Determination of in vivo acute inhalation toxicity of manganese chelate of lysine using Wistar Han rats

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Determination of *in vivo* acute inhalation toxicity of manganese chelate of lysine using Wistar Han rats

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Institute: Wageningen Food & Biobased Research

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Experiments were carried out by Charles River Laboratories Den Bosch BV

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Summary

A new product is developed by Metex Noovistago (Metex) and is aimed for animal nutrition. The new product is a manganese chelate of lysine. The evaluation of the acute inhalation toxicity, relevant to workers safety aspects for the handling of this product, is an important aspect for Metex. For this purpose, the test item was assessed in rats of both sexes following a single 4-hour nose-only exposure to one or more defined concentrations. The study design described in this report is based on the most recent guidelines [1-8].

The result of the 4 h acute inhalation study, LC_{50} value of manganese chelate of lysine in most susceptible sex (males) of Wistar Han rats was established to be within the range of >1 - ≤5 mg/L.

Based on these results and according to the guideline/regulations [6-7], manganese chelate of lysine:

- should be classified in Category 4 for acute toxicity by the inhalation route.
- and labeled as H332: Harmful if inhaled.

1 Introduction

A new product is developed by Metex Noovistago (Metex) with the aim to be used for animal nutrition. The new product is a manganese chelate of lysine. In this report, the above-described product will be referred as "manganese chelate of lysine" or as "test item".

The objective of this study was to assess the acute inhalation toxicity of the manganese chelate of lysine in Wistar Han rats of both sexes following a single 4-hour nose-only exposure to one or more defined concentrations. Animals were retained for a 14-day post exposure observation period. This study intends to provide information on the potential health hazards of the manganese chelate of lysine and data produced can be used for classification/labelling of the test item. This study provides a rational basis for risk assessment in humans.

The design of this study is based on the following study guidelines [1-4]:

- OECD Guideline 403. Acute Inhalation Toxicity, September 2009.
- EPA Health Effects Test Guideline OPPTS 870.1300. Acute Inhalation Toxicity, August 1998.
- EC No 440/2008, part B. Acute Toxicity (inhalation), May 2008, amended by COMMISSION REGULATION (EU) No 260/2014 (24 January 2014).
- Appendix to Director General Notification, No. 12-Nousan-8147. Agricultural Production Bureau, Ministry of Agriculture, Forestry and Fisheries of Japan (JMAFF), November 2000, including the most recent revisions.

In short, the test item was administered as an aerosol by nose-only inhalation for 4 hours to two groups of five male and five female Wistar Han rats at target concentrations of 1 and 5 mg/L, respectively. Mortality and clinical signs were monitored daily during the observation period and body weights were determined on days 1, 2, 4, 8 and 15. Macroscopic examination was performed on the day of death or after terminal sacrifice (day 15).

The experimental part was outsourced to a third party – Charles River Laboratories Den Bosch BV - while results and conclusions were assembled by WFBR in the present report.

2 Methods

2.1 Test system

The test system consisted of rats, WI (Han) strain, outbred, SPF-Quality, sourced from Charles River Deutschland, Sulzfeld, Germany. Exposure group consisted of 5 animals of both sexes, appr. 9-12 weeks old, including nulliparous and non-pregnant females.

The Wistar Han rat was chosen as the animal model for this study as it is a rodent species accepted by regulatory agencies for toxicity testing. The total number of animals to be used in this study was considered to be the minimum required to properly characterize the effects of the test item. This study was designed such that it did not require an unnecessary number of animals to accomplish its objectives. The study plan was reviewed and agreed by the Animal Welfare Body of Charles River Laboratories Den Bosch B.V.

Animal husbandry details including housing, environmental conditions, food, water, animal enrichment and veterinary care were closely monitored and are described in the report of Charles River Laboratories Den Bosch BV, which is provided in Annex 1.

2.2 Test item

The test item, manganese chelate of lysine, developed by Metex was delivered to Wageningen Food & Biobased Research (WFBR). WFBR stored the test item for 3 months at ambient conditions. 200g of the test item was shipped to Charles River Laboratories Den Bosch BV, a third party, which carried out the evaluation of the acute inhalation toxicity.

As no suitable test atmosphere could be generated by using the powder of the test item as delivered, it was mixed with water (Elix) at 33.33% (w/v) and heated up to approximately 40°C prior to the start of the exposure.

The experimental study began on 3rd May 2023 and was completed on 24th May 2023.

2.3 Experimental design and inhalation procedure

The study was performed following a stepwise exposure scenario. Target concentrations were based on the cut off concentration values specified in the UN and EC classification guidelines [6-7]. Five rats of each sex were exposed to a test item at target concentration of 1 mg/L. Based on the results, one additional group of most susceptible sex (males) was exposed to 5 mg/L, in accordance with the OECD 403 guidelines.

The test item was administered once via the inhalation route on day 1, by nose-only directed flow exposure for 4 hours.

The inhalation route of administration was selected because this route was defined as a possible route of human exposure. The starting exposure level was selected based on the available test item data and the one expected not to cause mortality. The animals were exposed to the test concentration selected over the exposure period, while also maintaining an acceptable particle size distribution: target mass median aerodynamic diameter (MMAD) in the range of 1-4 µm with a range of 1.5-3 for the geometric standard deviation (GSD).

2.4 Test atmosphere generation and characterisation

The rats were exposed to the test item in the exposure chamber, designed for directed flow nose-only inhalation. As no suitable test atmosphere could be generated by using the powder as delivered, the test item was mixed with water (Elix). An aerosol was generated by nebulization of the test item with pressurized air. The primary aerosol was diluted with pressurized air before it entered the exposure chamber. The nebulizer was placed in a water bath of approximately 40°C. The mixture in the nebulizer was stirred during the generation.

Representative samples were taken for determination of the actual concentration during exposure at 1 and 5 mg/L. Sample volumes were measured by means of a dry gas meter (type G 1.6, Actaris Meterfabriek B.V., Dordrecht, The Netherlands). The collected amount of test item in the air sample was measured gravimetrically. Subsequently, the time-weighted mean concentrations with the standard deviations were calculated.

The particle size distribution was characterized twice during each exposure period. The samples were drawn with a flow of 2 L/min from the test atmosphere. Amounts of test item collected were measured gravimetrically. Subsequently, the MMAD and the GSD values were determined based on OECD guidance document No 39 [8]. Graphs of the cumulative mass of test item collected (percentage of total collected) against the cut points of the impactor stages were drawn on log-normal paper.

2.5 In-life procedures, observations and measurements

Throughout the study, the rats were observed for general health/mortality and moribundity twice daily, in the morning and at the end of the working day.

The animals were checked for mortality, behavioural signs of distress and effects on respiration at least three times during exposure. Post exposure observations were performed at periodic intervals on the day of exposure (at least two times) and once daily thereafter. The observation period was 14 days. The onset, intensity and duration of these signs was recorded (if appropriate), particular attention being paid to the animals for the first hours after exposure.

The animals were weighed individually on day 1 (pre-exposure), 2, 4 and 8 and 15. Terminal body weights were collected from animals found dead or euthanized moribund after day 1.

All moribund animals and animals surviving to the end of the observation period were euthanized according to laboratories Standard Operating Procedures.

A detailed description on the test system preparation, handling and exposure details along with the specifics on data collection and calculations can be found on the report of Charles River Laboratories Den Bosch BV provided in Annex 1.

3 Results

The LC_{50} value of the test item was ranked within the following ranges: >0 - ≤0.05, >0.05 - ≤0.5, >0.5 - ≤1, >1 - ≤5 or as exceeding 5 mg/L, or the maximum achievable concentration. No statistical analysis was performed (the method used is not intended to allow the calculation of a precise LC_{50} value).

The results were evaluated according to:

- Globally Harmonized System of Classification and Labelling of Chemicals (GHS) of the United Nations (2021) [6].
- Regulation (EC) No 1272/2008 of the European Parliament and of the Council of 16 December 2008 on classification, labelling and packaging of items and mixtures [7].

3.1 Test atmosphere characterization

A total 13 and 18 representative samples were taken for determination of the actual concentration during exposure at 1 and 5 mg/L, respectively.

At 1 mg/L, the time-weighted mean actual concentration was 1.253 ± 0.032 mg/L. The nominal concentration (amount of test item used divided by the volume of pressurized air used) was 7 mg/L. This resulted in a generation efficiency (ratio of actual and nominal concentration) of 19%.

At 5 mg/L, the time-weighted mean actual concentration was 3.455 ± 0.069 mg/L. The nominal concentration (amount of test item used divided by the volume of pressurized air used) was 50 mg/L. This resulted in a generation efficiency (ratio of actual and nominal concentration) of 7%.

The concentration was measured at time points (n=13 and n=18 during exposure at 1 and 5 mg/L, respectively) that were equally distributed over the exposure period, the results of which demonstrated that the material was sufficiently stable. The variation in concentration was caused by adjustments to the generation equipment. The generations were interrupted on three occasions at 1 mg/L and one occasion at 5 mg/L, in order to replace the glassware with clean glassware. To compensate for these changes and interruptions, the generation times were elongated by 3 and 1 minute (for 1 and 5 mg/L, respectively), in order to achieve an actual exposure time of 240 minutes. By calculating the time-weighted mean concentration, effects of interruptions and variations were taken into account resulting in an actual reflection of the mean exposure concentration over time.

The particle size distribution was characterized during each exposure period. The samples were drawn with a flow of 2 L/minute from the test atmosphere and the results are presented in the Table 1.

Table 1 Mass median aerodynamic diameter (MMAD) and the geometric standard deviation (GSD) data.

Exposure (mg/L)	MMAD (µm)	GDS
1.0	3.9	2.1
	3.4	1.9
5.0	3.8	1.9
	3.5	2.1

For detailed information on data collection, calculations and recordings see Annex 1, which provides the report of Charles River Laboratories Den Bosch BV.

3.2 Observations

3.2.1 Mortality

At 1 mg/L exposure, no mortality occurred.

At 5 mg/L exposure, four males were found dead or were euthanized for animal welfare reasons between days 1 and 3. No mortality occurred in the females.

3.2.2 Clinical signs

At 1 mg/L, decreased respiratory rate was seen during the exposure in two males. After exposure, decreased and/or increased respiratory rate, hunched posture and erected fur were noted in all animals between days 1 and 6. In addition, one female and all males showed partly closed eyes and decreased activity on day 1. Two males also showed abnormal breathing sounds on day 1.

At 5 mg/L, increased and/or decreased and irregular respiratory rate and/or shallow breathing was seen during exposure in all animals. After exposure, clinical signs consisted of labored breathing, irregular respiratory rate, abnormal breathing sounds, gasping, hunched posture, erected fur, partly/completely closed eyes, retching, discharge from the nostrils, brown staining of the fur and decreased activity.

3.2.3 Body weights

Body weight loss was noted for all animals at 1 and 5 mg/L exposure.

At 1 mg/L, moderate to severe body weight loss was noted for all animals on day 2. Four males and all females regained weight after day 2 and the remaining animal regained weight after day 4.

At 5 mg/L, moderate to severe body weight loss was noted for the surviving animals on day 2. All surviving animals regained weight after day 2.

3.2.4 Macroscopic findings

At 1 mg/L exposure, no abnormalities were found at macroscopic *post mortem* examination of the animals.

At 5 mg/L exposure, macroscopic *post mortem* examination revealed abnormalities of the lungs (failure to collapse, pale white foci), thymus (dark red foci), trachea (white frothy content), cecum (distended with gas), stomach (distended with gas), right testis (dark red discoloration) and autolysis. Macroscopic examination of the surviving animals at termination did not reveal any abnormalities.

For detailed information on data collection, calculations and recordings the reader is referred to the report of Charles River Laboratories Den Bosch BV provided in Annex 1.

4 Conclusions

The objective of this study was to assess the acute inhalation toxicity of manganese chelate of lysine in rats of both sexes following a single 4-hour nose-only exposure to one or more defined concentrations. Wistar Han rats were retained for a 14-day post exposure observation period. This data provided information on the potential health hazards of manganese chelate of lysine and data produced can be used for classification/labelling of the test item, thus a rational basis for risk assessment in human.

Two different concentrations were tested during the 4 hours nose-only exposure, which for the most susceptible sex (male) resulted in a LC_{50} value range of >1 - ≤5 mg/L.

Based on these results and according to the Globally Harmonized System of Classification and Labelling of Chemicals (GHS) [6] and Regulation (EC) No 1272/2008 [7], the manganese chelate of lysine should be:

- classified in Category 4 for acute toxicity by the inhalation route.
- and labeled as H332: Harmful if inhaled.

Literature

1. OECD Guideline 403. Acute Inhalation Toxicity, September 2009.
2. EPA Health Effects Test Guideline OPPTS 870.1300. Acute Inhalation Toxicity, August 1998.
3. EC No 440/2008, part B. Acute Toxicity (inhalation), May 2008, amended by COMMISSION REGULATION (EU) No 260/2014 (24 January 2014).
4. Appendix to Director General Notification, No. 12-Nousan-8147. Agricultural Production Bureau, Ministry of Agriculture, Forestry and Fisheries of Japan (JMAFF), November 2000.
5. Appendix 1 of project license AVD23600202216274 approved by the Central Authority for Scientific Procedures on Animals (CCD).
6. Globally Harmonized System of Classification and Labelling of Chemicals (GHS), United Nations, New York and Geneva (2021) (including all amendments).
7. Regulation (EC) No 1272/2008 on classification, labelling and packaging of items and mixtures (including all amendments).
8. OECD Guidance document on inhalation toxicity studies Series on Testing and Assessment No. 39 (Second Edition).

Annex 1 Report Charles River

FINAL REPORT

Test Facility Study No. 20418386

Assessment of Acute Inhalation Toxicity with Chélate-Manganese in the Wistar Han Rat (Nose-Only)

GLP

SPONSOR:
Wageningen Food & Biobased Research
P.O.Box 17,
6700 AA Wageningen
The Netherlands

TEST FACILITY:
Charles River Laboratories Den Bosch BV
Hambakenwetering 7
5231 DD 's-Hertogenbosch
The Netherlands

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QUALITY ASSURANCE STATEMENT

This report was inspected by the Test Facility Quality Assurance Unit (QAU) according to the Standard Operating Procedure(s). The reported method and procedures were found to describe those used and the report reflects the raw data. The Test Facility inspection program was conducted in accordance with Standard Operating Procedure. During the on-site process inspections, procedures applicable to this type of study were inspected.

