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RESEARCH PAPER

Human health no-effect levels of TiO_2 nanoparticles as a function of their primary size

Alexis Laurent · Jack R. Harkema · Elisabeth W. Andersen · Mikołaj Owsianiak · Eldbjørg B. Vea · Olivier Jolliet

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Abstract As engineered nanomaterials are increasingly introduced on the market into a broad range of commodities or nanoproducts, there is a need for operational, reliable tool, enabling to consistently assess the risks and impacts associated with the releases of nanoparticles. The lack of a developed metric that accurately represents their toxic effects while capturing the influence of the most relevant physicochemical properties is one of the major impediments. Here, we investigate the relationships between the toxic responses of nano-sized and micro-sized particles in in vivo toxicological studies and their physicochemical properties. Our results for TiO_2 particles indicate statistically significant associations between the primary particle size and their toxicity responses for combined inhalation and ingestion exposure routes, although the numerical values should be considered with care due to the inability to encompass influences from other relevant physicochemical properties like surface coatings. These findings allow for expressing mass-based adverse effect levels as a continuous function of the primary size of particles. This meaningful, exploratory metric can thus be used for screening purposes and pave the way for reaching adaptive, robust risk assessments of nanomaterials, e.g. for setting up consistent threshold levels, as well as consistent life cycle assessments of nanoproducts. We provide examples of such applications.

Keywords Titanium dioxide · Particle size · Toxicity · Nanotoxicology · Risk assessment · Life cycle assessment · Environmental and safety effects

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A. Laurent (✉) · M. Owsianiak · E. B. Vea
Division for Quantitative Sustainability Assessment, Department of Management Engineering, Technical University of Denmark, 2800 Kgs, Lyngby, Denmark
e-mail: alau@dtu.dk

J. R. Harkema
Department of Pathobiology and Diagnostic Investigation, College of Veterinary Medicine, Michigan State University, East Lansing, MI, USA

E. W. Andersen
Statistics and Data Analysis Section, Department of Applied Mathematics and Computer Science, Technical University of Denmark, 2800 Kgs, Lyngby, Denmark

O. Jolliet
Department of Environmental Health Sciences, School of Public Health, University of Michigan, Ann Arbor, MI, USA

Introduction

The commercialization of engineered nanomaterials has dramatically increased over the past years (Hendren et al. 2011; Keller et al. 2013; Mitrano et al. 2015). Simultaneously, the potential releases of nanoparticles and their consequent risks and impacts along the life cycle of nanoproducts (products embedding nanomaterials) have been raised in many studies (Grieger et al. 2012; Maynard et al. 2006; Nel et al. 2006; Oberdörster 2010; Oberdörster et al. 2005;

SCENIHR 2009; Stone et al. 2010a; Wiesner et al. 2006). Several works have thus attempted to perform human and ecological risk assessments of several nanomaterials, e.g. nano-scale titanium dioxide (Christensen et al. 2011; US-EPA 2010; Warheit 2013). Likewise, a number of studies have performed life cycle assessments of nanoproducts, quantifying the environmental impacts from their manufacture, use and disposal stages (e.g. Walser et al. 2011). However, these attempts do not gather sufficient robustness and reliability to allow for conclusive assessments of the risks and impacts stemming from the released nanoparticles because of difficulties in estimating their actual emissions and in identifying, tracking and evaluating the many parameters influencing their fate, transport and toxicity (Aschberger et al. 2011; Jolliet et al. 2014; Savolainen et al. 2010; Warheit 2013).

To support the evaluation of the health effects, a large number of toxicological studies have been conducted (see review by Krug 2014). Several reviews have been published over the years to synthesize current knowledge and give overviews of the toxic effects of fine particles and/or nanoparticles (e.g. see non-exhaustive list in Supplementary Methods; Krug 2014). Very few of these have performed comprehensive, quantitative analyses of the findings to identify possible common patterns. Most works provide thorough snapshots of existing studies at a given time, but limit their analyses to qualitative discussions. Among the most comprehensive ones, the study by Krug (2014) has thus analysed general trends observed over more than 10,000 publications and showed that, despite the sheer number of studies, a number of challenges still remains in their interpretation, particularly due to a lack of comparability across studies and a widespread omission of consistent characterization of the nanoparticles (Krug 2014). The generally poor reporting of physicochemical properties known to influence the toxicity of nanoparticles has often been raised (e.g. Clark et al. 2012; Krug 2014). Among the relevant physicochemical properties, the primary particle size, shape, specific surface area, surface chemistry and reactivity, composition, coating composition, crystallinity, charge, solubility and state of agglomeration and aggregation have been flagged as the most important (e.g. Maynard and Aitken 2007; MINChar 2008; Landsiedel et al. 2010; Oberdörster 2010; Stone et al. 2010a). The primary particle size, one of the most studied properties, has been demonstrated to significantly contribute to the toxic effects (Oberdörster et al. 2005). This relationship indicates that the mass of particles alone cannot be a sufficient metric to characterize their toxic effects since the intake (i.e. amount of nanoparticle entering the body) of the same mass of particles of different sizes may result in different toxic effects (Oberdörster et al. 2005). To date, there is still a need to better characterize the effects of nanoparticles on human health once they are inhaled or ingested as a function of their physicochemical properties (Jolliet et al. 2014).

In this study, we propose a methodology to quantitatively investigate the relationships between the non-carcinogenic effects of nano-sized and micro-sized particles and selected physicochemical properties so that it can ultimately serve as a support for risk assessment and life cycle assessment of nanomaterials and nanoproducts. We focus on nano-scale titanium dioxide (TiO_2), which is among the mostly used nanomaterials on the market and one of the mostly investigated in toxicological studies (Hendren et al. 2011; Keller et al. 2013; Mitrano et al. 2015). We specifically aim to (i) review the experimental settings and findings of all available in vivo studies published on this material that met selection criteria with respect to exposure routes, exposure time and observed toxic endpoints; (ii) analyse ways to investigate relationships between non-carcinogenic effects of nanoparticles and selected physicochemical properties, (iii) explore the derivation and application of no-observed adverse effect levels (NOAELs) for nano-sized and micro-sized particles to be used in risk assessment and life cycle assessment.

