	Group 8 - BaSO_4 low	Group 9 - BaSO_4 high
Group 10 - ZrO_2 low	Group 11 - ZrO_2 high	Group 12 - SiO_2 low
Group 13 - SiO_2 high		

MPh – Macrophages; PMN – Polymorphonuclear cells; Lympho - Lymphocytes

Analysis of Bronchoalveolar Lavage Fluid (BALF) – cont'd
 Sacrifice at day 28 following inhalation
 Differential cell count – means, absolute

Wistar Han		Leucocyte conc.(1/ml)	MPh (1/ml)	PMN (1/ml)	Lympho (1/ml)
Group					
1 Control Group	Mean	130938	129825	862	251
	Std	29221	29217	457	237
	N	6	6	6	6
2 μ -TiO ₂ low	Mean	76146	75620	450	76
	Std	37653	37784	566	186
	N	6	6	6	6
3 μ -TiO ₂ high	Mean	99792	99309	465	18
	Std	64654	64246	571	44
	N	6	6	6	6
4 nano-TiO ₂ P25 low	Mean	95938	89053	6636	248
	Std	44968	40667	13278	307
	N	6	6	6	6
5 nano-TiO ₂ P25 high	Mean	140000	111136	**28448	416
	Std	54440	34374	22995	619
	N	6	6	6	6
6 μ -Eu ₂ O ₃ low	Mean	**1549167	n.m.	n.m.	n.m.
	Std	1726744	n.m.	n.m.	n.m.
	N	6	6	6	6
7 μ -Eu ₂ O ₃ high	Mean	1148750	n.m.	n.m.	n.m.
	Std	845638	n.m.	n.m.	n.m.
	N	6	6	6	6

Statistics Test: Dunnett Test: * - 5%; ** - 1%; *** - 0.1% significance level

Group 1 - Control	Group 2 - μ -TiO ₂ low	Group 3 - μ -TiO ₂ high
Group 4 - nano-TiO ₂ low	Group 5 - nano-TiO ₂ high	Group 6 - μ -Eu ₂ O ₃ low
Group 7 - μ -Eu ₂ O ₃ high	Group 8 - μ -BaSO ₄ low	Group 9 - μ -BaSO ₄ high
Group 10 - μ -ZrO ₂ low	Group 11 - μ -ZrO ₂ high	Group 12 - nano-SiO ₂ low
Group 13 - nano-SiO ₂ high		

MPh – Macrophages; PMN – Polymorphonuclear cells; Lympho – Lymphocytes

Analysis of Bronchoalveolar Lavage Fluid (BALF) – cont'd
 Sacrifice at day 28 following inhalation
 Differential cell count – means, absolute

Wistar Han Males		Leucocyte conc.(1/ml)	MPh (1/ml)	PMN (1/ml)	Lympho (1/ml)
Group					
1 Control Group	Mean	98125	95292	2266	567
	Std	38452	38729	3606	753
	N	6	6	6	6
8 μ -BaSO ₄ low	Mean	98646	97947	516	183
	Std	25834	25642	302	240
	N	6	6	6	6
9 μ -BaSO ₄ high	Mean	63229	62405	698	127
	Std	23892	24504	715	159
	N	6	6	6	6
10 μ -ZrO ₂ low	Mean	79375	74764	4266	345
	Std	23164	24723	3070	416
	N	6	6	6	6
11 μ -ZrO ₂ high	Mean	*227188	145829	***79762	*1596
	Std	174349	113016	64986	1388
	N	6	6	6	6
12 SiO ₂ NM-200 low	Mean	133333	127315	5327	692
	Std	52813	50538	2755	422
	N	6	6	6	6
13 SiO ₂ NM-200 high	Mean	72292	69698	2258	335
	Std	25065	25413	2225	218
	N	6	6	6	6

Statistics Test: Dunnett Test: * - 5%; ** - 1%; *** - 0.1% significance level

Group 1 - Control	Group 2 - μ -TiO ₂ low	Group 3 - μ -TiO ₂ high
Group 4 - nano-TiO ₂ low	Group 5 - nano-TiO ₂ high	Group 6 - μ -Eu ₂ O ₃ low
Group 7 - μ -Eu ₂ O ₃ high	Group 8 - μ -BaSO ₄ low	Group 9 - μ -BaSO ₄ high
Group 10 - μ -ZrO ₂ low	Group 11 - μ -ZrO ₂ high	Group 12 - nano-SiO ₂ low
Group 13 - nano-SiO ₂ high		

MPh – Macrophages; PMN – Polymorphonuclear cells; Lympho – Lymphocytes

Analysis of Bronchoalveolar Lavage Fluid (BALF) – cont'd
Sacrifice at day 3 following inhalation
Biochemical parameters in the BALF supernatant – means

Males		LDH U/L	GLU U/L	TP mg/L	
1m	Mean	31.5	0.30	93.8	1 st control
	S.D.	7.8	0.09	12.4	
2m	Mean	32.7	0.18	93.5	
	S.D.	9.1	0.08	16.3	
3m	Mean	34.8	0.23	96.7	
	S.D.	7.1	0.10	18.2	
4m	Mean	45.3	0.28	114.7	
	S.D.	23.7	0.12	13.1	
5m	Mean	70.3*	0.37	154.0	
	S.D.	27.7	0.21	41.2	
6m	Mean	196.2**	15.18**	1068.0**	
	S.D.	19.2	1.30	143.4	
7m	Mean	214.2**	15.77**	1144.5**	
	S.D.	42.1	1.92	203.2	
1m	Mean	29.7	0.18	101.8	2 nd control
	S.D.	6.8	0.08	17.1	
8m	Mean	34.2	0.22	115.7	
	S.D.	9.2	0.08	17.7	
9m	Mean	32.7	0.18	110.0	
	S.D.	7.8	0.04	14.3	
10m	Mean	72.3**	0.50	237.0**	
	S.D.	8.8	0.11	35.7	
11m	Mean	122.8**	1.23**	478.2**	
	S.D.	17.9	0.29	115.5	
12m	Mean	98.8**	0.47	220.7**	
	S.D.	32.2	0.12	59.4	
13m	Mean	154.2**	0.90**	324.8**	
	S.D.	37.1	0.47	82.4	

N=6

Statistics Test: Dunnett Test: * - 5% significance level; ** - 1% significance level
 Group 1 – Control Group 2 - Bayertitan low Group 3 - Bayertitan high
 Group 4 - TiO₂ P₂₅ low Group 5 - TiO₂ P25 high Group 6 - Eu₂O₃ low
 Group 7 - Eu₂O₃ high Group 8 - BaSO₄ low Group 9 - BaSO₄ high
 Group 10 - ZrO₂ low Group 11 - ZrO₂ high Group 12 - SiO₂ low
 Group 13 - SiO₂ high

LDH: Lactic Dehydrogenase GLU: β-Glucuronidase TP: Total Protein

Analysis of Bronchoalveolar Lavage Fluid (BALF) – cont'd
Sacrifice at day 28 following inhalation
Biochemical parameters in the BALF supernatant – means

Males		LDH U/L	GLU U/L	TP mg/L	
1m	Mean	34.8	0.28	108.5	1 st control
	S.D.	6.3	0.08	9.1	
2m	Mean	33.0	0.23	107.3	
	S.D.	12.9	0.05	12.5	
3m	Mean	36.8	0.30	121.7	
	S.D.	11.4	0.09	31.7	
4m	Mean	37.8	0.33	116.0	
	S.D.	18.9	0.20	37.0	
5m	Mean	54.0	0.40	138.2	
	S.D.	25.8	0.18	37.3	
6m	Mean	249.3**	15.93**	1603.8**	
	S.D.	56.5	5.98	264.1	
7m	Mean	311.7**	19.85**	2074.5**	
	S.D.	68.5	4.51	341.9	
<hr/>					
1m	Mean	32.2	0.23	114.8	2 nd control
	S.D.	10.3	0.12	17.4	
8m	Mean	27.8	0.22	101.3	
	S.D.	7.7	0.08	25.4	
9m	Mean	27.5	0.22	110.5	
	S.D.	10.7	0.04	37.4	
10m	Mean	33.8	0.22	114.3	
	S.D.	7.3	0.04	15.1	
11m	Mean	62.2**	0.55	205.2*	
	S.D.	26.5	0.52	128.0	
12m	Mean	28.2	0.20	108.3	
	S.D.	3.9	0.09	14.6	
13m	Mean	29.3	0.20	102.5	
	S.D.	5.4	0.00	14.2	
<hr/>					
N=6					

Statistics Test: Dunnett Test: * - 5% significance level; ** - 1% significance level
 Group 1 – Control Group 2 - Bayertitan low Group 3 - Bayertitan high
 Group 4 - TiO₂ P₂₅ low Group 5 - TiO₂ P25 high Group 6 - Eu₂O₃ low
 Group 7 - Eu₂O₃ high Group 8 - BaSO₄ low Group 9 - BaSO₄ high
 Group 10 - ZrO₂ low Group 11 - ZrO₂ high Group 12 - SiO₂ low
 Group 13 - SiO₂ high

Appendix 3 Lung Weights of Animals used for BAL (day 3)

Group	Sex		Terminal Bweight g	Lung Weight g	Lung /Bodywt g/kg	
1	m	Mean	282.35	1.2240	4.33895	1 st control
		S.D.	9.45	0.0698	0.28695	
		N	6	6	6	
<hr/>						
2	m	Mean	277.33	1.2358	4.45790	
		S.D.	11.54	0.0818	0.27427	
		N	6	6	6	
<hr/>						
3	m	Mean	277.68	1.2700	4.56877	
		S.D.	16.83	0.1246	0.28295	
		N	6	6	6	
<hr/>						
4	m	Mean	284.53	1.4077*	4.95768*	
		S.D.	16.42	0.0768	0.33610	
		N	6	6	6	
<hr/>						
5	m	Mean	289.88	1.4842**	5.12407**	
		S.D.	19.57	0.0864	0.14950	
		N	6	6	6	
<hr/>						
6	m	Mean	281.58	1.8247**	6.49088**	
		S.D.	11.76	0.1133	0.49745	
		N	6	6	6	
<hr/>						
7	m	Mean	283.68	1.8908**	6.68355**	
		S.D.	31.51	0.1900	0.43980	
		N	6	6	6	
<hr/>						
1	m	Mean	301.62	1.3445	4.47065	2 nd control
		S.D.	17.73	0.0468	0.30419	
		N	6	6	6	
<hr/>						
8	m	Mean	275.07	1.2733	4.63655	
		S.D.	15.58	0.0686	0.28220	
		N	6	6	6	
<hr/>						
9	m	Mean	295.38	1.3837	4.69708	
		S.D.	29.35	0.0991	0.20759	
		N	6	6	6	
<hr/>						
10	m	Mean	287.37	1.5023	5.23607**	
		S.D.	11.13	0.0567	0.31502	
		N	6	6	6	
<hr/>						
11	m	Mean	286.08	1.7120**	5.98130**	
		S.D.	30.84	0.2106	0.28602	
		N	6	6	6	
<hr/>						
12	m	Mean	276.70	1.5393*	5.59567**	
		S.D.	23.49	0.0715	0.53505	
		N	6	6	6	
<hr/>						
13	m	Mean	283.10	1.6545**	5.84517**	
		S.D.	17.30	0.1183	0.25954	
		N	6	6	6	
<hr/>						

Lung weights are statistically significantly increased at day 3 in the TiO₂ P25, Eu₂O₃, ZrO₂ and SiO₂ groups, two each (Dunnett's test).

