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# GUIDANCE DOCUMENT ON INHALATION TOXICITY STUDIES

**Series on Testing and Assessment**
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# GUIDANCE DOCUMENT ON INHALATION TOXICITY STUDIES

**Second Edition**

IOMC
INTER-ORGANIZATION PROGRAMME FOR THE SOUND MANAGEMENT OF CHEMICALS
A cooperative agreement among FAO, ILO, UNDP, UNEP, UNIDO, UNITAR, WHO, World Bank and OECD

**Environment Directorate**
**ORGANISATION FOR ECONOMIC CO-OPERATION AND DEVELOPMENT**
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**OECD Environment Directorate,**
**Environment, Health and Safety Division**
**2, rue André-Pascal**
**75775 Paris cedex 16**
**France**

**Fax : (33-1) 44 30 61 80**

**E-mail : ehscont@oecd.org**

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# *FOREWORD*

In October 2011, an OECD Expert Meeting on Inhalation Toxicity Testing was held to discuss potential revisions needed on the OECD Test Guidelines on inhalation: TGs 403 (Acute Inhalation), 436 (Acute Inhalation – Acute Toxic Class), 412 (Subacute Inhalation Toxicity), and 413 (Subchronic Inhalation Toxicity), as well as on the Guidance Document on Acute Inhalation Toxicity Testing published in 2009. The discussion focused on the need for developing and/ or adapting existing guidance documents and OECD Test Guidelines for inhalation toxicity testing of nanomaterials.

The recommendations from the meeting were further discussed by the Working Party on Manufactured Nanomaterials (WPMN) and the Working Group of the National Coordinators of the Test Guidelines Programme (WNT), which identified the recommended revisions as a high priority. The OECD completed the adaptations in TGs 412 (Subacute Inhalation Toxicity), and 413 (Subchronic Inhalation Toxicity) for the safety testing of manufactured nanomaterials in 2017. In addition, the TG 433 (Fixed concentration procedure) was also developed in 2017. As such, the motivations for updating this Guidance Document were to accommodate the testing of nanomaterials for TG 412 and TG 413, and to reduce animal numbers and reflect animal welfare for TG 433.This new edition of Guidance Document 39 reflects changes made in these Test Guidelines and provides guidance relevant to the conduct of these TGs for the safety testing of nanomaterials. Although it is recognised that the current structure does not differentiate explicitly guidance applicable to acute studies from guidance applicable to long term studies, this revised edition will assist regulators in implementing the TGs 412 and 413 for nanomaterials safety testing. The reader will refer to the text of respective Test Guidelines where explicit requirements are typically provided.

The present document was approved by the Working Group of the National Co-ordinators of the Test Guidelines Programme (WNT) at its 30th meeting in April 2018. The Joint Meeting of the Chemicals Committee and the Working Party on Chemicals, Pesticides and Biotechnology agreed to its declassification on 30 June 2018.

This document is published under the responsibility of the Joint Meeting of the Chemicals Committee and the Working Party on Chemicals, Pesticides and Biotechnology of the OECD.

# 1. INTRODUCTION

## 1.1. Background

1. In 1981, the OECD adopted Test Guideline 403 (TG 403) (OECD, 1981), which describes how to perform a traditional acute inhalation LC50 study. Since OECD Test Guidelines and Guidance are periodically reviewed in the light of scientific progress and animal welfare considerations, the OECD adopted five new or updated test guidelines between 2009 and 2017:

- TG 403 – a revised TG that includes two protocols—a traditional $LC_{50}$ protocol and a C × t protocol (adopted in 2009)
- TG 412 – 28-day inhalation test guideline (last updated in 2017)
- TG 413 – 90-day inhalation guideline(last updated in 2017)
- TG 433 – fixed concentration procedure (adopted in 2017)
- 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 labelling according to the United Nations Globally Harmonized System (GHS) of Classification and Labelling of Chemicals (UN, 2007) (adopted in 2009).

2. Guidance Document (GD 39) was originally published in 2009 to provide detailed information on the conduct of inhalation studies of all durations. Yet another Guidance Document, GD 125 (OECD, 2010b), which provides histopathology guidance for TGs 412 and 413, was approved in 2010. Test Guidelines 412 and 413 were revised and a new TG 433 was developed in 2017. The motivations were to accommodate the testing of nanomaterials for TG 412 and TG 413, and to reduce animal numbers and reflect animal welfare for TG 433.

3. For a number of Test Guidelines, the inhalation route of exposure may be used but this is not mentioned specifically in the guidance. If the inhalation route is chosen, the present guidance document should be consulted in the design of the studies.

### *1.1.1. Global Regulations Utilising Acute Inhalation Data*

4. Alternative Test Guideline TG 436 is able to satisfy most regulatory needs for a range estimate for an LC50 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 a Traditional LC50 protocol and also a concentration x time (C × 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 (National Research Council, 2001). The C × t protocol of the revised TG 403 can provide additional information which may be useful

for certain purposes, such as the derivation of Acute Exposure Guideline Levels (AEGLs).

5. For a glossary of terms see Appendix I.

# 2. PURPOSE

6. The main purpose of this document is to assist the regulated community and regulators in selecting the most appropriate acute inhalation TG so that particular data requirements can be met while reducing animal usage and suffering. This Guidance Document also contains additional supporting information on the conduct and interpretation of studies performed using the inhalation test guidelines: TG 403, TG 436, TG 433, TG 412, and TG 413. As mentioned before, for a number of Test Guidelines, such as TG 451 (carcinogenicity), TG 452 (chronic toxicity) or the Test Guidelines on reproduction and neurologic endpoints, the inhalation route of exposure may be used but this is not mentioned specifically in the guidance. If the inhalation route is chosen, the present guidance document should be consulted in the design of the studies. The second purpose of this document is to provide the necessary guidance when testing nanomaterials via inhalation in the 28-d and 90-d toxicity studies, subsequent to the high priority activity undertaken at the OECD in 2011.

7. 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 temporally 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., equilibrium of liquid/solid aerosol and vapour). The method chosen to collect test atmospheres for the determination of actual 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. All 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 the acute toxicity guidelines TG 403, TG 433[1], or TG 436 and the repeated exposure guidelines TG 412 and TG 413 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.

[1] In light of the fact that TG 433 was recently adopted (2017), the guidance offered in this guidance is preliminary.

# 3. DATA NEEDS

## 3.1. Triggers of Inhalation Toxicity Testing

8. 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 ethical, 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.

9. Further guidance on the waiving of acute inhalation toxicity tests can be found in OECD GD 237 (OECD, 2016). 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 tests procedures are detailed elsewhere (Babrauskas, V. et al, 2008).

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, OECDa) or 90 (TG 413, OECDb) days (the latter covers approximately 10% of the lifespan of a rat). The data derived from the 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 entire concentration-response/effect relationship. At the lower end is the no-observed-adverse-effect concentration (NOAEC). The target concentrations selected should allow the identification of the target organ(s) and demonstrate a clear concentration-response:

- The high concentration level should result in a clear level of toxicity but not cause lethality or persistent signs that might lead to lethality or prevent a meaningful evaluation of the results. When testing aerosols, the high concentration may be the

maximally achievable level that can be reached while meeting the particle size distribution standard.

- The intermediate concentration level(s) should be spaced to produce a gradation of toxic effects between that of the low and high concentrations.
- The low concentration level, which will ideally be a NOAEC, should produce little or no evidence of toxicity.

## 3.2. Uses of the Inhalation Tests

11. Acute inhalation toxicity data are used to satisfy hazard classification and labelling requirements, to estimate the toxicity of mixtures, and to assess human health and environmental risk. The derivation of either a point estimate of the LC50 value (using TG 403) or a range estimate of the LC50 using TG 436 (OECD, 2009b) or an inferred LC50 using TG 433 (OECDc, 2017) generally meets the acute inhalation toxicity regulatory requirements for classification and labelling 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 to repel insects). 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 5.1.4). For highly volatile test chemicals, 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 (UN, 2007).

12. Repeated exposure inhalation toxicity data are used to satisfy hazard characterization requirements with focus on a NOAEC or a benchmark concentration (BMC). The NOAEC is deduced. This value is achieved by empirical data characterizing the concentration-response/effect relationship of relevant endpoints or benchmark analysis. Therefore, in these types of studies, the primary focus is not on the classification and labelling of substances/mixtures as being commercialized but rather on characterization of toxic mechanisms and exposure atmospheres causing health hazards to repeatedly exposed humans. Chapter 4. of this Guidance Document addresses these aspects in greater detail.

### *3.2.1. Instillation and aspiration studies*

13. Instillation and aspiration studies are occasionally used as an inexpensive and easy way to learn something about the toxicity of a test chemical. These studies cannot be used in risk assessments because they do not resemble normal inhalation (the upper respiratory tract is bypassed) and they do not provide external exposure concentrations. For further information refer to appendix VI.

## 3.3. Definition of the Exposure Metric

14. Acute inhalation toxicity studies should be based on mass concentrations to comply with the unit of 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. Additional metrics may also be recorded during an experiment (i.e. surface area and particle number) to facilitate understanding of toxicity mechanisms. This is particularly relevant when investigating nanomaterials, where the most appropriate dose metric may not be known (ECHA, 2017).

## 3.4. 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 5.1.9):

$$\frac{mg\ /\ L \times 24{,}200}{MW} = ppm \qquad \frac{mg\ /\ m^3 \times 24.20}{MW} = ppm$$

$$\frac{ppm \times MW}{24{,}200} = mg/L \qquad \frac{ppm \times MW}{24.20} = mg/\ m^3$$

MW = Molecular weight

16. These algorithms imply that 1 mole of gas at the specified temperature (22°C in the equations above) 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 (Patty′s Toxicology, 2001). The conversion constants 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 II.

**Table 1. Conversion constants for different temperatures**

| Temperature | Conversion Constants | |
|---|---|---|
| **20 C** | 24,050 mg/L | 24.05 mg/m3 |
| **22 C** | 24,200 mg/L | 24.20 mg/m3 |
| **25 C** | 24,450 mg/L | 24.45 mg/m3 |

## 3.5. 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 waive testing or to minimize the

animal usage and enhance the quality of the study. There are advantages and disadvantages to both head/nose-only and whole-body exposure methods. The head/nose-only exposure method minimises exposure or uptake by non-inhalation routes and allows testing of individual animals at high concentrations, as required for limit tests, without the need for large quantities of material. Further advantages include; ease of maintenance of a homogenous test atmosphere, less potential for test chemical instability (e.g., reaction with excreta or humidity), and faster equilibration of the chamber atmosphere due to the smaller volume required. However, the head/nose-only technique does require restraint of the animals throughout the exposure period, which is not necessary for whole-body exposures, and therefore may cause more stress than whole-body technique. However, it is easier to remove a distressed animal from a nose-only chamber than a whole-body chamber. The selected exposure model should be designed to minimise any pain, distress or suffering experienced by the animals, consistent with the scientific objective 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 quantitative structure-activity relationship (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 (UN, 2007) 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 (UN, 2007). 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.

## 3.6. Data Bridging

18. Some national and international regulatory systems estimate the acute inhalation 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, it is stated in the GHS that mixtures can be classified using either point or range estimates of the LC50 of each component (UN, 2007). However, 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 4. should be observed carefully.

19. When testing simple mixtures (e.g., agrochemical preparations) of well characterized components, the Finney equation as defined by WHO's Environmental Health Criteria (WHO, 1978 - Principles and Methods for Evaluating the Toxicity of Chemicals: Part 1) may be used to estimate an LC50 (see equation below and the Glossary of Terms), provided these components produce additive acute toxicity and have parallel regression lines of probit against log-concentrations. The estimated LC50 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.

20. Alternatively, the acute toxicity estimate (ATE) of the GHS (UN, 2007; chapter 3.1.3. "Classification Criteria for Mixtures") can be applied. 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 ATEmix value:

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

where:

Ci = concentration of the ingredient i of n ingredients, and i runs from 1 to n

ATEi = Acute Toxicity Estimate of ingredient i

21. Any conversion from experimentally obtained acute toxicity range values (such as ranges obtained by using TG 436 or inferred by TG 433) to acute toxicity point estimates should be based on the GHS (see Appendix II) (UN, 2007).

22. 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.

## 3.7. Feasibility of Testing Mixtures

23. 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 or TG 433.

## 3.8. Evidence from Humans

24. 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.

## 3.9. Applicability of the Test Guidelines for Testing Pharmaceuticals

25. Inhalation toxicity testing by TGs 403, 412, 413, 433 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 concentration detailed in Appendix II.

# 4. ACUTE TEST GUIDELINE SELECTION

## 4.1. Outline of the Exposure Methodology

26. Acute inhalation toxicity is the total of adverse effects caused by a test chemical following a single, uninterrupted exposure of non-fasted healthy young adult animals by inhalation 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 (see section 3.5) 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 (Zwart et al, 1990; 1992). While nose-only is the preferred mode of exposure in the Test Guidelines, special objectives of the study may be better achieved by using the whole-body mode of exposure.

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 for rodents), non-pregnant, and body weights for each sex should be within ±20% of the mean weight of all previously exposed animals at the same age and same gender (males are the default sex for TG 433). As the mean weight increases, respiratory minute volume will also increase, though not in a proportional manner (Alexander et al. 2008).

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, smog, fibres, and nanomaterials). 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 behavioural

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.

29. Test atmospheres in inhalation chambers may consist of a mixture of different phases (*e.g.,* vapour, liquid aerosol, or the equilibrium thereof). Because of the need to generate respirable particles, the fraction of airborne particles generated from a mixture of polydisperse particles may not mirror a test chemical's aerosol characteristics under conditions of use. These aspects should be considered when judging the toxicological significance of findings from acute inhalation toxicity tests. It is possible to combine such information to produce a combined plot, but this involves making assumptions about particle density in particular, which can be difficult to determine accurately.

30. Acute inhalation toxicity testing Guidelines and available technologies have improved significantly over time, both in terms of well-defined animal exposure and test atmosphere characterization. Especially for short-term inhalation studies, exposure paradigms have shifted from whole-body to nose-only modes with novel procedures that minimize the re-breathing of atmospheres, attain faster inhalation chamber concentration equilibrium, and optimize the uniformity (i.e., degree of dynamic mixing) of flows within an inhalation chamber. The availability of computer-supported real-time monitoring devices and increased analytical sensitivity allows for better attainment of a uniform, spatial dispersion and temporal stability of test chemicals in an inhalation chamber. This dependence on available technologies when exposing animals is unique to inhalation toxicology.

31. 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 are not a concern. 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 (Phalen R.F., 2009).

32. The characterization of solid and liquid aerosols in inhalation chambers frequently requires that an aerosol sample be conveyed to a measurement or collection device. This is accomplished by withdrawing a sample from an inhalation chamber such that the sample is representative of the aerosol in the animals' breathing zone and not affected by the sampling process. 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. One objective of this Guidance Document is to clearly specify the importance of particle size and to describe how to minimize sampling errors. This means that isokinetic sampling strategies to preserve chamber aerosol characteristics need to be considered so that all phases (solid, vapour, gas) and particle size fractions are

collected with high efficiency from the animals' breathing zone in order to obtain similar material mass balances from different procedures.

## 4.2. Prioritization of Test Guideline

33. TG 433 or TG 436 should be used in preference over TG 403 because these guidelines provide 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.*, $LC_{50}$), non-lethal threshold concentration (*e.g.*, $LC_{01}$), 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. All TGs include a requirement to follow the OECD Guidance Document No. 19 on Humane Endpoints (OECD, 2000) (see section 4.13), which should reduce the overall suffering of animals used in acute toxicity studies and provide useful data for human risk characterization.

34. The selection of a Test Guideline is based upon a test chemical's specific data requirements. 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. When testing a chemical that is known, or likely to be, a respiratory irritant TG 403 should be used using either the traditional or the C × t procedure.

35. A study director or principal investigator should consider the following scenarios described in the section 5 when selecting a Test Guideline for a given test chemical.

## 4.3. Existing Evidence

36. 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.

37. 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).

38. 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 433 or TG 436 study.

## 4.4. Regulatory needs

39. Regulatory requirements should be consulted to determine if results obtained from a TG 433 or TG 436 study will be adequate.

40. 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.

## 4.5. Test chemicals that are anticipated to be highly toxic

41. 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.

## 4.6. Test chemicals that are severely irritating or corrosive

42. When testing irritant chemicals for specific regulatory needs (e.g., for emergency planning purposes), it is necessary to use air concentrations that will yield the desired degree of toxicity. TG 403 should be used when exposing animals to irritants because it provides the study director 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 section 4.13). Pulmonary function and body temperature should be measured to identify and quantify respiratory reflexes.