Methodology

Overview of the methodology

The overall methodology consists of a 6-step approach, which includes (1) identification and selection of in vivo studies, (2) characterization of particles with respect to their reported physicochemical properties, (3) expression of the doses into "intake doses" to allow comparisons across exposure routes, (4) review of the displayed toxicity responses for each experiment using information on the endpoints reflecting adverse effects resulting from exposure to the nanoparticle, (5) statistical analyses of the relationships between the reported physicochemical properties and the incidence or absence of adverse effects, and (6) extrapolations to human

equivalent doses. Each of these steps is succinctly described in the following subsections.

In the following, the term 'experiment' refers to a test on a given species exposed to a specific type of particles (in terms of sizes, surface treatment and crystal form—see below section on particle characterization). An experiment may include several exposure levels (i.e. different doses). The term 'study' refers to a set of experiments performed by the same research group and may include different particle types, exposure pathways and test animals.

Identification, selection and classification of studies

The identification of in vivo studies was done through literature search engine and (see Supplementary Methods) complemented by the cross-checking of citing and cited literature as well as studies cited in review papers in the field of nanotoxicology. Complete documentation of those steps is available in Supplementary Methods.

In vitro studies were disregarded because they relate to acute toxicity and methods to use them for predicting chronic (i.e. long-term); *in vivo* toxicity are yet undeveloped (Oberdörster 2010). Ways of incorporating the large pool of data stemming from them should however be better investigated (Oberdörster 2010; Krug 2014). Out of the retrieved in vivo studies, filtering criteria were applied to retain (i) studies with sufficiently long exposure durations, (ii) studies for which the monitored toxic endpoints are comparable with other studies, (iii) studies, for which reporting contains sufficient information for particle characterization and analysis of the results, and (iv) studies addressing oral and inhalation exposure pathways, which are both considered the most relevant for risk assessment and life cycle assessment applications. For the latter, because no consensus currently exist on the correspondence between inhalation and intratracheal instillation studies, the intratracheal instillation tests, in which the particles are directly administered into the lower part of the respiratory tract of the animal under anaesthesia, were disregarded in the current study (see, e.g. Aitken et al. 2009; Bakand et al. 2012; Driscoll et al. 1991, 2000; Warheit et al. 2005). However, further work should continue exploring the comparability of the results in the large body of intratracheal instillation experiments (>50 studies) with the findings from inhalation studies to bring additional data for interpretation (Krug 2014).

With respect to exposure durations, the retrieved studies were classified into four groups, i.e. acute, subacute, subchronic or semi-chronic, and chronic. The categorisation is strongly dependent on the species, e.g. maximum lifetime (Vermeire et al. 1999). Supplementary Methods (Table M3) show the categories and their associated definitions that are assumed for studies on rats, mice and hamsters. Acute studies, i.e. with a repeated exposure of less than seven consecutive days, were disregarded in the current study, as the overall aim is to investigate chronic toxicity.

Only studies with a comprehensive report of the toxicity responses were included. Biodistribution and dosimetry-based studies were not considered when they did not investigate possible incidence of adverse toxic effects. Studies, in which only morphological effects of exposure to micro-sized or nano-sized particles were observed (e.g. weight), were disregarded. In addition, genotoxicity tests were excluded because of the difficult comparability with other toxicological studies and their linkage to potential mutagenicity carcinogenic effects, which are considered outside the scope of the study (Koedrith et al. 2014). Only studies including investigations of non-cancer effects were considered (although studies investigating cancer effects are also reported in Tables S1 and S2). Furthermore, all tests performed on animal models of human susceptibility, e.g. pregnant mice (Gao et al. 2011; Warheit et al. 2015) were excluded. Finally, all tests with responses and/or doses and/or particle characterization that could not be quantified properly were excluded.

Particle properties available for statistical analysis

Over the past decade, the field of nanotoxicology has identified a relatively large number of physicochemical properties of the nanoparticles that are accountable for their toxic effects. From the literature, about 10 generic physicochemical properties are frequently reported as influential to the fate and health effects of the nanoparticles, i.e. the primary particle size (incl. size distribution), the shape (aspect ratio), the specific surface area, the surface chemistry/reactivity, the composition (incl. impurities), the coating composition (if any), the crystal structure, the charge, the solubility, the state of agglomeration (Zeta potential) and aggregation (Landsiedel et al. 2010; Maynard and Aitken 2007; MINChar 2008; Oberdörster 2010; Stone et al. 2010a, b).

A case-by-case approach is advised when addressing the behaviour of nanoparticles in the environment and their impacts on human health (and ecosystems)—see e.g. Stone et al. (2010a) and SCENIHR (2009). Therefore, not all properties will play the same role whether carbon nanotubes or nano-TiO_2 are studied, for example. One of the major problems to analyse the influence of these properties is the lack of comprehensive documentation in the experimental studies, which render the difficult find of patterns (Clark et al. 2012). Although it is not directly addressed in this study, another issue consists in the contrast between the properties of pristine nanoparticles, which are manufactured nanomaterials and are the focus of most toxicological studies, and those of the nanoparticles eventually embedded in consumer products and potentially released to the environment in their use or disposals (Nowack et al. 2012). Properties of the latter categories of particles (and their changeability after releases) are more relevant to human health impact and risk assessments (Nowack et al. 2012).

With respect to TiO_2 nanoparticles, a number of studies have highlighted the influence on the toxicity responses of several physicochemical properties, including the primary particle size (e.g. Oberdörster et al. 2005), the surface treatment, e.g. presence and type of coatings (e.g. Warheit et al. 2005), and the crystal form (e.g. Jiang et al. 2008). To comprehensively analyse their influences and dependencies, a sufficiently detailed documentation of these properties is required for the majority of the retained studies. Unfortunately, because of the paucity of data across the retained studies, the surface-related characteristics, e.g. coatings of pigmentary particles, could not be integrated. Therefore, only the primary particle size and the crystal form were analysed in relation to the toxic responses, an assumption that affects the numeric estimates. Further works should address those gaps.