Lung Weights of Animals used for BAL (day 28) – cont'd

Group	Sex		Terminal Bweight	Lung Weight	Lung /Bodywt	1 st control
			g	g	g/kg	
1	m	Mean	340.55	1.4488	4.26510	1 st control
		S.D.	34.84	0.1194	0.22422	
		N	6	6	6	
		-----	-----	-----	-----	-----
2	m	Mean	333.67	1.4458	4.33503	
		S.D.	18.53	0.1068	0.24950	
		N	6	6	6	
		-----	-----	-----	-----	-----
3	m	Mean	347.13	1.6842	4.86685	
		S.D.	18.39	0.5298	1.59424	
		N	6	6	6	
		-----	-----	-----	-----	-----
4	m	Mean	339.48	1.6637	4.94063	
		S.D.	33.83	0.1450	0.67431	
		N	6	6	6	
		-----	-----	-----	-----	-----
5	m	Mean	344.55	1.5833	4.62715	
		S.D.	34.93	0.1162	0.51953	
		N	6	6	6	
		-----	-----	-----	-----	-----
6	m	Mean	352.32	2.4937**	7.07057**	
		S.D.	20.77	0.2151	0.26765	
		N	6	6	6	
		-----	-----	-----	-----	-----
7	m	Mean	333.33	2.7558**	8.26838**	
		S.D.	28.93	0.3183	0.57784	
		N	6	6	6	
		-----	-----	-----	-----	-----
1	m	Mean	342.25	1.4840	4.35038	2 nd control
		S.D.	36.18	0.1237	0.26801	
		N	6	6	6	
		-----	-----	-----	-----	-----
8	m	Mean	321.68	1.4287	4.44573	
		S.D.	21.65	0.1073	0.26777	
		N	6	6	6	
		-----	-----	-----	-----	-----
9	m	Mean	337.50	1.4415	4.27758	
		S.D.	24.18	0.1022	0.26058	
		N	6	6	6	
		-----	-----	-----	-----	-----
10	m	Mean	355.95	1.5890	4.48317	
		S.D.	38.73	0.1281	0.32352	
		N	6	6	6	
		-----	-----	-----	-----	-----
11	m	Mean	332.22	1.6183	4.87048*	
		S.D.	28.26	0.2084	0.43322	
		N	6	6	6	
		-----	-----	-----	-----	-----
12	m	Mean	337.22	1.5418	4.57725	
		S.D.	28.32	0.1309	0.23300	
		N	6	6	6	
		-----	-----	-----	-----	-----
13	m	Mean	336.72	1.6127	4.79950	
		S.D.	30.05	0.1027	0.15718	
		N	6	6	6	
		-----	-----	-----	-----	-----

Lung weights are statistically significantly increased at day 28 in the two Eu₂O₃ and the ZrO₂ high dose group (Dunnett's test). Full recovery was detected in the TiO₂ P25 and SiO₂ groups.

Appendix 4 Chemical analysis of lung loads (individual data)

Group 2: Titanium dioxide; μ -TiO ₂ Bayertitan T									Aerosol conc.: 27.6 mg/m ³		
Group	Animal	TiO ₂ - soluble (ionic)			TiO ₂ - unsoluble (particulate)			TiO ₂ – Total			
		Mean	ASD	RSD	Mean	ASD	RSD	Mean	ASD	RSD	
		(μ g/Organ)	(%)		(μ g/Organ)	(%)		(μ g/Organ)	(%)		
2 d3+	113	1,8	0,11	6,1	1184	2	0,1	1186	2	0,1	
	114	0,9	0,05	5,1	644	6	0,9	644	6	0,9	
	115	1,0	0,01	1,3	1307	7	0,5	1308	7	0,5	
	116	1,6	0,07	4,2	957	12	1,3	958	12	1,3	
	117	9,3	0,09	1,0	1118	13	1,2	1127	13	1,2	
	118	0,5	0,00	0,3	750	10	1,3	751	10	1,3	
	Mean	1,2	0,5	45,5	993	258	26,0	996	259	26,0	
Day 28 post instillation (d28+)											
Group	Animal	TiO ₂ - soluble (ionic)			TiO ₂ - unsoluble (particulate)			TiO ₂ – Total			
		Mean	ASD	RSD	Mean	ASD	RSD	Mean	ASD	RSD	
		(μ g/Organ)	(%)		(μ g/Organ)	(%)		(μ g/Organ)	(%)		
2 d28+	119	1,1	0,01	1,2	572	7	1,3	573	7	1,3	
	120	0,5	0,01	2,7	333	1	0,4	333	1	0,4	
	121	0,6	0,05	8,7	665	5	0,8	666	5	0,8	
	122	6,4	0,03	0,5	498	7	1,4	505	7	1,4	
	123	0,7	0,08	10,4	782	12	1,5	782	12	1,5	
	124	24,3	0,65	2,7	678	12	1,7	702	12	1,8	
	Mean	0,7	0,3	39,5	588	158	26,9	593	160	27,0	
Day 90 post instillation (d90+)											
Group	Animal	TiO ₂ - soluble (ionic)			TiO ₂ - unsoluble (particulate)			TiO ₂ – Total			
		Mean	ASD	RSD	Mean	ASD	RSD	Mean	ASD	RSD	
		(μ g/Organ)	(%)		(μ g/Organ)	(%)		(μ g/Organ)	(%)		
2 d90+	125	0,9	0,03	3,4	465	6	1,4	466	6	1,4	
	126	1,2	0,15	12,0	336	5	1,6	337	5	1,6	
	127	0,3	0,05	14,6	318	5	1,7	318	5	1,7	
	128	0,4	0,04	11,5	307	5	1,6	307	5	1,6	
	129	0,6	0,06	11,1	165	2	1,0	165	2	1,0	
	130	1,0	0,08	7,9	310	6	1,8	311	6	1,8	
	Mean	0,7	0,3	45,6	317	96	30,2	318	96	30,2	

Data in italics: Not included for mean value

ASD: Absolute standard deviation - RSD: Relative standard deviation

Chemical analysis of lung loads (individual data) – cont'd

Group 3: Titanium dioxide; μ -TiO ₂ Bayertitan T								Aerosol conc.: 85.2 mg/m ³		
Group	Animal	TiO ₂ - soluble (ionic)			TiO ₂ - unsoluble (particulate)			TiO ₂ - Total		
		Mean	ASD	RSD	Mean	ASD	RSD	Mean	ASD	RSD
		(μ g/Organ)	(%)		(μ g/Organ)	(%)		(μ g/Organ)	(%)	
3 d3+	113	1,8	0,1	7,4	4252	38	0,9	4254	38	0,9
	114	2,3	0,0	1,9	4276	37	0,9	4278	37	0,9
	115	2,0	0,0	1,2	3216	22	0,7	3218	22	0,7
	116	5,9	0,3	5,5	2807	7	0,2	2813	7	0,3
	117	2,4	0,0	1,3	2948	9	0,3	2950	9	0,3
	118	3,1	0,1	2,0	2586	14	0,5	2590	14	0,5
	Mean	2,3	0,5	22,7	3348	739	22,1	3351	738	22,0
Day 28 post instillation (d28+)										
Group	Animal	TiO ₂ - soluble (ionic)			TiO ₂ - unsoluble (particulate)			TiO ₂ - Total		
		Mean	ASD	RSD	Mean	ASD	RSD	Mean	ASD	RSD
		(μ g/Organ)	(%)		(μ g/Organ)	(%)		(μ g/Organ)	(%)	
3 d28+	119	10,0	0,1	1,1	1481	4	0,3	1491	4	0,3
	120	2,6	0,2	6,2	2091	1	0,1	2094	1	0,1
	121	7,5	0,4	5,5	1615	6	0,4	1623	7	0,4
	122	1,3	0,1	5,8	1636	4	0,3	1637	4	0,3
	123	1,3	0,1	4,6	1298	1	0,0	1299	1	0,1
	124	1,1	0,1	4,8	1493	0	0,0	1494	0	0,0
	Mean	1,6	0,7	44,0	1602	268	16,7	1606	268	16,7
Day 90 post instillation (d90+)										
Group	Animal	TiO ₂ - soluble (ionic)			TiO ₂ - unsoluble (particulate)			TiO ₂ - Total		
		Mean	ASD	RSD	Mean	ASD	RSD	Mean	ASD	RSD
		(μ g/Organ)	(%)		(μ g/Organ)	(%)		(μ g/Organ)	(%)	
3 d90+	125	0,9	0,1	6,9	1012	12	1,2	1013	12	1,2
	126	0,6	0,0	3,2	790	10	1,2	790	10	1,2
	127	4,2	0,1	2,2	913	17	1,9	918	17	1,9
	128	2,1	0,2	7,3	711	12	1,7	713	12	1,7
	129	0,8	0,0	3,6	950	16	1,7	951	16	1,7
	130	0,6	0,0	1,3	900	13	1,4	901	13	1,4
	Mean	1,5	0,1	3,5	879	110	12,5	881	110	12,5