## 4.7. Technical problems

43. Technical problems may be encountered that make it impractical to perform a TG 433 or TG 436 study with its fixed concentrations. For example, if it is difficult to achieve the target chamber atmosphere concentration during pre-testing (before animals are exposed), then a TG 403 study should be performed. A TG 403 study is less affected by deviations from target concentrations because statistical analysis considers whatever actual concentrations were achieved.

## 4.8. Future changes in the GHS category bands

44. Changes to GHS category bands in the future will require a reassessment of biometrical performance (target) of TG 433 or 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.

## 4.9. TG 403 Studies - Traditional protocol or C × t protocol?

45. If a fixed point estimate of lethality is needed (e.g., a 4 hr LC50), the Traditional LC50 protocol should be performed.

46. If an estimate of the effect of time on concentration is needed, a C × t protocol should be performed.

47. If information is needed on LC10 or LC01 values, a C × t protocol will provide better estimates than a Traditional LC50 protocol (Zwart et al, 1990; 1992).

48. 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.

## 4.10. Range-finding studies in Test Guidelines

49. **TG 403**: A range-finding 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.

50. **TG 433**: The purpose of the sighting study is to allow selection of the most sensitive sex and an appropriate starting concentration for the main study.

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

## 4.11. Main Studies

52. **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 $LC_{50}$ study or a C × t study. In a Traditional $LC_{50}$ 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 multiple concentrations and exposure durations (Zwart et al, 1990; 1992). 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.*, $LC_{50}$), confidence interval, and slope. Other regulatory requirements may require estimation of additional lethal toxicity indices (*e.g.*, $LC_{01}$, $LC_{10}$).

53. **TG 433:** Pre-specified fixed concentrations are used in the main study. Groups of 5 animals in a single sex (males unless females are deemed to be more susceptible sex) are simultaneously exposed in a stepwise manner, with the initial concentration being selected to produce evident toxicity in some animals. Depending on the presence or absence of evident toxicity or mortality, further groups of animals may be exposed at higher or lower fixed concentrations as set out in Annex 1 of TG 433 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 labelling, 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.

54. **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 unequivocally to classify the test chemical. Because accuracy in achieving each target concentration is paramount to assure accurate classification and labelling, 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.

## 4.12. Information Provided by Each Test Guideline

55. The results of tests conducted according to TG 403, TG 433, 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:

1. TG 436 and TG 433 provide an inferred range estimate of the $LC_{50}$ 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).
2. The Traditional $LC_{50}$ protocol in TG 403 provides a point estimate of the $LC_{50}$ 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 $LC_{50}$" The approximate $LC_{50}$ is defined as the geometric mean from these mortalities.
3. 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 in case of steep concentration-mortality relationships because a C × t study relies on concentrations and durations rather than on concentrations alone.

## 4.13. Animal Welfare Considerations

56. Ethical concern for the welfare of animals includes the alleviation of stress and suffering. In addition to allowing for classification and labelling, 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.

57. All three acute inhalation TGs (TG 403, TG 433 and TG 436) require that OECD Guidance Document No. 19 on Humane Endpoints (OECD, 2000) 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 433 and 436 have stopping rules which limit the number of animals used in a test. TG 433 uses evident toxicity rather than lethality as an endpoint.

58. Animals showing severe and enduring signs of distress and pain should be humanely killed as described in OECD Guidance Document no. 19 (OECD, 2000). When exposing animals to a test chemical with corrosive or strong irritant properties, the targeted concentrations should not induce severe irritation/corrosive effects, yet 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 actual 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[2].

## 4.14. Limitations of Particular Approaches

59. 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 to compare the performance of the three acute inhalation test guidelines (Price et al., 2011) demonstrates that all three methods perform well in the absence of gender differences for chemicals with steeper concentration-response curve slopes when the true LC50 value is not very close to the edge of one of the classification ranges. TG 403 and TG 436 have similar properties with TG 433 more likely to erroneously assign a chemical to a more stringent class and less likely to assign it to a less stringent class. All three methods have the potential to misclassify in the presence of unanticipated gender differences (Stallard et al., 2003; Price et al., 2011). The range-finding study in TG 433 was subsequently modified to first test both a male and female animal to identify potential differences in sensitivity, overcoming the potential for sex differences to influence the classification (Stallard et al., 2011). 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 or TG 433. 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.

60. Unlike the TG 403 approach where point values are estimated by applying established statistical procedures to whatever analytical concentrations animals are exposed to, TG 433 and TG 436 studies require a greater measure of accuracy and consistency in chamber atmosphere because they solely depend on the outcome at the targeted exposure cut-off. This is why a technical pre-test without animals is required for TG 433 and 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 433 and 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

[2] From UN GHS, chapter 3.2 (UN, 2007). "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".

classification boundaries (see Appendix II) and because statistical analysis uses the actual concentrations. Nevertheless, technical pre-tests are recommended when performing a TG 403 study to maximize the likelihood of successful tests.

61. Literature surveys of systemically acting test chemicals show that there is usually little difference in susceptibility between the sexes in oral acute toxicity studies (Lipnick, R.L., et all, 1995). There is little useful information on relative sex sensitivity in acute inhalation studies. Sex-related differences in 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.

# 5. CONDUCT OF INHALATION STUDIES

## 5.1. Principle of the Test

### *5.1.1. Technical feasibility of desired test atmospheres*

62. The feasibility of generating a targeted test atmosphere should be determined in a test without animals. Tests are mandatory for TG 436 and recommended for TG 403 and TG 433 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 test 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.

63. 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.

### *5.1.2. Control Group*

64. A concurrent negative (air) control group is not necessary for acute studies. A concurrent control group is required for non-acute inhalation tests. 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.

### *5.1.3. Vehicle*

65. 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.

### *5.1.4. Limit Test*

66. The limit test 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 actual limit concentration is in the range of the vapour saturation concentration). For aerosols, the mass median aerodynamic diameter (MMAD) of the test atmosphere should be considered if no deaths occur at the limit concentration or the maximum attainable concentration. If the MMAD exceeds 2 µm, further efforts should be employed to reduce the test chemical's particle size. If the test atmosphere achieves the recommended MMAD criterion of ≤2 µm with a σg 1-3, and less than 50% lethality occurs at the limit concentration or the maximum attainable concentration, no further testing is necessary.

67. The selection of limit concentrations usually depends on regulatory requirements. 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 (see Appendix II) (UN, 2007). The GHS limit concentrations are used in TG 433 and 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 (UN, 2007), and justifications should be given in the study report. In the case of potentially explosive test chemicals, care should be taken to avoid conditions favourable for an explosion. For safety reasons it is generally advisable to not exceed 50% of the published Lower Explosive Limit (LEL).

68. 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 ≤2 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:

- a decrease in the respirable particle size fraction (and thus reduced toxicity)

- increased fluctuation and variability in inhalation chamber concentrations accompanied by increased spatial inhomogeneities,
- overloading of equipment used to characterize test atmospheres, and
- a divergence of nominal and actual concentrations.

69. 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.

70. When testing aerosols, for acute toxicity, the primary goal should be to achieve a respirable particle size (MMAD $\leq$2 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.

71. 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 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.

72. 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 section 3.4 and Appendix II). For example, at 22ºC, 20 mg/L of a gas is equivalent to 24,200 ppm if the gas has a molecular weight of 20 g/mol, or 2,420 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 analysed by the procedures used.

### *5.1.5. Performance of the Traditional protocol and the C × t Protocol - TG 403*

73. Selection of the number of animals and the number of concentrations tested in the Traditional $LC_{50}$ protocol and the C × t protocol should be informed by the study

director's understanding of the test's needed performance. For additional information see the Performance Assessment of these two protocols (OECD, 2009a). 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.

74. 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 $LC_{50}$ estimates that are comparable with a Traditional $LC_{50}$ protocol in terms of bias and precision. Calculation of a value for n requires mortality and survivors at a single concentration/time-point. When using 2 animals/ sex in a C × t design, the chance to be able to calculate a value for n is increased substantially, especially when there may be sex differences. With 1 animal per sex (or 2 of the more susceptible sex) the performance with respect to $LC_{10}$ or $LC_{01}$ estimates is greater than one would expect from the Traditional $LC_{50}$ protocol, and reasonably reliable $LC_{10}$ or $LC_{01}$ estimates would usually be obtained for all durations within the tested time range (OECD, 2009a). 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 $LC_{50}$-test, the addition of 1 extra animal per sex per C × t combination will reward the experimenter with better estimates (Zwart, J.H.E. et al, 1992). 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 $5^{th}$ exposure concentration with 5 durations may also suffice.

### *5.1.6. Performance of Repeated Exposure Studies - TG 412 and TG 413*

75. 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) (OECD, 1981d), TG 452 (chronic toxicity)( OECD, 1981e), and TG 453 (combined chronic toxicity/carcinogenicity)( OECD, 1981f) recommend that TG 412, TG 413, and GD 39 should be consulted when designing inhalation studies of chronic duration. Similarly, TG 412, TG 413, and GD 39 should be consulted when designing developmental, reproductive toxicity and neurotoxicity studies using TG 422, TG 412, and TG 443 when exposure is by the inhalation route.

76. 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:

1) Bronchoalveolar lavage (BAL) and the analysis of BAL fluid (BALF) 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 section 8.4.8.

2) Measurements of lung burden are mandatory when a range-finding study or other information demonstrates that a poorly soluble aerosol is likely to be retained in the lung. Biosolubility may differ significantly from the solubility in water and varies depending on biological systems such as cells and fluids. In order to decide whether your material is poorly soluble, the solubility of a solid material may be assessed by measuring solubility in a simulated biofluid). A poorly soluble material is generally understood as having a solubility of less than 0.1 g dissolved in 100 ml dissolvent within 24 hours. Examples of simulated biofluids include artificial lung lining fluid that contains salts and proteins or in an acidic environment that mimics the lysosomal fluid of macrophages. It is noted that to date there is no corresponding standardized test system. Therefore, adequate justification of the method applied is necessary. In either case, it is important to provide data on dissolution rates rather than fixed values.

3) The 2017 versions of TG 412 and TG 413 require aerosols to have a mass median aerodynamic diameter (MMAD) of $\leq 2$ μm with a σg of 1-3. 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).

4) 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).

77. As depicted in the study designs for both TG 412 and TG 413 (see OECD, 2017a; 2017b) there are two options based on the nature of the 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.

78. **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 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.

79. **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 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. If needed, 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.

80. 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 as explained in the Option B of TG 412 and 413 (see Annex in OECD, 2017a and 2017b), such as BALF, toxicity (including recovery), and toxicokinetics. Other options include:

1) 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. Lung burden measurement at three time points allow curve fitting on post-exposure clearance kinetics..

2) 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.

3) 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).

81. 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 Annex in TG 412 and 413). A minimum of two lung burden measurements are necessary when investigating clearance kinetics. For curve-fitting, a third lung burden measurement is needed. As a default, the study director may consider scheduling a PEO for lung burden measurements at 60 days after exposure termination. There is considerable literature of half-time on inhaled poorly soluble particles. As indicated above, 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.

### *5.1.7. Selection of an Inhalation Chamber*

82. A dynamic, validated inhalation system with suitable control of all inhalation chamber parameters is required for inhalation toxicity studies. Dynamic inhalation systems include nose-only chambers and whole-body chambers. 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.

83. 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 (Phalen R.F., 2009). The preferred mode of exposure is nose-only (which term includes head-only, nose-only, or snout-only) for the following reasons:

a) Exposure and/or uptake by any other route than inhalation (oral route via preening or dermal route) are minimized, especially when testing aerosols.

b) Technician exposure from handling exposed animals is minimized.

c) A minimum of test chemical is needed due to low chamber volume.

d) High concentrations (*e.g.*, limit concentrations) are readily achieved.

e) The instability of test chemicals (*e.g.*, reactivity with excreta or humidity) and test atmosphere in-homogeneity are of minimal concern.

f) The time required to attain inhalation chamber equilibration ($t_{95}$) is negligible relative to the duration of exposure and therefore not an issue.

g) 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 × t protocol, utilizing the same exposure concentrations for multiple exposure durations).

h) The exposure of individual animals can be interrupted at any time during the course of exposure to avoid undue suffering of animals.

i) Animals are readily accessible for specific physiological measurements (*e.g.*, respiratory function, body temperature) or the collection of blood, if applicable.

j) 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.

84. The principal advantages and disadvantages of nose-only vs. whole body exposure have been detailed elsewhere (Phalen R.F., 2009). Immobilization stress associated with long-term, nose-only exposure has been examined (Pauluhn, J. et al, 1999). Apart from differences in food and water intake, it was concluded that mode of exposure-associated differences in cardiovascular endpoints and respiration did not occur. Test laboratories should demonstrate that the animal restrainers used do not cause undue stress to animals (see section 5.1.8). 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.

85. 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 (Cannon, W.C., et al, 1983; Moss, O.R., et al, 2006) in which the inhalation exposure air flow and the exhalation flow can mix and be re-breathed. When an animal is confined to a restraining tube the observation of its behaviour 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 (Lipnick, R.L., et al, 1995; Cannon, W.C., et al, 1983; Moss, O.R., et al, 2006; Pauluhn, J. et al., 2007).

### *5.1.8. Nose-Only Exposure Technique*

86. During exposure, animals are exposed to the test chemical while in restraining tubes. The restraining tubes should not impose undue physical, thermal, or immobilization 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.

87. 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 leakages 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.

88. Animals should 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 (Moss, O.R., et al, 2006; Pauluhn, J. et al, 2007).

### *5.1.9. Whole-Body Exposure Technique*

89. 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 slight 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. Due to the testing of very low concentrations and the higher number of animals per chamber (relative to acute studies) possible interferences/interactions of the test article with excreta/ammonia/exhaled air etc. needs to be considered.

90. 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 build-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 (Phalen R.F., 2009).

$$t_{95}\ (\text{min}) = 3 \times \left( \frac{chamber\ volume}{chamber\ airflow} \right)$$

### *5.1.10. Range Finding Study - TG 412 and TG 413*

91. 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 ensure a robust main study. This is especially true when testing poorly soluble solid aerosols. For TG 412 and TG 413, 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, lung burden and local area lymph node (LALN) 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.

92. A range-finding study in TG 412 or TG 413 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 (GD 19; OECD, 2000). 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.

# 6. MONITORING OF EXPOSURE CONDITIONS

## 6.1. Inhalation Chambers

### *6.1.1. Chamber Airflows*

93. 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 section 6.2.2.

### *6.1.2. Chamber Temperature and Relative Humidity*

94. 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.

### *6.1.3. Inhalation Chamber Sampling*

95. 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 x time), should be appropriately calibrated. Sampled volumes can also be directly obtained with wet gas meters. Possible 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 analyse 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 actual concentrations include all physical forms of the analyte examined.

96. 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 (ACGIH, 1984; Willeke K and Baron P.A., 1993) or the relationship shown in Appendix II (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.

## 6.2. Test Atmosphere Characterization

### 6.2.1. *Nominal Concentration*

97. 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 actual 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 actual concentrations.

98. The consistency of inhalation tests can be judged by a comparison of nominal and actual 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, actual concentrations can significantly deviate from nominal concentrations, even by orders of magnitude. Ratios of nominal to actual 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 sublimate 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.

### *6.2.2. Actual Concentration*

99. 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. Actual concentrations can either confirm that the animals were properly exposed or reveal test chemical generation problems. The actual concentration is the test chemical concentration at the animals' breathing zone in an inhalation chamber. Actual 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. 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.

100. 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.

101. Individual actual 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.

102. 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 section 5.1.9). 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.

103. 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 analyse 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 actual chamber concentration due to precipitation, non-homogenous mixtures, volatile components, or other factors, additional analyses of inert components may be necessary as detailed above.

104. 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% inert, the actual mixture concentration is the concentration of the active ingredient multiplied by ten. It is not necessary to analyse 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.

### *6.2.3. Aerosol Particle-Size Distribution*

105. 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 (Landsiedel R., et al, 2014; Pauluhn, J., 2010). When isolated nanoparticles are generated as an aerosol, additional nanoparticle-specific sizing methods should be considered (e.g. a differential mobility analyzing system (DMAS)). 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.

106. 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 electron microscopy (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 to demonstrate that the targeted agglomeration state and/or morphology can be gained.

107. Because aerosol particle size distribution determines the site of initial deposition 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.

108. Although the standard for acute studies (MMAD of ≤4 μm with a σg of 1-3) 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 $\sigma_g$ 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. 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 (Phalen R.F., 2009). 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.

109. 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.

110. 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 analyse 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 analyse particle size distribution.

111. In repeated inhalation exposure studies, primary methods, such as chemical analysis, and secondary methods, such as infrared-spectrophotometry, for the determination of actual concentrations may be used if primary results are confirmed by the simpler, less elaborate secondary methods.

### *6.2.4. Aggregate Density*

112. Aggregate densities are commonly characterized by mercury pycnometry for porosimetry according to the ISO-Standard 15901-1 (2005). This standard delineates that different pores can occur as 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).

