Committee for Risk Assessment

RAC

Annex 1

Background document

to the Opinion proposing harmonised classification and labelling at EU level of

ozone

EC Number: 233-069-2
CAS Number: 10028-15-6

CLH-O-0000007279-64-01/F

The background document is a compilation of information considered relevant by the dossier submitter or by RAC for the proposed classification. It includes the proposal of the dossier submitter and the conclusion of RAC. It is based on the official CLH report submitted to consultation. RAC has not changed the text of this CLH report but inserted text which is specifically marked as 'RAC evaluation'. Only the RAC text reflects the view of RAC.

Adopted

16 March 2023

CLH report

Proposal for Harmonised Classification and Labelling

Based on Regulation (EC) No 1272/2008 (CLP Regulation),
Annex VI, Part 2

International Chemical Identification:

Ozone

EC Number: **233-069-2**

CAS Number: **10028-15-6**

Index Number: **n.a.**

Contact details for dossier submitter:

Federal Institute for Occupational Safety and Health (BAuA)
Federal Office for Chemicals
Friedrich-Henkel-Weg 1-25
D-44149 Dortmund, Germany
ChemG@baua.bund.de

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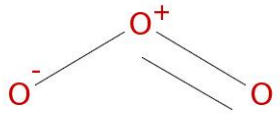
CONTENTS

1 IDENTITY OF THE SUBSTANCE

1.1 Name and other identifiers of the substance

Table 1: Substance identity and information related to molecular and structural formula of the substance

Name(s) in the IUPAC nomenclature or other international chemical name(s)	Trioxygen
Other names (usual name, trade name, abbreviation)	Ozone
ISO common name (if available and appropriate)	-
EC number (if available and appropriate)	233-069-2
EC name (if available and appropriate)	Ozone
CAS number (if available)	10028-15-6
Other identity code (if available)	
Molecular formula	O_3
Structural formula	
SMILES notation (if available)	[O-][O+]=O
Molecular weight or molecular weight range	47.9982 g/mol
Degree of purity (%) (if relevant for the entry in Annex VI)	100

1.2 Composition of the substance

Table 2: Constituents (non-confidential information)

Constituent (Name and numerical identifier)	Concentration range (% w/w minimum and maximum in multi-constituent substances)	Current CLH in Annex VI Table 3.1 (CLP)	Current self- classification and labelling (CLP)
Ozone CAS: 10028-15-6	100	-	Ox, Gas 1; H270 Skin Corr. 1B; H314 Eye Dam. 1; H318 Acute Tox. 1; H330 STOT RE 1; H372 Aquatic Acute 1; H400 Aquatic Chronic 1; H410

Table 3: Impurities (non-confidential information) if relevant for the classification of the substance

Impurity (Name and numerical identifier)	Concentration range (% w/w minimum and maximum)	Current CLH in Annex VI Table 3.1 (CLP)	Current self-classification and labelling (CLP)	The impurity contributes to the classification and labelling
-				

Table 4: Additives (non-confidential information) if relevant for the classification of the substance

Additive (Name and numerical identifier)	Function	Concentration range (% w/w minimum and maximum)	Current CLH in Annex VI Table 3.1 (CLP)	Current self-classification and labelling (CLP)	The additive contributes to the classification and labelling
-					

Table 5: Test substances (non-confidential information) (this table is optional)

Identification of test substance	Purity	Impurities and additives (identity, %, classification if available)	Other information	The study(ies) in which the test substance is used
-				

2 PROPOSED HARMONISED CLASSIFICATION AND LABELLING

2.1 Proposed harmonised classification and labelling according to the CLP criteria

Table 6: Proposed harmonised classification and labelling according to the CLP criteria

	Index No	International Chemical Identification	EC No	CAS No	Classification		Labelling			Specific Conc. Limits, M-factors	Notes
					Hazard Class and Category Code(s)	**Hazard statement Code(s)**	**Pictogram, Signal Word Code(s)**	**Hazard statement Code(s)**	**Suppl. Hazard statement Code(s)**		
Current Annex VI entry	No current Annex VI entry										
Dossier submitters proposal	TBD	Ozone	233-069-2	10028-15-6	Ox. Gas 1 Acute Tox. 1 Muta. 2 Carc. 2 STOT SE1 STOT SE3 STOT RE1 Aquatic Acute 1 Aquatic Chronic 1	H270 H330 H341 H351 H370 (nervous system) H335 H372 (cardiovascular, nervous, respiratory system) H400 H410	GHS03 GHS06 GHS08 GHS09 Dgr	H270 H330 H341 H351 H370 (nervous system) H335 H372 (cardiovascular, nervous, respiratory system H410		Inhalation ATE 10 ppm (gases) M = 100 M = 1	
Resulting Annex VI entry if agreed by RAC and COM					Ox. Gas 1 Acute Tox. 1 Muta. 2 Carc. 2 STOT SE1 STOT SE3 STOT RE1 Aquatic Acute 1 Aquatic Chronic 1	H270 H330 H341 H351 H370 (nervous system) H335 H372 (cardiovascular, nervous, respiratory system) H400 H410	GHS03 GHS06 GHS08 GHS09 Dgr	H270 H330 H341 H351 H370 (nervous system) H335 H372 (cardiovascular, nervous, respiratory system H410		Inhalation ATE 10 ppm (gases) M = 100 M = 1	

