Section A6.1.3 Acute Toxicity

Annex Point IIA6.1 **Acute Inhalation Toxicity Study in Rats – Defined LC_{50}**

Official use only

1 REFERENCE

1.1	**Reference**	[redacted] Acute Inhalation Toxicity Study in Rats – Defined LC_{50}, [redacted], Report No. 31452, 17 October 2012
1.2	**Data protection**	Yes
1.2.1	Data owner	Clariant Corporation
1.2.2		
1.2.3	Criteria for data protection	Data submitted to the MS after 13 May 2000 on existing a.s. for the purpose of its [entry into Annex I/IA / authorisation]

2 GUIDELINES AND QUALITY ASSURANCE

2.1	**Guideline study**	Yes U.S. EPA Health Effects Test Guidelines, OPPTS 870.1300
2.2	**GLP**	Yes
2.3	**Deviations**	No

3 MATERIALS AND METHODS

3.1	**Test material**	As given in section 2:- [redacted] BIT
3.1.1	Lot/Batch number	[redacted]
3.1.2	Specification	As given in section 2
3.1.2.1	Description	White powder
3.1.2.2	Purity	1,2 benzisothiazolin-3-one:- 84-85% Water:- ~ 15%
3.1.2.3	Stability	Test substance was expected to be stable for the duration of testing. Expiration Date: September 30, 2012
3.2	**Test Animals**	Non-entry field
3.2.1	Species	Rat
3.2.2	Strain	Sprague-Dawley derived, albino
3.2.3	Source	[redacted]
3.2.4	Sex	Male and Female
3.2.5	Age/weight at study initiation	Young adult (8-12 weeks)/males 232-403 grams and females 170-280 grams at experimental start.
3.2.6	Number of animals per group	5 males and 5 females were included in each of the 3 dose groups. A total of 15 Males and 15 Females were used in the testing.
3.2.7	Control animals	No
3.3	**Administration/**	Inhalation

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Exposure

3.3.1 Postexposure period: 14 days

Oral

3.3.2 Type: Not applicable

3.3.3 Concentration

3.3.4 Vehicle

3.3.5 Concentration in vehicle

3.3.6 Total volume applied

3.3.7 Controls

Inhalation

3.3.8 Concentrations

Gravimetric Chamber Concentrations

Exposure Level		Time of Sample (hour)	Total Test Substance Collected (mg)	Air Flow(liters)	Collection Time (min)	Chamber Conc. (mg/L)
Target (mg/L)	Actual (mg/L)					
0.05	0.054	0.5	1.8	4	7	0.064
		1	1.2	4	7	0.043
		2	1.6	4	7	0.057
		2.5	1.6	4	7	0.057
		3.5	1.5	4	7	0.054
		3.75	1.4	4	7	0.050
Average ± Standard Deviation						0.054 ± 0.007
0.5	0.55	0.5	4.7	4	2	0.59
		1	4.2	4	2	0.53
		2	4.3	4	2	0.54
		2.5	4.3	4	2	0.54
		3.5	4.4	4	2	0.55
		3.75	4.2	4	2	0.53
Average ± Standard Deviation						0.55 ± 0.02
2.0	2.21	0.5	18.7	4	2	2.33
		1	17.1	4	2	2.14
		2	20.1	4	2	2.51
		2.5	16.1	4	2	2.01
		3.5	16.9	4	2	2.11
		3.75	17.4	4	2	2.18
Average ± Standard Deviation						2.21 ± 0.18

Section A6.1.3 Acute Toxicity

Annex Point IIA6.1 **Acute Inhalation Toxicity Study in Rats – Defined LC_{50}**

Nominal Chamber Concentration

Exposure Level (mg/L)	Total Test Substance Used (g)	Average Total Airflow (Lpm)	Total Time of Exposure (min)	Nominal Concentration (mg/L)
0.054	4.4	31.6	241	0.578
0.55	28.2	31.6	241	3.7
2.21	48.8	31.7	241	6.39