The dates of Quality Assurance inspections are given below.

Test Facility Study No. 20418386

Type of Inspections	Phase/Process	Start Inspection date	End Inspection date	Reporting date to TFM and SD*
Study				
	Final Study Plan	01-May-2023	01-May-2023	01-May-2023
	Report	07-Aug-2023	09-Aug-2023	09-Aug-2023
	Final Report	21-Sep-2023	21-Sep-2023	21-Sep-2023
Process				
	Animal Facilities	03-Mar-2023	15-Mar-2023	17-Mar-2023
		05-Apr-2023	28-Apr-2023	28-Apr-2023
		05-May-2023	30-May-2023	30-May-2023
	Test Item Handling			
	Exposure			
	Observations/Measurements			
	Specimen Handling			
	Necropsy	12-Apr-2023	12-Apr-2023	17-Apr-2023
		17-May-2023	17-May-2023	17-May-2023
	Observations/Measurements			
	Specimen Handling			
	Test Item Formulation	02-May-2023	09-May-2023	09-May-2023
		24-May-2023	24-May-2023	24-May-2023
	Test Item Handling			
	Test Item Receipt	13-Dec-2022	15-Dec-2022	20-Dec-2022
		17-Mar-2023	27-Mar-2023	27-Mar-2023
	Test Item Handling			

*TFM=Test Facility Management SD = Study Director

All electronic signatures appear at the end of this Report upon finalization.

COMPLIANCE STATEMENT AND REPORT APPROVAL

The study was performed in accordance with the OECD Principles of Good Laboratory Practice as accepted by Regulatory Authorities throughout the European Union, United States of America (FDA and EPA), Japan (MHLW, MAFF and METI) and other countries that are signatories to the OECD Mutual Acceptance of Data Agreement.

Exceptions from the above regulations are listed below.

- No Certificate of Analysis or analytical report was available. However, sufficient information was available regarding the batch number, expiry date, composition, physical description and the test material was stored under the conditions described. Based on the available information it was considered that the study integrity was not affected by this.

This study was conducted in accordance with the procedures described herein. There were no deviations from the study plan and standard operating procedures. The report represents an accurate and complete record of the results obtained.

There were no deviations from the above regulations that affected the overall integrity of the study or the interpretation of the study results and conclusions.

All electronic signatures appear at the end of the document upon finalization.

1. RESPONSIBLE PERSONNEL

1.1. Test Facility

Role/Phase	Quality Assurance Unit	Name	Contact Information
Study Director	Charles River	Sandra van de Wiel, PhD	Address as cited for Test Facility Tel: +31 73 640 6700 E-mail: Sandra.vandeWiel@crl.com
Test Facility Management	Charles River	Harry Emmen, MSc	Address as cited for Test Facility Tel: +31 73 640 6700 E-mail: Harry.Emmen@crl.com
Test Facility QAU	Charles River	Lead QA	Address as cited for Test Facility Tel: +31 73 640 6700 E-mail: QADenBosch@crl.com

2. SUMMARY

The objective of this study was to assess the acute inhalation toxicity of Chélate-Manganese in rats of both sexes following a single 4-hour nose-only exposure to one or more defined concentrations. Animals were retained for a 14-day post exposure observation period.

The study was carried out based on the guidelines described in:

- OECD Guideline 403. *Acute Inhalation Toxicity,* September 2009.
- EPA Health Effects Test Guideline OPPTS 870.1300. *Acute Inhalation Toxicity*, August 1998.
- EC No 440/2008, part B. Acute Toxicity (inhalation), May 2008, amended by COMMISSION REGULATION (EU) No 260/2014 (24 January 2014).
- Appendix to Director General Notification, No. 12-Nousan-8147. Agricultural Production Bureau, Ministry of Agriculture, Forestry and Fisheries of Japan (JMAFF), November 2000.

Chélate-Manganese was administered as an aerosol by nose-only inhalation for 4 hours to one group of five male and five female Wistar Han rats at target concentrations of 1 and 5 mg/L. Mortality and clinical signs were observed daily during the observation period and body weights were determined on Days 1, 2, 4, 8 and 15. Macroscopic examination was performed on the day of death or after terminal sacrifice (Day 15).

At 1 mg/L, the time-weighted mean actual concentration was 1.253 ± 0.032 mg/L. The nominal concentration (amount of test material used divided by the volume of pressurized air used) was 7 mg/L. This resulted in a generation efficiency (ratio of actual and nominal concentration) of 19%. At 5 mg/L, the time-weighted mean actual concentration was 3.455 ± 0.069 mg/L. The nominal concentration (amount of test material used divided by the volume of pressurized air used) was 50 mg/L. This resulted in a generation efficiency (ratio of actual and nominal concentration) of 7%.

The particle size distribution was characterized during each exposure period. At 1 mg/L, the MMAD was 3.9 µm (gsd 2.1) and 3.4 µm (gsd 1.9). At 5 mg/L, the MMAD was 3.8 µm (gsd 1.9) and 3.5 µm (gsd 2.1)

At 1 mg/L, no mortality occurred. At 5 mg/L, four males were found dead or were euthanized for humane reasons between Days 1 and 3. No mortality occurred in the females at 5 mg/L.

At 1 mg/L, decreased respiratory rate was seen during the exposure in two males. After exposure, decreased and/or increased respiratory rate, hunched posture and erected fur were noted in all animals between Days 1 and 6. In addition, one female and all males showed partly closed eyes and decreased activity on Day 1. Two males also showed abnormal breathing sounds on Day 1. At 5 mg/L, increased, decreased and irregular respiratory rate and/or shallow breathing was seen during exposure in all animals. After exposure, clinical signs consisted of labored breathing, irregular respiratory rate, labored breathing, abnormal breathing sounds, gasping, hunched posture, erected fur, partly/completely closed eyes, retching, discharge from the nostrils, brown staining of the fur and decreased activity.

At 1 mg/L, moderate to severe body weight loss was noted for all animals on Day 2. Four males and all females regained weight after Day 2 and the remaining animal regained weight after Day 4. At 5 mg/L, moderate to severe body weight loss was noted for the surviving animals on Day 2. All surviving animals at 5 mg/L regained weight after Day 2.

No abnormalities were found at macroscopic *post mortem* examination of the animals at 1 mg/L. Macroscopic *post mortem* examination of the animals of 5 mg/L that died or were euthanized for humane reasons revealed abnormalities of the lungs (failure to collapse, pale white foci), thymus (dark red foci), trachea (white frothy content), cecum (distended with gas), stomach (distended with gas), right testis (dark red discoloration) and autolysis. Macroscopic examination of the surviving animals at 5 mg/L at termination did not reveal any abnormalities.

Based on the most susceptible sex (males), the inhalation $LC_{50, 4h}$ value of Chélate-Manganese in Wistar Han rats was established to be within the range of >1 - ≤5 mg/L.
Based on these results and according to the:

- Globally Harmonized System of Classification and Labelling of Chemicals (GHS), United Nations, New York and Geneva (2021) (including all amendments), Chélate-Manganese should be classified in Category 4 for acute toxicity by the inhalation route.
- Regulation (EC) No 1272/2008 on classification, labelling and packaging of items and mixtures (including all amendments), Chélate-Manganese should be classified as Category 4 and should be labeled as H332: Harmful if inhaled.

3. INTRODUCTION

The objective of this study was to assess the acute inhalation toxicity of Chélate-Manganese in rats of both sexes following a single 4-hour nose-only exposure to one or more defined concentrations. Animals were retained for a 14-day post exposure observation period. This study intends to provide information on the potential health hazards of Chélate-Manganese and data produced can be used for classification/labelling of the test material. This study provides a rational basis for risk assessment in human.

The design of this study is based on the following study guidelines:

- OECD Guideline 403. *Acute Inhalation Toxicity,* September 2009.
- EPA Health Effects Test Guideline OPPTS 870.1300. *Acute Inhalation Toxicity*, August 1998.
- EC No 440/2008, part B. Acute Toxicity (inhalation), May 2008, amended by COMMISSION REGULATION (EU) No 260/2014 (24 January 2014).
- Appendix to Director General Notification, No. 12-Nousan-8147. Agricultural Production Bureau, Ministry of Agriculture, Forestry and Fisheries of Japan (JMAFF), November 2000, including the most recent revisions.

The study plan is presented in Appendix 2.

Study Initiation Date:	26 Apr 2023
Initiation of Dosing:	03 May 2023
Completion of In-life:	24 May 2023
Experimental Start Date:	03 May 2023
Experimental Completion Date:	24 May 2023

4. MATERIALS AND METHODS

4.1. Test Material

The terms “item” and “material” (i.e. test item, control item) do have the same meaning. Therefore, both terms can be used interchangeably.

4.1.1. Test Material

Identification:	Chélate-Manganese
Batch (Lot) Number:	Chélate-Manganese
Expiry date:	01 June 2025
Physical Description:	Brown powder (determined by Charles River Den Bosch)
Purity/Composition:	Manganese: 19%, lysine: 23%, Sulfates: 39%
Storage Conditions:	At room temperature

Additional information

Test Facility test material number:	500476/A
Purity/Composition correction factor:	No correction factor required

Test material handling:	No specific handling conditions required
Solubility in vehicle:	
• Water	Not available
Stability in vehicle:	
• Water	Not available

4.1.2. Test Material Characterization

The Sponsor provided to the Test Facility documentation of the identity, purity, composition, and stability for the test material. The Sponsor has appropriate documentation on file concerning the method of synthesis, fabrication or derivation of the test material, and this information is available to the appropriate regulatory agencies should it be requested.

4.2. Reserve Samples

For each batch (lot) of test material, a reserve sample (about 0.5 gram) was collected and maintained under the appropriate storage conditions by the Test Facility.

4.3. Test and Reference Material Inventory and Disposition

Records of the receipt, distribution, and storage of test material were maintained. With the exception of reserve samples, all unused Sponsor-supplied test material will be discarded or returned to the Sponsor after completion of the scheduled program of work. Records of the decisions made will be kept at the Test Facility.

4.4. Preparation of Test Material

As no suitable test atmosphere could be generated by using the powder as delivered, the test material was mixed with water (Elix) at 33.33% (w/v) and heated up to approximately 40°C prior to the start of the exposure.

Any residual volumes were discarded.

4.5. Test System

Species:	Rat
Strain:	Crl: WI(Han)
Condition:	Outbred, SPF-Quality
Source:	Charles River Deutschland, Sulzfeld, Germany
Number of Animals:	5 animals of both sexes per exposure group. Two exposure groups. Females were nulliparous and non-pregnant.
Age at the Initiation of Dosing:	Young adult animals, approximately 9-12 weeks old.
Weight at the Initiation of Dosing:	Males: 274 to 316 g. Females: 159 to 213 g.

4.5.1. Justification for Test System and Number of Animals

The Wistar Han rat was chosen as the animal model for this study as it is a rodent species accepted by regulatory agencies for toxicity testing. The total number of animals to be used in this study was considered to be the minimum required to properly characterize the effects of the test material. This study was designed such that it did not require an unnecessary number of animals to accomplish its objectives.

The study plan was reviewed and agreed by the Animal Welfare Body of Charles River Laboratories Den Bosch B.V. within the framework of Appendix 1 of project license AVD23600202216274 approved by the Central Authority for Scientific Procedures on Animals (CCD) as required by the Dutch Act on Animal Experimentation (December 2014).

4.5.2. Animal Identification

At study assignment, each animal was identified using a subcutaneously implanted electronic identification chip.

4.5.3. Environmental Acclimation

The animals were allowed to acclimate to the Test Facility toxicology accommodation for at least 5 days before the commencement of dosing.

4.5.4. Selection, Assignment, Replacement, and Disposition of Animals

Animals were assigned to the study at the discretion of the coordinating biotechnician, with all animals within ± 20% of the sex mean body weights. Animals in poor health or at extremes of body weight range were not assigned to the study.

Before the initiation of dosing, a health inspection was performed and any assigned animal considered unsuitable for use in the study were replaced by alternate animals obtained from the same shipment and maintained under the same environmental conditions.

The disposition of all animals was documented in the study records.

4.5.5. Husbandry

4.5.5.1. Housing

On arrival and following assignment to the study, animals were group housed (up to 5 animals of the same sex and same exposure group together) in polycarbonate cages (Makrolon MIV type; height 18 cm.) containing sterilized wooden fibers as bedding material equipped with water bottles. The rooms in which the animals were kept were documented in the study records.

Animals were separated during designated procedures/activities. Each cage was clearly labeled.

4.5.5.2. Environmental Conditions

Target temperatures of 20 to 24°C with a relative target humidity of 40 to 70% were maintained. The actual daily mean temperature during the study period was 21 to 22°C with an actual daily mean relative humidity of 42 to 60%. A 12-hour light/12-hour dark cycle was maintained. Ten or greater air changes per hour with 100% fresh air (no air recirculation) were maintained in the animal rooms.

4.5.5.3. Food

Pelleted rodent diet (SM R/M-Z from SSNIFF® Spezialdiäten GmbH, Soest, Germany) was provided ad libitum throughout the study, except during designated procedures.

The feed was analyzed by the supplier for nutritional components and environmental contaminants. Results of the analysis were provided by the supplier and are on file at the Test Facility.

It is considered that there were no known contaminants in the feed that would interfere with the objectives of the study.

4.5.5.4. Water

Municipal tap-water was freely available to each animal via water bottles, except during designated procedures.

Periodic analysis of the water was performed, and results of these analyses are on file at the Test Facility.

It is considered that there were no known contaminants in the water that would interfere with the objectives of the study.

4.5.5.5. Animal Enrichment

For psychological/environmental enrichment, animals were provided with paper (Enviro-dri, Wm. Lillico & Son (Wonham Mill Ltd), Surrey, United Kingdom), except when interrupted by study procedures/activities.

