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PREAMBLE

BACKGROUND

1. Following the replacement of Test Guideline 401 on “Acute Oral Toxicity” with Test Guidelines 420, 423 and 425 in 2001, the WNT14 (Working Group of the National Co-ordinators of the Test Guidelines Programme) in 2002 found it timely also to develop alternative Test Guidelines for the “Acute Inhalation Toxicity Study” (TG 403), applying similar biostatistical approaches. The WNT14 approved the proposal from the UK for development of a Test Guideline on “Acute Inhalation Toxicity – Fixed Concentration Procedure (FCP)” (draft TG 433) as an alternative offering reduction and refinement compared to TG 403. The draft was accompanied by a Guidance Document on acute inhalation toxicity testing that was initially called GD No. 39 (renamed 39A after the WNT16).

2. At the WNT16, a second proposal for an alternative test method on “Acute Inhalation Toxicity - Acute Toxic Class (ATC) Method”, also offering reduction and refinement to TG 403, was presented by Germany and was approved by the WNT to be added to the rolling work-plan. This draft TG 436 also came with a German draft Guidance Document (No. 39B).

3. The 1st version of TG 433 was circulated in November 2002 together with the draft Guidance Document 39A. The 1st versions of the draft TG 436 and GD 39B were circulated in December 2004. A considerable amount of comments was received on both drafts.

4. Prior to the WNT17 meeting in April 2005, the US proposed updating the existing TG 403 so that it would cover all regulatory requirements, in addition to addressing some reductions in the use of laboratory animals. The WNT17 meeting was unable to reach any decision on the way forward for these projects, especially considering that the new methods had been on the rolling work-plan as alternatives to TG 403 for several years.

THE MEETINGS

5. The 1st Expert Consultation on Acute Inhalation Toxicity was held at the Federal Institute for Risk Assessment (BfR) in Berlin on 22-24 February 2006. The main purpose of the meeting was to revise the alternative draft Test Guidelines 433 and 436 in accordance to the comments received from member countries in previous circulation rounds and to develop a strategy to finalize the draft TGs as quickly as possible. Another important task of the meeting was to harmonize the alternative acute Test Guidelines with the newly proposed revised TG 403. The meeting was also asked to discuss the scope of the Guidance Document and whether it should be constructed to encapsulate the essence of all three draft acute Test Guidelines.

6. The Berlin meeting successfully addressed a multitude of issues and in general the discussions were constructive and focused on solving problems and harmonizing the three draft acute TGs to the extent possible. Since it was not possible to reach consensus at the meeting on the validation status and overall performance of draft TGs 433 and 436, a Performance Assessment Group (PAG) lead by Germany was established to assess the performance of the methods by biostatistical evaluations and simulations. Also a Guidance Document Drafting Group (GDDG) was established to merge the two draft GDs.

7. The GDDG had a meeting in Berlin at BfR, in June 2006, to discuss the merging of the GDs and to develop a work plan for future activities.
8. A second Expert Consultation Meeting was held at US EPA in Washington DC 7-9 November 2006. The main purpose of the meeting was to revise the existing TG 403 but issues regarding the alternative TGs 433 and 436, the PAG and the draft Guidance Document No. 39 were also addressed. The Test Guidelines were harmonized to the extent possible. The meeting agreed to preliminarily adopt the PAG report, to add the Netherlands’ C × t protocol to the draft TG 403 and to establish a C × t Performance Assessment Group (C × t PAG) that would execute a similar biostatistical analysis as was done for TGs 433 and 436. A number of issues remained unresolved, e.g., the use of evident clinical signs of toxicity and the use of one sex in TG 433.

9. The WNT19 generally agreed to the recommendations and the proposed work-plan by the expert meeting and endorsed the establishment of a C × t PAG. US EPA offered to host a final Expert Meeting in Washington DC in spring 2008 to resolve all remaining issues and come up with a final work plan for the finalization of the acute inhalation projects. To speed up the process with the C × t PAG, a statistician co-ordinated this work and reported to the Expert Group well ahead of the planned meeting in 2008. The strategy was overall approved by the WNT. Regarding the alternative methods, it became evident at the meeting that the supportive validation material for the draft TG 433 may not be satisfactory, and that actual testing or further analysis of the data may have to be done to be able to establish the performance of the TG 433 FCP for acute inhalation.

10. An Expert Consultation Meeting for revision of the Test Guidelines 403, 412 (28-day inhalation) and 413 (90-day inhalation) was held in De Bilt in the Netherlands on 18-19 June 2007. Because revisions to TGs 412 and 413 were added to the agenda, it was decided that GD 39 should be expanded to include inhalation studies of all durations, not just acute studies. Regarding the TG 403, the goal of the meeting was to have a clear insight on the regulatory needs for both protocols (Traditional LC50 and C × t). Additional agreements on the goals for the performance assessment of the C × t protocol and on a work plan, including time table and task assignment for the statistical analysis were discussed. The complete Performance Assessment Report of the C × t was available on 14 March 2008.

11. An Expert Consultation Meeting was held at US EPA in Washington DC 15-17 April 2008 for resolving the last remaining issues and to discuss the C × t PAG Report, in particular. The meeting approved the C × t PAG Report with some changes to the conclusions, as outlined in the C × t PAG Report. The meeting further made changes to the draft TGs and GD 39, and after in-depth discussions and revisions of the TG 436 PAG report, it was finally approved by the meeting.

ACKNOWLEDGEMENT

12. Many experts have participated in the OECD meetings and working groups and the Secretariat would especially like to mention the experts that have been involved in the development and drafting of this document, the two acute Test Guidelines 403 and 436, revisions to TGs 412 and 413, and the two Performance assessment Reports for the TG 436 and the 403/ C × t, including:

Bruce Allen, consultant
Patricia M. Bittner, US CPSC
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Finis Cavender, USA
Ron Crosier, US Army
Philippa Edwards, HPA, UK
Robin Fielder, HPA, UK
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Peter Bos, RIVM Bilthoven, NL
Iris Camacho, US EPA
Robert Combes, FRAME, UK
Michael Delorme, DuPont, USA
Ernest Falke, US EPA
Armin Gamer, BASF, Germany
The Secretariat gratefully acknowledges these experts and others who have contributed to the inhalation project for their professional assistance and their indispensible contributions to the finalisation of this important Guidance Document.

Comment [GA1]: This section could be removed as well, to be determined. Always a risk that someone is missed, and the current list is quite outdated (names not necessarily matching affiliations anymore).

Comment [GM2]: To be updated
I. INTRODUCTION

1. In 1981, the OECD adopted Test Guideline 403 (TG 403)\(^1\), which describes how to perform a traditional acute inhalation LC\(_{50}\) study, and also Test Guideline 412 (TG 412; 28-day inhalation) and Test Guideline 413 (TG 413; 90-day inhalation). Since OECD Test Guidelines and Guidance Documents are periodically reviewed in the light of scientific progress and animal welfare considerations, the OECD adopted four test guidelines in 2009:
   - TG 403 – a revised TG that includes two protocols—a traditional LC\(_{50}\) protocol and a C \(\times t\) protocol
   - TG 436 – a new Acute Toxic Class (ATC) test guideline that uses fewer animals than TG 403 by applying serial steps and fixed target concentrations to rank test chemical toxicity for classification and labeling according to the United Nations Globally Harmonized System (GHS) of Classification and Labeling of Chemicals\(^{11}\).
   - TG 412 – a revised 28-day inhalation test guideline
   - TG 413 – a revised 90-day inhalation guideline

2. TG 436, which is an alternative to TG 403, is able to satisfy most regulatory needs for a range estimate for an LC\(_{50}\) and GHS categorization. TG 436 also uses considerably fewer animals than TG 403. Because TG 436 cannot satisfy all regulatory and scientific needs, TG 403 may be used. TG 403 contains both a traditional LC\(_{50}\) protocol and a concentration \(\times\) time (C \(\times t\)) protocol. Both protocols provide maximum flexibility to characterize the entire range of the concentration-mortality relationship so that it can satisfy a variety of regulatory needs\(^{17}\). The C \(\times t\) protocol of TG 403 can provide additional information which may be useful for certain purposes, such as the derivation of Acute Exposure Guideline Levels (AEGLs).

3. Also in 2009, Guidance Document (GD 39) was drafted and approved. GD 39 provides detailed information on the conduct of inhalation studies of all durations. In 2010, yet another Guidance Document was approved—GD 125—which provides histopathology guidance for TGs 412 and 413.

4. Another round of revisions commenced in 2014 with TG 412, TG 413, GD 39, and GD 125 being revised to accommodate the testing of nanomaterials and to reflect the evolving state of the science. These documents were approved in [INSERT YEAR]. Because of time constraints, acute TGs 403 and 436 were not included in this revision effort.

5. There are no chronic or carcinogenicity study Test Guidelines specifically for the inhalation route but the following may be used for any route of exposure, including inhalation:
   - TG 451 – Carcinogenicity Studies (adopted 12th May 1981)\(^{14}\)
   - TG 452 – Chronic Toxicity Studies (adopted 12th May 1981)\(^{15}\)
   - TG 453 – Combined Chronic Toxicity/Carcinogenicity Studies (adopted 12th May 1981)\(^{16}\)

These TGs recommend that TGs 412 and 413 and GD 39 should be consulted when designing chronic inhalation studies.

6. A glossary of acronyms and terms can be found in Appendix I.
II. PURPOSE

7. The purpose of this document is to assist the regulated community and regulators in selecting the most appropriate acute and repeated exposure inhalation TGs so that particular data requirements can be met while reducing animal usage and suffering. This Guidance Document contains additional information on the conduct and interpretation of studies performed using TG 403, TG 436, TG 412, and TG 413.

8. For some test chemicals, reliability may be significantly affected if it is difficult to achieve a specific stable target concentration, so elaborate pre-tests without animals may be needed to achieve a specific temporarily stable atmosphere concentration and particle size distribution. It can also be difficult to achieve equivalent chamber concentrations and particle size distributions in the pre-test, range-finding study, and main study. This can result in inconsistent responses in the animal studies. The test chemical concentration can determine which part(s) of the respiratory tract are most affected. For example, a low concentration of a highly water soluble gas or vapour may cause nasal irritation, but a high concentration may cause nasal irritation and also lung oedema (which may be fatal). Many test chemicals are generated in two phases (e.g., an equilibrium of aerosols from liquid/solid material and vapour). The method chosen to collect test atmospheres for the determination of analytical concentrations should adequately collect all phases of the test chemical. As the ratio of these phases varies with concentration, so too does the site of deposition and toxicity. Portal-of-entry physiological responses (such as reflex bradypnea) may alter test chemical uptake due to hyper- or hypoventilation and metabolism. This can result in greater or lesser toxicity and an increase in inter-animal variability. In principle, the selection of a test guideline is driven by regulatory needs. However, the numbers of variables associated with inhalation tests show that a science-based selection is required to generate meaningful and robust data in order to achieve the desired objectives. These aspects are described in detail in chapter 4.
III. DATA NEEDS

Triggers of Inhalation Toxicity Testing

9. Acute inhalation toxicity studies are the ideal means for characterizing acute inhalation hazards, but there are circumstances when requiring an inhalation toxicity study is not justified for humane, scientific, or practical reasons. Testing in GHS category 5 is generally discouraged and should only be considered when there is a strong likelihood that results of such a test would have direct relevance for protecting human health (see Appendix II). As a rule, testing should be done unless there are compelling reasons for not testing, such as:

- There is little or no significant human exposure to a test chemical by the inhalation route as it is produced, marketed or used.
- A test chemical has low volatility and is not aerosolized under conditions of use.
- A test chemical is too large to be inhaled (e.g., non-friable granules) or resistant to attrition (i.e., milling).
- An aerosol for an end-use product or application method may be considered essentially non-inhalable provided >99% of the particles by mass are >100 μm in diameter at the point where humans are exposed.
- A test chemical cannot be generated as a gas, vapour, or aerosol in sufficient concentration to elicit animal toxicity in the optimal conditions of an inhalation chamber.

Further guidance on the waiving of acute inhalation toxicity tests can be found in OECD GD... The decision to test or not test should be considered on a case-by-case basis using a weight-of-the-evidence approach. However, toxicity associated with effluents of thermolysis or combustion of products otherwise not inhalable may be subject to testing. Principles of such test procedures are detailed elsewhere (18).

10. In contrast, the repeated exposure Test Guidelines enable the characterization of adverse effects following repeated daily or 5-times per week inhalation exposure to a test chemical for at least 28 (TG 412) (12) or 90 (TG 413) (13) days (the latter covers approximately 10% of the lifespan of a rat). The data derived from these studies and especially the sub-chronic (90 days) inhalation toxicity study can be used for quantitative risk assessments and for the selection of test concentrations for chronic studies. The objective of these studies is to reveal target organs and sensitive non-lethal endpoints characterizing toxicity, including an analysis of the cumulative concentration-response/effect relationship. At the lower end is the no-observed-adverse-effect concentration (NOAEC) and at the upper end is the maximum tolerated concentration (MTC). The MTC should not affect longevity of the animal nor induce undue distress. The design of repeated inhalation studies precludes such effects to occur based on adequately designed range-finding studies which are dealt with in the respective TG.

Uses of the Inhalation Tests

12. Acute inhalation toxicity data are used to satisfy hazard classification and labeling requirements, to estimate the toxicity of mixtures, and to assess human health and environmental risk. The derivation of either a point estimate of the LC$_{50}$ value (using TG 403) or a range estimate of the LC$_{50}$ (using TG 436) (2) generally meets the acute inhalation toxicity regulatory requirements for classification and labeling of industrial chemicals, consumer products, and many pesticide applications. Acute inhalation toxicity studies can also characterize hazards associated with end-user products (e.g., biocides used indoors, multipurpose spray cans, aerosolized cleansing agents, incense...
insect repellents). Non-lethal endpoints representing the lower end of the concentration-response curve may be as useful as lethal endpoints. The data needs of the majority of OECD member countries can be met by testing at the *limit concentration* or the *maximum attainable concentration* (depending on the specific properties of the test chemical; see paragraphs 63-67). For highly volatile, low-toxicity test chemicals (e.g., refrigerants), testing beyond the *limit concentration* may be necessary to meet specific regulatory needs. For animal welfare reasons, testing in excess of the limit concentration (*i.e.*, in the GHS Class 5 ranges) is discouraged and should only be considered when there is a strong likelihood that results of such a test would have direct relevance for protecting human health (11).

13. Repeated exposure inhalation toxicity data are used to satisfy hazard characterization requirements that focus on a NOAEC or a BMCL. This value is deduced from empirical data characterizing the concentration-response/effect relationship of relevant endpoints. It denotes the level of exposure at which there is no biologically or statistically significant increase in the frequency or severity of any adverse effects in the exposed population when compared to an appropriate control. A statistically interpolated NOAEC, the Point of Departure, from this relationship can be obtained by benchmark analysis. Testing paradigms focus on the duplication of exposure regimens and atmospheres which have relevance to exposure patterns humans are likely to incur. Therefore, in these types of studies, the primary focus is not on the classification and labeling of substances/mixtures being commercialized but rather on characterization of toxic mechanisms and exposure atmospheres causing health hazards to repeatedly exposed humans. Chapter 6 of this Guidance Document addresses these aspects in greater detail.

**Definition of the Exposure Metric**

14. Acute inhalation toxicity studies should be based on mass concentrations to comply with the unit of an analytical standard curve used for the analytical method. Thus, gas, vapour, and aerosol concentrations are expressed using a mass per volume metric, such as mg/L or mg/m³, where the mass concentration is related to the test chemical and not to an arbitrarily selected analyte. This allows for a direct comparison of test chemicals regardless of their physical state.

**Conversion of Units of Exposure Concentrations**

15. Although gases are always tested in mass units (*e.g.*, mg/L or mg/m³), mass units may be converted to volumetric gas units (parts per million, abbreviated “ppm” or “ppmV”) under standard conditions to comply with specific regulatory needs such as the GHS Classification System. The following algorithms may be used to perform conversions at 22°C and 101 kPa atmospheric pressure, the recommended conditions for animal testing (see paragraph 90):

\[
\frac{mg/L \times 24,200}{MW} = ppm \quad \frac{mg/m^3 \times 24.20}{MW} = ppm
\]

\[
\frac{ppm \times MW}{24,200} = mg/L \quad \frac{ppm \times MW}{24.20} = mg/m^3
\]

MW = Molecular weight

16. These algorithms imply that 1 mole of gas at the specified temperature and pressure occupies
a defined molar volume of an ideal gas. Unlike mass units, volumetric gases units (e.g., ppm) vary with temperature and pressure. The use of volumetric gas units is complicated by their inconsistent application. For example, gas concentrations are reported at 0°C by gas producers, 20°C by GHS, and 25°C by Patty’s Toxicology Handbook (19). The conversion factors in the table below can be substituted in the conversion algorithms above to perform conversions at 20°C, 22°C, and 25°C. For further details see ‘Conversion of units’ in Appendix I.

<table>
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<tr>
<th>Temperature</th>
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<tr>
<td>20°C</td>
<td>24,050 mg/L</td>
</tr>
<tr>
<td>22°C</td>
<td>24,200 mg/L</td>
</tr>
<tr>
<td>25°C</td>
<td>24,450 mg/L</td>
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### Optimizing the Performance of the Test

17. Before considering inhalation testing, all available information on the test chemical, including existing studies whose data would support not doing additional testing, should be considered by the testing laboratory to minimize the animal usage and enhance the quality of the study. This may also yield information on the most appropriate species, strain, sex and mode of exposure. Key information may include the identity and chemical structure of the test chemical, its composition (for mixtures) and physico-chemical properties (e.g., vapour pressure), the results of any relevant toxicity tests on the test chemical, available (Q)SAR data and toxicological data on structurally related test chemicals, and the anticipated use(s) of the test chemical. For acute studies, a test chemical’s physical state affects classification because the GHS classification boundaries (11) are dissimilar for gases, vapours, and aerosols (see Appendix II). Knowledge of dustiness and particle size for solid test chemicals will allow for selection of the ideal testing approach and starting concentration that will enhance respirability (e.g., through the use of micronization). Factors that enhance potential human exposure due to physico-chemical properties or a specific use pattern need to be considered. In this context, testing in GHS Class 5 should only be considered when there is a strong likelihood that results of such a test would have direct relevance for protecting human health (11). While nose-only is the preferred mode of exposure in all of the Test Guidelines, special objectives of the study may be better achieved by using the whole-body mode of exposure. The use of other modes of exposure should be based on the focus of the study and should be justified in the study report.

### Data Bridging

18. Some national and international regulatory systems estimate the toxicity of a mixture (formulation) using weighted averages of the LC₅₀ point estimates for each component when actual data on the mixture are not available. The resulting calculated toxicity values are then used for hazard classification. Especially for mixtures, available information should be utilized as “bridging principles” which enable suppliers to derive a sound classification of mixtures with a minimum of experimental animals. A concentration-response curve is sometimes needed for extrapolation and reliable identification of hazard and risk posed by mixtures. At present, agreed approaches for estimating the toxicity of mixtures using range data are only accepted in the EU and in some other countries. However, the GHS agreed in 2007 that mixtures can be classified using either point or range estimates of the LC₅₀ of each component (11). Inhalation testing may be required if the percentages of components in the test chemical differ appreciably following aerosolization or evaporation due to dissimilar physico-chemical properties. Therefore, the test principles detailed in ‘Chapter V: Acute Test Guideline Selection’ should be observed carefully.
When testing simple mixtures (e.g., agrochemical preparations) of well characterized components, the Finney equation as defined by WHO (20) may be used to estimate an LC$_{50}$ (see equation below and Appendix I), provided these components produce additive acute toxicity and have parallel regression lines of probit against log-concentrations. The estimated LC$_{50}$ can then be verified or refuted by performing a TG 436 study or this information can be used as a starting point for testing in place of a range-finding study.

Alternatively, the acute toxicity estimate (ATE) of the GHS (chapter 3.1.3.) “Classification Criteria for Mixtures” can be applied (11). For mixtures, it is necessary to obtain or derive information that allows the criteria to be applied to the mixture (of different particle sizes) for the purpose of classification. The following equation is used to derive an ATE$_{mix}$ value:

$$\frac{100}{ATE_{mix}} = \sum_{i=1}^{n} \frac{C_i}{ATE_i}$$

where:

- $C_i$ = concentration of the ingredient $i$ of $n$ ingredients, and $i$ runs from 1 to $n$
- $ATE_i$ = Acute Toxicity Estimate of ingredient $i$

Any conversion from experimentally obtained acute toxicity range values (such as ranges obtained by using TG 436) to acute toxicity point estimates should be based on the GHS classification system (11)(Chapter 3.1.3., Table 3.1.2). See Appendix II.

Before existing inhalation toxicity study data can be used for bridging purposes, the quality of the exposure data and the consistency of animal data should be assessed. Common pitfalls include inappropriate methodologies to generate respirable aerosols or characterize exposure atmospheres. When data from several acute inhalation toxicity studies are available, scientific judgment should be used in selecting the study that was best performed and characterized.

Feasibility of Testing Mixtures

Because a limit test (described below and in Appendices II and III) is commonly used when testing mixtures (end-use products), preference should be given to using TG 436.

Evidence from Humans

For classification purposes, reliable epidemiological data and experience on the effects of substances on humans (e.g., occupational data, data from accident data bases) should be considered in the evaluation of human health hazards. Human data that are reliable and of good quality will generally have precedence over other data. Human data will not necessarily supersede well-conducted animal studies, but rather the human and animal studies should both be assessed for their quality, the robustness of their data, and the impact of potentially confounding factors. Human testing solely for hazard identification purposes is not acceptable.

Applicability of Test Methods for Testing Pharmaceuticals

Acute inhalation toxicity testing by TGs 403 and 436 may not necessarily be relevant for inhalation pharmaceuticals. The International Committee on Harmonization (ICH) specifies pharmaceutical methods. Study designs for special purpose-driven studies differ from current OECD acute toxicity Guidelines, which are primarily designed for comparative evaluation and
assessment of acute (lethal) toxic potency. These studies typically characterize pharmaceuticals with very low toxicity and thus may require test concentrations above the respective limit concentrations detailed in Appendix II.
IV. COMPARISON OF ACUTE TEST GUIDELINES: TG 403 AND TG 436

Outline of the Exposure Methodology

26. Acute inhalation toxicity is the total of adverse effects caused by a test chemical following a single, uninterrupted inhalation exposure of non-fasted healthy young adult animals over a short period of time (less than 24 hours) to an adequately generated and characterized test chemical atmosphere. The total of adverse effects is best described by cumulative mortality. A fixed duration exposure of 4 hours is generally recommended but shorter or longer exposure durations may be appropriate to meet specific objectives. The limiting duration for nose-only exposure for rats is generally 6 hours. If other species are used, shorter exposure durations may be indicated to prevent undue species-specific distress. When using species other than rats, justification for exposure durations other than 4 hours should be provided. An observation period of at least 14 days after exposure, recording of body weights at regular intervals, and the necropsy of all animals is recommended. Technological details are addressed in chapter 6 of this document. Some authorities prefer that end-use products sold to the public should be tested in a way that reflects most closely the anticipated exposure pattern. Also the selection of a vehicle should be based on these considerations. If acute inhalation testing of the test chemical was omitted due to a lack of likelihood of exposure, then testing of the mixture becomes mandatory if its content in the mixture exceeds 0.1%. The preferred mode of exposure is nose-only. This particular exposure mode allows for the testing of multiple exposure durations using the same exposure atmosphere in order to obtain a range of concentration x time (C × t) relationships (21)/(22). While nose-only is the preferred mode of exposure, special objectives of the study may be better achieved by using the whole-body mode of exposure (20).

27. This Guidance Document primarily describes studies performed in commonly used rodent species (generally the rat), but it may also be adapted for studies in non-rodent species. Animals should be randomly assigned to the experimental groups. Most animal suppliers do not indicate litter mates so the Guidelines do not call for randomizing animals from a single litter across exposure groups. Females should be nulliparous and non-pregnant. On the exposure day, animals should be young adults 8 to 12 weeks of age, and body weights for each sex should be within ±20% of the mean weight of all previously exposed animals at the same age. As the mean weight increases, respiratory minute volume will also increase, though not in a proportional manner.

28. The determination of acute inhalation toxicity is usually an initial step in the assessment and evaluation of the toxic characteristics of an inhaled test chemical, whether it is a gas, vapour, or aerosol (e.g., dust, mist, smoke, fume, fog, or smog). It provides information on health hazards likely to arise from short-term exposure by the inhalation route. An evaluation of acute toxicity data should include the relationship, if any, between the animals’ exposure to a specific test chemical chamber concentration and the incidence and severity of all abnormalities, including behavioral and clinical effects, the reversibility of observed effects, gross lesions, body weight changes, effects on mortality, and any other toxic effects. Elaborate technical measures are often taken to maximize exposure to the entire respiratory tract, and to assure temporal and spatial stability of exposure concentrations.

Prioritization of Test Guideline

29. When range estimates are sufficient for estimating acute inhalation toxicity, TG 436 should be given preference because this alternative method provides significant reductions in the number of animals used (for details see Appendix III). Conversely, the focus of TG 403 is on the analysis of the entire concentration-response relationship ranging from non-lethal to lethal outcomes in order to derive a median lethal concentration (i.e., LC₅₀), non-lethal threshold concentration (e.g., LC₀₁), and slope. The higher level of information provided by the two protocols in TG 403 should be judiciously
counterbalanced by the number of animals used to achieve this objective. Both TGs include a requirement to follow the OECD Guidance Document No. 19 on Humane Endpoints (24) which should reduce the overall suffering of animals used in acute toxicity studies and provide useful data for human risk characterization.

30. The selection of a Test Guideline is based upon a test chemical’s specific data requirements. TG 436 should be given preference if it is able to satisfy regulatory or scientific needs. Whenever the objective of the test is to perform a limit test or a test at the maximum attainable concentration with an anticipated non-lethal outcome, TG 436 should be used. If there is a regulatory or scientific requirement for an assessment of the concentration response relationship, with or without a detailed analysis of the C × t relationship, then TG 403 is the preferred approach.

31. A study director or principal investigator should consider the following scenarios when selecting a Test Guideline for a given test chemical.

**Existing Evidence**
- An attempt should be made to predict the outcome of a test by read-across/bridging/(Q)SAR procedures, especially for mixtures with components of known toxicity.
- If such a prediction can be made with high confidence, testing should start with one single point estimate (e.g., an estimated LC₅₀ or a limit concentration).
- If the assumption regarding the toxicity at the tested value is refuted, the test result can be used to define the starting point for a TG 436 study.

**Regulatory needs**
- Regulatory requirements should be consulted to determine if results obtained from a TG 436 study will be adequate.
- A TG 403 study should be performed if there is a regulatory/consumer protection need for a lethality point estimate (e.g., an LC₅₀ or LC₁₀), a concentration-response analysis, and/or sex susceptibility quantification.

**Test chemicals anticipated to be highly toxic**
- Some highly toxic test chemicals may pose a unique health hazard. If a test chemical is classified as GHS Category 1 or 2 in a TG 436 study, or if there is information that suggests it will likely be classified as Category 1 or 2, then consideration should be given to performing a TG 403 study so its toxicity can be further characterized.

**Test chemicals that are severely irritating or corrosive**
- Testing corrosive and/or irritating test chemicals at concentrations that are expected to cause severe pain and/or distress should be avoided to the extent possible. The corrosive/irritating potential should be appraised by expert judgment using such evidence as human and animal experience (e.g., from repeated exposure studies performed at non-corrosive/irritant concentrations), existing in vitro data, pH values, information from similar substances or any other pertinent data, for the purpose of investigating whether further testing can be waived. For specific regulatory needs (e.g., for emergency planning purposes), the TG 403 may be used for exposing animals to these materials because it provides the study director or principal investigator with control over the selection of target concentrations. The targeted
concentrations should not induce severe irritation or corrosive effects, but should be sufficient to extend the concentration-response curve to levels that reach the regulatory and scientific objective of the test. These concentrations should be selected on a case-by-case basis and justification for concentration selection should be provided (see paragraph 39).

Technical problems

- Technical problems may be encountered that make it impractical to perform a TG 436 study with its fixed concentrations. For example, if pre-testing (before animals are exposed) reveals difficulty in achieving the target chamber atmosphere concentration during then a TG 403 study should be performed. A TG 403 study is less affected by deviations from target concentrations because statistical analysis uses the analytical concentrations to which the animals were actually exposed.

