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# Poorly soluble particulates: Searching for a unifying denominator of nanoparticles and fine particles for DNEL estimation

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ABSTRACT

Under the new European chemicals regulation, REACH (Registration, Evaluation, Authorization and Restriction of Chemicals) a Derived No-Effect Level (DNEL), i.e., the level of exposure above which humans should not be exposed, is defined. The focus of this paper is to develop a weight-of-evidence-based DNEL-approach for inhaled poorly soluble particles. Despite the common mode of action of inhaled insoluble, spherical particulate matter (PM), a unifying, most appropriate metric conferring pulmonary biopersistence and toxicity has yet not been demonstrated. Nonetheless, there is compelling evidence from repeated rat inhalation exposure studies suggesting that the particle displacement volume is the most prominent unifying denominator linking the pulmonary retained dose with toxicity. Procedures were developed to analyze and model the pulmonary toxicokinetics from short-term to long-term exposure. Six different types of poorly soluble nano- to submicron PMs were compared: ultrafine and pigmentary $TiO_2$, synthetic iron oxide ($Fe_3O_4$, magnetite), two aluminum oxyhydroxides (AlOOH, Boehmite) with primary isometric particles approximately of either 10 or 40 nm, and MWCNT. The specific agglomerate densities of these materials ranged from 0.1 g/cm$^3$ (MWCNT) to 5 g/cm$^3$ ($Fe_3O_4$). Along with all PM, due to their long retention half-times and associated biopersistence in the lung, even short-term inhalation studies may require postexposure periods of at least 3 months to reveal PM-specific dispositional and toxicological characteristics. This analysis provides strong evidence that pulmonary toxicity (sustained inflammation) is dependent on the volume-based cumulative lung exposure dose. Lung toxicity, evidenced by PMN in BAL occurred at lung doses exceeding 10-times the overload threshold. Furthermore, the conclusion is supported that repeated inhalation studies on rats should utilize an experimental window of cumulative volume loads of respirable PM in the range of 1 μl/lung (no-adverse-effect range); however, not exceeding ≈10 μl/lung that would lead to retention half-times increasing 1 year. This can be targeted best by computational toxicology, i.e., the modeling of particle deposition and lung retention biokinetics during the exposure and recovery periods. Inhalation studies exceeding that threshold volume may lead to meaningless findings difficult to extrapolate to any real-life scenario. In summary, this analysis supports a volume-based generic mass concentration of 0.5 μl $PM_{respirable}/m^3$ × agglomerate density, independent on nano- or submicron-sized properties, as a generic no-adverse effect level in both rats and humans.



## 1. Introduction

Results from numerous short-term inhalation/aspiration/instillation studies with various types of carbon nanotubes (CNT) have been published (for reviews see Donaldson et al., 2006; Madl and Pinkerton, 2009; Maynard, 2007; Oberdörster et al., 2005, 2007). The degree and kind of aggregation of CNT structures is determined by the rigidity and pliancy of nanotubes and whether their diameters are thin enough to allow their buckling and self-aggregation into low-density, particle-like, intertwined, and often coiled assemblages (Pauluhn, 2009a). Given the differences in the physical shape of agglomerate structures, a categorization into rigid and flexible CNT appears to be among the most straightforward discriminative variable. In addition, the type of assemblage structure and whether it is stabilized by mere agglomeration or some kind of inter-tubular aggregation (physical entanglement) needs to be appreciated. Hence, depending on these characteristics, agglomerate structures of nanotubes may differ appreciably from thin-walled to thick-walled, rigid MWCNT. These properties may be decisive for hazard assessment as the critical toxic principle may either emerge from the individual tube structure (e.g., fiber) or the collective behavior of inhalable assemblages of nanotubes.

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Unlike conventional poorly soluble crystalline particle structures, MWCNT are present as submicronsized agglomerated arrangements of closely packed CNT which increase the void-space volume creating a novel type of composite low-density PM-structures. Consequently, when phagocytized by alveolar macrophages, much less particle mass is needed to exceed the volumetric overload limit for the diminution of macrophage-mediated clearance (Morrow, 1988, 1992, 1994). Based on Morrow's hypothesis of the volumetric overload of alveolar macrophages, the particle displacement volume rather than surface area appears to be the most critical metric for these types of materials. Hence, agglomerated nanoparticles present in submicronsized form may cause a volumetric overload of alveolar macrophages at lower exposure doses as compared to their micron-sized crystalline counterparts. Yet, no single particle characteristic as a hallmark indicator directing fate and pulmonary toxicity has been identified (Madl and Pinkerton, 2009). However, emerging views suggest that the assemblage displacement volume of MWCNT, which is critical to the impairment of alveolar macrophage-mediated clearance and elicitation of pulmonary inflammation, may dictate the fate and pulmonary response to this type of structures. Considerable efforts have been expended in measuring and modeling pulmonary deposition of inhaled particles in rodents and other species, and several comprehensive reviews have been published (Miller, 2000; Brown et al., 2005; Oberdörster et al., 1992; Oberörster, 2002). This retrospective comparison focuses on a PM-volume-based metric as the most apt denominator to compare PMs of different size, effective density, and structure. The focus of previous approaches was limited to submicronsized, high-density particles (Pauluhn, 2009b) whereas this analysis utilized additional data from recent repeated exposure inhalation studies on rats with nanostructured, low-density materials.

In the context of pulmonary toxicity of poorly soluble particles surface area is often considered to be the leading metric. However, it is hard to believe that the gauge commonly used to determine surface area ($N_2$) (Klobes et al., 2006) is at any rate reflective of the competitive adsorption of the numerous peptides and proteins present in the lining fluids of the lung. In other words, the biologically effective surface areas are dependent on the gauge size of the most avidly binding endogenous polypeptide, including its competitive displacement. Likewise, especially for particle structures in the submicron to nanometer range, the aggregation properties of assemblages of PM may be changed by preparation and collection. For such complex and irregular shape three-dimensional aggregated objects it becomes increasingly difficult to assign one single physical qualifier for an unequivocal characterization. The phenomena occurring during the contact between nanoparticles and cellular media or biological fluids (dispersion, agglomeration/aggregation, protein adsorption) in relation to the surface properties of the nanoparticles considered are discussed elsewhere in detail (Fubini et al., 2010). From that perspective, the three-dimensional characteristics 'volume' appears to be a better qualifier than PM number or surface area. In this context and following the hypothesis of AM overload, the most directly accessible and mechanism-based variable is the displacement volume of PM within the available pool of phagocyte or AM (volume of distribution, $V_d$). Therefore, the following risk analysis is solely focused on this variable. Accordingly, surface area has not been considered in this paper as a previous analysis with higher-density particles of different surface areas has demonstrated that the PM mass concentrations and pulmonary inflammation correlated better than surface area concentrations (Pauluhn, 2009b).

The objective of this paper is to analyze whether the somewhat unique pulmonary inflammatory potency of MWCNT assemblages share some unifying characteristics with their higher-density granular biopersistent counterparts when using a volume metric. Based on this rationale, the composite volume of aggregates appears to be the most critical variable of dose. Hence, it is timely to analyze as to which extent current testing paradigms need to be modified to the advancement in toxicological knowledge to appropriately identify and rank the hazards of PMs. Computational toxicology (modeling) has been utilized to better design and predict the outcome of repeated rat inhalation studies. This latter aspect serves the additional objective to improve the design and dose-rationalization of inhalation bioassays across different laboratories. Especially for lung toxicity, the Guidance given in the new REACH regulation (ECHA, 2008a,b,c,d) is lacking prescriptive instructions to arrive at scientifically reasonable assessment factors and NELs (no-effect levels) for the derivation of Occupational Exposure Levels (OELs) for these types of substances. This paper attempts to rationalize a mode-of-action-based, scientific approach.

## 2. Methods

### 2.1. Study design and experimental variables

It is important to keep in mind that the model system or testing regimen themselves may influence measured responses irrespective of the particulate material investigated. At present, most of the studies are concerned with pulmonary pathology patterns following single to short-term high-dose bolus dosing. These types of studies have ample room for experimental artifacts which include localized pathology due to particle clumping and irregular particle distribution as a result of dosing of poorly characterized materials and inhomogeneous particle suspensions. Most studies addressing the translocation of particles to extrapulmonary organs do not attempt to critically associate inflammation-related barrier-disruption with increased particle translocation. Compelling experimental evidence supports the view that the study of PM-translocation requires extended postexposure periods and should also include the time-dependent PM-translocation to the draining lung (hilus) lymph-nodes as reference (Pauluhn, 2009c, 2010a,b; Bermudez et al., 2002, 2004). Particle retention and biokinetics, including their dose-dependence, did not receive a great deal of attention yet. Commonly, the procedures used to generate and characterize atmospheres in inhalation studies provide a much higher dosing accuracy and reproducibility than attained by the alternative instillation methods. Therefore, issues related to dosimetry and kinetics can most appropriately be addressed by inhalation protocols.

Published evidence demonstrates that ultrafine $TiO_2$ particles caused more inflammation in rat lungs than exposure to fine $TiO_2$ (Ferin et al., 1992; Bermudez et al., 2002, 2004). These differences in toxic potencies seem to be a result of their unique size, surface area/activity and/or crystal properties (Warheit et al., 2005, 2007; Warheit, 2008). In rats, pulmonary inflammatory responses increase precipitously under conditions of apparent decompensated lung overload, a particle-induced depression of clearance as a consequence of an exceedingly high volumetric overload of the alveolar macrophages and associated loss of alveolar macrophage mobility (Morrow, 1988, 1992; Stöber and McClellan, 1997). Overload has been loosely defined as the alveolar burden causing a two- to four-times reduction in alveolar clearance of rats relative to normal clearance rates (ILSI, 2000). Accordingly, it is timely to analyze existing data from repeated exposure inhalation studies in rats to consider as to whether more robust criteria and thresholds for lung overload can be identified and whether the underlying toxic principles apply to humans as well.

This retrospective analysis analyzed and compared different types of PMs examined in 4- and especially 13-week repeated exposure rat inhalation bioassays (for details see Table 1). Studies conducted in compliance with OECD-413 and OECD-GD#39 (OECD, 2009) were given preference. The PM primary particle size ranged from nano- to submicronsized covering an effective density of PM from 0.1 to 5 g/cm$^3$. The lowest density was represented by multi-walled carbon nanotubes (Baytubes®). Pigment grade magnetite ($Fe_3O_4$) was the particle with the highest density. Aluminum oxyhydroxide (AlOOH) consisted of agglomerated arrangements of closely packed submicron aggregates consisting of nanoparticles of 10–40 nm. These structures are technically designed to disintegrate into their nanostructures in molten plastic. The principal range of structures addressed in this analysis is illustrated in Fig. 1.