3.3.9 Particle size

Particle Size Distribution 0.054 mg/L

Stage	Effective Cutoff Diameter (µm)	% of Total Particles Captured (by weight)	Cumulative (%)
Sample 1			
0	9.0	7.6	92.4
1	5.8	14.7	77.7
2	4.7	11.4	66.3
3	3.3	20.1	46.2
4	2.1	19.0	27.2
5	1.1	19.0	8.2
6	0.7	2.2	6.0
7	0.4	3.3	2.7
F	0.2	2.7	0.0
Sample 2			
0	9.0	5.4	94.6
1	5.8	18.3	76.3
2	4.7	18.3	58.1
3	3.3	21.5	36.6
4	2.1	17.2	19.4
5	1.1	12.9	6.5
6	0.7	1.1	5.4
7	0.4	2.2	3.2
F	0.2	3.2	0.0

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Annex Point IIA6.1 **Acute Inhalation Toxicity Study in Rats – Defined LC_{50}**

Particle Size Distribution 0.55 mg/L

Stage	Effective Cutoff Diameter (µm)	% of Total Particles Captured (by weight)	Cumulative (%)
Sample 1			
0	9.0	9.7	90.3
1	5.8	16.2	74.0
2	4.7	13.0	61.0
3	3.3	24.4	36.7
4	2.1	16.9	19.8
5	1.1	16.9	2.9
6	0.7	1.6	1.3
7	0.4	0.6	0.6
F	0.2	0.6	0.0
Sample 2			
0	9.0	6.7	93.3
1	5.8	11.3	82.0
2	4.7	14.7	67.3
3	3.3	25.7	41.6
4	2.1	18.3	23.2
5	1.1	20.8	2.4
6	0.7	0.9	1.5
7	0.4	0.6	0.9
F	0.2	0.9	0.0

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Annex Point IIA6.1 **Acute Inhalation Toxicity Study in Rats – Defined LC_{50}**

Particle Size Distribution 2.21 mg/L

Stage	Effective Cutoff Diameter (µm)	% of Total Particles Captured (by weight)	Cumulative (%)
Sample 1			
0	9.0	7.4	92.6
1	5.8	9.9	82.7
2	4.7	14.8	67.9
3	3.3	23.8	44.1
4	2.1	23.5	20.7
5	1.1	16.4	4.3
6	0.7	2.8	1.5
7	0.4	0.3	1.2
F	0.2	1.2	0.0
Sample 2			
0	9.0	9.0	91.0
1	5.8	11.3	79.7
2	4.7	19.5	60.2
3	3.3	21.3	38.8
4	2.1	18.8	20.1
5	1.1	16.7	3.3
6	0.7	2.3	1.0
7	0.4	1.0	0.0
F	0.2	0.0	0.0

3.3.10	Type or preparation of particles	The test substance was ground in a 6 litre (1.6-gallon) urethane-lined milling jar (Abbethane, Paul O'Abbe) with porcelain grinding media (1.27 cm balls) for 24 hours. After milling, the substance was sieved through a ~1 cm (⅜") polyethylene sieve to separate it from the grinding media and any other large particles that remained. The ground test substance was aerosolized using a modified Wright Dust Generator driven by a variable speed motor (Dayton, Model #4Z538A) D.C. speed control with 0-100 potentiometer. The test substance was packed into the dust container (Wright, Model DF183A SS or DF183) and compressed to 0.205 (2.0 mg/L), 0.088 (0.05 mg/L) or 0.219 (0.5 mg/L) kg/m^2 using a lab press (Carver, Model C). The container was then fitted with a stainless steel cutting head (Model DF DF193 SS or 194SS) and cutting blade (Model DF190 SS or DF191SS). Compressed air was supplied to the dust generator at 30 psi. The aerosolized dust was then fed directly into the chamber through the dust outlet assembly.
3.3.11	Type of exposure	nose only
3.3.12	Vehicle	None
3.3.13	Concentration in vehicle	Not applicable
3.3.14	Duration of exposure	4 h