Future changes in the GHS category bands

- Changes to GHS category bands in the future will require a reassessment of biometrical performance (target) of TG 436 studies. Such changes will not alter performance of TG 403 studies because the concentrations tested are not fixed to GHS cut-off values.

TG 403 Studies - Traditional LC₅₀ protocol or C × t protocol?

- If a fixed point estimate of lethality is needed (e.g., a 4 hr LC₅₀), the Traditional LC₅₀ protocol should be followed.
- If an estimate of the effect of time on concentration is needed, the C × t protocol should be followed.
- If information is needed on LC₁₀ or LC₀₁ values, a C × t protocol will provide better estimates than a Traditional LC₅₀ protocol (21)(22).
- It is the responsibility of the investigator in consultation with appropriate regulatory authorities to determine whether the desired objectives are better achieved with the Traditional LC₅₀ protocol or the C × t protocol.

Range-finding Studies

32. **TG 403**: A range-finding (sighting) study may be used to estimate test chemical potency, to identify sex differences in susceptibility, and to assist in selecting exposure concentration levels for the main study. A range-finding study using up to three animals/sex/concentration (for details see Appendix III) may be needed to choose an appropriate starting concentration for the main study and to minimize the number of animals used. It may be necessary to use three animals/sex to establish a sex difference. The feasibility of generating adequate test atmospheres should be assessed during technical pre-tests without animals. It is generally not necessary to perform a range-finding study if mortality data are available from a TG 436 study. When selecting the initial target concentration in a TG 403 study, the study director should consider the mortality patterns observed in any available TG 436 studies for both sexes and for all concentrations tested.

33. **TG 436**: This Guideline does not call for a range-finding study.

Main Studies

34. **TG 403**: This Guideline allows a study director or principal investigator to choose between two types of studies depending on regulatory and scientific needs: a Traditional LC₅₀ study or a C × t
study. In a Traditional LC50 study, 5 rats per sex and concentration are exposed in a stepwise procedure. The lowest selected concentration is expected to produce low levels of mortality, and the highest concentration is expected to be lethal to most of the animals. The C × t study tests a matrix of multiple concentrations and exposure durations (21)(22). Each exposure atmosphere can be used to obtain a range of concentration x time (C × t ) relationships by periodically placing and removing animals in a nose-only chamber for predetermined durations. For both study designs, testing should be performed in a single sex if one is known to be more susceptible. GHS toxicity classification with TG 403 is based on mortality and the derivation of a statistically obtained median lethal concentration (i.e., LC50), confidence interval, and slope. Other regulatory requirements may require estimation of additional lethal toxicity indices (e.g., LC01, LC10).

35. **TG 436**: Pre-specified fixed concentrations are used in the main study. Groups of 3 animals/sex (or 6 animals of the more susceptible sex) are simultaneously exposed in a stepwise manner, with the initial concentration being selected to produce mortality in some animals. Depending on the presence or absence of mortality, further groups of animals may be exposed at higher or lower fixed concentrations as set out in Annexes 1-3 of TG 436 until it is possible to unequivocally assign a GHS class to the test chemical. Because accuracy in achieving each target concentration is paramount to assure accurate classification and labeling, a technical pre-test without animals is mandatory. Although most studies will be 4 hours in duration, other exposure durations may be used to serve specific regulatory purposes.

**Information Provided by Each Test Guideline**

36. The results of tests conducted according to TG 403 and TG 436 allow a test chemical to be classified according to all the systems in current use, including the GHS Classification System. In addition:

- TG 436 provides a range estimate of the LC50 instead of a point estimate. The ranges, as defined by GHS classification cut-off values, are different for each physical state of the test chemical under test conditions (gas, vapour, aerosol) (see Appendix II).
- The Traditional LC50 protocol in TG 403 provides a point estimate of the LC50 value with confidence intervals when at least 3 data points (concentration levels) are available with finite probabilities of mortality. In case there are only two data points with mortality close to 0% and 100% available (i.e., a very steep concentration-mortality relationship), they can be used to estimate an “approximate LC50.” The approximate LC50 is defined as the geometric mean from these mortalities.
- The C × t protocol in TG 403 yields a matrix of data points for a range of concentrations and durations that can yield point estimates for a variety of durations. The C × t protocol works well when there are steep concentration-mortality relationships because a C × t study relies on both concentrations and durations rather than concentrations alone.

**Animal Welfare Considerations**

37. Ethical concern for the welfare of experimental animals includes the alleviation of stress and suffering. In addition to allowing for classification and labeling, acute inhalation toxicity studies may provide important information regarding potential hazards that may be associated with the use of consumer products (e.g., indoor biocides, multipurpose spray cans, aerosolized cleansing agents, insect repellent incense). To this end, the non-lethal endpoints at the lower end of the concentration-response curve might be as useful as lethal endpoints. Whenever this objective can be achieved by using alternative test methods, which use fewer animals, this approach should be taken.
38. Both TG 403 and TG 436 require that OECD Guidance Document No. 19 on Humane Endpoints (24) must be followed, which should reduce the overall suffering of animals used in acute inhalation toxicity testing. TG 403 uses a range-finding study to minimize the number of animals needed in a main study. TG 436 has stopping rules which limit the number of animals used in a test.

39. Animals showing severe and enduring signs of distress and pain should be humanely killed as described in OECD Guidance Document no. 19 (24). When exposing animals to a test chemical with corrosive or strong irritant properties, the targeted concentrations should not induce severe irritation/corrosive effects, but should be sufficient to extend the concentration-response curve to levels that reach the regulatory and scientific objective of the test. Test chemicals that are eye/skin irritants may also be respiratory tract irritants at high exposure concentrations. Due to markedly different methodological approaches, the results from eye/skin corrosivity tests may not be readily translated to inhalation exposure concentrations delivered over a specified time period. Therefore, corrosive test chemicals should be assessed and tested following expert judgment on a case-by-case basis.

Limitations of Particular Approaches

40. A performance assessment against actual data and statistical simulations identified areas where TG 436 may have outcomes which result in a more or less stringent classification than that based on the “true” LC50 value (as obtained by TG 403) due to the fact that the ranges are defined by GHS cut-off values. Comparative statistical analysis (25) demonstrates that a method that provides a range estimate of the LC50 is likely to perform poorly for chemicals with shallow concentration-response slopes. Some test chemicals cause delayed deaths (e.g., 5 days or more after exposure to the test chemical), which may have an impact on the practicality of conducting a study using TG 436. The finding of a delayed death may require additional lower concentration levels to be used or a study to be repeated. The GHS classification boundaries are not equidistant across classification classes, and they are inconsistent between gases, vapours, and aerosols (dusts and mists), so the required reliability/precision changes from one class to another. Therefore, scientific judgment is needed to decide which of the acute TGs will best achieve the objective of the test.

41. Unlike the TG 403 approach where point values are estimated by applying established statistical procedures to whatever analytical concentrations animals are exposed to, TG 436 studies require a greater measure of accuracy and consistency in chamber atmosphere because they depend solely on the outcome at the targeted exposure cut-off. This is why a technical pre-test without animals is required for TG 436 studies. Although this may be time-consuming and result in a protraction of the study, it is necessary to assure that the target concentration and particle size (for aerosols) are attained. Appendix III details the variation that should not be exceeded for the targeted point estimates used in TG 436 studies. A protracted study may both increase the day-to-day variability of testing and affect the body weights of pre-assigned animals. These factors are of less concern when using TG 403 because the incremental steps and the associated changes in the physical characteristics of exposure atmospheres are commonly smaller than the cut-off limits of classification boundaries (see Appendix II) and because statistical analysis uses the analytical concentrations. Nevertheless, technical pre-tests are recommended when performing a TG 403 study to maximize the likelihood of successful tests.

1 From UN GHS, chapter 3.2 (11). “In addition to classification for inhalation toxicity, if data are available that indicate that the mechanism of toxicity was corrosivity of the substance or mixture, certain authorities may also choose to label it as corrosive to the respiratory tract. Corrosion of the respiratory tract is defined by destruction of the respiratory tract tissue after a single, limited period of exposure analogous to skin corrosion; this includes destruction of the mucosa. The corrosivity evaluation could be based on expert judgment using such evidence as: human and animal experience, existing (in vitro) data, pH values, information from similar substances or any other pertinent data.”
42. Literature surveys of systemically acting test chemicals show that there is usually little difference in susceptibility between the sexes in oral acute toxicity studies (26). There is little useful information on relative sex sensitivity in acute inhalation studies. Sex-related differences in metabolic rates, body weights, and the body weight-related increase in ventilation can lead to a higher inhaled dose in males as compared to females of the same age. When there is a need to test both sexes, simultaneous testing of both sexes is recommended because it is difficult to exactly reproduce identical exposure atmospheres when testing is sequential, especially with aerosols.
VI. CONDUCT OF INHALATION STUDIES

ANIMAL SELECTION AND HUSBANDRY

Animal Selection and Assignment

43. Animals are randomly selected, marked for individual identification, and kept in their cages for at least 5 days prior to the start of the test to allow for acclimatization to laboratory conditions. Although several mammalian test species may be used, the preferred species is the rat. Usage of common laboratory strains is recommended. If another mammalian species is used, the tester should provide justification for its selection. On the first day of exposure, young adult rats should be 8-12 weeks old in acute studies or 6-8 weeks old in repeated exposure studies.

44. The time interval between single exposure treatment groups is determined by the onset, duration, and severity of toxic signs. Commencement of an exposure should be delayed until one is reasonably confident of the outcome of previously treated animals. The exposure of animals at the next lower or higher concentration should be based on previous experience and scientific judgment.

Animal Husbandry

45. Each animal should be assigned a unique identification number. A system is required to randomly assign animals to test and control groups. The animals generally should be group-caged by sex, but the number of animals per cage should not interfere with clear observation of each animal and should minimize losses due to cannibalism and fighting. The nature of a test chemical or toxic effects (e.g., morbidity, excitability) may indicate a need for individual caging to prevent cannibalism. Animals should be housed individually in whole-body inhalation chambers during exposure to aerosols to prevent ingestion of test chemical due to grooming of cage mates. For feeding, conventional and certified laboratory diets may be used with an unlimited supply of municipal drinking water.

KEY PRINCIPLES OF INHALATION TESTS

Selection of an Inhalation Chamber

46. A dynamic, validated inhalation system with suitable control of all inhalation chamber parameters is required for acute inhalation toxicity studies. Dynamic inhalation systems include nose-only chambers and whole-body chambers. Experimental animals may either be exposed whole-body (horizontal and vertical flow type chambers, small, medium, and large size chambers with laminar, circular or turbulent flow arrangements to enhance the homogeneity of inhalation chamber concentrations) or nose-only (in mixed-flow, directed-flow, or flow-past inhalation chambers) with positive, negative, or zero flow gradients across the animals’ breathing zones. Each arrangement may require specific considerations which are partially addressed in this document. Historical data should demonstrate that horizontal/vertical concentration gradients in the inhalation chamber and bias airflows which dilute breathing zone atmospheres do not occur to any appreciable extent.

47. The following should be considered when choosing an inhalation chamber: 1) reactivity of test chemical with humidity and/or ammonia, 2) temporal stability of test atmosphere (e.g., minimization of particle growth and coagulation/aggregation), 3) prevention of re-breathing of test atmospheres, and 4) measurements and/or collection of biological specimens during the course of exposure. The preferred mode of exposure is nose-only (which term includes head-only, nose-only, or snout-only) for
the following reasons:

- Exposure and/or uptake by any other route than inhalation (oral route via preening or dermal absorption) are minimized, especially when testing aerosols.
- Technician exposure from handling exposed animals is minimized.
- A minimum of test chemical is needed due to low chamber volume.
- High concentrations (e.g., limit concentrations) are readily achieved.
- The instability of test chemicals (e.g., reactivity with excreta or humidity) and test atmosphere in-homogeneity are of minimal concern.
- The time required to attain inhalation chamber equilibration ($t_{eq}$) is negligible relative to the duration of exposure and therefore not an issue.
- Adding or removing animal restraining tubes during exposure to a fixed steady state chamber concentration allows for multiple exposure durations in one single test (the $C \times t$ protocol, utilizing the same exposure concentrations for multiple exposure durations).
- The exposure of individual animals can be interrupted at any time during the course of exposure to avoid undue suffering of animals.
- Animals are readily accessible for specific physiological measurements (e.g., respiratory function, body temperature) or the collection of blood, if applicable.
- The pre-conditioning of air prior to entering the inhalation chamber (e.g., in order to eliminate ubiquitous environmental constituents such as ozone, nitrogen oxides, hydrocarbons, and particulates, or to allow testing under defined humidity or gas conditions) is technically less demanding with nose-only chambers than with larger whole-body inhalation chambers.

48. The principal advantages and disadvantages of nose-only vs. whole body exposure have been detailed elsewhere (23). An examination of the nose-only mode of exposure in repeated exposure studies showed that the mild immobilization stress of rodents in properly designed restraining tubes did not cause any exposure-associated differences in cardiovascular endpoints or respiration, though these animals had no access to food or water during the exposure period. (27). The design of animal restrainers may differ from one laboratory to another. When atypical restraining-tube designs are used, test laboratories should demonstrate that they do not cause undue stress to exposed animals (see also paragraph 50). While nose-only is the preferred mode of exposure, special objectives of the study may give preference to the whole-body mode of exposure. The use of other modes of exposure should be based on the focus of the study and should be justified in the study report.

49. In directed-flow (flow-past) nose-only inhalation chambers, the inhalation exposure air flow and the exhalation flow are separated so the exhaled air from one rat cannot be inhaled by another. Directed-flow chambers are preferable to chambers of small volume using a mixed-flow operation principle (28)(29) in which the inhalation exposure air flow and the exhalation air flow can mix and be re-breathed. When an animal is confined to a restraining tube the observation of its behavior and physical condition is somewhat restricted. Subtle clinical signs may be obscured due to impaired locomotion and limited capability to evoke specific neurobehavioral responses. If the focus of a study is on neurobehavioral changes over the course of an exposure, this is sufficient justification for using an alternative exposure mode such as whole-body exposure. A detailed analysis and recording of clinical signs should be made, but not limited to, the time when maximal systemic toxicity is expected, which is usually on the exposure day. Details have been published elsewhere (26)(28)(29)(30). Because of the study design of the $C \times t$ protocol, a nose-only chamber should always be used when
performing the study.

**Nose-Only Exposure Techniques**

50. During exposure, animals are exposed to the test chemical while in restraining tubes. The restraining tubes should not impose undue physical, thermal, or immobilisation stress on the animals. Restraint may affect physiological endpoints such as body temperature (hyperthermia) and/or respiratory minute volume. If generic data are available to show that no such changes occur to any appreciable extent, then pre-adaptation to the restraining tubes is not necessary. When precise dosimetry is the objective of the study, however, pre-adaptation may decrease inter-animal variability. Urine and faeces should escape from the restrainer during the course of exposure.

51. To provide optimal exposure of animals, a slight positive balance of air volumes supplied to and extracted from the exposure system should be ensured to prevent dilution of the test chemical at the animals' breathing zone. The design of the restraining tube and the pressure difference should make it impossible for animals to avoid inhalation exposure. If leakage from the inhalation equipment cannot be excluded by design, the inhalation equipment should be operated in a well-ventilated chemical hood to avoid harming laboratory personnel. Maintenance of slight negative pressure inside the hood will prevent leakage of the test chemical into the surrounding area.

52. Animals should be exposed in flow-past inhalation equipment designed to sustain a dynamic airflow that ensures an adequate air exchange of at least 2-3 times the respiratory minute volume of animals exposed (i.e., at least 0.5 L/min per exposure port for rats). Each exposure port should have similar exposure conditions with an oxygen concentration of at least 19% and a carbon dioxide concentration not exceeding 1%. The design and operating conditions of the chamber should minimize the re-breathing of exhaled atmosphere. A significant disturbance of airflow dynamics during the collection of test atmosphere should be avoided (29)(30).

**Whole-Body Exposure Techniques**

53. Animals should be tested with inhalation equipment designed to sustain a dynamic airflow of at least 10 air changes per hour. Higher airflow rates may be useful to meet specific requirements imposed by the test chemical. An oxygen concentration of at least 19%, a carbon dioxide concentration not exceeding 1%, and an evenly distributed exposure atmosphere should be ensured. Where concerns might apply, these gas levels should be measured in the vicinity of the animals' breathing zone. All animals should be individually housed to preclude them from breathing through the fur of their cage mates, thus reducing their aerosol exposure. To ensure stability of a chamber atmosphere, the total "volume" of the test animals should not exceed 5% of the chamber volume. Maintenance of a slightly negative pressure inside the chamber will prevent leakage of test chemical into the surrounding area. Food and drinking water should be accessible for exposures exceeding 8 hours.

54. In a dynamic whole-body chamber, the test chemical concentration initially rises rapidly, and then slowly approaches a theoretical equilibrium provided; 1) the output of the test chemical is constant; and, 2) the test chemical is instantaneous and thoroughly mixed throughout the chamber. Under these conditions, an exponential built-up of concentration is seen throughout the chamber. The time to 95% atmosphere equilibrium ($t_{95}$) in minutes is calculated using the following simplified formula. More details are presented elsewhere (23).
\[ t_{95} \text{ (min)} = 3 \times \left( \frac{\text{chamber volume}}{\text{chamber airflow}} \right) \]

Technical feasibility of generation a target atmosphere

55. The feasibility of generating a targeted atmosphere should be determined without animals. This is mandatory for TG 436 and recommended for TG 403, TG 412, and TG 413 to prevent useless animal exposures. Each test chemical may pose unique physical challenges and/or require vehicle systems to generate and characterize the test atmosphere. This preliminary step can show that a stable inhalation chamber atmosphere can be generated at the target concentration and particle size (for aerosols; see below). Collection efficiency and sampling error of equipment used to characterize an atmosphere should be ascertained. The equipment used to sample chamber atmospheres (e.g., flow-limited critical orifices, gas meters, or flow controllers) should be regularly calibrated. Evaporated constituents from the test atmosphere or the collection medium (e.g., glass bubblers containing volatile solvents) should not interfere with the precise determination of the sampled volume. Ideally, the comparison of results obtained from different equipment should identify technical inconsistencies and verify that sampling errors do not occur to any appreciable extent.

56. In the case of highly reactive materials (reaction potential with moisture, oxygen etc.) the test atmosphere should be fully characterised and its relevance to the potential human exposure situation should be considered. For example, it may be acceptable to expose animals to degradation products in air as this will represent the actual overall hazard to humans in the workplace/environment. Controlled dried air is always used for generation during inhalation studies, and normally the moisture content is low enough not to result in delivery issues. Diluent air, if used, is dried to a lesser degree and may also be humidified to a level consistent with ambient to emulate the hazard environment. In repeated inhalation studies using generally markedly lower concentrations than in acute inhalation studies the stability and homogeneity of atmospheres needs to be verified by appropriate analytical methodologies.

Control Groups

57. A concurrent negative (air) control group is not necessary for acute studies. When a vehicle other than water is used to assist in generating the test atmosphere, a vehicle control group should be used when historical inhalation toxicity data are not available. If a toxicity study of a test chemical formulated in a vehicle reveals no toxicity, it follows that the vehicle is also non-toxic at the concentration tested so there is no need for a vehicle control. To allow for statistical comparisons of non-lethal endpoints, adequate historical data from a similarly exposed control group may help in distinguishing between specific effects caused by the test chemical and non-specific effects associated with the method of exposure.

Vehicles

58. If the targeted concentration cannot be attained using the undiluted test chemical, a vehicle should be used. The selection of the vehicle should be based on previous experience, the pattern of use or physical restraints (solubility and stability of test chemical, particle size). A vehicle may also be considered to enhance the dustiness of solid test chemicals (powders). The kind and concentration of vehicle should not interfere with the outcome of the study with regard to the airborne test chemical’s analytical stability or toxicity. Ideally, the vehicle selected should be non-toxic with water being given first preference. When a vehicle other than water is used, a vehicle control group should only be used when historical inhalation toxicity data are not available. If a concurrent vehicle control is to be
avoided, historical data should show that the vehicle does not interfere with the outcome of the study.

**Exposure Duration**

59. The duration of exposure should be specified. For whole-body chambers, the exposure time is defined as the time between the \( t_{95} \) equilibration of the chamber concentration and the \( t_{95} \) chamber concentration decay. Chamber equilibration and decay are assumed to be nearly instantaneously in nose-only chambers. For longer exposure durations, whole-body chambers are recommended.

**TEST GUIDELINES**

**TG 403 – Performance of the Traditional LC50 and C × t Protocols**

60. Selection of the number of animals and the number of concentrations tested in the Traditional LC50 protocol and the C × t protocol should be informed by the study director’s understanding of the performance needs of the test. For additional information see the Performance Assessment of these two protocols (25). It used simulated and real data sets to describe the strengths and weaknesses of both protocols, and the effect on point estimates that result from using an assortment of animal numbers, concentrations, and durations. Anyone who selects one of these protocols for a particular regulatory need is urged to consider carefully this landmark assessment.

61. Normally, two animals per C × t interval (one per sex using both sexes or two of the more susceptible sex) will be adequate. The Performance Assessment simulation analysis, which tested 4 concentrations and 5 durations per concentration, demonstrated that performing a C × t protocol with 1 animal/sex or 2 animals of the more susceptible sex will provide LC50 estimates that are comparable with a Traditional LC50 protocol in terms of bias and precision. With 1 animal per sex (or 2 of the more susceptible sex) the performance with respect to LC10 or LC01 estimates is greater than one would expect from the Traditional LC50 protocol, and reasonably reliable LC10 or LC01 estimates would usually be obtained for all durations within the tested time range (25). Under some circumstances, the study director may elect to utilize two rats per sex per C × t interval. The same simulation analysis demonstrated that testing 1 animal per sex per C × t combination may not be sufficient in all cases, even when testing 4 concentrations and 5 durations per concentration. Using 2 animals per sex per C × t interval (or 4 animals of the susceptible sex) may reduce bias and variability, increase the estimation success rate, and improve confidence interval coverage. If one is interested in the additional estimates available from a C × t experiment (e.g., the one-hour LC values) not estimable from a Traditional LC50-test, the addition of 1 extra animal per sex per C × t combination will reward the experimenter with better estimates (24). However, in case of an insufficiently close fit to the data (when using 1 animal per sex or 2 animals of the more susceptible sex per C × t interval) a 5th exposure concentration with 5 durations may also suffice.

62. In order to observe contemporary animal welfare regulations it may be prudent to merge results from past and new data sets. This could be achieved following the approach suggested by ten Berge (41,42) and Zwart et al. (22) by introducing the toxic load exponent (\( n \)) to combine the exposure concentration (C) and exposure time (t) using the following equation: \( C^n \times t = k \) where k is a numerical effect-based constant. Exponential weighing factors are incorporated to better mathematically describe the relative contribution of C and t. This equation can be generalized as follows: \( y_{\text{probit}} = b_0 + b_1 \log(C) + b_2 \log(t) \) where \( n = b_1/b_2 \). The ‘\( n \)’ is considered to be a chemical-specific and toxic endpoint-specific exponent. The ‘dependent variable’ ‘\( y \)’ which represents the effect, is commonly probit transformed and \( b_0, b_1, \) and \( b_2 \) are empirically derived constants. The definite advantage of this approach is that the entire matrix of empirical data can be used to calculate any LC\(_{50}\) and any fraction thereof, including the non-lethal threshold concentration (LC\(_{10}\)) which is
taken as POD commonly for deriving Acute Emergency Response Guideline Values (46)(47).

**TG 403 and TG 436 - Limit Test**

63. The limit test in acute studies is primarily used when the test chemical is known to be virtually non-toxic, i.e., eliciting a toxic response only above the regulatory limit concentration. Limit tests evaluate the targeted limit concentration or, if technically not achievable due to the test chemical’s physicochemical nature, the maximum attainable concentration. For gases and vapours, there is no need for further testing if less than 50% lethality occurs at the limit concentration or the maximum attainable concentration (in case the analytical limit concentrations is in the range of the vapour saturation concentration). If the MMAD significantly exceeds 4 µm (see technical feasibility section above), further efforts should be employed to reduce the test chemical’s particle size. For aerosols, the MMAD of the test atmosphere should be considered if no deaths occur at the limit concentration or the maximum attainable concentration. If the test atmosphere achieves the recommended MMAD standard of 1-4 µm and less than 50% lethality occurs at the limit concentration or the maximum attainable concentration, no further testing is necessary.

64. The selection of limit concentrations usually depends on regulatory requirements. When the GHS Classification System is used, the limit concentrations are 20,000 ppm for gases, 20 mg/L for vapours, and 5 mg/L for liquid and solid aerosols (see Appendix II)(11). The GHS limit concentrations are used in TG 436 to set the upper classification boundaries for GHS Class 4 test chemicals. The GHS limit concentrations may also be used for other inhalation toxicity studies. For animal welfare reasons GHS discourages testing in excess of a limit concentration. The limit concentration should only be considered when there is a strong likelihood that results of such a test would have direct relevance for protecting human health (11), and justifications should be given in the study report. In the case of potentially explosive test chemicals, care should be taken to avoid conditions favorable for an explosion. For safety reasons it is generally advisable to not exceed 50% of the published Lower Explosive Limit (LEL).

65. Achieving the GHS limit concentration of 5 mg/L is technically challenging for most aerosols and greatly exceeds real-world human exposure. It can be difficult or impossible to generate a respirable (MMAD of 1-4 µm) liquid or solid aerosol at this concentration without encountering experimental shortcomings. As aerosol concentration increases, particle size also increases due to the aggregation of solid particles or coalescing of liquid particles. The usual consequences are 1) a decrease in the respirable particle size fraction and thus reduced toxicity, 2) increased fluctuation and variability in inhalation chamber concentrations accompanied by increased spatial inhomogeneity, 3) overloading of equipment used to characterize test atmospheres, and 4) a divergence of nominal and analytical concentrations. At very high concentrations, dry powder aerosols and chemically reactive liquid aerosols (e.g., polymers) tend to form conglomerates in the proximal nose causing physical obstruction of the animals’ airways (e.g., dust loading) and impaired respiration which may be misdiagnosed as a toxic effect. When testing aerosols, the primary goal should be to achieve a respirable particle size (MMAD of 1-4 µm). This is possible with most test chemicals at a concentration of 2 mg/L. Aerosol testing at greater than 2 mg/L should only be attempted if a respirable particle size can be achieved. As stipulated in TG 403, dilutions of corrosive test chemicals may be tested at exposure concentrations sufficient to extend the concentration-response curve to levels that reach the objective of the test and thus serve regulatory and scientific needs, however, the targeted concentrations should not induce severe irritation/corrosive effects. These concentrations should be selected on a case-by-case basis and justification for concentration selection should be provided.

66. If the targeted regulatory limit concentration cannot be achieved by the initial technical
procedures, then at least one alternative generation method should be used, ideally using different physical principles but with established methodologies. A reasonable attempt should be made to generate the test chemical, but extreme technical solutions are not recommended. An explanation and supportive data should be provided that explains why the regulatory limit concentration could not be achieved. Information about a test chemical’s toxicity can be derived from data about similar test chemicals or similar mixtures or products, taking into consideration the identity and percentage of components known to be of toxicological significance. If TG 403 is to be used, and there is little or no toxicity information, or if the test chemical is expected to be toxic, a range-finding study and a main study should be performed.

67. Those using the GHS Classification System should note that it uses units of mg/L to classify vapours, but units of ppm to classify gases even though gases and vapours are both gaseous when humans and animals are exposed to them. The conversion between mg/L and ppm is based on the molecular weight of a test chemical (see equations in paragraph 15 and Appendix I). For example, at 22°C, 20 mg/L of a gas is equivalent to 24200 ppm if the gas has a molecular weight of 20 g/mol, or 2420 ppm if it has a molecular weight of 200 g/mol. Gases and volatile test chemicals with a vapour saturation concentration that can exceed 20 mg/L (at approximately 22˚C) should be tested at the limit concentration of 20 mg/L. This limit should only be exceeded when there is a compelling reason, and the reason should be explained in the study report. For volatile liquids with a vapour saturation concentration in the range of 2-20 mg/L (at approximately 22˚C), the maximum chamber concentration should be at least in the range of this vapour saturation concentration. Commonly, this is achieved by generating a liquid aerosol, which then equilibrates with the vapour phase. Under such circumstances each phase needs to be appropriately collected and analyzed by the procedures used.