113. Particle deposition within the respiratory tract may be estimated by using the Multiple-path Particle Model MPPD v3.04 (or higher). MPPD requires the correct agglomerate density to calculate the regional deposition. This software 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. The software is open access and can be downloaded from this website: https://www.ara.com/products/multiple-path-particle-dosimetry-model-mppd-v-304. New versions are announced and available when launched.

## 6.3. Test Atmosphere Generation

### *6.3.1. Gases*

114. 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.

### *6.3.2. Vapours*

115. 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 aerosol droplets at concentrations above vapour saturation because this results in biphasic exposure (vapour and aerosol) and thus requires particle sizing. 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 conditions of test.

## 6.4. Aerosols from liquids

116. 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.

### *6.4.1. Aerosols from solid materials*

117. 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.

118. 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.

119. 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 (Phalen R.F., 2009).

120. The optimization of dustiness and particle size must account for the following constraints: 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.

## 6.5. Animal Exposure

### *6.5.1. Animal Selection and Assignment*

121. 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. At the beginning of a study, young adult rats should be approximately 8-12 weeks of age for acute studies, and 7–9 weeks of age old for long-term studies) should be used (further details are given in section 4.1)

122. In acute studies, the time interval between 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.

### *6.5.2. Animal Husbandry*

123. Each animal should be assigned a unique identification number. A system is required to randomly assign animals to test groups and a control group (if applicable). 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 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.

### *6.5.3. Exposure Time*

124. The duration of exposure should be specified. 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 and decay. Chamber equilibration and decay are assumed to be nearly instantaneous in nose-only chambers. For longer exposure durations, whole-body chambers are recommended.

# 7. OBSERVATIONS OF ANIMALS

## 7.1. Clinical Signs

125. Animals should be observed frequently during the exposure period with an emphasis on observing the time of onset of toxic signs. 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. For humane reasons and to ensure proper clinical oversight, observations may be performed at least hourly when animals are exposed to a known irritant, corrosive, or oxidant. Additional observations are made if the animals continue to display signs of toxicity. Observations include changes in skin and fur, eyes and mucous membranes, and also respiratory, circulatory, autonomic and central nervous systems, and somato-motor activity and behaviour patterns. Attention should be directed to observations of tremors, convulsions, salivation, diarrhoea, lethargy, coma, irregular respiration, hypoactivity, and bodyweight loss (>10%) (Sewell et al. 2015). The measurement of rectal temperatures may provide supportive evidence of reflex bradypnea, the Paintal reflex, 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 found elsewhere (Gad, S.C. and Chengelis, C.P., 1998) and objective measurements that are indicative of impending death and/or severe pain and/or distress are available in OECD Guidance Document No. 19 (OECD, 2000).

126. The duration of the observation period is not fixed, but should be determined by the nature and time of onset of clinical signs and length of the recovery period. 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.

127. 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 misjudgement, need to be carefully balanced. Expert judgment is needed to justify the respective procedure.

## 7.2. Body Weight

128. 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. 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.

## 7.3. Pathology

129. 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 (see section 7.6). 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.

## 7.4. Respiratory Reflexes

130. Laboratory rodents—but not humans—have two respiratory reflexes that allow them to markedly reduce their minute volume and thus reduce their exposure to inhaled irritants by entering a reversible, hibernation-like state. Failing to account for reduced rodent exposure may result in a risk assessment that is not health protective for humans. The potential impact of these reflexes on risk assessments has not received the attention it deserves from toxicologists and risk assessors, largely because test guidelines did not require measurements that could quantify the extent of these reflexes. TG 403, TG 412, and TG 413 now require pulmonary function and body temperature measurements when testing chemicals that are known, or likely to be, sensory irritants.

131. The first respiratory reflex—reflex bradypnea—is initiated by nociceptive stimulation of trigeminal nerves in the mucosa of the upper respiratory tract and eyes. It is triggered by water soluble sensory irritants such as aldehydes, ammonia, isocyanates, and pyrethroids that tend to be retained in the upper respiratory tract (URT); though excessive concentrations may overwhelm the scrubbing capacity of the URT and result in lower respiratory tract (LRT) exposure. Reflex bradypnea 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.

132. Sensory irritants with low water solubility, such as ozone and phosgene, can trigger the Paintal reflex by nociceptive stimulation of vagal C-fibres in the LRT. Although the signs of reflex bradypnea and the Paintal reflex are similar, the Paintal reflex can be distinguished by apneic pauses between breaths, which are then followed by rapid, shallow breathing. Because hypothermia occurs in rodents experiencing reflex bradypnea and the Paintal reflex, body temperature should always be measured if breathing measurements are performed, using either a subcutaneously implanted transponders or a rectal thermometer..

133. The profound physiological manifestations caused by these rodent-specific reflexes are often misinterpreted as systemic chemical toxicity or as adverse developmental, neurologic, or behavioural effects. A human risk assessment may not be health protective if it does not account for reduced rodent test chemical exposure caused by one of these respiratory reflexes. Further information on these reflexes can be found in Appendix V.

## 7.5. Pulmonary Function Tests

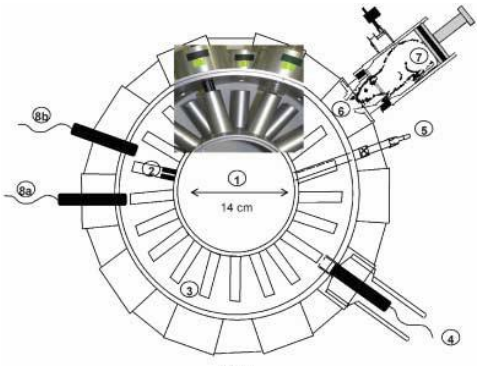
134. Pulmonary function tests (PFT) provide a non-invasive means for assessing how well the lungs are working. These tests may include measurements of lung volumes, capacities, rates of flow, compliance, and gas exchange. Pulmonary function data can be used to identify obstructive and restrictive lung disorders. They can also reveal when, and to what extent, rodents experience a respiratory reflex when exposed to a sensory irritant. When evaluating respiratory reflexes, measurements should include respiratory rate (breaths/minute), air flow, tidal volume, and minute volume. These parameters can be used to identify whether a reflex occurred in the URT (reflex bradypnea) or the LRT (Paintal reflex), and to show the extent of reduced rodent exposure to an irritant. TG 403, TG 412, and TG 413 require measurements of pulmonary function and body temperature when testing chemicals that are known, or likely to be, sensory irritants.

135. Respiratory reflexes 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 or rectal thermometers. 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.

### *7.5.1. Pulmonary Function Measurements Using Volume Displacement Plethysmography:*

136. The head-only volume displacement plethysmograph is preferred when measuring respiratory reflexes because it can be fitted into the port of a nose-only chamber as shown in the upper right of Figure 1, thus allowing pulmonary function tests to be performed while an animal is exposed to a test chemical. 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 robust 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 1. Head-only volume displacement plethysmograph in the port of a nose-only chamber**




*Note*: This diagram illustrates the use of a head-only volume displacement plethysmograph in the port of a nose-only chamber
*Source*: Pauluhn and Thiel, 2007

137. 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 compartments 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 time periods of about 45 seconds. 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 Appendix V).

### *7.5.2. Pulmonary Function Measurements Using Whole-Body Plethysmography*

138. The primary disadvantage of whole-body barometric plethysmography is an inability to expose animals while measuring pulmonary function. Other limitations include a lack of precision, sensitivity to temperature and humidity, algorithms that use a default body temperature of 37 ℃, faulty measurement of hypothermic animals (i.e., those experiencing reflex bradypnea or the Paintal reflex), and the need for frequent recalibrations when measuring tidal volume. If justified by the principle mode of action, however, 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.

139. The occurrence and prolongation of apnea periods can be measured by the composite lung function endpoint enhanced pause (Penh). Penh 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 tidal volume in volume-displacement plethysmographs, which require restraint, the composite endpoint, Penh is proposed to be a more attractive endpoint utilizing whole-body plethysmography without any restraint or complex interventions (Pauluhn, 2004).

140. 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 Penh. Measurement of Penh by unrestrained plethysmography does not provide a direct assessment of any specific physiologic variable. Penh 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 (Mitzner et al., 1998, 2003; Pauluhn, 2004):

$$Penh = \frac{PEF}{PIF} \times \left( \frac{ET}{Rt} - 1 \right)$$

## 7.6. Bronchoalveolar Lavage

141. When there is evidence that the lower respiratory tract (i.e., the alveoli and/or the tracheobronchial region) is the primary site of deposition and retention, then BAL is the technique of choice to quantitatively analyze dose-effect relationships for various parameters focusing on alveolitis, pulmonary inflammation, and phospholipidosis. This allows for dose-response and time-course changes of alveolar injury to be suitably probed. BAL fluid (BALF) analyses are particularly useful when the response is generalized and occurring in the luminal parts of the lower respiratory tract, but it may also be of limited relevance for focal responses or areas/interstitial responses not accessible by the lavage fluid. Because severe inflammation can lead to airway plugging, the most severely damaged location may not be accessible via the lavage fluid. Thus, measurements in BALF generally complement the results from histopathology examinations but cannot replace them. Either one could be the more sensitive under a given circumstance. Rather, the two approaches are complementary and are best used in concert as some changes may preferentially occur in the alveolus while others (e.g., fibrosis) occur in the mesenchymal tissue compartment not accessible by BAL. The examination of statistical correlates between BAL data and data for morphological changes derived from the scoring of lesions is a particularly powerful approach to quantitative analysis of data.

142. A great deal of valuable information can be obtained from the acellular component of BALF including levels of immunoglobulins, enzymes, inflammatory mediators, and surfactant (Henderson, 1989). However, variable dilutions of BALF can cause both inaccuracy of quantification and difficulties detecting trace amounts of solute.

Therefore, acellular components are most concentrated. There are many BAL-associated details to be further determined, including the issue of erythrocytes. BALF contains some erythrocytes when lung is not fully exsanguinated, and it may contain erythrocytes even when an inflamed lung is exsanguinated fully.

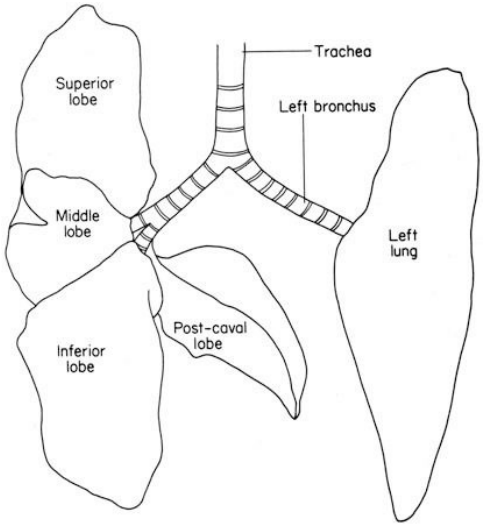
143. The alveolar surface of the lungs is lined with a complex and highly surface-active material: pulmonary surfactant. The lung appears to be one of the most sensitive organs for changes in phospholipid homeostasis, since the turnover of phospholipids is highest in this organ due to the anabolism, catabolism, storage and recycling of pulmonary surfactant. This material consists of 90% lipids (including glycerol) and 10% surfactant-specific proteins. The lipids are mainly phospholipids; among the most important being phosphatidylcholine (approximately 60%), which is mainly responsible for lowering surface tension. The water-soluble film of surfactant phospholipids and apoproteins determines both structure and homeostasis of the surfactant. The surface film that lines the alveoli prevents alveolar collapse. The functions of surfactant, including its surfactant apoproteins, in the alveolar lining layer are diverse. By stabilization of the fluid balance and reducing the contractile forces in the curved air-liquid interface, it prevents transudation of fluid into the alveoli (Van Golde et al., 1988). Disturbance of the surfactant system by noxious agents can take place at different stages (Van Golde et al., 1988). A compromised surfactant layer may lead to an increased permeability of the air-blood barrier and subsequent extravasation of plasma proteins (Nieman, 1985, Reasor, 1981). While the physicochemical properties and behaviour of nanomaterials can be accurately characterized under idealized conditions, this is no longer the case in complex physiological environments. Site of deposition specific molecules can be adsorbed at the nanomaterial-bio interface to form a corona that critically affects the particles' (patho) biological entities (Docter et al., 2015).

144. The strength of BAL is that it integrates the overall intraluminal response to injury in a readily quantifiable manner by determining cellular endpoints (cell viability, cytodifferentiation and activation) and acellular endpoints originating from local inflammatory processes or from plasma as a result of alveolar barrier disruption. While this method is suitable to probe for diffuse injuries, it has limited resolution for focal injuries. Histopathology changes are more complex to integrate area- and intensity-wise but they are the most suitable means to unequivocally identify the anatomical locations of structural injury and the response to injury. Protocols that focus on the instant response to particle exposure may benefit from BALF-analysis as the intraseptal changes may occur secondary to alveolitis and acute injury. At that point in time where structural remodelling of lung tissue occurs, histopathology will be the method of choice. From that perspective, it can be concluded that BAL and histopathology each have their particular advantages and shortcomings and should be viewed as complementary.

145. Laboratories should follow their own standard operating procedures when collecting BALF. In the absence of an SOP, the following procedures may be used. A procedure to obtain BALF obtained from rats: Animals should be weighed before euthanization (e.g. using ketamine (75–100 mg/kg)/xylazine (10 mg/kg) anaesthesia). The abdomen and the thorax should be opened and blood should be withdrawn from the abdominal aorta with a syringe. A cannula should be placed in the trachea and the diaphragm should be opened. One half of the lung is tied off and then used for weighing and/or histopathology as described above (in case of rats commonly the left lung lobe), and the other half (cranial lobe/l. cranialis, median lobe/l. medius, caudal Lobe/l. caudalis, accessory lobe/l. accessorius) is lavaged. The left lung should be clipped, while the right lung should be flushed once using 26-28 mL/kg body weight (approximately

60% of the total lung capacity) of ($Ca^{2+}$/$Mg^{2+}$-free phosphate-buffered) saline (pH 7.4). The flush consisted of three up and down movements. Only BALF data with a recovery >60% of the total flush volume should be included. The reason for lower recovery rates includes accidental damage to the lung before or during flushing. The BALF should be centrifuged at 400 × g at 4 °C for 10 min. Erythrocytes can affect the acellular component of BAL and could lead to faulty cell count results. A possible solution is to separate cells from the fluid as quickly as possible by centrifugation. The supernatant can be used to determine e.g. lactate dehydrogenase (LDH), albumin, and total protein. The cell pellet should be suspended in 1 mL of phosphate-buffered saline for total cell counts and cell differential counts of 400 cells prepared as cytospins.

**Figure 2. Rodent trachea and lungs**




*Note*: The (left) lung to be used for histopathology is instilled by an appropriate fixative using an instillation pressure of 20-30 cm of water and further processing. This procedure is to be used whenever the analysis is more qualitative (e.g., mechanism-related or proof-of-principle-related).
*Source*: http://www.informatics.jax.org/cookbook/figures/figure53.shtml

146. The following endpoints are considered **mandatory**: LDH activity and total protein or albumin levels in acellular BALF, and cell counts and differentials for alveolar macrophages, lymphocytes and neutrophils:

a) Measurement of LDH activity and protein or albumin Level in the acellular BAL fluid: BALF samples for LDH should be kept at room temperature, not frozen, and measured the same day as lavage. LDH activity can be quantified by detection of the oxidation of lactate coupled to the reduction of $NAD^+$ by spectrometry at 340 nm. Protein or albumin level can be quantified by the method of Lowry et al. (1951) using an autoanalyzer. Various methods are available for total protein or albumin determinations.

b) Measurement of cell counts and differentials: Add a drop of the BAL cell suspension onto a hemocytometer and count under low power light microscopy. Add $10^5$ cells and spin onto a slide using a centrifuge. Stain with Romanowsky stain (the black precipitate formed from the addition of aqueous solutions of methylene blue and eosin, dissolved in methanol) or equivalent. Conduct cell differentials by identifying a minimum of 400 cells using low power light

microscopy. The number of alveolar macrophages, lymphocytes, and neutrophils are quantified by multiplying the % per cell type by the total number of BAL cells.

147. A large list of optional measurements can be given. Measurement of eosinophil count from BAL has been suggested to evaluate allergic response. This could be done simply by identifying the % of eosinophils on the cytospin slides discussed above. Measurement of cytokines, chemokines, and mediators has also been suggested. This could be done using a sample of the first acellular BALF discussed above. Mediator levels can be quantified using ELISA kits. These mediators could be evaluated at a later time on frozen BALF samples without increasing the number of rats used in the study.