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3.3.15	Controls	Not applicable
		Dermal
3.3.16	Area covered	Not applicable
3.3.17	Occlusion	
3.3.18	Vehicle	
3.3.19	Concentration in vehicle	
3.3.20	Total volume applied	
3.3.21	Duration of exposure	
3.3.22	Removal of test substance	
3.3.23	Controls	
3.3.24	Vehicle	
3.3.25	Concentration in vehicle	
3.3.26	Total volume applied	
3.3.27	Controls	
3.4	**Examinations**	All animals were observed for mortality during the exposure period. The surviving animals were examined for signs of gross toxicity, and behavioral changes upon removal from the exposure tube and at least once daily thereafter for 14 days or until death occurred. Observations included gross evaluation of skin and fur, eyes and mucous membranes, respiratory, circulatory, autonomic and central nervous systems, somatomotor activity and behavior pattern. Particular attention was directed to observation of tremors, convulsions, salivation, diarrhea, and coma. Body weights were recorded prior to exposure and again on Days 1, 3, 7 and 14 (termination) or after death.
3.5	**Method of determination of LD_{50}**	Finney Probit analysis
3.6	**Further remarks**	None

Section A6.1.3 Acute Toxicity

Annex Point IIA6.1 **Acute Inhalation Toxicity Study in Rats – Defined LC_{50}**

4 RESULTS AND DISCUSSION

4.1 Clinical signs

Summary of Mortality Data

Exposure Level (mg/L)	Mortality		
	Males	Females	Total
0.054	0/5	0/5	0/10
0.55	3/5	3/5	6/10
2.21	5/5	4/5	9/10

Individual Body Weights (0.054 mg/L)

Animal No	Sex	Body Weight (g)				
		Initial	Day 1	Day 3	Day 7	Day 14
3311	M	352	326	340	350	367
3312	M	371	356	370	375	403
3313	M	383	365	379	392	409
3314	M	365	349	361	368	394
3315	M	403	390	402	414	449
3316	F	280	268	284	284	291
3317	F	260	261	269	273	282
3318	F	263	260	256	261	277
3319	F	258	249	264	269	258
3320	F	246	237	252	263	255

Individual Body Weights and Mortalities (0.55 mg/L)

Animal No.	Sex	Body Weight (g)					Mortality	
		Initial	Day 1	Day 3	Day 7	Day 14	Day	Weight (g)
3321	M	243	215	196	230	265	E	-
3322	M	243	-	-	-	-	0	236
3323	M	232	-	-	-	-	1	219
3324	M	239	210	188	214	260	E	-
3325	M	246	-	-	-	-	0	240
3326	F	172	158	-	-	-	2	156
3327	F	187	168	158	183	206	E	-
3328	F	176	159	137	120	-	9	108
3329	F	187	180	180	192	209	E	-
3330	F	170	149	-	-	-	2	141

E = euthanized via CO_2 inhalation on Day 14

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Individual Body Weights and Mortalities (2.21 mg/L)

Animal No.	Sex	Body Weight (g)					Mortality	
		Initial	Day 1	Day 3	Day 7	Day 14	Day	Weight (g)
3301	M	282	-	-	-	-	0	274
3302	M	300	-	-	-	-	0	289
3303	M	271	-	-	-	-	0	265
3304	M	257	-	-	-	-	0	249
3305	M	281	-	-	-	-	0	271
3306	F	206	-	-	-	-	0	202
3307	F	211	201	204	215	239	E	-
3308	F	208	-	-	-	-	0	202
3309	F	214	-	-	-	-	0	210
3310	F	216	-	-	-	-	0	204

E = euthanized via CO_2 inhalation on Day 14

Individual cage-side observations found the animals to be active and healthy in the 0.054 mg/l Group.

Individual Cage-Side Observations (0.55 mg/L)

Animal Number	Findings	Day of Occurrence
MALES		
3321	Gasping	CR[1]-1
	Hypoactivty	CR-2
	Rales (moist)	CR-3
	Irregular respiration	CR-5
	Facial staining (red), ocular discharge (red)	1
	Active and healthy	6-14
3322, 3325	Dead	CR
3323	Irregular respiration, rales (moist), gasping, hypoactivity	CR-0 (1 hr)
	Dead	1
3324	Gasping	CR-1
	Rales (moist), hypoactivity	CR-5
	Irregular respiration	CR-9
	Nasal discharge (red)	1-2
	Ocular discharge (red)	1-3
	Opacity in both eyes[2]	3-14

