OECD/OCDE 433

Adopted:
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OECD Guideline for Testing of Chemicals

Acute Inhalation Toxicity: Fixed Concentration Procedure

INTRODUCTION

1. OECD Guidelines for the Testing of Chemicals are periodically reviewed in the light of scientific progress and animal welfare considerations. The original acute inhalation Guideline 403 was adopted in 1981. Development of an Inhalation Fixed Concentration Procedure (FCP) was considered appropriate, following adoption of the revised oral Fixed Dose Procedure (FDP), OECD Guideline 420 in December 2001 and the deletion of the oral toxicity test, OECD Guideline 401. This FCP guideline will allow the use of a series of fixed concentrations for the determination of acute inhalation toxicity in only one sex.

2. Traditional methods for assessing acute toxicity use death/moribundity of animals as the sole endpoint. In 1984, a new approach to acute toxicity testing was suggested by the British Toxicology Society based on the administration of test chemical at a series of fixed concentration levels [1]. This approach avoided using death/ moribundity of animals as either an exclusive or an intended endpoint by incorporating evident clinical signs of toxicity at one of a series of fixed dose levels, as an endpoint on which to base classification of the test chemical. This approach is also taken for this guideline. In agreement with the OECD Guidance Document on Humane Endpoints [2] and definitions contained therein refinements are introduced in order to minimize any suffering and distress by the animals and, to the extent feasible, reduce the number of animals used. Evident toxicity is a general term describing clear signs of toxicity following the administration of a test chemical, such that at the next highest fixed concentration either severe pain and enduring signs of severe distress, moribund condition or probable mortality in most animals can be expected. Guidance on the recognition of evident toxicity has been provided by Sewell et al (2015) [3]: Evident toxicity has been reached if one or more animals display any one of the listed signs (from the day after exposure onwards): tremors, hypoactivity, irregular respiration or bodyweight loss (>10% pre-study value). Should there be lack of clarity regarding the evident toxicity, it can be concluded that the toxicity is not evident, and that further testing should be considered. The statistical properties of the FCP have been evaluated using mathematical modelling [4-6].

3. Guidance on the conduct and interpretation of acute inhalation studies can be found in the Guidance Document No. 39 on Acute Inhalation Toxicity Testing [7].

In accordance with the decision of the Council on a delegation of authority to amend Annex I of the decision of the council on the Mutual Acceptance of Data in the assessment of chemicals [C(2018)49], this Guideline was amended by the OECD's Joint Meeting of the Chemicals Committee and the Working Party on Chemicals, Pesticides and Biotechnology by written procedure on 25 June 2018..

4. Definitions used in the context of this Guideline can be found in Guidance Document No. 39 on Acute Inhalation Toxicity Testing [7].

5. The method provides information on the hazardous properties and allows the test chemical to be ranked and classified according to the United Nations (UN) Globally Harmonized System of Classification and Labelling of Chemicals (GHS) for the classification of chemicals which cause acute toxicity [8].

INITIAL CONSIDERATIONS

6. All available information on the test chemical should be considered by the testing facility prior to conducting the study. Such information will include the identity and chemical structure of the test chemical; its physico-chemical properties; the results of any other *in vitro* or *in vivo* toxicity tests on the test chemical; available (Q)SAR data and toxicological data on structurally related substances; the anticipated use(s) of the test chemical and the potential for human exposure. Some of this information will assist in the selection of an appropriate starting concentration (e..g. through read-across from toxicity of structurally-related chemicals /those of same chemical class, and/or data from predictive software), or will allow further testing to be avoided if available information is sufficient. Before use of the Test Guideline on a mixture for generating data for an intended regulatory purpose, it should be considered whether, and if so why, it may provide adequate results for that purpose. Such considerations are not needed, when there is a regulatory requirement for testing of the mixture.

PRINCIPLE OF THE TEST

7. It is a principle of the method that only moderately toxic concentrations are used so that 'evident toxicity' (described in more detail below), rather than death/moribundity is used as an endpoint, and concentrations that are expected to be lethal are avoided. Also, concentrations that are expected to cause marked pain and distress, due to corrosive[1] or severely irritant actions, should not be administered. When testing an irritating or corrosive chemical refer to GD39 [7] for guidance. Moribund animals, or animals obviously in pain or showing signs of severe and enduring distress shall be humanely killed, and are considered in the interpretation of the test results in the same way as animals that died on test. Criteria for making the decision to kill moribund or severely suffering animals, and guidance on the recognition of predictable or impending death, are the subject of a separate OECD Guidance Document [2].

8. Groups of animals of a single sex are exposed for a short period of time to the test chemical in a stepwise procedure using the appropriate fixed concentrations for vapours, dusts/mists (aerosols) or gases as set out in Annex 1. The initial concentration level is selected on the basis of existing information or a sighting study at the concentration expected to produce evident toxicity, clear signs of toxicity without causing severe toxic effects or mortality, that predict exposure to the next highest concentration will cause severe toxicity or death/moribundity in most animals. Analysis by Stallard et al., (2011) provides the rationale for the sighting study [6]. Guidance on the recognition of evident toxicity is provided in section 40 and has been described by Sewell et al (2015) [3]. It is important to note that 'evident toxicity' occurs prior to the clinical signs and conditions

[1] Determined using a validated test methods (e.g., TG430 or 431) or an acceptable prediction.

associated with pain, suffering, and impending death, that are described in the OECD Guidance Document on humane end-points [2]. This document describes signs and conditions in animals in which evident toxicity has already been exceeded. Further groups of animals may be tested at higher concentrations in the absence of signs of evident toxicity or mortality at lower concentrations.

9. This procedure continues until the concentration causing evident toxicity or no more than one death/ moribund animal is identified, or when no effects are seen at the highest concentration or when deaths/ moribundity occur at the lowest concentration. Depending on the outcome of the test (i.e. evident toxicity or mortality/moribundity), testing at one concentration level may be sufficient to allow judgement on the acute toxicity of the test chemical. Evident toxicity is defined as clear signs of toxicity following the administration of a test chemical, such that at the next highest fixed concentration either severe pain and enduring signs of severe distress, moribund condition or probable mortality in most animals can be expected'. Analysis by Sewell *et al.* (2015) [3] has shown that evident toxicity has been reached if one or more animals display any one of the listed signs (from the day after exposure onwards): tremors, hypoactivity, irregular respiration or bodyweight loss (>10% pre-study value). However, the analysis also includes information on other more rarely observed signs that also have high predictivity. This guidance should be used in conjunction with study director experience and judgement. For further guidance please see GD39 [7].