For primary particle size, the values reported by the authors of the studies were considered as such, although some discrepancies might occur in their characterization across studies. It is noteworthy that in most studies, data about size distribution was missing or largely insufficient to allow for a comprehensive accounting of this aspect. When available, such information could however be useful to investigate the influence of the particle aggregation state on the potential toxicity of nanoparticles and should thus be encompassed in future studies comparable to the current one.

Expression of animal doses

For all included experiments, each tested dose and its associated responses were individually treated. A similar method as the one developed by Gold et al. (1984) (CPDB website at http://potency.berkeley.edu/methods.html) was applied. All reported doses were translated into average daily chronic dose rates expressed in a mass unit of the particle intake per day (d) and kg body weight (kg-bw) for ingestion and inhalation exposure—see Eqs. 1 and 2, respectively:

$$ID_{a,\mathrm{chronic}} = \frac{ID_{a,i} \times CF_{\mathrm{exp}}}{BW_a \times CF_i} \quad (1)$$

$$ID_{a,\mathrm{chronic}} = \frac{C_{a,i} \times IR_a \times CF_{\mathrm{exp}}}{BW_a \times CF_i} \quad (2)$$

where $ID_{a\,,\,chronic}$ is the average daily chronic intake dose (e.g. mg/kg-bw/day) for a given animal a; $ID_{a\,,\,i}$ is the daily ingested dose (e.g. mg/day) used in the test with animal a and exposure duration i; CF_{exp} is the correction factor for exposure time to translate the discontinuous regimen in animal test into an assumed continuous daily exposure used as target exposure (e.g. CF_{exp} = D/7 × H/24, with D = days of weekly exposure and H = hours of daily exposure); BW_a is the body weight (kg-bw) of animal a (reported in studies or default values taken from US-EPA (1988)); CF_i is the dimensionless correction factor for duration of the exposure i (subacute, subchronic, chronic; see below); $C_{a\,,\,i}$ is the concentrations (mg/m^3) used in the test with animal a and exposure duration i; IR_a is the inhalation rate of test animal a in m^3/d (reported in US-EPA 1988).

The correcting factors CF_i from Vermeire et al. (1999, 2001) were used to adjust the duration of the exposure, with values for subacute-to-chronic factor of 5 and subchronic-to-chronic ratio of 2. They are derived for oral NOAEL data but Vermeire et al. (2001) report their assumed applicability to systemic effects caused by inhalation or dermal exposures. These extrapolation factors were derived from investigating chemicals effects. In the absence of any data with regard to particles, it is assumed valid for the purpose of this study. Further work is needed to verify and/or refine that assumption.

It is noteworthy that the approach to express doses as intake doses for both exposure routes differs from that used in some other studies (e.g. Brown et al. 2005; Kuempel et al. 2006; Pauluhn 2011; Oller and

Oberdörster 2010). In these, the dose expression also encompasses some elements of absorption by the receiving body. For example, in particle inhalation studies, Jarabek et al. (2005), Kuempel et al. (2006) and Oller and Oberdörster (2010) include the deposited fractions of particles in the lungs, which also depend on the agglomeration/aggregation state and can be calculated via e.g. a multiple-path particle dosimetry model (e.g. Asgharian et al. 1995, 2001; Asgharian and Price 2007). In the current study, we intend to bring results from all exposure pathways on an equal basis. In practice, this can be done before or after absorption processes (e.g. after absorption from GI tract for ingestion route or from the depositions in the lungs). However, the absorption mechanisms are dependent on several parameters, including characteristics specific to both the particle type and the receiving animal/human body. Based on the often incomplete data available in the retrieved studies and the general lack of knowledge in the mechanisms governing particle absorptions, the determination of absorption fractions was disregarded for all considered exposure routes. All doses for inhalation and ingestion were therefore expressed as intake doses. The possibility to harmonize all doses as uptake doses, reflecting the amount of nanoparticles absorbed in the body via the lungs or the gastrointestinal tract, should however be explored in further studies, as it is deemed more consistent.

Review of observed toxic responses

As the toxic endpoints vary considerably across the selected studies, the observed toxic responses were reviewed for each single dose tested with focus on ensuring comparability and harmonization across the studies. General selection criteria were thus defined, including (i) evaluation of the incidence of adverse effects and not their severities, hence, disregarding post-exposure monitoring/recovery periods, which could yield different toxicity characterization in the data set but could difficultly be harmonized across studies (e.g. studies not addressing recovery vs. studies addressing it); (ii) evaluation of the toxic responses based on the stained sections and micrographs of exposed organs and tissues (e.g. incidence of necrosis) and/or the reported levels of serum biochemical values and haematological parameters (based on statistical significance when compared to controls), and the interpretation of histopathological findings reported by the authors of the studies; (iii) emphasis to identify actual adverse effects. With respect to the latter, the accumulation of macrophages was thus not deemed an adverse effect, because it was regarded as a defence mechanism, which could be triggered by other causes than the exposure to the (nano) particles. Many experiment results were analysed based on the reported incidence of necrosis or apoptosis, both indicative of induced inflammation. Chronic alveolar inflammation was considered an adverse effect for lung toxicity. Statistically different (from controls) levels of neutrophils (PMN) or some enzymes, e.g. aspartate transaminase (AST) or alanine transaminase (ALT), indicative of liver toxicity and injury, were also considered as markers of adverse effects. All toxic endpoints were considered equally in this review and were not differentiated in further analysis to allow retaining a sufficiently large pool of data. However, ways to account for their large diversities, and thus render the different doses (e.g. NOAELs), should be explored in future studies (ECHA 2017).