**TG 412 and TG 413 – Performance of Repeated Exposure Studies**

68. There are two test guidelines for repeated inhalation exposure studies: TG 412 for 28 day (subacute) studies and TG 413 for 90 day (subchronic) studies. Although there are no test guidelines specifically for inhalation studies of chronic duration, TG 451 (carcinogenicity)\(^1\), TG 452 (chronic toxicity)\(^2\), and TG 453 (combined chronic toxicity/carcinogenicity)\(^3\) recommend that TG 412, TG 413, and GD 39 should be consulted when designing inhalation studies of chronic duration.

69. The 2009 versions of TG 412 and TG 413 have been revised to reflect the evolving state-of-the-science and to accommodate the testing of nanomaterials. It is noted that nanoparticles (~1–100 nm) and fine particles (~0.1–2.5 µm) coexist as a continuum and that samples of engineered nanoparticles commonly consist of aggregated/agglomerated structures in the micrometer range rather than as isolated nanoparticles. TG 412 and TG 413 are identical in all respects except for study duration (28 v 90 days) and the number of animals used in the main study (5/sex/group in TG 412 v 10/sex/group in TG 413). Animal numbers in satellite groups are identical for the two TGs. The most notable changes in the revised TGs are as follows:

- Bronchoalveolar lavage (BAL) and the evaluation of BAL fluid (B Alf) is required for all test chemicals. The mandatory BALF parameters are:
  - lactate dehydrogenase (LDH)
  - total protein or albumin
  - total leukocyte count, absolute cell counts, and calculated differentials for alveolar macrophages, lymphocytes, neutrophils, and eosinophils.
Additional optional BALF parameters that may be evaluated are described in paragraph 122.

- Measurements of lung burden are mandatory when a range-finding study or other information demonstrates that a poorly soluble solid aerosol is likely to be retained in the lung. Because there is no defined method for ascertaining whether solid aerosol particles are soluble in the lung, water solubility is used as an approximation. The water solubility of an aerosol may be assessed using GD 105 (OECD, 1995), by using an artificial lung lining fluid that contains salts and proteins, or by testing in an acidic environment that mimics the lysosomal fluid of macrophages.

- The 2009 versions of TG 412 and TG 413 required particulate aerosols to have a mass median aerodynamic diameter (MMAD) of 1-3 \( \mu \)m with a geometric standard deviation (GSD or \( \sigma_g \)) of 1.5-3.0. To accommodate the testing of nano-range aerosols and to enhance deposition in the pulmonary region, a new standard is recommended: MMAD of \( \leq 2 \) \( \mu \)m with a \( \sigma_g \) of 1-3. Justification should be provided in the study report if this standard cannot be met, including a description of efforts taken to meet it, such as milling (refer to GD 39). Justification should be provided in the study report if this MMAD criterion cannot be met, including a description of measures taken in an attempt to meet it (e.g., milling).

- When an inhaled test chemical is known or likely to be a sensory irritant, periodic measurements of pulmonary function and body temperature should be performed to identify and quantify the impact of reflexes in the upper and/or lower respiratory tract (e.g., reflex bradypnea and the Paintal reflex), as described in Appendix V.

70. As depicted in Appendix IV, the study designs for both TG 412 and TG 413 demonstrate two options based on the nature of the test chemical. A decision flow chart is used to determine which option should be used for a given test chemical. Option A is used for test chemicals that are not likely to be retained in the lungs, that is, gases, vapours, liquid aerosols, and soluble solid aerosols. Option B is used when testing poorly soluble solid aerosols that are likely to be retained in the lungs.

71. Option A: The main study in Option A consists of 5 animals/sex/concentration in TG 412 or 10 animals/sex/concentration in TG 413. These animals are sacrificed within a day after the final test chemical exposure, which is designated Post Exposure Observation period 1 (PEO-1). Additional satellite groups of 5 animals/sex/concentration (TG 412 and TG 413) are exposed concurrently with the main study groups and sacrificed at PEO-2, a post-exposure duration determined by the study director based on what was learned in a range-finding study. Both the main study and satellite group animals are assessed for clinical observations, body weight measurements, food and water consumption, clinical pathology, gross pathology, organ weights, lung weight (left lung) histopathology (left lung) and BALF analysis (right lung). While the TGs provide recommendations for which lung should be used for histopathology and BALF analysis, a study director may choose to reverse the lung order. Option A uses a total of 80 animals (TG 412) or 120 animals (TG 413) at the maximum, i.e. when satellite groups are included.

72. Option B: The main study in Option B consists of 5 animals/sex/concentration (TG 412) or 10 animals/sex/group (TG 413), which are subject to the same observations as in Option A (clinical observations, body weight measurements, food and water consumption, etc.) and additional groups of 5 males/concentration that are used for lung burden measurements. Males are used because they have higher minute volumes than females and are thus likely to have higher lung burdens. All main study animals are sacrificed one day following the last test chemical exposure (PEO-1) to allow for rapid particle clearance via mucociliary transport. A study director has the option to include one or two satellite groups to obtain information on clearance kinetics. These groups are exposed concurrently.
with the main study animals and sacrificed at PEO-2 and PEO-3. Since at least three time points are generally required to provide information on clearance kinetics, lung burden measurements are performed at exposure termination (PEO-1) and at 2 additional PEOs (PEO-2 and PEO-3). The post-exposure duration for the satellite groups are determined by the study director. The PEO-2 satellite groups consist of 5 males and 5 females/concentration. The females are used for histopathology (left lung) and BALF evaluation (right lung), and the males are used for histopathology (left lung) and lung burden measurements (right lung). As with Option A, the lung order may be reversed.

A third satellite group of 5 males/group is sacrificed at PEO-3, and right lungs are assessed for lung burden. The study director may also use the satellite groups for additional assessments (designated at TBD in Appendix IV, Option B), such as BALF, toxicity (including recovery), and toxicokinetics. Other options include:

- The study director has the option to schedule PEO-3 before the recovery group (PEO-2, if included).
- If the use of two post-exposure time points is considered sufficient, lung burden measurements may be performed at PEO-1 (main study) and at PEO-2 (recovery group) only, if timing for evaluation of recovery and lung clearance can be aligned to one another. The satellite group at PEO-3 can then be omitted from the study.
- The study director may choose to perform lung burden measurements at PEO-1 (main study) and at PEO-3 (satellite group) and to use both sexes of the recovery groups (PEO-2) for BALF measurements.

Thus, Option B provides a study director with a lot of flexibility. Option B uses a maximum of 120 animals (TG 412) or 160 animals (TG 413).

Measurements of bronchoalveolar lavage fluid (BALF) and lung burden are performed preferably within 24 hr after exposure termination (to allow for rapid particle clearance) and may be performed at one or two additional post-exposure intervals (see Appendix IV). A minimum of two lung burden measurements are necessary when investigating clearance kinetics. Lung burden measurement at three time points allow curve fitting on post-exposure clearance kinetics. The need for additional post-exposure observations, the duration of the post-exposure interval and the timing of the post-exposure observations (PEOs) are determined by the study director based upon results from, among others, the range-finding study. Lung burden and BALF are measured for all concentrations.

The design of the main study is greatly dependent on information learned during a range-finding study. A range-finding study should be performed unless sufficient information already exists to perform a robust main study. While the primary purpose of a range-finding study is to inform the selection of concentration levels for a main study, it may also provide additional information that can assure a robust main study. This is especially true when testing poorly soluble solid aerosols. A range-finding study may, for example, provide information regarding analytical methods, particle size distribution, systemic toxicity, toxicokinetics, test chemical solubility in the lung, translocation of particles, discovery of toxic mechanisms, clinical pathology (i.e., haematology/clinical chemistry), histopathology, biomarkers of lung injury, gender sensitivity, BALF data, and estimates of what may be the No Observed Adverse Effects Concentration (NOAEC), Lowest Observed Adverse Effects Concentration (LOAEC), Maximum Tolerated Concentration (MTC), and the benchmark concentration (BMC) in a main study. The study director should use a range-finding study to identify the upper concentration that is tolerated without undue stress to the animals, and the parameters that will best characterize a test chemical’s toxicity. BAL may be performed at a range-finding study’s...
exposure termination and periodically during a post-exposure period. When testing an aerosol of a solid material, an assessment of the test chemical solubility in water and post-exposure lung burden are recommended to inform a decision on the duration of the main study post-exposure period and the spacing of post-exposure observation (PEO) time points. Also, LALN burden measurements may provide information on translocation. The rationale for the selection of target concentrations for the main study should be provided in the study report of the main study.

76. A range-finding study may consist of one or more test chemical concentration levels and a control group. Depending on the endpoints chosen, no more than 5 males and 5 females should be exposed at each concentration level. A range-finding study should last a minimum of 5 days and generally no more than 28 days, and may include a post-exposure period and animal numbers need to be adjusted accordingly. The rationale for the selection of concentrations for the main study should be provided in the study report. The objective of the main study is to demonstrate a concentration-response relationship based on what is anticipated to be the most sensitive endpoint. The low concentration should ideally be a no observed-adverse effect concentration while the high concentration should elicit unequivocal toxicity without causing undue stress to the animals or affecting their longevity (24). When testing poorly soluble particles, it may be necessary for a range-finding study to be longer than 14 days to allow for a robust assessment of test chemical solubility and lung burden. Further details on the conduct of 28 and 90-day inhalation studies can be found in TG 412 and TG 413.

TEST ATMOSPHERE GENERATION

Gases

77. Certified gases are available for testing from pressurized cylinders. The targeted concentration of the chemical should be produced by mixing the test gas with dry air (chemicals that may react with water vapour) or humidified air. The test chemical/air mixing ratio must be precisely controlled by either a precision gas metering devices or by calibrated (ideally digital) mass flow controllers. Whatever flow device is used for metering the test substance, it may require daily re-calibration using soap bubble calibration devices. Ambient temperatures and barometric pressures must be recorded day by day.

Vapours

78. Vapours are preferentially generated from the liquid vapour phase at controlled temperatures. When using elevated temperatures to increase the vapour saturation concentration, caution is advised to avoid producing condensations aerosols, which become increasingly stable at concentrations above vapour saturation. Small gas bubblers or other controlled evaporation devices can be used to generate a constant flow of vapour phase in a carrier gas (e.g., dry nitrogen for highly reactive chemicals) or humidified air. The targeted concentrations can be produced as described for gases. Emphasis should be directed to calculate the mass-loss of liquid chemical relative to the flow of carrier gas through the evaporation device. Under ideal conditions, the resultant calculated concentration should be similar to the thermodynamically calculated vapour saturation concentration under the condition of test.

Aerosols from liquids

79. Most frequently, liquid aerosols are generated by atomization (a nozzle connected to a metering pump with attached baffle system to eliminate large particles) or nebulization (e.g. a collison nebulizer that generates a constant spray volume from the liquid in a small reservoir. Larger aerosols may be impacted on the wall of this reservoir which serves as baffle. More volatile constituents may
evaporate with subsequent changes of the composition and concentrations of chemicals contained in the reservoir. When operated at elevated temperatures (e.g., a viscous liquid that is aerosolized or evaporated), vapours can condense in the cooling chimney to form condensation aerosols. Depending on the composition of test chemical, each of these devices may produce its own ratio of liquid aerosol and vapour phase. Partial enrichment of the more volatile fraction cannot be avoided when trapping large aerosol particles to enhance their respirability. For mixtures containing materials with different vapour pressures, atomization principles should be given preference to nebulization principles. Some end-use formulations are technically optimized for controlled discharge, e.g., evaporation devices or spray cans. It is not recommended to dismantle such devices to make inhalation testing possible. Rather, it is prudent to test such systems in their end-use configuration under maximum technically feasible conditions. All of these generation systems may require further optimization of respirability by cyclones, impaction, or gravitational separation systems.

**Aerosols from solid materials**

80. Solid aerosols may range from tangled bundles of fibrous material with essentially no dustiness, compact solid smelts with negligible vapour pressure, granulate formulations designed to be resistant to attrition up to readily dispersible fine dusts. This wide range of physical properties require multiple generation systems adapted to the physical properties of the test chemical and the desired concentration to be attained in inhalation chambers. Generation principles can be stratified as follows: The first step is to increase dustiness with a minor focus on respirability so that the test chemical can be reproducibly metered into any dust generation/particle-size optimization system. This may be achieved by ‘milling’ of any dry, non-sticky material or ‘sonication’ of the material dispersed in a carrier fluid. For milling, the ball mill is a type of grinder that may provide the highest degree of controlled grinding with minor electrostatic charging. However, it must be kept in mind that any milling procedure may possibly deteriorate any complex over-structure of the test chemical and should therefore be used for increasing dustiness only. Sonication may not only change the material’s morphology but it may also cause leaching-off of surface-bound impurities.

81. The degree of achieved dustiness and the target concentration determine the kind of dry-dust generation principle to be used. Multiple devices are commercially available, e.g., Wright dust feeder, fluidized bed generator, rotating-brush generators, and other push/pull dispersion systems. All of these systems require further optimization of respirability. By default, aerodynamic separation procedures should be given preference because they do not impose undue mechanical stress or an electrostatic charge to particulate structures. Commonly applied methods are cyclones and systems that dry-aerosolize the test chemical with a classifying airstream by gravitational and/or centrifugal forces. Ionizers may be required to prevent or minimize the agglomeration of the airborne dry dust. Wet dispersed dry aerosol can also be generated as described for liquid aerosols. Post-aerosolization drying of atmospheres is required, either by dry dilution air or by diffusion dryers.

82. It is beyond the scope of this guidance document to elaborate on more research-based specialized generation methods of pristine nanoparticles, e.g., metal fumes or combustion aerosol. Such fumes can readily be produced by a combination of combustion/condensation/dilution devices, such as spark generators fitted with metal tipped electrodes or combustion engines. Their particle-size is highly dependent on the time elapsed to dilution to prevent or promote particle coagulation and agglomeration. Depending on the carrier gas, e.g. oxygen vs. argon, metal or metal oxide fumes are produced. Due to the low concentrations generated, the particle number concentration and count median diameters are given preference to any mass-based metric. Apparatuses commonly used in inhalation studies for atmosphere generation are reviewed in details elsewhere (23).

83. The optimization of dustiness and particle size must account for the following constrains: For
isometric particles, mass and rodent-adjusted respirability should be the major focus. For non-isometric morphologies, such as fibres or aggregated structures thereof, any specific aspect ratio, fibre length or over-structure of agglomerates—in the absence of overwhelmingly high granular fractions—should thoughtfully be observed. Hence, dry dust generation of complex materials remains a balancing act between the technical feasibility to make complex structures testable in rodent inhalation studies and those structures to which humans may actually be exposed. Thus, it is essential to keep these aspects in mind when validating feed-back loops of the aerosolized test chemicals in inhalation chambers.

MONITORING OF EXPOSURE CONDITIONS

Chamber Airflow

84. Airflow into dynamic inhalation chambers (e.g., pressurized air to disperse a test chemical, atmospheric air to evaporate a volatile test chemical, and dilution and conditioning airflows) and airflow at the chamber exhaust port should be controlled and monitored to obtain stable conditions throughout the exposure period. Pressure may also be measured within the chamber. Devices should be calibrated under conditions of use (e.g., by using bubble meters, wet test meters, dry gas meters). A technical description of the calibration of devices that measure airflows should be documented and described in the study report. Further guidance is provided in paragraph 94.

Chamber Temperature and Relative Humidity

85. The chamber temperature should be maintained at 22 ± 3°C. The relative humidity in the animals’ breathing zone, for both nose-only and whole-body exposures, should be monitored regularly and recorded at least three times during each exposure. The relative humidity should ideally be maintained in the range of 30 to 70%, but this may not be possible when testing water based test chemicals, or may not be measurable due to test chemical interference with the test method. The proper performance of devices should be demonstrated, e.g., by using calibrated reference probes or saturated salt solution probes for measuring relative humidity. A technical description of the calibration of equipment used to measure inhalation chamber temperature and relative humidity, including the location of probes relative to the exposed animals, should be documented and described in the study report.

Test Atmosphere Characterization

86. When assessing exposure concentrations (mass/volume of air), both the mass determined and the volumes of air sampled from the inhalation chamber and passed through the collection device should be precisely measured. Flow meters, critical orifices, or dry gas meters used to define the sampled volume as a function of airflow \((rate \times time)\), should be appropriately calibrated. Sampled volumes can also be directly obtained with wet gas meters. Potential sampling errors, such as those caused by inappropriate collection efficiency, instability of the test chemical in solvents or on adsorbents, or a poor recovery from the collection medium, should be considered when designing a specific strategy to analyze components from inhalation chambers. Solvents evaporating from a collection device may cause volume errors. The collection efficiency depends markedly on the physical characteristics of the test chemical (gas, vapour, aerosol, particle size). Therefore, precautions should be taken to minimize size-selective sampling errors, and to assure that analytical concentrations include all physical forms of the analyte examined.

87. Chamber atmosphere samples should be taken from the vicinity of the animals’ breathing zone. During sampling, the airflow should be monitored at regular intervals to detect changes caused
by an increased resistance in the adsorbent used. If impingers or gas bubblers containing volatile liquids (other than water) are used during sampling of test atmosphere, evaporation of the solvent should be taken into account. Many mechanisms that affect representative sampling depend on aerosol particle size and airflow rates. A given sampling system may exhibit representative sampling over a specific particle size range but may not be able to characterize particles larger or smaller than that range. Isokinetic sampling strategies to preserve chamber aerosol characteristics need to be considered so that all phases and particle size fractions of a specific analyte are collected with high efficiency from the animals’ breathing zone in order to obtain similar material mass balances from different procedures. Sampling ports should be designed in such a way that potential sampling errors as a result of non-isokinetic sampling or by size-selective sampling are minimized. The tolerance limits for the radius of the sample probes may be calculated according to published formulas (31)(32) or the relationship shown in Appendix I (Representative sampling of atmospheres). The collection efficiency of the equipment used to characterize exposure atmospheres should be measured. This information is of relevance when different devices used in a study provide inconsistent measurement results.

**Analytical Concentrations**

88. The goal of every inhalation study is for test animals to be exposed to constant chamber concentrations that are close to the desired target concentration. Analytical concentrations can either confirm that the animals were properly exposed or reveal test chemical generation problems. The analytical concentration is the test chemical concentration at the animals’ breathing zone in an inhalation chamber. Analytical concentrations can be obtained either by specific methods (e.g., direct sampling, adsorptive or chemical reactive methods, and subsequent analytical characterisation) or by non-specific methods such as gravimetric filter analysis. The use of gravimetric analysis is acceptable only for single component powder aerosols or aerosols of low volatility liquids, and should be supported by appropriate pre-study characterisation. Potentially reactive test chemicals should be assessed by methods specific for the test chemical that will not interfere with any degradation product. For single component test atmospheres, specific methods should be used for gaseous and volatile test chemicals, including liquid aerosols. Volatile test chemicals may exist as a vapour at low concentrations and as a vapour-aerosol equilibrium at higher concentrations. The applied sampling technology should integrate all phases. Non-specific methodologies may be appropriate for solid and liquid aerosols with low volatility provided the percentage of the vapour phase under testing conditions does not exceed 1% of the total concentration.

89. The exposure atmosphere shall be held as constant as practicable and monitored continuously and/or intermittently depending on the method of analysis. When intermittent sampling is used, chamber atmosphere samples should be taken at least twice in a four-hour study. If marked sample-to-sample fluctuations occur, the next concentrations tested should use four samples per exposure. For very short exposure durations, the time required for atmosphere collection may exceed the animals’ exposure duration. When testing very low aerosol concentrations, it may be technically difficult to accomplish this sampling frequency due to long sampling periods and the limited airflow rate typically used to extract samples from small inhalation chambers.

90. Individual chamber concentration samples should deviate from the mean chamber concentration by no more than ±10% for gases and vapours, and by no more than ±20% for liquid or solid aerosols. In addition to the variability of chamber equilibrium concentrations, these error boundaries also comprise errors from other sources, e.g., variability related to the analytical method and variability in the sampling and collection of the analyte.

91. Ideally, analytical data obtained by intermittent sampling should be complemented by non-specific, real-time monitoring data (e.g., recorded by aerosol photometers for particulates or a total hydrocarbon analysers for volatile materials). These data can demonstrate that temporally stable
exposure conditions prevailed, and that the time required to reach the inhalation chamber equilibrium concentration is negligible in relation to the total duration of exposure, or is adequately taken into account. Time to attain inhalation chamber equilibration ($t_{95}$) should be calculated and reported. The duration of an exposure spans the time that the test chemical is generated. This takes into account the times required to attain chamber equilibration ($t_{95}$) (see paragraph 54). It should be noted that monitoring of the test atmosphere is an integral measurement of all dynamic inhalation chamber parameters and hence provides an indirect, though integrative, measure of inhalation chamber control. Therefore, the frequency of airflow measurements may be reduced to one single measurement at the start of an exposure. The characterization of test atmosphere should be representative for the atmosphere to which animals are exposed. Real-time monitoring instruments may not be suitable if their sensing units become covered with excessive quantities of test chemical or if they are subject to being destroyed by the test chemical. If they cannot be used, expert judgement should be made as to whether the monitoring of physical chamber parameters generates relevant data. Care should be taken to avoid generating explosive concentrations.

For very complex mixtures consisting of vapours/gases, and aerosols (e.g., combustion atmospheres and test chemicals propelled from purpose-driven end-use products/devices), both phases may behave differently in an inhalation chamber. Therefore, at least one indicator substance (analyte) normally the principal active in the tested product formulation, of each phase (vapour/gas and aerosol) should be selected. The back-calculation to the test chemical should utilize that analyte with the greatest precision, typically the one present in the highest concentration. For simple mixtures of known characteristics, e.g., pesticide formulations, the gravimetric filter analysis should be given preference since this requires the least number of assumptions. It is not necessary to analyze inert ingredients provided the mixture at the animals’ breathing zone is analogous to the formulation prior to aerosolization; the grounds for this conclusion should be provided by expert judgement. If there is some difficulty in measuring analytical chamber concentration due to precipitation, non-homogenous mixtures, volatile components, or other factors, additional analyses of inert components may be necessary as detailed above.

Whenever the test chemical is a mixture (e.g., a formulation), the analytical concentration should be reported for the total formulation and not just for the active ingredient or the component (analyte). In the case of simple mixtures, the percentage of potentially volatile components (i.e., those presumed to be present as vapours in the inhalation chamber upon aerosolization of a liquid) relative to those components recovered by the filter should be determined. The mass concentrations obtained by filter analysis can then be back-calculated to the mass concentration of the test chemical. If gravimetric analysis is not suitable due to unstable gravimetric conditions (e.g., continuous change in filter weight over a specified time of filter conditioning), the analysis of an appropriate component (analyte) of that mixture can then serve to back-calculate the actual test chemical concentration. If, for example, a simple mixture (e.g., a pesticide formulation) contains 10% active ingredient and 90% inerts, the actual mixture concentration is the concentration of the active ingredient multiplied by ten. It is not necessary to analyze inert ingredients provided the mixture at the animals’ breathing zone is analogous to that of the formulation. The grounds for this conclusion should be described in the study report.

Nominal Concentrations

Nominal concentrations (mass of test chemical disseminated into the exposure system during the generation period divided by the total airflow through the inhalation chamber during the same time period) and analytical concentrations (measured mass concentration of test chemical recovered from the breathing zone of the exposed animal) should be determined. The nominal concentration is not used to characterize the animals’ exposure. For gases or highly volatile substances, nominal concentrations are useful to judge the consistency of analytical concentrations.
95. The consistency of inhalation tests can be judged by a comparison of nominal and analytical concentrations for volatile liquid and gaseous test chemicals. However, this comparison is of limited relevance for aerosols (solid or liquid) due to significant losses of particles in pre-separator systems and particle deposition on chamber and tubing walls. This is due to the fact that technically demanding measures should be taken for liquid and solid aerosols to remove large particle-size fractions from the air stream. Consequently, analytical concentrations can significantly deviate from nominal concentrations, even by orders of magnitude. Ratios of nominal to analytical concentrations are difficult to predict as they are contingent upon the apparatus used for aerosolization and particle size optimization, and they are dependent on the physico-chemical properties of the test chemical (e.g., viscosity, volatility, and ability to sublime or to co-distill with any carrier material). For liquid aerosols, the particle size distribution may decrease with the decreasing concentration. To achieve comparable particle size distribution within a wide range of concentrations (e.g., from 2 mg/L to 0.02 mg/L) dilution systems may be used. In this case the nominal concentration does not reflect the generation efficiency and is thus not meaningful.

**Aerosol Particle-Size Distribution**

96. Current classifications of solid substances call for a mass-based metric. Occupational standards follow this paradigm with the exception of fibres. Accordingly, the targeted, nominal, and analytical concentrations utilized in inhalation studies are mass-based by default. The objective of particle-size analysis is to calculate the percentage of the mass of particles in exposure atmospheres likely to be deposited in the nasopharyngeal, tracheobronchial, and pulmonary (alveolar) airways of rats. Similar estimations can be made for humans, making a dosimetric adjustment between these species possible. Submicrometer nanoparticles have a tendency to agglomerate and to then behave aerodynamically as micrometer-sized particles. When isolated nanoparticles are expected to occur in toxicologically significant quantities, additional nanoparticle-specific sizing methods should be considered, e.g., a scanning mobility particle sizer (SMPS). However, these devices measure the thermodynamic equivalent diameter (the diameter of a unit-density sphere having the same diffusion coefficient as the particle of interest), which is count-based rather than mass-based. A particular disadvantage of this method is that thermodynamic equivalent diameter concerns particles with a diameter from a few nanometers to 1 μm. This means only a fraction of the entire mass-based particle-size distribution is actually measured. Such small sub-fraction of the total distribution with large surface area:mass relationship may be amenable to the rate of solubilization. This then may call for a mass-metric again, bearing in mind that the solubilization-rate is linked to surface area. Therefore, the guidance given in the following sections is mandatory for all particle inhalation studies in the absence of robust experimental evidence showing the opposite.

97. The particle size distribution should be determined at least once during a single exposure study for each concentration level using an appropriate method of measurement. For repeated exposure studies targeted exposure levels have to be attained by more elaborate pre-exposures (without animals) for the validation of methods used. For particles in the nano-/micrometer-sized range, a critical orifice cascade impactor or an appropriate alternative real-time method may be considered. When a wide range of concentrations must be covered, cascade impactors with different sampling flow rates may become necessary to provide representative time-weighted average sampling observing the upper specification limit of each stage to prevent particle bouncing and re-entrainment into the next cascade impactor stage. Real-time devices with short sampling periods may be used when temporally and spatially stable and reproducible conditions can be attained. In case where this cannot be met, repeated measurements per exposure session may become necessary. Under reproducible and stable conditions and uniformity of the mass concentrations measured by filter and cascade impactor analyses, measurements should take place at least weekly; however, measurements should be taken daily when marked day-by-day fluctuations in particle size occur. The use of EM is useful for complex and/or
critical morphologies of a test specimen that may affect the outcome of study. This method should be considered at least once during the pre-validation study to demonstrate that the targeted agglomeration state and/or morphology can be gained. Especially for non-isometric solid aerosols or fibres, a more frequent analysis is recommended at least one at the beginning, midterm, and at the end of 28 and 90 day studies. For longer studies, measurements every 3 months may be considered if stable conditions can be demonstrated during the first 3 months of study.

98. Because aerosol particle size distribution determines the site of initial deposition, retention, and clearance in the respiratory tract, the particle-size distribution should allow for exposure of all relevant regions of the respiratory tract. Deposition and/or damage to any region of the respiratory tract may potentially induce toxicity or lethality, so it is not possible to predict, a priori, the most responsive region of the respiratory tract or the most harmful particle-size. The mass median aerodynamic diameter (MMAD) standard for aerosols had been 1-4 µm for acute studies and 1-3 µm for repeated exposure studies, with a geometric standard deviation (GSD or σG) of 1.5-3.