### 2.2. Exposure regimen and duration of exposure and postexposure periods

The extremes of exposure regimens commonly involved in inhalation toxicity testing are illustrated in Figs. 2 and 3. These representations depict the often experienced experimental challenges involved with repeated exposure inhalation testing: (1) the 'loading phase' may be too short and, due to the surface activity of PMs, surfactant adsorption onto accessible particle surfaces may result in instant, site of initial deposition-dependent surfactant dysfunction and acute alveolitis. In addition to the acute, deposition-related changes, short-term high-dose regimens require thoughtful considerations on dosimetry to arrive at meaningful multiples of

**Table 1**
Characteristics of test substances.

| Substance | Concentration (mg/m$^3$) | $t_{1/2}$ (days) | NO(A)EL (mg/m$^3$) | MMAD (μm) | GSD | MPPD2 (%resp.) | Density (g/cm$^3$) |
|---|---|---|---|---|---|---|---|
| $Fe_3O_4$ | 4.7 | | 4.7 | 1.3 | 2.0 | | 5.1 |
| 13-w | 16.6 | n.d. | (BAL-PMN) | 1.3 | 2.0 | 10 | |
| | 52.1 | | | 1.3 | 2.0 | | |
| $Fe_3O_4$ | 10.1 | 53 | 10.1 | 1.53 | 2.02 | | 5.1 |
| 4-w | 19.7 | 79 | (BAL-PMN) | 1.46 | 2.03 | 10 | |
| | 45.6 | 109 | | 1.53 | 2.08 | | |
| | 95.8 | 165 | | 1.53 | 2.13 | | |
| $Fe_3O_4$-2-w | 185.6 | 208 | – | 1.52 | 1.95 | 10 | 5.1 |
| CB | 1.1 | 107 | 1.1 | 1.4 | 2.5 | | 1.8 |
| 13-w | 7.6 | 329 | (BAL-PMN) | 1.6 | 2.7 | 8 | |
| | 50.3 | 667 | | 1.5 | 2.5 | | |
| u$TiO_2$ | 0.52 | 63 | 0.52 | | | | |
| 13w | 2.1 | 132 | (BAL-PMN) | 1.44 | 2.60 | 8 | 1.6 |
| | 10.5 | 395 | | | | | |
| AlOOH-40 nm | 0.4 | 50 | 3.3 | 0.59 | 2.47 | | 2.9 |
| 4-w | 3.3 | 43 | (BAL-PMN) | 0.57 | 2.63 | 11 | |
| | 28 | 94 | | 0.63 | 2.56 | | |
| AlOOH-10 nm | 0.4 | 42 | 3.1 | 1.75 | 2.71 | | 2.9 |
| 4-w | 3.1 | 58 | (BAL-PMN) | 1.65 | 2.79 | 8 | |
| | 28.3 | 177 | | 1.68 | 2.72 | | |
| MWCNT | 0.1 | 151 | 0.1 | 1.67 | 1.7 | | 0.1–0.3 |
| 13-w | 0.45 | 350 | (BAL-PMN) | 1.91 | 1.68 | 8 | |
| | 1.62 | 318 | | 1.93 | 1.67 | | |
| | 5.98 | 375 | | 2.19 | 1.76 | | |
| p$TiO_2$ | 9.6 | 50 | 9.6 | | | | |
| 13-w | 47.7 | 324 | (BAL-PMN) | 1.44 | 1.71 | 10 | 4.3 |
| | 239.1 | 838 | | | | | |
| p$TiO_2$ | 5.4 | 68 | 5.4 | 1.9 | 2 | | |
| 4-w | 51.9 | 110 | (BAL-PMN) | 1.7 | (Estimated) | 9 | 4.3 |
| | 252.2 | 330 | | 1.44 | | | |

*Abbreviations*: w: week, CB: carbon black, n.d.: not determined, MMAD: mass median aerodynamic diameter, GSD: geometric standard deviation, Resp.: respirable fraction deposited in the pulmonary region. References: CB: Elder et al. (2005), 13-week ultrafine and pigmentary $TiO_2$ Bermudez et al. (2002, 2004), AlOOH Pauluhn (2009c), iron oxides Pauluhn (2008); Pauluhn (2009a,b), 4-week pigmentary $TiO_2$ Warheit et al. (1997), and MWCNT Pauluhn (2010a).

the overload threshold. (2) This is opposed to occupational exposures where recurrent chronic encounters to PMs may take place and lung injury is manifested above the retention-related overload threshold. Accordingly, protocols devised to attain this threshold during short dosing sessions may not necessarily manifest that specific phenotype of lung injury typical for PMs occurring during recurrent, chronic occupational exposure patterns. To study the response to deposited and retained PMs extensive postexposure periods are required to better understand the relationship of biokinetics and sustained pulmonary inflammation and recovery (Fig. 4). Some granular biopersistent particles (quartz) show a remarkable self-amplification of inflammation during the postexposure phase. Hence, to experimentally reveal a quartz-like mode of action a postexposure period in the range of 3 months is required (Pauluhn, 2009a).

Likewise, extreme doses of PMs may entirely overwhelm the capacity of the pool of phagocytes to remove the particle load from the alveolar surface with resultant aberrant kinetics and toxicity. Therefore kinetic modeling and judicious dose selection are of paramount importance in the context of study design and what it can deliver. The axis of ordinates shown in Fig. 4 illustrate convincingly that mass-concentrations must be put into a volumetric perspective to adequately simulate the extend of lung-overload and to select a meaningful and nevertheless balanced duration of postexposure period. Especially for low-density materials, even rela-

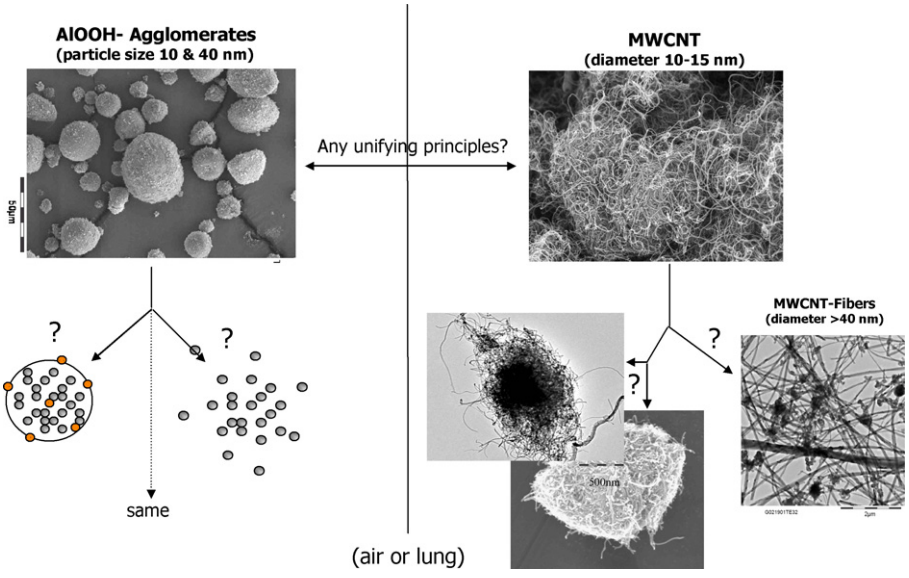



**Fig. 1.** This analysis addressed nanosized and submicronsized (pigmentary) particle structures (not shown). The two types of nanostructures addressed were aluminum oxyhydroxide consisting of 10 nm or 40 nm primary particle sizes (Pauluhn, 2009c) which tend to agglomerate into micronsized aggregates. MWCNT (10–15 nm) as pristine material (intertwined structure) and following dispersion into inhalation chambers (coiled structure). More rigid MWCNT (>40 nm) were present as tubes (fibers).

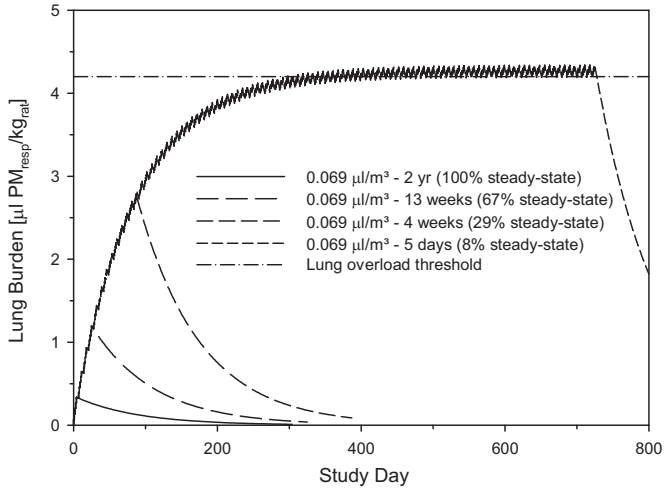



**Fig. 2.** Modeling of exposure-related increase in volumetric lung burdens of rats exposed to constant respirable particle ($PM_{resp}$) concentrations at 0.069 µl/m$^3$ at variable exposure durations (5 days/week) of 1, 4, 13, or 104 weeks. The overload threshold is defined as 4.2 µl $PM_{resp}$/$kg_{rat}$. From the percentages given the study duration-to-steady state time adjustment factors can be calculated. These are 12.5, 3.4, 1.5 and 1 for 1, 4, 13, or 104 weeks, respectively.

tively low exposure concentrations may cause a delay in clearance so that the rats' longevity may be insufficient to cover even one single retention half-time ($t_{1/2}$). Hence, in regard to chronic inflammatory pulmonary changes, adequately designed 4- and 13-week inhalation studies followed by postexposure periods of 3–6 months (or even longer) may deliver the highest level of information to better appreciate the primary toxic principle of the PM examined. As for the loading period, only minimal trade-offs can be made for the duration of postexposure period as at least one retention half-time needs to be covered to generate a scientifically meaningful set of both biokinetic and lung injury data.

### 2.3. Pulmonary biokinetics of poorly soluble particles

Elimination is usually a logarithmic process – that is, a constant proportion of the substance is eliminated per unit time which is described by a first-order relationship: $C_t = C_0 e^{-kt}$ where $C_t$ is the concentration after the time $t$, $C_0$ is the initial concentration at $t = 0$, and $k$ is the elimination constant ($k_e$). The relationship between the elimination rate constant ($k_e$) and half-time is given by the following Eq. (1):

$$k_e = \frac{\ln 2}{t_{1/2}} \quad (1)$$

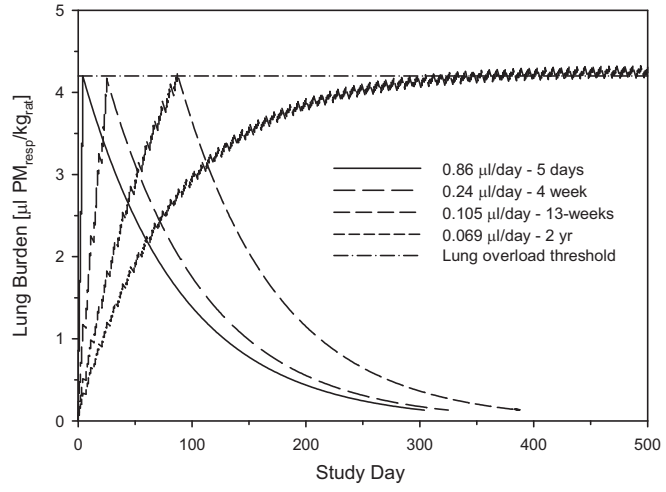
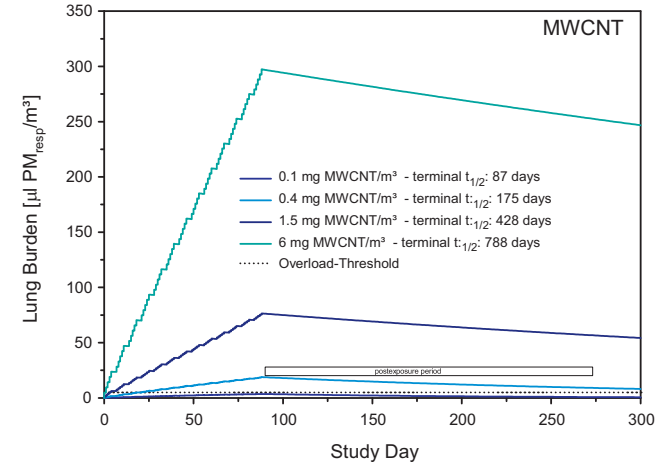
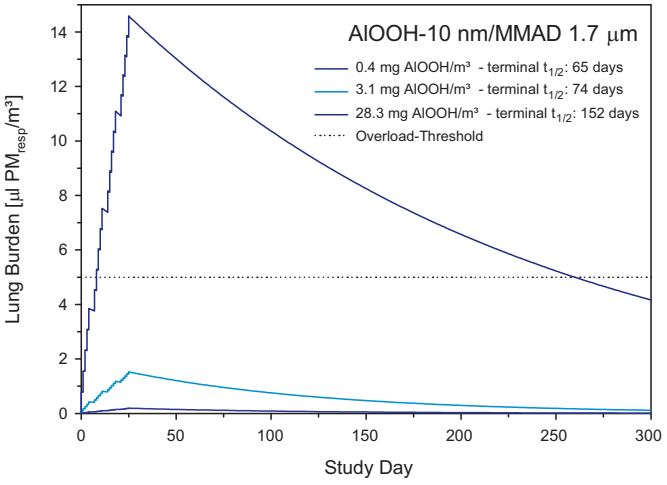