Each dose-specific experiment was thus flagged as either a NOAEL or a lowest-observed adverse effect level (LOAEL), or was not flagged if the experiment dose was lower (higher) than an already-flagged NOAEL (LOAEL) for the same experiment. Although tests within each single experiment were flagged as NOAEL or LOAEL (or not flagged), the data are in fact interval-censored, and all flagged tests should be distinguished according to three groups, i.e. (1) those with only an identified NOAEL (i.e. left-censored), (2) those with only an identified LOAEL (i.e. right-censored) and (3) those with both identified NOAEL and LOAEL in the same experiment (i.e. termed 'interval-censored NOAEL/LOAEL' in the following). Therefore, the level at which an adverse effect occurs lies between NOAELs and LOAELs of tests belonging to group 3 (hence 'interval censoring'), or lies above NOAELs of group 1 (how far above is unknown, hence 'right-censoring') or below LOAELs of group 2 (how far below is unknown, hence 'left-censoring'). In the reporting and analysis of the results, the distinction between the NOAELs of groups 1 and 3 as well as that between the LOAELs of groups 2 and 3 were made by considering either the whole set of NOAEL/LOAEL data or the data set limited to interval-censored NOAEL/LOAELs.

Regression analyses

Several parametric regression analyses were performed to investigate the relationships between the incidence of adverse effects and the primary size and the crystallinity of the TiO_2 particles: (i) a preliminary analysis of

variance (ANOVA), (ii) multiple linear regression analyses, and (iii) a regression analysis accounting for the censored nature of the data (i.e. differentiating left-censored, right-censored and interval-censored data). Statistical software from the R system, version 3.2.3 (R Core Team, Vienna, AT), and statistical software Stata, v. 13 (StataCorp LP, College Station, TX, USA) were used to perform these analyses.

Based on the review of the toxic responses (see section "Review of observed toxic responses"), ANOVA tests were carried out using the whole set of NOAEL/LOAEL data, testing the influence of the primary size of the particles, the exposure route and the type of toxicity response to explain TiO_2 toxicity. For these ANOVA tests, the particles in our data set were grouped into two size groups (nano-range, i.e. below 100 nm, and micro-range, above 100 nm).

Multiple linear regression (MLR) analyses were computed on the NOAEL and LOAEL identified through the review (see section "Review of observed toxic responses"), and encompassed the following numerical and categorical variables: (i) the primary size of the particles, (ii) the crystal form (relevant to TiO_2), (iii) the exposure route, (iv) the tested animal and (v) the type of toxicity response (NOAEL or LOAEL). The analyses were separately conducted on the entire set of data as well as on the data set limited to interval-censored NOAEL/LOAEL values (see section "Review of observed toxic responses"). In these regressions, the primary size of the particles was included as continuous variable. The generic model of the regression analysis describes NOAEL and can be expressed for an observation i with Eq. 3:

$$\log_{10}(NOAEL_i) = \alpha + \beta_{size}\ \log_{10}(d_i) + \beta_{species-route}\ I_{species-route} + \beta_{cryst-an}\ X_{cryst-an} + \varepsilon_i \quad (3)$$

with d the primary particle size, β_{size} the parameter expressing the slope for the dependence on primary particle size, $\beta_{species-route}$ the parameter for given species and exposure route, conditioned with the Boolean variable $I_{species-route}$ (0, 1), and $\beta_{cryst-an}$ the parameter for anatase crystal form, conditioned with the content of anatase $X_{cryst-an}$ (%) and ε_i expressing a normal distribution with mean 0. When only exposure route was considered as a variable (i.e. no species differentiation; see section "Relationships between toxic effects and primary particle size of TiO_2"), the parameter $\beta_{species-route}$ in Eq. 3 is substituted by β_{route}.

To integrate the censored nature of the data into the regression analysis, an additional parametric regression analysis of the censored data was conducted (Klein and Moeschberger 2003). Such type of models is commonly used for accelerated failure time modelling, and its explorative use here aims to test the relationships between the size variable and the absence or incidence of adverse effects defined as interval-censored, left-censored or right-censored data. The model expression for that censoring-based regression (CR) is the same as described in Eq. 3. In addition to testing the statistical significance of the variables, the tested model can also describe the point where adverse effects start occurring, i.e. virtually the upper achievable NOAEL.

The results of all regression tests were examined and interpreted based on the statistical significance of the parameters and the model as a whole (p values <0.05). In addition, multiple linear regression models were also validated using leave-one-out cross-validation procedure and characterized with the predictive squared correlation coefficient Q2.

Extrapolations to human equivalent doses

Equivalent human intake doses, i.e. NOAELs for humans, were extrapolated from average daily chronic intake doses for the selected animal for ingestion and inhalation exposure routes—see Eq. 4.

$$NOAEL\ {}^{ex}_{hum} = \frac{NOAEL\ {}^{ex}_{a}}{AF_a} \times BW_{hum} \quad (4)$$

With NOAEL $^{ex}_{hum}$ being the NOAEL expressed as the average daily intake dose for humans (in mg/day/person) for chronic exposure route *ex*; NOAEL $^{ex}_{a}$ the NOAEL expressed as the average daily intake dose for animal a and chronic exposure route *ex* (in mg/kg-bw/day); AF_a the interspecies allometric factor for animal a; and BW_{hum} the body weight (kg) of humans (70 kg; US-EPA 1988).

Interspecies extrapolation from animals to humans was performed by applying allometric factors (Gold et al. 1984; Jarabek et al. 2005; Rosenbaum et al. 2011; Vermeire et al. 1999, 2001; Vermeire et al. 1999). As defined by Vermeire et al. (2001), the interspecies factors include (i) a default distribution to account for variability in specific toxicokinetics and

toxicodynamics, and (ii) a default factor to account for systemic differences between species caused by differences in body size and related basal metabolic rate. Vermeire et al. (2001) report a geometric mean of the latter equal to 1, thus, reflecting the biological assumption that all species are equally sensitive. The interspecies allometric factors AF_a thus express the systemic differences between species after exposure. Three methods are commonly used to determine these factors, whether the extrapolations are based on body weight, surface area or caloric demand (Vermeire et al. 1999). In the current study, the recommendations of Vermeire et al. (1999, 2001), who indicate the preference of extrapolations based on calorific demands, were followed, with default values of AF_a equal to 4.1 (rats), 7.3 (mice) and 4.7 (hamster; own calculation). It is noteworthy that this is in contrast to some previous studies on nano-sized and micro-sized particles (e.g. Jarabek et al. 2005; Kuempel et al. 2006), where the allometric factor is defined by the ratios of body weights between humans and animals for systemic effects, or by the ratios of lung masses or lung surface areas between humans and animals for effects in the respiratory tract.