Data in italics: Not included for mean value

ASD: Absolute standard deviation - RSD: Relative standard deviation

Chemical analysis of lung loads (individual data) – cont'd

Group 4: nano-Titanium dioxide; TiO₂ P25 (EU/JRC) **Aerosol conc.:** 9.7 mg/m³

Day 3 post instillation (d3+)										
Group	Animal	TiO₂ - soluble (ionic)			TiO₂ - unsoluble (particulate)			TiO₂ - Total		
		Mean	ASD	RSD	Mean	ASD	RSD	Mean	ASD	RSD
		(µg/Organ)	(%)		(µg/Organ)	(%)		(µg/Organ)	(%)	
4 d3+	113	1,2	0,03	2,6	486	1	0,1	487	1	0,1
	114	2,2	0,05	2,1	565	5	0,9	568	5	0,9
	115	1,9	0,13	7,1	191	2	1,3	193	3	1,3
	116	2,0	0,03	1,6	457	6	1,3	459	6	1,3
	117	3,5	0,03	0,9	383	6	1,4	386	6	1,4
	118	2,1	0,01	0,3	555	8	1,4	557	8	1,4
	Mean	2,1	0,8	35,3	436	155	35,5	438	155	35,3
Day 28 post instillation (d28+)										
Group	Animal	TiO₂ - soluble (ionic)			TiO₂ - unsoluble (particular)			TiO₂ - Total		
		Mean	ASD	RSD	Mean	ASD	RSD	Mean	ASD	RSD
		(µg/Organ)	(%)		(µg/Organ)	(%)		(µg/Organ)	(%)	
4 d28+	119	3,0	0,22	7,2	107	1	0,7	110	1	0,9
	120	1,3	0,01	0,7	255	2	1,0	256	2	1,0
	121	1,1	0,01	0,7	259	3	1,0	260	3	1,0
	122	1,6	0,01	0,6	375	5	1,4	377	5	1,4
	123	0,9	0,01	0,9	189	3	1,3	190	3	1,3
	124	1,3	0,02	1,5	259	4	1,4	261	4	1,4
	Mean	1,5	0,8	49,7	241	89	37,0	242	89	36,6
Day 90 post instillation (d90+)										
Group	Animal	TiO₂ - soluble (ionic)			TiO₂ - unsoluble (particulate)			TiO₂ - Total		
		Mean	ASD	RSD	Mean	ASD	RSD	Mean	ASD	RSD
		(µg/Organ)	(%)		(µg/Organ)	(%)		(µg/Organ)	(%)	
4 d90+	125	0,7	0,01	0,9	68,2	0,5	0,7	68,8	0,5	0,7
	126	0,5	0,01	1,3	173	2	1,1	173	2	1,1
	127	0,8	0,00	0,2	100	1	0,7	100	1	0,6
	128	0,5	0,02	3,5	81,4	0,4	0,5	82,0	0,4	0,5
	129	0,5	0,01	1,5	104	1	0,7	104	1	0,7
	130	0,5	0,00	0,9	152	1	0,6	152	1	0,6
	Mean	0,6	0,1	18,9	113	41	36,1	113	41	35,8

ASD: Absolute standard deviation - RSD: Relative standard deviation

Chemical analysis of lung loads (individual data) – cont'd

Group 5: nano-Titanium dioxide; TiO ₂ P25 (EU/JRC)									Aerosol conc.: 29.1 mg/m ³		
Day 3 post instillation (d3+)											
Group	Animal	TiO ₂ - soluble (ionic)			TiO ₂ - unsoluble (particulate)			TiO ₂ - Total			
		Mean	ASD	RSD	Mean	ASD	RSD	Mean	ASD	RSD	
		(µg/Organ)	(%)		(µg/Organ)	(%)		(µg/Organ)	(%)		
5 d3+	113	5,3	0,22	4,2	1615	9	0,6	1621	9	0,6	
	114	3,5	0,25	7,1	1285	8	0,6	1288	8	0,6	
	115	4,8	0,34	7,2	1462	7	0,5	1467	8	0,5	
	116	1,7	0,13	7,2	1811	14	0,8	1813	14	0,8	
	117	1,1	0,07	6,5	1444	12	0,9	1445	12	0,9	
	118	2,4	0,02	0,8	1308	16	1,2	1310	16	1,2	
	Mean	3,1	1,7	53,6	1488	198	13,3	1491	198	13,3	
Day 28 post instillation (d28+)											
Group	Animal	TiO ₂ - soluble (ionic)			TiO ₂ - unsoluble (particulate)			TiO ₂ - Total			
		Mean	ASD	RSD	Mean	ASD	RSD	Mean	ASD	RSD	
		(µg/Organ)	(%)		(µg/Organ)	(%)		(µg/Organ)	(%)		
5 d28+	119	6,1	0,14	2,3	1505	16	1,1	1512	16	1,1	
	120	4,7	0,11	2,3	1469	13	0,9	1474	13	0,9	
	121	1,2	0,04	3,5	1003	9	0,9	1004	9	0,9	
	122	2,7	0,09	3,4	1218	9	0,8	1220	10	0,8	
	123	2,8	0,12	4,2	897	6	0,6	899	6	0,6	
	124	2,2	0,13	5,7	1510	5	0,3	1512	5	0,4	
	Mean	3,3	1,8	53,9	1267	270	21,3	1270	272	21,4	
Day 90 post instillation (d90+)											
Group	Animal	TiO ₂ - soluble (ionic)			TiO ₂ - unsoluble (particulate)			TiO ₂ - Total			
		Mean	ASD	RSD	Mean	ASD	RSD	Mean	ASD	RSD	
		(µg/Organ)	(%)		(µg/Organ)	(%)		(µg/Organ)	(%)		
5 d90+	125	3,4	0,12	3,6	861	9	1,0	865	9	1,1	
	126	3,8	0,13	3,5	955	13	1,4	959	13	1,4	
	127	2,1	0,07	3,5	796	8	1,0	798	8	1,0	
	128	4,3	0,20	4,5	929	6	0,7	933	6	0,7	
	129	3,7	0,17	4,56	655	7	1,1	659	7	1,1	
	130	4,8	0,24	5,0	691	7	1,0	696	7	1,1	
	Mean	3,7	0,9	24,8	815	123	15,2	818	123	15,1	

ASD: Absolute standard deviation - RSD: Relative standard deviation

Chemical analysis of lung loads (individual data) – cont'd

Group 6: Europium oxide ($\mu\text{-Eu}_2\text{O}_3$)									Aerosol conc.: 31,9 mg/m³		
Day 3 post instillation (d3+)											
Group	Animal	Eu_2O_3 - soluble (ionic)			Eu_2O_3 - unsoluble (particulate)			Eu_2O_3 - Total			
		Mean	ASD	RSD	Mean	ASD	RSD	Mean	ASD	RSD	
		($\mu\text{g/Organ}$)	(%)		($\mu\text{g/Organ}$)	(%)		($\mu\text{g/Organ}$)	(%)		
6 d3+	113	55,5	0,2	0,4	1085	5	0,4	1141	5	0,4	
	114	173	0,8	0,5	918	1	0,1	1092	1	0,1	
	115	154	0,1	0,1	1378	1	0,1	1532	1	0,1	
	116	137	0,1	0,1	795	2	0,2	932	2	0,2	
	117	79,0	0,1	0,1	1393	1	0,1	1472	2	0,1	
	118	37,1	0,1	0,2	1132	12	1,1	1169	13	1,1	
	Mean	106	56,2	53,1	1117	240	21,5	1223	232	19,0	
Day 28 post instillation (d28+)											
Group	Animal	Eu_2O_3 - soluble (ionic)			Eu_2O_3 - unsoluble (particulate)			Eu_2O_3 - Total			
		Mean	ASD	RSD	Mean	ASD	RSD	Mean	ASD	RSD	
		($\mu\text{g/Organ}$)	(%)		($\mu\text{g/Organ}$)	(%)		($\mu\text{g/Organ}$)	(%)		
6 d28+	119	946	0,3	0,0	744	0	0,0	1691	1	0,0	
	120	407	1,0	0,2	990	1	0,1	1397	2	0,1	
	121	219	0,4	0,2	994	1	0,1	1213	2	0,1	
	122	218	1,3	0,6	949	1	0,1	1167	2	0,2	
	123	117	0,4	0,4	1020	0	0,0	1138	1	0,1	
	124	14,8	0,1	0,4	1024	0	0,0	1039	0	0,0	
	Mean	320	333	104	954	106	11,1	1274	236	18,5	
Day 90 post instillation (d90+)											
Group	Animal	Eu_2O_3 - soluble (ionic)			Eu_2O_3 - unsoluble (particulate)			Eu_2O_3 - Total			
		Mean	ASD	RSD	Mean	ASD	RSD	Mean	ASD	RSD	
		($\mu\text{g/Organ}$)	(%)		($\mu\text{g/Organ}$)	(%)		($\mu\text{g/Organ}$)	(%)		
6 d90+	125	706	1,0	0,1	700	1	0,1	1406	2	0,1	
	126	425	1,7	0,4	905	2	0,2	1330	2	0,1	
	127	444	1,6	0,4	691	6	0,8	1135	7	0,7	
	128	95,7	0,4	0,5	1066	0	0,0	1162	1	0,1	
	129	349	0,5	0,1	822	1	0,1	1171	2	0,1	
	130	96,3	0,2	0,2	866	4	0,5	962	4	0,5	
	Mean	353	233	65,9	842	140	16,7	1194	156	13,1	

ASD: Absolute standard deviation - RSD: Relative standard deviation

Chemical analysis of lung loads (individual data) – cont'd

Group 7: Europium oxide ($\mu\text{-Eu}_2\text{O}_3$)									Aerosol conc.: 92.8 mg/m ³		
Day 3 post instillation (d3+)											
Group	Animal	Eu ₂ O ₃ - soluble (ionic)			Eu ₂ O ₃ - unsoluble (particulate)			Eu ₂ O ₃ - Total			
		Mean	ASD	RSD	Mean	ASD	RSD	Mean	ASD	RSD	
		($\mu\text{g/Organ}$)	(%)		($\mu\text{g/Organ}$)	(%)		($\mu\text{g/Organ}$)	(%)		
7 d3+	113	176	0,0	0,0	3336	3	0,1	3512	3	0,1	
	114	113	0,2	0,1	3192	4	0,1	3305	4	0,1	
	115	180	2,8	1,6	2328	4	0,2	2508	7	0,3	
	116	108	0,3	0,3	4139	3	0,1	4247	3	0,1	
	117	14,4	0,3	2,0	4017	2	0,1	4031	2	0,1	
	118	41,0	0,3	0,7	3983	2	0,1	4024	3	0,1	
	Mean	105	68,0	64,5	3499	693	19,8	3604	644	17,9	
Day 28 post instillation (d28+)											
Group	Animal	Eu ₂ O ₃ - soluble (ionic)			Eu ₂ O ₃ - unsoluble (particulate)			Eu ₂ O ₃ - Total			
		Mean	ASD	RSD	Mean	ASD	RSD	Mean	ASD	RSD	
		($\mu\text{g/Organ}$)	(%)		($\mu\text{g/Organ}$)	(%)		($\mu\text{g/Organ}$)	(%)		
7 d28+	119	406	1,4	0,4	3153	8	0,2	3559	9	0,3	
	120	629	1,4	0,2	2482	4	0,2	3112	5	0,2	
	121	364	7,1	1,9	3496	1	0,0	3861	8	0,2	
	122	200	0,2	0,1	2615	5	0,2	2815	5	0,2	
	123	210	0,0	0,0	3387	44	1,3	3598	44	1,2	
	124	424	2,6	0,6	2839	2	0,1	3263	4	0,1	
	Mean	372	159	42,6	2995	415	13,9	3368	378	11,2	
Day 90 post instillation (d90+)											
Group	Animal	Eu ₂ O ₃ - soluble (ionic)			Eu ₂ O ₃ - unsoluble (particulate)			Eu ₂ O ₃ - Total			
		Mean	ASD	RSD	Mean	ASD	RSD	Mean	ASD	RSD	
		($\mu\text{g/Organ}$)	(%)		($\mu\text{g/Organ}$)	(%)		($\mu\text{g/Organ}$)	(%)		
7 d90+	125	594	1,4	0,2	2369	4	0,2	2963	5	0,2	
	126	800	1,3	0,2	3043	1	0,0	3843	2	0,1	
	127	460	4,9	1,1	1725	3	0,2	2186	8	0,4	
	128	2043	1,6	0,1	1079	0	0,0	3122	2	0,1	
	129	68,7	0,8	1,2	2003	5	0,3	2072	6	0,3	
	130	139	0,0	0,0	2417	3	0,1	2556	3	0,1	
	Mean	684	720	105,3	2106	671	31,9	2790	661	23,7	