**Fig. 3.** Modeling of exposure-related constant volumetric lung burdens of rats exposed to variable respirable particle ($PM_{resp}$) concentrations at 0.86, 0.24, 0.105, and 0.069 µl/m$^3$ 5 days/week for 1, 4, 13, or 104 weeks. The overload threshold is defined as 4.2 µl $PM_{resp}$/$kg_{rat}$.



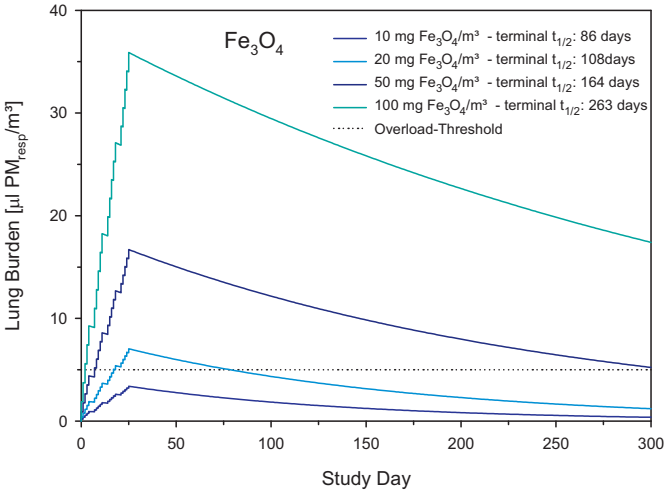




**Fig. 4.** Modeling of time- and volume-dependent lung burdens of respirable particle ($PM_{resp}$) of rats exposed for either 4 weeks (AlOOH) or 13 weeks (MWCNT, $Fe_3O_4$) using a 6 h/day, 5 days/week exposure regimen followed by a 6 months postexposure period. The overload threshold is defined as 5 µl $PM_{resp}$/m$^3$. The MWCNT and $Fe_3O_4$ studies were designed to attain this threshold in the lowest group at the end of the exposure period. At the outset of the AlOOH study it was believed that the kinetics of this nanosized material is similar to that of ultrafine $TiO_2$. This did not materialize and the concentrations selected were too low.

**Table 2**
Extrapolation of AM volume load from rat to man.

| | Average AM volume ($\mu m^3$) | Average AM counts in lung | AM volume per lung ($\mu m^3$/kg bw) |
|---|---|---|---|
| Rat (kg-based) | 1166 | $6 \times 10^7$ | $7 \times 10^{10}$ |
| Human (70 kg) | 4990 | $7.0 \times 10^9$ | $5 \times 10^{11}$ |

AM: alveolar macrophage. AM volumes were from Krombach et al. (1997). AM counts in the lungs of rats represent data determined by morphometric evaluation of histological preparations and considered the publications from Rehn et al. (1992), Morgan et al. (1980), Lehnert et al., 1985, Dethloff et al. (1987), Crapo et al. (1983), and Stone et al. (1992). Opposite to the AM volume, the AM counts per lung appear to be body weight dependent. Therefore, normalizations used the respective body weights given by these authors. Based on all publications the average count of AM/kg bw-rat was $6 \times 10^7$. The average AM count for humans (70 kg) was from Oberdörster (1995). The AM volume determined in this study using Wistar rats was calculated as follows: AM-volume (Fig. 5) × total cell counts of controls (Fig. 7) × 1/0.45 $kg^{-1}$ bw (average body weight during the 6 months postexposure period used in the MWCNT study (Pauluhn, 2010a) × adjustment of retrieved BAL-cells (≈20%, Pauluhn, 2009c) = $1.16 \times 10^3$ $\mu m^3 \times 5 \times 10^6 \times 2.2\ kg^{-1} \times 5 = 6.4 \times 10^{10}$ $\mu m^3$/kg bw.

Half-time is dependent on the clearance (CL) and the volume of distribution ($V_d$) and are combined using the following relationships:

$$t_{1/2} = \frac{\ln 2 \times V_d}{CL} \quad (2)$$

with

$$CL = V_d \times k_e \quad (3)$$

There is a common strong relationship between $V_d$ and body weight across species (Mahmood, 2007). This aspect has been observed when adjusting this endpoint across species. Any increase in the volume of distribution also increases the mean residence time of retained particulate matter.

Following exposure to insoluble particles there is an adaptive influx of alveolar macrophages. Thus, this concept does not consider possible changes in the alveolar macrophage mobility kinetics as already addressed by other authors (Yu et al., 1989) rather than the dynamic increase in the phagocyte pool with increasing lung particle burdens. The clearance of deposited PM via phagocytosis is an important aspect of lung defense. Rapid removal lessens the time available to cause direct tissue interaction and damage. Retention is the actual amount of inhaled particles found in the lungs at any postexposure time and is determined by the relative rates of deposition and clearance. PM deposited in the alveoli are primarily phagocytized by alveolar macrophages which are ultimately transported to the mucociliary escalator and cleared mechanically via the airways. A small fraction of particles is also cleared via the lymphatic system draining the lung. The translocation of particles to the draining hilar lymph-nodes (LALNs) commonly increases significantly at lung burdens high enough to cause pulmonary inflammation and barrier disruption (Pauluhn, 2009a). In regard to their alveolar and interstitial retention as well as lymphatic drainage species differences between rats and humans exist. In rats PM are retained predominately in the airspaces, whereas in humans chronically inhaled PM are retained in the interstitium (Nikula et al., 2001).

In previous inhalation studies with AlOOH a high correlation ($r^2 = 0.94$) of BAL-cell and lung tissue PM burdens was found (Pauluhn, 2009c). This appears to suggest that the particle lung burden may not necessarily originate from interstitialization rather than from particle-laden resident alveolar macrophages that were not retrieved by bronchoalveolar lavage (BAL). This interpretation is further corroborated by lung histopathology. Compartmentalized kinetic analyses support the conclusion that only ≈20% of the particle-laden phagocytic cells were recovered by BAL whereas the balance accounts for the lung burden, i.e., PM-laden alveolar macrophages (AM) not retrievable by BAL using four lavage cycles (Pauluhn, 2009a,c). Similar observations have been made by Rehn et al. (1992) who report a 14% recovery of AM using six lavages. In addition, these authors demonstrate increased number of macrophages in the lung as a result of the particle-induced pathogenetic process. At the same time, the recovery of cells by BAL was significantly increased as well. This means increased activation lead to a reduced adhesion, thereby increasing their accessibility by lavage. This finding demonstrates that a direct correlation of the total cell count of BAL-cells and the total cell population in the alveolus is not a simple, straightforward relationship. Therefore, the AM counts referred in Table 2 are based on morphometric methodologies while the AM volume was from Coulter® counter measurements of lavaged cells (for details see Krombach et al., 1997).

As a corollary of their function, alveolar macrophages engulf and retain PM. With increasing lung burdens this may lead to a macrophage-load dependent increase in cellular volume and/or an increased recruitment of phagocytic cells. Both relationships are presented in Fig. 5. Data are from rats exposed for 13 weeks to aerosolized MWCNT followed by a postexposure period of 6 months (Pauluhn, 2010a). At the highest exposure level, measurements of total cell counts and alveolar macrophage counts in BAL fluid yielded a 10- to 12-fold increase relative to the control. The

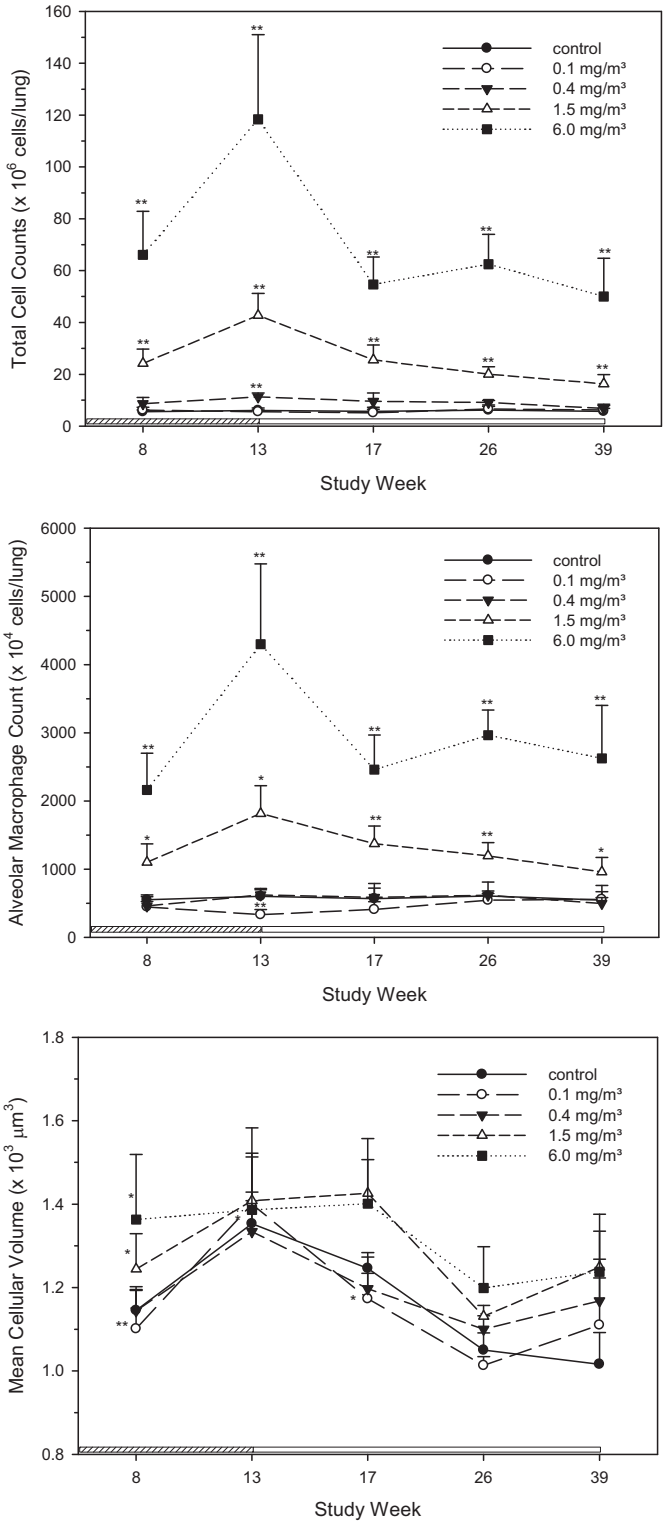



**Fig. 5.** Comparison of cellular inflammatory endpoints (total cell count, total macrophage count, mean cellular volume) in bronchoalveolar lavage (BAL) from rats exposed for 8 weeks (interim sacrifice) and 13 weeks. For exposure details see Table 1. Data points represent the mean ± S.D. of six male rats examined on study weeks 13 (postexposure day 1), 17, 26, and 39. Asterisks denote statistical significance to the time-matched control (*$P < 0.05$, **$P < 0.01$).