Results and discussion

Review results

The application of selection criteria to identify relevant in vivo studies for the review (see "Methodology" section) led to shortlisting a total of 181 collected studies in 209 scientific publications to a number of 21 retained in vivo studies addressing subacute, subchronic and chronic exposure to TiO_2 particles via ingestion and inhalation routes (32 scientific publications; see Tables S1 and S2 and Supplementary Methods). The retained data correspond to 60 different tests, in which 17 NOAELs and 26 LOAELs were identified (see Table 1). The range of particle sizes over the entire data set is 4–450 nm. The review details of these tests are documented in Tables S1 and S2 for ingestion and inhalation exposure routes, respectively.

Relationships between toxic effects and primary particle size of TiO_2

Unlike anticipated (see, e.g. Jiang et al. 2008), none of the statistical analyses of the correlation between the toxicity of TiO_2 particles and their physicochemical properties showed that the crystallinity of TiO_2 particles expressed a statistically significant influence on their toxicity when the primary particle size was also considered. A strong correlation was observed between the crystallinity and the primary size of the particles in the retained experiments (see Table S3), since small-sized particles were associated with higher proportions of anatase whereas larger-sized particles were dominantly in a rutile form. The crystallinity was therefore disregarded from further analysis in this study, although its relationship with toxicity of the particles should still be explored in future research (Jiang et al. 2008). In the following subsections, the analysis was therefore centred on studying the relationships between the primary size and the toxicity of the particles.

The overall trend illustrated in Fig. 1a suggests an influence of particle size when classifying the data set into two size groups (nano-range, i.e. below 100 nm, and micro-range, above 100 nm). The two-way ANOVA analysis indeed revealed statistically significant differences between the size groups, classified into absence/occurrence of adverse effects, i.e. NOAEL or LOAEL (see Fig. 1a; all data considered, regardless of their interval-censored nature) when all routes were combined ($F_{2,40} = 2.96$; p values of 0.039 and 0.27 for the effects of size and absence/occurrence of adverse effects, respectively). When only inhalation data are considered, both the size and the absence/occurrence of adverse effects become statistically significant ($F_{2,30} = 14.4$; $p < 0.005$ in both cases; see Fig. 1b). This suggests that in addition to the influence of the size, there might be some influence of the exposure route on nanoparticle toxicity. This influence was not found to be statistically significant in this ANOVA analysis probably due to a low number of data points.

Multiple linear regressions were performed to further refine the ANOVA test (see section "Regression analyses"). Table 2 provides the results for four analyses made on either the entire data set ($n = 43$) or the data set limited to interval-censored NOAEL/LOAEL ($n = 14$), with and without species differentiation. The considered variables are able to explain between 88 and 92% (adjusted R^2) of the data variability when restricting the dataset to the most reliable data with that interval-censored NOAEL/LOAEL. When considering the entire dataset, data variability increases, and the fraction explained is reduced to approximately 65%.

Table 1 Summary of reviewed in vivo studies with ranges of NOAEL and LOAEL for TiO_2 particles

Exposure routes	Number of studies (papers)[a]	Number of tests[a]	Number of left-censored NOAEL data	Number of right-censored LOAEL data	Number of interval-censored NOAEL/LOAEL data	NOAEL (LOAEL) ranges (mg/kg-bw/d)
Ingestion	6 (6)	15	3	5	1/1	40–24,000 (8–1000)
Inhalation	15 (26)	45	7	14	6/6	0.0836–4.05 (0.0171–10.5)
Total retrieved	21 (32)	60	10	19	7/7	–

[a] Studies refer to a set of experiments performed by the same research group. The results of a study are sometimes disseminated in several papers, hence, the higher number of papers than that of studies. Tests refer to experiments conducted on a given species exposed to a specific type of particles with a specific exposure level

Effect of particle size Statistically significant associations are observed between NOAEL and size-variable for the restricted interval-censored data set, with significant (i.e. below 0.05) p values of 0.004 (test with exposure route differentiation) and 0.001 (test with both species and exposure route differentiation). When considering the entire dataset, the association with the size-variable is significant ($p = 0.009$) when only exposure route is differentiated and marginally significant ($p = 0.049$) when both route and species are differentiated. These results suggest a firm correlation between the primary size of the particle and its toxicity. Likewise, a marked differentiation between the absence and occurrence of adverse effects (i.e. NOAEL or LOAEL) is overall observed (see Table 2).

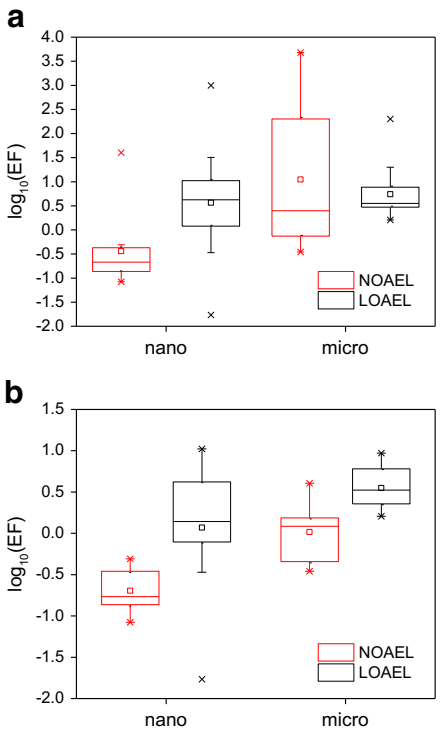

Fig. 1 Influence of the primary size on the occurrence of adverse toxic effect of TiO_2 particles in nano- and micro-sized ranges for (**a**) the entire data set, and (**b**) the data set restricted to the inhalation exposure route. EFs indicated on the *y*-axis are either NOAEL or LOAEL data points (adjusted to average daily chronic intake doses in mg/kg-bw/d; see Table 1). *Boxes* indicate the 25th and 75th percentiles, *square and horizontal lines within the boxes* indicate mean and median, respectively. *Whiskers* indicate inner and outer fence values assuming a default coefficient of 1.5, so that data points outside the fence values (in our study one data point) are considered outliers. Crosses indicate 25th and 95th percentiles