99. Although the standard for acute studies (MMAD of 1-4 µm with a σG of 1.5-3) remains unchanged at this time, the standard for repeated-exposure studies has been changed to accommodate the testing of nano-range aerosols and to enhance deposition in the pulmonary region. The new recommended standard for repeated exposure studies is: MMAD of ≤2 µm with a σG of 1-3. Justification should be provided in the study report if this standard cannot be met, including a description of efforts taken to meet it, such as milling. Although a reasonable effort should be made to meet the acute and repeated-exposure MMAD standards, expert judgment should be provided if they cannot be achieved. For example, electrostatically charged particles, fibrous particles, and hygroscopic materials (which increase in size in the moist environment of the respiratory tract) may exceed these standards. It can also be difficult for aerosols to meet these standards at high concentrations due to the tendency for solid aerosols to agglomerate and for liquid aerosols to coalesce. Especially for particles deemed to be innocuous and biologically “inert,” emphasis should be given to generating particle size distributions amenable to preferentially depositing in the lower respiratory tract.

100. As alluded to above, particle size analyses should, by default, use a mass-based metric that allows for direct comparison with mass-based analytical concentrations. Multistage cascade impactors should be given preference. They should be designed to collect and classify the entire range of particle sizes present in the inhalation chamber that exceed approximately 0.1 µm. To maintain accurate cut-point size ranges for each stage of a cascade impactor, it is imperative that precise air flow rates be maintained throughout the sample collection period. If different air flow rates are used during a collection, new cut-points for stages should be determined. Other devices or physical principles may be used if equivalence to the cascade impactor can be shown (with regard to MMAD and GSD, including the mass concentration sensed) or when required by the nature of the test chemical (e.g., combustion atmospheres, smoke). Particle sizing should also be performed in test atmospheres where condensation aerosols may be formed from vapour atmospheres. For non-adhesive aerosols, such as dry powders, the individual impactor stages should be covered with an adhesive stage coating (e.g., silicone spray) if particle bounce and re-entrainment are expected. For high-concentrations of liquid aerosols, the stages may be covered by an adsorptive filter to prevent run-off of liquid deposits.

101. As shown in Appendix VI, the MMAD of the aerosol collected in the cascade impactor can be calculated. The steps are as follows:

a) Calculate the total mass of test chemical collected in the cascade impactor. Start with test chemical collected on the stage that captures the smallest particle-size fraction (this would be the back-up filter if one is used), then divide this test chemical mass by the total mass found above.
b) Multiply this quotient by 100 to convert to percent. Enter this percent opposite the effective cut-off diameter of the stage above it in the impactor stack. Repeat these steps for each of the remaining stages in ascending order.

c) For each stage, add the percentage of mass found to the percentage of mass of the stages below it.

d) Plot the percentage of the cumulative mass less than the stated size versus particle size using a log probability scale, and draw a straight line that best fits the plotted points (see Appendix VI). Established statistical procedures should be used to achieve the best fit.

e) Note the particle size at which the line crosses the 50% mark. This is the estimated Mass Median Aerodynamic Diameter (MMAD).

f) For calculation of the Geometric Standard Deviation (GSD) refer to the log probability graph used to calculate the MMAD. Provided that the line is a good fit to the data, the size distribution is log-normal and the calculation of GSD is appropriate. Note the particle size at which the line crosses the 84.1% mark and the 50% mark. Calculate the GSD as follows:

\[
GSD = \frac{84.1\% \text{ mark}}{50\% \text{ mark}}
\]

g) Algorithms for the calculation of particle size characteristics have been published (35)(36)(37)(38). A representative analysis of particle size data is shown in Table 1 and Figure 2 (Appendix VI).

102. The mass concentration obtained by particle size analysis should be within reasonable limits of the mass concentration obtained by filter analysis. Equivalence demonstrates that there were no sampling errors (especially an under-sampling of larger particles) or particle losses within the device used to analyze particle size distribution. Non-equivalence in the presence of a highly loaded stage collecting the largest particle size might be taken as indirect evidence for the existence of particles too large to be collected by the device used to analyze particle size distribution.

103. When the aerosol particle size distribution does not appreciably exceed 1 µm, scanning mobility particle sizers, differential mobility analyzers, or mobility particle sizers are preferred for non-fibrous and isometric nanomaterials, both for aerosol exposure particle counts and size distributions. Particle count cannot be balanced when mass-based toxicities occur. Critical orifice cascade impactors deliver the highest degree of information. Micro-orifice uniform-deposit impactors may be used for fibrous materials to determine the exposure concentrations in terms of mass, though fibrous material may obstruct/clog nozzles. Where technically feasible, two different methods of determining quantitative particle exposure (i.e., particle counts, size distribution, or particle mass) should be used. Aerosols produced using nanomaterials often agglomerate. Due to the laws of physics, higher concentrations of nanoparticles tend to coagulate and aggregate. When aggregation occurs, nano-sized non-agglomerated particles cannot be produced without destroying the most likely structure to which humans may be exposed. Transmission electron microscopy of alveolar macrophages should be used in 28 and 90-day studies to ascertain the predominate retained structures. The consistency of these findings and those obtained by particle analyses may provide important additional information to further refine particle analyses.
Aggregate Density

104. Aggregate densities are commonly characterized by mercury pyknometry for porosimetry according to the ISO-Standard 15901-1 (2005). This standard delineates that different pores can occur as either apertures, channels, or cavities within a solid body or as space (e.g., interstices or voids) between solid particles in a bed, compact or aggregate (ISO 15901-1, 2005. Pore size distribution and porosity of solid materials by mercury porosimetry and gas adsorption -- Part 1: Mercury porosimetry. Available at: http://www.iso.org/iso/home/store/catalogue_tc/catalogue_detail.htm?csnumber=32620)

105. Particle deposition within the respiratory tract may be estimated by using the Multiple-path Particle Model MPPD v3.04 (or higher). This software was developed by Applied Research Associates, Inc. and The Hamner Institutes for Health Sciences (formerly CIIT and CIIT Centers for Health Research), in collaboration with the National Institute of Public Health and the Environment (RIVM), the Netherlands, and the Ministry of Housing, Spatial Planning and the Environment, the Netherlands, and can be downloaded free of charge. This software calculates relative aerosol deposition percentages in the nasopharyngeal, tracheobronchial, and pulmonary (alveolar) airways in rodents and humans. This software may also be used to calculate the accumulated lung burdens when the lung burden-dependent elimination half-time is known.

INSTILLATION AND ASPIRATION STUDIES

Instillation and Aspiration Exposure Techniques

106. For hazard identification and screening purposes, animals may be dosed with test chemicals by intratracheal instillation or aspiration. These exposure techniques are particularly useful for testing nanoparticles and fibres, and they offer a way to rapidly screen the hazard of nanomaterials for the ranking of relative toxicities between materials. While the use of instillation or aspiration exposure is a simple and inexpensive way to screen test chemicals and to compare the relative toxicity of multiple chemicals, it may be necessary to account for numerous experimental factors and artifacts. Most notably, the exposure methods—test chemical delivery directly to the respiratory tract as a suspension—and delivered dose kinetics do not replicate normal respiration. During inhalation, particles may be deposited throughout the entire respiratory tract, including the nose and lungs, allowing for a slow build-up of the dose and for normal clearance processes to occur. By contrast, instillation bypasses the upper respiratory tract and results in different sites of pulmonary deposition, exposure kinetics, and clearance mechanisms/rates. Instillation results in exceedingly high dose and application rates over a short period of time. The use of vehicles, such as saline, and surfactants add an additional level of uncertainty since the defense systems of the lung can be affected. It should be demonstrated that controls exposed solely to the vehicle do not differ to any appreciable extent from negative controls. Because nanomaterials tend to agglomerate in aqueous media, rigorous dispersion measures are required prior to instillation. Analytical procedures should be applied to demonstrate that major changes in the morphology of the test material or partial dislodgement of more soluble constituents can be ruled out. It is for these reasons that data from instillation and aspiration studies cannot be used in human risk assessments.

107. A review that compared instillation and inhalation studies with the same initial lung burden revealed a similarity in induced pulmonary inflammation and related factors such as cell analysis, chemokine, proinflammatory cytokine, and oxidative stress in BALF (Morimoto et al., 2016). There was also a tendency for the inflammatory response in the instillation studies, as well as a delay in clearance from the lung, to be greater than or equal to that seen in inhalation studies. This suggests that instillation studies may be sufficiently sensitive to be useful for screening the hazard of test chemicals. Nevertheless, GD 36 (OECD, 2012) recommends that instillation/aspiration studies should be backed
up by an appropriate and representative inhalation study. Further technical guidance on instillation and aspiration can be found in GD 36 (OECD, 2012) and elsewhere (48, 49, 50) (Nakanishi et al., 2015).

While the methods used in inhalation toxicity studies are highly controlled and standardized, alternative dosing methodologies have numerous variables that can be difficult to control. For example:

- Sample preparation: Test chemicals must be dispersed in a vehicle, and stable suspensions can only be achieved in the presence of additives. High-energy dispersion may be conducive to solubilization of poorly soluble constituents, and disagglomerated particle structures may reagglomerate prior to dosing. Especially when testing nanomaterials, this can result in agglomeration sizes that may not be encountered in an inhalation study. These circumstances require additional verification of the soluble : insoluble fractions of poorly soluble instilled particles. Similarly, whole lung microscopy is needed, preferably confocal laser microscopy, to account for agglomerated structures adhering to the intraluminal surfaces of the lung that are too large to undergo phagocytosis relative to those being engulfed by alveolar macrophages. When pulmonary toxicity is typified by non-inhalable structures, alternative dosing methods should be replaced by inhalation studies.

- Dose volume: The instillation volume should ideally be in the range of 1 mL/kg-bw but should not exceed 2 mL/kg-bw. Data are required to demonstrate that the instillation procedure does not produce marked elevations in BAL-endpoints (e.g. total protein, LDH, PMN, and total cell counts) relative to sham controls (air exposure only).

- Instillation procedure: Instillation methodologies may vary from one laboratory to another. Once anesthetized, the animals are placed on an angled board by hanging the upper incisor teeth on an incisor loop of 45° to <90° (supine head up). Injury to the epiglottis can be prevented by using an otoscope fitted with a speculum. A mechanical laboratory animal ventilator should be fitted to the instillation device for about 1 minute at an appropriate tidal volume and frequency to allow for a re-distribution of the instilled bolus within the airways of the lung.

- Accounting for lung dead space: The anatomical dead-space of the lung is approximately 30% of the total lung volume. Hence, it cannot be ruled out that this fraction of volume is not retained in the lower airways and will rapidly be cleared from the airways into the GI-tract. Notably, this retained fraction is highly dependent on the instilled volume and instillation method applied. Therefore, calculated (nominal) total lung doses should be used to empirically verify total lung burden. Measurements should be made on the first post-administration day.

- Inadvertent lobar dosing: Method-specific inadvertent lobar dosing may produce heterogeneous dosing patterns. Accordingly, estimations of lobar lung burdens and back-calculation to total lung burden measurement is discouraged.

- Aspiration-induced alveolar inflammation: In aspiration studies of nanoparticles, there is anecdotal evidence, which is not reported by all laboratories, that alveolar inflammation induced by bacterial rinsing has been an undesired effect of pharyngeal aspiration in rats (OECD, 2012).

- A lack of vehicle-induced alveolar inflammation: In the mouse, there are numerous published studies demonstrating a lack of alveolar inflammation in vehicle-exposed mice after pharyngeal aspiration of nanoparticles. With regards to the unusually high doses to bronchioles, this may
likely be an observation related to lack of adequate particle dispersion in the lung and/or morphology of a particular nanoparticle, and not a general effect of pharyngeal aspiration (OECD, 2012).

- Variation between laboratories: As opposed to inhalation methods, retained lung burdens in instillation studies can only be judged by nominal settings which may vary from one laboratory to another. This shortcoming can be overcome by lung burden measurements shortly after and 1 day after instillation/aspiration.

- Alternative dosing techniques: When alternative dosing techniques are used in repeated administration studies, a non-treated group is required to demonstrate that the dosing procedure did not affect the susceptibility of animals.

Dose Selection in Instillation and Aspiration Studies

109. The alveolar surface is lined with a complex and highly surface-active material—the pulmonary surfactant. Surfactant is a naturally occurring complex of phospholipids, neutral lipids, and several specific proteins secreted by type II pneumocytes. When depleted of surfactant by the adsorptive forces of high surface area nanoparticles, the alveolar surface of the lung develops a marked increase in surface tension, which causes the lung to become very noncompliant and to collapse at low transpulmonary pressure. Hence, any marked disturbances of the surfactant system, either by noxious agents or by excessive doses or dose-rates of particulates that adsorb constituents from surfactant, will inevitably result in a compromised surfactant layer with increased permeability of the air-blood barrier and changes in lung mechanics. It is of ultimate importance to consider that a high dose rate can cause instant lung damage when the rate of surfactant reconstitution by the type II pneumocytes is overwhelmed. The lung is morphologically structured for the exchange of air but not to handle large amounts of fluids or solids dosed directly into the lung or designed to bypass its tracheobronchial clearance system. A better option is judiciously designed repeated exposure inhalation studies with a focus on not overwhelming the intricate lung physiology. Doses and dose-rates delivered to the lung must be low enough to not mask substance-specific outcomes by derailed compensatory responses. Instillation studies of poorly soluble low-toxicity particles, whether micro-sized or nano-sized, do not generate simple relationships of dose and toxicity that are readily applicable for concentration-selection in repeated exposure inhalation studies (48). Because poorly soluble substances are handled by the lung using common kinetic principles, the selection of test concentrations requires a full understanding of the physiological limits and mechanisms of the clearance of particles from the lung in order to generate meaningful data for human risk assessment (51) (52).

110. For single and repeated exposure studies, the decision trees in Appendix III and IV should be considered. In case the test chemical is an upper respiratory tract irritant or has corrosive properties, a sighting study on the non-lethal endpoint of “reflex-bradypnea” (see Appendix V) is recommended to minimize undue distress to experimental animals. For potentially corrosive substances, this method is recommended for justifying the selection of exposure concentration in repeated exposure studies. The threshold causing lower respiratory tract irritation up to the MTC can readily be probed in animals exposed for 6-hours with bronchoalveolar lavage the following day (53) (54) (55) (56) (57). Such pre-study prevents the occurrence of ‘acute-on-chronic’ inflammatory exacerbations and sets a frame of concentrations to minimize irritant-related distress in experimental animals.
ANIMAL OBSERVATIONS

Clinical Signs

111. Animals should be observed frequently during the exposure period. Observation may be hindered when animals are in nose-only restraining exposure tubes, however. Following exposure, careful clinical observations should be made at least twice on the day of exposure, or more frequently when indicated by the animals’ response to treatment, and at least once daily thereafter during the post-exposure period. Additional observations are made if the animals continue to display signs of toxicity. In repeated exposure studies, clinical observations should be made prior to and after exposure and once a day on exposure-free days. Observations include changes in skin and fur, eyes and mucous membranes, and also respiratory, circulatory, autonomic and central nervous systems, and somatomotor activity and behavior patterns. Attention should be directed to observations of tremors, convulsions, salivation, diarrhoea, lethargy, sleep and coma. Rectal temperature measurements may provide supportive evidence of reflex bradypnea or treatment-/confinement-related hypo-/hyperthermia. Signs suggestive of mild neurotoxicity may be more difficult to observe in nose-only restrainers than in whole-body chambers. Guidance on clinical signs can be elsewhere (38) and objective measurements that are indicative of impending death and/or severe pain and/or distress are available in OECD Guidance Document No. 19 (24).

112. The duration of the observation period is not fixed, but should be determined by the nature and time of onset of clinical signs, the length of the recovery period, and the endpoints chosen, e.g., sequential analyses interrelating time-course changes in BALF and lung burden. The times at which signs of toxicity appear and disappear are important, especially if there is a tendency for signs of toxicity to be delayed. All observations are systematically recorded with individual records being maintained for each animal. Animals found in a moribund condition and animals showing severe pain and/or enduring signs of severe distress should be humanely killed for animal welfare reasons. When animals are killed for humane reasons or found dead, the time of death should be recorded as precisely as possible.

113. Care should be taken when conducting examinations for clinical signs of toxicity that initial poor appearance and transient respiratory changes, resulting from the exposure procedure, are not mistaken for treatment-related effects. Animals killed in a moribund state are considered in the interpretation of the test results in the same way as animals that died on test. Some test chemicals may have effects with delayed onset, such as an obliterating bronchiolitis. Animal welfare aspects, and the likelihood of scientific misjudgment, need to be carefully balanced. Expert judgment is needed to justify the respective procedure.

Body Weight

114. Individual animal weights should be recorded on the day of exposure prior to exposure (day 0), and at least on days 1, 3, and 7 (and weekly thereafter), and at the time of death or euthanasia if exceeding day 1. In repeated exposure studies body weights should be recorded twice weekly (e.g., on Mondays and Fridays) during the exposure period, and once a week during the recovery period. Surviving animals are weighed and humanely killed at the end of the post-exposure period. Animals should be observed for a minimum of 14 days. Extended observation periods may be necessary if toxic effects fail to reverse or are delayed in onset. A sustained decrement in body weight is recognized as a critical indicator of moribundity and should therefore be closely monitored. At the end of the test, surviving animals are weighed and then humanely killed.
**Pulmonary Function, Reflex Bradypnea, and Lower Respiratory Tract Reflexes**

115. Reflex bradypnea, due to sensory irritation in the upper respiratory tract, and the apnea reflex (Painal reflex), due to sensory irritation in the lower respiratory tract, are protective reflexes that allow rodents—but not humans—to significantly reduce their exposure to inhaled sensory irritants. The potential impact of these reflexes on human health risk assessments has not received the attention it deserves from toxicologists and risk assessors, largely because testing guidelines have not required examination of pulmonary function and body temperature.

Reflex bradypnea is initiated by stimulation of trigeminal nerves in the mucosa of the upper respiratory tract and eyes. It is triggered by water soluble irritants such as aldehydes, ammonia, isocyanates, and pyrethroids. It is manifested by immediate decreases in the metabolic rate, CO₂ production, and demand for oxygen. This is followed by rapid decreases in respiratory rate (breaths/minute), body temperature (as much as 11°C in rats and 14°C in mice), minute volume, heart rate, blood pressure, and activity level. Reflex bradypnea also results in decreased blood pO₂ and pCO₂ and increased blood pH. Irritants with low water solubility can trigger the Painal or apnea reflex by stimulation of vagal C-fibres in the lower respiratory tract. Rodents exposed to irritants such as ozone and phosgene initially have apneic pauses between breaths, which are then followed by rapid, shallow breathing, bradycardia, hypotension, bronchoconstriction, laryngospasm, airway mucus secretion, bronchial and nasal vasodilation, and hypothermia. Hence, such highly rodent-specific reflex-mediated nociceptive changes may be misinterpreted as systemic toxic substance-induced effects. Dosimetric adjustments from reflexively stimulated rodents to humans utilizing ‘default respiratory parameters’ may become increasingly incorrect. Prior knowledge of the concentration-dependent occurrence of these reflexes provides a sound means to select exposure concentrations that will not be confounded by undue irritation. Methodological details can be found in Appendix V.

**Pathology**

116. All test animals, including those which die during the test or are removed from the study for animal welfare reasons, should be subjected to complete exsanguination (if feasible) and gross necropsy. Necropsies should be performed as soon as possible. If a necropsy cannot be performed immediately after a dead animal is discovered, the animal should be refrigerated (not frozen) at temperatures low enough to minimize autolysis. All gross pathological changes should be recorded for each animal with particular attention to any changes in the respiratory tract. Determination of lung weight and microscopic examination may be considered for organs showing evidence of gross pathology in animals surviving 24 or more hours. Microscopic examination may also be considered for the respiratory tract if it is likely to be affected because it may yield useful information, such as evidence of irritation. For test chemicals that may cause tissue destruction at the site of initial deposition within the respiratory tract, microscopic examination of the entire respiratory tract should be considered. Tissues should be adequately fixed and the examination should include sections of the nasal tissues, larynx, trachea, main bronchi and lung lobes. Microscopic examination of these tissues may provide useful information on the test chemical’s pattern of deposition within the entire respiratory tract and mode of action. Further information on pathologic evaluation can be found in GD 125 (OECD, 2000).

**Bronchoalveolar Lavage (BAL)**

117. Bronchoalveolar lavage (BAL) probes the intra-alveolar lumen and surfaces of the lung, which are in intimate contact with both deposited and retained particles. The evaluation of bronchoalveolar lavage fluid (BALF) may provide useful early and quantitative information for inhaled dose (concentration x time)-related changes. BALF can suitably be used for dose-response and time-course
analyses to probe for processes reflecting substance-specific adversity and/or the response of the lung to injury. The evaluation of BALF has been used successfully for decades in rats exposed to lower respiratory tract irritant gases and aerosols (53) (54) (55) (56) (57) as well as to toxic and low-toxic soluble and poorly soluble particles (61) (62) (63) (64) (65) (66) (67) (68). BALF evaluation has become a common means to evaluate pulmonary inflammation and damage in rats, mice, and guinea pigs (69) (70) (71). In all these species, BALF endpoints have been shown to be highly reproducible within and among different laboratories (72) (73) (74) (75) (76).

118. A major advantage of evaluating BALF parameters is that they provide quantifiable and objective measures allowing continuous data to be expressed as means ± standard deviation/error. Hence, data sets can readily be compared subjected to statistical and benchmark analyses. In contrast, classical histopathological evaluation of lung response is categorical and employs scoring for severity (as a reflection of an intensity and abundance or distribution) and incidences on a non-continuous scale (e.g., 1-4 or 1-5). Because scoring is subjective, variation is seen among pathologists. In addition, BAL provides a simple means to probe the luminal site of the alveoli and obtains an integrated measure of inflammation and damage responses from the lung. Standard fixation techniques used for pathology might dilute changes occurring in the alveoli. For these reasons, significant changes in BALF parameters can be appreciated better and more focused as compared to pathological changes in this particular region of the lung. For example, in a 6 month inhalation study of rats exposed to crystalline silica, statistically significant elevations in BALF neutrophils, LDH activity, and albumin level were reported at earlier exposure time points and lower lung burdens than for histopathological indicators of inflammation and lung damage (69). The ability of BALF evaluation to provide reproducible and quantifiable information on dose and time dependence of particle-induced lung injury and inflammation is of great value for quantitative risk assessment and determination of relationships between nanoparticle potency and physicochemical properties. Specific information on animal numbers and the timing of BAL in repeated exposure studies can be found in TG 412 and TG 413 and also in paragraphs 71 and 72 of this guidance document.

**Bronchoalveolar Lavage (BAL) Method**

119. Bronchoalveolar lavage is applied either *in situ* or using excised wet lungs. Although the latter method allows weighing of the total lung to identify an imbalanced distribution of fluids in the lung, the testing schemes used in TG 412 and 413 make this impossible since the right and left lungs are processed separately. For irritants and also low-toxicity poorly soluble substances, BALF evaluation provides an invaluable means to judge the overall significance of findings, including those from histopathology. Partial lung lavage requires ligation of the bronchus leading to the left lung lobe or the right lobes. When the right lung is used for lavage, the left lung lobes can be spared for additional examinations, such as weight and histopathology without increasing animal numbers; however, with the trade-off of higher variability of data. TGs 412 and 413 describe performing BAL on the right lung using either 5 or 10 animals/sex/concentration in the main study. Rats from repeated inhalation studies are examined at the end of the exposure period, followed or not by serial sacrifices during the recovery period.

120. A laboratory should follow its standard operating procedures when performing BAL. Typically, the rat lung is carefully lavaged with the same lavage fluid at least 2 times with about 5 ml saline or adjusted medium per lavage cycle; that is, the same volume is instilled and retrieved at least twice to prevent excessive dilution. The instillation pressure and number of lavage cycles should be thoughtfully selected to avoid epithelial desquamation or increased counts of red blood cells (RBC) due to high washing forces. Cells are commonly centrifuged at 800 xg at 4 °C for 10 min. After re-suspension in an appropriate medium, cytospots of adequate cell density are prepared (e.g. 2 x 10^5 per cytospot) and differentiated by light microscopy (at least 300 cells per cytospot). The wash-out is
collected and the retrieved pooled fluids should not be lower than ≈80%. For cytological analyses, cells in the BAL-fluid are counted using an automatic cell analyzer. An analyzer may or may not count all cells, including the epithelial or red blood cells. Vigorous lavage increases an analyzer’s RBC count. Conversely, manual cytology does not consider this as cells to be incorporated in the balance, taken as non-existing. This percentage is then used to back-calculate to absolute counts:

\[ \text{Absolute PMN count} = (\text{total cell count} + \text{RBC}) \times \text{PMN percentage (RBC not counted)} \]

**Bronchoalveolar Fluid (BALF) - Mandatory and Optional Parameters**

121. Mandatory BALF parameters include lactate dehydrogenase (LDH); total protein or albumin; total leukocyte count, absolute cell counts and calculated differentials for alveolar macrophages, lymphocytes, neutrophils, and eosinophils. Abnormal alveolar macrophages (e.g. those with foamy appearance) should be accounted for as well. Aliquots of the acellular supernatant are examined with an automatic analyzer for total protein or albumin, and lactate dehydrogenase (LDH).

122. Optional BALF parameters may include cytokines, chemokines, and mediators, alkaline phosphatase, and β-N-acetyl-glucosaminidase or β-glucuronidase. For irritant test chemicals γ-glutamyltransferase may be useful to identify changes in Clara-cell activity. Soluble collagen may be a sensitive endpoint for probing an increased alveolo-capillary permeability \(^{56}\). Selected pro-inflammatory factors may be considered to better understand the likely source and cause of elevated endpoints.

**Presentation of Bronchoalveolar Lavage (BALF) Data**

123. Cells should be presented as total leukocyte count, absolute cell counts, and calculated differentials per lung or animal. Biochemical endpoints in the supernatant of the retrieved BAL-fluid should be normalized to the volume of the retrieved BAL-fluid. Pro-inflammatory factors determined in BAL-fluid may actually originate from a subclass of BAL-cells. The analysis of BAL-data requires careful consideration as to whether concentration-dependent changes originate from any localized injury or secondary outcomes, for instance, a decreased retrieval of BAL-fluid, an increased BAL-cell count, or increased exudation from capillary vessels.

124. A sizable body of information exists relating changes in BAL-cells and mediator levels from animal assay to humans and vice versa \(^{77}\). The consideration of this interrelationship may facilitate the translation of rat BAL data to humans. It is worth noting that, human BAL data have been used to support the decision making process for the regulation of ambient ozone levels \(^{78}\). Depending on the nature of the test chemical, it may be productive to consult published evidence in order to facilitate the extrapolation of data from animal bioassays to humans and vice versa.

**Lung Burden and Local Area Lymph Node (LALN) Burden Measurement**

126. Lung burden measurements performed in the course of repeated exposure studies in rats may help understand the toxicity of poorly soluble particles. This endpoint integrates all exposure-related variables of a study and is considered to be the all-encompassing metric of cumulative dose. However, each cumulative lung burden may have a different kinetic history due to burden-specific changes in clearance. A wealth of information is available comparing modeled and empirically determined lung burdens \(^{86-101}\). Lung burden measurements can be supplemented by time-course measurements of particles into the lymph nodes that drain the lungs, e.g. the lung-associated lymph nodes (LALN) and the liver, i.e., organs physiologically committed to sequestering particles from the lung or the systemic circulation. From these analyses, inhalation toxicologists deduce the
proportionality of accumulated lung burdens and exposure concentrations as well as the kinetic cornerstones on which substance-specific and particle overload are expected to occur.

127. Despite the use of a wide range of methods to generate and characterize exposure atmospheres, lung burden data provide a means to compare studies with different designs across laboratories. Most methods used to determine metals, metal salts, and oxides in lung tissue use destructive methods to ash the lung in order to attain soluble analytes (metal ions) with well-defined oxidation states. Carbonaceous isometric materials (carbon black) are also analyzed in digested lung tissue using light scattering methods. With the advent of complex isometric and tubular structures, the analytical procedures have been considerably refined, ranging from EC/OC analysis (Thermal-Optical Analysis for Organic & Elemental Carbon) to trace-spiked procedures. For metals the AAS (atomic absorption spectrometer) in graphite mode may be used. To date, the AAS method is replaced by the ICP-OES (Inductively Coupled Plasma – Optical Emission Spectroscopy) analysis. All methods are somewhat specific the type of test substance examined (48)(80)(81)(86)(102-107). For isometric test materials, data are normalized to the total organ (lung) or total wet organ weights of LALN and liver. For the more complex non-isometric tangled structures of nano-fibres and nano-tubes and structures substance-specific morphological characteristics have to be accounted for (80)(81).