BAL-cell volume of control rats examined during a time period of 6 months yielded volume of $1162 \pm 139\ \mu m^3$ (Fig. 5, lower panel). This matches exactly the respective cellular volume of $1166\ \mu m^3$ reported for AM retrieved by BAL from Krombach et al. (1997), see Table 2, and other authors ($1170\ cm^3$; Haley at al., 1991). AM volumes were not affected in any conclusive dose- or time-course related manner. Similar observations were made in other repeated exposure inhalation studies using nano- or submicronsized PM (data not shown).

### 2.4. Lung overload

Unlike other laboratory animals and humans, rats appear to be more susceptible to overload-related effects due to impaired macrophage-mediated alveolar clearance. Oberörster (2002) proposed that the threshold of causing particle-induced chronic effects is the pulmonary dose that results in a first reduction in macrophage-mediated clearance. Morrow (1998) suggested that a rats' macrophage-mediated clearance is impaired at an estimated volumetric loading of 6% or 1 μl/g-lung. Significant impairment has been postulated to occur at 10 μl/g-lung volumetric loading (Brown et al., 2005). The half-time of PM for alveolar clearance in rats under non-overloading conditions has been reported to be in the range of 50–80 days (Donaldson et al., 2008; Stöber and McClellan, 1997; Brown et al., 2005).

In 4-week (AlOOH, $Fe_3O_4$) and 13-week (MWCNT, $Fe_3O_4$) rat repeated inhalation exposure studies, the increased total cell counts in BAL and the decreased pulmonary PM elimination ($t_{1/2}$) coincided (Figs. 6 and 7). This relationship suggests an association between decreased clearance and increased recruitment of (phagocytic) cells in the absence of any conclusive change in AM volume. Accordingly, the lung burden-dependent decreased clearance appears to be exclusively caused by the increased pool of AM which then becomes the $V_d$ for deposited and phagocytosed PM. To equate the maximal increase in total cell counts and AM shown in Fig. 5 with the expected changes in retention half-times as a result of $V_d$-overload, a ≈15-fold increase of $t_{1/2} \approx 60$ (normal) to $t_{1/2} \approx 900$ days (maximal overload) can be expected. Thus, the relationship of the biokinetic cornerstones $V_d \times k_e$ shows that the overload-dependent decrease in clearance (CL) appears to be contingent upon the dynamic change in these kinetic variables. This line of evidence is followed more closely below. Interestingly, despite different methodological approaches and strains of rats, the $V_d$ calculated based on the average BAL-cell volume given in Fig. 5 (lower panel) and the number of lavaged cells of air-only exposed control rats (Fig. 7) assuming a ≈20% retrieval of the AMs resident in the lung, the body weight adjusted BAL-cell volume ($V_d$) was almost identical to that as shown in Table 2.

The relative weight of the exsanguinated lung to body weight in rats from subchronic nose-only inhalation studies is 0.4 and 0.5% for male and female rats, respectively. Accordingly, the fractional PM displacement volume of $V_d \times 6/100$ (6%) is considered to be the physiological point of departure for changes in $V_d$-related homeostasis. Based on the relationship given below, this is equivalent to $4.2 \times 10^9\ \mu m^3$ AM-volume per 4.5 g lung weight per kg-rat (see Eqs. (4) and (5)). As 1 μl is equal $10^9\ \mu m^3$, this cumulative volume threshold concentration equals ≈1 μl PM/g-lung. This estimate is similar to that estimated by Morrow (1988, 1992) utilizing an entirely different approach. Therefore, based on a 6 h/day, five-times/week exposure regimen for 13 consecutive weeks, the fractional PM volume deposited in the pulmonary region of lung per exposure day may not exceed 0.105 μl $PM/kg_{rat}$ to comply with these constraints (Fig. 3). Nose-only exposed rats had respiratory minute volumes ($V_E$) of about 0.8–1 l/min/$kg_{rat}$ (Pauluhn and Thiel, 2007; Mauderly, 1986). For a 6-h exposure period at 0.8 l/min-$kg_{rat}$ this is equal to $V_E = 0.29\ m^3$. Subtraction of the physiological dead-space reduces the total inhaled volume by 1/3 which yields the alveolar ventilation volume of $0.19\ m^3_{alv}$. This means 0.105 μl $PM_{resp}/0.19\ m^3_{alv}$ or 0.55 μl $PM_{resp}/m^3_{alv}$ per exposure day is considered the generic no-observed-(adverse)-effect-level (NO(A)EL) in rats exposed for 13-weeks to poorly soluble PM. The 2-year equivalent is 0.069 μl $PM_{resp}/0.19\ m^3_{alv}$ or 0.36 μl $PM_{resp}/m^3_{alv}$ (see Fig. 3). In terms of inhalation chamber concentrations and exposure durations (adjustment from alveolar ventilation to normal ventilation) this means that the above generic volumetric overload-threshold is attained when using daily exposure concentrations at 0.83 and 0.54 μl $PM_{resp}/m^3$ for 3 months and chronic repeated inhalation exposures, respectively. As already mentioned above, this displacement volume is that of the airborne aggregated structure. Volume concentration can readily be calculated from effective density of the aggregate by using appropriate methodologies (Klobes et al., 2006). However, caution must be exercised when doing so as assemblages from nanostructures may be morphologically much more complex than those from higher-density micron-sized crystals. Therefore, especially for the former, the dispersion technique applied may affect both compaction and effective density of the PM which needs to be taken to consideration.

In rats the most conservative threshold for toxic effects is considered to be the cumulative pulmonary dose that results in a reduction in clearance mediated by the increase in $V_d$ as a physiological reflection to the adaptively increase of phagocytic cells relative to the cumulative PM-load. Relating the total AM volume ($V_d$) to the cumulative overload threshold dose – which has been defined to be 6% – the overload threshold can be estimated as follows (for parameters see Table 2):

$$\text{total AM volume}\,(V_d) = \frac{AM_{volume}}{kg_{rat}} = 7 \times 10^{10}[\mu m^3] \times 10^{-9} = 70\left[\frac{\mu l}{kg_{rat}}\right] \quad (4)$$

$$\text{overload threshold}\,(V_{d\text{-thresh}}) = 70 \times 0.06 = 4.2\frac{\mu l}{kg_{rat}} \quad (5)$$

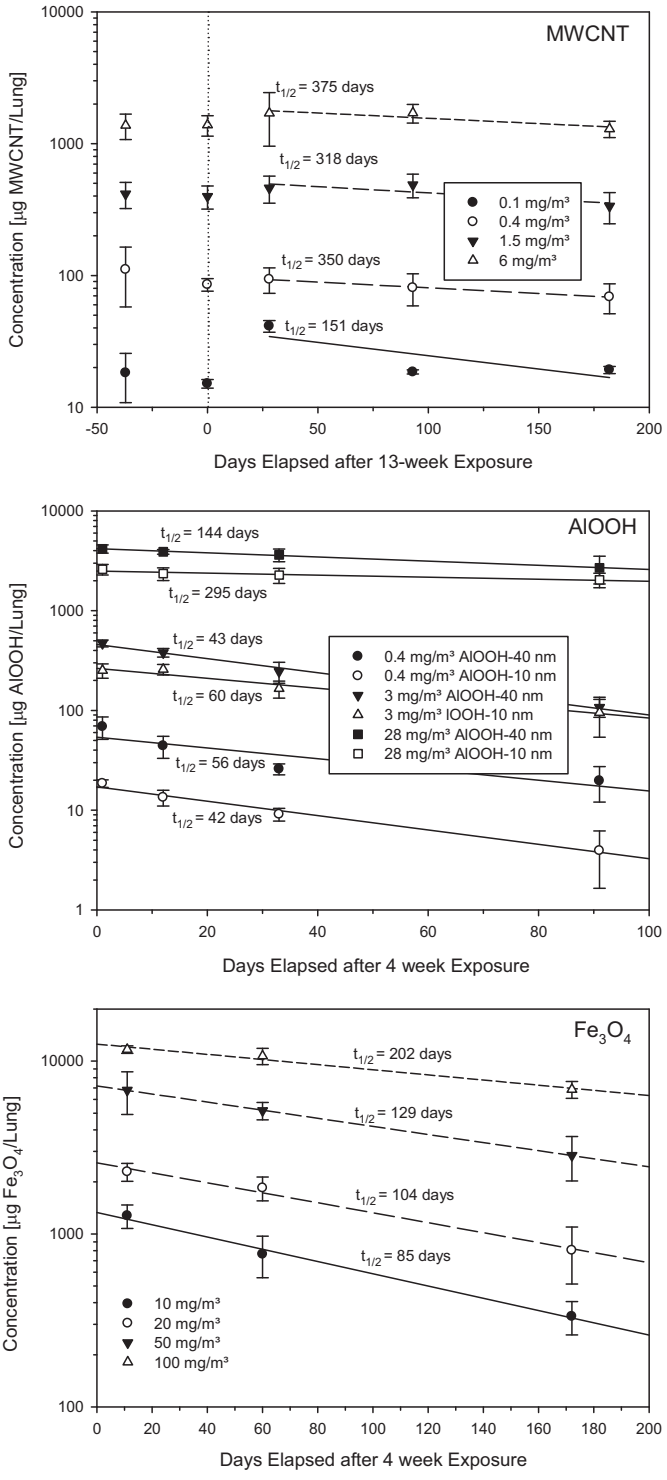



**Fig. 6.** Time- and concentration-related changes in lung burdens of rats exposed to respirable particle ($PM_{resp}$) of rats exposed for either 4 (AlOOH) or 13 weeks (MWCNT, $Fe_3O_4$). For exposure details see Table 1. Data points represent the mean ± S.D. of six male rats per group examined on multiple time points. The fate of inhaled particles was calculated based on a first-order elimination rate ($k = \ln(2)/t_{1/2}$ [$days^{-1}$]).