148. Alternative measurements are encouraged that might inform mode of action (MOA) or (adverse outcome pathway (AOP) and pathways initiated in pathogenesis process for the inhaled agent. Other parameters that may be considered are those indicative of lysosomal injury, phospholipidosis, fibrosis, and irritant or allergic inflammation which may include the determination of pro-inflammatory cytokines/chemokines. Parameters often reported in the literature include measurement of the following:

c) Soluble collagen levels in acellular BALF as an indicator of a dysfunctional alveolar air/blood barrier.

d) □-Glutamyltranspeptidase in acellular BALF as an indicator of an increased Clara-cell and also Type II pneumocyte activities.

e) β-NAG (β-N-acetylglucosaminidase) in acellular BALF is most probably the lysosomal enzyme released from resident alveolar macrophages engaged with particle endocytosis.

f) Cytokine, chemokine, or other mediator levels in acellular BALF as indicators of (pro-) inflammatory, proliferative, or (pro-)fibrogenic states. Release of these mediators from BAL cells may also be determined.

g) Although the precise mechanism by which any pulmonary phospholipidosis occurs still needs to be elucidated, determination of phospholipids in BALF including the cells as surfactant constituents may be useful to assess surfactant dysfunction because these endpoints appear to indicate changes most significantly. Emphasis could also be directed to foamy macrophages.

## 7.7. Toxicokinetics

149. Although toxicity testing is aimed at characterization of a test chemical's toxicity, inclusion of the measurements of toxicokinetic parameters (e.g., ADME) will provide valuable information for multiple reasons, including supporting read across and grouping. Therefore, inclusion of toxicokinetic parameters in an inhalation toxicity study can be considered provided that 1) this is of added value for the conduct of additional inhalation toxicity studies and/or the risk assessment, 2) there is no interference with the outcome of the inhalation toxicity study and 3) there is preferably no use of additional animals. The suggestions provided here are guidance for vapours, gases and soluble particles. Particles that poorly dissolve and are likely to retain in the lungs require a different approach, i.e., focus will then be on lung burden measurements for the understanding of lung clearance kinetics.

150. Several aspects need to be taken into account when considering inclusion of toxicokinetic measurements in an inhalation study. Blood sampling can be performed during studies with nose-only exposure, e.g., by taking blood samples from the tail vein. However, in case of whole body exposure sampling can only be performed after the end of the study, which limits the possibilities of informative toxicokinetic measurements. Further, the possible effect of the sampling on the outcome the study shall be taken into consideration. For example, blood sampling in studies including Functional Observational Battery (FOB) may affect the animal behaviour and is therefore not recommended.

151. Toxicokinetic information obtained in a range-finding study may support for the design of follow-up studies by providing useful information to determine concentrations for the main study (OECD, 2010a).

152. Information on toxicokinetics can contribute to refinement of the risk assessment by providing a better understanding of the relationship between external exposure and the toxicity observed. 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, 2010a; Brandon et al., 2015). Internal concentrations of the parent compound and/or metabolites may contribute in the identification of the toxic agent and its relationship with observed effects and thus in the understanding of the mode of action.

153. Considerations of the OECD TG 417 (OECD, 2010a) are also applicable when including toxicokinetics in a toxicity test. Inclusion of the measurements of toxicokinetic parameters in blood samples can be performed to obtain estimates of basic parameters such as e.g., Cmax, Tmax, elimination half-life (t1/2), AUC, for the test chemical. Parameters can be easily measured in blood, for example by sampling from the tail vein. The volume and number of blood samples which 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 analysed for each individual animal. In some circumstances (e.g., metabolite characterization), it might be necessary to pool samples from more than one animal. Pooled samples should be clearly identified and an explanation for pooling provided.

154. 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 vapours are soluble enough to gain access to the systemic circulation with rapid metabolization 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.

155. However, the design of repeated exposure 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. Alveolar macrophages are typically the dominant pathway of elimination of poorly soluble particles from the lung. For normal clearance 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 normal clearance 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 (Brown, J.S., et al, 2005).

156. Poorly soluble, low toxicity micrometer particles or nanoparticles are deposited in the alveolar region and are retained by endocytotic 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 approximately 6% of the pooled volume of alveolar macrophages represents the level at which impaired clearance starts to occur in rats, which is the most commonly used species for inhalation toxicity studies. .

157. In case of submicron particulate chemicals, observations on agglomeration state and cellular compartmentalization should be reported and particles should be quantified in target tissues, if technically feasible. Chemicals with fibre geometry may need additional specific observations, such as interstitial and pleural detection and possibly quantification, depending on fibre dimensions. This can be done for instance by Dark-field electron microscopy (Mercer RR, et al. 2018; Wagner, et al. 2014).

158. There is extensive literature on kinetic modelling from inhalation studies (Keller J., et al., 2014; Pauluhn J, et al, 2003; Pauluhn J, 2011a)

## 7.8. Lung Burden Measurement

159. Lung burden measurements performed in the course of repeated exposure studies in rats, provide a metric of retained dose and may be helpful in understanding the toxicity of poorly soluble particles. However, each retained lung burden may have a different kinetic history due to burden-specific changes in clearance. A wealth of information is available comparing modelled and empirically determined lung burdens. 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 retained lung burdens in relation to exposure concentrations, exposure duration, and clearance.

160. 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 digest the lung in order to attain soluble analytes (metal ions) with well-defined oxidation states. Carbonaceous isometric materials (carbon black) are also analysed 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 Couples Plasma – Optical Emission Spectroscopy) analysis. All methods are somewhat specific to the type of test substance examined (Baisch B.L., et al., 2014; Bellmann, B., et al., 1991; Cassinelli ME, 1998; Donaldson K, et al., 2010; Doudrick K, et al. 2013; ILSI, 2005; Kasai T, et al.,

2016; Tamura M, et al., 2011; Saxena et al. 2008 and 2009). 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 substance-specific morphological characteristics have to be accounted for (ILSI, 2005; Kasai T, et al., 2016).

161. 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 analyte 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 hilus 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 are used as analytical standard.

162. The typical metric for recording lung burden is mg of test chemical per g lung tissue.

## 7.9. The Value and Utility of Lung Burden Measurements in Risk Assessments

163. 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. 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.

164. Lung burden data can be used for the risk assessment of poorly soluble particles (e.g. as obtained from tests according to TG 412 and TG 413). When pulmonary effects are driving the human health risk assessment, risk assessors need to evaluate whether the occurrence of the pulmonary effects are better characterised by exposure concentration or by retained dose in the lungs. The human equivalent dose and lifetime human exposure may be calculated for risk estimation. Applications of such principles are available in literature e.g.: NIOSH (2013) and Oberdörster et al. (2015).

165. Another value of lung burden data is the possibility of reading across hazard data from studies using various sizes of the same materials (Oberdörster and Kuhlbusch, 2018). The same external concentrations can result in differences in retained dose. Conversely, different external concentrations can result in the same retained dose for different particle sizes.

166. Although lung burden measurement is mandatory at only one post-exposure observation period in Option B (at PEO-1), 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.

## 7.10. Animal Welfare – Bronchoalveolar Lavage, Lung Burden, and Histopathology

167. 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.

168. 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; unless a laboratory has demonstrated proficiency in this procedure.

# 8. STATISTICAL ANALYSIS OF DATA

## 8.1. Median Lethal Concentration ($LC_{50}$) and Other Percentiles

169. 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 (Schaper, M.M., et al, 1994; ten Berge, W.F., et al 1986; and ten Berge, W.F. and Zwart, A. 1989).

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

1) Actual exposure concentrations
2) The number of animals exposed
3) The number that died.

171. 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 (LC01, LC10, ...) 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-LC01 and LC99, 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.

172. When experimental/mathematical procedures require the estimation of median lethal concentration values from multiple exposure durations ($LCt_{50}$) this is accomplished by the C × t protocol combining the exposure concentration (C), exposure time (t) and the toxic load exponent (n), using the following equation: $k = C^n \times t$ where k is a numerical constant (Gad, S.C. and Weil, C.S., 1989; Sokal, R.R. and Rohlf, P.J., 1969). This equation can be generalized using a two-variate-surface plot relating toxicity (mortality) and time as follows:

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

$$\text{where } n = b_1/b_2$$

173. Here, y is the Probit value and b0, b1 and b2 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 × t relationships evaluated in one single matrix. From the constants of the two-variate surface plot, the respective LCt50 and LCt01 (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 × t relationships based on exposure durations of less than 15 minutes should be judged carefully.

## 8.2. Body Weights and Non-Lethal Endpoints

174. 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 analyse this, and in what form to analyse the organ weight data (as absolute weights, weight changes, or percentages of body weight), have been dealt with elsewhere (Schaper, M.M., et al, 1994). 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 analysed 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 (Schaper, M.M., et al, 1994; Witschi, H-P. and Last, J.A., 1996).

175. The analysis of pathology data is best analysed by ANOVA followed, if called for, by a *post hoc* test. Repetitively measured data should be analysed 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 analysed, 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 (Witschi, H-P. and Last, J.A., 1996). However this transformation is not appropriate for continuous endpoints like absolute body weights or absolute body weight gains.

# 9. REFERENCES

American Conference of Governmental Industrial Hygienists (ACGIH), (1984). Particle Size-Selective Sampling in the Workplace. Pub. No. 0830. ACGIH, Cincinnati, OH.

Alexander, DJ et al. Association of Inhalation Toxicologists (AIT), (2008). Working party recommendation for standard delivered dose calculation and expression in non-clinical aerosol inhalation toxicology studies with pharmaceuticals. Inhal Toxicol 2008; 20:1179-89.

ASTM (2012). ASTM E981 - 04(2012) Barrow et al. (1986). Sensory irritation: Studies on 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

Babrauskas, V., Gann, R., and Grayson, S. (2008). Hazards of combustion products: Toxicity, opacity, corrosivity and heat release. Interscience communications, London, UK

Baisch B.L., Corson N.M., Wade-Mercer P., Gelein R., Kennell A.J., Oberdörster G., Elder A., (2014). Equivalent titanium dioxide nanoparticle deposition by intratracheal instillation and whole body inhalation: the effect of dose rate on acute respiratory tract inflammation. *Part Fibre Toxicol.* 11: 5.

Barrow, CS; Steinhagen, WH; Chang, JCF. (1983). Formaldehyde sensory irritation. In: Formaldehyde Toxicity. Ed: J.E. Gibson. Washington, D.C., Hemisphere Publishing Corporation. 16-25.

Barrow, C.S., Buckley, L.A., James, R.A., Steinhagen, W.H., and Chang, J.C.F. (1986). Sensory 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 Craig S. Barrow, Hemisphere Publishing Corp., Washington D.C. pp. 101-122.

Bellmann, B., Muhle, H., Creutzenberg, O., Dasenbrock, C., Kilpper, R., MacKenzie, J.C., Morrow, P., and Mermelstein, R., (1991). Lung clearance and retention of Toner, utilizing a tracer technique, during chronic inhalation exposure in rats. Fundam. Appl. Toxicol. 17, 300-313.

Bide, R.W., Armour, S.J., and Yee, E. (2000). Allometric respiration/body mass data for animals to be used for estimates of inhalation toxicity to young adult humans. *J. Appl. Toxicol.* 20: 273-290.

Bos, P.M.J.; Busschers, M; and Arts, J.H.E. (2002). Evaluation of the sensory irritation test (Alarie test) for the assessment of respiratory tract irritation. JOEM. Vol. 44. No. 10. October 2002. 968-976.

Bos, P.M.J.; Zwart, A.; Reuzel, P.G.J.; and Bragt, P.C. (2008) Evaluation of the sensory irritation test for the assessment of occupational health risk. Critical Reviews in Toxicology. Vol 21. Issue 6. 423-450.

Brown, J.S., Wilson, W.E., and Grant, L.D. (2005). Dosimetric comparisons of particle deposition and retention in rats and humans. *Inhal. Toxicol*. 17: 355-385.

Cannon, W.C., Blanton, E.F., and McDonald, K.E. (1983). The flow-past chamber: An improved nose-only exposure system for rodents. *Am. Ind. Hyg. Assoc. J.* 44:923-928.

Cassinelli ME, O'Connor PF, eds. (1998). NIOSH Method 5040. In: NIOSH Manual of Analytical Methods (NMAM), Second Supplement to NMAM. 4th ed. DHHS (NIOSH) Publication No. 94-113.

Cincinnati, OH:National Institute for Occupational Safety and Health. Available: http://www.cdc.gov/niosh/nmam/pdfs/5040.pdf.

Chan P.K. and Hayes A.W. (1994). Acute Toxicity and Eye Irritancy. In: Principles and Methods of Toxicology. A.W Hayes, Ed. Raven Press Ltd, New York.

Chang, JCF and Barrow, CS. (1984). Sensory irritation tolerance and cross-tolerance in F-344 rats exposed to chlorine or formaldehyde gas. Tox & Appl. Pharm. 76, 319-327.

Clark P, Koehler KA, Volckens J. An improved model for particle deposition in porous foams. J Aerosol Sci. (2009);40:563–72

Diener W., Siccha L., Mischke U., Kayser D. and Schlede E. (1994). The Biometric Evaluation of the Acute-Toxic-Class Method (Oral.) Arch. Toxicol. 68, 599-610.

Diener W., Mischke U., Kayser D. and Schlede E. (1995). The Biometric Evaluation of the OECD Modified Version of the Acute-Toxic-Class Method (Oral). Arch. Toxicol. 69, 729-734.

Diener W., Kayser D. and Schlede E. (1997). The Inhalation Acute-Toxic-Class Method; Test Procedures and Biometric Evaluations. *Arch. Toxicol.* 71, 537-549.

Diener W, and Schlede E (1999). Acute Toxic Class Methods: Alternatives to LD50/LC50 Tests. ALTEX 1: 129-134

Donaldson K, Murfy FA, Duffin R, Poland CA. (2010). Asbestos carbon nanotubes and the pleural mesothelium: a review of the hypothesis regarding the role of long fiber retention in the parietal pleura inflammation and mesothelioma. Part Fibre Toxicol 7:5, 1-17.

Doudrick K, Corson N, Oberdörster G, Elder AC, Herckes P, Halden RU, Westerhoff ) (2013). Extraction and Quantification of Carbon Nanotubes in Biological Matrices with Application to Rat Lung Tissue. ACSNano 7: 8849-8856.

Driscoll K.E., Costa D.L., Hatch G., Henderson R., Oberdörster G., Salem H., Schlesinger RB., (2000). Intratracheal instillation as an exposure technique for the evaluation of respiratory tract toxicity: Uses and limitations. Toxicological Sciences. 55, 24-35.

European Chemicals Agency (2017) Guidance on information requirements and chemical safety assessment Appendix R7-1 for nanomaterials applicable to Chapter R7a Endpoint specific guidance.

https://echa.europa.eu/documents/10162/13632/appendix_r7a_nanomaterials_en.pdf. Published May 2017

Gad, S.C. and Chengelis, C.P. (1998). Acute Toxicology Testing, second edition. Academic Press, New York.

Gad, S.C. and Weil, C.S. (1989). Statistics for Toxicologists. *Principles and Methods of Toxicology*, second Ed. Editor: A. Wallace Hayes. Raven Press Ltd., New York, pp 435-483.

Gordon, CJ; Spencer, PJ; Hotchkiss, J; Miller, DB; Hinderliter, PM; Pauluhn, J. (2008). Thermoregulation and its influence on toxicity assessment. Toxicology 244, 87-97.

Hasegawa-Baba Y., Kubota H., Takata A., Miyagawa M., (2014). Intratracheal Instillation Methods and the Distribution of Administered Material in the Lung of the Rat. J Toxicol Pathol 27, 197-204.

Holzhütter, H-G, Genschow, E., Diener, W., and Schlede, E (2003) Dermal and Inhalation Acute Toxicity Class Methods: Test Procedures and Biometric Evaluations for the Globally Harmonized Classification System. Arch. Toxicol. 77, 243-254

Holzum,B; Schmidt, U; Pauluhn, J; et al. (1994). FCR 1275: Explanatory report on results and mechanistic studies for embryotoxic effects in rats after inhalation: Lab Project Number: 106369; 23219: 22581. Unpublished study prepared by Bayer Ag.

ILSI (2005). Special Issue – Testing of fibrous particles: Short-term assays and strategies: Report of an ILSI Risk Science Institute Working Group. Inhal. Toxicol. 17, 497-537.

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)

Jaeger, RJ and Gearhart, JM. (1982). Respiratory and metabolic response of rats and mice to formalin vapor. Toxicology. 25, 299-309.

Kane, LE; Barrow, CS; Alarie, Y. (1979). A short-term test to predict acceptable levels of exposure to airborne sensory irritants. Am. Ind. Hyg. Assoc. J (40); 207-229.

Kane, L.E. and Alarie, Y. (1977). Sensory Irritation to Formaldehyde and Acrolein during Single and Repeated Exposures in Mice. American Industrial Hygiene Association Journal, 38, 509-522.