4.5.5.6. Veterinary Care

Veterinary care was available throughout the course of the study; however, no examinations or treatments were required.

4.6. Experimental Design

The study was performed following a stepwise exposure scenario. Target concentrations were based on the cut off concentration values specified in the UN and EC classification guidelines. Five animals of each sex were exposed to a test material target concentration of 1 mg/L. Based on the results, one additional group of the most susceptible sex (males) was exposed to 5 mg/L, as in accordance with the OECD 403 guidelines.

4.6.1. Administration of Test Material

The test material was administered once via the inhalation route on Day 1, by nose-only directed flow exposure for four hours.

4.6.2. Justification of Route and Dose Levels

The inhalation route of administration was selected because this route was defined as a possible route of human exposure. Nose-only exposure was used since this was the most applicable method of exposure for the test model while minimizing concurrent exposure by the oral and dermal routes. Exposure levels were selected based on the EC and UN classification guidelines. The starting exposure level was selected based on the available test material data and was one expected not to cause mortality.

The animals were exposed to the test concentration selected over the exposure period while also maintaining an acceptable particle size distribution: target mass median aerodynamic diameter (MMAD) in the range of 1-4 µm with a range of 1.5-3 for the geometric standard deviation (gsd).

4.7. Inhalation Exposure Procedure

4.7.1. Animal Husbandry on the Day of Exposure

The animals were moved to the inhalation area to in order to perform the exposure. During exposure, there was no access to food and water. After exposure, animals were returned to their cages which were placed in a fume cupboard for a short time period to allow test material remnants to evaporate. At the end of the exposure day the surviving animals were returned to the animal room.

4.7.2. Exposure Chamber

Animals were exposed to the test material via the nose-only inhalation route. For this purpose the animals were placed in polycarbonate restraining tubes, which were connected to the exposure chamber. Animals were allowed to acclimatize for at least fifteen minutes after the last animal has been placed.

The eyes of the animals were treated with crème (Opthosan, AST Farma BV, Oudewater, The Netherlands) in order to protect the eyes from possible adverse effects on the eyes before they were placed in the tubes.

The design of the exposure chamber is based on the directed flow nose-only inhalation chamber (Am. Ind. Hyg Assoc. J. 44(12): 923-928, 1983). The chamber consists of animal sections with eight animal ports each. Each animal port has its own test atmosphere inlet and exhaust outlet. The number of animal sections and number of open inlets were adapted to the air flow in such a way that at each animal port the theoretical air flow was at least 1 L/min. The main inlet of the test atmosphere was located at the top section and the main outlet was located at the bottom section. The direction of the flow of the test atmosphere guaranteed a freshly generated atmosphere for each individual animal. The placement of the individual animals in the inhalation chamber is shown in Figure 1. All components of the exposure chamber in contact with test material were made of stainless steel, glass, rubber or plastic. To avoid exposure of the personnel and contamination of the laboratory the exposure chamber was placed in a fume hood, maintained at a slight negative pressure.

4.8. Test Atmosphere Generation and Characterization

4.8.1. Test Atmosphere Generation

As no suitable test atmosphere could be generated by using the powder as delivered, the test material was mixed with water (Elix). An aerosol was generated by nebulization of the test material with pressurized air. The primary aerosol was diluted with pressurized air before it entered the exposure chamber (Figure 1). The nebulizer was placed in a water bath of approximately 40°C. The mixture in the nebulizer was stirred during the generation. The mean total airflows were 23 and 14 L/minute for the 1 and 5 mg/L exposure groups, respectively.

From the exposure chamber the test atmosphere was passed through a filter before it was released to the exhaust of the fume hood.

4.8.2. Test Atmosphere Concentration Sampling

A total of 13 and 18 representative samples were taken for determination of the actual concentration during exposure at 1 and 5 mg/L, respectively. Samples were drawn from the test atmosphere through a tube mounted in one of the free animal ports of the exposure chamber. Samples were drawn through a glass fiber filter (type Glass microfiber filter GF/C™ Whatman Amersham place, Little Chalfont, Buckinghamshire, UK). Sample volumes were measured by means of a dry gas meter (type G 1.6, Actaris Meterfabriek B.V., Dordrecht, The Netherlands). The filters were dried in a stove set at 50 °C for 5 minutes in order to evaporate the vehicle (Elix water). The collected amount of the dried test material in the air sample was measured gravimetrically. Subsequently the time-weighted mean concentrations with the standard deviations were calculated.

4.8.3. Particle Size Distribution Investigations

The particle size distribution was characterized twice during each exposure period. The samples were drawn with a flow of 2 L/min. from the test atmosphere through a tube mounted in one of the free animal ports of the exposure chamber (Figure 1).

The samples were collected with an 8 stage Marple personal cascade impactor containing fiber glass filters (TE-290-GF. Tisch Environmental, Cleves, Ohio, USA) and a fiber glass back-up filter (SEC-290-F1, Westech, Upper Stondon, Bedfordshire, England). Amounts of test material collected were measured gravimetrically. Subsequently the Mass Median Aerodynamic Diameter (MMAD) and the Geometric Standard Deviation (GSD) were determined based on OECD guidance document No 39. Graphs of the cumulative mass of test material collected (percentage of total collected) against the cut points of the impactor stages were drawn on log-normal paper. When drawing the graphs more weight was given to the cut points where the cumulative mass sampled was within the range of 5 to 95%. The Mass Median Aerodynamic Diameter (MMAD), i.e. the particle size where 50% of the particle mass was borne by particles smaller than the MMAD and the σ84%, (the particle size where 84% of the particle mass was borne by particles smaller than the σ84%) was read from the graph. The geometric standard deviation (gsd) was calculated as σ84% / MMAD.

4.8.4. Pre - Exposure Atmosphere Characterization Investigations

The performance characteristics of the test atmosphere generation and exposure system to be used was assessed during trial generations. These trials were performed according to test facilities SOP's and since these trials were performed prior to preparation of the study plan no GLP was claimed for these trials. This was considered as no exception of the GLP guidelines since the measurements and observations were not used for interpretation for the outcome of this study. For the same reason, these results are not reported and kept in the raw data of this study. These investigations were undertaken to establish:

Aerosol concentration assessment
Temporal variation in chamber concentration
Test material utilization
Particle size distribution measurements (within the target MMAD range 1-4 µm)

Trial generation results showed that it was not possible to generate a suitable test atmosphere using the powder as delivered. Instead, the test item was mixed with water to generate aerosols.

4.8.5. Nominal Test Atmosphere Concentration

The nominal concentration was calculated by dividing the amount of test material used by the volume of pressurized air (average air flow times exposure time) entering the exposure chamber used for exposure of the animals. Due to the small volume of the exposure chamber the equilibrium time was negligible. The volume of air was calculated from the average air flow (which was measured by means of thermal mass flow meters and recorded regularly, preferably in 30-minute intervals) and the exposure time.

4.8.6. Test Atmosphere Stability Monitoring

It was considered that the opacity of the test atmosphere could not be reliably monitored by means of an aerosol monitoring system. An indication of stability of the test atmosphere was obtained from the concentration measurements equally distributed over time.

4.8.7. Temperature and Relative Humidity of the Test Atmosphere

The temperature and relative humidity were measured with a humidity and temperature indicator (E+E Elektronik, Engerwitzdorf, Austria) and recorded after the animals were connected to the exposure chamber and at 30-minute intervals after initiation of exposure. The probe was inserted in a tube mounted in one of the free animal ports of the exposure chamber (Figure 1). The temperature of the atmosphere during the exposures were between 22.1 and 26.3°C. The relative humidity was between 69 and 100% which was considered appropriate for this relatively short 4-hour exposure duration.

4.9. In - Life Procedures, Observations, and Measurements

4.9.1. Mortality/Moribundity Checks

Throughout the study, animals were observed for general health/mortality and moribundity twice daily, in the morning and at the end of the working day. Animals were not removed from cage during observation, unless necessary for identification or confirmation of possible findings. Animals showing pain, distress or discomfort, which was considered not transient in nature or is likely to become more severe, were sacrificed for humane reasons based on OECD guidance document on the recognition, assessment, and use of clinical signs as humane endpoints for experimental animals used in safety evaluation (ENV/JM/MONO/ 2000/7).

4.9.2. Clinical Observations

4.9.2.1. Observations During Exposure

Animals were checked for mortality, behavioral signs of distress and effects on respiration at least three times during exposure.

4.9.2.2. Post Exposure Observations

Post exposure observations were performed at periodic intervals on the day of exposure (at least two times) and once daily thereafter. The observation period was 14 days.

All the animals were examined for reaction to exposure. The onset, intensity and duration of these signs was recorded (if appropriate), particular attention being paid to the animals for the first hours after exposure.

4.9.3. Body Weights

Animals were weighed individually on Day 1 (pre-exposure), 2, 4 and 8 and 15. Terminal body weights were collected from animals found dead or euthanized moribund after Day 1.

4.10. Terminal Procedures

All moribund animals and animals surviving to the end of the observation period were euthanized according to laboratories Standard Operating Procedures. All animals assigned to the study were subjected to necropsy and descriptions of all internal macroscopic abnormalities were recorded. On completion of the necropsy, the animals' carcasses were disposed of, and no tissues were retained.

5. ANALYSIS

All results presented in the tables of the report are calculated using values as per the raw data rounding procedure and may not be exactly reproduced from the individual data presented.

The LC_{50} value of the test material will be ranked within the following ranges: >0 - ≤0.05, >0.05 - ≤0.5, >0.5 - ≤1, >1 - ≤5 or as exceeding 5 mg/L or the maximum achievable concentration. No statistical analysis will be performed (the method used is not intended to allow the calculation of a precise LC_{50} value).

The results were evaluated according to:

- Globally Harmonized System of Classification and Labelling of Chemicals (GHS) of the United Nations (2021) (including all amendments).
- Regulation (EC) No 1272/2008 of the European Parliament and of the Council of 16 December 2008 on classification, labelling and packaging of items and mixtures (including all amendments).

6. COMPUTERIZED SYSTEMS

Computerized systems used in the study are listed below. All computerized systems used in the conduct of this study have been validated; when a particular system has not satisfied all requirements, appropriate administrative and procedural controls were implemented to assure the quality and integrity of data.

Text Table 1
Computerized Systems

System Name	Description of Data Collected and/or Analyzed
M-Files®	Reporting and collection of 21 CFR Part 11 compliant signature
REES Centron	Temperature, relative humidity and/or atmospheric pressure monitoring Animal and Laboratory facilities
Dispense	Test material receipt, accountability and/or formulation activities
Provantis	In-life and *post mortem* (mortality; clinical observations; body weights; macroscopic findings) data collection

7. RETENTION AND DISPOSITION OF RECORDS

All study-specific raw data, documentation, study plan and final report from this study were archived at the Test Facility at finalization of the report. At least two years after issue of the final report, the Sponsor will be contacted.

Electronic data generated by the Test Facility were archived as noted above, except that data collected using Provantis and files stored on M-Files® (Study Plan, documentation and reporting files) were archived at the Charles River Laboratories facility location in Wilmington, Massachusetts, USA.

8. RESULTS

8.1. Test Atmosphere Characterization

8.1.1. Concentration

(Table 1 and Figure 1)

At 1 mg/L, the time-weighted mean actual concentration was 1.253 ± 0.032 mg/L. The nominal concentration (amount of test material used divided by the volume of pressurized air used) was 7 mg/L. This resulted in a generation efficiency (ratio of actual and nominal concentration) of 19%.

At 5 mg/L, the time-weighted mean actual concentration was 3.455 ± 0.069 mg/L. It showed to be technically unfeasible to obtain an actual concentration of 5 mg/L. The nominal concentration (amount of test material used divided by the volume of pressurized air used) was 50 mg/L. This resulted in a generation efficiency (ratio of actual and nominal concentration) of 7%.

The concentration was measured at time points (n=13 and n=18 for 1 mg/L and 5 mg/L, respectively) that were equally distributed over the exposure period, the results of which demonstrated that the material was sufficiently stable. The variation in concentration was caused by adjustments to the generation equipment. The generation was interrupted on three occasions (1 mg/L) or on one occasion (5 mg/L) in order to replace the glassware with clean glassware. To compensate for these interruptions, the generation time was elongated by three minutes at 1 mg/L and by one minute at 5 mg/L minutes to achieve an actual exposure time of 240 minutes. By calculating the time-weighted mean concentration, effects of interruptions and variations were taken into account resulting in an actual reflection of the mean exposure concentration over time.

8.1.2. Particle Size

(Table 1)

The particle size distribution was characterized during each exposure period. At 1 mg/L, the MMAD was 3.9 µm (gsd 2.1) and 3.4 µm (gsd 1.9). At 5 mg/L, the MMAD was 3.8 µm (gsd 1.9) and 3.5 µm (gsd 2.1)

8.2. Observations

8.2.1. Mortality

(Table 2)

At 1 mg/L, no mortality occurred.

At 5 mg/L, four males were found dead or were euthanized for humane reasons between Days 1 and 3. No mortality occurred in the females.

8.2.2. Clinical Signs

(Table 2)

At 1 mg/L, decreased respiratory rate was seen during the exposure in two males. After exposure, decreased and/or increased respiratory rate, hunched posture and erected fur were noted in all animals between Days 1 and 6. In addition, one female and all males showed partly closed eyes and decreased activity on Day 1. Two males also showed abnormal breathing sounds on Day 1.

At 5 mg/L, increased, decreased and irregular respiratory rate and/or shallow breathing was seen during exposure in all animals. After exposure, clinical signs consisted of labored breathing, irregular respiratory rate, labored breathing, abnormal breathing sounds, gasping, hunched posture, erected fur, partly/completely closed eyes, retching, discharge from the nostrils, brown staining of the fur and decreased activity.

8.2.3. Body Weights

(Table 2)

At 1 mg/L, moderate to severe body weight loss was noted for all animals on Day 2. Four males and all females regained weight after Day 2 and the remaining animal regained weight after Day 4.