ASD: Absolute standard deviation - RSD: Relative standard deviation

Chemical analysis of lung loads (individual data) – cont'd

Group 8: Barium sulfate ($\mu\text{-BaSO}_4$)									Aerosol conc.: 41.7 mg/m³		
Day 3 post instillation (d3+)											
Group	Animal	BaSO₄ - soluble (ionic)			BaSO₄ - unsoluble (particulate)			BaSO₄ - Total			
		Mean	ASD	RSD	Mean	ASD	RSD	Mean	ASD	RSD	
		($\mu\text{g/Organ}$)	(%)		($\mu\text{g/Organ}$)	(%)		($\mu\text{g/Organ}$)	(%)		
8 d3+	113	5,2	0,34	6,5	1202	31	2,6	1207	31	2,6	
	114	5,6	0,05	0,8	973	12	1,2	978	12	1,2	
	115	6,2	0,07	1,1	964	14	1,5	970	14	1,5	
	116	3,8	0,01	0,2	920	7	0,7	924	7	0,7	
	117	7,0	0,03	0,4	752	39	5,1	759	39	5,1	
	118	7,9	0,42	5,3	738	64	8,7	746	65	8,7	
	Mean	6,0	1,4	23,9	925	170	18,4	931	170	18,2	
Day 28 post instillation (d28+)											
Group	Animal	BaSO₄ - soluble (ionic)			BaSO₄ - unsoluble (particulate)			BaSO₄ - Total			
		Mean	ASD	RSD	Mean	ASD	RSD	Mean	ASD	RSD	
		($\mu\text{g/Organ}$)	(%)		($\mu\text{g/Organ}$)	(%)		($\mu\text{g/Organ}$)	(%)		
8 d28+	119	2,1	0,04	1,9	406	2	0,5	408	2	0,6	
	120	1,5	0,01	0,4	179	6	3,1	181	6	3,1	
	121	1,5	0,02	1,1	206	4	2,1	207	4	2,1	
	122	1,5	0,02	1,1	176	1	0,6	177	1	0,6	
	123	1,7	0,02	1,2	214	2	0,8	215	2	0,8	
	124	4,1	0,05	1,3	147	2	1,2	151	2	1,2	
	Mean	2,1	1,0	50,3	221	93	42,2	223	93	41,8	
Day 90 post instillation (d90+)											
Group	Animal	BaSO₄ - soluble (ionic)			BaSO₄ - unsoluble (particulate)			BaSO₄ - Total			
		Mean	ASD	RSD	Mean	ASD	RSD	Mean	ASD	RSD	
		($\mu\text{g/Organ}$)	(%)		($\mu\text{g/Organ}$)	(%)		($\mu\text{g/Organ}$)	(%)		
8 d90+	125	0,5	0,00	0,1	13,0	0,0	0,4	13,5	0,0	0,4	
	126	0,9	0,01	1,3	26,1	0,1	0,3	26,9	0,1	0,3	
	127	0,1	0,00	0,9	14,4	0,0	0,3	14,6	0,0	0,3	
	128	0,3	0,00	1,2	23,7	0,0	0,2	24,0	0,1	0,2	
	129	0,2	0,00	1,7	24,4	0,1	0,2	24,6	0,1	0,2	
	130	0,3	0,00	1,4	15,3	0,0	0,2	15,5	0,0	0,2	
	Mean	0,4	0,3	71,6	19,5	5,8	30,0	19,9	6,0	30,0	

ASD: Absolute standard deviation - RSD: Relative standard deviation

Chemical analysis of lung loads (individual data) – cont'd

Group 9: Barium sulfate ($\mu\text{-BaSO}_4$)								Aerosol conc.: 135.8 mg/m ³		
Day 3 post instillation (d3+)										
Group	Animal	BaSO_4 - soluble (ionic)			BaSO_4 - unsoluble (particulate)			BaSO_4 - Total		
		Mean	ASD	RSD	Mean	ASD	RSD	Mean	ASD	RSD
		($\mu\text{g/Organ}$)	(%)		($\mu\text{g/Organ}$)	(%)		($\mu\text{g/Organ}$)	(%)	
9 d3+	113	6,5	0,18	2,8	936	14	1	943	14	1,5
	114	6,6	0,27	4,1	1683	1	0	1690	1	0,1
	115	7,5	0,15	2,0	1120	1	0	1127	1	0,1
	116	13,2	0,16	1,2	1233	1	0	1246	1	0,1
	117	12,9	0,19	1,5	926	26	3	939	26	2,8
	118	15,0	0,26	1,7	1732	2	0	1747	3	0,2
	Mean	10,3	3,8	37,2	1271	357	28,1	1282	358	27,9
Day 28 post instillation (d28+)										
Group	Animal	BaSO_4 - soluble (ionic)			BaSO_4 - unsoluble (particulate)			BaSO_4 - Total		
		Mean	ASD	RSD	Mean	ASD	RSD	Mean	ASD	RSD
		($\mu\text{g/Organ}$)	(%)		($\mu\text{g/Organ}$)	(%)		($\mu\text{g/Organ}$)	(%)	
9 d28+	119	4,0	0,05	1,3	473	30	6	477	30	6,3
	120	2,3	0,05	2,2	446	2	0	448	2	0,4
	121	4,2	0,07	1,6	535	24	5	539	25	4,6
	122	3,2	0,08	2,4	636	1	0	639	1	0,2
	123	2,8	0,04	1,4	356	3	1	359	3	0,9
	124	3,4	0,05	1,4	403	14	4	407	14	3,5
	Mean	3,3	0,7	21,9	475	100	21,0	478	100	20,9
Day 90 post instillation (d90+)										
Group	Animal	BaSO_4 - soluble (ionic)			BaSO_4 - unsoluble (particulate)			BaSO_4 - Total		
		Mean	ASD	RSD	Mean	ASD	RSD	Mean	ASD	RSD
		($\mu\text{g/Organ}$)	(%)		($\mu\text{g/Organ}$)	(%)		($\mu\text{g/Organ}$)	(%)	
9 d90+	125	0,8	0,01	1,4	57,2	2	2,7	58,0	1,5	2,7
	126	0,8	0,01	1,4	54,0	1	2,7	54,7	1,5	2,7
	127	0,5	0,01	1,8	38,9	1	2,6	39,3	1,0	2,6
	128	1,4	0,02	1,2	143	4	2,7	145	3,9	2,7
	129	1,2	0,02	1,9	333	1	0,2	334	0,7	0,2
	130	0,3	0,00	1,4	49,5	2	3,2	49,8	1,6	3,2
	Mean	0,8	0,4	52,2	113	114	101,5	113	115	101,1

Data in *italics*: Not included for mean value

ASD: Absolute standard deviation - RSD: Relative standard deviation

Chemical analysis of lung loads (individual data) – cont'd

Group 10:		Y-stabil. Zirconium oxide; YSZ (μ -ZrO ₂)						Aerosol conc.: 53.4 mg/m ³		
Day 3 post instillation (d3+)										
Group	Animal	ZrO ₂ - soluble (ionic)			ZrO ₂ - unsoluble (particulate)			ZrO ₂ - Total		
		Mean	ASD	RSD	Mean	ASD	RSD	Mean	ASD	RSD
		(μ g/Organ)	(%)		(μ g/Organ)	(%)		(μ g/Organ)	(%)	
10 d3+	113	9,0	0,1	1,2	1782	4	0,2	1791	4	0,2
	114	11,6	0,2	2,0	1526	0	0,0	1538	1	0,0
	115	7,5	0,2	3,3	1148	0	0,0	1156	1	0,1
	116	6,5	0,2	3,6	1085	4	0,4	1092	4	0,4
	117	7,5	0,2	3,1	1363	6	0,4	1371	6	0,4
	118	8,9	0,2	2,2	1229	1	0,1	1238	2	0,1
	Mean	8,5	1,8	21,1	1356	262	19,3	1364	263	19,3
Day 28 post instillation (d28+)										
Group	Animal	ZrO ₂ - soluble (ionic)			ZrO ₂ - unsoluble (particulate)			ZrO ₂ - Total		
		Mean	ASD	RSD	Mean	ASD	RSD	Mean	ASD	RSD
		(μ g/Organ)	(%)		(μ g/Organ)	(%)		(μ g/Organ)	(%)	
10 d28+	119	6,1	0,2	3,2	1451	8	0,6	1457	9	0,6
	120	7,0	0,2	2,8	1167	7	0,6	1174	7	0,6
	121	6,2	0,2	2,7	1029	3	0,3	1035	3	0,3
	122	6,5	0,3	3,9	1639	6	0,4	1646	7	0,4
	123	6,4	0,2	3,5	1038	2	0,2	1044	2	0,2
	124	5,6	0,2	3,1	954	3	0,3	960	3	0,3
	Mean	6,3	0,5	7,5	1213	273	22,5	1219	273	22,4
Day 90 post instillation (d90+)										
Group	Animal	ZrO ₂ - soluble (ionic)			ZrO ₂ - unsoluble (particulate)			ZrO ₂ - Total		
		Mean	ASD	RSD	Mean	ASD	RSD	Mean	ASD	RSD
		(μ g/Organ)	(%)		(μ g/Organ)	(%)		(μ g/Organ)	(%)	
10 d90+	125	2,8	0,1	2,7	710	2	0,3	712	2	0,3
	126	4,1	0,1	3,0	767	3	0,4	771	3	0,4
	127	3,6	0,1	2,7	709	3	0,4	713	3	0,4
	128	1,7	0,1	2,9	608	1	0,2	610	1	0,2
	129	3,0	0,1	3,4	822	3	0,3	825	3	0,4
	130	3,8	0,1	2,6	759	2	0,3	763	2	0,3
	Mean	3,2	0,9	27,2	729	73	10,0	732	73	10,0