128. As with all analytical methods, substance specific validation is required. Excised lungs must be spiked with well-characterized test material to establish a reference curve of any representative analyse exactly duplicating the processes of digestion, extraction, dilution, and analysis. The total recovery should be as high as experimentally feasible. The metric of calibration is determined by toxicological needs, i.e., mass-, count- and/or structure-based. Although it is beyond the scope of this guidance document to present any specific methodology of digestion and analysis, the following example demonstrates the principle course taken: The exsanguinated lung and excised LALNs from the hulus region are weighed and then the total (ideal, least error-prone condition) organ is digested in inorganic acids and microwaved. Aliquots are then used for analytical determinations. Procedural blank solutions and a standard solution of a well-specified analytical reference is used as analytical standard.

The Value and Utility of Lung Burden Measurements in Risk Assessments

129. Animal studies have routinely demonstrated adverse lung effects, and sometimes systemic toxicity, upon inhalation exposure to various poorly soluble particles, including nanoparticles. These effects include inflammation, oxidative stress, fibrosis, and carcinogenesis.

131. The U.S. National Institute for Occupational Safety and Health (NIOSH) conducted a qualitative risk assessment for carbon nanotubes (CNT) based on pulmonary effects (NIOSH, 2013). Although the NIOSH assessment was for carbon nanotubes, the general principles are relevant to any solid particles that are retained in the lungs. The NIOSH risk assessment provides an example of how lung effects, along with lung burden, can be used for the risk evaluation of nanomaterials tested with TG 413. Even though the risk assessment used estimated lung burden, a similar process would be applicable when lung burden is measure in the animal study. The process consists of the following general steps, which are described in detail in NIOSH (2013) and Oberdoster et al. (2015).

a. Evaluation of available hazard and dose-response data in animals and identification of adverse health effects that are associated with exposure to a nanomaterial.

b. Selection of a dataset that best describes the relationship between exposure to a nanomaterial and pulmonary effects in animals that are relevant to humans.

Comment [RIVM9]: This section (par 129-137) is for the largest part almost literally similar to Document#1 that has been discussed at the Arlington meeting. Document#1 was rather a discussion document than a guidance document. It does not provide guidance how in general lung burden measurements should be taken into account for risk assessment. Several questions were asked and doubts were raised during the discussion in Arlington that are not addressed in the present text. Among others:

- Reference is only made to CNT; its relevance for other nanomaterials (or PSP in general) was questioned.
- It is stated that lung deposition may be altered in various pathological states. This may imply that deposition is different for each exposure concentration since toxicity will vary in type and severity of effect between exposure concentrations. How should this be accounted for in the interpretation of lung burden and subsequently in risk assessment?
- The text is not in accordance with the TCG. For instance, paragraph 132 c mentions that lung burden measurements are required to distinguish between a highly soluble, semi-soluble and poorly soluble substance. However, since LB measurements are only performed for PSPs this paragraph is redundant.

Comment [GA10]: Debate on whether the CNT case may be applicable to other NM in granular form.

Comment [RIVM11]: Delivered or retained dose at the end of the last exposure day?

Comment [RIVM12]: So what are the parameters that describe the effects? LDL? PMN influx? Fibrosis severity?
c. Estimation of the total deposited lung dose (measured lung burden would be used in this step when collected in an animal study, such as TG 413).

d. Animal-dose response modelling to estimate critical effect level.

e. Calculation of human equivalent dose and working lifetime exposure for risk estimation.

132. When pulmonary effects are driving the human health risk assessment, it is assumed that the lung effects are associated with the total dose deposited and retained in the lungs (step c above). In the absence of measured lung burden data in an animal study, a risk assessor may use the following equation to estimate the lung dose. The calculation takes into account the exposure concentration and duration as reported in the animal study, the species-specific minute ventilation, and the alveolar deposition fraction (Pauluhn and Rosenbruch, 2014). This equation assumes constant minute volume (MV) and body weight (bw). Because it considers linear cumulative C × t dosing but zero clearance, it will likely yield an exaggerated lung burden:

\[
\text{Lung burden} \left(\frac{\mu g}{\text{rat lung}}\right) = \text{conc.} \left(\frac{\mu g}{L}\right) \times MV_{\text{rat}} \left[\frac{L}{6 \text{ hrs}}\right] \times \text{alv. fraction}_{\text{MPPD2.11}} \times \text{bw} (kg)
\]

133. There are several uncertainties when estimating lung burden instead of measuring in an animal study:

a) Actual values for minute ventilation in rodents are variable and differ greatly in the literature due to laboratory-specific experimental variables. In addition, some particles may evoke reflex bradypnea, causing minute ventilation to be significantly lower than literature values. However, most inhalation studies employing the above equation to calculate lung burden use literature values for minute ventilation and do not measure minute ventilation throughout the inhalation exposure period, because this would require specialized instrumentation and is labor intensive.

b) An estimation of lung deposited dose assumes no clearance kinetics during the inhalation exposure (e.g., a 90-day exposure). One could over-estimate lung burden in a repeated exposure study if, for example, test particles are semi-soluble. A study director can use a range-finding study to identify whether particles are highly soluble, semi-soluble, and poorly-soluble.

c) Estimation of lung deposited dose assumes no clearance kinetics during the inhalation exposure.

134. Given these uncertainties, an inhalation risk assessment of retained particles is strengthened by using actual measurements of lung burden rather than estimations of lung burden. This has been acknowledged in the scientific literature. One recent review paper by Oberdoster et al. (2015) discusses the importance of collecting lung burden measurements at the end of inhalation exposure and following the exposure. Such measurements support human health risk assessment by understanding exposure-dose-response relationships and clearance kinetics of retained particles.

135. Oberdoster et al. (2015) also discussed the value of post-exposure recovery or observation...
periods for subacute, subchronic, or chronic inhalation exposures. Such information provides evidence about the clearance of the retained lung burden and the progression or reversibility of effects observed at the end of the exposure (Oberdoster et al., 2015).

136. Although lung burden measurement is mandatory at only one post-exposure observation period in Option B (at PEO-1), three or more lung burden measurements may be needed to provide information on clearance kinetics and persistence/progression response, especially for poorly soluble particles. For example, one could measure lung burden 1 day post-exposure (PEO-1), 7-14 days post-exposure (PEO-2), and 90 days post-exposure (PEO-3), or whatever sampling times a study director considers appropriate for a given test chemical. This would allow evaluation of the clearance rate from the conducting zone as well as from the respiratory zone. Suggested sampling times for semi-soluble soluble particles with no persistent pulmonary effects could be much shorter, or perhaps measurement only at PEO-1 may be sufficient. A study director may use data from a range-finding study to determine the appropriate post-exposure duration as well as the optimal number and timing of sampling intervals for a repeated exposure inhalation study.

137. Tabulated lung burden data should be provided in the study report. When feasible, lung burden measurements should be used for concentration-response modelling (e.g., BMC). Details of the statistical analysis should be included in the study report.

Preparation of Lung Homogenates

138. While BALF analysis primarily probes for processes occurring within the terminal intralveolar region, analyses in lung homogenates may provide a better reflection of changes occurring in lung tissue. Thus, the determination of biomarker of tissue remodeling or fibrosis, including genomics, may be better probed by this analysis. Flash frozen (in liquid nitrogen), total, or right lung tissues are homogenized on ice in an appropriate buffer for a short duration (anti-proteolytic substances may be added) and centrifuged. Selected proteins can then be measured in the supernatant.

Animal Welfare – Bronchoalveolar Lavage, Lung Burden, and Histopathology

139. BALF measurements are required for all gases, vapours, and aerosols. Lung burden measurements are not required for soluble aerosols, but are mandatory for what are known or likely to be poorly soluble particles as determined during a range-finding study. Although the addition of satellite groups for lung burden measurements requires more animals than the 2009 versions of TG 412 and TG 413, adding bronchoalveolar lavage to TG 412 and TG 413 should have no impact on animal welfare or usage because it does not affect the normal course taken for euthanasia and necropsy procedures. This is because the lungs, which would have been used solely for histopathology, can be shared for both procedures, e.g., typically the left lung for histopathology and the right lung for BAL.

140. The practicality of evaluating histopathology, BALF, and lung burden in the same animal has been considered as a way to reduce animal usage. This procedure requires the use of separate lung lobes for evaluations of histopathology (right apical, intermediate, and cardiac lobes), BALF (left lung), and lung burden (right diaphragmatic lobe). This procedure would be very labor intensive, requiring technical skill to tie off not only the right main bronchus prior to lavage of the left lung but also the lobar bronchus to the right diaphragmatic lobe prior to fixation of the other right lobes. Although technically feasible, the practical feasibility of this procedure when processing many rat lungs at a time has not been evaluated in the current literature, nor validated. A loss of animal data due to technician error could result in a repeat study and the use of more animals than are saved by using this procedure. Another concern is that only by using the diaphragmatic lobe of the right lung (27.9% of the inhaled volume or deposited particle load), as opposed to the whole right lung, might the
particle load approach the detection limit for quantification at low exposures. For all these reasons, the same animal should not be used for three evaluations—histopathology, BALF, and lung burden.

Toxicokinetics

141. Although toxicity testing is aimed at characterization of a test chemical’s toxicity, the inclusion of toxicokinetic measurements in an inhalation study might provide valuable information for multiple reasons. The inclusion of toxicokinetic parameters may be considered when 1) they can inform and enhance the conduct of additional inhalation toxicity studies and/or a risk assessment, 2) there is no interference with the outcome of an inhalation toxicity study, and 3) there is preferably no use of additional animals. The suggestions provided here are guidance for gases, vapours, and soluble particles. Particles that are poorly soluble and likely to be retain in the lungs require a different approach, i.e., an emphasis on lung burden measurements for the understanding of lung clearance kinetics.

142. Several aspects need to be taken into account when considering inclusion of toxicokinetic measurements in an inhalation study. While blood can be collected from the tail vein during nose-only exposure, sampling can only be performed at the end of whole-body exposures, which limits the possibilities of informative toxicokinetic measurements. Further, the possible effect of blood sampling on the outcome the study shall be taken into consideration. For example, blood sampling in studies that include a Functional Observational Battery (FOB) may affect the animal behavior and is therefore not recommended.

143. Toxicokinetic information obtained in a range-finding study may support the design of follow-up studies by providing useful information to determine concentrations for the main study (OECD 417).

144. Information on toxicokinetics can contribute to the refinement of a risk assessment by providing a better understanding of the relationship between external exposure and observed toxicity. The information may also contribute to a better-founded extrapolation of the animal data to humans and thereby improve human hazard and/or risk assessment (OECD 417; Brandon et al., 2015). Internal concentrations of the parent compound and/or metabolites may contribute in the identification of the toxic agent and thus in the understanding of the mode of action.

145. OECD TG 417 considerations are applicable when including toxicokinetics in a toxicity test. The inclusion of toxicokinetic parameter measurements in blood samples can provide estimates of basic parameters for the test chemical, such as $C_{\text{max}}$, $T_{\text{max}}$, elimination half-life ($t_{1/2}$), and area under the curve (AUC). The volume and number of blood samples that can be obtained per animal will be limited by potential effects of repeated sampling on animal health/physiology and/or the sensitivity of the analytical method. Samples should be analyzed for each individual animal. In some circumstances (e.g., metabolite characterization), it may be necessary to pool samples from more than one animal. Pooled samples should be clearly identified and an explanation for pooling provided.

146. Toxicokinetics should be considered for chemicals that accumulate in the lung or translocate into specific accumulating organs following repeated exposures. The accumulated dose is partly a function of clearance. The low molecular weight chemicals typically examined in inhalation studies as aerosol or vapour are soluble enough to gain access to the systemic circulation with rapid metabolism and elimination. This prevents any marked carry-over of inhaled doses from one exposure to another. Although ‘biomarkers of exposure’ may be of interest for facilitating the dosimetric adjustment from animals to humans, the measurement of such specialized endpoints is beyond the scope of the guidance document.

147. However, the design of repeated inhalation studies with poorly soluble, low toxicity particles depends on the retention kinetics of the test chemical in the lung. The clearance of such aerosol, which
is mediated by alveolar macrophages and the exposure level, may not be fast enough to prevent their time-dependent accumulation in the lung. Particle clearance may be further slowed as a result of increasing lung burdens and particle residence times in the lung. This aggravates any cumulative particle dose-specific, and not necessarily test chemical-specific, pulmonary inflammation. Therefore, the design of repeated inhalation studies with these types of solid aerosols needs to be targeted so that the accumulated lung burden does not jeopardize the physiological functions of the lung to an extent that would preclude any meaningful translation to humans. This objective is achieved by appropriate range-finding studies revealing the coherence of simulated and kinetically predicted data. Exposure concentrations should be selected to cover the entire range of lung burdens, i.e., those which do not delay clearance up to those which produce a reasonable delay of particle clearance. Alveolar macrophages are typically the dominant pathway of elimination of poorly soluble particles from the lung. For non-overload conditions, elimination half-times of particles retained in the lung are in the range of $t_{1/2} = 60$ to 90 days. Accordingly, post-exposure periods should not be shorter than one generic elimination half-time at non-overload conditions. Lung burden and respirable particle size are interrelated. Therefore, attempts should be made to maximize lung burdens while maintaining a GSD large enough to expose the entire respiratory tract (34).

148. Poorly soluble, low toxicity micrometer particles or nanoparticles are deposited in the alveolar region and are retained by endocytic uptake within the dynamic pool of alveolar macrophages. As deduced from a wealth of experimental data from rats, this pool of macrophages in the alveolar region increases homeostatically with increased particle burdens. The accumulated 'displacement volume' of particles exceeding 6% of the pooled volume of alveolar macrophages represents the POD at which this type of 'kinetic lung overload' occurs. This term is used to define the volume of assembled structures displaced by particles within the pool of alveolar phagocytes. It is important to recall that this pool is controlled by cell number but not by increased volume of individual macrophages. This POD is empirically verified by increased total cell counts retrieved in bronchoalveolar lavage fluid (BALF).

149. In case evidence of facilitated or enhanced dissolution exists, a $t_{1/2} < 60$ days is a likely outcome and would challenge any "poorly soluble" particle paradigm (Figs. 2 and 3) (51). Depending on the kinetics of dissolution, a $t_{1/2} \approx 1$-3 days may be envisaged in a 1-week (5 days x 6 hours/day) study in rats exposed to micrometer-sized ZnO (84) whereas a $t_{1/2} \approx 17$ days could be deduced for minimally soluble CeO$_2$ (85) as illustrated in Figure 2. Dissolution of CeO$_2$ may occur following deposition on the protein-rich, wet surfaces anywhere within the airways of the lung (pulmonary and tracheobronchial regions). However, the endocytosis of particles by alveolar macrophages is restricted to the pulmonary region. With increased dissolution, scavenger proteins may become saturated. This then thermodynamically stabilizes the particle phase. The mechanisms determining the dissolution rate change progressively towards kinetic processes (51). This explains why the solubilized fraction is essentially the same to entirely different lung burdens of CeO$_2$ (Figure 2). Due to the much faster dissolution kinetics relative to the macrophages 'shuttle' kinetics, post-exposure periods must be long enough to cover at least one-to-two half-times of the typical macrophage-mediated retention kinetics of $t_{1/2} = 60$ to 90 days. These lung burdens can then be used to separate the macrophage-related retention kinetics from that related to mere dissolution (Figure 2).
Figure 2: Modeling of 1- and 4-weeks inhalation study in rats exposed to CeO$_2$ of unit density 6 hours/day on 5 consecutive days per week. The model assumed that dissolution was dependent on the deposition of particles in both the pulmonary (P) and tracheobronchial regions (TB) and that the alveolar macrophage-related clearance is restricted to the pulmonary (P) region. Data points represent actually determined lung burdens as published (85).

150. The example depicted in Figure 2, illustrates that the sampling frequency for lung burden measurements can be elaborated best by a thoughtfully conceptualized 4-week inhalation study (51). As shown in Figure 2, a 1-week inhalation study may reveal the dissolution kinetics but may substantially underestimate that related to the macrophage kinetics. Ideally, for each category of kinetic clearances at least 3 appropriately time-spaced lung burden measurements are necessary to conclude on the impact of each kinetic characteristic, including the threshold at which ‘kinetic lung overload’ is expected to occur.

151. Once the overload-condition is attained, this is indicated by half-times exceeding $t_{1/2} \approx 90$ days (Figure 3). An appropriately designed and executed pilot 4-week repeated exposure inhalation study can serve the purpose to verify or refute that the test chemical under consideration can truly be regarded as “poorly soluble” under in vivo conditions.
Figure 3: Modeling of a 13-week inhalation study in rats exposed to poorly soluble particles (PM) of unit density 6 hours/day on 5 consecutive days per week. Concentrations represent those that are deposited in the pulmonary region (PMd). The left axis represents the calculated respirable concentration. The right axis translates from exposure concentrations to cumulative lung burdens. The lowest exposure level represents that at the transition from non-overload to overload ($t_{1/2} = 96$ days). Exposure to the same deposited inhaled dose of a slightly soluble PM is indicated by a more rapid clearance and shorter half-time ($t_{1/2} = 30$ days). Kinetic overload of poorly soluble PM increases the elimination $t_{1/2}$ from 96 to 156 and 359 days at respirable exposure concentrations of 0.3 and 1 mg/m³, respectively. These simulations illustrate that ‘dissolution’ can readily be detected and quantified during a post-exposure period of 60-90 days but may remain undetected or misjudged as reduced respirability when analyzed during the actual exposure period. Elimination half-times above the overload-threshold demonstrate an overload-related decreased clearance and the duration of post-exposure periods must be adopted accordingly. Half-times as short as 30-days demonstrate a dissolution rate not compatible with the poorly soluble particle paradigm (51, modified).

152. As conceptualized in Figure 3, toxicokinetics is focused solely to identify that cumulative dose level of the deposited and retained particulate matter that does not yet exceed the overload-threshold to any appreciable. If exceeded, a cumulative dose-dependent overload-dependent adversity occurs. This adversity must be judged to be generic particle- rather than substance-specific. Beyond this threshold, any accumulated particle displacement volume-induces lung inflammation and alveolar barrier disruption. Thus, translocation of particles into extrapulmonary compartments may be overload-specific rather than substance-specific. Accordingly, repeated inhalation exposure studies deliver the highest degree of substance-specific information in the range of doses from the POD that induce a minimal to a reasonable degree of kinetic lung overload. Accumulated lung burden that will not exceed a $t_{1/2}$ of 1 year fulfill this criterion. Just for clarification, ‘kinetic overload’ cannot be equaled with any ‘impairment’ of the constitutive alveolar clearance as such. The physiology-driven increase of the accumulating compartment of alveolar macrophages increases its turn-over time with increased pool size. The adversity observed is not necessarily substance-specific, that is, caused by longer residence times of particles in the lung and frustrated phagocytosis initiating and promoting sustained...

Comment [RIVM22]: This is irrelevant from a RA point of view. As long as effects are the result of the exposure the effects are relevant for RA, independent whether the effects are particle or substance specific. The term “overload-specific” should not be used since this is often associated with “not being relevant for humans”.

As conceptualized in Figure 3, toxicokinetics is focused solely to identify that cumulative dose level of the deposited and retained particulate matter that does not yet exceed the overload-threshold to any appreciable. If exceeded, a cumulative dose-dependent overload-dependent adversity occurs. This adversity must be judged to be generic particle- rather than substance-specific. Beyond this threshold, any accumulated particle displacement volume-induces lung inflammation and alveolar barrier disruption. Thus, translocation of particles into extrapulmonary compartments may be overload-specific rather than substance-specific. Accordingly, repeated inhalation exposure studies deliver the highest degree of substance-specific information in the range of doses from the POD that induce a minimal to a reasonable degree of kinetic lung overload. Accumulated lung burden that will not exceed a $t_{1/2}$ of 1 year fulfill this criterion. Just for clarification, ‘kinetic overload’ cannot be equaled with any ‘impairment’ of the constitutive alveolar clearance as such. The physiology-driven increase of the accumulating compartment of alveolar macrophages increases its turn-over time with increased pool size. The adversity observed is not necessarily substance-specific, that is, caused by longer residence times of particles in the lung and frustrated phagocytosis initiating and promoting sustained...
lung inflammation and remodeling. ADME-type kinetic studies do not deliver any substance-specific information at frank overload-conditions due to overload-specific inflammation and barrier disruption.
VII. STATISTICAL ANALYSIS OF DATA

Median Lethal Concentration (LC$_{50}$) and Other Percentiles

153. Dosage-effect relationships can usually be described by cumulative frequency distributions, mathematically represented by sigmoid curves. For each substance, a dosage (concentration)-effect relationship is examined which is assumed to be characteristic for a specific effect and species. In order to quantify this relationship, the term “median lethal concentration” (LC$_{50}$) was suggested as a measure of acute inhalation toxicity. The median lethal concentration is defined as the concentration that kills half of a suitably large number of animals exposed for a specified duration. Determination of the LC$_{50}$ requires a mathematical description of the concentration-effect curve. It is assumed, the concentration-effect curve can be transformed into a linear function by a log-concentration probit-cumulative mortality relationship. Other mathematical transformations that have been employed to linearize the concentration-effect curve include the use of the logistic function, angular transformation, and moving averages and interpolation (40)(41)(42).

154. The prerequisite to calculating the median lethal concentration or other percentiles is the availability of the following data:

- Analytical exposure concentrations
- The number of animals exposed
- The number that died.

In tests with few animals per exposure level the Thompson's method of moving averages may be the most efficient methodology and can give a sufficiently accurate solution if equally spaced test concentrations are used. If, however, one wishes to estimate a number of toxicity percentiles (LC$_{01}$, LC$_{10}$, ...) and is interested in more precisely establishing the slope of the concentration/lethality curve, sufficient exposure levels with the log/probit regression technique are required, and Thompson’s method cannot be used. The method used should allow the calculation of 95% confidence intervals at any point on the regression line. Tests of significance between two or more slopes of mortality curves derived in this way may readily be done by t-type tests. Note that the confidence interval at any one point will be different from the interval at other points since it depends on the exposure level and should be calculated separately. Additionally, the nature of the probit transform is such that toward the extremes of exposure-LC$_{01}$, and LC$_{99}$, for example, the confidence intervals will “balloon”; that is, they become very wide. Because the slope of the fitted line in these assays has a very large uncertainty in relation to the uncertainty of the LC$_{50}$ (the midpoint of the distribution), a great deal of caution should be exercised with calculated LC$_x$ values, where x is either very small or very large.

155. When experimental/mathematical procedures require the estimation of median lethal concentration values from multiple exposure durations (LC$_{t_{50}}$) this is accomplished by the C x t protocol in TG 403 by combining the exposure concentration (C), exposure time (t), and the toxic load exponent (n), using the following equation: $k = C^n x t$ where k is a numerical constant (43)(44). This equation can be generalized using a two-variate-surface plot relating toxicity (mortality) and time as follows:

$$y = b_0 + b_1 \ln(C) + b_2 \ln(t)$$

where $n = b_1/b_2$

Here, y is the Probit value and $b_0$, $b_1$, and $b_2$ are empirically derived constants. It should be recognized that C does not have inherent exponential properties, but t might have such properties because toxicity, under non-ideal conditions, is a function of at least two independent time-scales, one being
the half-life of the rate-determining step of the intoxication, and the other being the intensity of exposure. When sufficient data are available, the empirical constants shown above can be suitably solved mathematically by iterative mathematical procedures combining all \( C \times t \) relationships evaluated into one single matrix. From the constants of the two-variate surface plot, the respective \( LC_{50} \) and \( LC_{0.1} \) (or any other response values), including their confidence intervals, can readily be estimated. Short exposure times (less than 15 minutes) may lead to a transiently decreased inhaled dose after onset of exposure and, accordingly, underestimation of toxicity. Therefore, trigger values estimated from \( C \times t \) relationships based on exposure durations of less than 15 minutes should be judged carefully.

**Body Weights and Non-Lethal Endpoints**

156. Among the sets of data commonly collected in acute inhalation studies are body weights, the weights of selected organs, body temperature, and selected clinical pathology parameters in studies where the focus is on non-lethal endpoints. In fact, body weight (or the rate of body weight gain) is frequently the most sensitive indication of an adverse effect. How to best analyze this, and in what form to analyze the organ weight data (as absolute weights, weight changes, or percentages of body weight), have been dealt with elsewhere (40). Both absolute body weights and body weight gains (calculated as changes from a baseline measurement value, which is traditionally the animal's weight immediately prior to the exposure to test material) are almost universally best analyzed by ANOVA followed, if called for, by a *post hoc* test. Comparisons should be made against equally exposed historical control groups. Due to sequential exposure sessions, shifts in baseline body weights across exposure groups are inevitable in acute inhalation studies. Therefore, the statistical analysis of body weight gains should be given preference. The advantage is an increase in sensitivity because the adjustment of starting points (the setting of initial body weights as a relative zero value) acts to reduce the amount of initial variability. In this case, Bartlett's test is performed first to ensure homogeneity of variance and the appropriate sequence of analysis follows. With smaller sample sizes, the normality of the data becomes increasingly uncertain, and nonparametric methods such as Kruskal-Wallis may be more appropriate (40)(45).