At 5 mg/L, moderate to severe body weight loss was noted for the surviving animals on Day 2. All surviving animals regained weight after Day 2.

8.3. Macroscopic Findings

(Table 2)

No abnormalities were found at macroscopic *post mortem* examination of the animals of 1 mg/L.

Macroscopic *post mortem* examination of the animals of 5 mg/L that died or were euthanized for humane reasons revealed abnormalities of the lungs (failure to collapse, pale white foci), thymus (dark red foci), trachea (white frothy content), cecum (distended with gas), stomach (distended with gas), right testis (dark red discoloration) and autolysis. Macroscopic examination of the surviving animals at termination did not reveal any abnormalities.

9. CONCLUSION

Based on the most susceptible sex (males), the inhalation $LC_{50,\,4h}$ value of Chélate-Manganese in Wistar Han rats was established to be within the range of >1 - ≤5 mg/L.

Based on these results and according to the:

- Globally Harmonized System of Classification and Labelling of Chemicals (GHS), United Nations, New York and Geneva (2021) (including all amendments), Chélate-Manganese should be classified in Category 4 for acute toxicity by the inhalation route.
- Regulation (EC) No 1272/2008 on classification, labelling and packaging of items and mixtures (including all amendments), Chélate-Manganese should be classified as Category 4 and should be labeled as H332: Harmful if inhaled.

10. REFERENCES

OECD Guideline 403. Acute Inhalation Toxicity, September 2009.

EPA Health Effects Test Guideline OPPTS 870.1300. Acute Inhalation Toxicity, August 1998.

EC No 440/2008, part B. Acute Toxicity (inhalation), May 2008, amended by COMMISSION REGULATION (EU) No 260/2014 (24 January 2014).

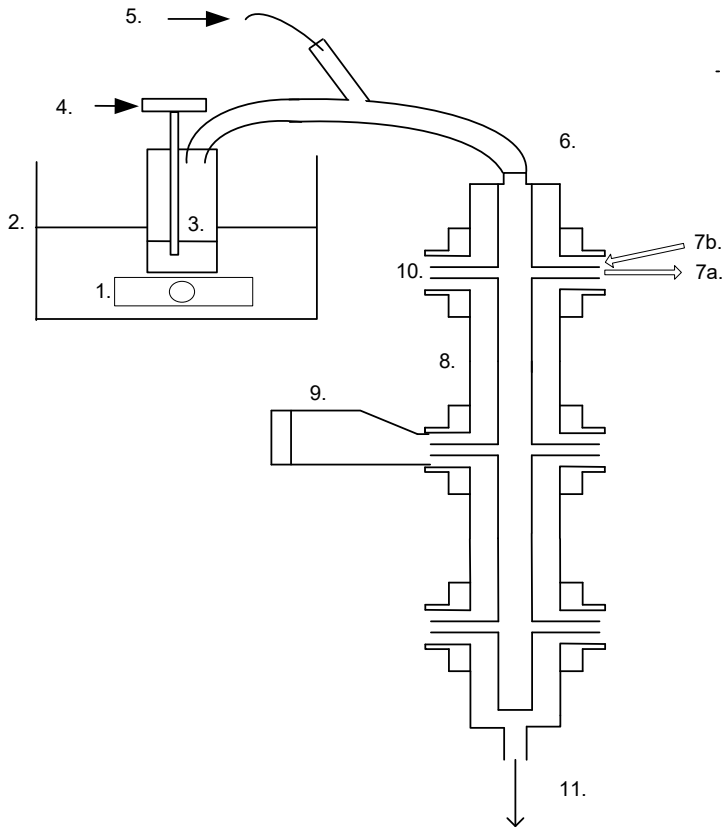
Appendix to Director General Notification, No. 12-Nousan-8147. Agricultural Production Bureau, Ministry of Agriculture, Forestry and Fisheries of Japan (JMAFF), November 2000.

Appendix 1 of project license AVD23600202216274 approved by the Central Authority for Scientific Procedures on Animals (CCD)

Appendix 1 Figures and Tables

Figure 1 Test Atmosphere Generation Set-up

Figure 1.1 Schematic Presentation of the Experimental Set-up Used for Exposure

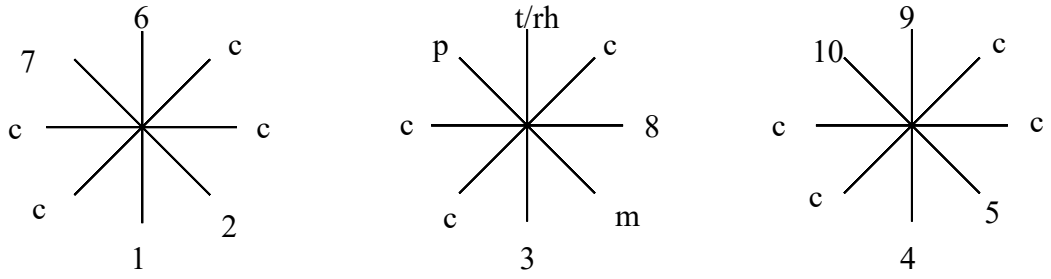


1. Heating plate
2. Water bath
3. Collison nebulizer with test item formulation
4. Pressurized air inlet (→)
5. Pressurized air for dilution (→) *
6. Main inlet exposure chamber
7a. Test atmosphere inlet to animal
7b. Exhaust outlet from animal
8. Exposure chamber, three levels (No 1 at the top, No 3 at the bottom)
9. Animal restrainer
10. Openings used for concentration, particle size, temperature and relative humidity measurements
11. Main exhaust outlet of exposure chamber to vacuum pump

*Note: Pressurized air for dilution is applicable for the 1 mg/L exposure only.

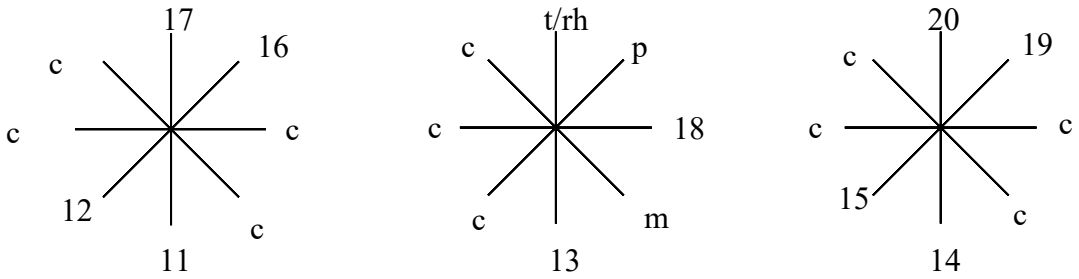
Figure 1.2 Schematic Presentation of the Placement of the Animals and the Location of the Sampling Point During Exposure

1 mg/L exposure group



Level 1 (top) Level 2 Level 3

5 mg/L exposure group

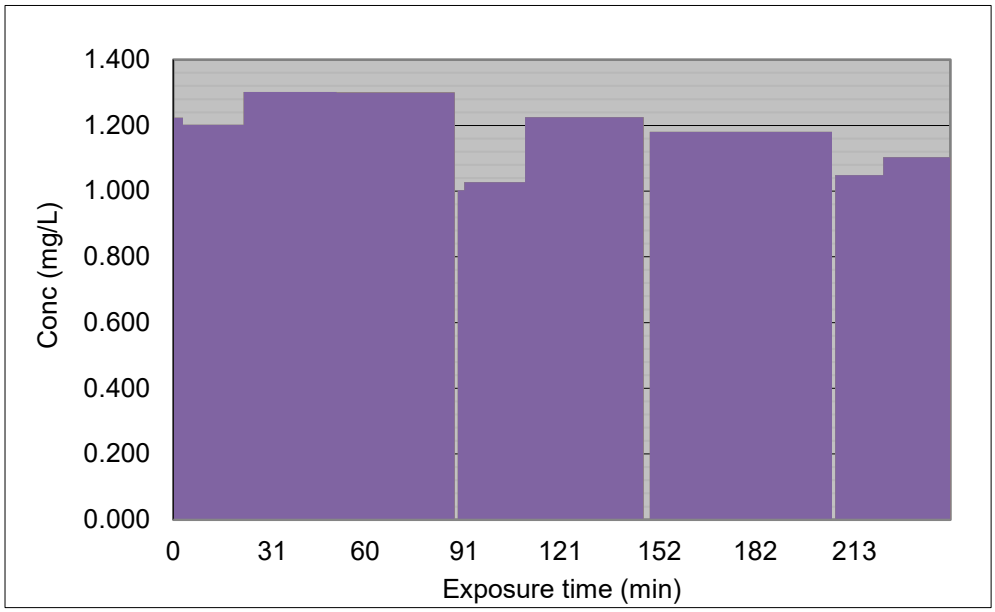


Level 1 (top) Level 2 Level 3

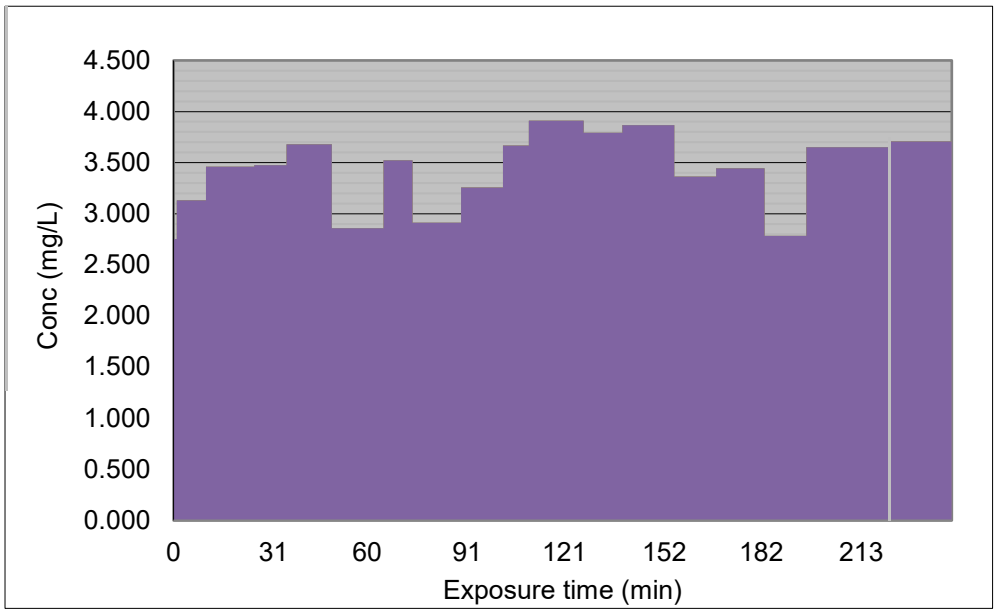
1.-20 animal number.
c closed atmosphere inlet to animal port.
o open atmosphere inlet to animal port.
p pressure gauge (inlet closed).
m opening used for total concentration and particle sizing (inlet open).
t/rh temperature and relative humidity (inlet open).

Figure 1.3 Stability of the Test Atmosphere

1 mg/L exposure group



5 mg/L exposure group



The concentration was measured at time points (n=13 and n=18 for 1 mg/L and 5 mg/L, respectively) that were equally distributed over the exposure period, the results of which demonstrated that the material was sufficiently stable. The variation in concentration was caused by adjustments to the generation equipment. The generation was interrupted on three occasions (1 mg/L) or on one occasion (5 mg/L) in order to replace the glassware with clean glassware. To compensate for these interruptions, the generation time was elongated by three minutes at 1 mg/L and by one minute at 5 mg/L minutes to achieve an actual exposure time of 240 minutes. By calculating the time-weighted mean concentration, effects of interruptions and variations were taken into account resulting in an actual reflection of the mean exposure concentration over time.

Table 1 Test Atmosphere Generation and Characterization

Table 1.1 Gravimetrical Concentration of Test Material

1 mg/L exposure group	
Start of generation of test atmosphere:	09:23
End of generation of test atmosphere:	13:26

Time (hh:mm)	Action	Sample Volume (L)	Mass Sampled (mg)	Concentration (mg/L)		% of Total Exposure Time		Weight Concentration (mg/L)
09:23	start of generation	n.a.	n.a.	n.a.		0.0		n.a.
09:27	start of sampling	6	7.34	1.223		1.7		0.020
09:45	start of sampling	6	7.21	1.202		7.5		0.090
10:00	start of sampling	6	7.81	1.302		6.3		0.081
10:15	start of sampling	6	7.81	1.302		6.2		0.081
10:28	start of sampling	6	7.80	1.300		5.4		0.070
10:51	stop generation	n.a.	n.a.	1.300	1)	9.6		0.125
10:52	start of generation	n.a.	n.a.	n.a.		n.a.		n.a.
10:54	start of sampling	6	6.02	1.003		0.8		0.008
11:14	start of sampling	7	7.19	1.027		8.3		0.086
11:34	start of sampling	6	7.35	1.225		8.3		0.102
11:51	stop generation	n.a.	n.a.	1.225		7.1		0.087
11:52	start of generation	n.a.	n.a.	n.a.		n.a.		n.a.
11:58	start of sampling	19	19.02	1.001		10.0		0.100
12:19	start of sampling	6	6.32	1.053		8.7		0.092
12:41	start of sampling	6	7.08	1.180		9.2		0.108
12:49	stop generation	n.a.	n.a.	1.180	1)	3.3		0.039
12:50	start of generation	n.a.	n.a.	n.a.		n.a.		n.a.
13:05	start of sampling	6	6.29	1.048		6.3		0.066
13:20	start of sampling	6	6.62	1.103		6.2		0.069
13:26	end of generation	n.a.	n.a.	1.103	1)	2.5		0.028
			Time-weighted mean concentration					1.253
				Standard deviation				0.032
				Number of samples				13

1) Assumed concentration, based on the last sample.
n.a.= not applicable

Table 1.1 (Continued) Gravimetrical Concentration of Test Material

5 mg/L exposure group	
Start of generation of test atmosphere:	08:38
End of generation of test atmosphere:	12:39