DESCRIPTION OF THE METHOD

Selection of animal species

10. The preferred rodent species is the rat, although on occasion other rodent species may be used. Justification should be provided for the use of other rodent or non-rodent species. Existing information (if available) or a sighting study using one male and one female animal may be performed to select the most sensitive sex for use in the main study [6]. This sighting study is not compulsory. The main purpose of the sighting study is to select an appropriate starting concentration for the main study but it can also inform the choice of sex. Males should be used as a default if there is no apparent difference in sensitivity, and females should only be used if they appear to be more sensitive than males. However, available knowledge of the toxicological or toxicokinetic properties of structurally related chemicals should also be taken in to account and adequate justification of sex selection should be provided.

11. Healthy young adult animals of commonly used laboratory strains should be employed. If females are used they should be nulliparous and non-pregnant. Each animal, at the commencement of testing, should be between 8 and 12 weeks old and its weight should fall within an interval of ±20% of the mean body weight *of any previously exposed animals*.

Housing and feeding conditions

12. The temperature of the experimental animal room should be 22 ± 3ºC. Although the relative humidity should be at least 30% and preferably not exceed 70% other than during room cleaning the aim should be 45-65%. Lighting should be artificial, the sequence being 12 hours light, 12 hours dark. For feeding, conventional laboratory diets may be used with an unlimited supply of drinking water. Feed should be provided ad

libitum where possible. Animals should be group-caged by concentration, but the number of animals per cage should not interfere with clear observations of each animal.

Preparation of animals

13. The animals are acclimatised to the laboratory conditions for at least five days prior to the start of exposure. Animals are randomly selected for use in the study and marked to provide individual identification.

Mode of exposure

14. Animals are exposed to the test chemical as a gas, vapour, aerosol, or a mixture thereof. The physical state to be tested depends on the physico-chemical properties of the test chemical, the selected concentration, and/or the physical form most likely present during the handling and use of the test chemical. Both head/nose-only and whole-body exposure techniques may be used. 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. The head/nose-only technique does, however, require restraint of the animals throughout the exposure period, which is not necessary for whole-body exposures, and therefore causes more stress than whole-body technique. However, it easier to remove a distressed animal from a nose-only chamber than 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 [2].

Head/nose-only exposure technique

15. During exposure, the animals are exposed to the test chemical in exposure tubes. The animal restraining tubes should not impose undue stress on the animal, should be constructed in such a way as to avoid hyperthermic stress for the animal and should make it impossible for the animal to avoid inhalation exposure. However, if a negative balance of air volumes supplied and extracted cannot be avoided, a dilution of test atmosphere by bias-airflow (via exposure tubes) should be prevented. The inhalation chamber should be operated in well ventilated chemical hoods. The animals should be tested with inhalation equipment designed to sustain a dynamic air flow which exceeds at least twice the respiration ventilation volume of all animals in the inhalation device. An adequate oxygen content of at least 19% and a carbon dioxide concentration not exceeding 1%, with similar exposure conditions at each exposure port should be ensured. During the sampling of the test atmosphere, a significant disturbance of the airflow dynamics should be avoided. The rate of air flow should be adjusted to ensure that conditions throughout the equipment are essentially the same. The principles of the nose-only exposure technique and its particular advantages and disadvantages are described in GD 39 [7].

Whole-body exposure technique

16. The animals should be tested using inhalation equipment designed to sustain a dynamic air flow of approximately 12 to 15 air changes per hour. Other air flow rates may be useful to meet specific requirements imposed by the test chemical. However, an adequate oxygen content of at least 19%, a carbon dioxide concentration not exceeding

1%, and an evenly distributed exposure atmosphere should be ensured. As a general rule to ensure the stability of a chamber atmosphere, the total volume of the test animals should not exceed five per cent of the volume of the test chamber. The principles of the whole-body exposure technique and its particular advantages and disadvantages are described in GD 39 [7].

Exposure conditions

17. A fixed duration of exposure of four hours, excluding equilibration time, is recommended. Other durations may be needed to meet specific requirements.

18. To establish suitable exposure concentrations, a technical trial test without animals is mandatory. It is technically difficult to generate test atmospheres to accurately meet specified fixed exposure concentrations. Therefore, to prevent unnecessary repeat testing, individual chamber concentration samples should not deviate from the mean chamber concentration by more than ±10% for gases and vapours, and by no more than ± 20% for liquid or solid aerosols.In the case of potentially explosive test chemicals, care should be taken to avoid favourable conditions for explosions. Further guidance can be found in GD 39 [7].

Particle size

19. As it is difficult to predict the most responsive region of the respiratory tract or the most harmful particle size, the particle size distribution of dusts and aerosols should be such that exposure of all regions of the tract can be achieved. An aerosol with a mass median aerodynamic diameter (MMAD) ≤4 μ m and a geometric standard deviation (GSD) in the range of 1 to 3.0 is recommended to ensure that comprehensive respiratory tract exposure occurs [10]. In case a laboratory deviates from the recommended MMAD, an explanation and justification should be given. For example, metal fumes may be smaller than this standard, and charged particles, fibres, and hygroscopic materials (which increase in size in the moist environment of the respiratory tract) may exceed this standard.

Generation of test atmospheres

20. Where necessary, a suitable vehicle may be added to the test chemical to help generate an appropriate concentration and respirability of the test chemical in the atmosphere. Where a vehicle is used to help generate an appropriate concentration of the substance in the atmosphere, the acute inhalation toxicity of the vehicle should be known. A concurrent vehicle (or any other) control group is not considered necessary for well characterised control vehicles. Particulate material may be subjected to mechanical processes to achieve the required particle size distribution, however, care should be taken not to decompose or alter the test chemical, which should be analytically verified, as rigorous mechanical processes could change particle size distribution from that of pristine materials. Adequate care should be taken not to contaminate the test chemical. It is not necessary to test non-friable granular materials which are purposefully formulated to be un-inhalable. An attrition test should be used to demonstrate that respirable particles are not produced when the granular material is handled. If an attrition test produces respirable articles, an inhalation toxicity test should be performed.

MONITORING OF EXPOSURE CONDITIONS

Chamber airflow

21. The flow of air through the exposure chambers should be monitored continuously and recorded at least three times during each exposure.