Likewise, to calculate the cumulative PM-volume load per lung at the end of a repeated inhalation exposure studies, the following Eq. (6) has been used:

$$\text{cumulative volume concentration} = C_i \times F_a \times \frac{E_d}{\rho_e}\left[\frac{\mu l}{m^3}\right] \quad (6)$$

where $C_i$, actual mass concentration of PM in the vicinity of the breathing zone of rats, mg/$m^3$; $F_a$, fractional deposition of PM in the alveolar region, $PM_{resp}$, as

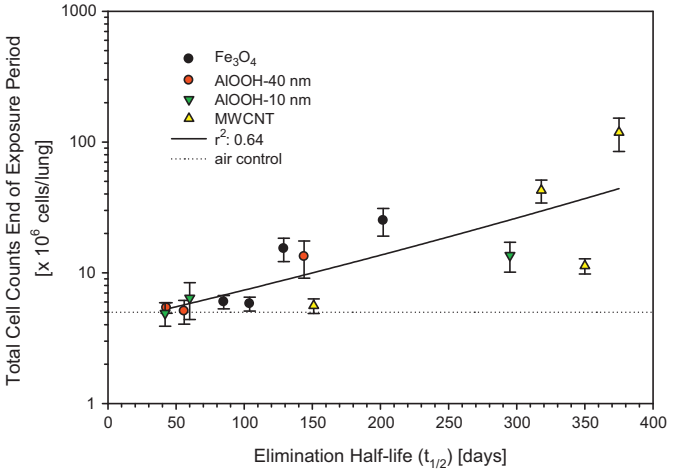



**Fig. 7.** Dependence of total cell counts in bronchoalveolar lavage (BAL) on the retention half-time. For exposure details see Table 1.

estimated by MPPD2 calculations; $E_d$, number of exposure days; $\rho_e$, effective density of agglomerated PM in the vicinity of the breathing zone of rats, g/cm$^3$.

$PM_{resp}$ is calculated using MPPD2 software (Anjilvel and Asgharian, 1995; RIVM, 2002) based on the empirically determined mass median aerodynamic diameter (MMAD) and geometric standard deviation (GSD) obtained during rat nose-only inhalation studies. While the densities of MWCNT and uTiO$_2$ (as the test article and not following dispersion into inhalation chambers) were determined by mercury pycnometry, all other densities represent published 'material density' data without specific reference to the method used for determination.

PM-related pulmonary toxicity appears to be contingent upon the cumulative volumetric lung dose that exceeds the homeostatic range of particle accumulation in pulmonary phagocytes. At this threshold the normal phagocyte-mediated elimination half-time does not exceed a $t_{1/2}$ of 60–80 days. Based on the relationships detailed above, this threshold should be similar to 1 μl PM-volume/g-lung and can be converted to a mass-based generic NO(A)EL for repeated rat inhalation studies using Eq. (7):

$$\mathrm{NO(A)EL}_{\mathrm{predicted}_i} = \frac{1\,\mu\mathrm{l}}{0.29\,\mathrm{m}^3} \times \frac{\rho}{f_{vi}} \times \frac{100}{\mathrm{PM}_{\mathrm{resp}}} \left[\frac{\mathrm{mg}}{\mathrm{m}^3}\right] \quad (7)$$

where 1 μl, threshold volume of AM at which 6% of the $V_d$ is consumed, $f_v$, fractional volumetric daily exposure dose to attain steady state (study duration specific accumulation factor for PM volume). This study duration-dependent fraction '*i*' can be calculated from Fig. 3 using the ratios of the concentration at steady state and the respective daily dose. For 1-, 4-, 13-, and 104-week studies this factor becomes 4.9, 17.5, 40, and 61, respectively. The pulmonary deposited dose '$PM_{resp}$', is estimated by MPPD2 calculation, $\rho$, effective density of agglomerated PM, ideally from breathing zone samples of rats, g/cm$^3$.

When accounting for the inhaled volume of exposure atmosphere per day ($V_E \times 360$ min) and kg$_{rat}$ (0.29 m$^3$), the effective agglomerate density ($\rho$), the fraction of PM-volume ($f_v$) that has to be inhaled per exposure day to attain the cumulative overload threshold of 4.2 μl $PM_{resp}$/kg$_{rat}$ (Eq. (5)) at the end of study (Fig. 3), and the total PM concentration potentially inhalable (100/$PM_{resp}$) in inhalation chambers, then the NO(A)EL can be estimated at the outset of study. Eq. (7) also demonstrates that the particle size range of MMAD 1–3 μm must be closely observed (OECD, 2009), otherwise NOAELs increase with decreasing respirability of PMs.

### 2.5. Particle volume burden and pulmonary inflammation

As long as particles are deposited and retained in the lung in a homogeneous manner, the elevation of polymorphonuclear neutrophilic granulocytes (PMN) in BAL is generally considered to be the most sensitive and quantitative endpoint to probe for PM-induced pulmonary inflammation. In their primary function in host defense, PMNs have been likened to a watchman function, searching for invading microbial pathogens that they phagocytose and destroy. The generation of oxygen-derived free radicals, catalyzed by a membrane-bound NADPH oxidase, represents one of the main systems by which phagocytes (PMN, AM, monocytes) kill particle-like microorganisms. These intermediates of oxygen reduction are formed by phagocytes during the engulfment of particulate matter. The peculiar metabolic pathway of activated phagocytes is called "respiratory burst" (Bellavite, 1988). While it is clear that neutrophils can emigrate from the vasculature into the alveoli via interepithelial migration tunnels without causing injury, there is compelling evidence that in pathologic circumstances, neutrophils are primary perpetrators of inflammatory injury to the lung. Their activation may be excessive and/or prolonged, leading to extracellular release of cytotoxic compounds that can induce a spectrum of responses in neighboring cells ranging from activation to injury and death (Zemans et al., 2009). The armamentarium available in PMNs and AMs of causing oxidative stress has to be judged judiciously relative to that triggered by the PM *per se*. Uptake of the PM by phagocytes has been shown to stimulate an endogenous respiratory burst, but most studies have not made the difficult distinction between exogenous or endogenous sources of oxidants when measuring intracellular oxidation (Bellavite, 1988; Tao et al., 2003; Yokota et al., 2005). The increase in neutrophil counts in concert with decompensated overload supports the common view held, *viz.* that the endogenous sources of oxidants appear to be quantitatively more critical than those attributable to the retained PM.

### 2.6. Validation and verification of procedures

The objective of this analysis is to derive a generic $PM_{nano\text{-}to\text{-}submicron}$ "*worker-OEL long-term for inhalation route-local*" (ECHA, 2008a,b,c,d). The kinetic model devised utilized empirical data from subacute (4 weeks) and subchronic (13 weeks) rat inhalation studies, most of them using a directed-flow nose-only mode of exposure (Pauluhn and Thiel, 2007). Therefore, the algorithms developed on studies up to 13 weeks were used to predict the outcome of two chronic 2-year rat whole-body inhalation studies reported by Muhle et al. (1991) and Bellmann et al. (1991). The data of this study were not used in the prior parameterization of the model. In this study rats were exposed to either toner (1, 4, and 16 mg PM/m$^3$) or pigmentary TiO$_2$ (5 mg/m$^3$) on a 6 h/day, five-times/week exposure regimen for 2 years. Toner and TiO$_2$ have a specific density of 1.2 and 4.3 g/cm$^3$, respectively. The MMAD (GSD) of toner and TiO$_2$ were 4 μm (1.5) and 1.1 μm (1.6), respectively. The dose descriptors of this study were defined, *inter alia*, by changes in the lung-overload dependent retention kinetics as well as the influx in BAL-PMNs. Accordingly, in regard to the range of particle sizes and particle densities examined as well as the toxicological endpoints addressed this study was considered most appropriate to challenge the modeling procedures applied.

The predicted cumulative volumetric lung doses and associated changes in BAL-PMN are summarized in Fig. 8. Regarding the kinetic predictions in terms of attaining the overload-threshold and multiples thereof, the prediction matched the empirical data reported by Muhle et al. (1991) and Bellmann et al. (1991). For toner (1, 4, and 16 mg/m$^3$) and TiO$_2$ (5 mg/m$^3$) the following retention half-times (confidence interval, 95%) were reported: 81 (75–88), 173 (135–241), 491 (306–1000)), and 94 (78–117) days. The respective predicted half-times were 90, 278, 757, and 167 days (see Fig. 8, upper panel). At 1 mg/m$^3$ the clearance half-time was not prolonged to any appreciable extent and did not exceed the 5 μl $PM_{resp}$/m$^3$ volumetric threshold. Therefore, this level was predicted to be the NO(A)EL of study which matched the conclusions of Muhle et al. (1991). The BAL-PMN (%) measured within the last quarter of study were for the toner (0, 1, 4, and 16 mg/m$^3$) and TiO$_2$ (5 mg/m$^3$) 0.5–1.9, 0.7–7.0, 6.7–9.7, 33–43, and 2.3–4.9%. The respective predicted percentages of PMNs were ≤2, 1.2, 20.6, 40, and 13%. Taking into account that bronchoalveolar lavage is differently executed among laboratories, the predictions in PMN levels have striking similarities with a tendency to be slightly over-predicted. From that one may conclude that a chronic exacerbation of PMN-influx did not occur (Fig. 8, lower panel). Similar conclusions can be drawn for the high correlation of the observed and predicted lung burdens at the end of the exposure period of 4-, 13-, and 104-week repeated exposure rat inhalation studies (Fig. 9). The prediction of the mass-based lung burden from the calculated volumetric lung particle burden took into account the 'cumulative volume exposure intensity scale' [μl $PM_{resp}$/m$^3$] adjusted for the inhaled volume per exposure day (0.29 m$^3$/kg$_{rat}$) and the actual rats' body weight (equation depicted in Fig. 9). The high correlation of the BAL-PMN influx and lung PM burdens based on the PM displacement volume relative to PM mass (Fig. 10) shows that the inflammatory response scales more favorably with the PM-volume rather than PM-mass. This dependency corroborates the conclusion that the PM-induced pulmonary inflammation is contingent upon the generic particle volume and the ensuing endogenous response to it.

Collectively, the data characterizing the pulmonary dose and associated inflammatory effect are conclusive with a minimal tendency to "over-prediction". This may be attributable to multiple factors, *inter alia*, the cumulative lung dose may be higher in rats utilizing a directed-flow nose-only mode of exposure (see studies from Pauluhn and coworkers) as compared to conventional whole body mode of exposure (chronic studies). Similarly, older rats inflicted with PM-induced lung injury may change their respiratory minute volumes with resultant changes of the inhaled dose.

### 2.7. Data analyses

For simulation of the exposure duration-related accumulation of particles in the lung a one-compartment, first-order elimination kinetics is assumed. The elimination constant $k_e$ was either empirically determined from time-course lung burden analyses of the study postexposure period or, alternatively, was estimated on the dependence of the elimination half-time on the cumulative PM-volume lung burden using an algorithm to dynamically adjust $k_e$ based on the degree of volumetric PM-burden. The maximum estimated elimination half-times always refer to the $k_e$ after the last exposure day. A Fortran computer code was used for calculations. Non-linear curve fitting utilized SigmaPlot 11.0 software (Systat Software Inc., Point Richmond, CA).