ASD: Absolute standard deviation - RSD: Relative standard deviation

Chemical analysis of lung loads (individual data) – cont'd

Group 11:		Y-stabil. Zirconium oxide; YSZ (μ -ZrO ₂)						Aerosol conc.: 164.0 mg/m ³		
Day 3 post instillation (d3+)										
Group	Animal	ZrO ₂ - soluble (ionic)			ZrO ₂ - unsoluble (particulate)			ZrO ₂ - Total		
		Mean	ASD	RSD	Mean	ASD	RSD	Mean	ASD	RSD
		(μ g/Organ)	(%)		(μ g/Organ)	(%)		(μ g/Organ)	(%)	
11 d3+	113	16,6	0,3	1,9	3923	84	2,1	3940	84	2,1
	114	19,2	0,6	3,3	4267	83	2,0	4287	84	2,0
	115	28,6	1,2	4,1	4257	85	2,0	4285	86	2,0
	116	21,7	0,8	3,9	3497	67	1,9	3519	68	1,9
	117	20,3	1,0	4,8	3630	70	1,9	3650	71	2,0
	118	14,7	0,8	5,4	3030	48	1,6	3045	49	1,6
	Mean	20,2	4,8	23,9	3767	479	12,7	3788	482	12,7
Day 28 post instillation (d28+)										
Group	Animal	ZrO ₂ - soluble (ionic)			ZrO ₂ - unsoluble (particulate)			ZrO ₂ - Total		
		Mean	ASD	RSD	Mean	ASD	RSD	Mean	ASD	RSD
		(μ g/Organ)	(%)		(μ g/Organ)	(%)		(μ g/Organ)	(%)	
11 d28+	119	11,0	0,4	4,0	3508	74	2,1	3519	74	2,1
	120	10,8	0,5	4,2	3370	66	2,0	3381	66	2,0
	121	12,2	0,5	4,1	3514	64	1,8	3527	65	1,8
	122	11,5	0,4	3,1	4099	103	2,5	4111	104	2,5
	123	14,9	0,6	4,2	4285	92	2,2	4300	93	2,2
	124	9,1	0,4	4,2	3494	60	1,7	3503	60	1,7
	Mean	11,6	1,9	16,5	3712	381	10,3	3723	382	10,3
Day 90 post instillation (d90+)										
Group	Animal	ZrO ₂ - soluble (ionic)			ZrO ₂ - unsoluble (particulate)			ZrO ₂ - Total		
		Mean	ASD	RSD	Mean	ASD	RSD	Mean	ASD	RSD
		(μ g/Organ)	(%)		(μ g/Organ)	(%)		(μ g/Organ)	(%)	
11 d90+	125	6,7	0,2	2,5	2957	52	1,8	2964	52	1,8
	126	3,1	0,1	2,6	2022	34	1,7	2025	34	1,7
	127	8,8	0,2	2,8	3691	78	2,1	3700	78	2,1
	128	4,7	0,2	3,7	2283	35	1,6	2288	36	1,6
	129	5,6	0,2	3,0	2674	50	1,9	2680	50	1,9
	130	4,5	0,1	3,2	2683	49	1,8	2688	49	1,8
	Mean	5,6	2,0	35,3	2718	580	21,3	2724	581	21,3

ASD: Absolute standard deviation - RSD: Relative standard deviation

Chemical analysis of lung loads (individual data) – cont'd

Group 12:		amorphous Silicon dioxide (nano-SiO ₂ NM-200)						Aerosol conc.: 11.1 mg/m ³		
Group	Animal	SiO ₂ - soluble (ionic)			SiO ₂ - unsoluble (particulate)			SiO ₂ - Total		
		Mean	ASD	RSD	Mean	ASD	RSD	Mean	ASD	RSD
		(µg/Organ)	(%)		(µg/Organ)	(%)		(µg/Organ)	(%)	
12 d3+	113	7,2	0,1	1,3	247	6	2,4	254	6	2,4
	114	4,8	0,2	3,9	172	3	1,6	177	3	1,7
	115	8,0	0,2	2,0	162	5	3,2	170	5	3,2
	116	5,0	0,0	0,6	178	2	1,2	183	2	1,2
	117	2,8	0,2	6,8	171	7	3,9	174	7	3,9
	118	4,7	0,2	3,7	212	7	3,1	217	7	3,1
	Mean	5,4	1,9	34,9	191	33	17,1	196	33	16,9
Day 28 post instillation (d28+)										
Group	Animal	SiO ₂ - soluble (ionic)			SiO ₂ - unsoluble (particulate)			SiO ₂ - Total		
		Mean	ASD	RSD	Mean	ASD	RSD	Mean	ASD	RSD
		(µg/Organ)	(%)		(µg/Organ)	(%)		(µg/Organ)	(%)	
12 d28+	119	3,2	0,1	4,1	75,9	2,4	3,2	79,2	2,5	3,2
	120	4,3	0,2	4,4	117	1,6	1,3	122	1,8	1,4
	121	6,2	0,2	3,1	66,3	2,9	4,4	72,5	3,1	4,3
	122	5,4	0,1	2,7	107	1,6	1,5	112	1,7	1,5
	123	3,1	0,0	0,9	85,4	2,7	3,2	88,5	2,7	3,1
	124	3,9	0,1	3,4	91,1	5,3	5,8	95,0	5,4	5,7
	Mean	4,4	1,2	28,0	90,5	19,0	21,0	94,9	19,0	20,0
Day 90 post instillation (d90+)										
Group	Animal	SiO ₂ - soluble (ionic)			SiO ₂ - unsoluble (particulate)			SiO ₂ - Total		
		Mean	ASD	RSD	Mean	ASD	RSD	Mean	ASD	RSD
		(µg/Organ)	(%)		(µg/Organ)	(%)		(µg/Organ)	(%)	
12 d90+	125	2,9	0,1	4,0	24,7	1,7	6,8	27,6	1,8	6,5
	126	3,2	0,1	2,3	13,3	2,1	16,0	16,5	2,2	13,3
	127	3,3	0,1	2,6	31,9	1,3	4,2	35,2	1,4	4,0
	128	3,1	0,1	1,7	15,9	1,0	6,5	18,9	1,1	5,7
	129	2,5	0,1	2,6	14,4	2,5	17,0	16,9	2,5	14,9
	130	2,9	0,0	1,4	26,7	1,1	4,0	29,5	1,1	3,8
	Mean	3,0	0,3	10,0	21,1	7,7	36,2	24,1	7,7	32,1

ASD: Absolute standard deviation - RSD: Relative standard deviation

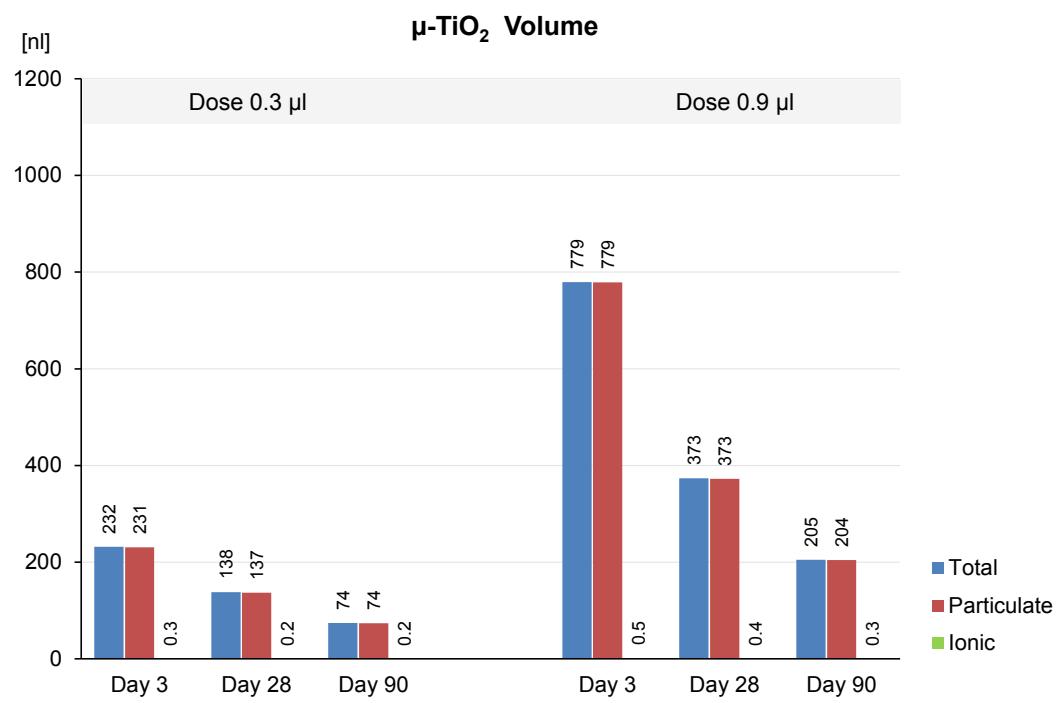
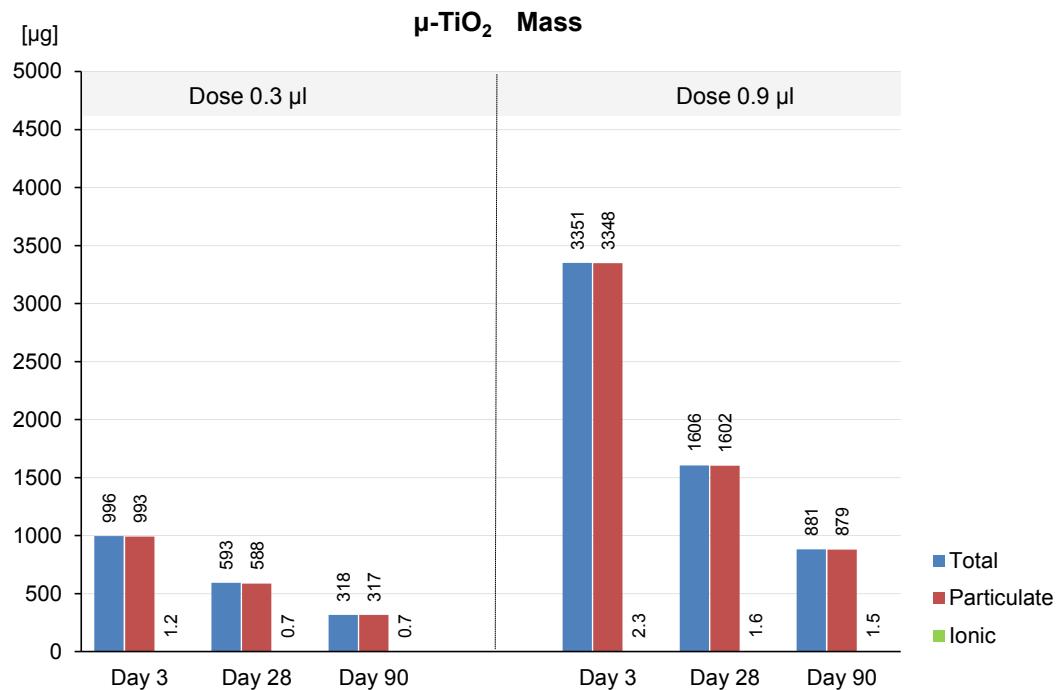
Chemical analysis of lung loads (individual data) – cont'd