Chamber temperature

22. The air temperature in the animal's breathing zone should be monitored continuously and recorded at least three times during each exposure. Ideally the temperature should remain within the range 22±3°C. Deviations from this range should be commented upon with an assessment of the effect, if any, on the outcome of the exposure.

Relative humidity

23. The relative humidity in the animal's breathing zone, for both the head/nose only and the whole body exposures, should be monitored continuously and recorded three times during each exposure where possible. The RH should ideally be maintained in the range of 30-70% but it is recognised that under certain circumstances this may either be unattainable (e.g. when testing water based formulations) or may not be measurable due to interference by the test chemical with the test method.

Concentration of test chemical

24. Actual concentrations of the test chemical should be measured in the breathing zone of the rats in both the head/nose only and the whole body exposures. During the exposure period, the actual concentrations of the test chemical shall be held as constant as practicable (see paragraph 17) and monitored continuously or intermittently depending on the method of analysis. If intermittent sampling is used at least five samples should be taken at approximately hourly intervals. If not feasible due to limited air flow rates or low concentrations, fewer samples may be collected, with a minimum of one sample may be collected over the entire exposure period. For single component solid aerosols and liquids that are of extremely low volatility, gravimetric analysis is acceptable. When performing gravimetric sampling at the higher exposure concentrations used in these studies, care should be taken to calibrate the flow meter (or dry gas meter) used to determine sampled volume as a function of the pressure drop across the filter (based upon the relationship pressure x volume = constant). A calibration volume curve should be generated for each flow meter or dry gas meter used.

25. For aerosols of liquid formulations that can be evaporated to a constant weight, gravimetric analysis of the dried residue may be used. Appropriate extrapolation to calculate the weight of formulation should be applied to the gravimetric data. It is not necessary to analyse inert ingredients provided the mixture at the animal's breathing zone is analogous to the formulation; the grounds for this conclusion should be provided in the study report. If there is some difficulty in measuring chamber analytical concentration due to precipitation, non-homogenous mixtures, volatile components, or other factors, additional analyses of inert components may be necessary.

26. Where gravimetric analysis is unsuitable and the test atmosphere contains more than one component, chemical analysis of the major active ingredient followed by

extrapolation to the concentration of formulation may be acceptable but should be justified.

27. Whenever the test chemical is a formulation, the analytical concentration should be reported for the total formulation and not just for the active ingredient.

Particle size distribution

28. The particle size distribution of the test aerosol should be determined at least twice during each 4-hour exposure. A range of sampling devices is suitable but the device selected should allow calculation of the MMAD (See paragraph 18). Geometric Mean Diameter (GMD) may be determined by using a Scanning Mobility Particle Sizer (SMPS). In the case of multi-component aerosols the principles given above for determination of concentration should be applied. Adequate information should be available within the testing facility to demonstrate that such samplers collect an atmospheric sample that is representative of the atmosphere to which the animals are exposed.

Nominal concentration

29. The nominal exposure chamber concentration should be determined by recording the amount of test chemical disseminated into the exposure chamber/tube during the generation period and dividing this by the total airflow through the chamber/tube during the same period.

PROCEDURE

Sighting study

30. The purpose of the sighting study is to allow selection of the most sensitive sex and an appropriate starting concentration for the main study. However, a sighting study may not be warranted if prior information is available to guide the choice of a starting concentration and/or the most sensitive sex. Therefore, this study should not be considered compulsory. The test chemical is administered to two animals (one male and one female) simultaneously at a chosen starting concentration for a period of at least four hours. Testing continues in a sequential manner depending on the outcome following the flow charts in Annex 1 [6]. If both animals demonstrate the same response of death, non-fatal evident toxicity or no effects, the sighting study either stops and leads to a main study conducted in males or continues to test two animals (one male and one female) at the next concentration. If, at any concentration, a sex difference is indicated, the main study will be conducted using the sex that is shown to be the more sensitive, and the sighting study continues with that sex alone in such a way as to determine an appropriate main study starting concentration. The sighting study is completed when a decision on the starting concentration for the main study can be made, based on signs of evident toxicity or if a death/moribundity is seen at the lowest fixed concentration. Males should be used for the main study if there is no apparent difference in sensitivity between sexes.

31. The starting concentration for the sighting study is selected from the fixed concentration levels found in Annex 1 as a concentration expected to produce evident toxicity based, when possible, on evidence from existing data on the same chemical and/or structurally related chemicals. In the absence of such information, the starting concentration will be 10 mg/L, 1 mg/L or 2500 ppm for vapours, dusts/mists (aerosols) and gases, respectively.

32. A period of at least 24 hours will be allowed between the testing of each pair of animals. All animals should normally be observed for at least one week.

33. In cases where an animal tested at the lowest fixed concentration level in the sighting study dies or exhibits clear clinical signs of toxicity, the normal procedure is to terminate the study and assign the test chemical to GHS [8] Category 1 without proceeding to main study testing (as shown in Annex 1). However, if further confirmation of the classification is required (i.e. if death/moribundity of only one sex occurs), an optional supplementary procedure may be conducted, as follows. An additional animal of the most sensitive sex is tested at the lowest fixed concentration. If this animal dies, then GHS Category 1 will be confirmed and the study will be immediately terminated. If the animal survives, then a maximum of three additional animals will be tested at this concentration. Because there will be a high risk of mortality, these animals should be tested in a sequential manner to protect animal welfare. The time interval between exposure of each animal should be sufficient to establish that the previous animal is likely to survive. If an additional death/moribundity occurs, the testing sequence will be immediately terminated and no further animals will be tested. The classification will be as shown in Annex 1: Category 1 if there are two or more deaths/moribundities (outcome A), or Category 2 if there is one death/moribundity (outcome B).

MAIN STUDY

Numbers of animals and concentration levels

34. The action to be taken following testing at the starting concentration level is indicated by the flow charts in Annex 1. Depending on the outcome of each study, one of three actions will be required; either stop testing and assign the appropriate hazard classification class, test at a higher fixed concentration or test at a lower fixed concentration. However, a concentration level, which caused death/moribundity in the sighting study, will not be revisited in the main study, to protect animal from unnecessary suffering (see Annex 1). Experience has shown that the most likely outcome at the starting concentration level will be that the test chemical can be classified and no further testing will be necessary. When testing a descending series and 2-3 deaths are observed (within the scope of outcome A), then in the interests of animal welfare the test should be halted and the test chemical classified according to outcome C of the next concentration in the series. Guidance on the recognition of evident toxicity (within the scope of outcome B) is provided in section 40 and described by Sewell et al. (2015) [3]

35. A total of five animals of one sex (the most sensitive sex as indicated in the sighting study, or males only) will normally be used for each concentration level investigated, in addition to the pairs of animals used in the sighting study.