The best estimate of the size parameter slope β_{size} that expresses the increase in the $\log_{10}$(NOAEL) as a function of the $\log_{10}$(particle size) varies between 0.46 and 0.87 (see Table 2). This supports the observations that toxic effects continuously decrease as the primary particle size increases. These results imply that an exposure to TiO_2 nanoparticles of 10-nm primary size would lead to toxic effects approximately 2.9–7.5 times higher than the same exposure to TiO_2 particles of 100-nm primary size (range of 1–19 when considering the positive 95% CIs reported in Table 2). This finding, especially the numerical estimates, should be considered with care because physicochemical properties other than the particle size and that are not investigated in the present study might significantly alter the reported trends. For example, the surface coatings is known to significantly influence the toxicity of the nanoparticles, and even though our review disregarded coated nanomaterials, the tested particles may still happen to be doped and/or affected by the test media, as these aspects are not always monitored and reported transparently in toxicological studies (Clark et al. 2012; Warheit et al. 2005; Yang et al. 2014).

Table 2 Results of MLR analysis for inhalation and ingestion exposure to TiO_2 particles

Parameter	Only interval-censored NOAEL/LOAEL (n = 14) Exposure route differentiation	Only interval-censored NOAEL/LOAEL (n = 14) Species and exposure route differentiation	All NOAEL/LOAEL data (n = 43) Exposure route differentiation	All NOAEL/LOAEL data (n = 43) Species and exposure route differentiation
Intercept	−1.80 (−2.69; −0.90)**	−2.31 (−3.32; 1.29)**	−1.20 (−2.00; −0.40)**	−1.234 (−2.02; −0.43)**
Log size	0.74 (0.30; 1.17)**	0.87 (0.47 1.28)**	0.57 (0.15; 0.99)**	0.46 (0.002; 0.91)**
Inhalation	0 (reference, see intercept)	NA	0 (reference, see intercept)	NA
Ingestion	1.76 (1.23; 2.30)**	NA	1.90 (1.42; 2.39)**	NA
Ingestion, mouse	NA	1.97 (1.37; 2.57)**	NA	2.08 (1.36; 2.80)**
Ingestion, rat	NA	NA	NA	2.53 (1.64; 3.43)**
Inhalation, mouse	NA	0.51 (0.04; 1.05)*	NA	0 (reference, see intercept)
Inhalation, rat	NA	0.141 (0.36; 0.64)	NA	0.47 (−0.13; 1.08)
Inhalation, hamster	NA	0 (reference, see intercept)	NA	0.21 (−0.64; 1.05)
LOAEL	0.73 (0.36; 1.09)**	0.72 (0.40; 1.04)**	0.31 (−0.12; 0.73)	0.21 (−0.25; 0.67)
Adj. R2	0.884	0.918	0.648	0.656
Q2 (LOO)	0.841	0.894	0.589	0.524
p value for model	1.47E-05**	5.55E-05**	1.41E-09**	2.79E-08**

NA: not applicable

Statistically significant results, assumed with $p < 0.05$, are indicated by asterisks '**'

Results with $0.05 < p < 0.1$ are indicated by '*'.

95% confidence intervals (95% CI) are provided in brackets.

This trend of a positive slope β_{size} can also be observed when performing regression analyses taking into account the censored nature of the data (i.e. Censoring-based regression—CR analysis; see section "Regression analyses"), where positive values of the slope β_{size} are obtained (0.80 and 0.41 for species-route differentiation and only route differentiation, respectively; see Tables S4 and S5). However, it should be noted that these CR tests, although deemed the most consistent when taking the entire set of available data, did not reveal any statistical significance with p values above 0.05 (Tables S4 and S5). A strong dependence on the inclusion and exclusion of data in this statistical test (data not shown) suggests that further attempts at the CR application should be made when larger and more consistent data sets become available.

Exposure route Figure 2 plots the size-differentiated NOAEL functions obtained from the results of the different regression analyses. As reflected by the highly significant coefficient for ingestion (versus inhalation) of close to 1.8–1.9 in both experiment only differentiating the exposure route, the NOAEL values are approximately 60–80 times lower for inhalation exposure than for ingestion. As also illustrated in Fig. 2, the variations between the estimates of the slope β size are strongly dependent on the data set considered and whether or not only interval-censored NOAEL/LOAEL data are considered (thus disregarding left- and right-censored NOAEL and LOAEL data points). Accounting for the entire data set (i.e. 43 data points) substantially extends the number of data, whereas the data set limited to interval-censored data (i.e. 14 data points) is deemed more accurate.

Species differentiation Differentiating the number of species in addition to the exposure route only slightly increases the adjusted R^2 for the restricted set limited to interval-censored NOAELs/LOAELs, whereas it does not bring any increase in adjusted R^2 when considering

the entire data set. With consideration to the restricted data set, for which there are little data for a relatively large number of variables, a regression analysis made with only a differentiation between exposure routes seems more appropriate.

Albeit their limitation to the case of TiO_2 nanoparticles, our findings thus provide two major advances in the assessment of the toxic effects of nanoparticles: (i) a quantitative measure of the association between toxic effects and primary sizes of nanoparticles; and (ii) an expression of toxic effects in a meaningful metric. Many studies have reported the different magnitudes of effects on animals following exposure to different sizes of nanoparticles or to either nano-scale or micro-scale particles, but none managed to quantify this difference for entire size ranges, thus providing continuity and allowing for useful predictions. Furthermore, the utilization of mass-based metrics alone have been demonstrated not to be valid for capturing the effects of nanoparticles, and other complementary metrics based on surface area or particle numbers have been proposed to account for the particle sizes (Oberdörster et al. 2005). The above findings advance towards the determination of a meaningful, operational metric to express exposure levels, even though it only relies on the study of the primary size and ignores potential influences of other physicochemical properties of nanoparticles. The mass-based exposure levels (translated into intake doses) are expressed as a function of the primary particle size, thus, implicitly accounting for the differences in the surface areas and particle numbers. Even for the inhalation pathway, in which the absorption is strongly dependent on the state of agglomeration and aggregation, aggregates of same sizes but with different primary sizes can lead to different toxic responses (Ferin et al. 1992), thus, indicating possible disaggregation mechanisms after intake and attesting the strong influence of the primary particle size in the toxic effects. This therefore supports our assertion that, given the current state of knowledge, the expression of NOAEL as a function of the primary size as illustrated in Fig. 2 can adequately capture the nano-scale-specific toxicity of TiO_2 particles while also allowing characterization of micro-sized particles, and thus address the metrics issue raised in earlier studies. Further research is however needed to explore how the inclusion of more physicochemical properties can refine this expression of the NOAEL and to what extent this finding applies to other nanoparticles.