Time (hh:mm)	**Action**	**Sample Volume (L)**	**Mass Sampled (mg)**	**Concentration (mg/L)**		**% of Total Exposure Time**		**Weight Concentration (mg/L)**
08:38	start of generation	n.a.	n.a.	n.a.		0.0		n.a.
08:40	start of sampling	5	13.76	2.752		0.8		0.023
08:49	start of sampling	5	15.66	3.132		3.7		0.117
09:04	start of sampling	5	17.30	3.460		6.3		0.216
09:13	start of sampling	5	17.38	3.476		3.8		0.130
09:28	start of sampling	5	18.39	3.678		6.2		0.230
09:43	start of sampling	5	14.30	2.860		6.3		0.179
09:52	start of sampling	5	17.62	3.524		3.8		0.132
10:07	start of sampling	5	14.56	2.912		6.2		0.182
10:20	start of sampling	5	16.28	3.256		5.4		0.176
10:29	start of sampling	5	18.34	3.668		3.7		0.138
10:45	start of sampling	5	19.55	3.910		6.7		0.261
10:58	start of sampling	5	18.96	3.792		5.4		0.205
11:13	start of sampling	5	19.33	3.866		6.3		0.242
11:27	start of sampling	5	16.81	3.362		5.8		0.196
11:42	start of sampling	5	17.22	3.444		6.3		0.215
11:54	start of sampling	5	13.92	2.784		5.0		0.139
12:02	start of sampling	5	18.25	3.650		3.3		0.122
12:20	stop generation	n.a.	n.a.	3.650	1)	7.5		0.274
12:21	start of generation	n.a.	n.a.	n.a.		n.a.		n.a.
12:23	start of sampling	5	18.54	3.708		0.8		0.031
12:39	end of generation	n.a.	n.a.	3.708	1)	6.7		0.247
			Time-weighted mean concentration					3.455
				Standard deviation				0.069
				Number of samples				18

1) Assumed concentration, based on the last sample.
n.a.= not applicable

Table 1.2 Aerodynamic Particle Size Distribution in the Test Atmosphere

1 mg/L exposure group	
Start of generation of test atmosphere:	09:23
End of generation of test atmosphere:	13:26
Sampling speed (L/min):	2

measurement 1:

Sampling time: 10:40

Sample volume (L): 25

Stage	**Cut Point (µm)**	**Mass Sampled (mg)**	**Relative Mass (%)**	**Cumulative Mass (% of total sampled)**
1	21.0	0.00	0.00	100.00
2	15.0	0.00	0.00	100.00
3	10.0	2.37	6.34	93.66
4	6.0	6.88	18.42	75.24
5	3.5	14.53	38.89	36.35
6	2.0	9.36	25.05	11.30
7	0.9	3.05	8.16	3.13
8	0.5	0.87	2.33	0.80
Back up	0.25	0.30	0.80	0.00
MMAD[1] (µm):		3.9		
gsd[2]:		2.1		

measurement 2:

Sampling time: 12:30

Sample volume (L): 25

Stage	**Cut Point (µm)**	**Mass Sampled (mg)**	**Relative Mass (%)**	**Cumulative Mass (% of total sampled)**
1	21.0	0.00	0.00	100.00
2	15.0	0.00	0.00	100.00
3	10.0	1.37	3.99	96.01
4	6.0	5.08	14.80	81.21
5	3.5	11.17	32.54	48.67
6	2.0	11.28	32.86	15.82
7	0.9	4.07	11.86	3.96
8	0.5	1.00	2.91	1.05
Back up	0.25	0.36	1.05	0.00
MMAD[1] (µm):		3.4		
gsd[2]:		1.9		

[1] Mass Median Aerodynamic Diameter
[2] Geometric standard deviation

Table 1.2 (Continued) Aerodynamic Particle Size Distribution in the Test Atmosphere

5 mg/L exposure group	
Start of generation of test atmosphere:	08:38
End of generation of test atmosphere:	12:39
Sampling speed (L/min):	2

measurement 1:

Sampling time: 10:01

Sample volume (L): 4

Stage	**Cut Point (µm)**	**Mass Sampled (mg)**	**Relative Mass (%)**	**Cumulative Mass (% of total sampled)**
1	21.0	0.04	0.16	99.84
2	15.0	0.00	0.00	99.84
3	10.0	1.66	6.77	93.07
4	6.0	4.37	17.82	75.24
5	3.5	8.92	36.38	38.87
6	2.0	7.35	29.98	8.89
7	0.9	1.61	6.57	2.32
8	0.5	0.49	2.00	0.33
Back up	0.25	0.08	0.33	0.00
MMAD[1] (µm):		3.8		
gsd[2]:		1.9		

measurement 2:

Sampling time: 12:15

Sample volume (L): 4

Stage	**Cut Point (µm)**	**Mass Sampled (mg)**	**Relative Mass (%)**	**Cumulative Mass (% of total sampled)**
1	21.0	0.04	0.12	99.88
2	15.0	0.00	0.00	99.88
3	10.0	1.57	4.77	95.11
4	6.0	5.17	15.70	79.42
5	3.5	11.03	33.49	45.93
6	2.0	10.79	32.76	13.18
7	0.9	3.26	9.90	3.28
8	0.5	0.86	2.61	0.67
Back up	0.25	0.22	0.67	0.00
MMAD[1] (µm):		3.5		
gsd[2]:		2.1		

[1] Mass Median Aerodynamic Diameter
[2] Geometric standard deviation

Table 2 In-life Observations and Macroscopy

Table 2.1 Individual Mortality

Sex: Male Day(s): - Relative to Start Date

1 mg/L Group 1	Day of Death	Removal Date	Path Removal Reason
1	15	17-May-2023	TERM
2	15	17-May-2023	TERM
3	15	17-May-2023	TERM
4	15	17-May-2023	TERM
5	15	17-May-2023	TERM

Sex: Female Day(s): - Relative to Start Date

1 mg/L Group 1	Day of Death	Removal Date	Path Removal Reason
6	15	17-May-2023	TERM
7	15	17-May-2023	TERM
8	15	17-May-2023	TERM
9	15	17-May-2023	TERM
10	15	17-May-2023	TERM

Sex: Male Day(s): - Relative to Start Date

5 mg/L Group 2	Day of Death	Removal Date	Path Removal Reason
11	2	11-May-2023	UNSC
12	2	11-May-2023	FD
13	3	12-May-2023	UNSC
14	1	10-May-2023	UNSC
15	15	24-May-2023	TERM

Sex: Female Day(s): - Relative to Start Date

5 mg/L Group 2	Day of Death	Removal Date	Path Removal Reason
16	15	24-May-2023	TERM
17	15	24-May-2023	TERM
18	15	24-May-2023	TERM
19	15	24-May-2023	TERM
20	15	24-May-2023	TERM

TERM = Terminal Euthanasia
FD = Found Dead
UNSC = Unscheduled Termination

Table 2.2 Individual Clinical Observations

Group 1 Sex: Male 1 mg/L																			
	Observation Type: All Types	Day(s) Relative to Start Date																	
		E1	E2	E3	1 1h	1 3h	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	Respiratory Rate Abnormal, Decreased	.	.	X	.	.	X	.	.	.	.	.	.	.	.	.	.	.	.	.
	Respiratory Rate Abnormal, Increased	.	.	.	X	X	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	Breathing, Abnormal Sounds	.	.	.	.	.	X	.	.	.	.	.	.	.	.	.	.	.	.	.
	Hunched Posture	.	.	.	X	X	X	X	X	X	X	.	.	.	.	.	.	.	.	.
	Fur, Erected	.	.	.	X	X	X	.	.	.	.	.	.	.	.	.	.	.	.	.
	Eye Closed, Left, Partly	.	.	.	X	X	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	Eye Closed, Right, Partly	.	.	.	X	X	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	Activity Decreased	.	.	.	X	X	.	.	.	.	.	.	.	.	.	.	.	.	.	.
2	Respiratory Rate Abnormal, Decreased	.	.	.	.	.	X	.	.	.	.	.	.	.	.	.	.	.	.	.
	Respiratory Rate Abnormal, Increased	.	.	.	X	X	.	X	.	.	.	.	.	.	.	.	.	.	.	.
	Hunched Posture	.	.	.	X	X	X	X	X	X	X	.	.	.	.	.	.	.	.	.
	Fur, Erected	.	.	.	X	X	X	.	.	.	.	.	.	.	.	.	.	.	.	.
	Eye Closed, Left, Partly	.	.	.	X	X	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	Eye Closed, Right, Partly	.	.	.	X	X	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	Activity Decreased	.	.	.	X	X	.	.	.	.	.	.	.	.	.	.	.	.	.	.
3	Respiratory Rate Abnormal, Decreased	.	.	X	.	.	X	.	.	.	.	.	.	.	.	.	.	.	.	.
	Respiratory Rate Abnormal, Increased	.	.	.	X	X	.	X	.	.	.	.	.	.	.	.	.	.	.	.
	Breathing, Abnormal Sounds	.	.	.	.	.	X	.	.	.	.	.	.	.	.	.	.	.	.	.
	Hunched Posture	.	.	.	X	X	X	X	X	X	X	.	.	.	.	.	.	.	.	.
	Fur, Erected	.	.	.	X	X	X	.	.	.	.	.	.	.	.	.	.	.	.	.
	Eye Closed, Left, Partly	.	.	.	X	X	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	Eye Closed, Right, Partly	.	.	.	X	X	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	Activity Decreased	.	.	.	X	X	.	.	.	.	.	.	.	.	.	.	.	.	.	.

E1, E2, E3 = observations during exposure
X = Present

Table 2.2 Individual Clinical Observations (Continued)

Group 1 Sex: Male 1 mg/L																				
	Observation Type: All Types	Day(s) Relative to Start Date																		
		E1	E2	E3	1 1h	1 3h	2	3	4	5	6	7	8	9	10	11	12	13	14	15
4	Respiratory Rate Abnormal, Decreased	.	.	.	.	.	X	.	.	.	.	.	.	.	.	.	.	.		
	Respiratory Rate Abnormal, Increased	.	.	.	X	X	.	X	.	.	.	.	.	.	.	.	.	.		
	Hunched Posture	.	.	.	X	X	X	X	X	X	X	.	.	.	.	.	.	.		
	Fur, Erected	.	.	.	X	X	X	.	.	.	.	.	.	.	.	.	.	.	.	.
	Eye Closed, Left, Partly	.	.	.	X	X	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	Eye Closed, Right, Partly	.	.	.	X	X	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	Activity Decreased	.	.	.	X	X	.	.	.	.	.	.	.	.	.	.	.	.	.	.
5	Respiratory Rate Abnormal, Decreased	.	.	.	.	.	X	.	.	.	.	.	.	.	.	.	.	.	.	.
	Respiratory Rate Abnormal, Increased	.	.	.	X	X	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	Hunched Posture	.	.	.	X	X	X	X	X	X	X	.	.	.	.	.	.	.	.	.
	Fur, Erected	.	.	.	X	X	X	.	.	.	.	.	.	.	.	.	.	.	.	.
	Eye Closed, Left, Partly	.	.	.	X	X	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	Eye Closed, Right, Partly	.	.	.	X	X	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	Activity Decreased	.	.	.	X	X	.	.	.	.	.	.	.	.	.	.	.	.	.	.

E1, E2, E3 = observations during exposure
X = Present

Table 2.2 Individual Clinical Observations (Continued)

Group 1 Sex: Female 1 mg/L																				
	Observation Type: All Types	Day(s) Relative to Start Date																		
		E1	E2	E3	1 1h	1 3h	2	3	4	5	6	7	8	9	10	11	12	13	14	15
6	Respiratory Rate Abnormal, Decreased	.	.	.	.	.	X	.	.	.	.	.	.	.	.	.	.	.	.	.
	Respiratory Rate Abnormal, Increased	.	.	.	X	X	.	X	.	.	.	.	.	.	.	.	.	.	.	.
	Hunched Posture	.	.	.	X	X	X	X	X	X	.	.	.	.	.	.	.	.	.	.
	Fur, Erected	.	.	.	X	X	X	.	.	.	.	.	.	.	.	.	.	.	.	.
7	Respiratory Rate Abnormal, Decreased	.	.	.	.	.	X	.	.	.	.	.	.	.	.	.	.	.	.	.
	Respiratory Rate Abnormal, Increased	.	.	.	X	X	.	X	.	.	.	.	.	.	.	.	.	.	.	.
	Hunched Posture	.	.	.	X	X	X	X	X	X	.	.	.	.	.	.	.	.	.	.
	Fur, Erected	.	.	.	X	X	X	.	.	.	.	.	.	.	.	.	.	.	.	.
	Eye Closed, Left, Partly	.	.	.	X	X	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	Eye Closed, Right, Partly	.	.	.	X	X	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	Activity Decreased	.	.	.	X	X	.	.	.	.	.	.	.	.	.	.	.	.	.	.
8	Respiratory Rate Abnormal, Decreased	.	.	.	.	.	X	.	.	.	.	.	.	.	.	.	.	.	.	.
	Respiratory Rate Abnormal, Increased	.	.	.	X	X	.	X	.	.	.	.	.	.	.	.	.	.	.	.
	Hunched Posture	.	.	.	X	X	X	X	X	X	.	.	.	.	.	.	.	.	.	.
	Fur, Erected	.	.	.	X	X	X	.	.	.	.	.	.	.	.	.	.	.	.	.
9	Respiratory Rate Abnormal, Decreased	.	.	.	.	.	X	.	.	.	.	.	.	.	.	.	.	.	.	.
	Respiratory Rate Abnormal, Increased	.	.	.	X	X	.	X	.	.	.	.	.	.	.	.	.	.	.	.
	Hunched Posture	.	.	.	X	X	X	X	X	X	.	.	.	.	.	.	.	.	.	.
	Fur, Erected	.	.	.	X	X	X	.	.	.	.	.	.	.	.	.	.	.	.	.
10	Respiratory Rate Abnormal, Decreased	.	.	.	.	.	X	.	.	.	.	.	.	.	.	.	.	.	.	.
	Respiratory Rate Abnormal, Increased	.	.	.	X	X	.	X	.	.	.	.	.	.	.	.	.	.	.	.
	Hunched Posture	.	.	.	X	X	X	X	X	X	.	.	.	.	.	.	.	.	.	.
	Fur, Erected	.	.	.	X	X	X	.	.	.	.	.	.	.	.	.	.	.	.	.