36. The time interval between exposures at each level is determined by the onset, duration and severity of toxic signs. Exposure of animals at the next concentration should be delayed until there is confidence in the survival of the previously tested animals. A period of three or four days between the exposures at each concentration level is recommended to allow for the observation of delayed toxicity. The time interval may be adjusted as appropriate, *e.g.*, in case of inconclusive response.

Limit Test

37. The limit test is primarily used in situations where the study director has information indicating that the test chemical is likely to be non-toxic, *i.e.*, having toxicity

only above regulatory limit doses. Information about the toxicity of the test chemical can be gained from knowledge about similar tested compounds or similar tested mixtures or products, taking into consideration the identity and percentage of components known to be of toxicological significance. In those situations where there is little or no information about its toxicity, or in which the test chemical is expected to be toxic, the main test should be performed.

38. Using the normal procedure, a main study starting concentration of 20 mg/l, 5 mg/l or 20 000 ppm for vapours, dusts/mists (aerosols) and gases, respectively, followed by exposure of a further five animals at this level serves as a limit test for this guideline, if achievable. When testing aerosols, the primary goal should be to achieve a respirable particle size (i.e. an MMAD of ≤4 μm). This is possible with most test chemicals at a concentration of 2 mg/L. Aerosol testing at greater than 2 mg/L should only be attempted if a respirable particle size can be achieved. In some cases, as required by some regulatory authorities, testing up to the limit of GHS class 5 may be conducted. However, testing in animals in Category 5 ranges is discouraged and should only be considered when there is a strong likelihood that the results of such testing would have a direct relevance to the protection of human health [8].

Observations

39. During the exposure period the animals should be observed frequently. In addition, after exposure, careful clinical observations should be made at least twice on the day of exposure, or more frequently when indicated by the response of the animals to the treatment. Thereafter clinical observations should be made at least once daily for a total of 14 days. The length 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 and should therefore be recorded, especially for signs of toxicity that have a tendency to be delayed in onset. All observations should be 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 without delay. When animals are killed for humane reasons or found dead, the time of death should be recorded as precisely as possible. Animals that require euthanasia should be considered to have died (for the purposes of classifying outcome A and outcome B).

40. Additional observations will be necessary if the animals continue to display clinical signs of toxicity. Observations should include changes in skin and fur, eyes and mucous membranes, and also respiratory, circulatory, autonomic and central nervous systems, and somatomotor activity and behaviour pattern. If possible, a differentiation between local and systemic effects should be determined. Attention should be directed to observations of tremors, convulsions, salivation, diarrhoea, lethargy (hypoactivity), irregular respiration, sleep, coma and bodyweight loss (see notes 40 and 41 below). A clinical signs lexicon for signs that may be observed in acute inhalation studies can be found in Sewell et al. (2015) [3] . The principles and criteria summarised in the Humane Endpoints Guidance Document should be taken into consideration [2]. Animals found in a moribund condition and animals showing severe pain or enduring signs of severe distress should be humanely killed for animal welfare reasons. 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. 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. Therefore signs observed on the day of exposure should be treated with caution.

41. In accordance with outcome B, evident toxicity has been reached if one or more animals display any one of the listed signs (from the day after exposure onwards): tremors hypoactivity, irregular respiration or bodyweight loss (>10% pre-study value i.e. compared to the day of exposure, see paragraph 41). Guidance on the recognition of evident toxicity has been provided by Sewell et al. (2015) [3] where evidence from a large retrospective dataset showed that if any one of these signs is observed in at least one animal from the day after exposure, death of two or more animals is almost always observed at the next highest concentration. The analysis also includes information on other more rarely observed signs that also have high predictivity and can be taken into consideration. These recommendations on the recognition of evident toxicity should be used in this test guideline, but are not intended to overrule study director experience and judgement - they are meant to act as a guide only. If severe toxicity (including signs not explicitly listed above) is observed on the day of exposure, or at any time during the study, the study should be stopped and animals euthanized as appropriate. In the event that a decision between Outcome B and C is being made in the absence of death, and the study director decides there is uncertainty whether evident toxicity has been observed (i.e. triggering Outcome B), there is the option to test at the next higher concentration limit.

Body weight

42. Individual animal weights should be recorded a minimum of once during the acclimatization period, 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. Body weight is recognised as a critical indicator of toxicity and animals exhibiting a decrement of >10%, compared to the day of exposure), indicate evident toxicity has been reached. More frequent measurements of bodyweight should be undertaken if there are concerns surrounding the condition of an animal. Surviving animals should be weighed and humanely killed at the end of the post-exposure period.

Pathology

43. All test animals (including those that die during the test or are removed from the study for animal welfare reasons) should be subjected to gross necropsy. If 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. Necropsies should be performed as soon as possible, normally within a day or two. All gross pathological changes should be recorded for each animal. Microscopic examination of organs showing evidence of gross pathology in animals surviving 24 or more hours after the initial exposure may also be considered because it may yield useful information.

DATA AND REPORTING

Data

44. Individual animal data should be provided. Additionally, all data should be summarised in tabular form, showing for each test group the number of animals used, the number of animals displaying signs of toxicity, the number of animals found dead during the test or killed for humane reasons, time of death (or approximate time of death if the

exact time is unknown) of individual animals, a description and the time course of effects of toxicity and reversibility, and necropsy findings.

Test Report

45. The test report should include the following information, as appropriate:

Test chemical and control substance

- source, lot number, limit date for use, if available;
- stability of the test chemical itself, if known;
- solubility and stability of the test chemical in solvent, if known;
- measurement of pH, osmolality, and precipitate in the culture medium to which the test chemical was added, as appropriate

Mono-constituent substance:

- physical appearance, water solubility, and additional relevant physicochemical properties;
- chemical identification, such as IUPAC or CAS name, CAS number, SMILES or InChI code, structural formula, purity, chemical identity of impurities as appropriate and practically feasible, etc.

Multi-constituent substance, UVCBs and mixtures

- characterized as far as possible by chemical identity (see above), quantitative occurrence and relevant physicochemical properties of the constituents.
- Vehicle
- justification for use of vehicle and justification for choice of vehicle (if other than water).