Kasai T, Umeda Y, Ohnishi M, Mine T, Kondo H, Takeuchi T, Matsumoto M, Fukushima S., (2016). Lung carcinogenicity of inhaled multi-walled carbon nanotube in rats. Particle and Fibre Toxicology 13:53.

Keller J, Wohlleben W, Ma-Hock L, Strauss V, Gröters S, Küttler K, Wiench K, Herden C, Oberdörster G, van Ravenzwaay B, Landsiedel R., (2014). Time course of lung retention and toxicity of inhaled particles: short-term exposure to nano-Ceria. Arch Toxicol. 88, 2033–2059.

Kobayashi T., Oshima Y., Tsubokura Y., Hashizume N., Ajimi S., Kayashima T., Nakai M., Sasaki T., Kawaguchi K., Imatanaka N., (2016). Effects of Dose Volume and Delivery Device on Bronchoalveolar Lavage Parameters of Intratracheally Administered Nano-Sized TiO2 in Rats. Regul Toxicol Pharmacol 30, 233-241.

Landsiedel R., Ma-Hock L., Hofmann T., Wiemann M., Strauss V., Treumann S., Wohlleben W., Gröters S., Wiench K., van Ravenzwaay B., (2014). Application of short-term inhalation studies to assess the inhalation toxicity of nanomaterials. *Particle and Fibre Toxicology* 11:16.

Lee, LY and Pisarri, TE. (2001). Afferent properties and reflex functions of bronchopulmonary C-Fibers. Resp Physiol. 125, 47-65

Lipnick, R.L., Cotruvo, J.A., Hill, R.N., Bruce, R.D., Stitzel, K.A., Walker, A.P., Chu, I., Goddard, M., Segal, L., and Springer, J.A. (1995). Comparison of the Up-and-Down, Conventional $LD_{50}$ and Fixed Dose Acute Toxicity Procedures – *Fd. Chem. Toxicol.* 33: 223-231.

M.A. and Epstein R.A. (1978). A theoretical analysis of the barometric method for measurement of tidal volume. Respir. Physiol. 32: 105-120.

Mallet, M.L. (2002). Pathophysiology of accidental hypothermia. QJ Med. 95. 775-785.

Mautz, WJ; Kleinman, MT; Bhalla, DK; Phalen, RF. (2001). Respiratory tract responses to repeated inhalation of an oxidant and acid gas-particle air pollutant mixture. Toxicological Sciences 61, 331-341.

Mitzner W. and Tankersley C. (1998). Noninvasive measurement of airway responsiveness in allergic mice using barometric plethysmography. Am. J. Respir. Crit Care Med 158: 340-341.

Mitzner W, Tankersley C., Lundblad L.K., Adler A., Irvin C.G., and Bates J. (2003). Interpreting Penh in mice. J. Appl. Physiol. 94: 828-832.

Monfared, A.L. (2012). Histomorphological and ultrastructural changes of the placenta in mice exposed to formaldehyde. Tox & Industrial Health. 1-8.

Morimoto, Y.; Izumi, H.; Yoshiura, Y.; Fujishima, K.; Yatera, K.; Yamamoto, K. 2016. Usefulness of intratracheal instillation studies for estimating nanoparticle-induced pulmonary toxicity. Int J Mol Sci. 27:17(2). Pii: E165, doi: 10.3390/ijms17020165.

Moss, O.R., James R.A., and Asgharian, B. (2006). Influence of exhaled air in inhalation exposure delivered through a directed-flow nose-only exposure system. *Inhal. Toxicol.* 18:45-51

Nielsen, GD. (1991). Mechanisms of activation of the sensory irritant receptor by airborne chemicals. Crit. Rev. Toxicol.. 21(3)183-208.

NRC (National Research Council) (2001). Standing Operating Procedures for Developing Subcommittee on Acute Exposure Guideline Levels Committee on Toxicology Board on Environmental Studies and Toxicology. Commission on Life Sciences - Acute Exposure Guideline Levels for Hazardous Chemicals. URL: http://www.nap.edu

Oberdörster G, Castranova V, Asgharian B, Sayre P. Inhalation Exposure to Carbon Nanotubes (CNT) and Carbon Nanofibers (CNF): Methodology and Dosimetry. J Toxicol Environ Health B Crit Rev. (2015); 18(3-4):121-212.

Günter Oberdörster and Thomas A.J. Kuhlbusch (2018). *In vivo* effects: Methodologies and biokinetics of inhaled nanomaterials. NanoImpact Volume 10, April 2018, Pages 38-60

OECD (1981d). Test Guideline 451. OECD Guideline for Testing of Chemicals. Carcinogenicity studies.

[OECD (1981e). Test Guideline 452. OECD Guideline for Testing of Chemicals. Chronic Toxicity studies.

OECD (1981f). Test Guideline 453. OECD Guideline for Testing of Chemicals. Combined Chronic Toxicity/Carcinogenicity Studies.

OECD (2000). Guidance Document on the Recognition, Assessment and Use of Clinical Signs as Humane Endpoints for Experimental Animals Used in Safety Evaluation. Environmental Health and Safety Monograph Series on Testing and Assessment No. 19.

OECD (2001) Test Guideline 423. OECD Guideline for Testing of Chemicals. Acute Oral Toxicity – Acute Toxic Class. Available:

[http://www.oecd.org/document/22/0,2340,en_2649_34377_1916054_1_1_1_1,00.html]

OECD (2009) Test No. 403: Acute Inhalation Toxicity, OECD Publishing, Paris.

OECD (2009a) Performance Assessment: Comparison of 403 and C×T Protocols via Simulation and for Selected Real Data Sets. [ENV/JM/MONO(2009)8]

OECD (2009b), Test No. 436: Acute Inhalation Toxicity – Acute Toxic Class Method, OECD Publishing, Paris.

OECD, (2010a), Test No. 417: Toxicokinetics, OECD Publishing, Paris

OECD (2010b) Guidance document on histopathology for inhalation toxicity studies, supporting TG 412 (Subacute inhalation toxicity: 28-day study) and TG 413 (Subchronic inhalation toxicity: 90-day study). Series on Testing & Assessment. No. 125.

OECD (2012). Guidance on Sample Preparation and Dopsimetry for the Safety Testing of Manufactured Nanomaterials. Series on the Safety of MOECD, 2012anufactured Nanomaterials No36. [ENV/JM/MONO(2012)40]

OECD (2016). Guidance Document on Considerations for Waiving or Bridging of Mammalian Acute Toxicity Tests - Series on Testing & Assessment. No. 237

OECD (2017a). Test No. 412: Repeated Dose Inhalation Toxicity: 28-day or 14-day Study. OECD Publishing, Paris

OECD (2017b). Test No. 413: Sub-chronic Inhalation Toxicity: 90-day Study. OECD Publishing, Paris

OECD (2017c). Test No. 433: Acute Inhalation Toxicity, fixed concentration procedure, OECD Publishing, Paris.

Patty′s Toxicology (2001). Patty's Toxicology Handbook, Eds. Eula Bingham; Barbara Cohrssen; Charles H. Powell. ISBN-13: 9780471319436, Wiley–Blackwell, USA.

Pauluhn J. (1989). A mechanistic approach to assess the inhalation toxicity and hazard of methylisocyanate and related aliphatic monoisocyanates. In: Assessment of Inhalation Hazards, Integration and Extrapolation Using Diverse Data, Mohr U (ed). Springer-Verlag, Heidelberg. 119-128.

Pauluhn, J. (1996). Risk assessment of pyrethroids following indoor use. Tox Letters 88. 339-348.

Pauluhn, J. (1998). Hazard identification and risk assessment of pyrethroids in the indoor environment. Appl. Occup. Environ. Hyg 13(6). 469-478.

Pauluhn, J. (2003). Overview of testing methods used in inhalation toxicity: from facts to artifacts. Toxicology Letters. 140-141. 183-193.

Pauluhn, J. (2004). Comparative analysis of pulmonary irritation by measurements of $P_{enh}$ and protein in bronchoalveolar lavage fluid in Brown Norway rats and Wistar rats exposed to irritant aerosols. *Inhalation Toxicology* 16: 159-175.

Pauluhn, J. (2006). Acute nose-only Exposure of Rats to Phosgene. Part I: Concentration x Time Dependence of $LC_{50}$s and Non-Lethal-Threshold Concentrations and Analysis of Breathing Patterns. Inhalation Toxicology 18, 423-435.

Pauluhn, J. (2008). Comparative assessment of early acute lung injury in mice and rats exposed to 1,6-hexamethylene diisocyanate-polyisocyanate aerosols. Toxicology 247. 33-45.

Pauluhn, J. (2018). Upper respiratory tract nociceptor stimulation and stress response following acute and repeated Cyfluthrin inhalation in normal and pregnant rats: Physiological rat-specific adaptions can easily be misunderstood as adversities. Toxicology Letter. 282. 8-24.

Pauluhn J. and Ozaki, K. (2015). Transfluthrin: Comparative efficacy and toxicity of reference and generic versions. Regulatory Toxicology and Pharmacology 71, 78-92.

Pauluhn, J. and Thiel, A. (2007). A simple Approach to Validation of Directed-flow Nose-only Inhalation Chambers. *J. Appl. Toxicol.* 27, 160-167.

Pauluhn, J. and Mohr, U. (1999). Repeated 4-week inhalation exposure of rats: effect of low-, intermediate, and high-humidity atmospheres. *Exp Toxic. Pathol.* 51: 178-187.

Pauluhn, J., Emura, M., Mohr, U., and Rosenbruch M., 2003. Inhalation toxicity of propineb. Part II: results of mechanistic studies in rats. Inhalation Toxicology 5: 435-460

Pauluhn, J. and A. Thiel (2007). A simple Approach to Validation of Directed-flow Nose-only Inhalation Chambers. *J. Appl. Toxicol.* 27:160-167.

Pauluhn, J. (2004a). Acute inhalation studies with irritant aerosols: Technical issues and relevance for risk charac¬teri¬zation. Archives of Toxicology 78, 243-251.

Pauluhn, J., (2004b). Comparative analysis of pulmonary irritation by measure¬ments of Penh and protein in bronchoalveolar lavage fluid in Brown Norway rats and Wistar rats exposed to irritant aerosols. Inhalation Toxicology 16, 159-175.

Pauluhn, J., (2004c). Pulmonary irritant potency of polyisocyanate aerosols in rats: Comparative assessment of irritant-threshold concentrations by broncho¬alveolar lavage. Journal of Applied Toxicology 24, 231-247.

Pauluhn, J., (2006). Acute nose-only Exposure of Rats to Phosgene. Part II: Concentration x Time Dependence of Changes in Bronchoalveolar Lavage during a follow-up Period of 3 Months. Inhalation Toxicology 18, 595-607.

Pauluhn, J., (2005). Retrospective Analysis of Acute Inhalation Toxicity Studies: Comparison of Actual Concentrations obtained by Filter and Cascade Impactor Analyses. *Regul. Toxicol. Pharmacol.* 42:236-244.

Pauluhn, J. and A. Thiel, (2007). A simple Approach to Validation of Directed-flow Nose-only Inhalation Chambers. J. Appl. Toxicol. 27:160-167.

Pauluhn, J., (2010). Subchronic 13-week Inhalation Exposure of Rats to Multiwalled Carbon Nanotubes: Toxic Effects are determined by Density of Agglomerate Structures, not fibrillar Structures. Toxicological Sciences 113, 226-242.

Pauluhn J, 2011a, Poorly Soluble Particulates: Searching for a Unifying Denominator of Nanoparticles and Fine Particles for DNEL Estimation. Toxicology, 279, 176-188

Pauluhn, J. (2011b). Interrelating the Acute and Chronic Mode of Action of inhaled Methylenediphenyl Diisocyanate (MDI) assisted by Computational Toxicology. Regulatory Pharmacology and Toxicology 61, 351-364.

Pauluhn J, (2014). Derivation of occupational exposure levels (OELs) of Low-toxicity isometric biopersistent particles: how can the kinetic lung overload paradigm be used for improved inhalation toxicity study design and OEL-derivation? Part Fibre Toxicol. 11:72.

Phalen R.F., (2009). Inhalation Studies: Foundations and Techniques. (2nd Edition) Informa Healthcare, New York.

Price, C., N. Stallard, S. Creton, I. Indans, R. Guest, D. Griffiths, and P. Edwards. 2011. A statistical evaluation of the effects of gender differences in assessment of acute inhalation toxicity. *Human & experimental toxicology*. 30:217-238.

Rosiello, A.P., Essigman, J.M., and Wogan, G.N. (1977). Rapid and Accurate Determination of the Median Lethal Dose ($LD_{50}$) and its Error with Small Computer. *J. Tox. Environ. Health* 3: 797-809.

Rossant, J. and Cross, J. (2001). Placental development: Lessons from mouse mutants. Nature. 2. 538-548.

Saxena RK, Gilmour MI, Schladweiler MC.,(2008). Hays Isolation and quantitative estimation of diesel exhaust and carbon black particles ingested by lung epithelial cells and alveolar macrophages in vitro. BioTechniques 44, 799-805.

Saxena RK, Gilmour MI, Schladweiler MC, McClure M, Hays M, Kodavanti UP. 2009. Differential Pulmonary Retention of Diesel Exhaust Particles in Wistar Kyoto and Spontaneously Hypertensive Rats. Tox. Sciences 111: 392–401

Schaper, M.M., Thompson, R.D., and Weil, C.S. (1994). Computer programs for calculation of median effective dose ($LD_{50}$ or $ED_{50}$) using the method of moving average interpolation. *Arch. Toxicol.* 68: 332-337.

Schlede E., Mischke U., Roll R. and Kayser D. (1992). A National Validation Study of the Acute-Toxic-Class Method - An Alternative to the $LD_{50}$ Test. *Arch. Toxicol.* 66, 455-470.

Schlede E., Mischke U., Diener W. and Kayser D. (1994). The International Validation Study of the Acute-Toxic-Class Method (Oral). *Arch. Toxicol.* 69, 659-670.

Sewell, F., I. Ragan, T. Marczylo, B. Anderson, A. Braun, W. Casey, N. Dennison, D. Griffiths, R. Guest, T. Holmes, T. van Huygevoort, I. Indans, T. Kenny, H. Kojima, K. Lee, P. Prieto, P. Smith, J. Smedley, W.S. Stokes, G. Wnorowski, and G. Horgan. 2015. A global initiative to refine acute inhalation studies through the use of 'evident toxicity' as an endpoint: towards adoption of the Fixed Concentration Procedure. Regulatory toxicology and pharmacology : RTP.

Slade, R.; Watkinson, WP; Hatch, GE. (1997). Mouse strain differences in ozone dosimetry and body temperature changes. Am J Physiol. 1997 Jan; 272 (1 Pt 1):L73-7

Stallard, N., C. Price, S. Creton, I. Indans, R. Guest, D. Griffiths, and P. Edwards. 2011. A new sighting study for the fixed concentration procedure to allow for gender differences. Human & experimental toxicology. 30:239-249.

Stallard, N., A. Whitehead, and I. Indans. 2003. Statistical evaluation of the fixed concentration procedure for acute inhalation toxicity assessment. *Human & experimental toxicology*. 22:575-585.

Shvedova, A.A., Kisin E., Murray A.R., Johnson V. J., Gorelik O., Arepalli S., Hubbs A.F., Mercer R.R., Keohavong P., Sussman N., Jin J., Yin J., Stone S., Chen B.T., Deye G., Maynard A., Castranova V., Baron P.A., Kagan V. E., (2008). Inhalation vs. aspiration of single-walled carbon nanotubes in C57BL/6 mice: inflammation, fibrosis, oxidative stress, and mutagenesis. *Am J Physiol Lung Cell Mol Physiol.* 295, L552–L565.

Sokal, R.R. and Rohlf, P.J. (1969). The arcsin transformation. In Biometry, 386-387. San Francisco, CA; W.H. Freeman & Co

Song JA1, Yang HS, Lee J, Kwon S, Jung KJ, Heo JD, Cho KH, Song CW, Lee K , 2010. Standardization of bronchoalveolar lavage method based on suction frequency number and lavage fraction number using rats.Toxicol Res. 2010 Sep;26(3):203-8. doi: 10.5487/TR.2010.26.3.203.

Suzui M., Futakuchi M., Fukamachi K., Numano T., Abdelgied M., Takahashi S., Ohnishi M., Omori T., Tsuruoka S., Hirose A., Kanno J., Sakamoto Y., Alexander D.B., Alexander W.T., Jiegou X., Tsuda H., 2016. Multiwalled carbon nanotubes intratracheally instilled into the rat lung induce development of pleural malignant mesothelioma and lung tumors. Cancer Sci 107, 924-935.