E1, E2, E3 = observations during exposure
X = Present

Table 2.2 Individual Clinical Observations (Continued)

Group 1 Sex: Male 5 mg/L																				
	Observation Type: All Types	Day(s) Relative to Start Date																		
		E1	E2	E3	1 1h	1 3h	2	3	4	5	6	7	8	9	10	11	12	13	14	15
11*	Retching	.	.	.	.	.	X													
	Respiratory Rate Abnormal, Increased	X	.	.	.	.	.													
	Respiratory Rate Abnormal, Irregular	.	.	.	.	.	X													
	Breathing, Labored	.	.	.	X	X	.													
	Breathing, Abnormal Sounds	.	.	.	X	X	X													
	Breathing, Shallow	.	X	X	.	.	.													
	Hunched Posture	.	.	.	X	X	X													
	Fur, Erected	.	.	.	X	X	X													
	Fur, Staining, Site Not Recorded, Brown	.	.	.	X	X	X													
	Activity Decreased	.	.	.	X	.	.													
12**	Respiratory Rate Abnormal, Decreased	.	X	X	.	.														
	Respiratory Rate Abnormal, Irregular	X	.	.	.	.														
	Breathing, Labored	.	.	.	X	X														
	Breathing, Abnormal Sounds	.	.	.	X	X														
	Hunched Posture	.	.	.	X	X														
	Fur, Erected	.	.	.	X	X														
	Fur, Staining, Site Not Recorded, Brown	.	.	.	X	X														
	Eye Closed, Site Not Recorded, Completely	.	.	.	.	X														
	Eye Closed, Site Not Recorded, Partly	.	.	.	X	.														
	Activity Decreased	.	.	.	X	X														
	Gasping	.	.	.	X	X														

E1, E2, E3 = observations during exposure
X = Present
* Euthanized for humane reasons on Day 2
** Found dead on Day 2

Table 2.2 Individual Clinical Observations (Continued)

Group 1 Sex: Male 5 mg/L																				
	Observation Type: All Types	Day(s) Relative to Start Date																		
		E1	E2	E3	1 1h	1 3h	2	3	4	5	6	7	8	9	10	11	12	13	14	15
13*	Respiratory Rate Abnormal, Decreased	.	X	.	.	X	X	X												
	Respiratory Rate Abnormal, Irregular	X	.	X	X	.	.	.												
	Breathing, Labored	.	.	.	X	X	X	X												
	Breathing, Abnormal Sounds	.	.	.	X	X	X	X												
	Hunched Posture	.	.	.	X	X	X	X												
	Fur, Erected	.	.	.	X	X	X	X												
	Fur, Staining, Site Not Recorded, Brown	.	.	.	X	X	X	X												
	Eye Closed, Site Not Recorded, Partly	.	.	.	X	X	X	.												
	Discharge, Consistency, Nostril Left, Consistency Not Recorded	.	.	.	.	.	.	X												
	Discharge, Consistency, Nostril Right, Consistency Not Recorded	.	.	.	.	.	.	X												
	Activity Decreased	.	.	.	X	X	X	X												
14**	Respiratory Rate Abnormal, Decreased	.	X	X	.															
	Respiratory Rate Abnormal, Irregular	X	.	.	.															
	Breathing, Labored	.	.	.	X															
	Breathing, Abnormal Sounds	.	.	.	X															
	Hunched Posture	.	.	.	X															
	Fur, Erected	.	.	.	X															
	Fur, Staining, Site Not Recorded, Brown	.	.	.	X															
	Eye Closed, Site Not Recorded, Partly	.	.	.	X															
	Activity Decreased	.	.	.	X															
	Gasping	.	.	.	X															
15	Respiratory Rate Abnormal, Decreased	X	X	X	.															
	Breathing, Labored	.	.	.	X	X	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	Breathing, Abnormal Sounds	.	.	.	X	X	X	.	.	.	.	.	.	.	.	.	.	.	.	.
	Breathing, Shallow	X	X	X	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	Hunched Posture	.	.	.	X	X	X	X	.	.	.	.	.	.	.	.	.	.	.	.
	Fur, Erected	.	.	.	X	X	X	X	.	.	.	.	.	.	.	.	.	.	.	.
	Fur, Staining, Site Not Recorded, Brown	.	.	.	.	X	X	.	.	.	.	.	.	.	.	.	.	.	.	.

E1, E2, E3 = observations during exposure
X = Present
* Euthanized for humane reasons on Day 3. ** Euthanized for humane reasons on Day 1.

Table 2.2 Individual Clinical Observations (Continued)

Group 1 Sex: Female 5 mg/L																				
	Observation Type: All Types	Day(s) Relative to Start Date																		
		E1	E2	E3	1 1h	1 3h	2	3	4	5	6	7	8	9	10	11	12	13	14	15
16	Respiratory Rate Abnormal, Decreased	.	.	X	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	Respiratory Rate Abnormal, Increased	X	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	Respiratory Rate Abnormal, Irregular	.	X	.	X	X	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	Breathing, Abnormal Sounds	.	.	.	X	X	X	X	.	.	.	.	.	.	.	.	.	.	.	.
	Hunched Posture	.	.	.	X	X	X	X	.	.	.	.	.	.	.	.	.	.	.	.
	Fur, Erected	.	.	.	X	X	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	Fur, Staining, Site Not Recorded, Brown	.	.	.	X	X	X	.	.	.	.	.	.	.	.	.	.	.	.	.
17	Respiratory Rate Abnormal, Decreased	.	.	.	.	X	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	Respiratory Rate Abnormal, Irregular	X	X	X	X	.	X	.	.	.	.	.	.	.	.	.	.	.	.	.
	Breathing, Abnormal Sounds	.	.	.	X	X	X	.	.	.	.	.	.	.	.	.	.	.	.	.
	Hunched Posture	.	.	.	X	X	X	X	.	.	.	.	.	.	.	.	.	.	.	.
	Fur, Erected	.	.	.	X	X	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	Fur, Staining, Site Not Recorded, Brown	.	.	.	X	X	X	.	.	.	.	.	.	.	.	.	.	.	.	.
	Activity Decreased	.	.	.	X	X	.	.	.	.	.	.	.	.	.	.	.	.	.	.
18	Respiratory Rate Abnormal, Increased	.	.	.	.	.	X	X	.	.	.	.	.	.	.	.	.	.	.	.
	Respiratory Rate Abnormal, Irregular	.	X	X	X	X	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	Breathing, Labored	.	.	.	X	X	.	X	.	.	.	.	.	.	.	.	.	.	.	.
	Breathing, Abnormal Sounds	.	.	.	X	X	X	X	.	.	.	.	.	.	.	.	.	.	.	.
	Hunched Posture	.	.	.	X	X	X	X	X	X	.	.	.	.	.	.	.	.	.	.
	Fur, Erected	.	.	.	X	X	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	Fur, Staining, Site Not Recorded, Brown	.	.	.	X	X	X	X	X	X	.	.	.	.	.	.	.	.	.	.
	Activity Decreased	.	.	.	.	X	.	.	.	.	.	.	.	.	.	.	.	.	.	.

E1, E2, E3 = observations during exposure
X = Present

Table 2.2 Individual Clinical Observations (Continued)

Group 1 Sex: Female 5 mg/L																				
	Observation Type: All Types	Day(s) Relative to Start Date																		
		E1	E2	E3	1 1h	1 3h	2	3	4	5	6	7	8	9	10	11	12	13	14	15
19	Respiratory Rate Abnormal, Decreased	X	X	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	Respiratory Rate Abnormal, Irregular	.	.	X	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	Breathing, Shallow	.	.	.	X	X	X	X	.	.	.	.	.	.	.	.	.	.	.	.
	Hunched Posture	.	.	.	X	X	X	X	.	.	.	.	.	.	.	.	.	.	.	.
	Fur, Erected	.	.	.	X	X	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	Fur, Staining, Site Not Recorded, Brown	.	.	.	X	X	X	X	X	X	.	.	.	.	.	.	.	.	.	.
20	Respiratory Rate Abnormal, Decreased	X	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	Respiratory Rate Abnormal, Increased	.	.	.	X	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	Respiratory Rate Abnormal, Irregular	.	X	X	.	X	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	Breathing, Shallow	.	.	.	X	X	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	Hunched Posture	.	.	.	X	X	X	X	X	X	.	.	.	.	.	.	.	.	.	.
	Fur, Erected	.	.	.	X	X	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	Fur, Staining, Site Not Recorded, Brown	.	.	.	X	X	X	.	.	.	.	.	.	.	.	.	.	.	.	.

E1, E2, E3 = observations during exposure
X = Present

Table 2.3 Individual Body Weights

Sex: Male Bodyweight (g)

1 mg/L Group 1	Day(s) Relative to Start Date				
	1	2	4	8	15
1	293	266	280	292	300
2	316	296	310	323	333
3	305	266	281	302	331
4	274	246	265	276	298
5	313	276	273	297	321
Mean	300.2	270.0	281.8	298.0	316.6
SD	17.1	18.2	17.0	17.0	16.7
N	5	5	5	5	5

Sex: Female Bodyweight (g)

1 mg/L Group 1	Day(s) Relative to Start Date				
	1	2	4	8	15
6	184	177	178	178	188
7	213	200	207	214	221
8	209	206	214	218	222
9	196	184	191	199	208
10	202	196	201	203	206
Mean	200.8	192.6	198.2	202.4	209.0
SD	11.4	11.9	14.1	15.7	13.8
N	5	5	5	5	5

Sex: Male Bodyweight (g)

5 mg/L Group 2	Day(s) Relative to Start Date				
	1	2	4	8	15
11	292	248	-	-	-
12	302	-	-	-	-
13	301	261	-	-	-
14	311	-	-	-	-
15	294	280	291	314	332
Mean	300.0	263.0	291.0	314.0	332.0
SD	7.5	16.1	-	-	-
N	5	3	1	1	1

Sex: Female Bodyweight (g)

5 mg/L Group 2	Day(s) Relative to Start Date				
	1	2	4	8	15
16	180	169	185	194	201
17	182	174	185	190	202
18	166	153	164	178	184
19	159	153	167	176	187
20	181	177	190	201	211
Mean	173.6	165.2	178.2	187.8	197.0
SD	10.5	11.5	11.8	10.6	11.2
N	5	5	5	5	5

Table 2.4 Individual Macroscopic Pathology

Animal: 1
Group: 1
Sex: Male
Dose: 1 mg/L
Removal Reason: Terminal Euthanasia
Study Day (Week) of Death: 15 (3)
Gross Status: Complete
Gross Pathology Observations:
No observations found

Animal: 2
Group: 1
Sex: Male
Dose: 1 mg/L
Removal Reason: Terminal Euthanasia
Study Day (Week) of Death: 15 (3)
Gross Status: Complete
Gross Pathology Observations:
No observations found

Animal: 3
Group: 1
Sex: Male
Dose: 1 mg/L
Removal Reason: Terminal Euthanasia
Study Day (Week) of Death: 15 (3)
Gross Status: Complete
Gross Pathology Observations:
No observations found

Animal: 4
Group: 1
Sex: Male
Dose: 1 mg/L
Removal Reason: Terminal Euthanasia
Study Day (Week) of Death: 15 (3)
Gross Status: Complete
Gross Pathology Observations:
No observations found

Animal: 5
Group: 1
Sex: Male
Dose: 1 mg/L
Removal Reason: Terminal Euthanasia
Study Day (Week) of Death: 15 (3)
Gross Status: Complete
Gross Pathology Observations:
No observations found

Table 2.4

Individual Macroscopic Pathology (Continued)

Animal: 6
Group: 1
Sex: Female
Dose: 1 mg/L
Removal Reason: Terminal Euthanasia
Study Day (Week) of Death: 15 (3)
Gross Status: Complete
Gross Pathology Observations:
No observations found

Animal: 7
Group: 1
Sex: Female
Dose: 1 mg/L
Removal Reason: Terminal Euthanasia
Study Day (Week) of Death: 15 (3)
Gross Status: Complete
Gross Pathology Observations:
No observations found

Animal: 8
Group: 1
Sex: Female
Dose: 1 mg/L
Removal Reason: Terminal Euthanasia
Study Day (Week) of Death: 15 (3)
Gross Status: Complete
Gross Pathology Observations:
No observations found

Animal: 9
Group: 1
Sex: Female
Dose: 1 mg/L
Removal Reason: Terminal Euthanasia
Study Day (Week) of Death: 15 (3)
Gross Status: Complete
Gross Pathology Observations:
No observations found

Animal: 10
Group: 1
Sex: Female
Dose: 1 mg/L
Removal Reason: Terminal Euthanasia
Study Day (Week) of Death: 15 (3)
Gross Status: Complete
Gross Pathology Observations:
No observations found

Table 2.4

Individual Macroscopic Pathology (Continued)

Animal: 11
Group: 2
Sex: Male
Dose: 5 mg/L
Removal Reason: Unscheduled Euthanasia
Study Day (Week) of Death: 15 (3)
Gross Status: Complete
Gross Pathology Observations:
LUNG : Failure to collapse
LUNG : Focus, pale; white, multifocal
THYMUS : Focus, dark; red, multifocal
TRACHEA : Abnormal content; frothy : (Comment) White

Animal: 12
Group: 2
Sex: Male
Dose: 5 mg/L
Removal Reason: Found Dead
Study Day (Week) of Death: 2 (1)
Gross Status: Complete
Gross Pathology Observations:
WHOLE ANIMAL : Abnormal appearance : (Comment) autolysis

Animal: 13
Group: 2
Sex: Male
Dose: 5 mg/L
Removal Reason: Unscheduled Euthanasia
Study Day (Week) of Death: 3 (1)
Gross Status: Complete
Gross Pathology Observations:
LARGE INTESTINE, CECUM : Abnormal content : (Comment) distended with gas
LUNG : Failure to collapse
LUNG : Focus, pale; white, multifocal
STOMACH : Abnormal content : (Comment) distended with gas
TESTIS : Discoloration, dark; red, right