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Animal Number	Findings	Day of Occurrence
FEMALES		
3326	Rales (moist), gasping	CR[1]-0 (1 hr)
	Irregular respiration, hypoactivity	CR-1
	Ocular and nasal discharge (red)	1
	Dead	2
3327	Gasping	CR-1
	Rales (moist), hypoactivity	CR-2
	Irregular respiration	CR-5
	Nasal discharge (red)	1
	Ocular discharge (red to black)	1-3
	Opacity in left eye[2]	3-14
3328	Rales (moist)	CR-0 (1 hr)
	Gasping	CR-1, 5-8
	Hypoactivity	CR-5
	Irregular respiration	CR-8
	Nasal discharge (clear)	1
	Ocular discharge (black)	1-8
	Ano-genital staining	4-8
	Dead	9
3329	Gasping, hypoactivity	CR-0 (1 hr)
	Rales (moist)	CR-2
	Irregular respiration	CR-8
	Reduced fecal volume	9-10
	Active and healthy	11-14
3330	Rales (moist)	CR-0 (1 hr)
	Irregular respiration, gasping, hypoactivity	CR-1
	Ocular and nasal discharge (red), ano-genital staining	1
	Dead	2

Individual Cage-Side Observations (2.21 mg/L)

Animal Number	Findings	Day of Occurrence
MALES		
3301 - 3305	Dead	CR[1]
FEMALES		
3306, 3308, 3309, 3310	Dead	CR
3307	Rales (moist), hypoactivity	CR
	Irregular respiration	CR-3
	Active and healthy	4-14

4.2 Pathology No gross abnormalities were found in any of the tissues or organs in the 0.054 mg/L group.

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Individual Necropsy Observations (0.55 mg/L)

Animal Number	Tissue	Findings
MALES		
3321, 3324	All tissues and organs	No gross abnormalities
3322	Liver Intestines	Slightly darkened Slightly distended
3323	Liver Stomach Intestines Kidneys	Slightly darkened Moderately distended Moderately distended Slightly darkened
3325	Liver Intestines	Moderately darkened Slightly distended
FEMALES		
3326	Lungs Liver Intestines	Extremely red Extremely dark in color Slightly yellow, slightly distended
3327, 3329	All tissues and organs	No gross abnormalities
3328	Lungs Intestines	Moderately red Extremely distended
3330	Lungs Liver Intestines	Extremely red Moderately dark in color Slightly yellow, slightly distended

Individual Necropsy Observations (2.21mg/L)

Animal Number	Tissue	Findings
MALES		
3301	Lungs Stomach	Moderately red Distended
3302, 3303	Lungs Stomach	Slightly red Distended
3304	Lungs Stomach	Partially discolored Distended
3305	Lungs Stomach	Discolored Distended
FEMALES		
3306	Stomach	Distended
3307	All tissues and organs	No gross abnormalities
3308	Lungs Stomach	Slightly red Distended
3309	Lungs	Slightly red
3310	Lungs	Discolored

4.3 Other None

4.4 LD_{50} 0.50 mg/L in male rats with 95% confidence intervals of 0.25 mg/L (lower) to 1.00 mg/L (upper)

0.57 mg/L in female rats with 95% confidence intervals of 0.05 mg/L

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(lower) and 2.94 mg/L (upper).

Combined sexes is 0.50 mg/L with confidence intervals of 0.18 mg/L (lower) and 0.98 mg/L (upper).

5 APPLICANT'S SUMMARY AND CONCLUSION

5.1 Materials and methods

Pre-test trials were conducted prior to initiation of the full inhalation study and established that the test substance, [redacted] BIT, should be prepared by being ground in a ball mill for 24 hours and sieved through a 10 mm polyethylene sieve prior to being aerosolized.

A nose-only inhalation chamber with an internal volume of approximately 6 L was used for the exposure test. Animals were individually housed in polycarbonate holding tubes which seal to the chamber with an "O" ring during exposure. The base unit terminated the chamber with a 1.27 cm diameter tube for discharged air. Filtered air was supplied by an air compressor to the dust generator and additional compressed mixing air was introduced into the chamber to help uniformly distribute the test atmosphere by creating a vortex at the chamber inlet. The chamber airflow was monitored throughout the exposure period.