Test animals

- species/strain used;
- microbiological status of the animals, when known;
- acclimatisation period;
- number, age and sex of animals (including, where appropriate, a rationale for use of females instead of males);
- source, housing conditions, historical data, diet etc.;

Test conditions

- details of test chemical preparation, including details of any procedures used to reduce the particle size of powders or to prepare solutions of the test chemical;
- a description (preferably including a diagram) of the equipment used to generate the test atmosphere and to expose the animals to the test atmosphere;

- details of the equipment used to monitor chamber temperature, humidity and airflow;
- details of the equipment used to collect samples for determination of chamber concentration and particle size distribution;
- details of the chemical analytical method used and method validation (including efficiency of recovery of test chemical from the sampling medium
- details for time needed for equilibrium of exposure concentration before animal exposure;
- method of randomisation in assigning animals to test and control groups;
- details of food and water quality (including diet type/source, water source);
- the rationale for the selection of the starting concentration.

Results

- tabulation of chamber temperature, humidity and airflow;
- tabulation of chamber nominal and actual concentration data;
- tabulation of particle size data including analytical sample collection data, particle size distribution and calculations of the MMAD and GSD;
- tabulation of response data and concentration level for each animal (i.e. animals showing signs of toxicity including mortality, nature, severity, and duration of effects);
- individual weights of animals at the day of exposure, in weekly intervals thereafter, and at time of death or euthanasia; date and time of death if prior to scheduled euthanasia, time course of onset of signs of toxicity and whether these were reversible for each animal;
- necropsy findings and histopathological findings for each animal, if available.

Discussion and interpretation of results

- Should there be uncertainty as to whether evident toxicity has been observed and judgment was required to reach a result then this should be explained in full in the discussion. Justification should be provided for where additional testing was conducted.

Conclusions

Literature

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8. UN, Globallly Harmonized System of Classification and Labelling of Chemicals (GHS), sixth revised edition ST/SG/AC.10/30/Rev.6, New York and Geneva. 2015.
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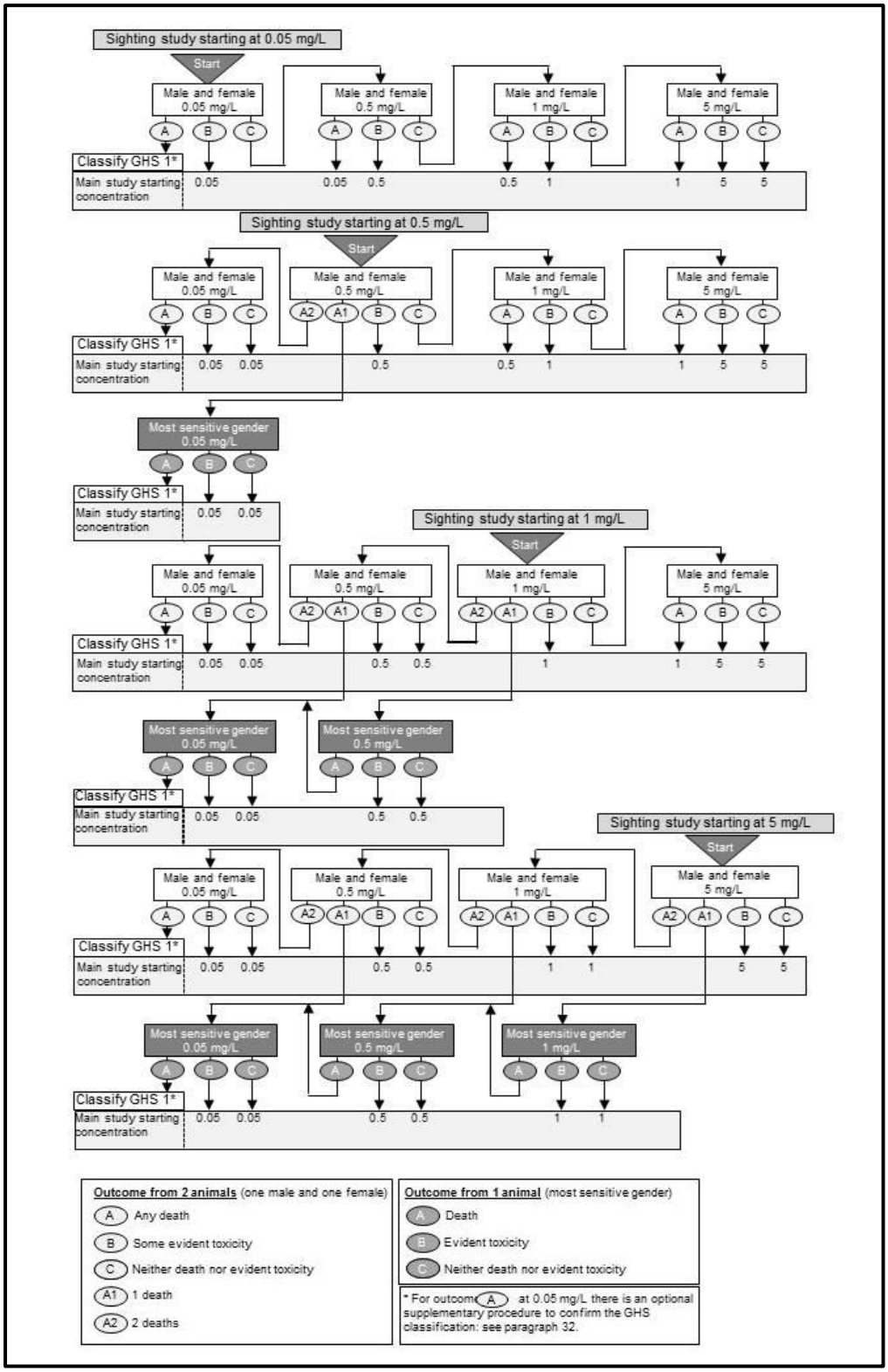
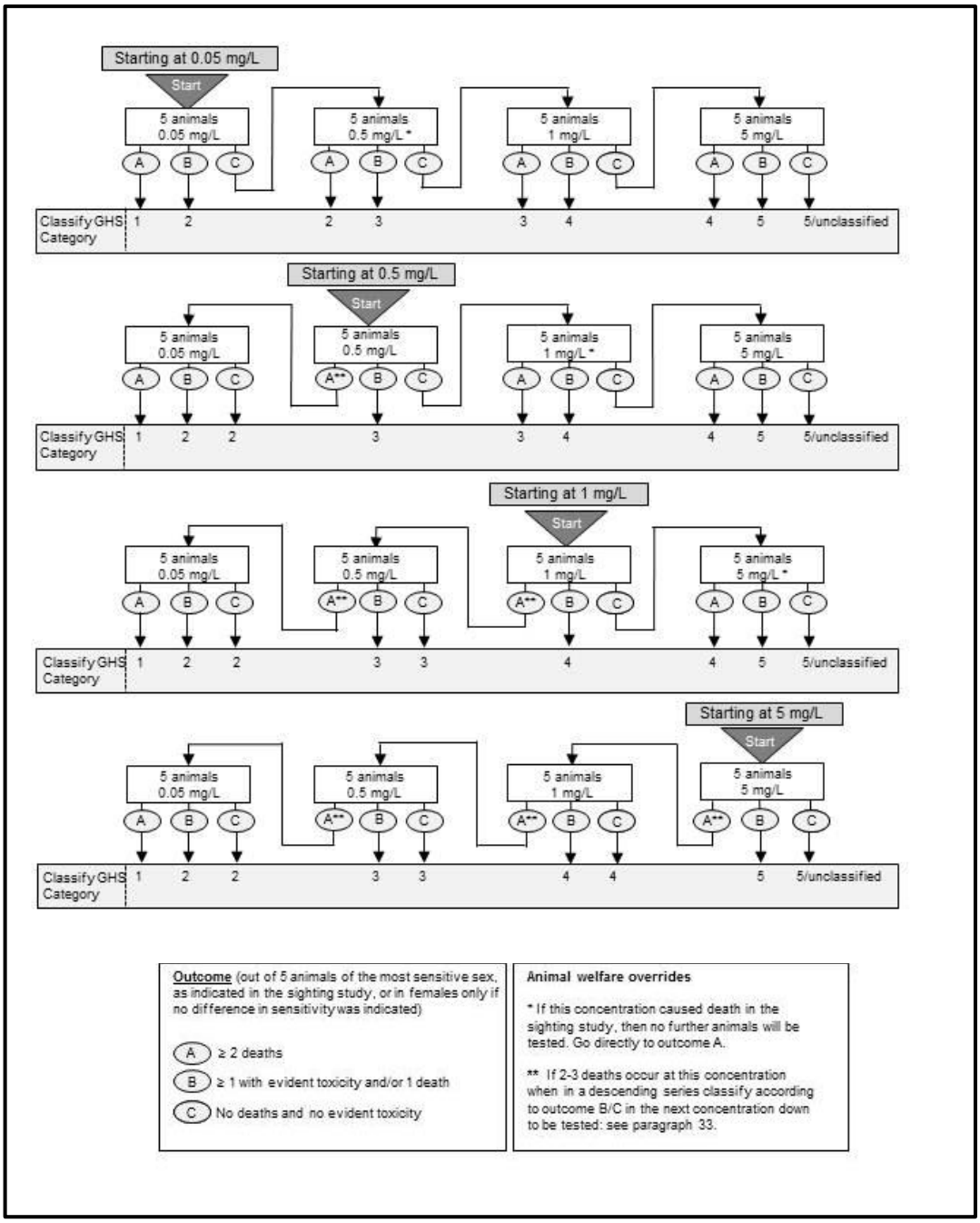
ANNEX FLOW CHARTS