Implications for assessing human health risks and impacts

Derivation of human NOAEL

A direct consequence of the aforementioned findings is the opportunity to determine NOAEL values for humans as function of the primary particle sizes. Based on the regression analyses made in Section "Relationships between toxic effects and primary particle size of TiO_2", the statistical results from the data set limited to the interval-censored NOAEL/LOAEL data that only distinguish between exposure routes without species differentiation were retained as basis for deriving the human NOAEL. These results presented high statistical significance for the different variable estimates. As reflected in Fig. S1, they also show conservative estimates once the animal data were converted into human-equivalent-exposure levels, compared to the use of the entire data set. Within the data-defined size range (4–450 nm), Eqs. 5 and 6 express these relationships for TiO_2 for both inhalation and ingestion routes, respectively (with d the primary size of the particles in nm; and $NOAEL_{hum}$ in mg/person/day).

$$\begin{aligned} \mathrm{NOAEL}_{hum}^{inh} &= 70 \left[10^{(-2.620 \; + \; 0.796 \; \times \; \log \; (d))} \right] \\ &= 1.680 \times 10^{-1} \times \; d^{0.796} \end{aligned} \tag{5}$$

$$\begin{aligned} \mathrm{NOAEL}_{hum}^{ing} &= 70 \left[10^{(-1.028 \; + \; 0.796 \; \times \; \log \; (d))} \right] \\ &= 6.567 \times \; d^{0.796} \end{aligned} \tag{6}$$

It should be noted that the conversion to human exposure levels was performed on the original data. Therefore, the run of new regression analyses was required to obtain the parameter estimates, although the results are very similar to those reported in Table 2 (i.e. statistical significance observed for all parameters and slope changed from 0.74 to 0.80; see Table S6). Because of the large uncertainties inherent to the determination of Eqs. 5 and 6 (see "Methodology" section and regression result analysis in Section "Relationships between toxic effects and primary particle size of TiO_2"), the above equations are not intended to model and predict human

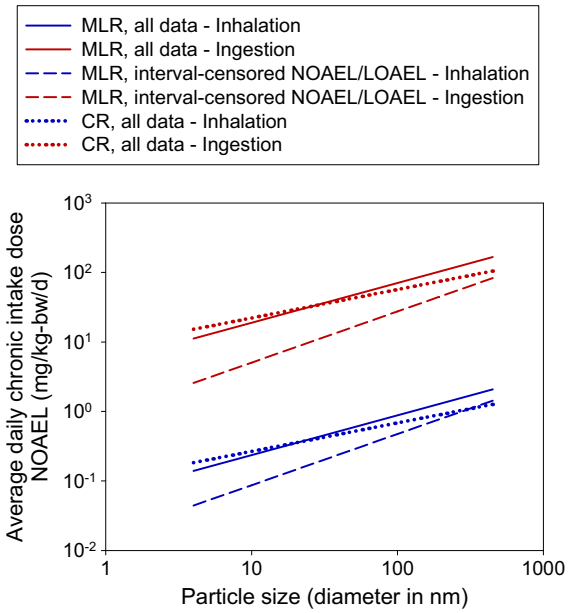

Fig. 2 NOAEL resulting from regression analyses for inhalation and ingestion exposure to TiO_2 particles (size range: 4–450 nm). Inclusion of different data sets (all data or interval-censored NOAEL/LOAEL only) and regression tests (multiple linear regression, MLR, or censoring-based regression, CR) differentiate the results from the regression tests, i.e. parameter values in Eq. 4, and hence the different curves. Interval-censored regression data accounting for the censored nature of the data are only presented for indicative purposes as no statistically significant slope for the size was observed (see Tables S4 and S5)

NOAELs as a default method. However, they are believed to present a useful and complementary approach to existing approaches encompassing reviews and selections of specific toxicological test results (e.g. Christensen et al. 2011) and are deemed relevant for screening purposes in the evaluation of risks and impacts of TiO_2 nanoparticles (see following section "Possible use in RA").

Possible use in RA

A major concern about health effects of nanoparticle stems from potential occupational and consumer exposure (via inhalation and ingestion). As illustrated in Fig. 3, the findings of this study show a relatively good agreement with the exposure limits recommended by the National Institute of Occupational Safety and Health (NIOSH) once these are translated into intake doses (Supplementary Methods and NIOSH 2011). For inhalation of TiO_2 (blue curves), the discontinuous exposure NIOSH thresholds and ranges, which differentiate the nano-scale and the micro-scale domains based on particle archetypes (NIOSH 2011), closely follow the proposed continuous size-dependent NOAELs. When considering the data ranges of the present study (i.e. 4–450 nm), the current study tends to yield more conservative estimates in the lower nano-sized and micro-sized ranges for inhalation. Overall, these comparisons therefore suggest that the findings are consistent with existing recommendations (e.g. NIOSH 2011) and epidemiological observations (e.g. Boffetta et al. 2004), although adjustments in recommended exposure thresholds should be envisaged to integrate a continuous size dependency and more conservative estimations.