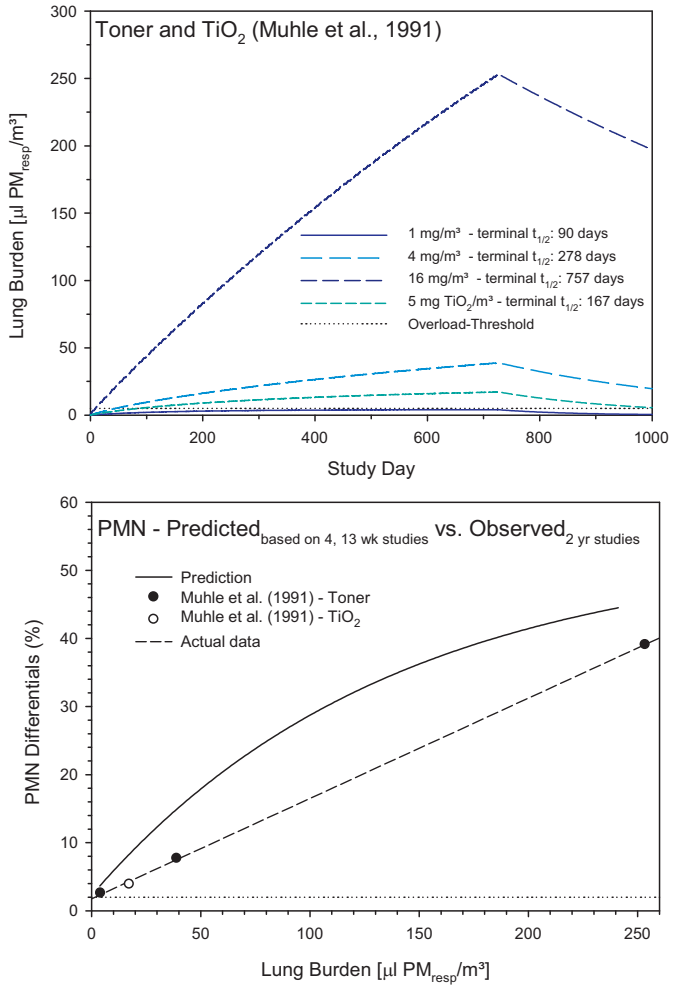



**Fig. 8.** Modeling of time- and volume-dependent lung burdens of respirable particle ($PM_{resp}$) of rats exposed for 104 weeks to toner and $pTiO_2$ (upper panel) based on the data published by Muhle et al. (1991). The overload threshold is defined as 5 µl $PM_{resp}/m^3$. The predicted and empirical PMN percentages in BAL are compared in the lower panel (filled circles: toner, open circles: $pTiO_2$). The predicted PMN, based on the relationship shown in Fig. 12, are represented be the solid line. The dotted line represents the lower bound of the 95% confidence interval given in Fig. 13.

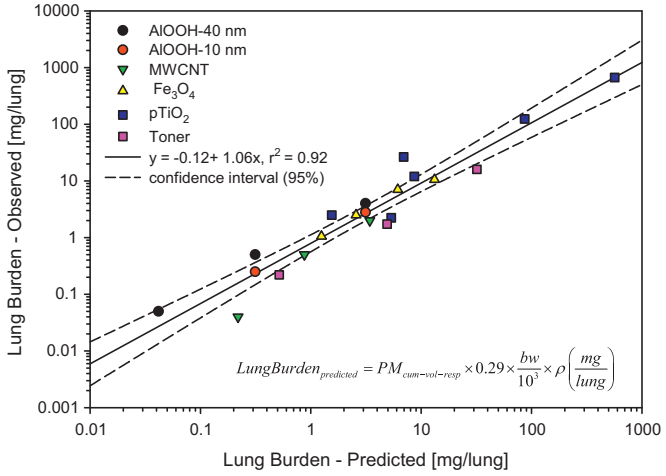



**Fig. 9.** Comparison of predicted and observed retention lung burdens of the from 4- and 13-week repeated exposure inhalation studies (see Table 1) with published data from chronic 2-year inhalation studies with $pTiO_2$ and toner from Muhle et al. (1991) and Bellmann et al. (1991) and $pTiO_2$ from Lee et al. (1986).

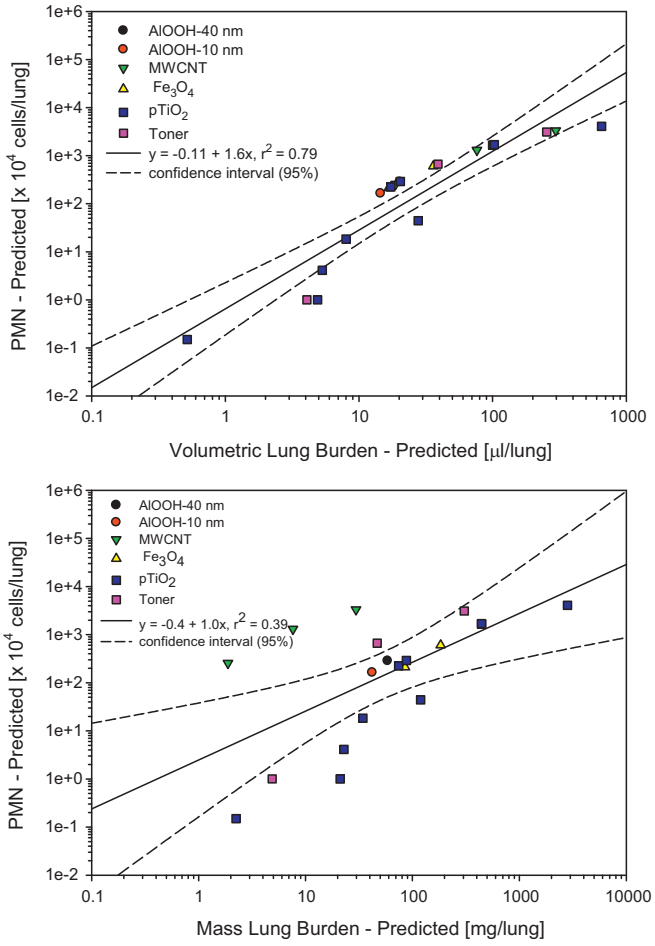



**Fig. 10.** Comparison of predicted BAL-PMN influx relative to the predicted terminal cumulative PM volume-based lung burden (upper panel) and the predicted terminal cumulative PM mass-based lung burden (lower panel).

## 3. Results and discussion

### 3.1. Pulmonary biokinetics, volume of distribution, and particle clearance

The retention $t_{1/2}$ of nanosized ($uTiO_2$, AlOOH, MWCNT) and submicronsized ($pTiO_2$, CB, $Fe_3O_4$) particles from 4 and 13 weeks inhalation studies (see Table 1) on the cumulative PM-volume concentrations are compared in Fig. 11. This illustration delineates a clear association of an increase in the cumulative volume concentrations and the increased retention $t_{1/2}$. Thus, using the volume metric, all data converge into one single relationship yielding a threshold estimate of $t_{1/2}$ = 64 days (Fig. 11) which is essentially similar to the published overload threshold of $t_{1/2} \approx 60$ days. Normalized to the lung weight, a PM-volume equal to or exceeding ≈1 µl PM/lung characterizes the range at which the onset of an increased $t_{1/2}$ occurs, while at ≈10 µl PM/lung an aberrantly longer elimination kinetic of $t_{1/2} \geq 1$ year occurs. PM-volume burdens beyond 10 µl PM/lung decrease the clearance further toward a $t_{1/2}$ of 800–900 days.

Interestingly, the theoretical approach defined by Eqs. (4) and (5) matches the empirical data shown in Fig. 11 as it converges into the same volumetric overload range as already derived by Morrow (1988). The reported 10-fold range from the onset of delayed clearance to almost complete stasis is concordant with the 5–50 µl $PM_{resp}/m^3$ or 1–10 µl PM/lung range as scaled in Fig. 11. Accordingly, the conclusions drawn from this relationship are in

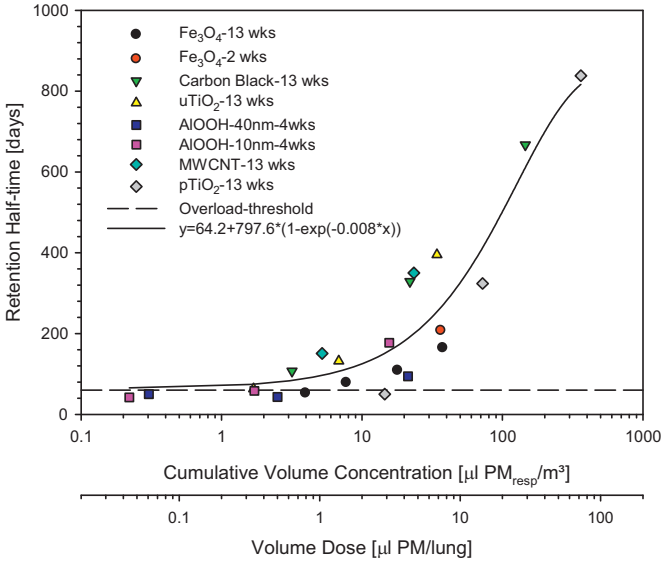



**Fig. 11.** Dependence of the retention half-time on the cumulative volume concentration of respirable particles ($PM_{resp}$). For exposure details see Table 1.

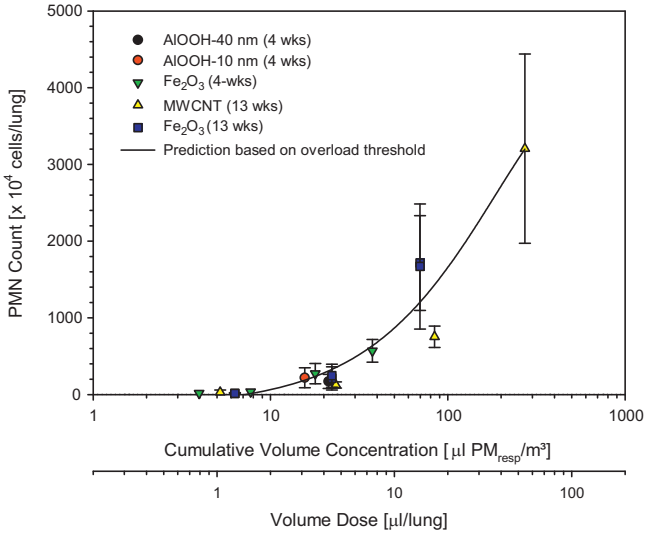



**Fig. 12.** Dependence of the relative cytodifferentiation of neutrophils (PMN) on 300 counted cells per cytospot and absolute neutrophil (PMN) count in BAL. For exposure details see Table 1.

agreement with Morrow's hypothesis, namely that the physiological array of volumetric overload of AMs occurs between 1 and 10 µl PM/lung. Half-times greater than 1 year were observed in few studies using postexposure periods of ≈1 year (Bermudez et al., 2002, 2004; Elder et al., 2005). This supports the notion that the scatter of data shown in Fig. 11 appears to be primarily related to experimental factors, such as differences in exposure modes and techniques to generate and characterize PM exposure atmospheres and insufficient length of postexposure periods at long retention half-times (see Figs. 4 and 6), including the unavailability of actual agglomerate densities of the aerosolized PMs. Similarly, especially for MWCNT, it cannot be ruled out that the dispersion techniques applied had affected the effective density of assemblages in inhalation chamber atmospheres as well.