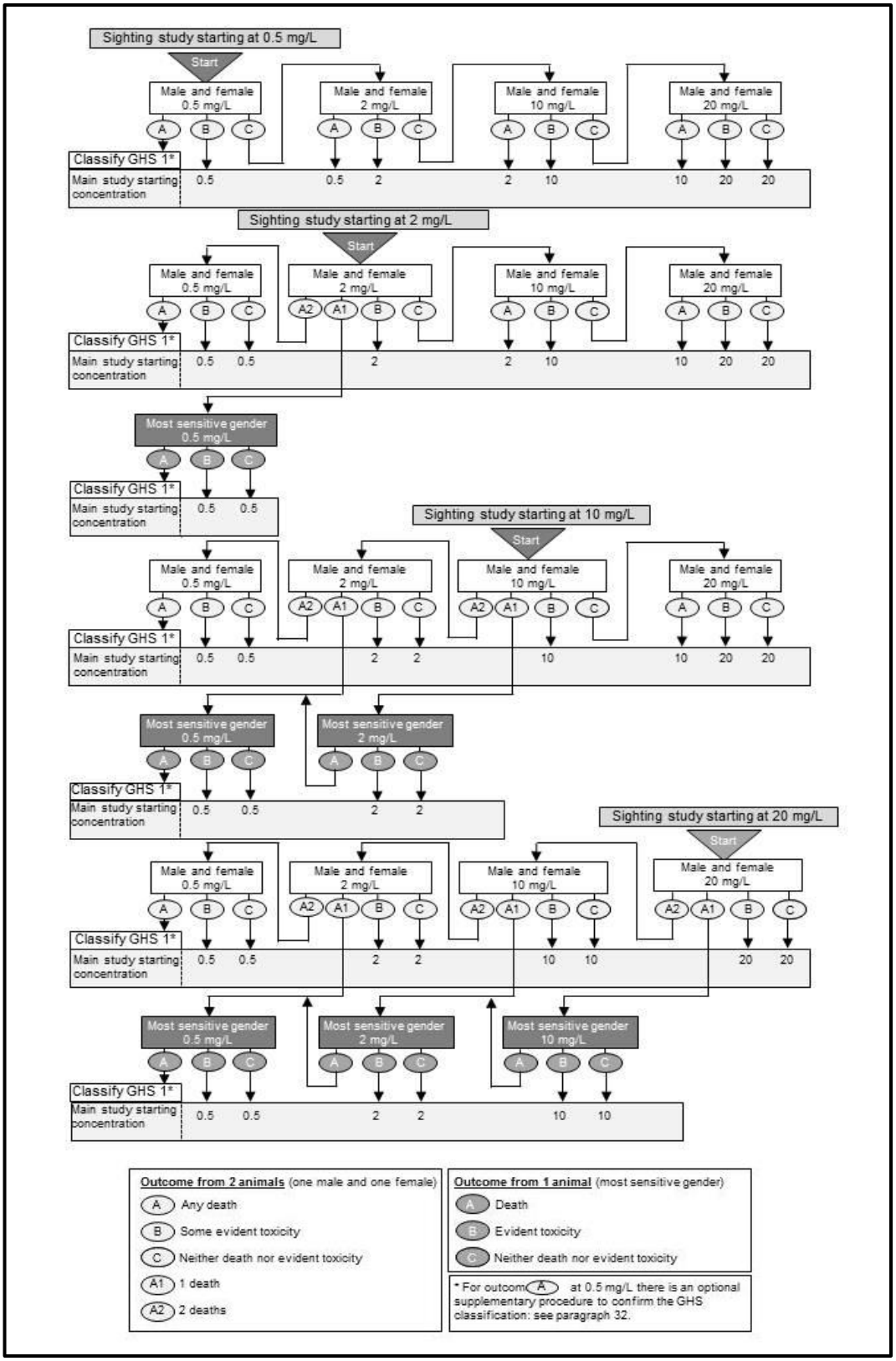
Flow chart for sighting study – dusts/mists (aerosols)