The temperature and relative humidity within the exposure tube as well as the room were monitored every 15 minutes for the first hour of exposure and every 30 minutes thereafter.

The ground test substance was aerosolized using a modified Wright Dust Generator driven by a variable speed motor. The test substance was packed into the dust container and compressed to 0.205 (2.0 mg/L), 0.088 (0.05 mg/L) or 0.219 (0.5 mg/L) kg/m2 using a lab press. The container was then fitted with a stainless steel cutting head and cutting blade. Compressed air was supplied to the dust generator at 30 psi. The aerosolized dust was then fed directly into the chamber through the dust outlet assembly.

Gravimetric samples were withdrawn at six intervals from the breathing zone of the animals during each exposure. Samples were collected using 37 mm glass fibre filters in a filter holder attached by 6.35 mm tygon tubing to a vacuum. Filter papers were weighed before and after collection to determine the mass collected. This value was divided by the total volume of air sampled to determine the chamber concentration. Sample airflows were measured using a Mass Flowmeter.

An eight-stage ACFM Andersen or Westech Ambient Particle Sizing Sampler was used to assess the particle size distribution of the test atmosphere. Samples were withdrawn from the breathing zone of the animals at two intervals for each exposure level.

For each exposure level, ten rats (five male and five female not previously tested) were selected for each exposure group. The animals were exposed to the targeted chamber concentration for at least 4 hours. At each level, the exposure period was extended beyond 4 hours to allow the chamber to reach equilibrium (T99). At the end of each exposure period, the generation of aerosolized test substance was terminated and the chamber was operated for a further 15-17 minutes with clean air. Following this period the animals were removed from the exposure tube and any excess test substance removed from their fur prior to being returned to their cages.

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Individual body weights of the animals were recorded prior to test substance exposure (initial) and again on Days 1, 3, 7 and 14 (termination) or after death.

All animals were observed for mortality during the exposure period. The surviving animals were examined for signs of gross toxicity, and behavioural changes upon removal from the exposure tube and at least once daily thereafter for 14 days or until death occurred. Observations included gross evaluation of skin and fur, eyes and mucous membranes, respiratory, circulatory, autonomic and central nervous systems, somatomotor activity and behaviour pattern. Particular attention was directed to observation of tremors, convulsions, salivation, diarrhoea, and coma.

Surviving rats were euthanized via CO2 inhalation on Day 14. Gross necropsies were performed on all decedents and euthanized animals. Tissues and organs of the thoracic and abdominal cavities were examined.

Probit Analysis (Finney, D.J., Probit Analysis, 3rd ed., Cambridge University Press, Cambridge, Great Britain, 1971, pp.1-333) was used to determine the LC50 and confidence limits.

5.2 Results and discussion

<u>0.054 mg/L</u>

All animals survived exposure to the test atmosphere at the 0.054 mg/L test concentration. The gravimetric and nominal chamber concentrations were 0.054 and 0.578 mg/L respectively. The mass median aerodynamic diameter was calculated to be 3.2 µm based on graphic analysis of the particle size distribution as measured with an ACFM Andersen Ambient Particle Sizing Sampler.

Immediately following exposure to the test atmosphere and throughout the 14-day observation period, all animals appeared active and healthy. There were no signs of gross toxicity, adverse pharmacologic effects, or abnormal behaviour. Although all five males and four females lost body weight by Day 1 and one animal on Day 3, all animals showed a weight gain thereafter through Day 14. No gross abnormalities were noted for any of the animals when necropsied at the conclusion of the 14-day observation period.

<u>0.55 mg/L</u>

Six animals died following exposure to the test atmosphere at the 0.55 mg/L test concentration. The gravimetric and nominal chamber concentrations were 0.55 and 3.7 mg/L respectively. The mass median aerodynamic diameter was calculated to be 3.6 µm based on graphic analysis of the particle size distribution as measured with an ACFM Andersen Ambient Particle Sizing Sampler.

Two males were found dead upon removal from the exposure tube. Following exposure, one male and three females were found dead by Day 9. The surviving rats exhibited ocular and/or nasal discharge, facial staining, abnormal respiration, hypoactivity, ano-genital staining and/or reduced faecal volume. On Day 3, opacity was evident in both eyes of one surviving male and in the left eye of one surviving female. It was noted on Day 13 that the affected eyes of both of these rats also appeared to bulge and have an irregular shape. Apart from the visual opacity and irregularity persisting in the eyes of the above rats, all surviving animals recovered from all other clinical signs by Day 11.