With regard to ingestion exposure, some concerns have emerged with the ingestion of TiO_2 as food additive (i.e. E171; primarily micro-sized). Although it could not establish an acceptable daily intake, the European Food Safety Authority (EFSA) Scientific Panel on Food Additives and Nutrient Sources recently highlighted a NOAEL of 2250 mg TiO_2/kg-bw/d obtained for rats exposed in a chronic study (103 weeks) to ingestion of E171 (EFSA 2016; NCI 1979). When translated into human-equivalent intake doses (see "Extrapolations to human equivalent doses" section), this resulted in a human NOAEL 1–2 orders of magnitude higher than our results for the same size range—see Fig. 3. While indicating the conservative nature of this NOAEL, this difference may be explained by the different properties of the food additive E171 and the TiO_2 nanoparticles tested in the other ingestion exposure studies supporting our results (e.g. rutile form, etc.; see Table S1; see also Yang et al. 2014). It is also noteworthy that in actual exposure situations, an important proportion of the particles would remain sorbed to the food matrix during the digestion process and may thus not be available for absorption through the gastrointestinal tract. In contrast, nanoparticles used in toxicological studies are typically not bound to any matrix, which may result in a higher absorption rate. Further investigation is therefore required to evaluate the actual toxic effects of nanoparticles present in consumer products (Nowack et al. 2012; EFSA 2016; Yang et al. 2014).

The dependence of the NOAEL on the primary size of the particles, including 95% confidence intervals, allows making refined, case-specific human health risk assessments, bringing more consistency to earlier attempts (Christensen et al. 2011; Kuempel et al. 2012; Som et al. 2013). Exposure situations, which are typically defined for a specific type of nanoparticles, can

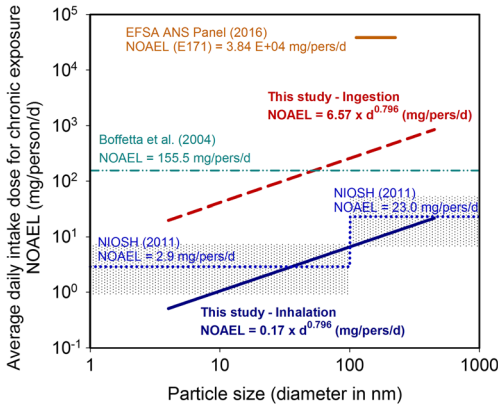

Fig. 3 Comparisons of NOAEL for humans for chronic inhalation and ingestion exposure to nano-sized and micro-sized TiO_2 particles (NOAEL expressed as average daily intake doses in mg/person/day), and assuming that coatings on pigmentary particles do not lead to artefacts (see section "Particle properties available for statistical analysis" in "Methodology"). The slope value for TiO_2 particles is 0.796 (95% CI: 0.43–1.16). External data were used to compare with the results from the current study (ingestion: NOAEL value for E171 highlighted by EFSA ANS Panel (2016); inhalation: epidemiological study by Boffetta et al. (2004); inhalation: recommended thresholds by NIOSH (2011)). The hatched area illustrates the range of inhalation exposure levels corresponding to NOAELs for noncarcinogenic effects as identified by NIOSH (2011); the *dark-blue-dotted line* indicates the recommended NIOSH thresholds relating to cancer effects and regarded as default (NIOSH 2011). Background details pertaining to these graphs are documented in Table S6 and in Supplementary Methods

thus be compared to the human NOAEL obtained across a range of primary sizes. Table 3 illustrates the application of the approach to occupational exposures to TiO_2 nanoparticles. Derived margins of exposure are observed to be well below potential uncertainty factors of 100 or 1000 for occupational studies. It suggests that present TiO_2 exposure may be high for workers, although occupational risks may be mitigated by the use of respiratory protection (see Table 3).

Possible use in LCIA

In line with common practice in life cycle impact assessment (e.g. Rosenbaum et al. 2011), Eqs. 5 and 6 can also be used to calculate effective doses ED50, i.e. chronic doses causing an adverse effect probability of 50%, to allow the calculation of characterization factors for TiO_2 particles, see for example Ettrup et al. (2017).

Conclusions and outlook

By demonstrating that it is feasible to integrate physicochemical properties into the definition of NOAEL, our proposed approach and its application to TiO_2 nanoparticles, albeit limited due to the difficulties surrounding coatings, can provide support for risk assessment of nanomaterials and life cycle assessment of nanoproducts. Until more comprehensive occupational human exposure and response data become available, our work can aid check and/or develop risk and life cycle assessment guidelines to ensure low risk exposures for consumers and workers. We therefore regard this study as the first step towards making use of the already large and increasing body of toxicological studies on nanoparticles and thus enable more consistent risk assessments and life cycle assessments.

However, our study clearly reflected that more data are required to (i) refine the assumptions performed for translating and harmonizing the tested doses across different experimental settings (e.g. harmonizing the

Table 3 Illustrative application of the developed NOAEL approach for human health risk assessment of TiO_2 occupational exposure situations

Exposure situations	Average daily intake (mg/person/day)	$NOAEL_{up}$—intake (mg/person/day)	Margin of exposure
Collection of TiO_2 (manufacturing)—without respiratory protection	6.3E-2	1.2 (0.5–3.0)	19.3 (7.8–47.5)
Collection of TiO_2 (manufacturing)—with respiratory protection	3.4E-6	1.2 (0.5–3.0)	3.6E + 5 (1.4E + 5–8.8E + 5)
Bagging of TiO_2 (manufacturing)	3.2E-1	2.5 (0.7–8.7)	7.9 (2.3–27.0)

Background details pertaining to these results are documented in Supplementary Methods; 95% confidence intervals are given in brackets for NOAEL and resulting margins of exposure. Data for occupational exposure data extracted from Koivisto et al. (2012a, 2012b). Primary sizes of 12 and 30 nm were considered for collection and bagging of TiO_2, respectively (see details in Supplementary Methods and Eqs. 5 and 6)

diversity of toxic endpoints) and for deriving chronic NOAELs for humans (see "Methodology" sections and Supplementary Methods); (ii) match the tested particles with those that are present in consumer products or subject to worker exposure; and (iii) integrate in the proposed methodology more toxicological data and encompass more physicochemical properties. Increasing consistency in reporting practice for toxicological studies, as recommended by Clark et al. (2012), should allow for studying a larger set of relevant particle properties, e.g. surface properties like coatings. The present approach should also be applied to other relevant types of nanoparticles, like silica, silver or carbon-based nanoparticles, ultimately contributing to holistic appraisals of the risks and impacts of nanotechnologies.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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