157. The analysis of clinical pathology data is best analyzed by ANOVA followed, if called for, by a *post hoc* test. Repetitively measured data should be analyzed by a one-way repeated measures analysis of variance (RM-ANOVA). All data are then compared against the pre-exposure data, if applicable. For data that pass the normality and equal variance tests, Dunnett’s *post hoc* multiple comparisons procedure is used to isolate the time points that differ from pre-exposure data. The criterion for statistical significance should be \( P < 0.05 \). Some concentration-effect relationships may be associated with concentration-dependent increases in variability. It may be that this can be compensated for by the logarithmic transformation of data. When percentages or proportions, where concentrations (combined with time, if applicable) result in zero responses relative to control, are analyzed, the outcomes should be transformed prior to analysis using the arcsine square-root function. This transformation is appropriate for percentages and proportions because the transformed data more closely approximate a normal distribution than do the non-transformed proportions (45). This transformation is not appropriate for continuous endpoints like absolute body weights or absolute body weight gains, however.
VIII. REFERENCES


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OECD (1995) *OECD Guidelines for the Testing of Chemicals, Section 1 - Physical-Chemical*


## APPENDIX I
### Abbreviations & Acronyms

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
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<tbody>
<tr>
<td>♂, ♀</td>
<td>male, female</td>
</tr>
<tr>
<td>µm</td>
<td>micrometer (formerly micron)</td>
</tr>
<tr>
<td>σ_g</td>
<td>geometric standard deviation (GSD)</td>
</tr>
<tr>
<td>AAS</td>
<td>atomic absorption spectrometer</td>
</tr>
<tr>
<td>ADME</td>
<td>absorption, distribution, metabolism, excretion</td>
</tr>
<tr>
<td>AEGL</td>
<td>acute exposure guideline level</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
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<tr>
<td>AT</td>
<td>apnea time</td>
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<tr>
<td>ATC</td>
<td>acute toxic class</td>
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<tr>
<td>ATE</td>
<td>acute toxicity estimate</td>
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<tr>
<td>atm</td>
<td>atmosphere (a unit of pressure)</td>
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<tr>
<td>AUC</td>
<td>area under the curve</td>
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<tr>
<td>BAL</td>
<td>bronchoalveolar lavage</td>
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<tr>
<td>BALF</td>
<td>bronchoalveolar lavage fluid</td>
</tr>
<tr>
<td>bar</td>
<td>bar (a unit of pressure)</td>
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<tr>
<td>BMC</td>
<td>benchmark concentration</td>
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<tr>
<td>bw</td>
<td>body weight</td>
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<tr>
<td>C</td>
<td>concentration</td>
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<tr>
<td>°C</td>
<td>degrees Celsius</td>
</tr>
<tr>
<td>CAS</td>
<td>Chemical Abstract Services</td>
</tr>
<tr>
<td>CHE</td>
<td>cholinesterase</td>
</tr>
<tr>
<td>CIIT</td>
<td>Chemical Industry Institute of Toxicology</td>
</tr>
<tr>
<td>CMD</td>
<td>count median diameter</td>
</tr>
<tr>
<td>CNT</td>
<td>carbon nanotubes</td>
</tr>
<tr>
<td>COHb</td>
<td>carboxyhaemoglobin</td>
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<tr>
<td>C × t</td>
<td>concentration × time</td>
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<tr>
<td>DMA</td>
<td>differential mobility analyzer</td>
</tr>
<tr>
<td>ECD</td>
<td>effective cut-off diameter</td>
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<tr>
<td>EM</td>
<td>electron microscopy</td>
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<tr>
<td>ET</td>
<td>expiratory time</td>
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<tr>
<td>EU</td>
<td>European Union</td>
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<tr>
<td>ev</td>
<td>extravascular</td>
</tr>
<tr>
<td>g/mol</td>
<td>grams/mole (unit of molecular weight)</td>
</tr>
<tr>
<td>GC</td>
<td>gas chromatography</td>
</tr>
<tr>
<td>GD</td>
<td>guidance document</td>
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<tr>
<td>GHS</td>
<td>Globally Harmonized System</td>
</tr>
<tr>
<td>GI</td>
<td>gastrointestinal tract</td>
</tr>
<tr>
<td>GSD</td>
<td>geometric standard deviation (σ_g)</td>
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<tr>
<td>HPLC</td>
<td>high-performance liquid chromatography</td>
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<tr>
<td>hr</td>
<td>hour</td>
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<td>hrs</td>
<td>hours</td>
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<tr>
<td>ICH</td>
<td>International Committee on Harmonization</td>
</tr>
<tr>
<td>ICP-OES</td>
<td>inductively coupled plasma-optical emission spectroscopy</td>
</tr>
<tr>
<td>IOMC</td>
<td>Interorganisation Programme for the Sound Management of Chemicals</td>
</tr>
<tr>
<td>IT</td>
<td>inspiratory time</td>
</tr>
<tr>
<td>iv</td>
<td>intravenous (injection)</td>
</tr>
<tr>
<td>J</td>
<td>Joule</td>
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<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>(Q)SAR</td>
<td>quantitative structure–activity relationship</td>
</tr>
<tr>
<td>RB</td>
<td>reflex bradypnea</td>
</tr>
<tr>
<td>RBC</td>
<td>red blood cell (erythrocyte)</td>
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<tr>
<td>RIVM</td>
<td>Rijksinstituut voor Volksgezondheid en Milieu (Netherlands National Institute for Public Health and the Environment)</td>
</tr>
<tr>
<td>RM-ANOVA</td>
<td>repeated measures analysis of variance</td>
</tr>
<tr>
<td>Rt</td>
<td>relaxation time</td>
</tr>
<tr>
<td>SAR</td>
<td>structure-activity relationship</td>
</tr>
<tr>
<td>S.D.</td>
<td>standard deviation</td>
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<tr>
<td>SI</td>
<td>International System of Units</td>
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<tr>
<td>SMPS</td>
<td>scanning mobility particle sizer</td>
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<tr>
<td>t</td>
<td>time</td>
</tr>
<tr>
<td>T</td>
<td>temperature</td>
</tr>
<tr>
<td>t₁/₂</td>
<td>time one-half</td>
</tr>
<tr>
<td>t₉₅</td>
<td>time to 95% inhalation chamber equilibrium</td>
</tr>
<tr>
<td>TG</td>
<td>test guideline</td>
</tr>
<tr>
<td>Torr</td>
<td>Torr (a unit of pressure)</td>
</tr>
<tr>
<td>UN</td>
<td>United Nations</td>
</tr>
<tr>
<td>URT</td>
<td>upper respiratory tract</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>Vₐ</td>
<td>volume of distribution</td>
</tr>
<tr>
<td>VMD</td>
<td>volume median diameter</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>xg</td>
<td>times gravity (e.g. 1000 xg)</td>
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</tbody>
</table>
**Glossary of Terms**

**Absolute temperature**: The absolute temperature (T) at 0 °C is 273.15 Kelvin [°K]. Thus, T [°K] = 273.15 + degrees Celsius.

**Absorbed dose**: Amount (of a substance) taken up by an organism or into organs or tissues of interest (synonym: internal dose).

**Absorption (in biology)**: Penetration of a substance into an organism by various processes, some specialized, some involving expenditure of energy (active transport), some involving a carrier system, and others involving passive movement down an electrochemical gradient: in mammals, absorption is usually through the respiratory tract, gastrointestinal tract, or skin.

**Actual concentration**: See Analytical concentration.

**Acute inhalation toxicity**: The adverse effects caused by an airborne test chemical following a single uninterrupted inhalation exposure of less than 24 hours. Most acute inhalation toxicity studies are 4 hours in duration.

**Adverse effect**: Change in biochemistry, morphology, physiology, growth, development, or lifespan of an organism which results in impairment of functional capacity or impairment of capacity to compensate for additional stress or increase in susceptibility to other environmental influences.

**Aerodynamic diameter**: The diameter of a unit density sphere having the same terminal settling velocity as the particle in question, whatever its size, shape, and density. It is used to predict where in the respiratory tract such particles may be deposited.

**Aerodynamic particle sizer**: A particle spectrometer that uses an acceleration system to differentiate particles by aerodynamic diameter and a laser velocimeter to detect particles. See also Cascade impactor.

**Aerosol**: A relatively time-stable suspension of small solid or liquid particles in a gas. The diameter size range of aerosol particles is about 0.001 to 100 µm. See also dust, fog, fume, haze, mist, smog, and smoke.

**Agglomerate**: A group of particles held together by van der Waals forces or surface tension.

**Aggregate**: A heterogeneous particle in which the various components are not easily broken apart.

**Alveolar**: The portion of the respiratory system in which gas exchange occurs; alveoli are small sacs at the end of the bronchioles.

**Analytical concentration**: The concentration of a test chemical in the test animal’s breathing zone. The sampled mass of the test chemical is determined by characterizing one or more constituents using either an analytical method specific for a selected component (e.g., chromatography) or a nonspecific, integrating method which addresses all non-volatile components, such as the total mass obtained by filter analysis (see gravimetric concentration). The terms actual concentration and analytical concentrations are commonly used interchangeably. The analytical or gravimetric concentration (not the nominal concentration) is generally used for hazard assessment. The analytical concentration is commonly expressed in mass units per unit volume of air (mg/L, mg/m³). The mass of test chemical per unit mass of test animal (e.g., mg/kg), or inhaled dose, is difficult to define in inhalation toxicity studies since the fraction of test chemical deposited/absorbed/retained in the respiratory tract is dependent on a number of variables often not defined or measured in acute inhalation studies. Due to these uncertainties, exposure should be defined in terms of the “analytical exposure concentration” and not the “exposure dose.”
Apoptosis: Active process of programmed cell death requiring metabolic energy, often characterized by fragmentation of DNA, and without associated inflammation. See also necrosis.

Aspiration: A dosing procedure in which a needle is passed into the trachea or a bronchus so a known quantity of test chemical can be blown into a specific area of the lungs, bypassing the upper respiratory tract. Also called Pharyngeal Aspiration or Insufflation.

Aspiration efficiency: The fraction of particles entering an inlet from an inhalation chamber. Non-isokinetic sampling losses may cause the aspiration efficiency to be less than 1.

Atomizer: A device used to produce liquid aerosols by mechanical disruption of a bulk liquid. Usually this consists of a metering pump connected to a nozzle.

Attrition: The wearing down of coarse powders and granules into airborne dust due to mechanical abrasion or stress. See also ‘Dustiness.’

Benchmark concentration: Statistical lower confidence limit on the concentration that produces a defined response (called the benchmark response or BMR, usually 5 or 10 %) for an adverse effect compared to background, defined as 0 %.

Bioaccumulation: Progressive increase in the amount of a substance in an organism or part of an organism which occurs because the rate of intake exceeds the organism’s ability to remove the substance from the body.

Bioavailability (general): Extent of absorption of a substance by a living organism compared to a standard system. Synonym: biological availability.

Bioavailability (in pharmacokinetics): Ratio of the systemic exposure from extravascular (ev) exposure to that following intravenous (iv) exposure as described by the equation:

\[ F = \frac{A_e \times D_{ev}}{B_e \times D_{iv}} \]

... where F is the bioavailability, A and B are the areas under the (plasma) concentration-time curve following extravascular and intravenous administration respectively, and D_{ev} and D_{iv} are the administered extravascular and intravenous doses.

Biodurability: The tendency to resist chemical and biochemical alteration through dissolution and enzymatic biodegradation or chemical disintegration within biological and environmental media leading to structural alteration, which in turn may be differentiated between those that are amenable and those that are resistant to dissolution, biodegradation, and/or disintegration.

Biokinetics (in toxicology): Biokinetics plays a critical role in dose response. Any thorough toxicity evaluation (Hazard Assessment) for a substance, and subsequently Risk Assessment, must consider biokinetics and biotransformation. Studies to examine these processes serve at least two general functions in the Risk Assessment process: First, the data collected can be used to help elucidate the mechanism of toxicity, define the target organs, and estimate critical exposure levels such as the Maximum Tolerated Dose (MTD) and the No-Observable-Effect Level (NOEL). The second is to help select the appropriate experimental animal model and procedure to extrapolate from animal toxicity to man (translational toxicology).

Biopersistence: The ability of particles and fibres to reside long-term in tissues.
**Biotransformation**: Chemical conversion (usually enzymatic) of a substance of interest into a different chemical within the body. Synonymous with 'metabolism.' (refer to TG 417)

**Bronchoalveolar lavage (BAL)**: The collecting of cells, particles, and secretions by flushing the small airways and alveoli of the lungs with saline while the animal is anesthetized.

**Bubble meter**: A tube with a defined volume into which bubbles are injected to measure airflow rate.

**Cascade impactor**: A device that uses a series of impaction stages with decreasing particle cut size so that particles can be separated into relatively narrow intervals of aerodynamic diameter; used to measure aerodynamic particle size (24).

**Chronic**: Long-term (in relation to exposure or effect). (1) In experimental toxicology, Chronic refers to mammalian studies lasting considerably more than 90 days or to studies occupying a large part of the lifetime of an organism. (2) In clinical medicine, long established or long lasting.

**Clearance (in toxicology)**: (1) Volume of blood or plasma or mass of an organ effectively cleared of a substance by elimination (metabolism and excretion) divided by the time of elimination. Total clearance is the sum of the clearances of each eliminating organ or tissue for a given substance. (2) In pulmonary toxicology, the volume or mass of lung cleared divided by the time of elimination is used qualitatively to describe removal of any inhaled substance which deposits on the lining surface of the lung.

**Coagulation**: An aerosol growth process resulting from the collision of aerosol particles.

**Concentration**: The mass of test chemical per unit volume of air (e.g., mg/L, mg/m$^3$), or the unit volume of test chemical per unit volume of air (e.g., ppm, ppb).

**Conversion of units - mg/m$^3$ to ppm**: The volume (liters) of a mole (gram molecular weight) of a gas or vapour is 24.45 at a pressure of 1 atmosphere (760 torr or 760 mm Hg) and a temperature of 25°C. To convert mg/m$^3$ to ppm at other temperatures and pressures, one should calculate the volume of 1 gram molecular weight of an airborne contaminant (e.g., 92.13 grams of toluene) by using the formula:

\[
V = \frac{RT}{P}
\]

… where \( R \) is the ideal gas constant; \( T \), the temperature in kelvins (273.16 + T°C); and \( P \), the pressure in mm Hg. This information can be substituted in the formulas for converting between mg/m$^3$ and ppm.

\[
Concentration (mg/m^3) = \frac{P \times MW \times \text{concentration in ppm}}{62.4 \times (273.2 + T°C)}
\]

and

\[
Concentration in ppm = \frac{62.4 \times (273.2 + T°C) \times \text{concentration in mg/m}^3}{P \times MW}
\]

… where the value of \( R \) is 62.4 when the temperature (\( T \)) is in kelvins, K (=273.16 + T°C), the pressure is expressed in units of mm Hg, and the volume is in liters. There are different values for the gas constant \( R \) if the temperature is expressed in degrees Fahrenheit (°F) or if other units of pressure (e.g., atmospheres, kilopascals) are used.
Corrosion: Commonly defined in dermal tests using a defined volume of test chemical per surface area (0.5 ml/6.25 cm²) under semi-occlusive exposure conditions. Skin corrosion is the production of irreversible damage to the exposed skin, namely, visible necrosis through the epidermis and into the dermis, following the application of a test chemical for up to 4 hours. Corrosive reactions are typified by ulcers, bleeding, bloody scabs, and, by the end of observation at 14 days, discolouration due to blanching of the skin, complete areas of alopecia, and scars.

Corrosivity: Test chemical-induced destruction of tissue at the portal-of-entry (e.g., oral, dermal, ocular, inhalation). Test chemicals defined as corrosive to gastrointestinal, dermal, or ocular tissues may not necessarily be corrosive to the respiratory tract. Because corrosivity in the respiratory tract may be site specific, the identification of affected sites may provide important information. Unlike skin testing (see 'Corrosion'), inhalation testing involves an incremental dosing procedure over time and potentially over a large surface area (e.g., a 0.35 m² lung surface area in a 250 g rat). Thus, in quantitative terms, results from a skin bolus test cannot be readily translated to the respiratory tract. This issue is complicated further as the site of primary injury (upper/lower respiratory tract, airways) may depend on the physical properties of the substance under consideration.

Critical organ (in toxicology): Organ that attains the critical concentration of a substance and exhibits the critical effect under specified circumstances of exposure and for a given population.

Critical organ concentration of a substance: Mean concentration of a substance in the critical organ at the time the substance reaches its critical concentration in the most sensitive type of cell in the organ.

Critical orifice: An orifice through which there is a constant flow when a sufficient pressure drop across the orifice causes sonic flow (32).

Cyclone: A mechanic device shaped as a conical cylinder that uses geometry and centrifugal acceleration to separate suspended particles from a gaseous stream on the basis of aerodynamic particle size.

Disposition: Total of the process of absorption of a chemical into the circulatory system, distribution throughout the body, biotransformation, and excretion.

Dissolution: Mathematical models for the dissolution of solid particles involve accounting for the complicated changes in the surface area and/or shape which occur during dissolution. Solid particles in liquids can be modeled using Nernst-Brunner type kinetics which is an extension of the Noyes and Whitney dissolution kinetics (Brunner and Tolloczko, 1900; Brunner, 1900; Nernst, 1904; Wong, 2007):\[
\frac{dM}{dt} = -\frac{D}{V_m h} S_A (C_s - C)
\]

... where M is the mass of solid material at a given time t, S_A is the area available for mass transfer, D is the diffusion coefficient of the dissolving material, V_m is the dissolution medium volume, h is the diffusion boundary layer thickness, C is the concentration, and C_s is the substances saturation solubility. Diffusion-controlled models were further refined for single spherical particle dissolution under sink conditions and pseudo steady-state of the kinetic release of a particles homogeneously dispersed in a matrix into a medium under perfect sink conditions (Wong, 2007). Polydisperse particle sizes and coated particles retained in an inflammatory milieu of the lung may add another dimension of complexity to any model. Due to the longer life-time of humans, time- and dissolution-related changes in particle properties are biased to underestimate the contribution of clearance by slow dissolution. For more details on the distinction between thermodynamic and kinetic equilibrium solubility, and how one can exceed the equilibrium solubility to yield a supersaturated solution,
specialized literature should be consulted (Dokoumetzidis and Macheras, 2006; Brittain, 2014; Wong, 2007).

**Distribution volume:** Theoretical volume of a body compartment throughout which a substance is calculated to be distributed.

**Dust:** Dry solid particles dispersed in a gas as a consequence of mechanical disruption of a bulk solid material or powder formed from a single component or mixture. Dust particles are generally irregular and larger than 0.5 µm (32).

**Dustiness:** Tendency of dry materials to liberate dust into the air when handled under specified conditions. It is restricted to materials transfer and processing operations and does commonly not include, for example, the generation of dust during machining or deliberate comminution. It would however include the dust which could result from previous machining or comminution. It must be stressed that dustiness methods are devised to estimate the dust liberation potential of products under specific conditions. Not all conditions possible will be mirrored. These methods may be qualitative or quantitative, relative or absolute. It is important to recognize that the use of dust reduced products has benefits to industry beyond that of health. Dustiness methods do not take toxicity of the mixture or individual component of the mixture into account. Therefore, especially for products containing highly toxic components, the kind and concentration of potentially toxic components have to be accounted for. This can either be achieved by analytical methodologies or, if too complex or imprecise, by acute inhalation exposure studies.

**Dynamic inhalation chamber:** A type of push-and-pull inhalation chamber with a constant airflow in which the atmosphere and test chemical are held constant so that inhalation chamber equilibrium is attained. Unlike a static chamber which has no airflow, a dynamic chamber has a steady state test chemical concentration, oxygen concentration, carbon dioxide concentration, temperature, and relative humidity for the duration of the exposure period. See also **Equilibrium concentration**.

**Effective Cut-off Diameter (ECD):** The upper particle size limit for a given stage of a cascade impactor.

**Elimination (in toxicology):** Disappearance of a substance from an organism or a part thereof, by processes of metabolism, secretion, or excretion.

**Elimination rate:** Differential with respect to time of the concentration or amount of a substance in the body, or a part thereof, resulting from elimination.

**Elutriator:** A device used to separate fine (respirable) particles from large particles.

**Endocytosis:** Uptake of material into a cell by invagination of the plasma membrane and its internalization in a membrane-bounded vesicle.

**Equilibrium concentration:** In dynamic systems, the test atmosphere is continuously delivered to and exhausted from the animal exposure chamber in a flow-through manner; the test chemical is not recirculated. After an initial rise, the chamber concentration will approach and maintain a stable equilibrium concentration if the air flow rates (in/out) and the generation rate are constant. Prediction of this equilibrium concentration requires accurate information on generation rate, losses of test chemical in various parts of the system, and flow rates as exemplified by the following formula:

\[
C_t = C_0 \left(1 - e^{-\frac{F}{V}}\right)
\]

… where \( C_t \) = concentration at the time \( t \), \( C_0 \) = equilibrium chamber concentration, \( F \) = total flow through the chamber, and \( V \) = chamber volume. For practical purposes, the inhalation chamber
equilibrium is attained at the time $t_{95}$ which is when $C_t = 95\% C_0$.

**Equilibrium solubility:** The maximum quantity of a substance that can be completely dissolved at a given temperature and pressure in a given amount of solvent, and that is thermodynamically valid as long as a solid phase exists, which is in equilibrium with the solution phase.

**Equivalence diameter:** The median equivalence diameter may reflect the number of particles, as in the count median diameter (CMD), reflect the mass, as in the mass median diameter (MMD), or reflect the volume, as in the volume median diameter (VMD). Small particles (< 0.5 µm) diffuse like gases and are defined by diffusion-equivalence diameter (thermodynamic), while larger particles respond to inertial forces and are defined by aerodynamic diameter.

**Evaporation:** 1. The transition from the liquid phase to the vapour phase. 2. The condition in which more vapour molecules are leaving a particle’s surface than arriving at the surface, resulting in shrinkage of a liquid particle. See also **Sublimation**.

**Exposure chamber:** A closed system used to expose animals to a gas, vapour, or aerosol of a test chemical. See Dynamic inhalation chamber, Nose-only inhalation chamber, and Whole-body inhalation chamber.

**Extrathoracic:** The portion of the respiratory tract before the thorax including the nose, mouth, nasopharynx, oropharynx, laryngopharynx, and larynx.

**Fines:** Airborne particles that are smaller than coarse particles and which have an aerodynamic diameter of approximately 0.1 to 2.5 µm (i.e., PM$_{2.5}$). Particles smaller than 0.1 µm (PM$_{0.1}$) in at least one dimension are referred to as ultrafine particles or nanoparticles.

**Finney equation:** This established relationship may be used to estimate an LC$_{50}$ for a mixture, provided all components produce additive acute toxicity and have parallel regression lines of probit against log doses (19).

$$\frac{1}{LC_{50}} = \left(\frac{\% \text{ ingredient}_1}{LC_{501}}\right) + \left(\frac{\% \text{ ingredient}_2}{LC_{502}}\right) + \cdots + \left(\frac{\% \text{ ingredient}_n}{LC_{50n}}\right)$$

**Fog:** A dense mist which impairs visibility. It is typically formed by condensation of supersaturated vapour. See also **Mist**.

**Friable:** Solid material easily crumbled. See also ‘Dustiness’.

**Fume:** Small solid particles that are usually the result of condensed vapour, with subsequent agglomeration. Fumes are often the result of combustion, welding, and other high temperature processes (34).

**Gas:** The state of matter distinguished from the solid and liquid states by relatively low density and viscosity, relatively great expansion and contraction with changes in pressure and temperature, the ability to diffuse readily, and the spontaneous tendency to become distributed uniformly throughout any container.

**Geometric standard deviation (σ$_g$ or GSD):** A unitless number used to portray the range of particle sizes. A particle distribution is considered to be monodisperse when the σ$_g$ is 1.0-1.2, and polydisperse when the σ$_g$ is >1.2 (37).

**GHS Globally Harmonized System of Classification and Labeling of Chemicals:** A system for the classification of chemicals according to standardized types and levels of physical, health and environmental hazards, and addressing corresponding communication elements, such as pictograms,
signal words, hazard statements, precautionary statements and safety data sheets, so as to convey information on their adverse effects with the intent to protect people and the environment. A joint activity of OECD (human health and the environment), UN Committee of Experts on Transport of Dangerous Goods (physico-chemical properties) and ILO (hazard communication) and co-ordinated by the Interorganisation Programme for the Sound Management of Chemicals (IOMO).

**Gravimetric concentration:** An inexpensive integrating method for measuring total aerosol concentrations in which test atmosphere sampled from the animals’ breathing zone is passed through a filter system. The total gravimetric concentration is calculated by dividing the mass of test chemical collected on the filter by the volume of air passed through the filter. Although gravimetric measurements are acceptable for dusts and liquids with low vapour pressures, other sampling and analytical methods (such as GC, HPLC, etc) should be used to measure chamber concentrations of gases, vapours, and liquids with moderate to high vapour pressures. Especially for moderately volatile test chemicals which exist as an equilibrated atmosphere of a liquid aerosol or dust (sublimation) and a vapour phase, the collection principle and the analytical determination should integrate all phases of a specific component.

**Haber’s rule:** The relationship between concentration and time to response for any given chemical is a function of the physical and chemical properties of the test chemical and the unique toxicologic and pharmacologic properties of the individual test chemical. The relationship according to Haber is \( C \times t = k \), where \( C \) = analytical exposure concentration, \( t \) = exposure duration \( (\geq t_{95}) \), and \( k \) = a constant. This concept states that exposure concentration and exposure duration may be reciprocally adjusted to maintain a cumulative exposure constant \( (k) \) and that this cumulative exposure constant will always reflect a specific quantitative and qualitative response. This relationship can also be expressed by the equation \( C^n \times t = k \), where \( n \) represents a chemical-specific, and even a toxic endpoint specific, exponent. The relationship described by this equation is basically in the form of a linear regression analysis of the log-log transformation of a plot of \( C \) vs. \( t \). Ten Berge et al. (42) found that the empirically derived value of \( n \) ranged from 0.8 to 3.5 among a group of chemicals examined.

**Haze:** A combination of vapour, dust, fume, and mist.

**Humane end point:** A humane endpoint can be defined as the earliest indicator in an animal experiment of severe pain, severe distress, suffering, or impending death.

**Impending death:** When a moribund state or death is expected prior to the next planned time of observation. Signs indicative of this state in rodents could include convulsions, lateral position, recumbence, and tremor (see the Humane Endpoint Guidance Document (24) for more details).

**Impinger:** A device in which particles are removed by impacting aerosol particles into a liquid.

**Inhalable aerosol:** Fraction of an aerosol that can enter the human respiratory system through the nose and mouth.

**Inhalable diameter:** The aerodynamic diameter of particles which can be inhaled through the nose and/or mouth of a given organism and deposited anywhere along the respiratory tract.

**Inhalation:** Exposure to a test chemical by normal respiration. The entire respiratory tract can be exposed.

**Inhalation chamber equilibrium:** see Equilibrium concentration.

**Instillation:** The deposition of a test chemical in the respiratory tract of an anesthetized animal, usually by transorally inserting a catheter or ball-tipped needle into the tracheal lumen. Rodents are typically instilled in the tracheal lumen. Large animals may be instilled directly into a specific lung lobe. Instillation bypasses the nasopharyngeal region.

**Insufflation:** A dosing procedure in which a needle is passed into the trachea or bronchus so a
known quantity of test chemical can be blown into a specific area of the lungs, bypassing the upper respiratory tract. Also called Aspiration or Pharyngeal aspiration.

Isokinetic sampling: Sampling condition in which the air flowing into an inlet has the same velocity and direction as the air flow at the sample collection point (see also Representative sampling of atmospheres).

Kelvin effect: Increase in partial vapour pressure for a particle’s curved surface required to maintain mass equilibrium relative to the vapour pressure above a flat liquid surface. This means that molecules tend to evaporate faster from small particles than from a flat liquid surface (see also vapour).

Kelvin: see Absolute temperature.

Kinetic lung overload: The increase of the pool of macrophages as a result of increased alveolar macrophage-related endocytosis of poorly soluble particles is the first indicator of the “kinetic lung overload” of low-toxicity, poorly soluble isometric particles. Along with the increased in the “volume of distribution, Vd”, of particles deposited and retained in the lung the elimination half-time increases above the normal t\textsubscript{1/2} of 60-90 days (in rats).

LC\textsubscript{50} (median lethal concentration): A time dependent, statistically derived estimate of a test chemical concentration that can be expected to cause death during exposure or within a fixed time after exposure in 50% of animals exposed for a specified time. The LC\textsubscript{50} value is expressed as mass of test chemical per unit volume of air (mg/L, mg/m\textsuperscript{3}) or as a unit volume of test chemical per unit volume of air (ppm, ppb). The exposure duration should always be specified (e.g., 4-hour LC\textsubscript{50}).

LC\textsubscript{50} (median lethal concentration per minute): The product of the concentration of a toxic gas, vapour, or aerosol and the exposure time causing lethality in 50% of test animals. For details see LC\textsubscript{50} (median lethal concentration). The LC\textsubscript{50} is expressed as mg/m\textsuperscript{3}\cdot min.

Limit concentration: The maximum concentration required for an inhalation toxicity study, depending on the physical state of the test chemical. When the GHS Classification System is used, the limit concentrations for gases, vapours, and aerosols are 20000 ppm, 20 mg/L and 5 mg/L, respectively, (or the maximum attainable concentration).

Limit test: An inhalation toxicity study performed using a single group of animals exposed to the test-specific limit concentration.

Lowest-observed-adverse-effect concentration (LOAEC): Lowest concentration or amount of a substance (dose), found by experiment or observation, which causes an adverse effect on morphology, functional capacity, growth, development, or life span of a target organism distinguishable from normal (control) organisms of the same species and strain under defined conditions of exposure.

Lung burden: A measure of the cumulative inhaled dose of poorly soluble particles retained in the lungs in a repeated exposure study due to an excessive exposure level and/or overwhelmed clearance mechanisms.

Lung overload: A continuously increasing prolongation of lung clearance of poorly soluble particles when the retained lung burden exceeds a certain threshold, primarily due to impaired alveolar macrophage clearance.

Macrophage: Migratory and phagocytic cell found in many tissues, especially in areas of inflammation, derived from blood monocytes and playing an important role in host defense mechanisms.

Manufactured nanomaterials: Nanomaterials intentionally produced to have specific properties or specific composition. [Working definition; ENV/CHEM/NANO(2007)]
Mass median aerodynamic diameter (MMAD): Mass median of the distribution of mass with respect to aerodynamic diameter. The median aerodynamic diameter and the geometric standard deviation are used to describe the particle size distribution of an aerosol, based on the mass and size of the particles. Fifty percent of the particles by mass will be smaller than the median aerodynamic diameter, and 50% of the particles will be larger than the median aerodynamic diameter. See also Equivalence diameter.