Animal: 14
Group: 2
Sex: Male
Dose: 5 mg/L
Removal Reason: Unscheduled Euthanasia
Study Day (Week) of Death: 1 (1)
Gross Status: Complete
Gross Pathology Observations:
LUNG : Focus, pale; white, multifocal
THYMUS : Focus, dark; red, multifocal

Animal: 15
Group: 2
Sex: Male
Dose: 5 mg/L
Removal Reason: Terminal Euthanasia
Study Day (Week) of Death: 15 (3)
Gross Status: Complete
Gross Pathology Observations:
No observations found

Table 2.4

Individual Macroscopic Pathology (Continued)

Animal: 16
Group: 2
Sex: Female
Dose: 5 mg/L
Removal Reason: Terminal Euthanasia
Study Day (Week) of Death: 15 (3)
Gross Status: Complete
Gross Pathology Observations:
No observations found

Animal: 17
Group: 2
Sex: Female
Dose: 5 mg/L
Removal Reason: Terminal Euthanasia
Study Day (Week) of Death: 15 (3)
Gross Status: Complete
Gross Pathology Observations:
No observations found

Animal: 18
Group: 2
Sex: Female
Dose: 5 mg/L
Removal Reason: Terminal Euthanasia
Study Day (Week) of Death: 15 (3)
Gross Status: Complete
Gross Pathology Observations:
No observations found

Animal: 19
Group: 2
Sex: Female
Dose: 5 mg/L
Removal Reason: Terminal Euthanasia
Study Day (Week) of Death: 15 (3)
Gross Status: Complete
Gross Pathology Observations:
No observations found

Animal: 20
Group: 2
Sex: Female
Dose: 5 mg/L
Removal Reason: Terminal Euthanasia
Study Day (Week) of Death: 15 (3)
Gross Status: Complete
Gross Pathology Observations:
No observations found

Appendix 2 Study Plan

FINAL STUDY PLAN

Test Facility Study No. 20418386

Assessment of Acute Inhalation Toxicity with Chélate-Manganese in the Wistar Han Rat (Nose-Only)

GLP

SPONSOR:
Wageningen Food & Biobased Research
P.O.Box 17,
6700 AA Wageningen
The Netherlands

TEST FACILITY:
Charles River Laboratories Den Bosch B.V.
Hambakenwetering 7
5231 DD 's-Hertogenbosch
The Netherlands

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1. OBJECTIVE(S)

The objective of this study is to assess the acute inhalation toxicity of Chélate-Manganese in rats of both sexes following a single 4 hour nose-only exposure to one or more defined concentrations. Animals will then be retained for a 14 day post exposure observation period. This study is intended to provide information on the potential health hazards of Chélate-Manganese and data produced can be used for classification/labelling of the test material. This study should provide a rational basis for risk assessment in man.

2. PROPOSED STUDY SCHEDULE

Proposed study dates are listed below. Actual dates will be included in the Final Report.

Experimental Starting Date:	15 May 2023 (Week 20) (First date of study-specific data collection)
Initiation of Dosing:	22 May 2023 (Week 21)
Completion of In-life:	09 Jul 2023 (Week 27) (Last date of necropsy)
Experimental Completion Date:	16 Jul 2023 (Week 28) (Last date on which data are collected)
Unaudited Draft Report:	23 Jul 2023 (Week 29)

3. SPONSOR

Role	Name	Contact Information
Sponsor Representative Name	Theo Verkleij	Address as cited for Sponsor Tel: +31 317 481 096 E-mail: theo.verkleij@wur.nl
Study Monitor Name	Panagiotis Voudouris	Address as cited for Sponsor Tel: + 31 317 48 6758 E-mail: panagiotis.voudouris@wur.nl

4. RESPONSIBLE PERSONNEL

Role/Phase	Quality Assurance Unit	Name	Contact Information
Study Director	Charles River	Sandra van de Wiel, PhD	Address as cited for Test Facility Tel: +31 73 640 6700 E-mail: Sandra.vandeWiel@crl.com
Test Facility Management	Charles River	Harry Emmen, MSc	Address as cited for Test Facility Tel: +31 73 640 6700 E-mail: Harry.Emmen@crl.com
Test Facility QAU	Charles River	Lead QA	Address as cited for Test Facility Tel: +31 73 640 6700 E-mail: QADenBosch@crl.com

5. TEST MATERIALS

5.1. Test Material Characterization

The Sponsor will provide to the Test Facility documentation of the identity, strength, purity, composition, and stability for the test material. A Certificate of Analysis or equivalent documentation may be provided for inclusion in the Final Report.

The Sponsor has appropriate documentation on file concerning the method of synthesis, fabrication or derivation of the test material, and this information is available to the appropriate regulatory agencies should it be requested.

5.2. Test Material Identification

5.2.1. Test Material

Identification:	Chélate-Manganese
Batch (Lot) Number:	Chélate-Manganese
Expiry date:	01 June 2025
Physical Description:	Brown powder (determined by Charles River Den Bosch)
Purity/Composition:	Manganese: 19%, lysine: 23%, Sulfates: 39%
Storage Conditions:	At room temperature
Additional information	
Test Facility test material number:	500476/A
Purity/Composition correction factor:	No correction factor required
Test material handling:	No specific handling conditions required
Solubility in vehicle:	
• Water	Not indicated
Stability in vehicle:	
• Water	Not indicated

5.3. Reserve Samples

For each batch (lot) of test material and if practically possible, a reserve sample will be collected and maintained under the appropriate storage conditions by the Test Facility.

5.4. Test Material Inventory and Disposition

Records of the receipt, distribution, storage, and disposition of test materials will be maintained.

5.5. Safety

The following safety instruction(s) apply to this study:

- Standard safety precautions specified in Charles River Den Bosch procedures

6. DOSE FORMULATION

6.1. Preparation of Formulations

The test material will be mixed with water. This will be done within 4 hours prior to the start of the exposure.

Details will be recorded in the raw data and reported accordingly.

Any residual volumes from each dosing occasion will be discarded unless otherwise requested by the Study Director.

6.2. Sample Collection and Analysis

The test material will be used as received from the Sponsor; therefore, samples for dose formulation analysis will not be collected by the Test Facility.

7. TEST SYSTEM

Species:	Rat
Strain:	Crl: WI(Han))
Condition:	Outbred, SPF-Quality
Source:	Based on availability, one of the following sources will be used and specified in the report: ● Charles River France, L'Arbresle, France ● Charles River Deutschland, Sulzfeld, Germany
Number of Animals:	5 animals of both sexes or 5 animals of the most sensitive sex (if identified) per exposure group. Females will be nulliparous and non-pregnant.
Target Age at the Initiation of Dosing:	Between 8 and 12 weeks old. Animals to be used within the study will be of approximately the same age.
Target Weight at the Initiation of Dosing:	230 to 380 g (males) and 130 to 280 (females).

The actual age and weight of the animals at the initiation of dosing will be listed in the Final Report.

7.1. Animal Identification

Method: Each animal will be identified using a subcutaneously implanted electronic identification chip and/or any other unique identification mark, to be documented in the study file. The actual animal identification method used will be specified in the Final Report.

7.2. Environmental Acclimation

The animals will be allowed to acclimate to the Test Facility toxicology accommodation for at least 5 days before the commencement of dosing.

7.3. Selection, Assignment, Replacement, and Disposition of Animals

Selection: Animals will be assigned to the study at the discretion of the coordinating biotechnician, with all animals within ± 20% of the sex mean body weights. Animals in poor health or at extremes of body weight range will not be assigned to the study.

Replacement: Before the initiation of dosing, a health inspection will be performed and any assigned animals considered unsuitable for use in the study will be replaced by alternate animals obtained from the same shipment and maintained under the same environmental conditions. The alternate animals may be used as replacements on the study within 1 day.

Disposition: The disposition of all animals will be documented in the study records.

8. HUSBANDRY

8.1. Housing

Caging: Animals will be group housed (up to 5 animals of the same sex and same dosing group together) in polycarbonate cages (Makrolon type IV, height 18 cm) containing sterilized wooden fibers as bedding material equipped with water bottles.

These housing conditions will be maintained unless deemed inappropriate by the Study Director and/or Clinical Veterinarian. The room(s) in which the animals will be kept will be documented in the study records.

Cage Identification: Cage card indicating at least Test Facility Study No., group, animal number(s).

8.2. Animal Enrichment

Animals will be socially housed for psychological/environmental enrichment and may be provided with items such as devices for hiding in, paper and/or objects for chewing, except when interrupted by study procedures/activities. Results of analysis for contaminants are provided by the supplier and are on file at the Test Facility. It is considered that there are no known contaminants that would interfere with the objectives of the study.

8.3. Environmental Conditions

The targeted conditions for animal room environment will be as follows:

Temperature:	20 to 24°C
Humidity:	40 to 70%
Light Cycle:	12 hours light and 12 hours dark (except during designated procedures)
Ventilation:	Ten or more air changes per hour

8.4. Food

Diet:	SM R/M-Z from SSNIFF® Spezialdiäten GmbH, Soest, Germany
Type:	Pellets (alternate diet may be provided on individual animal basis as warranted as approved by the Study Director).
Frequency:	Ad libitum, except during designated procedures.
Analysis:	Results of analysis for nutritional components and environmental contaminants are provided by the supplier and are on file at the Test Facility. It is considered that there are no known contaminants in the feed that would interfere with the objectives of the study.

8.5. Water

Type:	Municipal tap water.
Frequency/Ration:	Freely available to each animal via water bottles, except during designated procedures.
Analysis:	Periodic analysis of the water is performed, and results of these analyses are on file at the Test Facility. It is considered that there are no known contaminants in the water that could interfere with the outcome of the study.

8.6. Veterinary Care

Veterinary care will be available throughout the course of the study and animals will be examined by the veterinary staff as warranted by clinical signs or other changes. In the event that animals show signs of illness or distress, the responsible veterinarian may make initial recommendations about treatment of the animal(s) and/or alteration of study procedures, which must be approved by the Study Director. Treatment of the animal(s) for minor injuries or ailments may be approved without prior consultation with the Sponsor representative when such treatment does not impact fulfillment of the study objectives. If the condition of the animal(s) warrants significant therapeutic intervention or alterations in study procedures, the Sponsor representative will be contacted, when possible, to discuss appropriate action. If the condition of the animal(s) is such that emergency measures must be taken, the Study Director and/or attending veterinarian will attempt to consult with the Sponsor representative prior to responding to the medical crisis, but the Study Director and/or veterinarian has authority to act immediately at his/her discretion to alleviate suffering. The Sponsor representative will be fully informed of any such events.

9. EXPERIMENTAL DESIGN

The study will be performed following a stepwise exposure scenario. Target concentrations are based on the cut off concentration values specified in the UN and EC classification guidelines for dusts and mists (0.05, 0.5, 1 or 5 mg/L.), increased with 10% in order to avoid the actual mean concentrations to fall below the cut off values due to experimental variation.

Test material data indicate that severe effects may have to be expected at the highest target concentration as indicated above. Therefore, the study will start at 1 mg/L.

If test material related mortality of more than 50% is produced at one step, the Sponsor will be contacted on the further progress of the study and testing of a lower target concentration will be considered.

If test material related mortality of less than 50% is produced at one step, the Sponsor will be contacted on the further progress of the study and testing of a higher target concentration will be considered.

No further exposure levels will be tested as soon as classification and labeling of the test material is possible.

9.1. Administration of Test Material

Dose Route:	Inhalation
Frequency:	Once on Day 1
Target Exposure Duration:	4 hours
Method:	Nose-only directed flow exposure.

9.2. Inhalation Exposure Procedure

9.2.1. Animal Husbandry on the Day of Exposure

The animals will be moved to the inhalation area in order to perform the exposure. During the exposure, there will be no access to food and water. After exposure, the animals will be put back into their cages which will be placed in a fume cupboard for a short time period to allow test material remnants to evaporate. A sheet of filter paper may be used to cover the bedding material to prevent suffocation in case of bad health condition and in order to recover and to aid the clinical observations. The sheet of paper will be removed as soon as considered appropriate. Before the end of the exposure day, the animals will be returned to the animal room. In case of (evaporable) test material remnants, the animals may be kept in the inhalation area for a longer time period. This will be specified by the Study Director in the raw data.

9.2.2. Exposure Chamber

Animals will be exposed to the test material via the nose-only inhalation route. For this purpose the animals will be placed in polycarbonate restraining tubes, which will be connected to the exposure chamber. Animals will be allowed to acclimatize for at least fifteen minutes after the last animal has been placed.

The eyes of the animals will be treated with protective crème (specified in raw data and report) in order to protect them from possible adverse effects for the eyes before the animals are placed in the tubes.

The design of the exposure chamber is based on the directed flow nose-only inhalation chamber (Am. Ind. Hyg Assoc. J. 44(12): 923-928, 1983). The chamber consists of animal sections with eight animal ports each. Each animal port has its own test atmosphere inlet and exhaust outlet. The number of animal sections and number of open inlets will be adapted to the air flow in such a way that at each animal port the theoretical air flow is at least 1 L/min. The main inlet of the test atmosphere is located at the top section and the main outlet is located at the bottom section. The direction of the flow of the test atmosphere guarantees a freshly generated atmosphere for each individual animal. All components of the exposure chamber in contact with the test material are made of stainless steel, glass, rubber or plastic. To avoid exposure of the personnel and contamination of the laboratory the exposure chamber is placed in a fume hood.

9.3. Test Atmosphere Generation and Characterization

9.3.1. Test Atmosphere Generation

An aerosol will be generated by nebulization of the test material formulation. The test material formulation may be sonicated and heated at approximately 40 degrees Celsius. The type of nebulizer to be used depends on the properties of the test material. The primary aerosol may be diluted with pressurized air. Subsequently the aerosol will be passed through the exposure chamber.

From the exposure chamber the test atmosphere will be passed through a filter before it is released to the exhaust of the fume hood.