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		Although all surviving rats lost body weight through Day 3 or 7, all survivors showed a continued weight gain thereafter through Day 14. Gross necropsy of the decedents revealed discoloration of the lungs and/or intestines, a darkened liver and/or kidney, and/or distension of the stomach and/or intestines. No gross abnormalities were noted for any of the euthanized animals necropsied at the conclusion of the 14-day observation period. 2.21 mg/L Nine animals died following exposure to the test atmosphere at the 2.21 mg/L test concentration. The gravimetric and nominal chamber concentrations were 2.21 and 6.39 mg/L, respectively. The mass median aerodynamic diameter was calculated to be 3.5 µm based on graphic analysis of the particle size distribution as measured with an ACFM Westech Ambient Particle Sizing Sampler. All five males and four females were found dead upon removal from the exposure tubes. Following exposure to the test atmosphere, the surviving female exhibited abnormal respiration and appeared hypoactive, but recovered from these symptoms by Day 4 and appeared active and healthy for the remainder of the observation period. Although this surviving female lost body weight by Day 1, it showed continued weight gain thereafter through Day 14. Gross necropsy of the decedents revealed discoloration of the lungs and/or distention of the stomach. No gross abnormalities were noted for the euthanized animal necropsied at the conclusion of the 14-day observation period.
5.3	**Conclusion**	Non-entry field
5.3.1	Reliability	1
5.3.2	Deficiencies	No

Evaluation by Competent Authorities	
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	Tuesday, 08 September 2015
Materials and Methods	Applicant version is adopted
Results and discussion	Applicant version is adopted
Conclusion	Applicant version is adopted
Reliability	1
Acceptability	Acceptable
Remarks	
	COMMENTS FROM ...
Date	*Give date of comments submitted*
Materials and Methods	*Discuss additional relevant discrepancies referring to the (sub)heading numbers*

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Annex Point IIA6.1 **Acute Inhalation Toxicity Study in Rats – Defined LC_{50}**

	and to applicant's summary and conclusion. *Discuss if deviating from view of rapporteur member state*
Results and discussion	*Discuss if deviating from view of rapporteur member state*
Conclusion	*Discuss if deviating from view of rapporteur member state*
Reliability	*Discuss if deviating from view of rapporteur member state*
Acceptability	*Discuss if deviating from view of rapporteur member state*
Remarks	

Table A6_1_3-1 Table for Acute Toxicity

Dose [mg/L]	*Number of dead / number of investigated*	*Time of death (range)*	*Observations*
0	Not applicable	-	-
0.054	0/10 (0/5 ♂; 0/5 ♀)	Not applicable	-
0.55	6/10 (3/5 ♂; 3/5 ♀)	0 – 9 days	Observations included:- gasping, hypo-activity, rales (moist), irregular respiration, facial staining (red), ocular and nasal discharge (red or clear), opacity in one or both eyes, ocular discharge (red to black), ano-genital staining, reduced faecal volume. Gross necropsy of the decedents revealed discoloration of the lungs and/or intestines, a darkened liver and/or kidney, and/or distension of the stomach and/or intestines. No gross abnormalities were noted for any of the euthanized animals necropsied at the conclusion of the 14-day observation period.
2.21	9/10 (5/5 ♂; 4/5 ♀)	0 days	Observations included:- rales (moist), hypo-activity, irregular respiration. Gross necropsy of the decedents revealed discoloration of the lungs and/or distention of the stomach. No gross abnormalities were noted for the euthanized animal necropsied at the conclusion of the 14-day observation period.
LD_{50} value	0.50 mg/L in male rats with 95% confidence intervals of 0.25 mg/L (lower) to 1.00 mg/L (upper) 0.57 mg/L in female rats with 95% confidence intervals of 0.05 mg/L (lower) and 2.94 mg/L (upper). Combined sexes is 0.50 mg/L with confidence intervals of 0.18 mg/L (lower) and 0.98 mg/L (upper).		