Tamura M, Inada M, Nakazato T, Yamamoto K, Endo S, Uchida K, Horie M, Fukui H, Iwahashi H, Kobayashi N, Morimoto Y, Tao H., 2011. A determination method of pristine multiwall carbon nanotubes in rat lungs after intratracheal instillation exposure by combustive oxidation-nondispersive infrared analysis. Talanta 84, 802-808.

Technical Committee of the Inhalation Specialty Section, SOT. (1992) Recommendations for the Conduct of Acute Inhalation Limit Tests. *Fund. Appl. Toxicol.* 18, 321-327.

ten Berge, W.F., Zwart, A., and Appelman, L.M. (1986). Concentration-time mortality response relationship of irritant and systemically acting vapours and gases. J. Hazard. Mater. 13: 301-309.

ten Berge, W.F. and Zwart, A. (1989) More efficient use of animals in acute inhalation toxicity testing. *J. Hazard. Mat.* 21: 65-71.

United Nations (UN) (2007). Globally Harmonized System of Classification and Labelling of Chemicals (GHS), ST/SG/AC.10/30, UN New York and Geneva. Available: [http://www.unece.org/trans/danger/publi/ghs/ghs_welcome_e.html]

USP XXII (1992). The United States Pharmacopeia, 22nd revision, Suppl. 7, NF XVII, Physical Tests and Determinations pp. 3122 - 3129.

WEELs and ERPGs, 2015. Emergency Response Planning Guidelines (ERPG) and Workplace Environmental Exposure Levels (WEELS) Handbook. American Industrial Hygiene Association (AIHA) Guideline Foundation, Falls Church, VA.

WHO (1978). Environmental Health Criteria 6 – Principles and Methods for Evaluating the Toxicity of Chemicals: Part 1, World Health Organization, Geneva, pp.29.

Widdicombe, J. (2006). Reflexes from the lungs and airways: historical perspective. JU. Appl. Physiol. 101:628-634.

Willeke K and Baron P.A. (1993). Aerosol Measurement. Principles, Techniques, and Applications. Van Nostrand Reinhold, New York.

Witschi, H-P. and Last, J.A. (1996). Casarett and Doull's Toxicology – The Basic Science of Poisons, *Toxic Responses of the Respiratory System (Chapter 15)*, McGraw-Hill, New York, pp. 443.

Yokley, K. (2012). Sensory Irritation Response in Rats II: Recovery and Dose-Dependence. Bull Math Biol. 74(7):1673-90.

Zwart, J.H.E. Arts, J.M. Klokman-Houweling, and E.D. Schoen. (1990) Determination of Concentration-Time-Mortality Relationships to Replace LC50 Values. *Inhal. Toxicol*. 2, 105-117

Zwart, J.H.E. Arts, W.F. ten Berge, and L.M. Appelman. (1992) Alternative Acute Inhalation Toxicity Testing by Determination of the Concentration—Time—Mortality Relationship: Experimental Comparison with Standard LC50 Testing. *Reg. Toxicol. .Pharmacol.* 15, 278-290.

# APPENDIX I. ABBREVIATION, ACRONYMS AND GLOSSARY

## Abbreviations & Acronyms

| | |
|---|---|
| ♂,♀ | male, female |
| µm | micrometer (formerly micron) |
| $\sigma_g$ | geometric standard deviation (GSD) |
| AAS | atomic absorption spectrometer |
| ADME | absorption, distribution, metabolism, excretion |
| AEGL | acute exposure guideline level |
| ANOVA | analysis of variance |
| AT | apnea time |
| ATC | acute toxic class |
| ATE | acute toxicity estimate |
| atm | atmosphere (a unit of pressure) |
| AUC | area under the curve |
| BAL | bronchoalveolar lavage |
| BALF | bronchoalveolar lavage fluid |
| bar | bar (a unit of pressure) |
| BMC | benchmark concentration |
| bw | body weight |
| C | concentration |
| °C | degrees Celsius |
| CAS | Chemical Abstract Services |
| CHE | cholinesterase |
| CIIT | Chemical Industry Institute of Toxicology |
| CMD | count median diameter |
| CNT | carbon nanotubes |
| COHb | carboxyhaemoglobin |

| | |
|---|---|
| C × t | concentration × time |
| DMA | differential mobility analyzer |
| ECD | effective cut-off diameter |
| EM | electron microscopy |
| ET | expiratory time |
| EU | European Union |
| ev | extravascular |
| g/mol | grams/mole (unit of molecular weight) |
| GC | gas chromatography |
| GD | guidance document |
| GHS | Globally Harmonized System |
| GI | gastrointestinal tract |
| GSD | geometric standard deviation ($\sigma_g$) |
| HPLC | high-performance liquid chromatography |
| hr | hour |
| hrs | hours |
| ICH | International Committee on Harmonization |
| ICP-OES | inductively coupled plasma-optical emission spectroscopy |
| IOMC | Interorganisation Programme for the Sound Management of Chemicals |
| IT | inspiratory time |
| iv | intravenous (injection) |
| J | Joule |
| K | Kelvin |
| kPa | kilopascal |
| LALN | lung-associated lymph nodes |
| LB | lung burden |
| $LC_{01}$ | lethal concentration - 1% |
| $LC_{10}$ | lethal concentration - 10% |
| $LC_{50}$ | lethal concentration - 50%; median lethal concentration |
| $LC_{99}$ | lethal concentration - 99% |
| $LC_x$ | lethal concentration - x% |
| $LCt_{01}$ | lethal concentration per minute– 1% |

| | |
|---|---|
| $LCt_{50}$ | median lethal concentration per minute |
| LDH | lactate dehydrogenase |
| LOAEC | lowest observed adverse effect concentration |
| LRT | lower respiratory tract |
| m | mass |
| M | molecular mass |
| MetHb | methemoglobin |
| mg/L | milligrams/liter |
| $mg/m^3$ | milligrams/cubic meter |
| MMAD | mass median aerodynamic diameter |
| MMD | mass median diameter |
| MPPD | Multiple-Path Particle Dosimetry Model |
| MPS | mobility particle sizer |
| MTC | maximum tolerated concentration |
| MTD | maximum tolerated dose |
| MW | molecular weight |
| MWCNT | multiwall carbon nanotubes |
| n | toxic load exponent in the equation $k = C^n \times t$;<br>number of animals |
| N | Newton |
| NALT | nasal-associated lymphoid tissue |
| NIOSH | U.S. National Institute for Occupational Safety and Health |
| nm | nanometer |
| NOAEC | no adverse effect concentration |
| OECD | Organisation for Economic Co-operation and Development |
| P | calculated probability |
| Pa | Pascal |
| $pCO_2$ | partial pressure carbon dioxide |
| $P_{enh}$ | enhanced pause (in respiration) |
| PEF | peak expiratory flow |
| PEO | post-exposure observation |
| PIF | peak inspiratory flow |
| $PM_{0.1}$ | particulate matter – 0.1 µm |

| | |
|---|---|
| $PM_{2.5}$ | particulate matter – 2.5 µm |
| PMd | particulate matter deposition |
| PMN | polymorphonuclear (cell) |
| $pO_2$ | partial pressure oxygen |
| POD | point of departure |
| ppb | parts per billion |
| ppm | parts per million |
| ppmV | parts per million volume |
| PSP | poorly soluble particle |
| (Q)SAR | quantitative structure–activity relationship |
| RB | reflex bradypnea |
| RBC | red blood cell (erythrocyte) |
| RIVM | Rijksinstituut voor Volksgezondheid en Milieu (Netherlands National for Public Health and the Environment) |
| RM-ANOVA | repeated measures analysis of variance |
| Rt | relaxation time |
| SAR | structure-activity relationship |
| S.D. | standard deviation |
| SI | International System of Units |
| SMPS | scanning mobility particle sizer |
| t | time |
| T | temperature |
| $t_{1/2}$ | time one-half |
| $t_{95}$ | time to 95% inhalation chamber equilibrium |
| TG | test guideline |
| Torr | Torr (a unit of pressure) |
| UN | United Nations |
| URT | upper respiratory tract |
| UV | ultraviolet |
| $V_d$ | volume of distribution |
| VMD | volume median diameter |
| WHO | World Health Organization |
| ×g | times gravity (e.g. 1000 ×g) |

## Glossary of Terms

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

**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:** 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 actual concentration is commonly expressed in mass units per unit volume of air (mg/L, mg/m$^3$). 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 "actual exposure concentration" and not the "exposure dose".

**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.

**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 (Cannon, W.C., et al, 1983). 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 (Cannon, W.C., et al, 1983).

**Aggregate:** A heterogeneous particle in which the various components are not easily broken apart (Cannon, W.C., et al, 1983).

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

**Analytical concentration:** See actual concentration.

**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.

**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 %.

**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 (OECD, 2000).

**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:

$$V = (RT/ P)$$

… 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.

and

$$\text{Concentration in mg/m}^3 = (P/RT) \times MW \times (\text{concentration in ppm})$$

$$= \frac{\text{P x MW x (concentration in ppm)}}{\text{62.4 x (273.2 + T°C)}}$$

and

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

… 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 orifice:** An orifice through which there is a constant flow when a sufficient pressure drop across the orifice causes sonic flow (Willeke K and Baron P.A., 1993).

**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):

$$\frac{dM}{dt} = -\frac{D}{V_m h} S_A \times (C_S - C)$$

… 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 Cs 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; Britztain, 2014; Wong, 2007).

**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 (Willeke K and Baron P.A., 1993).

**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 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:

$$1) \quad C_t = C_0\left(1 - e^{-\frac{F}{V}t}\right)$$

… 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 (MMD). 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**.

**Evident toxicity**: Evident toxicity is a general term describing clear signs of toxicity following the administration of a test chemical, such that at the next higher fixed concentration either severe pain or enduring signs of severe distress, moribund condition or probable mortality in most animals can be expected[3].

**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**.

**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 (Patty′s Toxicology, 2001).

**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 (Brown, J.S., et al.; 2005).

[3] As accepted in OECD TG 420

**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 ($\sigma_g$ or GSD):** A unit less number used to portray the range of particle sizes. A particle distribution is considered to be monodisperse when the $\sigma_g$ is 1.0-1.2, and polydisperse when the $\sigma_g$ is >1.2 (Chan P.K. and Hayes A.W.,1994).

**GHS Globally Harmonized System of Classification and Labelling 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. This was 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 Inter-organisation Programme for the Sound Management of Chemicals (IOMC) (UN, 2007).

**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.

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

**Humane endpoint**: 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 OECD Guidance Document No.19: Humane Endpoint in for more details -OECD, 2000).

**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.

**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.**

**$LC_{50}$ (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 $LC_{50}$ value is expressed as mass of test chemical per unit volume of air (mg/L, $mg/m^3$) 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 $LC_{50}$).

**$LCt_{50}$ (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 **$LC_{50}$ (median lethal concentration).** The $LCt_{50}$ is expressed as $mg/m^3$•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:** The amount of test chemical that is present in the lung at a given time point due to an excessive exposure level and/or overwhelmed clearance mechanisms.

**Impaired clearance:** A continuously increasing prolongation of lung clearance of poorly soluble particles when the retained lung burden exceeds a certain threshold. For rats this is 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)4]

**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. MMADs of ≤4 μm are recommended for acute inhalation toxicology studies. 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 do not increase the concentration of respirable aerosol to any appreciable extent.

**Maximum tolerated concentration (MTC):** High concentration used in repeated exposure toxicity testing that is expected to produce a clear level of but not cause lethality or persistent signs that might lead to lethality or prevent a meaningful evaluation of the results 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.

**Micronization**: Mechanical procedure to reduce particle size. Mechanical stress due to milling, grinding or breakdown of particles may produce artifacts, such as surface activation and test chemical degradation.

**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 (Cannon, W.C., et al., 1983). A dense mist which impairs visibility is called a **fog**.

**Mixtures:** see **Test chemical.**

**MMAD:** 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 (Brown, J.S., et al., 2005). See also **Geometric Standard Deviation**.

**Nanoscale:** Size range from approximately 1 nm to 100 nm. **(ISO)**

**Nanomaterial:** Material with any external dimension in the nanoscale or having internal structure or surface structure in the nanoscale (ISO)

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

**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 adverse effect concentration (NOAEC):** Highest 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.

**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 Pa = $10^{-5}$ bar = 0.987 $10^{-5}$ atm = 0.0075 Torr.

**Particle bounce:** The rebounding of particles that fails 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.

**Pharyngeal aspiration:** See **Aspiration**.

**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 (Gad, S.C. and Chengelis, C.P., 1998). 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.

**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. Note that it is also called *Sighting study* in some OECD TGs.

**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, $CO_2$ 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 $pO_2$ and $pCO_2$ 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 (Pauluhn, J. and A. Thiel, 2007) 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.

$$5 \times \sqrt[3]{\frac{flow \times \tau}{4 \times \pi}} \le r_p \le \frac{1}{5} \times \sqrt[2]{\frac{flow}{g \times \tau \times \pi}}$$

$r_p$ = radius of the sample probe ($r_p$) in cm; flow = flow rate ($cm^3$ x $sec^{-1}$),
$\tau$ = relaxation time (sec), g = gravity constant = 980 $cm/sec^2$

Example calculation:

The targeted sampling airflow rate from an inhalation chamber is 3 L/min (50 $cm^3$/sec) and the probe sampling collection efficiency needs to be considered for particles up to 20 μm. Under these conditions the relaxation time for the largest particle of interest is approximately 0.001 sec.

$$5 \times \sqrt[3]{\frac{50 \times 0.001}{4 \times \pi}} \le r_p \le \frac{1}{5} \times \sqrt[2]{\frac{50}{g \times 0.001 \times \pi}} \Rightarrow 0.79 \le r_p \le 0.81\,cm$$

On the other hand, for particle up 15 μm (relaxation time 6 x 10-4) the inlet radius should meet the following conditions: $0.67 \le r_p \le 1.04\,cm$. 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 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 (tenBerge, et al., 1986).

**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.

**Sensory irritant:** A noxious substance that triggers a nociceptive response that is perceived as pain. Some, but not all, sensory irritants cause cellular damage.

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

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

**Sublimation:** 1) The transition from the solid phase directly to the vapour phase without passing through a liquid phase (*e.g.*, dry ice); and 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 **Actual concentration**.

**Test chemical:** A product, substance, preparation or mixture (a formulation of multiple components) used for inhalation testing. Some test chemicals may be thermally decomposed for the purpose of testing, as in combustion toxicology tests. Atmospheres that result from thermal decomposition are considered to be mixtures. In all other circumstances where a non-destructive test is used, the term test chemical should be used.

**Thermodynamic equivalent diameter:** The diameter of a spherical particle with the same diffusion coefficient as the particle of interest. The thermodynamic equivalent diameter concerns particles with a diameter from a few nanometres to 1 µm.

**$t_{95}$**: 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 particles:** Particles larger than 1 nm and smaller than 100 nm in at least one dimension.

**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 $\bullet 10^2$ J. J (Joule) is the unit of energy in N(Newton) •m. 1 Pa (Pascal) ≈ 1.0 $J \bullet L^{-1}$. The unit of Pa is $N \bullet m^{-2}$.

Temperature: T [K] = 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:

$$C_{sat} = \frac{2 \times 10^{-3} \times 100 \times 10^{3}}{8.314 \times (273.15 + 20)}\left(\frac{J \times mg \times K \times Mol}{Mol \times J \times K \times L}\right) = 0.082\left(\frac{mg}{L}\right)$$

**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.

**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 ($LC_{50}$)

176. 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) $LC_{50}$ 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) (UN, 2007).

**Table 2. The GHS system for classification of acute inhalation toxicity**

| | $LC_{50}$ | | |
|---|---|---|---|
| **GHS Class** | **Gases (ppm)[a]** | **Vapours (mg/L)** | **Aerosols (dusts and mists) (mg/L)** |
| **1** | 100 | 0.5 | 0.05 |
| **2** | > 100 and 500 | > 0.5 and 2 | > 0.05 and 0.5 |
| **3** | > 500 and 2500 | > 2 and 10 | > 0.5 and 1 |
| **4** | > 2500 and 20000 | > 10 and 20 | > 1 and 5 |
| **5** | > 20000 | > 20 | > 5 |

[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.
*Source*: United Nations (UN) (2007). Globally Harmonized System of Classification and Labelling of Chemicals (GHS), ST/SG/AC.10/30, UN New York and Geneva:
http://www.unece.org/trans/danger/publi/ghs/ghs_welcome_e.html

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

**Table 3. Conversion of Gases**

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

| Classification Class or Experimentally Obtained Acute Toxicity Risk Estimate (ppm) | Converted Acute Toxicity Point Estimate (ppm)a |
|---|---|
| 0 < Class 1 = 100 | 10 |
| 100 < Class 2 = 500 | 100 |
| 500 < Class 3 = 2500 | 700 |
| 2500 < Class 4 = 20000 | 4500 |
| Class 5 > 20000a | See note b |

*Source*: United Nations (UN) (2007). Globally Harmonized System of Classification and Labelling of Chemicals (GHS), ST/SG/AC.10/30, UN New York and Geneva: http://www.unece.org/trans/danger/publi/ghs/ghs_welcome_e.html.