A detailed description of the precise methods used for exposure of the animals will be included in the raw data and the report of the study.

9.3.2. Test Atmosphere Concentration Sampling

The concentration will be determined at least eight times during each exposure period. Sample intervals will be appropriately spaced over the exposure period, but additional samples might be taken in case delivery rate of test material to the air stream or the air flow is readjusted. Samples will be drawn through glass fiber filters. The filters will be dried in the stove in order to evaporate the vehicle (water). The amount of dry test material collected will be measured gravimetrically. The volume of the air sample will be measured by means of a dry gas meter. The actual concentration in each sample will be calculated from the amount of test material collected and the volume of the sample. A correction factor, established during trial generations, will be applied. The time-weighted mean concentration(s) and standard deviation(s) will be calculated.

9.3.3. Particle Size Distribution Investigations

In principle, the particle size distribution will be characterized twice during each exposure period. Additional samples might be taken if considered necessary. Samples will be drawn from the test atmosphere through a tube mounted in one of the free animal ports of the middle section of the exposure chamber. Samples will be collected in an eight stage personal Marple cascade impactor on glass fiber filters. The amount of test material collected will be measured gravimetrically. The mass median aerodynamic diameter (MMAD) and geometric standard deviation (gsd) will be determined for each sample as follows:

Based on OECD guidance document No 39 graphs of the cumulative mass of test material collected (percentage of total collected) against the cut points of the impactor stages will be drawn on log-normal paper. When drawing the graphs more weight will be given to the cut points where the cumulative mass sampled is within the range of 5 to 95%. The Mass Median Aerodynamic Diameter (MMAD), i.e. the particle size where 50% of the particle mass is borne by particles smaller than the MMAD and the σ84%, (the particle size where 84% of the particle mass is borne by particles smaller than the σ84% will be read from the graph. The geometric standard deviation (gsd) will be calculated as σ84% / MMAD.

In case during method development it is observed that the test material contains a relative large fraction of small particles (less than 0.25 µm, the smallest cut-point of the cascade impactor) the sample flow rate will be increased to obtain a better characterization of the particle size distribution at the smaller size range. The cut points of the cascade impactor will be calculated according to the following formula:

$$D_p = \left(\sqrt{(2 \ / \ flow}\right) \times D_{p,2}$$

D_p = cut point at increased sample flow rate, $D_{p,2}$ = cut point at 2 l/min the standard flow rate.

9.3.4. Pre-exposure Atmosphere Characterization Investigations

The performance characteristics of the test atmosphere generation and exposure system to be used will be assessed prior to the commencement of exposure during trial generations. These investigations will be undertaken to establish:

Aerosol concentration assessment (including appropriate collection media – glass fiber filters will be used as default. However, in cases where this is not appropriate, other collection media may be used. e.g. sorbent tubes)
Temporal variation in chamber concentration
Test material utilization (where appropriate)
Particle size distribution measurements (within the target MMAD range 1-4 µm)

These investigations have a non-GLP status and will be carried out in the quality assured environment of Charles River Den Bosch GLP test facility. These investigations showed that it is technically not possible to genetrate a suitable dust. Therefore, the test material was mixed with water and generated as an aerosol.

9.3.5. Nominal Test Atmosphere Concentration

The nominal concentration will be calculated by dividing the amount of test material used by the volume of pressurized air (average air flow times exposure time) entering the exposure chamber used for exposure of the animals. Due to the small volume of the exposure chamber the equilibrium time is negligible. The volume of air will be calculated from the average air flow (which is measured by means of thermal mass flow meters and will be recorded regularly, preferably in 30 minute intervals) and the exposure time.

9.3.6. Test Atmosphere Stability Monitoring

The stability of the test atmosphere may be monitored by means of a real time aerosol monitoring system. The probe of this system will be mounted on top of the exposure chamber. The data on the opacity will be used to illustrate the stability of the test atmosphere. No data on absolute concentrations will be generated with this system. The results will be expressed graphically. In case the test material deposits in the aerosol monitoring system or the opacity of the test atmosphere exceeds the range which can be measured with this aerosol monitor, the data generated by the system are regarded unreliable and will not be reported. Usually, this will turn out during method development. To avoid damage to the probe of the aerosol monitor, this will then be removed from the exposure chamber. In this case an indication of the stability of the test atmosphere will be obtained from the variation of the individual actual concentration measurements.

9.3.7. Temperature and Relative Humidity of the Test Atmosphere

The temperature and relative humidity will be measured with a humidity and temperature indicator (E+E Elektronik) and will be recorded after the animals are placed in the experimental set up and regularly, preferably in 30 minute intervals after initiation of the generation of the test atmosphere. The probe of the indicator will be inserted in one of the free animal ports of the exposure chamber.

The temperature in the exposure chamber will be maintained at 22 ± 3°C. If possible, the relative humidity will be maintained between 40 and 70%, preferably 50 to 60%. The method of test atmosphere generation, the use of water as vehicle or the aerosolization of high concentrations of dry particulate matter may preclude this condition. The minimum and maximum values during exposure will be reported.

10. IN-LIFE PROCEDURES, OBSERVATIONS, AND MEASUREMENTS

The in-life procedures, observations, and measurements listed below will be performed for all animals.

In-life Assessments

Parameter	Frequency (minimum required)	Comments
Mortality/ Moribundity	At least twice daily during the observation period	Animals will be observed for general health/mortality and moribundity. Animals will be observed within their cage unless necessary for identification or confirmation of possible findings.
Clinical Observations (during exposure)	At least three times	Animals will be observed within the restraining devices unless necessary for identification or confirmation of possible findings. Animals are checked for mortality, behavioral signs of distress and effects on respiration.
Clinical Observations (post exposure)	Twice on the day of exposure (preferably 1 and 3 hours after termination of exposure) and at least once daily thereafter.	Animals will be observed within their cage unless necessary for identification or confirmation of possible findings.
Individual Body Weights	On Days 1 (pre exposure), 2, 4, 8 and 15.	Terminal body weights will also be collected from animals if found dead or euthanized moribund after Day 1.

11. TERMINAL PROCEDURES

11.1. Unscheduled Euthanasia

Animals to be euthanized for humane reasons before the scheduled time will be sacrificed by an intra-peritoneal injection of pentobarbital and subjected to necropsy. Descriptions of all internal macroscopic abnormalities will be recorded. Body weight will not be recorded after release for euthanasia.

11.2. Scheduled Euthanasia

Animals surviving until scheduled euthanasia will be sacrificed by an intra-peritoneal injection of pentobarbital and subjected to necropsy. Descriptions of all internal macroscopic abnormalities will be recorded.

12. ANALYSIS

The LC_{50} value of the test material will be ranked within the following ranges:
>0 - ≤0.05, >0.05 - ≤0.5, >0.5 - ≤1, >1 - ≤5 or as exceeding 5 mg/L or the maximum achievable concentration. No statistical analysis will be performed (the method used is not intended to allow the calculation of a precise LC_{50} value).

The results can be evaluated according to the Globally Harmonized System of Classification and Labelling of Chemicals (GHS) of the United Nations (including all revisions) and the Regulation (EC) No 1272/2008 of the European Parliament and of the Council of 16 December 2008 on classification, labelling and packaging of materials and mixtures.

13. COMPUTERIZED SYSTEMS

The following computerized systems may be used in the study. The actual computerized systems used will be specified in the Final Report.

Computerized Systems

System Name	Description of Data Collected and/or Analyzed
M-Files®	Reporting and collection of 21 CFR Part 11 compliant signature
Provantis	In-life and postmortem (mortality; clinical observations; body weights; macroscopic findings) data collection
REES Centron	Temperature and humidity (animal and laboratory facilities) data collection

14. REGULATORY COMPLIANCE

The study will be performed in accordance with the OECD Principles of Good Laboratory Practice as accepted by Regulatory Authorities throughout the European Union, United States of America (FDA and EPA), Japan (MHLW, MAFF and METI), and other countries that are signatories to the OECD Mutual Acceptance of Data Agreement.

15. QUALITY ASSURANCE

15.1. Test Facility

The Test Facility Quality Assurance Unit (QAU) will monitor the study to assure the facilities, equipment, personnel, methods, practices, records, and controls are in conformance with Good Laboratory Practice regulations. The QAU will review the Study Plan, conduct inspections at intervals adequate to assure the integrity of the study, and audit the Final Report to assure that it accurately describes the methods and standard operating procedures and that the reported results accurately reflect the raw data of the study.

16. AMENDMENTS AND DEVIATIONS

Changes to the approved Study Plan shall be made in the form of an amendment, which will be signed and dated by the Study Director. Every reasonable effort will be made to discuss any necessary Study Plan changes in advance with the Sponsor. The Study Director will notify the Sponsor of deviations that may result in a significant impact on the study as soon as possible.

17. RETENTION AND DISPOSITION OF RECORDS, SAMPLES, AND SPECIMENS

All applicable study-specific raw data, electronic data, documentation, Study Plan, retained samples and specimens, and Final Reports will be archived at finalization of the report. All materials generated by Charles River from this study will be transferred to a Charles River archive. At least 2 years after issue of the Final Report, the Sponsor will be contacted.

Records to be maintained will include, but will not be limited to, documentation and data for the following:

- Study Plan, Study Plan amendments, and deviations
- Study schedule
- Study-related correspondence
- Test system receipt, health, and husbandry
- Test material receipt, identification and preparation
- In-life measurements and observations

18. REPORTING

A comprehensive Draft Report will be prepared following completion of the study and will be finalized following consultation with the Sponsor. The report will include all information necessary to provide a complete and accurate description of the experimental methods and results and any circumstances that may have affected the quality or integrity of the study.

The Sponsor will receive an electronic version of the Draft Report. The Final Report will be provided in Adobe Acrobat PDF format (hyperlinked and searchable). The PDF document will be created from native electronic files to the extent possible, including text and tables generated by the Test Facility. Report components not available in native electronic files and/or original signature pages will be scanned and converted to PDF image files for incorporation.

Reports should be finalized within 6 months of issue of the Draft Report. If the Sponsor has not provided comments to the report within 6 months of draft issue, the report will be finalized by the Test Facility unless other arrangements are made by the Sponsor.

19. JUSTIFICATIONS AND GUIDELINES

19.1. Justification of Test System and Number of Animals

At this time, studies in laboratory animals provide the best available basis for extrapolation to humans and are required to support regulatory submissions. Acceptable models that do not use live animals currently do not exist.

The Wistar Han rat was chosen as the animal model for this study as it is a rodent species accepted by regulatory agencies for toxicity testing. The total number of animals to be used in this study is considered to be the minimum required to properly characterize the effects of the test material. This study has been designed such that it does not require an unnecessary number of animals to accomplish its objectives.

19.2. Justification of Route and Dose Levels

The inhalation route of administration has been selected because this route has been defined as a possible route of human exposure. Nose-only exposure will be used since this is the most applicable method of exposure for the test model. This exposure technique is used to minimize concurrent exposure by the oral and dermal routes. Exposure levels will be selected based on the EC and UN classification guidelines. The starting exposure level was selected based on the available test material data and is one expected not to cause mortality.

The animals will be exposed to the test concentration selected over the exposure period while also maintaining an acceptable particle size distribution: target mass median aerodynamic diameter (MMAD) in the range of 1-4 µm with a range of 1.5-3 for the geometric standard deviation (gsd).

19.3. Guidelines for Study

The design of this study was based on the study objective(s), the overall product development strategy for the test material, and the following study design guidelines:

- OECD Guideline 403. *Acute Inhalation Toxicity,* September 2009.
- EPA Health Effects Test Guideline OPPTS 870.1300. *Acute Inhalation Toxicity*, August 1998.
- EC No 440/2008, part B. *Acute Toxicity (inhalation),* May 2008, amended by COMMISSION REGULATION (EU) No 260/2014 (24 January 2014)
- Appendix to Director General Notification, No. 12-Nousan-8147. Agricultural Production Bureau, Ministry of Agriculture, Forestry and Fisheries of Japan (JMAFF), November 2000, including the most recent revisions.

20. ANIMAL WELFARE

This Study Plan was reviewed and agreed by the Animal Welfare Body of Charles River Laboratories Den Bosch B.V. within the framework of Appendix 1 of project license AVD23600202216274 approved by the Central Authority for Scientific Procedures on Animals (CCD) as required by the Dutch Act on Animal Experimentation (December 2014).

Animals showing pain, distress or discomfort, which is considered not transient in nature or is likely to become more severe, will be sacrificed for humane reasons based on OECD guidance document on the recognition, assessment, and use of clinical signs as humane endpoints for experimental animals used in safety evaluation (ENV/JM/MONO/2000/7).

By approving this Study Plan, the Sponsor affirms that this study is required by a relevant government regulatory agency and that it does not unnecessarily duplicate any previous experiments.

ATTACHMENT A

Distribution List

Electronic copies will be supplied unless otherwise specified below.

Version	Recipient	
Original	Study Director	
1 Copy	Sponsor Representative / Study Monitor	
1 Copy	QAU / Management	Qaumailboxher;
1 Copy	Formulations	Tsfher;
1 Copy	Coordinating Biotechnician	Raaso, Halfdan; HER-DL-DIE-DB-rodents-C@crl.com;
1 Copy	Necropsy	Her/necropsy;
1 Copy	Provantis Support	her-db-provantissupport@crl.com

SPONSOR APPROVAL

The Study Plan was approved by the Sponsor by e-mail on the date designated below. The correspondence giving approval will be archived, as appropriate with other Sponsor communications.

25 Apr 2023

Date of Sponsor Approval

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TFM Approval-GLP:	I approve the Study Director identified in this document and management's responsibility to the study as defined by the relevant GLP.
Name:	**Lourens, Nicky**
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	26-Apr-2023 14:50:46 (UTC+00:00)
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Study Director Approval:	I approve this document.
Name:	**van de Wiel, Sandra**
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QA Approval:	I approve the Quality Assurance Statement for this report.
Name:	**de Kok, Pita**
	de Kok, Pita
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Study Director Approval:	I approve this Report.
Name:	**van de Wiel, Sandra**
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To explore the potential of nature to improve the quality of life

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