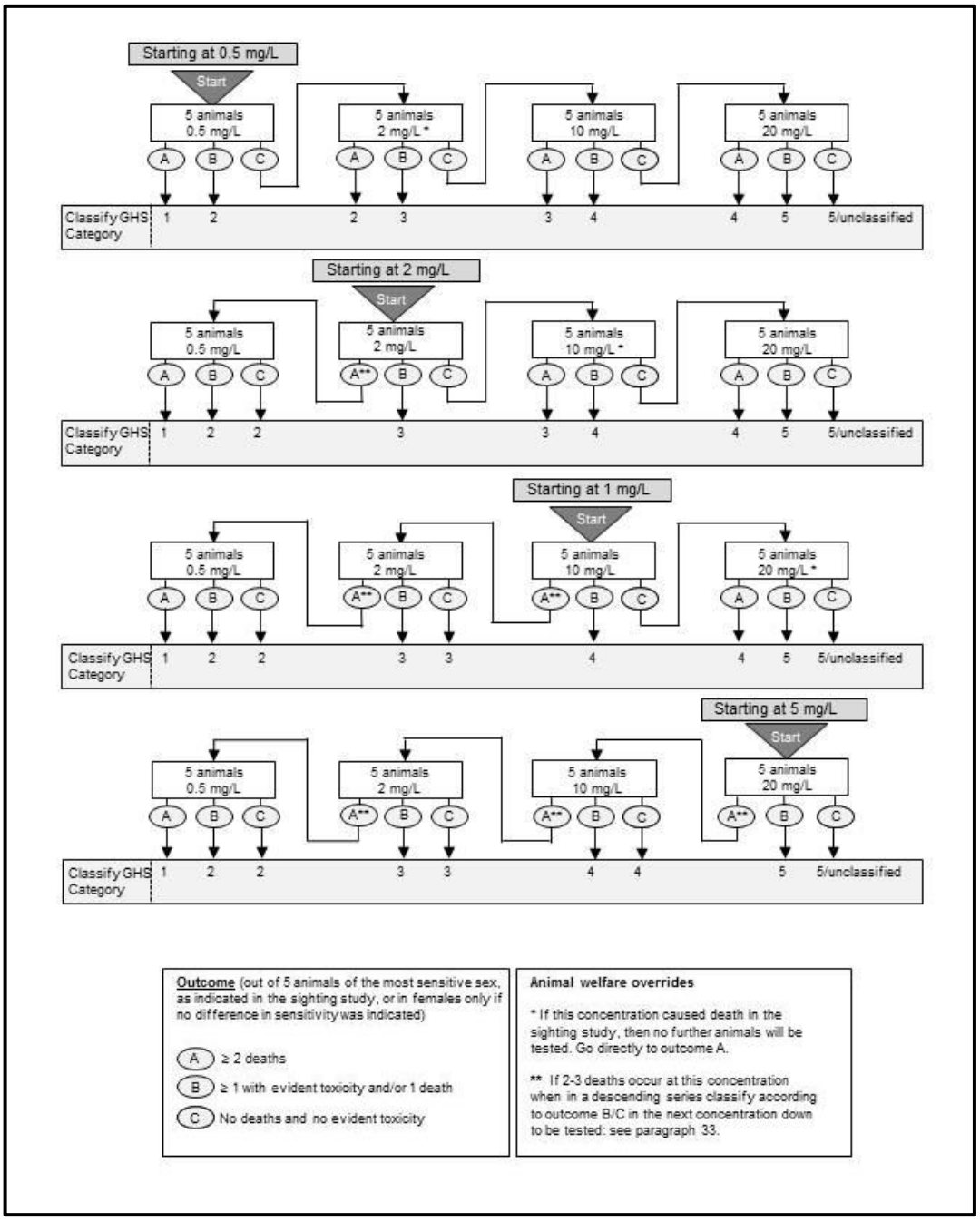
Annex 1 (continued)
Flow chart for main study – dusts/mists (aerosols)