Group 13:		amorphous Silicon dioxide (nano-SiO ₂ NM-200)						Aerosol conc.: 32.7 mg/m ³		
Day 3 post instillation (d3+)										
Group	Animal	SiO ₂ - soluble (ionic)			SiO ₂ - unsoluble (particulate)			SiO ₂ - Total		
		Mean	ASD	RSD	Mean	ASD	RSD	Mean	ASD	RSD
		(µg/Organ)	(%)		(µg/Organ)	(%)		(µg/Organ)	(%)	
13 d3+	113	5,5	0,1	2,0	379	23	5,9	384	23	5,9
	114	6,0	0,1	1,0	321	14	4,5	326	15	4,5
	115	10,0	0,2	1,7	305	13	4,1	315	13	4,0
	116	6,4	0,2	2,4	256	10	3,9	262	10	3,9
	117	7,6	0,2	2,4	381	13	3,5	389	14	3,5
	118	8,2	0,1	1,2	296	10	3,5	304	10	3,4
	Mean	7,3	1,7	23,1	323	49	15,3	330	49	14,8
Day 28 post instillation (d28+)										
Group	Animal	SiO ₂ - soluble (ionic)			SiO ₂ - unsoluble (particulate)			SiO ₂ - Total		
		Mean	ASD	RSD	Mean	ASD	RSD	Mean	ASD	RSD
		(µg/Organ)	(%)		(µg/Organ)	(%)		(µg/Organ)	(%)	
13 d28+	119	4,9	0,2	3,7	152	8	5,6	157	9	5,5
	120	5,1	0,1	2,9	92,2	6	6,4	97,3	6	6,2
	121	23,5	0,0	0,1	62,3	3	4,9	85,8	3	3,6
	122	5,4	0,2	2,8	173	3	1,6	178	3	1,6
	123	3,1	0,2	7,0	137	2	1,3	140	2	1,4
	124	3,4	0,1	3,9	103	2	1,7	106	2	1,7
	Mean	4,4	1,0	23,8	120	41	34,4	127	37	28,7
Day 90 post instillation (d90+)										
12 d90+	125	5,1	0,1	1,1	42,5	2,2	5,3	47,6	2,3	4,8
	126	2,4	0,1	2,9	31,3	1,7	5,3	33,6	1,7	5,2
	127	3,1	0,1	2,2	25,3	2,2	8,8	28,4	2,3	8,0
	128	3,2	0,0	1,6	42,0	3,4	8,0	45,2	3,4	7,5
	129	2,3	0,0	1,4	28,2	2,1	7,6	30,5	2,2	7,1
	130	16,3	0,2	1,1	33,0	1,6	4,9	49,3	1,8	3,6
	Mean	3,2	1,1	35,3	33,7	7,1	21,1	39,1	9,3	23,7

Data in italics: Not included for mean value

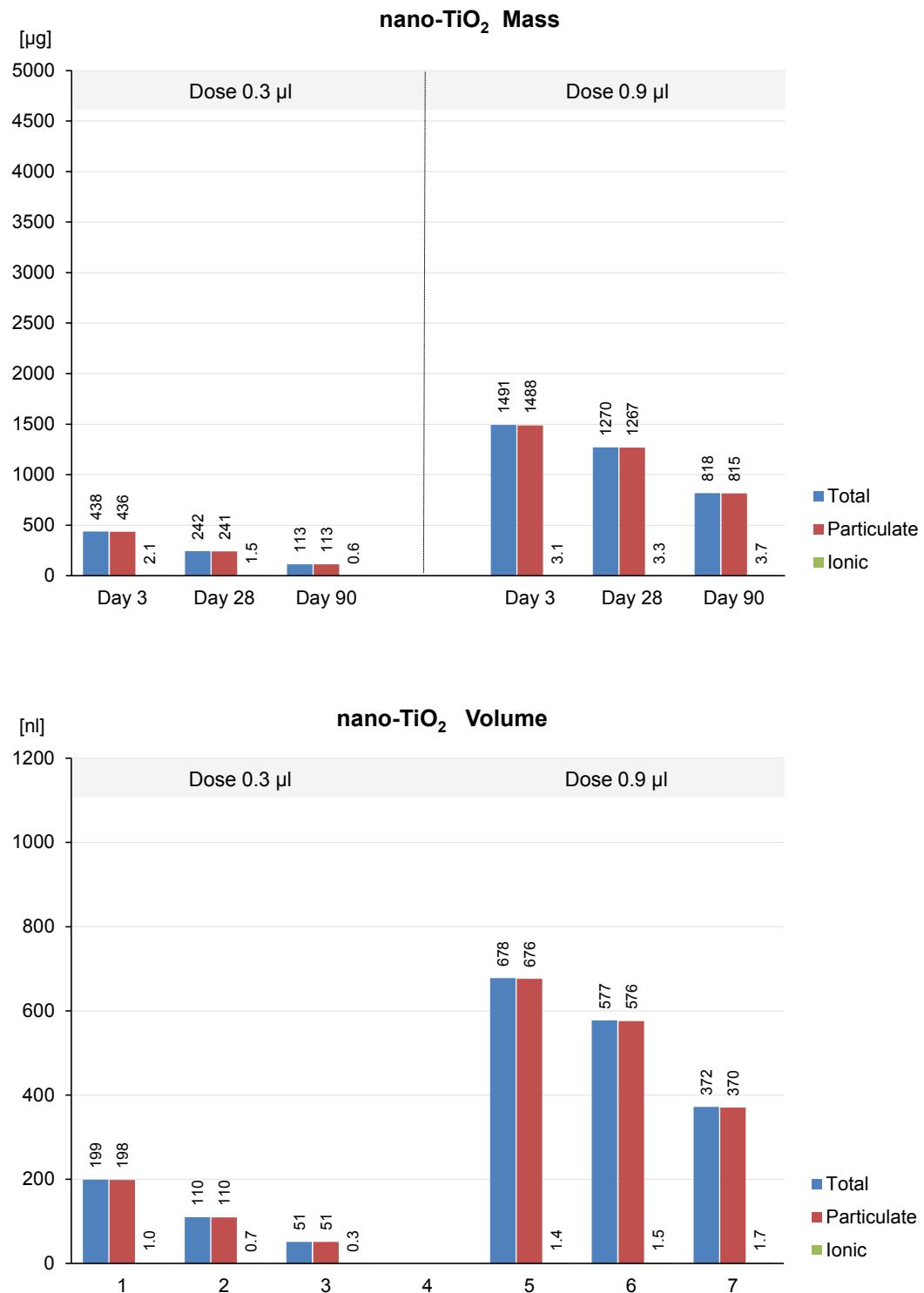
ASD: Absolute standard deviation - RSD: Relative standard deviation

Appendix 5 Chemical analysis of lung loads - means (figures)



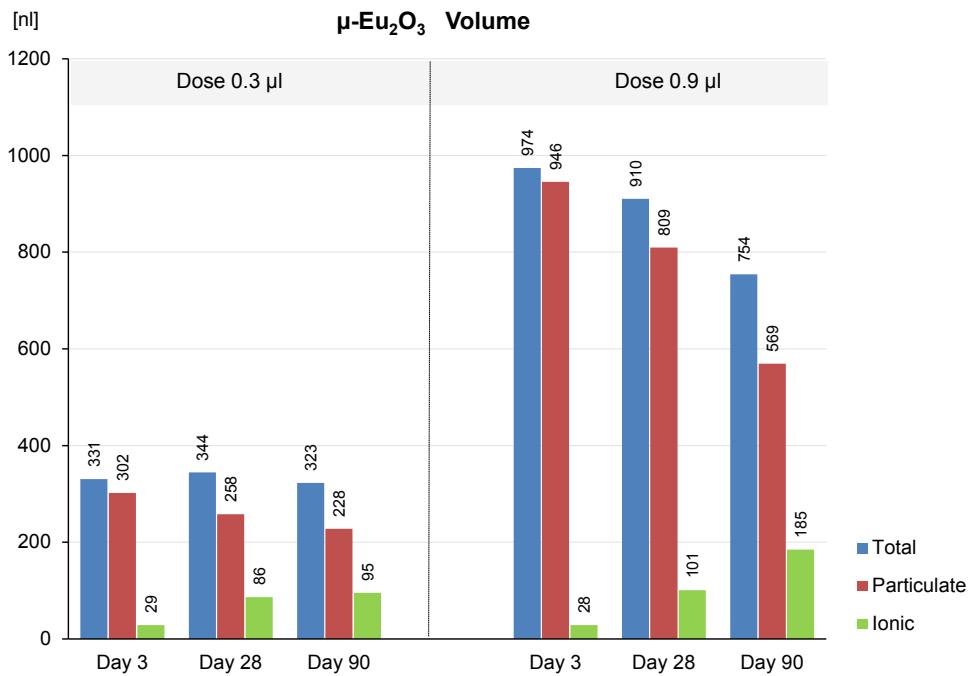
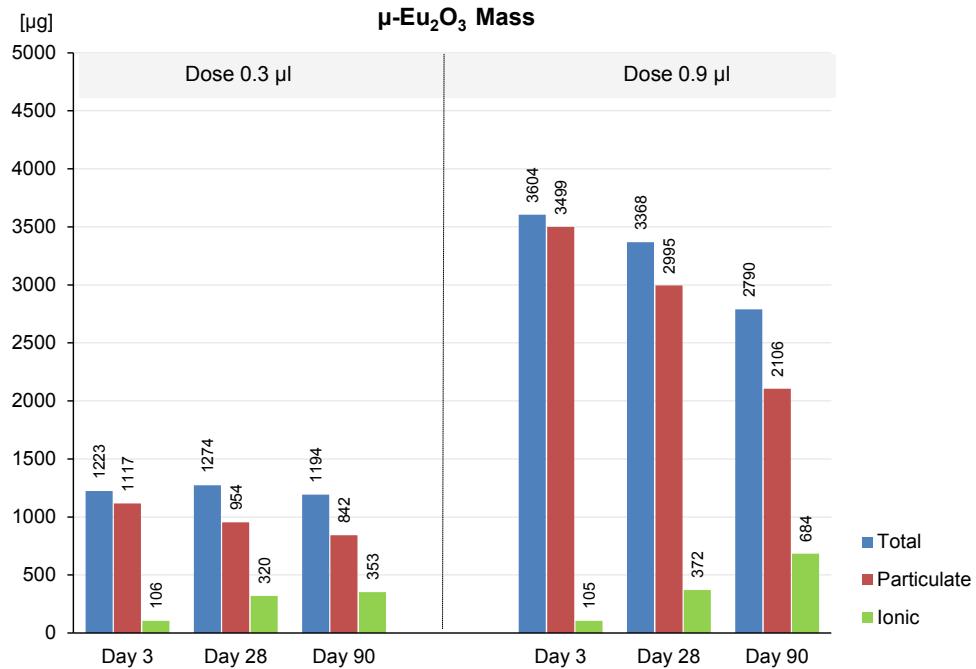
App. 5, Fig. 1a/b Titanium dioxide Bayertitan T - Total, particulate and ionic mass in lungs ($\mu\text{g/lung}$ or nl/lung \rightarrow $0.3 \mu\text{l}$: non-overload - $0.9 \mu\text{l}$ overload (volumetric))