Maximum attainable concentration: For vapour atmospheres, this concentration depends on the vapour saturation concentration of a test chemical under test conditions. For liquid and solid aerosols this concentration depends on a test chemical’s physical properties and also the type of equipment used to generate the aerosol. The maximum attainable concentration is generally defined such that any change of equipment and/or further increase of the nominal test chemical supply rate into the inhalation exposure system does not increase the concentration of respirable aerosol to any appreciable extent.

Maximum tolerated dose (MTD): High dose used in repeated exposure (chronic) toxicity testing that is expected on the basis of an adequate subchronic study to produce limited toxicity when administered for the duration of the test period. It should not induce overt toxicity, e.g. appreciable death of cells or organ dysfunction, or toxic manifestations that are predicted materially to reduce the life span of the animals except as the result of neoplastic development, or 10% or greater retardation of body weight gain as compared with control animals. For particle-induced pulmonary effects, an elimination half-time exceeding 1 year in rats is commonly considered to cause a dysfunction in clearance fulfilling the MTD criterion.

Milling: The grinding of solid materials or large particles into fine particles, as with a ball mill.

Mist: A liquid aerosol, typically formed by condensation of supersaturated vapours or by physical shearing of liquids, such as in nebulization, spraying, or bubbling. A dense mist which impairs visibility is called a fog.

Mixtures: see Test chemical.

MMA: See Mass Median Aerodynamic Diameter.

Monodisperse aerosol: Particles that are uniform in size. For practical purposes, an aerosol with a GSD < 1.2 may be considered monodisperse. See also Polydisperse aerosol and Geometric Standard Deviation.

Moribund status: Being in a state of dying or inability to survive, even if treated. (See the Humane Endpoint Guidance Document for more details).

Mucociliary escalator: A rapid means for removing foreign material from the respiratory tract. Particles captured in the mucus lining the respiratory tract are transported by constantly beating cilia posteriorly from the nose or anteriorly from the lungs toward the pharynx, where they are then swallowed.

Nanoform: A nanoscale form of something.

Nanoscale: The size range typically between 1 nm and 100 nm.

Nanomaterial: Material which is either a nano-object or is nanostructured.
**Nano-object** [Working definition; ENV/CHEM/NANO(2007)4]: Material confined in one, two, or three dimensions at the nanoscale.

**Nanoparticle**: A single nanoscale particle typically between 1 nm and 100 nm.

**Nanostructured** [Working definition; ENV/CHEM/NANO(2007)4]: Having an internal or surface structure at the nanoscale.

**Nebulizer**: A device in which droplet aerosols are produced by dispersion of a bulk liquid in a system that allows larger particles to be impacted and smaller particles to escape from the system (e.g., collision nebulizer).

**Necrosis**: Sum of morphological changes resulting from cell death by lysis and/or enzymatic degradation, usually affecting groups of cells in a tissue. See also apoptosis.

**No observed effect level (NOEL)**: Greatest concentration or amount of a substance, found by experiment or observation, that causes no alterations of morphology, functional capacity, growth, development, or life span of target organisms distinguishable from those observed in normal (control) organisms of the same species and strain under the same defined conditions of exposure.

**Nominal concentration**: The concentration of test chemical introduced into a chamber system. It is calculated by dividing the mass of test chemical generated by the volume of air passed through the chamber. The nominal concentration does not necessarily reflect the concentration to which an animal is exposed. The resultant actual concentration cannot be predicted from the nominal concentration by default because of its dependence on laboratory-specific technical variables. See also Actual concentration.

**Nose-Only Inhalation Chamber**: An inhalation chamber system that minimizes dermal exposure and oral exposure (via licking of contaminated fur). Animals are place in a restraining tube during the course of exposure. The design of this tube should not interfere with the thermoregulation of the animal to any appreciable extent. Head-only and snout-only are synonyms of nose-only.

**One-compartment model**: Kinetic model, where the whole body is thought of as a single compartment in which the substance distributes rapidly, achieving an equilibrium between blood and tissue immediately.

**Paintal reflex**: Poorly water soluble irritants (e.g., ozone and phosgene) can trigger the Paintal reflex in rodents via stimulation of vagal C-fibres in the lower respiratory tract. The Paintal reflex is initially manifest as apneic pauses between breaths, which are then followed by rapid, shallow breathing, bradycardia, hypotension, bronchoconstriction, laryngospasm, airway mucus secretion, bronchial and nasal vasodilation, and hypothermia.

**Pascal**: A unit of pressure used to define atmospheric pressure and vapour pressure. It is interrelated to other pressure units as follows: $1 \text{ Pa} = 10^{-5} \text{ bar} = 0.987 \times 10^{-5} \text{ atm} = 0.0075 \text{ Torr}$.

**Particle bounce**: The rebounding of particles that fail to adhere after impacting on the collecting surface of a cascade impactor stage. Compare with Re-entrainment.

**Particle size** - see Aerodynamic particle size.

**Particle size distribution**: A description of how much of an aerosol is in each of a set (or continuum) of size intervals.

**Particokinetics**: The dynamic biological behavior of nanoparticles at the molecular level (including gravitational sedimentation, dispersion, aggregation, and interaction with biomolecules in suspending media), cellular level (including cellular uptake, transport, biotransformation, and elimination), and whole-organism level (including absorption, distribution, metabolism, and excretion in vivo).
Pharyngeal aspiration: See Aspiration.

Pilot study: See Range-finding study.

Pinocytosis: Type of endocytosis in which soluble materials are taken up by the cell and incorporated into vesicles for digestion.

Polydisperse aerosol: An aerosol composed of particles with a range of sizes. A particle distribution is considered to be monodisperse when the GSD is 1.0-1.2, and polydisperse when the GSD is >1.2 (38). See also Monodisperse aerosol and Geometric Standard Deviation.

Poorly soluble particle (PSP): Solid aerosol particles deposited in the lung that do not undergo rapid dissolution and clearance.

Portal-of-entry effect: A local effect produced at the tissue or organ of first contact between the toxicant and a biological system. For the inhalation route, the portal-of-entry can be any part of the respiratory tract from the nose to the terminal alveoli of the lung.

Protein corona: Nanoparticles in a biological fluid (plasma, or otherwise) associate with a range of biopolymers, especially proteins, organized into the “protein corona” that is associated with the nanoparticle and continuously exchanging with the proteins in the environment.

Pulmonary (PU): Pertaining to the lungs, including the respiratory bronchioles, alveolar ducts, and alveoli.

Preparation: Formulation of multiple components. See Test chemical.

(Q)SARs (Quantitative Structure-Activity Relationships): Theoretical models for making predictions of physicochemical properties, environmental fate parameters, or biological effects (including toxic effects in environmental and mammalian species). They can be divided into two major types, QSARs and SARs. QSARs are quantitative models yielding a continuous or categorical result while SARs are qualitative relationships in the form of structural alerts that incorporate molecular substructures or fragments related to the presence or absence of activity.

Range-finding study: A preliminary study performed using a minimum of animals for the purpose of selecting concentrations to be used in a main study.

Re-entrainment: Return of particles to an air stream after deposition on a collecting surface of a cascade impactor stage. Compare with Particle bounce.

Reflex bradypnea: A reflex in rodents that is initiated by water soluble irritants (e.g., aldehydes, ammonia, isocyanates, and pyrethroids) via stimulation of trigeminal nerves in the mucosa of the upper respiratory tract and eyes. It is manifest by immediate decreases in the metabolic rate, CO₂ production, and demand for oxygen. This is followed by rapid decreases in respiratory rate (breaths/minute), body temperature (as much as 11°C in rats and 14°C in mice), minute volume, heart rate, blood pressure, and activity level. Reflex bradypnea also results in decreased blood pO₂ and pCO₂ and increased blood pH.

Relaxation time: Relaxation time is a parameter used to describe the settling behaviour of particles. The gravitational force effectively removes larger particles from the suspending gas.

Representative sampling of atmospheres: Tolerance limits for the sample probe orifice (r_p) can be calculated using formulas with varying complexity (30) in order to obtain optimal inlet efficiency for a specified sampling flow rate. The inlet efficiency is the fraction of airborne particles that is delivered to the aerosol transport section of a sampling system by the inlet. It is the product of the aspiration and transmission efficiencies. The formula shown below may be applicable to most conditions utilized in inhalation toxicology (at 293.15 Kelvin, 101.3 kPa, particles suspended in relatively calm air). This formula is arbitrarily selected and other, more complex formulas also may be more applicable for
specialized purposes.

\[ 5 \times \sqrt{\frac{\text{flow} \times \tau}{4 \times \pi}} \leq r_p \leq \frac{1}{5} \times 2 \sqrt{\frac{\text{flow}}{g \times \tau \times \pi}} \]

\( r_p \) = radius of the sample probe (\( r_p \)) in cm; \( \text{flow} \) = flow rate (\( \text{cm}^3 \times \text{sec}^{-1} \)),

\( \tau \) = relaxation time (sec), \( g \) = gravity constant = 980 cm/sec²

**Example calculation:**
The targeted sampling airflow rate from an inhalation chamber is 3 L/min (50 cm³/sec) and the probe sampling collection efficiency needs to be considered for particles up to 20 \( \mu \)m. Under these conditions the relaxation time for the largest particle of interest is approximately 0.001 sec.

\[ 5 \times \sqrt{\frac{50 \times 0.001}{4 \times \pi}} \leq r_p \leq \frac{1}{5} \times 2 \sqrt{\frac{50}{g \times 0.001 \times \pi}} \Rightarrow 0.79 \leq r_p \leq 0.81 \text{cm} \]

On the other hand, for particle up 15 \( \mu \)m (relaxation time 6 \times 10⁻⁴) the inlet radius should meet the following conditions: 0.67 \( \leq r_p \leq 1.04 \text{cm} \). These examples show that larger particles may not be sampled representatively if the sampling flow rate relative to the probe diameter does not match the required relationship.

**Respirable diameter:** The aerodynamic diameter of particles which are capable of reaching the gas-exchange region in the lungs (the alveoli) for the organism under study.

**Respirable fraction:** Fraction of aerosol that can reach the gas exchange region of the respiratory system (i.e., the alveoli). For details see European Standard EN 481:1993 (42).

**Respirable particulate mass:** The mass of material that is deposited in the gas-exchange region of the lungs for the organism under study.

**Retention:** The amount of deposited particles that are not cleared from the respiratory tract at a particular time after exposure.

**Retention (lung):** Amount of a substance that is left in the lung following deposition from the absorbed or cleared fraction after a certain time following exposure.

**Rotameter:** An airflow rate meter.

**Scanning mobility particle sizer (SMPS)** - A spectrometer that employs a continuous, fast-scanning technique to provide high-resolution measurements of the size and number concentration of aerosol particles with diameters ranging from approximately 2.5 nm to 1000 nm.

**Sedimentation:** Movement of particles by the influence of gravity.

**Sighting study:** See Range-finding study.

**Smog:** A word combination of smoke and fog; a combination of gases and aerosols formed during UV irradiation of hydrocarbons and oxides of nitrogen, ozone, etc.

**Smoke:** A solid and/or liquid aerosol which is the result of incomplete combustion or condensation of supersaturated vapour. Most smoke particles are sub-micrometer in size.

**Solubility** of any substance is normally determined during the pre-testing stage, and it is crucial to know whether the determined values represent genuine equilibrium solubilities (i.e., thermodynamic
values) or whether they represent the values associated with a metastable condition (i.e., kinetic values). An understanding of the distinction between thermodynamic and kinetic solubility requires one to determine if and when the substance is undergoing a physical change during the measurement period, and how any solubility values are to be assigned as reflecting either equilibrium solubility or metastable solubility. The equilibrium solubility of a compound is defined as the maximum quantity of that substance which can be completely dissolved at a given temperature and pressure in a given amount of solvent, and is thermodynamically valid as long as a solid phase exists which is in equilibrium with the solution phase. It is necessary for an investigator to understand the distinction between thermodynamic and kinetic solubility, and to know when a particular measurement represents an equilibrium solubility value, or if the determined value simply represents some type of metastable condition.

**Solubility equilibrium** is a type of dynamic equilibrium. It exists when a chemical compound in the solid state is in chemical equilibrium with a solution of that compound. The equilibrium is an example of dynamic equilibrium in that some individual molecules migrate between the solid and solution phases such that the rates of dissolution and precipitation are equal to one another. When equilibrium is established, the solution is said to be saturated. The concentration of the solute in a saturated solution is known as the solubility. Units of solubility may be molar (mol dm$^{-3}$) or expressed as mass per unit volume, such as μg ml$^{-1}$.

**Soluble particle**: Solid aerosol particles that undergo rapid dissolution and clearance in the lung.

**Speciation**: Distribution of an element amongst chemical species in a system.

**Static inhalation chamber**: An inhalation chamber without a source of fresh air. Static chambers cannot be used for Guideline studies because test chemical and oxygen concentrations decrease, and carbon dioxide concentration, humidity, and chamber temperature increase as the study progresses. Compare with Dynamic inhalation chamber.

**Sublimation**: 1. The transition from the solid phase directly to the vapour phase without passing through a liquid phase (e.g., dry ice, parafomaldehyde). 2. The condition in which more vapour molecules are leaving a solid particle’s surface than arriving at the surface, resulting in shrinkage of the particle. The opposite of sublimation is Deposition.

**Target concentration**: The desired chamber concentration. See also Nominal concentration and Analytical concentration.

**Test chemical**: A single chemical, chemical formulation, fibre, or biological substance that is tested in a toxicology study. In an inhalation study, a test chemical may be generated as a gas, vapour, liquid aerosol, or solid aerosol.

**Thermodynamic equivalent diameter**: The diameter of a unit-density sphere having the same diffusion coefficient as the particle of interest. The thermodynamic equivalent diameter characterizes particles with diameters from a few nanometres to 1 μm.

**Thoracic Fraction**: Fraction of aerosol that can reach the lung airways and the gas-exchange region. See also Respirable fraction, Inhalable aerosol.

**t$_{95}$**: see Equilibrium concentration.

**Threshold**: Dose or exposure concentration below which an effect will not occur.

**Toxicokinetics**: Process of the uptake of potentially toxic substances by the body, the biotransformation they undergo, the distribution of the substances and their metabolites in the tissues, and the elimination of the substances and their metabolites from the body.

**Ultrafine or nano-particles**: Particles larger than 1 nm and smaller than 100 nm in at least one
**Vapour:** The gaseous phase of a test chemical, including mixtures, which is normally in a liquid or solid state at ambient temperature and pressure. The vapour phase over a liquid is a diffusivity-dependent balance of evaporation and condensation. As a consequence of surface tension, vapour pressure is greater for small liquid droplets than for a plane surface (see Kelvin effect). See also Evaporation.

**Vapour saturation concentration:** For a vapour, the mass (m) and the molecular mass (M) of the evaporated liquid equilibrate as shown below. The approximate vapour saturation concentration can be estimated as follows:

$$C_{sat} = \frac{pM}{RT} \left( \frac{mg}{L} \right)$$

... where p is the vapour pressure (atm) at the specified absolute temperature T (K), M is the molecular mass (mg), and R is the gas constant which is R = 0.082 (L atm)/(K Mol) or in SI units R = 8.314 J/(K Mol) where 1 L atm = 1.01328 \(\times\)10^5 J. J (Joule) is the unit of energy in N(Newton) \(\times\)m. 1 Pa (Pascal) ≈ 1.0 J•L^{-1}. The unit of Pa is N•m^{-2}.

Temperature: T [ºK] = 273.15 + degree Celsius

Pressure conversions: 1 Pa = 10^{-5} bar = 0.987 10^{-5} atm = 0.0075 Torr.

**Example calculation:**

The molecular mass of a test chemical is 100 g and its vapour pressure at 20 ºC is 2 Pa.

$$C_{sat} = \frac{0.987 \times 10^{-5} \times 2 \times 100 \times 10^3}{0.082 \times (273.15 + 20)} \left( \frac{atm \times mg \times K \times Mol}{Mol \times L \times atm \times K} \right) = 0.082 \left( \frac{mg}{L} \right)$$

or in SI units:

$$C_{sat} = \frac{2 \times 10^3 \times 10^3}{8.314 \times (273.15 + 20)} \left( \frac{J \times mg \times K \times Mol}{Mol \times J \times K \times L} \right) = 0.082 \left( \frac{mg}{L} \right)$$

**Volume of distribution:** Apparent (hypothetical) volume of fluid required to contain the total amount of a substance in the body at the same concentration as that present in the plasma, assuming equilibrium has been attained.

**Wall loss:** Deposition of particles in a sampler on surfaces other than those designed for particle collection (e.g., chamber and tubing walls).

**Whole-body chamber:** An inhalation chamber that exposes the whole animal. Especially for aerosols, this results not only in inhalation exposure, but also dermal exposure and oral exposure (via licking of the fur).
APPENDIX II

GHS classification System for Acute Inhalation Toxicity

In the Globally Harmonized System of Classification and Labelling of Chemicals (GHS), substances can be allocated to one of the five toxicity categories based on acute toxicity by the inhalation route according to the numeric cut-off criteria shown below. Acute toxicity values are expressed as (approximate) LC₅₀ values or as Acute Toxicity Estimates (ATE). The concentrations to be used in limit tests are the upper bounds of Class 4 (20000 ppm for gases, 20 mg/L for vapours, and 5 mg/L for aerosols) (11).

Table 1: The GHS system for classification of acute inhalation toxicity.

<table>
<thead>
<tr>
<th>GHS Class</th>
<th>Gases (ppm)</th>
<th>Vapours (mg/L)</th>
<th>Aerosols (dusts and mists) (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>≤ 100</td>
<td>≤ 0.5</td>
<td>≤ 0.05</td>
</tr>
<tr>
<td>2</td>
<td>&gt; 100 and ≤ 500</td>
<td>&gt; 0.5 and ≤ 2</td>
<td>&gt; 0.05 and ≤ 0.5</td>
</tr>
<tr>
<td>3</td>
<td>&gt; 500 and ≤ 2500</td>
<td>&gt; 2 and ≤ 10</td>
<td>&gt; 0.5 and ≤ 1</td>
</tr>
<tr>
<td>4</td>
<td>&gt; 2500 and ≤ 20000</td>
<td>&gt; 10 and ≤ 20</td>
<td>&gt; 1 and ≤ 5</td>
</tr>
<tr>
<td>5</td>
<td>&gt; 20000</td>
<td>&gt; 20</td>
<td>&gt; 5</td>
</tr>
</tbody>
</table>

a) The use of units of ppm for gases in the GHS Classification System leads to a disparity of classification between gases and vapours (which are in units of mg/L) even though both are gaseous. The disparity increases beyond the molecular weight of 122. For a molecular weight of 122, the conversion factor from ppm to mg/L is 0.005.

Note: For some substances or mixtures the test atmosphere will not just be a vapour but will consist of a concentration-dependent phase equilibrium of liquid and vapour phase.

GHS Conversions from Acute Toxicity Range Values to Acute Toxicity Point Estimates

Table 2a: Conversion from experimentally obtained acute toxicity range values (or acute toxicity hazard classes) to acute toxicity point estimates for classification of gases.

<table>
<thead>
<tr>
<th>Classification Class or Experimentally Obtained Acute Toxicity Risk Estimate (ppm)</th>
<th>Converted Acute Toxicity Point Estimate (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 &lt; Class 1 ≤ 100</td>
<td>10</td>
</tr>
<tr>
<td>100 &lt; Class 2 ≤ 500</td>
<td>100</td>
</tr>
<tr>
<td>500 &lt; Class 3 ≤ 2500</td>
<td>700</td>
</tr>
<tr>
<td>2500 &lt; Class 4 ≤ 20000</td>
<td>4500</td>
</tr>
<tr>
<td>Class 5 &gt; 20000</td>
<td>See note b</td>
</tr>
</tbody>
</table>

Table 2b: Conversion from experimental obtained acute toxicity range values (or acute toxicity hazard classes) to acute toxicity point estimates for classification of vapours.
<table>
<thead>
<tr>
<th>Classification Class or Experimentally Obtained Acute Toxicity Risk Estimate (mg/L)</th>
<th>Converted Acute Toxicity Point Estimate (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 &lt; Class 1 ≤ 0.5</td>
<td>0.05</td>
</tr>
<tr>
<td>0.5 &lt; Class 2 ≤ 2.0</td>
<td>0.5</td>
</tr>
<tr>
<td>2.0 &lt; Class 3 ≤ 10.0</td>
<td>3</td>
</tr>
<tr>
<td>10.0 &lt; Class 4 ≤ 20.0</td>
<td>11</td>
</tr>
<tr>
<td>Class 5 &gt; 20.0</td>
<td>See note b</td>
</tr>
</tbody>
</table>

Table 2c: Conversion from experimental obtained acute toxicity range values (or acute toxicity hazard classes) to acute toxicity point estimates for classification of aerosols (dusts and mists).

These values are designed to be used in the calculation of the ATE for classification of a mixture based on its components and do not represent test results. The values are conservatively set at the lower end of the range of Classes 1 and 2, and at a point approximately one tenth from the lower end of the range for Classes 3-5.

From GHS (2007) (11) “…Criteria for Category 5 are intended to enable the identification of substances which are of relatively low acute toxicity hazard but which under certain circumstances may present a danger to vulnerable populations. These substances are anticipated to have an oral or dermal LD$_{50}$ in the range of 2000-5000 mg/kg bodyweight and equivalent doses for inhalation. The specific criteria for Category 5 are:

(i) The substance is classified in this Category if reliable evidence is already available that indicates the LD$_{50}$ (or LC$_{50}$) to be in the range of Category 5 values or other animal studies or toxic effects in humans indicate a concern for human health of an acute nature.

(ii) The substance is classified in this Category, through extrapolation, estimation or measurement of data, if assignment to a more hazardous category is not warranted, and:

- reliable information is available indicating significant toxic effects in humans; or

- any mortality is observed when tested up to Category 4 values by the oral, inhalation, or dermal routes; or

- where expert judgement confirms significant clinical signs of toxicity, when tested up to Category 4 values, except for diarrhoea, piloerection or an ungroomed appearance; or

- where expert judgement confirms reliable information indicating the potential for significant acute
effects from other animal studies.

Recognizing the need to protect animal welfare, testing in animals in Category 5 ranges is discouraged and should only be considered when there is a strong likelihood that results of such a test would have a direct relevance for protecting human health.”
**APPENDIX III**

**Comparison of Acute Test Guidelines**

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>Traditional LC₅₀ study</strong></td>
<td><strong>C × t study</strong></td>
<td><strong>Range estimate determination</strong></td>
</tr>
<tr>
<td>Study objective</td>
<td>Concentration response for lethal and non-lethal endpoints (endpoints are system independent)</td>
<td>Concentration response for lethal and non-lethal endpoints (endpoints are system independent)</td>
<td>Derivation of the toxic load exponent ( n ) in ( C^n \times t )</td>
</tr>
<tr>
<td>Major endpoint</td>
<td>Mortality</td>
<td>Mortality</td>
<td>Mortality</td>
</tr>
<tr>
<td>Use of data</td>
<td>Classification &amp; labeling by multiple systems including the GHS System</td>
<td>Classification and labeling by multiple systems including the GHS System.</td>
<td>Classification and labeling by the GHS System only (the fixed concentrations used in this Test Guideline are based on GHS cut-offs)</td>
</tr>
<tr>
<td>Use of data</td>
<td>Derivation of LC₁ values and slope for one specific duration (usually 4 hours) for specific regulatory requirements</td>
<td>Derivation of LC₁ values and slope for multiple exposure durations for specific regulatory requirements</td>
<td>A range estimate of LC₅₀ values for one specific exposure duration (usually 4 h)</td>
</tr>
<tr>
<td>Mode of exposure</td>
<td>Nose-only (preferred) or whole-body</td>
<td>Nose-only (whole-body chambers cannot be used)</td>
<td>Nose-only (preferred) or whole-body</td>
</tr>
<tr>
<td>Concentrations tested</td>
<td>Variable—selected by the study director.</td>
<td>Variable—selected by the study director</td>
<td>Gases: 100, 500, 2500, 20000 ppm</td>
</tr>
<tr>
<td>Concentrations tested</td>
<td></td>
<td></td>
<td>Vapours: 0.5, 2.0, 10.0, 20.0 mg/L</td>
</tr>
<tr>
<td>Concentrations tested</td>
<td></td>
<td></td>
<td>Aerosols: 0.05, 0.5, 1.0, 5.0 mg/L</td>
</tr>
<tr>
<td>Atmosphere: concentration variability</td>
<td>Gases and vapours: ±10% Aerosols: ±20%</td>
<td>Gases and vapours: ±10% Aerosols: ±20%</td>
<td>Gases and vapours: ±10% Aerosols: ±20%</td>
</tr>
<tr>
<td>Atmosphere: stability</td>
<td>Monitor continuously or hourly</td>
<td>Monitor continuously or hourly</td>
<td>Monitor continuously or hourly</td>
</tr>
<tr>
<td>Particle sizing (method)</td>
<td>At least twice during 4 hour exposure (cascade impactor)</td>
<td>At least twice during 4 hour exposure (cascade impactor)</td>
<td>At least twice during 4 hour exposure (cascade impactor)</td>
</tr>
<tr>
<td>Concentrations tested</td>
<td>Limit test: 1 Main study: At least 3</td>
<td>Limit test: 1 Main study: 4-5</td>
<td>1 or more</td>
</tr>
<tr>
<td>Exposure duration</td>
<td>Variable (generally 4 hours)</td>
<td>5 durations per concentration</td>
<td>4 hours</td>
</tr>
<tr>
<td>Particle size (aerosols)</td>
<td>MMAD: 1-4μm GSD: 1.5-3</td>
<td>MMAD: 1-4 μm GSD: 1.5-3</td>
<td>MMAD: 1-4 μm GSD: 1.5-3</td>
</tr>
<tr>
<td>Observation period</td>
<td>At least 14 days</td>
<td>At least 14 days</td>
<td>At least 14 days</td>
</tr>
<tr>
<td>-----------------------------------</td>
<td>---------------------------------------------</td>
<td>---------------------------------</td>
<td>--------------</td>
</tr>
<tr>
<td>Vehicle control group</td>
<td>Not generally required (historical data required if interactions cannot be excluded)</td>
<td>Not generally required (historical data required if interactions cannot be excluded)</td>
<td>Not generally required (historical data required if interactions cannot be excluded)</td>
</tr>
</tbody>
</table>

**Animals Tested:**

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Limit test</td>
<td>≤ 3 ♂ and ≤ 3 ♀ (or ≤ 3 of the known susceptible sex) per concentration.</td>
<td>≤ 3 ♂ and ≤ 3 ♀ per concentration</td>
<td>3 ♂ and 3 ♀ (or 6 of the known susceptible sex)</td>
</tr>
<tr>
<td>Range-finding study</td>
<td>In case of 2 animals/sex/(Cxt) point: * Both sexes: 20; Susceptible sex = 20</td>
<td>In case of 2 animals/sex/(Cxt) point: * Both sexes: 20; Susceptible sex = 20</td>
<td>0 (range-finding studies are not used)</td>
</tr>
<tr>
<td>Main study</td>
<td>1 or 2 animals/sex/(Cxt) point (or 2 or 4 animals of the susceptible sex per (Cxt) point) 5 durations per concentration)*</td>
<td>5 ♂ and 5 ♀ (or 5 of the known susceptible sex) per concentration.</td>
<td>3 ♂ and 3 ♀ (or 6 of the known susceptible sex) per concentration</td>
</tr>
<tr>
<td>Total animals used in a non-limit study</td>
<td>5 animals x 4 groups = 20 animals.</td>
<td>If 4 concentrations are tested:* Both sexes: 40; Susceptible sex: 40 In case of 2 animals/sex/(Cxt) point Both sexes: 80; Susceptible sex = 80</td>
<td>If 1 concentration is tested: 6 If 2 concentrations are tested: 12 If 3 concentrations are tested: 18</td>
</tr>
</tbody>
</table>

* Refer to paragraph 61 regarding the number of animals to be used per C × t interval (18).
APPENDIX IV

Study Designs for TG 412 and TG 413

Decision Flowchart for Lung Burden

Do results from range-finding studies and/or other considerations indicate that exposure to the test chemical is likely to result in lung retention?