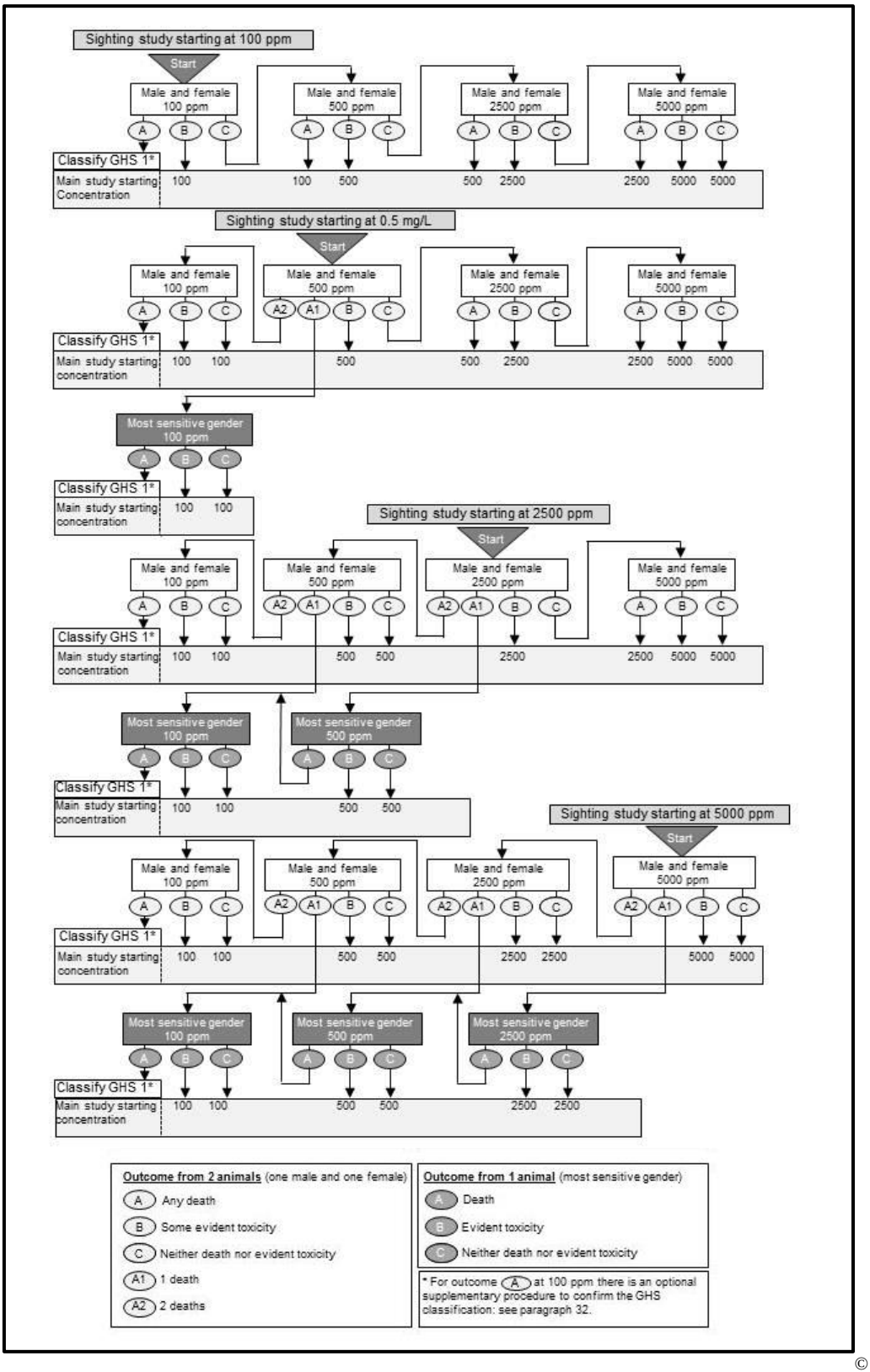
Annex 1 (continued)
Flow chart for sighting study – vapours



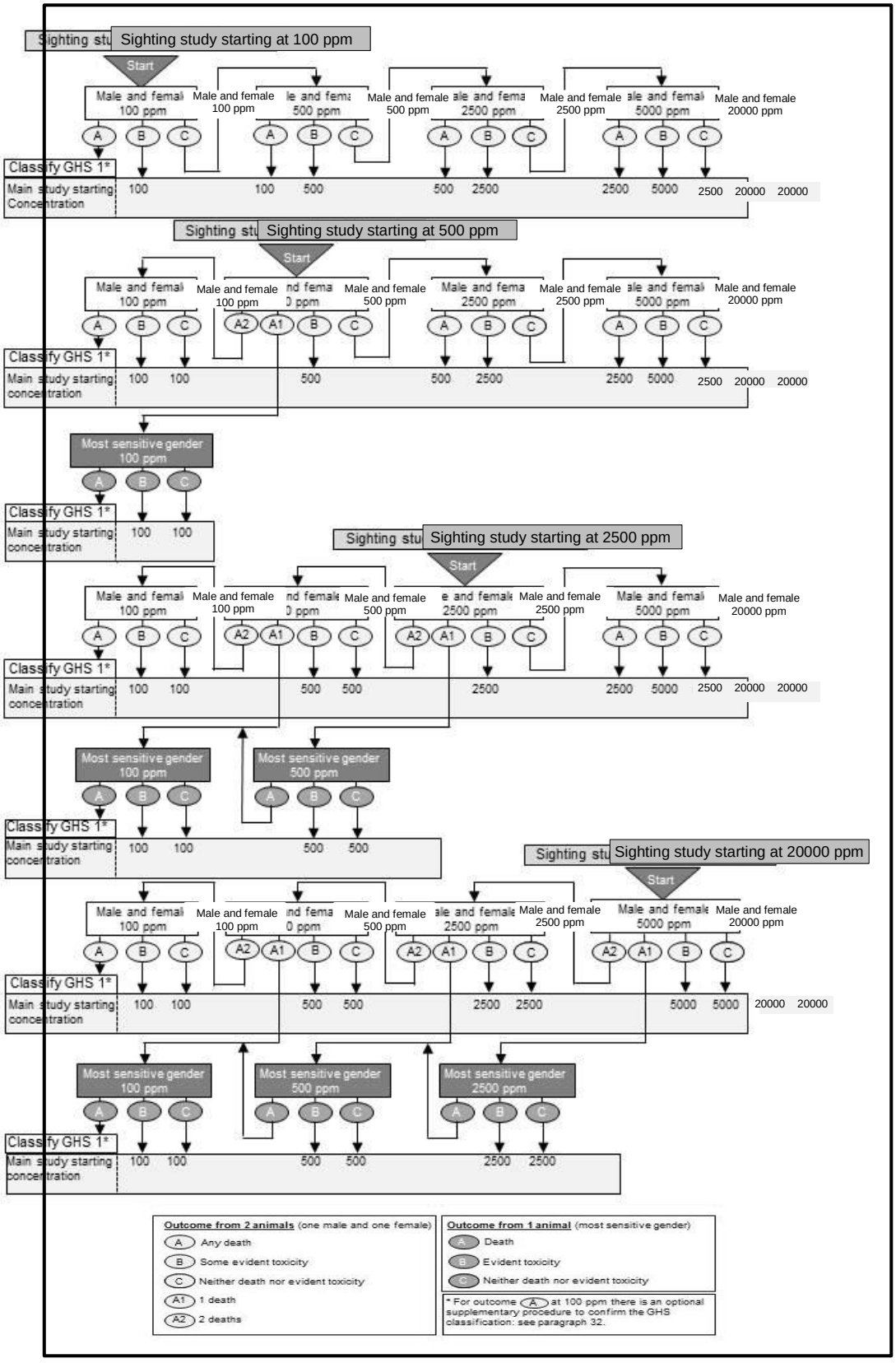
Annex 1 (continued)
Flow chart for main study – vapours



Annex 1 (continued)
Flow chart for sighting study – gases



Annex 1 (continued)
Flow chart for main study – gases



Annex 1 (continued)
Flow chart for main study – gases