### 3.2. Pulmonary particle volume burden and inflammation

The relationship of neutrophil counts in BAL, obtained by exactly identical methodological procedures (Pauluhn, 2009b,c, 2010a), and volumetric PM-lung burdens are analyzed in Fig. 12. Absolute BAL-PMN counts per lung and the relative counts obtained by cytodifferentiation are compared in Fig. 13. As for the increased retention half-times (Fig. 11), also the neutrophil counts were scaled to cumulative volumetric concentration as calculated by Eq. (6) with further adjustment to the particle volume/lung. This analysis shows that the pulmonary inflammatory changes, as indicated by changes in BAL-PMN, scale favorably with the cumulative volumetric concentration (linear log-log scale: $r^2 = 0.91$; analysis not shown). Using this scale, a precipitous increased in PMN began at a PM-volume load of ≈10 µl PM/lung. The analysis depicted in Fig. 13 demonstrates that minimal elevations in BAL-PMN at the lower confidence interval (which is $\approx 1 \times 10^4$ PMN counts/lung or 2% PMN by cytodifferentiation) appears to be beyond the resolution of assay. Commonly, limited numbers of cells are manually counted by light microscopy (200–300 per cytospot) for cytodifferentiation. This means, any finding at or below 2% PMNs – which is similar to 4–6 neutrophilic granulocytes counted per cytospot – is considered to be of no toxicological significance whereas air exposed control may incidentally show zero-percentages of PMNs (Haley et al., 1991). Accordingly, PMNs below the 2% level were omitted from the analysis shown in Fig. 13. The relationship depicted in Fig. 12 supports the notion that the accumulated PM-volume lung burdens, rather than the recent dose, appear to trigger the more sustained type of pulmonary inflammation as indicated by increased neutrophils in BAL. As a result of that, the model devised is biased to over-predict the pulmonary response to inhaled PMs.

### 3.3. Prediction of NOAELs based on volumetric overload-threshold

The NOAELs were predicted based on Eq. (7) taking into account the external exposure indices from a range of repeated rat inhalation studies (4–104 weeks). All studies utilized a 6 h/day, five-times per week exposure regimen. The predicted NOAELs assumed that the attainment of the homeostatic threshold of 1 µl PM-volume/lung is equal the kinetic threshold. By comparing Figs. 11 and 12 one may anticipate that inflammation is secondary to this kinetic phenomenon. All studies summarized in Fig. 14 utilized bronchoalveolar lavage and cytodifferentiation, in addition to conventional histopathology, as endpoints whereas the PMN influx appeared to be the most sensitive criterion to define the threshold

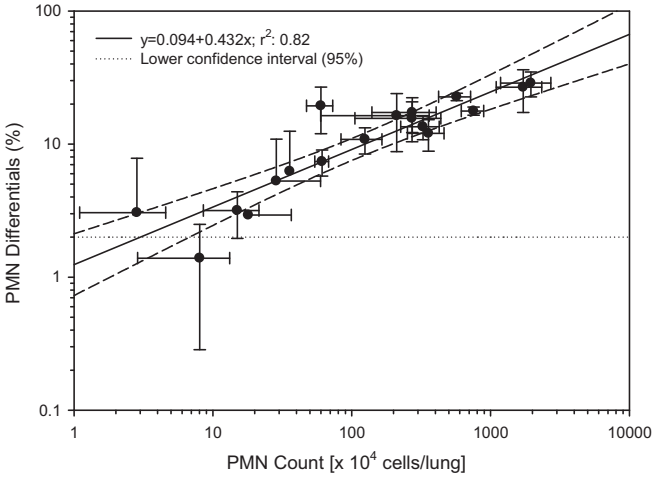



**Fig. 13.** Dependence of the absolute neutrophil (PMN) count in BAL on the cumulative volume concentration of respirable particles ($PM_{resp}$). Data points represent the mean ± S.D. of six male rats examined. In this analysis data from 4 weeks (AlOOH, $Fe_3O_4$) and 13 weeks (MWCNT, $Fe_3O_4$) were included (for exposure details see Table 1).

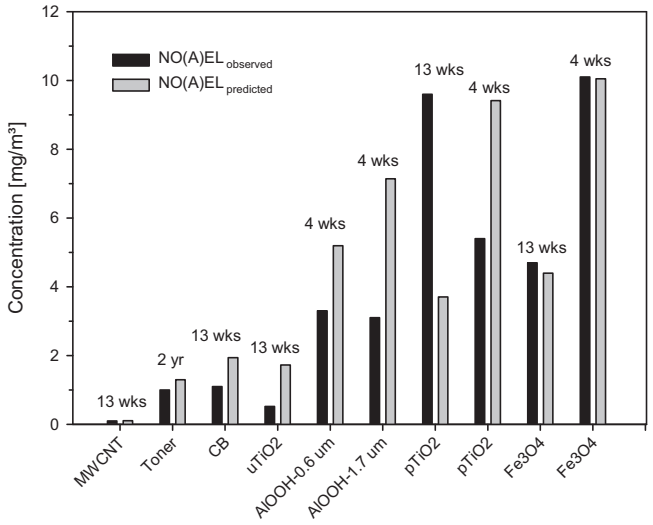



**Fig. 14.** Comparison of empirical and predicted NO(A)ELs from 4, 13, and 104 weeks rat repeated exposure inhalation studies. The 2-year toner study reproduced data from Muhle et al. (1991). For exposure details see Table 1. The predicted NO(A)ELs were calculated using Eq. (7).

for a statistically significant lung inflammation. Based on the comparison made in Fig. 14, the empirical (inflammation-based) and predicted (kinetic volumetric overload threshold-based) NO(A)ELs are in reasonable agreement. As far as differences occurred they appear to be caused by design factors, especially dose-selection. For the $pTiO_2$ 4 and 13 weeks inhalation studies the predicted NO(A)ELs were either lower or higher than the empirical NO(A)ELs given by the study authors (for details see Table 1). A statistically significant increase of BAL-PMNs occurred in the $pTiO_2$-13-week study at the lowest concentration of 9.6 mg/m$^3$ while in the $pTiO_2$-4-week study the lowest concentration was 5.4 mg/m$^3$ with the next higher concentration at 51.9 mg/m$^3$. The borderline NO(A)EL of 5 mg/m$^3$ obtained in the Muhle et al. (1991) chronic inhalation study on rats supports this conclusion (minimal influx of BAL-PMN and increased elimination half-time). Thus, it appears that the differences between actual and predicted NO(A)ELs occurred as a result of the wide spacing of exposure levels rather than any inaccuracy in the prediction (see also Fig. 4). The correct prediction from 4- to 104-week studies afforded by the derived accumulation factor $f_v$ gives further credence to the kinetic model devised, that means, time-adjustments need to be made relative to the attainment of the steady-state and not by using a prorated linear time-adjustment. Overall, this analysis supports the conclusion that all poorly soluble PM, whether nano- or submicronsized, arrive at the same unifying volumetric NO(A)EL of 0.5 μl $PM_{resp}$/m$^3$. Excess exposure of this threshold prompts an increase in the $V_d$ and residence time of retained particles.

### 3.4. Derivation of a human no-effect-level (DNEL)

Under the new European Chemicals Regulation, REACH (Registration, Evaluation, Authorization and Restriction of Chemicals), the chemicals industry is obligated to register all chemicals imported or produced in the European Union (EU) in volumes exceeding 1 tonne per annum (tpa) (EC, 2006). For volumes greater than 10 tpa, registrants must submit a Chemical Safety Report (CSR) (EC, 2006; ECHA, 2008a,b,c,d). Manufacturers, importers and downstream users should ensure that they manufacture/place on the market/use substances in such a way that they do not adversely affect human health. Manufacturers and importers are to assess and document that the risks arising from the substance they manufacture or import are controlled during manufacture and their own use(s) and that others further down the supply chain can control the risks. To achieve this objective for occupational settings, REACH defines a Derived No-Effect Level (DNEL) which is the level of occupational exposure above which humans should not be exposed. In the risk characterization, the exposure of each human population known to be or likely to be exposed is compared with the appropriate DNEL. The risk to humans can be considered to be controlled if the exposure levels estimated do not exceed the appropriate DNEL. To achieve these objectives, key elements from the respective US-EPA Human Equivalence Concentration (HEC) approach were adopted to be applicable to worker exposure (US-EPA, 2002). In this context, the acronym 'HEC' is used interchangeably with 'DNEL'.

Chronic lung tissue responses to inhaled particles, especially those which are not highly water soluble, reactive or toxic, may depend more on anatomic sites of particle retention than on sites of initial deposition. Anatomical differences between rats and humans could affect sites long-term particle retention. There are known differences in pulmonary compartmentalization showing that the relative amounts of interstitial and intraluminal PM retention differ markedly between humans and rats with a predominance of particles being found in the interstitium in man and intraluminarly in the rat (Kreyling and Scheuch, 2000; Nikula et al., 2001). Despite these known characteristics, the focus of this derivation is on the human pulmonary dose that prevents these types of changes to occur. Similar to rats, also for humans the key event is assumed to be the fractional exhaustion of the available phagocyte volume triggering the adaptive increase in $V_d$ and consequently a decrease in PM-clearance. To put rat data into human perspective multiple adjustments are needed. The body weight as key denominator for local effects occurring in the lung may be subject to challenge. However, no particular advantage appears to be given when using the lung volume, lung weight, lung surface area or any other pulmonary denominator because the variable determining the pulmonary dose is defined by the ventilation/kg bw and the respirability of PMs. Denominators such as the lung volume or alveolar surface area are allometrically scaled to body weight by slope factors of 1.06 or 0.95, respectively (Pinkerton et al., 1992). Thus, slope factors close to ≈1 do not appear to call for any further portal-of-entry specific adjustments. Therefore, this appraisal prefers to focus on the simplest, most straightforward and readily available denominator, which is the body weight.

Prevailing experimental evidence suggests that the onset of response commences with the adaptive increase in the phagocyte pool ($V_d$). As illustrated in Fig. 11, for rats this onset occurs at a cumulative volume concentration of ≈5 μl $PM_{resp}$/m$^3$ or 1 μl PM/lung with a proportional increase up to ≈50 μl $PM_{resp}$/m$^3$ or 10 μl PM/lung. The point of departure for destructive effects to occur either due to increased deterioration of AMs or free PM not gaining instant access to the pool of AMs seems to occur beyond this cumulative lung burden. The focus of human risk assessment is on the first yet homeostatic event that precedes the occurrence of pulmonary inflammation. At PM lung burdens above 10 μl PM/lung an entirely decompensated PM-clearance ($t_{1/2}$ > 1 year) seems to take place. Therefore, this cut-off is considered to be the MTD (maximum tolerated dose) for PM-related lung injury. Opposite to rat inhalation studies where mass-based total lung burdens can readily be determined and validated against modeling procedures, the determination of human lung burdens has to rely upon modeling alone. The estimation of the HEC or better the cumulative target organ dose capitalizes on the estimation of a generic occupational exposure level (OEL or DNEL) for poorly soluble PM ranging from nanosized to submicronsized. Suffice it to say, this approach focuses exclusively on that type of pulmonary toxicity conveyed by the physical properties of PM. Accordingly, it implicitly assumes that

adequate experimental evidence is available that excludes adverse effects to occur at extrathoracic deposition sites and that systemic bioavailability does not occur to any appreciable extent.