Chemical analysis of lung loads - means (figures) – cont'd



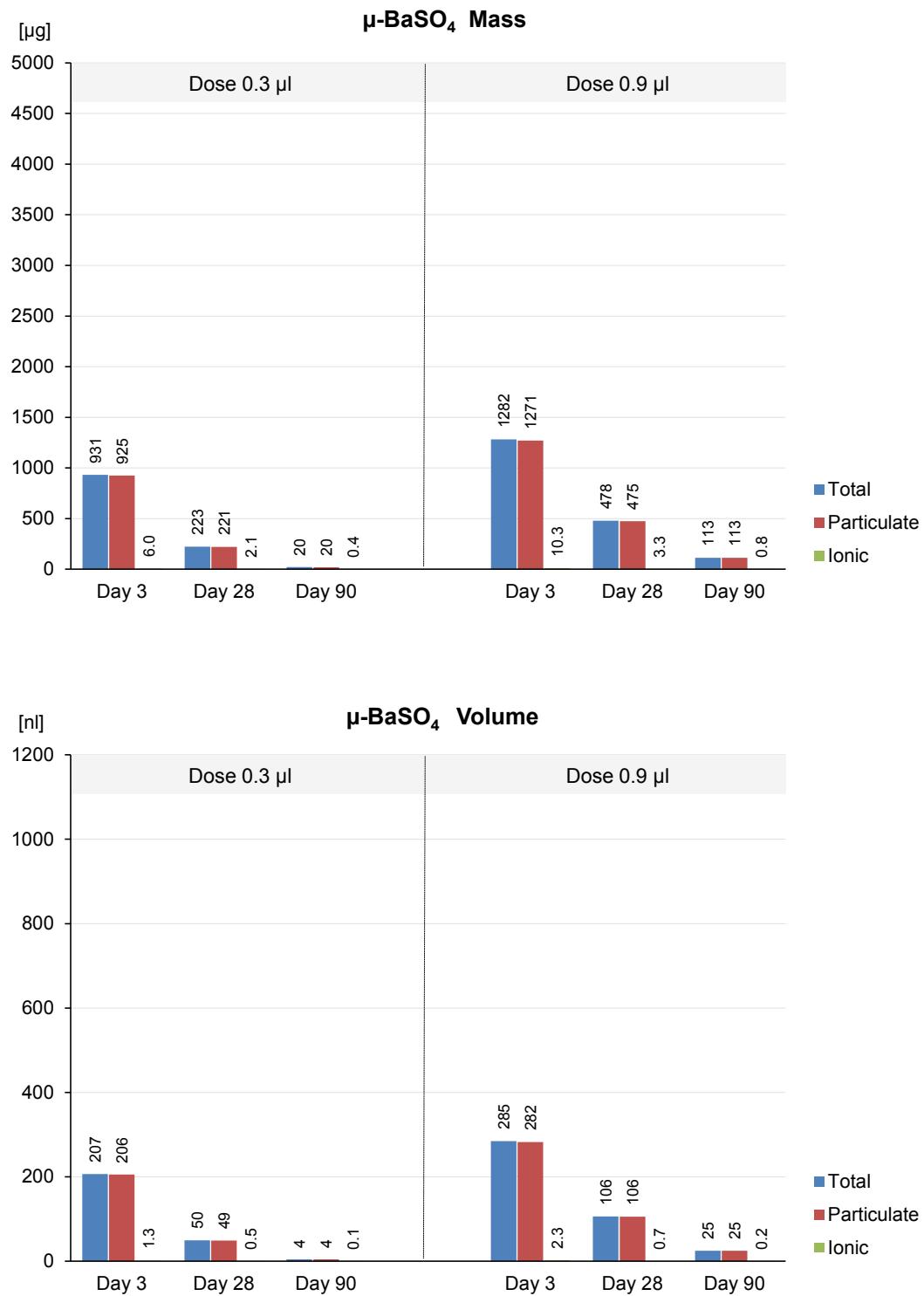
App. 5, Fig. 2a/b Titanium dioxide P25 - Total, particulate and ionic mass in lungs ($\mu\text{g/lung}$ or nl/lung → 0.3 μl : non-overload – 0.9 μl overload (volumetric))

Chemical analysis of lung loads - means (figures) – cont'd



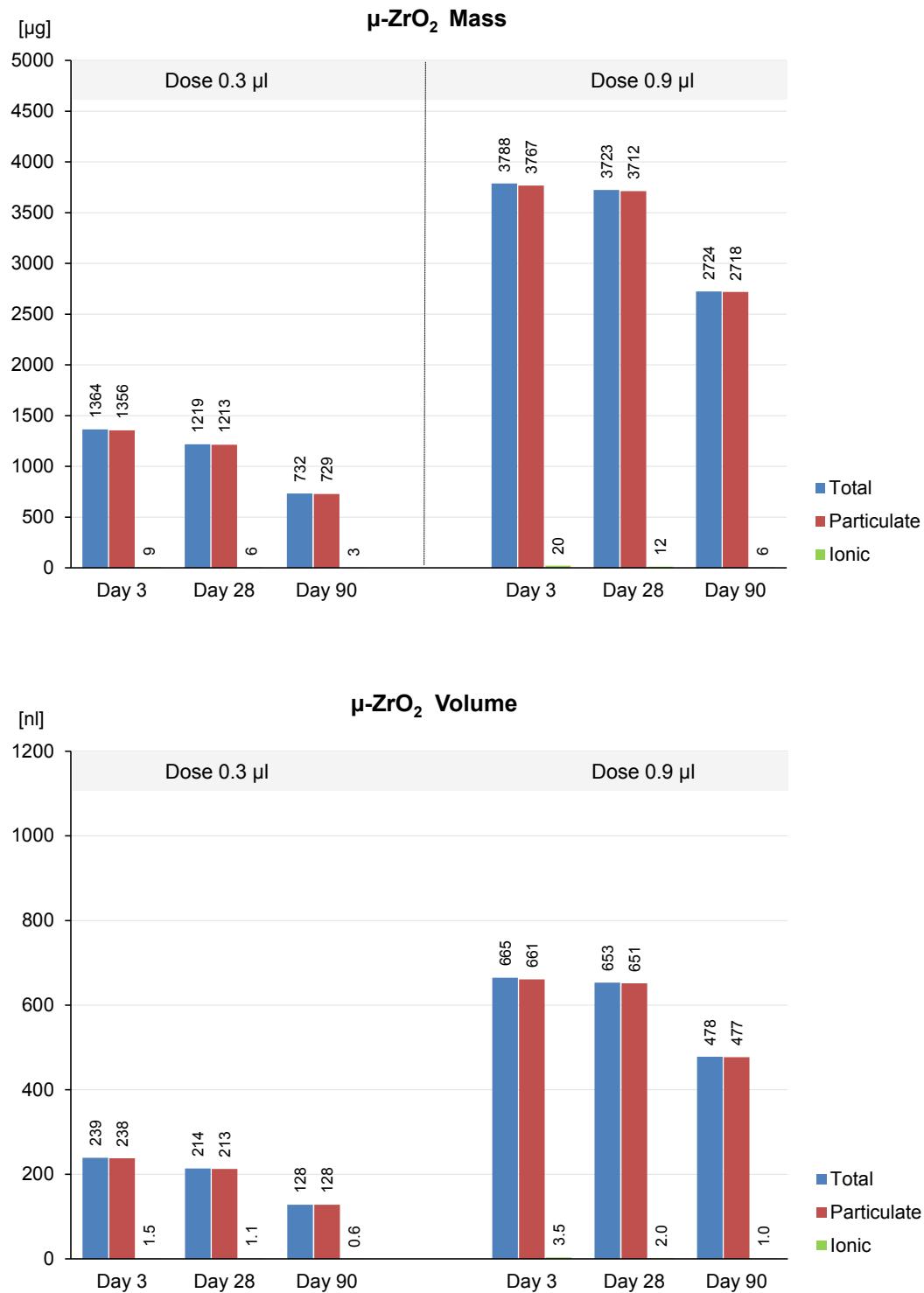
App. 5, Fig. 3a/b Europium oxide -Total, particulate and ionic mass in lungs ($\mu\text{g/lung}$ or nl/lung → 0.3 μl : non-overload – 0.9 μl overload (volumetric)