**Table 4. Conversion of Vapours**

Conversion from experimental obtained acute toxicity range values (or acute toxicity hazard classes) to acute toxicity point estimates for classification of vapours.

| Classification Class or Experimentally Obtained Acute Toxicity Risk Estimate (mg/L) | Converted Acute Toxicity Point Estimate (mg/L)a |
|---|---|
| 0 < Class 1 = 0.5 | 0.05 |
| 0.5 < Class 2 = 2.0 | 0.5 |
| 2.0 < Class 3 = 10.0 | 3 |
| 10.0 < Class 4 = 20.0 | 11 |
| Class 5 > 20.0a | See note b |

*Source*: United Nations (UN) (2007). Globally Harmonized System of Classification and Labelling of Chemicals (GHS), ST/SG/AC.10/30, UN New York and Geneva: http://www.unece.org/trans/danger/publi/ghs/ghs_welcome_e.html

**Table 5. Conversion of Aerosols**

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).

| Classification Class or Experimentally Obtained Acute Toxicity Risk Estimate (mg/L) | Converted Acute Toxicity Point Estimate (mg/L)a |
|---|---|
| 0 < Class 1 = 0.05 | 0.005 |
| 0.05 < Class 2 = 0.5 | 0.05 |
| 0.5 < Class 3 = 1.0 | 0.5 |
| 1.0 < Class 4 = 5.0 | 1.5 |
| Class 5 > 5.0a | See note b |

*Source*: United Nations (UN, 2007). Globally Harmonized System of Classification and Labelling of Chemicals (GHS), ST/SG/AC.10/30, UN New York and Geneva: http://www.unece.org/trans/danger/publi/ghs/ghs_welcome_e.html.

177. [a] 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.

178. [b] From GHS (UN, 2007) "…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 LD50 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 LD50 (or LC50) 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;
- 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.

179. 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

| Study Feature | TG 403 revised (2009)<br>Traditional $LC_{50}$ study | TG 403 revised (2009)<br>C × t study | TG 436 (2009) | TG 433 (2017)<br>Range estimate determination |
|---|---|---|---|---|
| **Study Design:** | | | | |
| **Major endpoint** | Mortality | Mortality | Mortality | |
| **Major objective** | • Concentration response for lethal and non-lethal endpoints (endpoints are system independent) | • Concentration response for lethal and non-lethal endpoints (endpoints are system independent).<br>• Derivation of n in $C^n$ x t | • Range estimate determination | • Evident toxicity and/or mortality |
| **Use of data** | • Classification & labeling by multiple systems including the GHS System.<br>• Derivation of $LC_x$ values and slope for one specific duration (usually 4 hours) for specific regulatory requirements | • Classification and labeling by multiple systems including the GHS System.<br>• Derivation of $LC_x$ values and slope for multiple exposure durations for specific regulatory requirements | • Classification and labeling by the GHS System only (the fixed concentrations used in this Test Guideline are based on GHS cut-offs).<br>• A range estimate of $LC_{50}$ values for one specific exposure duration (usually 4 h) | • Classification and labeling by the GHS System only (the fixed concentrations used in this Test Guideline are based on GHS cut-offs).<br>• An inferred range estimate of LC50 values for one specific exposure duration (usually 4 h). |
| **Mode of exposure** | Nose-only (preferred) or whole-body | Nose-only (whole-body chambers cannot be used) | Nose-only or whole-body | • Nose-only (preferred) or whole-body. |
| **Concentrations tested** | Variable—selected by the study director. | Variable—selected by the study director. | Gases:<br>100, 500, 2500, 20000 ppm<br>Vapours: 0.5, 2.0, 10.0, 20.0 mg/L | • Gases: 100, 500, 2500, 20000 ppm<br>• Vapours: 0.5, 2.0, 10.0, 20.0 |

| Study Feature | TG 403 revised (2009) Traditional $LC_{50}$ study | TG 403 revised (2009) C × t study | TG 436 (2009) | TG 433 (2017) Range estimate determination |
|---|---|---|---|---|
| | | | Aerosols: 0.05, 0.5, 1.0, 5.0 mg/L | mg/L<br>• Dusts and mists (aerosols): 0.05, 0.5, 1.0, 5.0 mg/L |
| **Atmosphere: concentration variability** | Gases and vapours: ±10%<br>Aerosols: ±20% | Gases and vapours: ±10%<br>Aerosols: ±20% | Gases and vapours: ±10%<br>Aerosols: ±20% | • Gases and vapours: ±10.0%<br>• Dusts and mists (aerosols): ±20.0% |
| **Atmosphere: stability** | Monitor continuously or hourly | Monitor continuously or hourly | Monitor continuously or hourly | • Monitor continuously or hourly |
| **Particle sizing (method)** | At least twice during 4 hour exposure (cascade impactor) | At least twice during 4 hour exposure (cascade impactor) | At least twice during 4 hour exposure (cascade impactor) | • At least twice during 4 hour exposure (cascade impactor) |
| **Concentrations tested** | Limit test: 1<br>Main study: At least 3 | Limit test: 1<br>Main study: 4-5 | 1 or more | • 1 or more |
| **Exposure duration** | Variable (generally 4 hours) | 5 durations per concentration | 4 hours | • 4 hours |
| **Particle size (aerosols)** | MMAD: 1-4μm<br>GSD: 1-3 | MMAD: 1-4μm<br>GSD: 1-3 | MMAD: 1-4μm<br>GSD: 1-3 | • MMAD: ≤4 μm<br>GSD: 1-3 |
| **Observation period** | At least 14 days | At least 14 days | At least 14 days | • At least 14 days |
| **Vehicle control group** | Not generally required (historical data required if interactions cannot be excluded) | Not generally required (historical data required if interactions cannot be excluded) | Not generally required (historical data required if interactions cannot be excluded) | Not generally required (historical data required if interactions cannot be excluded) |
| **Animals Tested:** | | | | |
| **Limit test** | 3 ♂ and 3 ♀ (or 5 of the known susceptible sex) | In case of 1 animal/sex/(Cxt) point: * Both sexes: 10; Susceptible sex: 10<br>In case of 2 animals/sex/(Cxt) point: * Both sexes = 20; Susceptible sex = 20 | 3 ♂ and 3 ♀ (or 6 of the known susceptible sex) | • 5 ♂ (or 5 of the known susceptible sex) |
| **Range-finding** | ≤ 3 ♂ and ≤ 3 ♀ (or ≤ 3 of the known | ≤ 3 ♂ and ≤ 3 ♀ per concentration | 0 (range-finding studies are not | • 0 (range-finding studies are |

| Study Feature | TG 403 revised (2009) Traditional $LC_{50}$ study | TG 403 revised (2009) C × t study | TG 436 (2009) | TG 433 (2017) Range estimate determination |
|---|---|---|---|---|
| **study** | susceptible sex) per concentration. At least 3 ♂ and 3 ♀ per concentration to test sex differences if not already known. | | used) | not used)<br>• an optional sighting study may be carried out. This will involve testing 1 male and 1 female animal per concentration. |
| **Main study** | 5 ♂ and 5 ♀ (or 5 of the known susceptible sex) per concentration | 1 or 2 animals/sex/(Cxt) point (or 2 or 4 animals of the susceptible sex per (Cxt) point) 5 durations per concentration)* | 3 ♂ and 3 ♀ (or 6 of the known susceptible sex) per concentration | • 5 ♂ (or 5 of the known susceptible sex) per concentration |
| **Total animals used in a non-limit study** | If 4 concentrations are tested:<br>Both sexes = 40<br>Known susceptible sex = 20 (if used for classification and labeling) | If 4 concentrations are tested:*<br>In case of 1 animal/sex/(Cxt) point:<br>Both sexes: 40; Susceptible sex: 40<br>In case of 2 animals/sex/(Cxt) point<br>Both sexes = 80; Susceptible sex = 80 | • If 1 concentration is tested: 6<br>• If 2 concentrations are tested: 12<br>• If 3 concentrations are tested: 18 | • If 1 concentration is tested: 5<br>• If 2 concentrations are tested: 10<br>• If 3 concentrations are tested: 15<br>• If 4 concentrations are tested: 20 |

* Refer to section 5.1.5 regarding the number of animals to be used per C × t interval (Babrauskas, V., et al., 2008).

# APPENDIX IV. PARTICLE SIZE DISTRIBUTION

180. 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 IV). 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 (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:

$$GSD = \frac{84.1\%\ mark}{50\%\ mark}$$

g) Algorithms for the calculation of particle size characteristics have been published (USP XXII, 1992; Pauluhn, J., 2005; Chan P.K. and Hayes A.W., 1994; Gad, S.C. and Chengelis, C.P., 1998). A representative analysis of particle size data is shown in the Table and Figure below.

181. To verify graphically that an aerosol is in fact unimodal and log-normally distributed, the normalized mass per stage ($f_H'$) is evaluated as a histogram. $\Delta \log D_p$ is equal to the difference $\log D_{p+1} - \log D_p$, 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 $f_H'$ by this equation:

.

$$f'_H = N_f \times \frac{mass / stage}{\Delta \log D_p} \qquad \textbf{(1)}$$

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

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

and use the normalization factor (Nf):

$$N_f = \left( \frac{\Sigma mass}{\log GSD \times \sqrt{2\pi}} \right)^{-1} \qquad \textbf{(3)}$$

An example calculation is provided in Table 6 Example for Cascade Impactor Analyses and Figure 3 Example calculation.

For non-modal particle size distributions other modes of evaluation may apply.

**Table 6. Example for Cascade Impactor Analyses**

| N | Impactor Stage (m - m) | Cut-Off Diameter (µm) | Mass/Stage (mg) | Relative Mass (%) | Cumulative Mass (%) |
|---|---|---|---|---|---|
| 1 | 0.06 -0.12 | 0.60 | 0.003 | 0.03 | 0.00 |
| 2 | 0.12-0.25 | 0.120 | 0.007 | 0.07 | 0.03 |
| 3 | 0.25-0.49 | 0.250 | 0.214 | 2.04 | 0.10 |
| 4 | 0.49-0.90 | 0.490 | 1.132 | 10.82 | 2.14 |
| 5 | 0.90-1.85 | 0.900 | 4.398 | 42.02 | 12.96 |
| 6 | 1.85-3.69 | 1.850 | 3.454 | 33.00 | 54.98 |
| 7 | 3.69-7.42 | 3.690 | 1.224 | 11.70 | 87.98 |
| 8 | 7.42-14.80 | 7.420 | 0.034 | 0.32 | 99.68 |
| 9 | 14.80-30.00 | 14.800 | 0.000 | 0.00 | 100.00 |

*Note:* Mass Median Aerodynamic Diameter (MMAD): 1.66 um
Geometric standard deviation (GSD): 1.80
*Source*: Add the source here. If you do not need a source, please delete this line.

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

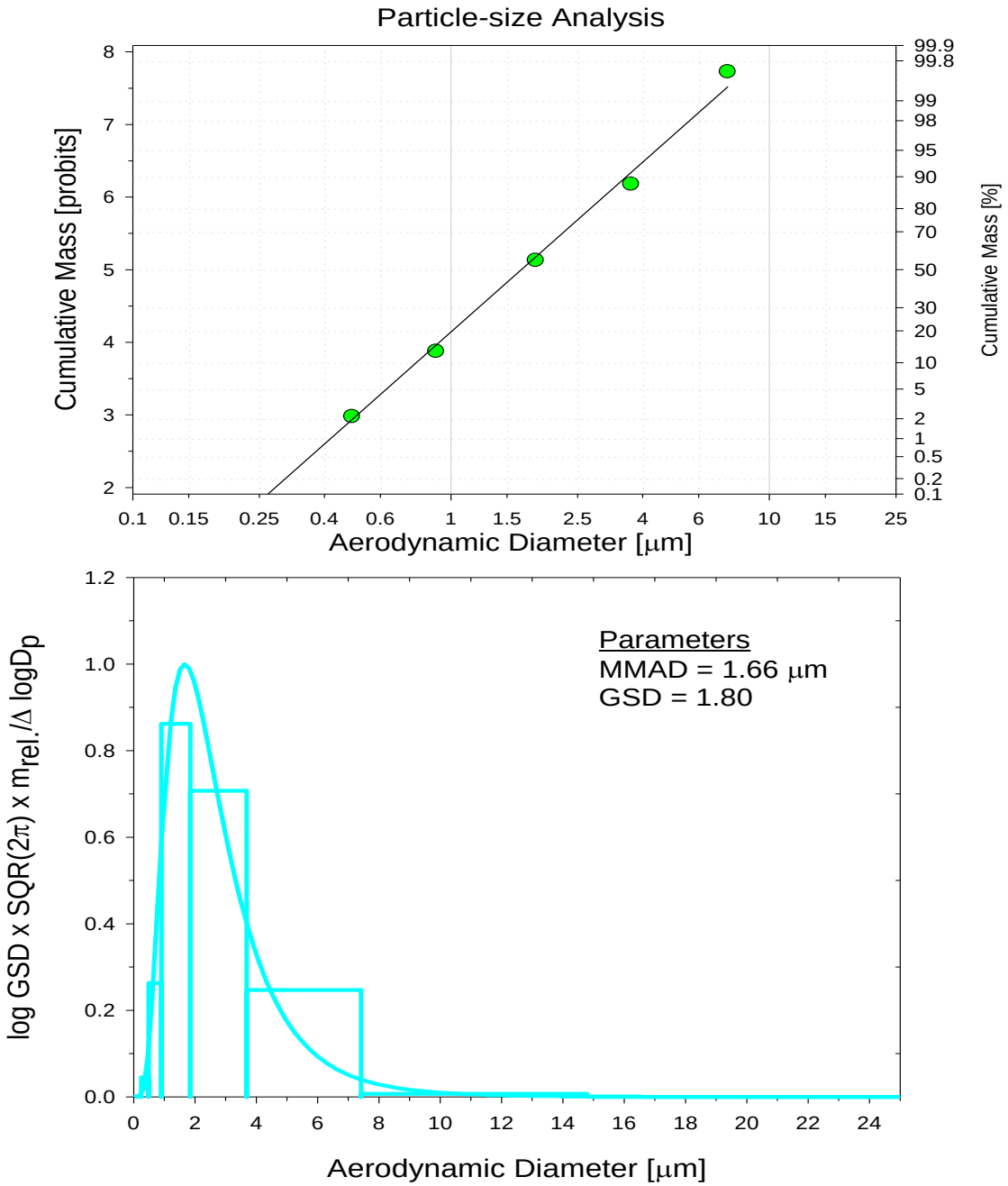
Respirability (percent < 1.0 um):
---
Mass related: 19.7 %

Respirability (percent < 3.0 um):
---
Mass related: 84.1 %

Respirability (percent < 5.0 um):
---
Mass related: 96.9 %

**Figure 3. Concentration-related hypothermia and decreased respiratory rate in mice due to reflex bradypnea**

(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)



# APPENDIX V. RESPIRATORY REFLEXES: REFLEX BRADYPNEA AND THE PAINTAL REFLEX

## Introduction

182. Laboratory rodents have two respiratory reflexes that allow them to markedly reduce their minute volume and thus reduce their exposure to sensory irritants by entering a reversible, hibernation-like state. Reflex bradypnea protects rodents from upper respiratory tract (URT) irritants and the Paintal reflex protects them from lower respiratory tract (LRT) irritants. Pulmonary function and body temperature measurements can be used to quantify the extent of these reflexes. Because humans do not experience these reflexes, a risk assessment may not be health protective if it fails to account for reduced test chemical exposure in rodents experiencing one or both of these reflexes. OECD test guidelines TG 403, TG 412, and TG 413 require measurements of pulmonary function and body temperature when a test chemical is known or likely to be a sensory irritant.

## Reflex Bradypnea

183. Reflex bradypnea (RB; brad″e-ne´ah), also known as the Kratschmer reflex, is a protective reflex that allows laboratory rodents—but not humans—to significantly reduce their exposure to URT irritants such as aldehydes, ammonia, isocyanates, and pyrethroid insecticides (Gordon et al., 2008). It is a reversible, hibernation-like state that allows rodents to adapt to environmental stress through the use of torpor due to reflexively suppressed metabolic demand and hypothermia.

### *Mechanism*

184. Trigeminal nociceptive (sensory) neurons in the mucosa of the URT and eyes can trigger this reflex in rodents. This same mechanism is thought to mediate avoidance behaviour in humans. Some sensory irritants cause cellular damage in the URT but others do not (e.g., Cyfluthrin, a pyrethroid). It is important to note that RB is triggered by nociception—not cell damage (Bos et al., 1992, 2002; Nielsen, 1991; Pauluhn, 2018). RB is regulated by a complex feedback response (Yokley, 2012) and the extent of RB depends on the air concentration of the irritant (Gordon et al., 2008).