The OEL is assumed to be protective for discontinuous exposures utilizing normalization steps for interspecies-specific differences in ventilation, pulmonary particle deposition, and clearance to arrive at defined pulmonary volumetric PM-burdens in rats and humans at given exposure durations per day. Each adjustment requires an appropriate normalizing factor. The choice of the most appropriate normalizing factor must be defined by the nature of the pathogenesis process, i.e., defined according to the mechanism of action for the effect under consideration (Jarabek et al., 2005). Under the premise that equal volumetric lung doses might produce similar across-species responses, time-dependent differences in the retained lung PM-volume dose have to be appreciated due to the apparent differences of pulmonary clearance kinetics of rats and humans bearing in mind that the clearance is dependent on both $V_d$ and $k_e$. For the retention of particles in humans variable half-times have been reported (Snipes, 1989). This author summarized half-times from single exposures of humans, dogs, and rats using a two-exponential kinetic model. From this data, for humans a median retention half-time of $t_{1/2}$ of 325 days was estimated. Instead of this the retention half-time of $t_{1/2}$ of 400 days published by Oberdörster (1995) was used which is consistent with similar data published by Kreyling and Scheuch (2000). Of note is that the terminal clearance in humans is reported be in the range of 700 days which is plausible as humans have a higher clearance into the lymphatic system. As detailed by Kreyling and Scheuch (2000) and Nikula et al. (2001), rats retain particles primarily within macrophages located within the lumens of alveolar ducts and alveoli whereas in the human lung the major fraction of particulate material is located in the interstitium. This is consistent with the higher clearance via the lymphatics in this species (Snipes, 1989). Interestingly, taking into account the 7-fold greater AM-pool ($V_d$) of humans relative to rats (Table 2) this should result in a proportionally longer clearance, i.e., of approximately 420 days. Hence, the $t_{1/2}$ of 400 days for the alveolar clearance in humans appears to be plausible.

The following equation and normalization factors have been used to adjust for exposure duration-related and species-specific differences in the retained pulmonary mass-burden of PM ($AF_{lung\ burden}$) under the conditions commonly applied in OECD-TG413/GD#39 (OECD, 2009) compliant nose-only rat inhalation studies (index '*A*') and occupational seetings (index '*H*'):

$$AF_{\text{lung burden}-A/H} = \frac{V_{E-A} \times F_{a-A}}{V_{E-H} \times F_{a-H}} \times \frac{BW_H}{BW_A} = \frac{0.29 \times 0.075 \times 70}{10 \times 0.164 \times 1} = 0.93 \quad (8)$$

where $V_E$ = respiratory minute volume inhaled by the subject during the time of exposure/day, $m^3$, $F_a$ = fractional deposition of PM in the alveolar region, $PM_{resp}$, as estimated by MPPD2 calculations, BW = body weight, kg. Note: rat ventilation data are normalized to 1 kg bw and exposure duration of 6 h.

The first step in this process is to take into account the ventilation of nose-only exposed rats which is 0.8–1 l/min per kg-rat (Pauluhn and Thiel, 2007; Mauderly, 1986) relative to workers (generic default value = 10 $m^3$/human per working day). The resultant average ventilation per 6-h exposure day was estimated to be 0.29 $m^3$/kg-day-rat. The alveolar deposition fraction was calculated using the most representative particle size attributes (MMAD and GSD) given in Table 1. The fractional alveolar deposition '$F_a$' in Eq. (8) was calculated using MPPD2 software based on an average MMAD of 1.8 μm and a GSD of 2 (assuming unit particle density and oronasally breathing humans). In regard to the '$F_a$' it was assumed that particle size distributions in inhalation chambers and at workplaces are similar. Consequently, the factor to adjust for the dose delivered to the target organ under adjusted exposure scenarios and species-specific inhaled-dose adjustments is 0.93. As this AF is close to 1 it does not call for any ventilation-deposition-related dosimetric adjustment between rats and humans, as long as the particle size distributions in inhalation chambers and occupational settings are roughly identical.

Of note is that inhalation animal studies usually employ homogeneous aerosols of small particle diameter. By contrast, workers are usually exposed to coarser and more heterogeneous aerosols. Yet, differences in deposited doses between animals and humans due to particle size differences of aerosols have to be taken into account in risk assessment (Oller and Oberdörster, 2010). This uncertainty commonly is overcome as measurements of PM at occupational settings differentiate between total and alveolar dust fractions.

Species specific differences in the pulmonary clearance of retained particles ($AF_{clearance}$) are described as

$$AF_{\text{clearance}-A/H} = \frac{V_{d-A} \times k_{e-A}}{V_{d-H} \times k_{e-H}} = \frac{V_{d-A} \times t_{1/2-H} \times \ln 2}{V_{d-H} \times t_{1/2-A} \times \ln 2} = \frac{7 \times 10^{10} \times 400_H}{50 \times 10^{10} \times 60_R} = 0.93 \quad (9)$$

where $V_d$ = pulmonary volume of distribution of PM retained in alveolar macrophages, μl, $t_{1/2}$ = retention half-time of PM from the lung, days. Note: retention half-times in rats (*A*) were calculated utilizing a first-order kinetics following repeated inhalation exposures. Retention half-times of humans utilized a two-exponential kinetic model following one single inhalation exposure.

The toxicokinetic normalization factor $AF_{clearance\text{-}A/H}$ has to be used reciprocal to the $AF_{lung\ burden\text{-}A/H}$. Thus, the $NO(A)EL_{HEC}$ for insoluble, approximately spherical agglomerated PM can be estimated as shown in Eq. (10):

$$NO(A)EL_{HEC} = NO(A)EL_A \times \frac{AF_{\text{lung burden}-A/H}}{AF_{\text{clearance}-A/H}} \times \frac{1}{AF_{\text{study duration}}} \approx NO(A)EL_A \times 1 \quad (10)$$

where $AF_{study\ duration}$ = the respective assessment factors required to adjust for the actual duration of study to the attainment of steady-state following chronic exposure can be derived from Fig. 2. The respective AFs are for sub-chronic (13 weeks) to chronic, subacute (4 weeks) to chronic, and subacute to sub-chronic 1.5, 3.4, and 2.4, respectively.

Taking the key features of interspecies alveolar dosimetry and mechanism-driven biokinetics into account, the basic principles of particle biokinetics in rats and humans appear to be remarkably similar, justifying an interspecies adjustment factor of ≈1. The objective of the HEC estimation is to prevent overloading-like conditions to occur at the attainment of steady state. As evident from Fig. 2, Eq. (10) must be adjusted for the duration of key study which serves as basis for the $NO(A)EL_A$ estimation. This means for a 90-day inhalation study, this requires a time-adjustment factor of 1.5 to attain steady-state. Accordingly, the $NO(A)EL_A$ from subchronic inhalation studies must be divided by the respective factor to derive the $NO(A)EL_{HEC}$ which, in this particular circumstance, is equivalent to the occupational exposure level (OEL). In chronic 2-year inhalation studies pulmonary steady-state is attained during the course of study (Fig. 3), therefore, further time-adjustments can be omitted. It has to be borne in mind that chronic phenotypes cannot necessarily be manifested during study periods as short as 4 weeks. However, this approach implicitly assumes postexposure periods of at least one retention half-time. Such a time period is considered adequate to manifest non-neoplastic, chronic types of lung disease. Thus, the

volume-based generic mass concentration of 0.54 μl $PM_{resp} \times \rho/m^3$ appears to be scientifically justified and defensible as a generic OEL for preventing PM-induced pulmonary overload-like conditions to occur in workers.

Intraspecies adjustments are not deemed to be required for insoluble and chemically non-reactive PM due to the lack of any significant systemic bioavailability at non-overloading lung burdens. At occupational settings this type of portal-of-entry related toxicity (as a result of particle overload) is independent on any local metabolism. Furthermore, the cornerstones of particle clearance appear to be well conserved in mammalian species and do not call for any specific rat-to-human adjustments. Indeed, changes of biokinetic parameters and deposition patterns can be expected in humans inflicted with severe pulmonary disease. However, the type of population addressed in this appraisal is not believed to fall into this category.

## 4. Conclusions

In regard to particle-related pulmonary toxicity diverse views have been articulated over the past decades on the most critical physical/physicochemical property determining the biopersistence and ensuing cumulative dose of deposited nano- and/or micrometer-sized particles in the lung (Maynard, 2007). This also includes the lead mechanism to initiate and sustain pulmonary inflammation and possible chronic sequelae thereof under conditions of various degrees of lung overload obtained in experimental rat models (Brown et al., 2005; Donaldson et al., 2008; Madl and Pinkerton, 2009; Oberdörster et al., 2007). The experimental evidence obtained in the most sensitive bioassay (rat) with granular biopersistent particles supports the view that the prevention of overload-like conditions may also prevent secondary long-term effects to occur (ILSI, 2000). The emphasis of this analysis was to follow these expert deliberations and to relate the empirically determined with the modeled cumulative lung particle dose as follows: (1) to categorize objectively the magnitude of lung overload and whether the state of overload is yet physiological or decompensated, (2) whether the 1 μl PM-volume/lung threshold dose is preceding intraluminal pulmonary inflammation (influx of neutrophils characterized by bronchoalveolar lavage), and (3) whether any kinetic relationship can be identified to better predict the NO(A)EL and MTD leading to a decompensated lung overload at the outset of repeated exposure inhalation studies.

Along with the objective to further refine and extend a past derivation of an OEL for MWCNT (Pauluhn, 2010b), attempts were made to identify common mechanistic denominators between higher- and lower-than-unit density, biopersistent nanosized and submicronsized insoluble PM. Based on the results of this analysis, it appears that the potency of PM to induce inflammation-related sustained lung injury is solely dependent on biokinetic rather than PM-inherent properties. The derivation of assessment factors took into account the spirit of current European regulations (ECHA, 2008c,d) as well as those promulgated by US-EPA (2002). The major differences of these two approaches appear to reside in how to deal with the "respiratory system", that is to say that it is dealt with in its entirety (ECHA) or logically stratified into the "nasopharyngeal", "tracheobronchial", and "pulmonary (alveolar)" regions (US-EPA). Nonetheless, the REACH regulation gives preference to an expert judgment-based approach focusing on the substance-specific mode of action to estimate DNELs. Occupationally, poorly soluble particulates are commonly regulated as one class with similar OELs. The outcome of this analysis is concordant with this approach; however, it would support to be using the aggregate PM-volume as the key unifying denominator.

Published evidence suggests that repeated exposure inhalation studies on rats represent the most sensitive bioassays in regard to granular biopersistent particulate matter. This analysis suggests that the prevention of any overload-like condition may also prevent adverse effects to occur from secondary inflammatory responses or long-term sequelae. This conclusion matches the deliberations of expert groups convened to address PM-related chronic toxicity (ILSI, 2000). A volume-based generic concentration of 0.54 μl $PM_{resp}/m^3$ is considered to represent a defensible OEL based on both generic theoretical considerations as well as empirical evidence. Related mass concentrations can readily be calculated by multiplication of the volume concentration with the PM-agglomerate density ($\rho$) (mass concentration-mg/$m^3$ = 0.54 μl $PM_{resp}/m^3 \times \rho$). Comparative analysis of volumetric lung burdens with ensuing decrease in PM-clearance and increased inflammation support the conclusion that repeated inhalation studies on rats should utilize an experimental window of cumulative volume concentrations between 1 and 10 μl PM/lung. This range can be targeted best by the modeling of particle deposition and lung retention biokinetics. Inhalation studies exceeding that upper-volume limit may generate meaningless findings with no relevance any real-life scenario. This type of decompensated overload expected to occur at this cumulative lung burden may produce free particles and deterioration in lung function that has no physiologic counterpart at the lower end of the cumulative dose-response curve. Likewise, using modeling procedures for dose selection to arrive at well rationalized targeted retention half-times not exceeding 1 year provides a means to better compare findings across different laboratories and to characterize the toxic properties of insoluble nano- and submicronsized PM in the future using simplified but better targeted testing protocols with less experimental animals.

## Conflict of interest

There are none.

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