Chemical analysis of lung loads - means (figures) – cont'd



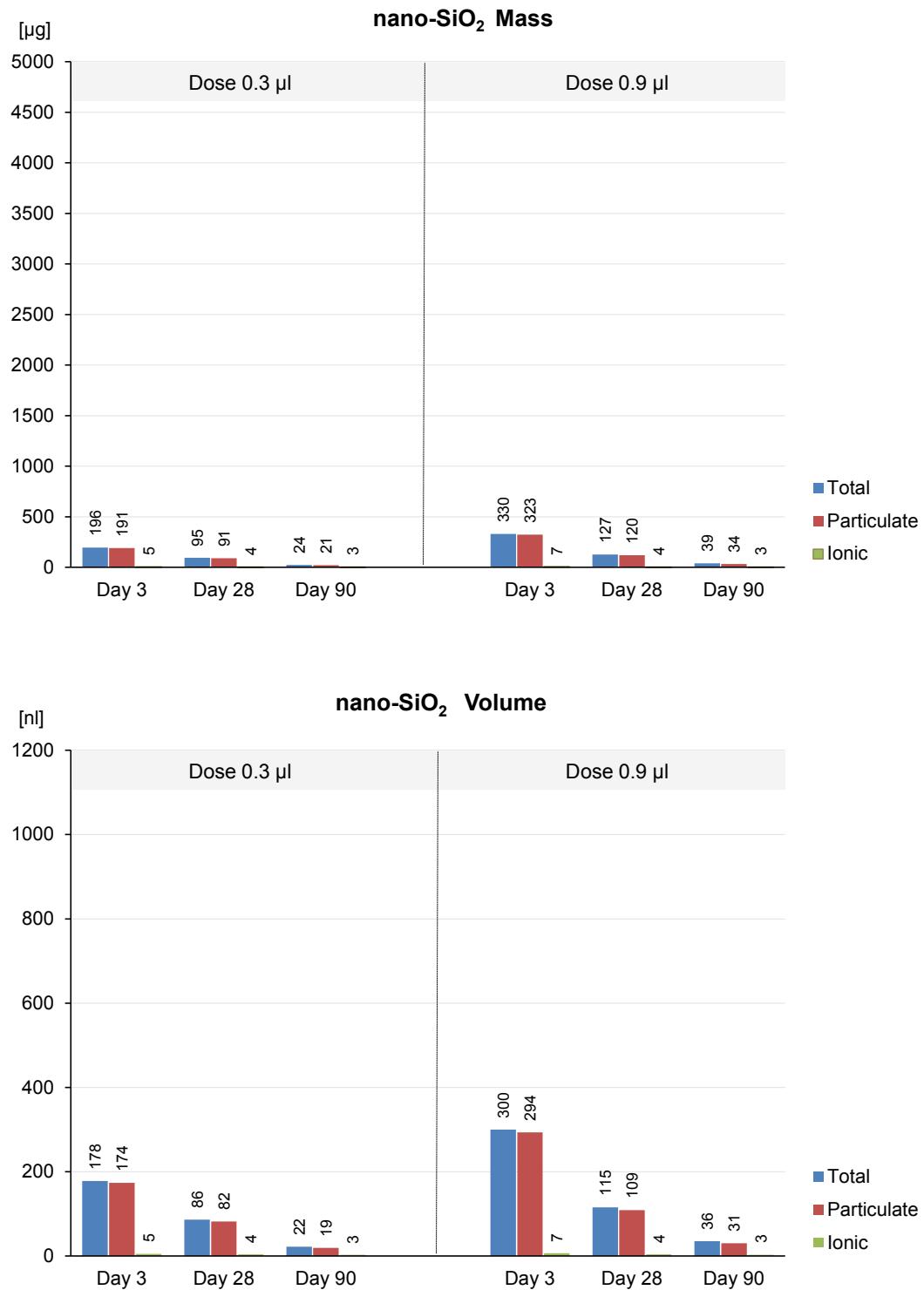
App. 5, Fig. 4a/b Barium sulfate - Total, particulate and ionic mass in lungs ($\mu\text{g/lung}$ or nl/lung → 0.3 μl : non-overload – 0.9 μl overload (volumetric)

Chemical analysis of lung loads - means (figures) – cont'd



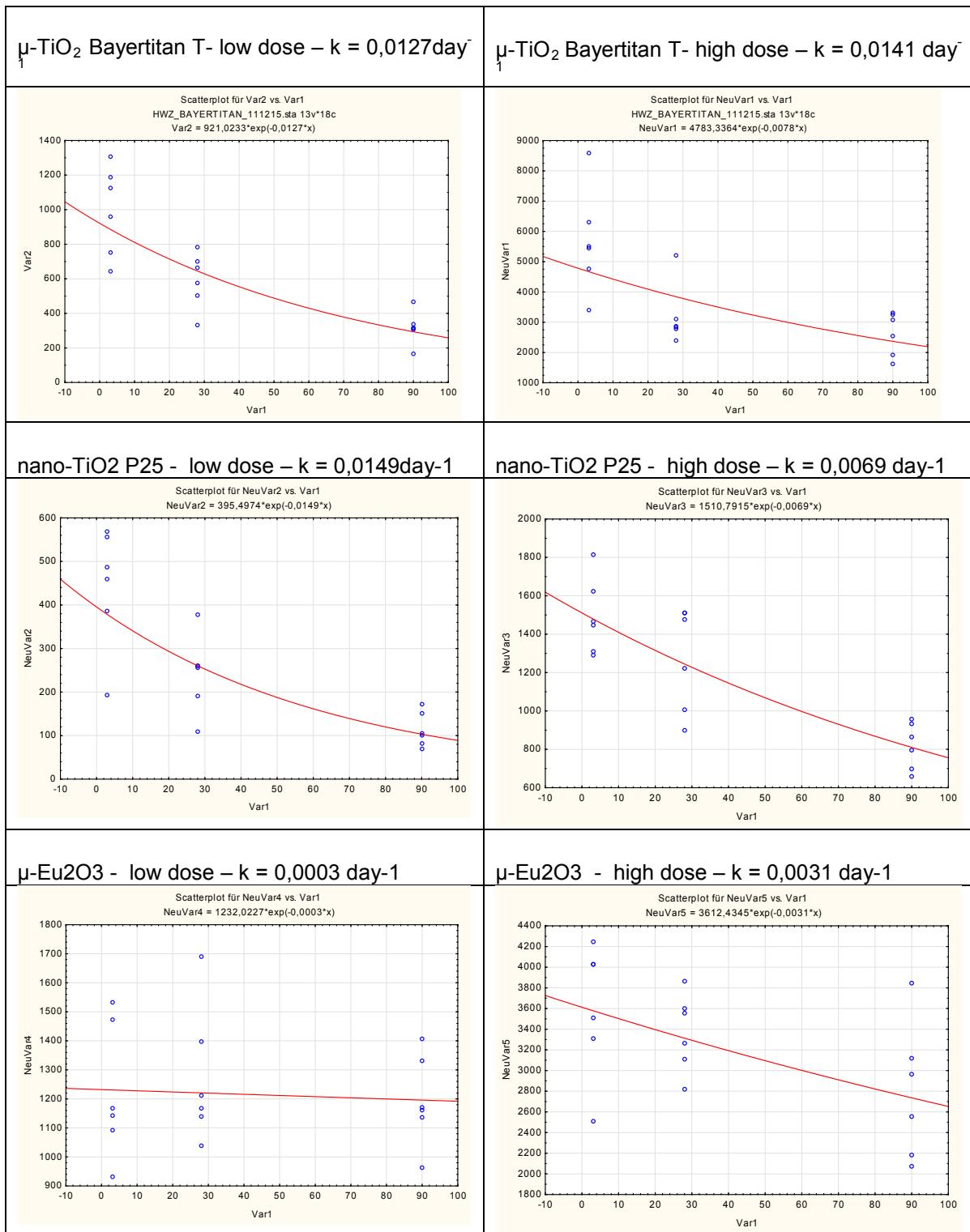
App. 5, Fig. 5a/b Zirconium oxide - Total, particulate and ionic mass in lungs ($\mu\text{g/lung}$ or nl/lung → $0.3\mu\text{l}$: non-overload – $0.9\mu\text{l}$ overload (volumetric)

Chemical analysis of lung loads - means (figures) – cont'd

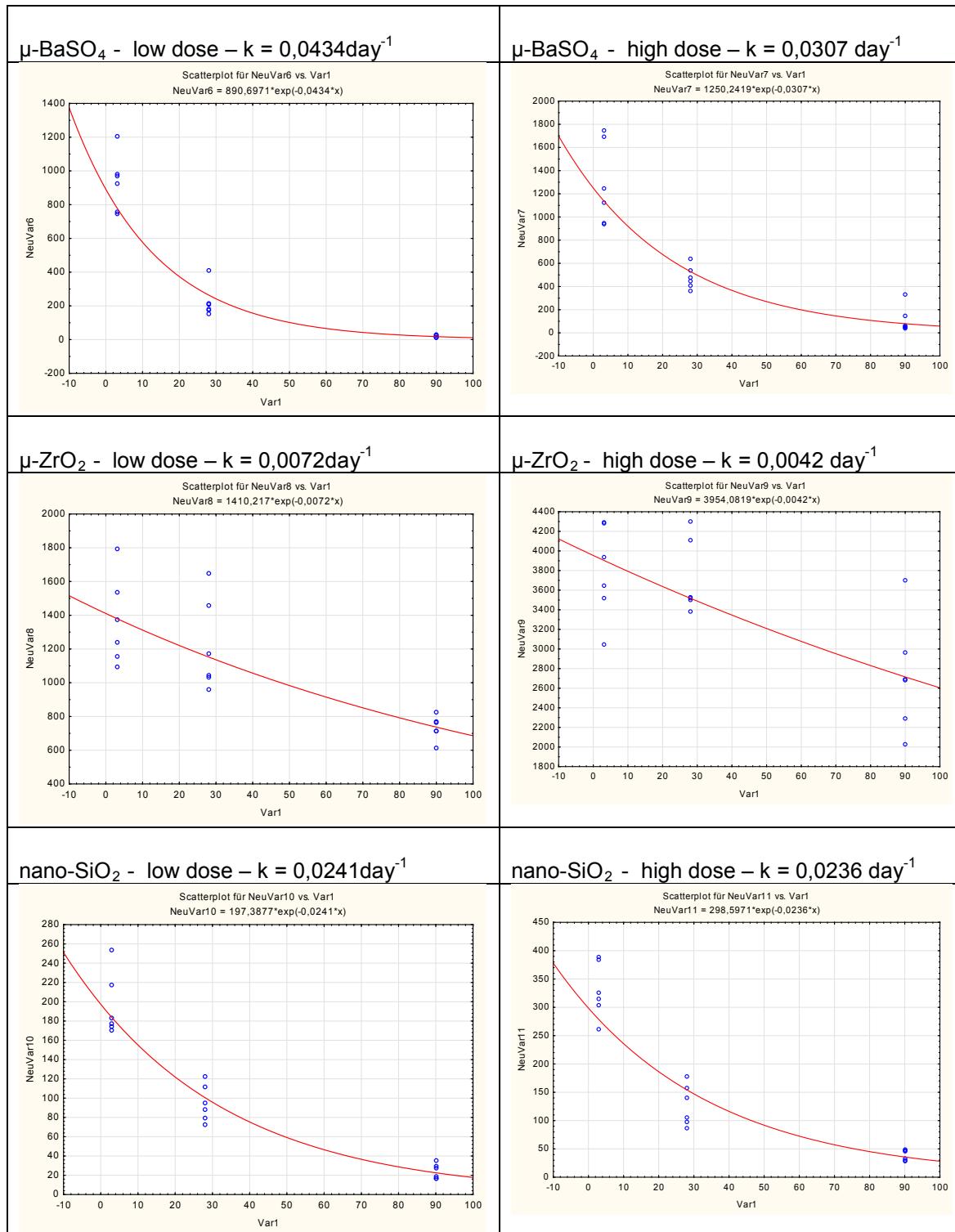


App. 5, Fig. 6a/b Amorphous silica - Total, particulate and ionic mass in lungs ($\mu\text{g/lung}$ or nl/lung → $0.3 \mu\text{l}$: non-overload - $0.9 \mu\text{l}$ overload (volumetric)

Appendix 6 Calculation of half-times $t_{1/2}$ (first order kinetics)



x axis: time (days)
y axis: $\mu\text{g/lung}$

Calculation of half-times $t_{1/2}$ (first order kinetics) – cont'd

x axis: time (days)
y axis: $\mu\text{g/lung}$