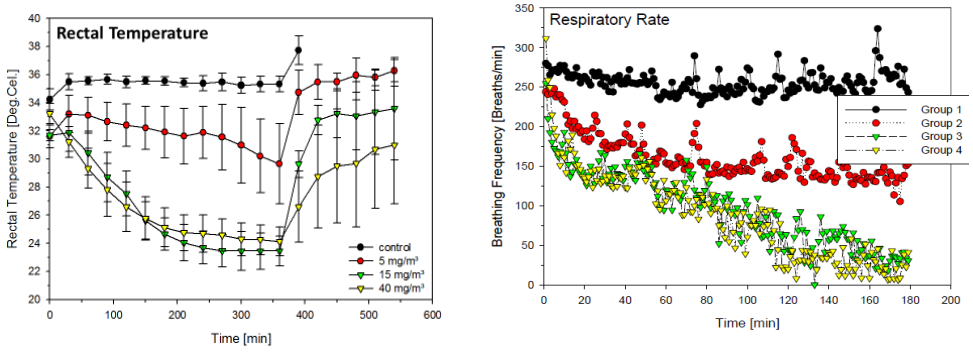
### *The signs of reflex bradypnea*

185. Reflex bradypnea in laboratory rodents is manifest by immediate decreases in the metabolic rate, CO2 production, and tissue demand for oxygen. This is quickly followed by decreases in respiratory rate (breaths/minute; Figure 4), body temperature (Tb; as much as 11°C in rats and 14°C in mice; Figure 4), minute volume (Figure 5), heart rate, blood pressure, cardiac output, oxygen delivery to tissues, and activity level (e.g., prostration). Due to increased gas solubility at lower temperatures and a reduced metabolic rate, RB also results in decreased blood pO2 and pCO2, increased blood pH, and compensatory respiratory alkalosis (Chang and Barrow, 1984; Gordon et al., 2008; Jaeger and Gearhart, 1982; Pauluhn, 1989, 1996, 2003, 2008, 2018). Additional signs seen in long-term studies include acidified urine and decreased body weight gain (Pauluhn, 2018). Figure 4 (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). As described later, RB can confound the interpretation of neurologic, behavioural, and developmental studies.

**Figure 4 Concentration-related hypothermia and decreased respiratory rate in mice due to reflex bradypnea**

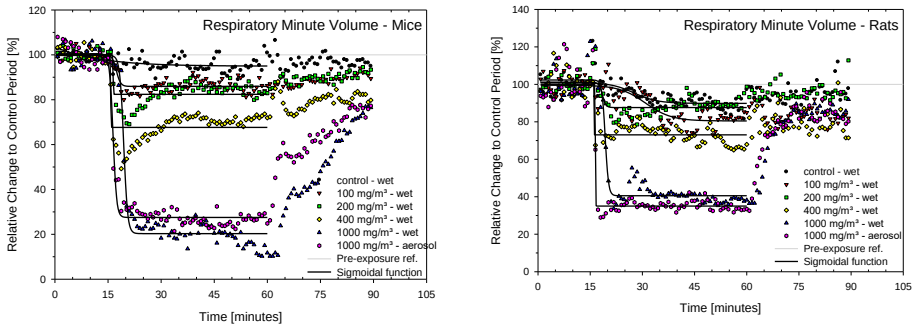
***Left panel***: Concentration-related hypothermia with gradual recovery following exposure. ***Right panel***: Concentration-related decreases in respiratory rate (breaths/minute).




*Note*: Note the correlation between the curves for rectal temperature and respiratory rate
*Source*: Gordon et al. (2008)

186. Figure 5 demonstrates the immediate onset of RB in mice and rats with a marked decrease in minute volume shortly after 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 may be unable to avoid exposure.

**Figure 5. Minute volumes for mice (left panel) and rats (right panel) measured in volume displacement nose-only plethysmographs**




*Note*: The rodents were sequentially exposed for 15 minutes to air, and 45 minutes to several concentrations of transfluthrin (a pyrethroid insecticide), followed by a 30 minute recovery period. 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

187. RB can only occur in small animals such as mice and rats that can rapidly lower their core body temperatures and oxygen demand. 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).

188. RB has been reported in the literature since the 1960s, but it is unknown to most toxicologists, which may be why so few inhalation studies were designed to identify and quantify RB. This is unfortunate because rodents are likely to experience RB when exposed to irritants at concentrations high enough to trigger nociceptors in the URT. For example, Swiss-Webster mice experienced 10% and 50% decreases in minute volume when exposed to formaldehyde at a concentration 5-fold and 38-fold higher than the human odor detection limit after only 10 minutes of exposure (Kane and Alarie (1977).

## The Paintal reflex

189. Lower respiratory tract (LRT) irritants, such as ozone and phosgene, can trigger a Paintal reflex. While the signs of the Paintal reflex are similar to those of RB, the primary signs are apnea following exhalations followed by reduced tidal volume and increased respiratory rate (i.e., rapid, shallow breathing with an overall reduction in minute volume), hypothermia, airway constriction, bradycardia, reduced cardiac output, and bronchial vasodilation (Lee and Pisarri, 2001; Mautz et al., 2001; Pauluhn, 2006; Slade et al., 1997; Widdicombe, 2006).

### *Mechanism*

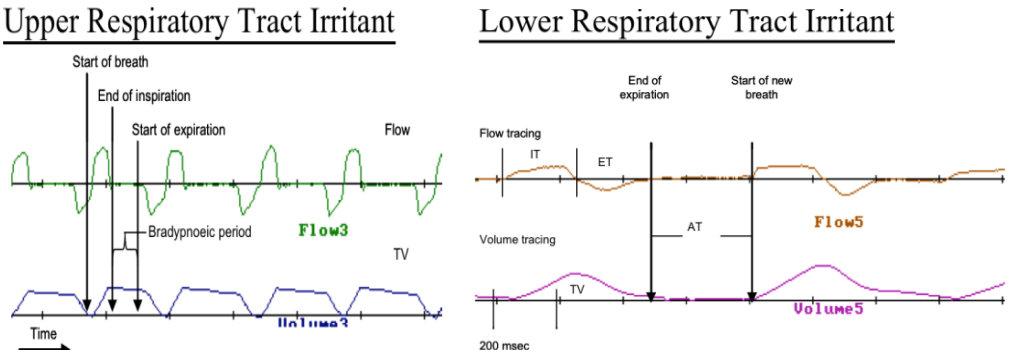
190. The Paintal reflex is triggered by vagal C-fibre stimulation. C-fibres, which have nerve endings located in the walls of the alveoli and airways in the LRT, have major controlling effects on spontaneous breathing. The nociceptive role of the Paintal reflex is to sense the onset of a pathophysiological condition within the LRT. The afferent activity arising from these vagal nerve fibres appears t play an important role in regulating cardiopulmonary function under both normal and abnormal physiological conditions. C-fibres are activated by irritants, foreign chemicals, and inflammatory mediators as well as pathological conditions including anaphylaxis, pneumonia, and microembolism (Widdicombe, 2006).

## How to Distinguish Between Irritation in the Upper v Lower Respiratory Tract

191. Because reflex bradypnea and the Paintal reflex cause similar physiological effects, pulmonary function data are needed to distinguish between the two reflexes. As shown in Figure 6, reflex bradypnea (URT) can be identified by a ‘bradypneic pause’ that occurs between the end of inspiration and the onset of expiration. Conversely, the Paintal reflex (LRT) can be identified by a reflexively-induced ‘apneic pause’ or breath-holding period after each exhalation. The bradypneic pauses of RB and the apneic pauses of the Paintal reflex can significantly reduce lung exposure to an inhaled irritant (Pauluhn, 2006).

**Figure 6. Reflex Bradypnea / the Paintal Reflex**

**Left Panel**: This demonstrates the bradypneic pause characteristic of reflex bradypnea. Notice that the bradypneic pause occurs after the animal inhaled ammonia. **Right Panel**: This 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.




*Note*: 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 in tidal breathing, respectively. The breath structure is characterized by three phases: inspiratory time (IT), expiratory time (ET), and apnea time (AT).
*Source*: Jürgen Pauluhn (Bayer Healthcare AG, Germany).

192. In rodents, reflex bradypnea is predominated by a time-independent decrease in breathing frequency (i.e., bradypnea) whereas the onset of reflex apnea (Paintal reflex) may be indicated by decreases in tidal volume (VT). 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.

## Sensory Irritation and the $RD_{50}$

193. 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 several 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 irritant chemical that causes a 50% decrease in respiratory rate (ASTM, 2012; Kane et al., 1979). Alarie tests are useful for **1)** identifying chemicals that are URT sensory irritants, **2)** quantifying irritating concentrations, and **3)** ranking chemicals for their sensory irritancy potential. Bos et al. (1992 and 2002) are excellent resources for $RD_{50}$ values for numerous respiratory irritants.

## Tolerance to URT sensory irritants

194. Most rodent studies that assessed RB have been acute Alarie tests lasting a few minutes to a few hours. Subacute studies demonstrate that RB persists in rats exposed to formaldehyde for 10 days (Chang and Barrow, 1984; Barrow et al., 1986) or to Cyfluthrin for 4 weeks (Pauluhn, 1998) with no indication of tolerance. No subchronic or chronic studies have investigated whether-or-when rodents develop a tolerance to URT irritants and begin to breathe normally. This is a serious data gap.

## The impact of Respiratory reflexes on Neurological and Behavioural Studies

195. The normal physiological effects of RB and the Paintal reflex can confound the interpretation of neurological and behavioural 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. As body temperature decreases, ataxia is progressively followed by a loss of fine motor control and reflexes, a reduction in cerebral blood flow and brain function, and eventually a loss of consciousness (Mallet, 2002). What may appear to be chemically-induced neurological or behavioural effects may actually be due to reflex-induced hypothermia, which protects rodents from inhaled irritants. For example, rodents can be expected to perform poorly in behavioural studies (e.g., rotarod or a maze) when they are hypothermic due to a respiratory reflex; but a risk assessor unfamiliar with these reflexes would ascribe the poor performance to chemical toxicity. These neurologic and behavioural effects in rodents are not relevant to humans, which do not experience reflex bradypnea or the Paintal reflex as rodents do. Only by measuring pulmonary function and body temperature in rodent studies can reflex-related neurologic effects be distinguished from chemical neurotoxicity.

### *The Impact of Respiratory Reflexes on Developmental Toxicity Studies*

196. Pregnant rodents experience an increase in minute volume to assure stable maternal blood gases ($O_2$ and $CO_2$) for their growing foetuses. When exposed to a sensory irritant, however, these dams experience the same respiratory reflexes as non-pregnant females. While these reflexes protect dams, they can harm their foetuses. Foetuses can experience developmental delays or defects due to impaired placental transfer of $O_2$ (hypoxia) and $CO_2$ (hypercapnia), foetal hypothermia, and malnutrition caused by reductions in maternal feeding, metabolism, and cardiac output. Foetuses cannot tolerate hypothermia and hypoxia as well as adults (Pauluhn, 1989, 2018). In adult rodents, decreased oxygen from the lungs is counterbalanced by reduced oxygen demand; but the same is not true for foetuses, which experience hypoxia due to **1)** reduced maternal respiration and **2)** a left-shift in maternal oxyhaemoglobin affinity caused by increased blood pH (Pauluhn, 2018).

197. Hypoxia is a normal regulator of placental development in both humans and rodents (Rossant & Cross, 2001). In studies of formaldehyde and Cyfluthrin in mice and rats, concentration-related decreases in foetal weight and placental weight were attributed to RB-induced hypothermia of the dams (Holzum et al., 1994; Monfared, 2012; Pauluhn, 2018). RB-induced developmental effects caused by foetal hypoxia, hypercapnia, hypothermia, and malnutrition are not relevant to humans. Only by measuring pulmonary function and body temperature in rodent studies can reflex-induced developmental effects be distinguished from chemical toxicity.

## The Impact of Respiratory Reflexes on Human Health Risk Assessments

198. The potential impact of respiratory reflexes on human health risk assessments has not received the attention it deserves from toxicologists and risk assessors, largely because testing guidelines did not require measurements of pulmonary function and body temperature until revised versions of TG 412 and TG 413 were adopted in 2017. The following are key points to consider in a risk assessment for a sensory irritant:

- The striking signs of RB and the Paintal reflex in rodents are normal physiological nociception responses to sensory irritation, so they must not be misconstrued as systemic toxicity.
- Because humans do not respond to respiratory irritants as rodents do, the signs of RB and the Paintal reflex must not be used to define points-of-departure (POD) in human risk assessments.
- When performing a risk assessment, it may be necessary to adjust the POD to account for reduced rodent exposure.
- Care should be taken to not misinterpret reflexively-induced physiological changes as adverse signs in neurological, behavioural, or developmental toxicity studies.
- Respiratory depression (RD) data may be used to estimate test chemical concentrations that may be irritating to rodents and humans.
- When evaluating studies of respiratory irritants that lack pulmonary function and body temperature data, a risk assessor is advised to look for clues that the rodents may have experienced a respiratory reflex. Word clues may include: cold-to-the-touch, sluggish, ataxia, lethargic, prostration, 'sleeping,' slow breathing, altered breathing, laboured breathing, impaired mobility, slow nerve conduction, delayed foetal development, reduced foetal weights, reduced placental weights, decreased $pO_2$ and $pCO_2$, increased blood pH, increased adrenal weights, and decreased thymus weights.

# APPENDIX VI. INSTILLATION AND ASPIRATION STUDIES

## Instillation and Aspiration Exposure Techniques

199. For hazard identification and screening purposes, animals may be dosed with test chemicals by intratracheal instillation or aspiration. These exposure techniques offer a way to rapidly screen and rank the hazard of solid materials, including fibres and nanomaterials (Nakanishi et al. 2015). While the use of instillation or aspiration exposure is a simple and inexpensive way to examine test chemicals, it is not a substitute for inhalation toxicity studies. Most notably, the exposure methods—test chemical delivery directly to the lower respiratory tract as a suspension—, bypassing the filtering capability of the nose, and delivered dose kinetics do not replicate normal respiration. During inhalation, particles may be deposited throughout the entire respiratory tract, including the nose, 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 rates. Instillation also results in high 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 should be used with caution in human risk assessments. Guidance on characterization of nanomaterials in suspension can be found in OECD (2012).

200. 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, the OECD Guidance on Sample Preparation and Dosimetry for the Safety Testing of Manufactured Nanomaterials (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 the Guidance on Sample Preparation and Dosimetry (OECD, 2012) and elsewhere (Baisch B.L., et al., 2014; Driscoll K.E., et al. 2000; Hasegawa-Baba et al., 2014; Kobayashi et al., 2016; Morimoto et al., 2016; Nakanishi et al., 2015; Shvedova, A.A., et al., 2008)

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

- ***Sample preparation:*** Test chemicals must be dispersed in a vehicle in stable condition. To achieve stable suspensions, additives are often used. 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 (Kobayashi et al., 2016). Data are required to demonstrate that the instillation procedure does not produce marked elevations in BAL-endpoints (e.g. total protein, LDH, polymorphonuclear cell (PMN), and total cell counts) relative to sham controls (air exposure only). When the concentration of prepared suspension is too low to deliver intended dose by one administration or when lower dose rate is preferred, a dose may be divided into multiple administrations.
- ***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 is desirable to 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. After the dosing, keep the animal being hanged on the restrained position for a while preventing immediate backflow of instilled suspension from the lung.
- ***Lung Burden***: The anatomical dead-space of the lung is approximately 30% of the total lung volume. So partial loss of the dosed suspension from lung could not be avoided in instillation although it can be minimized by complying the dose volume range (less than 2 mL/kg-bw) and keeping animals hanging on just after dosing (Kobayashi et al, 2016).Therefore, calculated (nominal) total lung doses should be empirically verified by initial lung burden, which would be measured soon after the administration. 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.
- ***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. The instillation procedure-specific dosing patterns can be evaluated by various

qualitative or quantitative techniques that evaluate the procedure-specific dosing distribution pattern of a variety of dyes or beads which can be evaluated by spectrophotometric, fluorimetric, radiological or gravimetric techniques. Such techniques can give an initial approximation of procedure/instrumentation-dependent dosing distribution patterns.

- 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). The alveolar inflammation by bacteria can be prevented by disinfect the instillation device with disinfectant such as alcohol at the time of injection.
- 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).

## Dose Selection in Instillation and Aspiration Studies

202. 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 (Baisch B.L., et al., 2014). 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 (Pauluhn J, 2011a; Pauluhn J, 2014).