- **No**
  - **Option A**
    - Conduct main study to test chemicals unlikely to result in lung retention
      (gases, vapours, liquid aerosols and soluble solid aerosols)
    - Mandatory Examinations:
      - Clinical observations
      - Body weight measurements
      - Food/water consumption
      - Clinical pathology
      - Gross pathology/organ weights
      - Histopathology
      - Bronchoalveolar lavage (BAL)

- **Yes**
  - **Option B**
    - Conduct main study to test chemicals likely to result in lung retention
      (poorly soluble solid aerosols)
    - Mandatory Examinations:
      - Same as those for Option A
      - Lung burden measurements at one time point.
      - Additional lung burden measurements are optional.

---

1. A successful main study depends on information obtained from a range-finding study or previous studies. GD39 describes how to decide between Options A and B based primarily on a particle’s solubility.
Test scheme for TG 412:

Abbreviations
PEO = post-exposure observations
PEO-1 = within one day after the last exposure day
PEO-2 = within x weeks after the last exposure day (recovery group)
PEO-3 = within y weeks after the last exposure day (satellite groups) (y can be < or > than x (PEO-2))
0 = control group
C_x = exposure concentration
BAL = bronchoalveolar lavage
HP = histopathology
LB = Lung burden
RL = Right lung
LL = Left lung
TBD = to be determined

TG 412 Option A - Test chemicals unlikely to result in lung retention

<table>
<thead>
<tr>
<th>Examination</th>
<th>Main study</th>
<th>Satellite Groups</th>
<th>Total animals</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PEO-1</td>
<td>PEO-2</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>HP (LL) + BAL (RL) 5 f/5 m</td>
<td>HP (LL) + BAL (RL) 5 f/5 m</td>
<td></td>
</tr>
<tr>
<td>C_1</td>
<td>HP (LL) + BAL (RL) 5 f/5 m</td>
<td>HP (LL) + BAL (RL) 5 f/5 m</td>
<td></td>
</tr>
<tr>
<td>C_2</td>
<td>HP (LL) + BAL (RL) 5 f/5 m</td>
<td>HP (LL) + BAL (RL) 5 f/5 m</td>
<td></td>
</tr>
<tr>
<td>C_3</td>
<td>HP (LL) + BAL (RL) 5 f/5 m</td>
<td>HP (LL) + BAL (RL) 5 f/5 m</td>
<td></td>
</tr>
<tr>
<td></td>
<td>∑ = 40</td>
<td>∑ = 40</td>
<td>∑ = 80</td>
</tr>
</tbody>
</table>

Examinations in the main study at PEO-1 [mandatory] and in the recovery group at PEO-2 [optional]:
- Clinical observations
- Body weight measurements
- Food/water consumption
- Clinical pathology
- Gross pathology/organ weights
- Lung weight-left lung
- Histopathology-left lung
- BAL-right lung
**TG 412 Option B – Test chemicals likely to result in lung retention**

### Examinations in the Main Study

**[mandatory]**

1. **PEO-1 (f and m)**
   - Clinical observations
   - Body weight measurements
   - Food/water consumption
   - Clinical pathology
   - Gross pathology/organ weights
   - Lung weight-left lung (f and m)
   - Histopathology–left lung (f and m)
   - BAL-right lung (f and m)

2. **PEO-1 (m only)**
   - Lung burden -right lung
   - Other parameters to be determined by the study director

### Exposure Groups

<table>
<thead>
<tr>
<th>MAIN STUDY</th>
<th>PEO-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>HP (LL) + BAL (RL)</td>
</tr>
<tr>
<td></td>
<td>5 f/5 m</td>
</tr>
<tr>
<td>C1</td>
<td>HP (LL) + BAL (RL)</td>
</tr>
<tr>
<td></td>
<td>5 f/5 m</td>
</tr>
<tr>
<td>C2</td>
<td>HP (LL) + BAL (RL)</td>
</tr>
<tr>
<td></td>
<td>5 f/5 m</td>
</tr>
<tr>
<td>C3</td>
<td>HP (LL) + BAL (RL)</td>
</tr>
<tr>
<td></td>
<td>5 f/5 m</td>
</tr>
</tbody>
</table>

**Total animals**

\[ \sum = 40 \] \[ \sum = 20 \] \[ \sum = 60 \]

### Examinations in the Satellite Groups

**[optional]**

- **PEO-2 (f and m):**
  - Lung weight-left lung (f and m)
  - Histopathology–left lung (f and m)
  - BAL-right lung (f only)
  - Lung burden-right lung (m only)

- **PEO-3 (m only):**
  - Lung burden (right lung)
  - Other parameters to be determined by the study director

### Exposure Groups

<table>
<thead>
<tr>
<th>SATELLITE GROUPS</th>
<th>PEO-2</th>
<th>PEO-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>HP (LL) + BAL (RL)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5 f</td>
<td></td>
</tr>
<tr>
<td>C1</td>
<td>HP (LL) + BAL (RL)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5 f</td>
<td></td>
</tr>
<tr>
<td>C2</td>
<td>HP (LL) + BAL (RL)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5 f</td>
<td></td>
</tr>
<tr>
<td>C3</td>
<td>HP (LL) + BAL (RL)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5 f</td>
<td></td>
</tr>
</tbody>
</table>

**Total animals**

\[ \sum = 40 \] \[ \sum = 20 \] \[ \sum = 60 \]
**Test scheme for TG 413:**

*Abbreviations*

- PEO = post-exposure observations
- PEO-1 = within one day after the last exposure day
- PEO-2 = within x weeks after the last exposure day (recovery group)
- PEO-3 = within y weeks after the last exposure day (satellite groups) (y can be < or > than x (PEO-2))
- 0 = control group
- \( C \_x \) = exposure concentration
- BAL = bronchoalveolar lavage
- HP = histopathology
- LB = Lung burden
- RL = Right lung
- LL = Left lung
- TBD = to be determined

**TG 413 Option A - Test chemicals unlikely to result in lung retention**

<table>
<thead>
<tr>
<th>Exposure Groups</th>
<th>Main study</th>
<th>Satellite Groups</th>
<th>Total animals</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>PEO-1</td>
<td>PEO-2</td>
<td></td>
</tr>
<tr>
<td></td>
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\( \Sigma = 80 \) \quad \( \Sigma = 40 \) \quad \( \Sigma = 120 \)
TG 413 Option B – Test chemicals likely to result in lung retention

### Examinations in the Main Study

[**Mandatory**]

1. **PEO-1 (f and m)**
   - Clinical observations
   - Body weight measurements
   - Food/water consumption
   - Clinical pathology
   - Gross pathology/organ weights
   - Lung weight-left lung (f and m)
   - Histopathology-left lung (f and m)
   - BAL-right lung (f and m)

   2. **PEO-1 (m only)**
      - Lung burden-right lung
      - Other parameters to be determined by the study director

### Exposure Groups

<table>
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<tr>
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<tr>
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<td>10 f/10 m</td>
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<tr>
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</table>

$$\sum = 80$$ $$\sum = 20$$ $$\sum = 100$$

### Examinations in the Satellite Groups

[**Optional**]

**PEO-2 (f and m):**
- Lung weight-left lung (f and m)
- Histopathology-left lung (f and m)
- BAL-right lung (f only)
- Lung burden-right lung (m only)

**PEO-3 (m only):**
- Lung burden (right lung)
- Other parameters to be determined by the study director

### Exposure Groups

<table>
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<tr>
<td>C1</td>
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<td></td>
<td>5 f</td>
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<tr>
<td>C2</td>
<td>HP (LL) + BAL (RL)</td>
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<td></td>
<td>5 f</td>
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<tr>
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$$\sum = 40$$ $$\sum = 20$$ $$\sum = 60$$
APPENDIX V

Pulmonary Function, Reflex Bradypnea, and Lower Respiratory Tract Reflexes

Reflex Bradypnea

Reflex bradypnea (RB; brad"e-ne'ah or brad-ip-ne'ā), also known as the Kratschmer reflex, is a protective reflex that allows laboratory rodents to significantly reduce their exposure to upper respiratory tract (URT) irritants such as aldehydes, ammonia, isocyanates, and pyrethroids (Gordon et al., 2008). This reflex is initiated by stimulation of trigeminal nerves in the mucosa of the URT and the eyes, and is associated with the chemosensitive part of the nociceptive system—the common chemical sense—that detects noxious airborne exposures (Nielsen, 1991).

The signs of reflex bradypnea

RB is manifest by immediate decreases in the metabolic rate, CO₂ production, and demand for oxygen. This is followed by rapid decreases in respiratory rate (breaths/minute; Figure 1), body temperature (as much as 11°C in rats and 14°C in mice; Figure 1), minute volume (Figure 2), heart rate, blood pressure, and activity level (e.g., prostration). RB also results in decreased blood pO₂ and pCO₂ and increased blood pH (Figure 3) (Pauluhn, 1989, 1996, 2003, 2008; Gordon et al., 2008; Jaeger and Gearhart, 1982; Chang and Barrow, 1984). RB can also impact neurologic, behavioral, and developmental studies.

RB is regulated by a complex feedback response (Yokley, 2012). The extent of RB depends on the concentration of the irritant (Gordon et al., 2008). As shown in Figure 1, after several hours of exposure to an isocyanate, mice in the high concentration group had a mean body temperature of 23°C and approximately 90% decreases in respiratory rate.

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**Figure 1:** Left panel: Concentration-related hypothermia in mice exposed to an isocyanate for 6 hours. Note the gradual recovery in body temperature after the exposure ended.

Right panel: Concentration-related decreases in respiratory rate in mice exposed to an isocyanate. Note the correlation between the curves for rectal temperature and respiratory rate over the course of 180 minutes.

**Source:** Gordon et al. (2008)
Figure 2: Minute volumes for mice (left panel) and rats (right panel) in volume displacement nose-only plethysmographs. The animals were sequentially exposed for 15 minutes to air, 45 minutes to different concentrations of transfluthrin (a pyrethroid), followed by a 30 minute recovery period. The solid lines were derived using a sigmoid model fitted to measurements from the pre-exposure and exposure periods to estimate the degree of respiratory depression. All data were normalized to the pre-exposure period (=100%). The responses in mice and rats were similar but the mice had a slightly greater decrease in minute volume.

Source: Data duplicated from Pauluhn & Ozaki, 2015

Figure 2 demonstrates that the onset of RB is immediate, with a marked decrease in minute volume in mice and rats shortly after formaldehyde exposure begins. Because reduced respiration lessens exposure to an irritating chemical, an animal’s toxicity is reduced and its survival is enhanced. This is important for the survival of rodents living in burrows and confined spaces that cannot avoid exposure. Figure 1 (left panel) demonstrates that the effects of RB are reversible following a 6 hour exposure, though it can take several minutes to several hours for all physiological parameters to return to pre-exposure conditions depending on the extent of hypothermia (Barrow et al., 1983; Pauluhn, 1996; Jaeger and Gearhart, 1982).

The physiological signs of RB in rodents can be striking, but they should not be misconstrued as signs of toxicity or be used to define an animal point-of-departure (POD). Also, the signs of RB in animals must not be considered relevant to humans since humans do not experience this reflex. RB can only occur in small animals such as mice and rats that can rapidly lower their core body temperatures and oxygen demand when their metabolic rates reflexively decrease. Even a mild decrease in body temperature can lessen the toxicity and metabolic activation of many chemicals, but it can also slow the excretion of toxicants. Overall, the protection from cellular toxicity afforded by RB-induced hypothermia outweighs the undesirable effect of a slower excretion rate (Gordon et al., 2008).

Even though RB has been reported in the literature since the 1960s, it is largely unknown to most toxicologists, which may be why so few inhalation studies attempt to identify and quantify RB.
This is unfortunate since RB likely occurs in most, if not all, rodent inhalation toxicity studies of URT irritants.

**Irritation, reflex bradypnea, and the RD_{50}**

A test for assessing sensory irritation was developed by Yves Alarie in the 1960s. In an Alarie test, rodent respiration is measured before, during, and after exposure to one or more concentrations of an irritant, and then respiratory depression (RD) is statistically quantified. The most commonly reported value in Alarie tests is the RD_{50}—the concentration of a sensory irritating chemical that causes a 50% decrease in the respiratory rate (ASTM, 2012; Kane et al., 1979). Alarie tests are useful for 1) identifying chemicals which are URT sensory irritants, 2) quantifying sensory irritating concentrations, and 3) ranking chemicals for their sensory irritancy potential.

**Tolerance to URT irritants**

Nearly all rodent studies that assessed RB have been acute Alarie tests lasting a few minutes to a few hours. Two subacute studies demonstrated that RB persists in rats exposed to formaldehyde for 10 days (Chang and Barrow, 1984; Barrow et al., 1986) or to cyfluthrin (a pyrethroid insecticide) for 4 weeks (Pauluhn, 1998) with no indication of tolerance. No long-term studies have investigated whether-or-when rodents develop a tolerance to URT irritants and eventually begin to breathe normally. This is a serious data gap.

**Reflex bradypnea and human health risk assessments**

The potential impact of RB on human health risk assessments has not received the attention it deserves from toxicologists and risk assessors, largely because testing guidelines did not require examination of RB-related endpoints, i.e., pulmonary function and body temperature. If, for example, RB causes rodents to reduce their exposure to an URT irritant during most or all of a long-term study, a human health risk assessment that fails to account for this reduced rodent exposure may be biased to underestimate the true human risk. Mouse studies are a particular concern because mice have a somewhat greater RB response than.

**The impact of reflex bradypnea on neurological and behavioral studies**

The normal physiological effects of RB can confound neurological and behavioral studies in rodents. Hypothermia causes reduced peripheral nerve conduction velocity due to an apparent reduced flux of potassium and chloride ions across axon membranes, and also prolonged synaptic delay time at neuromuscular junctions. A progressive decrease in body temperature results in ataxia, loss of fine motor control and reflexes, a reduction in cerebral blood flow and brain function, and eventually a loss of consciousness (Mallet, 2002). Thus, what appear to be chemically-induced neurological or behavioral effects may actually be due to RB-induced hypothermia. It should not be surprising that hypothermic rodents experiencing or recovering from RB may be too sluggish to perform well in behavioral studies such as rotarod or a swim maze. RB-induced neurologic and behavioral effects in rodents are not relevant to humans. Only by measuring pulmonary function and body temperature can RB-related neurologic effects be distinguished from chemical neurotoxicity.

**The impact of reflex bradypnea on developmental toxicity studies**

Pregnant dams are protected by RB, but their fetuses are not. Fetuses can experience
developmental delays or defects due to impaired placental transfer of O\(_2\) (hypoxia) and CO\(_2\) (hypercapnia), fetal hypothermia, and malnutrition due to reduced maternal metabolism. Fetuses do not tolerate hypothermia as well as adults (Pauluhn, 1989).

Figure 3: Hypothermia induced by reflex bradypnea results in a decrease in pCO\(_2\) (left panel) and an increase in blood pH (respiratory alkalosis; right panel). Rats were exposed to cyfluthrin, a pyrethroid insecticide.

Source: Jürgen Pauluhn (Bayer Healthcare AG, Germany)

When dams experience RB, their fetuses may experience hypoxia due to 1) reduced maternal respiration and 2) a left-shift in maternal oxyhemoglobin affinity caused by an increase in blood pH (respiratory alkalosis; Figure 3). Normal oxygen exchange to the fetus requires a gradient between maternal and fetal oxyhemoglobin affinities. When pregnant dams experience RB, their blood pH becomes more alkaline, resulting in a left shift in maternal oxyhemoglobin affinity. As demonstrated in Figure 4, a maternal left-shift results in the affinities of maternal and fetal oxyhemoglobin being indistinguishable, which impairs oxygen exchange to the fetus (hypoxia) and removal of CO\(_2\) (hypercapnia). Hypoxia is a normal regulator of placental development in both humans and mice (Rossant & Cross, 2001). In studies of formaldehyde and cyfluthrin in mice and rats, concentration-related decreases in fetal weight were attributed to RB-induced hypothermia of the dams (Monfared, 2012; Holzum et al., 1994). RB-induced developmental effects caused by fetal hypoxia, hypercapnia, hypothermia, and malnutrition are not relevant to humans. Only by measuring pulmonary function and body temperature can developmental and placental effects caused by RB be distinguished from chemical toxicity.
Figure 4: Normal oxygen exchange to the fetus requires a gradient between maternal (red line) and fetal (green line) oxyhemoglobin affinities. When reflex bradypnea causes maternal blood pH to increase from 7.35 to 7.6 (respiratory alkalosis), this causes a left shift in maternal oxyhemoglobin affinity (yellow line). Oxygen exchange to the fetus is impaired when the affinities of maternal oxyhemoglobin (yellow line) and fetal oxyhemoglobin (green line) become indistinguishable.

Source: Jürgen Pauluhn (Bayer Healthcare AG, Germany)

The Paintal reflex

Lower respiratory tract (LRT) irritants, such as ozone and phosgene, can trigger a Paintal reflex by the stimulation of vagal C-fibres (formerly known as J-fibres). C-fibres, which have nerve endings located in the walls of the alveoli and airways in the LRT, have major controlling effects on spontaneous breathing. The nociceptive role of the Paintal reflex is to sense the onset of a pathophysiological condition within the lower respiratory tract. The afferent activity arising from these vagal nerve fibres appears to play an important role in regulating cardiopulmonary function under both normal and abnormal physiological conditions (Widdicombe, 2006).

C-fibres are activated by irritants, foreign chemicals, and inflammatory mediators as well as pathological conditions including anaphylaxis, pneumonia, and microembolism. As shown in Figure 5, the initial characteristic of a Paintal reflex is a reflexively-induced ‘apneic pause’ or breath-holding period between each breath. This is in contrast to the ‘bradypneic pause’ typical of reflex bradypnea in the URT that occurs between the end of inspiration and the onset of expiration. These apneic pauses can significantly reduce lung exposure to an inhaled irritant (Pauluhn, 2006).
Figure 5: This figure shows how reflex bradypnea can be distinguished from the Paintal reflex. These examples are from rodent studies of ammonia (URT irritant) and phosgene (LRT irritant). Upper and lower analog tracings represent flow- and volume-derived changes, respectively. The breath structure is characterized by three phases: inspiratory time (IT), expiratory time (ET), and apnea time (AT). These phases can be used to distinguish between upper respiratory tract irritants. The upper graph demonstrates the bradypneic pause characteristic of reflex bradypnea. Notice that the bradypneic pause occurs after the animal has inhaled ammonia. The lower graph demonstrates the apneic pause characteristic of a Paintal reflex. The apneic pause occurs between breaths, that is, after the animal has exhaled and before it takes its next breath of phosgene.

Source: Jürgen Pauluhn (Bayer Healthcare AG, Germany)

Several minutes of apneic pause are followed by rapid, shallow breathing (figure 6), bradycardia, hypotension, bronchoconstriction, laryngospasm, airway mucus secretion, and bronchial and nasal vasodilatation (Widdicombe, 2006). As with RB, the Paintal reflex may result in hypothermia (figure 7; Slade et al., 1997).
Figure 6: Effects of oxidant gas-particle mixtures of ozone, NO₂, ammonium bisulfate, carbon black particles, and nitric acid on rat breathing frequency and tidal volume over 4 hours during the first day of exposure. Data are hourly means ± SE, n = 8. The aerosol MMAD was 0.3 µm.
Source: Mautz et al. (2001)

Figure 7: Core body temperatures of individual mice exposed to 2.0 ppm O₃ for 2 hours at an ambient temperature of 22°C. Core body temperatures were monitored using peritoneally implanted radiotelemetry transmitters. The arrow indicates the time when O₃ exposure began.
Source: Slade et al. (1997)
In rodents, reflex bradypnea is predominated by a time-independent decrease in breathing frequency whereas the onset of reflex apnea (Paintal reflex) may be indicated by changes in tidal volumes (TV). To better judge the relative contribution of reflexes originating in the upper and/or lower respiratory tract, it is recommended to compare these endpoints side-by-side.

The occurrence and prolongation of apnea periods can be measured by the composite lung function endpoint enhanced pause (P$_{enh}$). P$_{enh}$ is likely more related to changes in breathing control whether caused by stimulation of receptors or changes in the mechanical properties of lung parenchyma. As opposed to measurements of respiratory rate and TV in volume-displacement plethysmographs (see Figure 8), which require restraint, the composite endpoint, P$_{enh}$ is proposed to be a more attractive endpoint utilizing whole-body plethysmography without any restraint or complex interventions (Pauluhn, 2004).

In this type of plethysmograph, data are collected every minute and averaged over specified periods. Ideally, data collection periods range from end of exposure up to the next exposure day. The particular advantage of whole-body plethysmography is that it does not interfere with the inhalation exposure at all and animals are not subjected to any additional immobilization stresses. Food and water should be available ad libitum during measurement periods exceeding 6 hours. Commonly, the following respiratory parameters are evaluated: respiratory rate, tidal volume, respiratory minute volume, peak inspiratory and expiratory flow rates during tidal breathing, inspiratory and expiratory times, including the dimensionless parameter P$_{enh}$. Measurement of P$_{enh}$ by unrestrained plethysmography does not provide a direct assessment of any specific physiologic variable. P$_{enh}$ is derived during spontaneous tidal breathing from the dimensionless relationship combining peak inspiratory flow (PIF) and peak expiratory flow (PEF), expiratory time (ET), and relaxation time (Rt, defined as time to expire 65% of the inhaled volume) as follows:

$$\text{P}_{\text{enh}} = \frac{\text{PEF}}{\text{PIF}} \times \left( \frac{\text{ET}}{\text{Rt}} - 1 \right)$$

Further details are published elsewhere (Epstein & Epstein, 1978; Hamelmann et al., 1997; Liu et al., 2013; Mitzner et al., 2003; Mitzner and Tankersley, 1998; Pauluhn, 2004).

**Pulmonary Function Assessments**

**How to identify reflex bradypnea and Paintal reflex in inhalation toxicity studies**

Reflex bradypnea and the Paintal reflex are both involved in the physiological control of breathing but with different manifestations for upper and lower respiratory sensory irritants, respectively. Highly water soluble irritants tend to be retained in the URT; though excessive concentrations may overwhelm the scrubbing capacity of the URT and result in LRT exposure, potentially with lethal outcomes. Irritants with low water solubility, such as ozone and phosgene, tend to trigger a Paintal reflex in the LRT. The principle characteristics of typical breathing cycles need to be documented for each concentration in order to identify the concentration-dependent critical location at which “sensory irritation” occurs. Sensory irritation-related endpoints are of importance to better qualify the transition from mere sensory (neurogenic) irritation to cytotoxic irritation causing airway inflammation and obstruction.

Collectively, for measurements of ‘sensory irritation’ the focus should be on concentration-
dependent reflex-bradypnea using volume-displacement plethysmography. As long as this reflex is stimulated, a concentration-dependent change in breathing patterns can be expected (Figures 1 and 2). When there is combined stimulation of upper and lower respiratory tract reflexes, the complexity of superimposed reflexes precludes the use of RB as a meaningful endpoint for hazard assessment since LRT irritation and injury can occur. In this case, pulmonary function measurements should be supplemented with BAL analyses to better quantify effects attributable to the lower airways.

RB and the Paintal reflex can be readily assessed and quantified by periodically measuring pulmonary function. These measurements should always be complemented by body temperature measurements, e.g., by using subcutaneously implanted transponders. Measurements should be made in all groups (including controls) at least at the beginning and towards the end of a study, and periodically in subchronic and chronic studies. If justified by the principle mode of action, animals in repeated exposure studies may be placed into whole-body barometric plethysmographs to record changes in breathing patterns after cessation of exposure up to the next exposure day. However, if RB is the sole modus, rodent pulmonary function can be readily achieved by fitting a head-only volume displacement plethysmograph into the port of a nose-only chamber as shown in the upper right of Figure 8. The mode of exposure, position, and degree of restraint is indistinguishable from that in commonly used animal restraining tubes. In addition, this analysis provides a more exact measurement of any substance-induced change in respiratory rate and minute volume. Minute volume is needed to estimate the inhaled dose of the substance tested.

**Figure 8:** This diagram illustrates how a head-only volume displacement plethysmograph can be placed into the port of a nose-only chamber to measure pulmonary function changes induced by reflex bradypnea and the Paintal reflex

**Source:** (Pauluhn and Thiel, 2007)

**Respiratory Function Measurements Using Volume Displacement Plethysmography:**

Pulmonary function tests should use simultaneous measurements performed on at least four restrained, spontaneously breathing rodents in modified nose-only animal restrainers with wire-mesh style pneumotachographs. Fleisch tubes should only be used when heat transfer from the heated tube
to the plethysmograph can be prevented. Fluctuations of thoracic air flows should be measured with a
differential pressure transducer fitted directly onto the plethysmograph. The head and body
compartment should be separated using an adequately constructed neck seal. Precautions must be
taken to avoid artifacts due to restraint and tight fitting seals around the neck. Accordingly, air
exposed animals should not show drifts in measurements during the entire data collection period.
Volumes are digitally calculated by integration of the flow signal from the body compartment.

Plethysmographs should be calibrated prior to each exposure using a calibration pump with stroke
volumes and breathing cycles matching the animals examined (for baseline data see Bide et al., 2000).
Data should be digitally integrated and collected over about 45 second time-periods. This minimizes
undue fluctuations without losing any marked time-resolution. After acclimatization to the
plethysmograph, baseline parameters are collected during a pre-exposure period of about 15 minutes
(dry air), followed by the exposure period to the test chemical for at least 45 minutes. Recovery should
be analyzed during post-exposure measurements of at least 30 minutes (examples are shown in Figures
1 and 2).

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correlation to pathology, structure-activity, tolerance development, and prediction of species
differences to nasal injury. IN: Toxicology of the nasal passages. Ed. Craig S. Barrow. 101-122
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Corporation. 16-25.
irritation: studies on correlation to pathology, structure-activity, tolerance development, and
prediction of species differences to nasal injury. In Toxicology of the Nasal Passages, Edited by
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exposed to chlorine or formaldehyde gas. Tox & Appl. Pharm. 76, 319-327.
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538-548.


APPENDIX VI

Particle Size Distribution

To verify graphically that an aerosol is in fact unimodal and log-normally distributed, the normalized mass per stage \( f'(i) \) is evaluated as a histogram. \( \Delta \log D \) is equal to \( \log D_{p+1} - \log D_p \), whereas \( D_p \) is the lower cut-size limit and \( D_{p+1} \) the higher cut-size limit of the corresponding impactor stage. Calculate the histogram \( f' \) by this equation:

\[
f'(H) = N_f \times \frac{\text{mass}/\text{stage}}{\Delta \log D_p}
\]

(1)

Calculate the log-normal mass distribution \( y(D_{ae}) = N_f x y(D_{ae}) \) as a function of the aerodynamic diameter \( (D_{ae}) \) using this equation:

\[
y(D_{ae}) = \exp \left( -\frac{(\log D_{ae} - \log \text{MMAD})^2}{2 \times \log^2 \text{GSD}} \right)
\]

(2)

and use the normalization factor \( (N_f) \):

\[
N_f = \left( \frac{\Sigma \text{mass}}{\log \text{GSD} \times \sqrt{2\pi}} \right)^{-1}
\]

(3)

An example calculation is provided in Table 1 and Figure 2.

For non-modal particle size distributions other modes of evaluation may apply.
Table 1: Example table for cascade Impactor Analyses

<table>
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<th>N</th>
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<th>Cut-Off Diameter (µm)</th>
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<th>Relative Mass (%)</th>
<th>Cumulative Mass (%)</th>
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<td>0.03</td>
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<td>3.690</td>
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<td>87.98</td>
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<tr>
<td>9</td>
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<td>14.800</td>
<td>0.000</td>
<td>0.00</td>
<td>100.00</td>
</tr>
</tbody>
</table>

Mass Median Aerodynamic Diameter (MMAD): 1.66 µm
Geometric standard deviation (GSD): 1.80

System: CASCADE-IMPACTOR
Airflow: 5.85 L/min.
Sampling time: 60.00 seconds
Concentration (computed): 1789.06 mg/m³

Respirability (percent < 1.0 µm):
Mass related: 19.7%

Respirability (percent < 3.0 µm):
Mass related: 84.1%

Respirability (percent < 5.0 µm):
Mass related: 96.9%
Figure 2: Upper panel - plot of the percentage of mass less than the stated size (probability scale) versus aerodynamic particle size (log scale). Lower panel: Particle-size distribution hand histogram and log-normal distribution (equation 2).