Table 7: Reason for not proposing harmonised classification and status under public consultation

Hazard class	Reason for no classification	Within the scope of public consultation
Explosives	Hazard class not applicable	No
Flammable gases (including chemically unstable gases)		
Oxidising gases	Harmonised classification proposed	Yes
Gases under pressure	Hazard class not applicable	No
Flammable liquids		
Flammable solids		
Self-reactive substances		
Pyrophoric liquids		
Pyrophoric solids		
Self-heating substances		
Substances which in contact with water emit flammable gases		
Oxidising liquids		
Oxidising solids		
Organic peroxides		
Corrosive to metals		
Acute toxicity via oral route		
Acute toxicity via dermal route		
Acute toxicity via inhalation route	Harmonised classification is proposed	Yes
Skin corrosion/irritation	Data inconclusive	Yes
Serious eye damage/eye irritation		
Respiratory sensitisation	Data conclusive but not sufficient for classification	Yes
Skin sensitisation	Hazard class not applicable (gas)	No
Germ cell mutagenicity	Harmonised classification is proposed	Yes
Carcinogenicity		
Reproductive toxicity	Data inconclusive	Yes
Specific target organ toxicity-single exposure	Harmonised classification is proposed	Yes
Specific target organ toxicity-repeated exposure		
Aspiration hazard	Hazard class not applicable (gas)	No
Hazardous to the aquatic environment	Harmonised classification proposed	Yes
Hazardous to the ozone layer	Hazard class not applicable	No

3 HISTORY OF THE PREVIOUS CLASSIFICATION AND LABELLING

The substance has not been subject to harmonised classification and labelling before.

RAC general comment
Almost all studies describing human health hazard classes are from the public domain. The studies were submitted by the applicant for the draft risk assessment report (draft CAR for Biocidal Products Regulation, BRP) for ozone generated from oxygen in accordance with Regulation (EU) No. 528/2012. Therefore, the overall conclusions for each human health hazard class are based on open literature data. None of these studies complies with the relevant OECD TG recommendations. Accordingly, a weight of evidence approach was taken.

4 JUSTIFICATION THAT ACTION IS NEEDED AT COMMUNITY LEVEL

There is no requirement for justification that action is needed at Community level as ozone is an active substance in the meaning of Regulation (EU) No 528/2012 and therefore shall normally be subject to harmonised classification and labelling.

4.1 Explanation for not proposing specific concentration limits for specific target organ toxicity

For the hazard classes STOT SE (nervous system) and STOT RE (cardiovascular, nervous and respiratory system) specific concentration limits were derived in accordance with the guidance values of the CLP Regulation and the guidance on application of the CLP criteria. The current proposal for inclusion in Annex VI does not include the derived SCLs, though.

The harmonised classification is proposed in conjunction with the assessment of "ozone generated from oxygen" as an active substance under the biocides regime. There it is regarded as an *in-situ* substance, i.e. it will be produced at the site of use from ambient or compressed air pursuant to DIN EN 12876. As ozone is highly unstable and will not be placed on the market as part of a mixture, specific concentration limits for the classification of mixtures are not necessary.

Also, when comparing the LC_{50} values of ozone with the derived SCL it becomes apparent that the concentrations when a hypothetical mixture would have to be classified for (repeated) target organ effects is several magnitudes above the LC_{50}. While SCLs are set to classify mixtures they might be misinterpreted as ambient air limit values, below which the ozone poses no health threat. At present, the relevant ambient air limits set by legislators are as low as 120 $\mu g/m^3$ (0.06 ppm. Guideline value for ambient air for a maximum period of 8 hours per day, established as a level at which acute effects on public health are likely to be small. Air Quality Guidelines for Europe, World Health Organization, Regional Office for Europe Copenhagen, WHO Regional Publications, European Series, No. 91, 2nd edition, 2000).

As the classification of mixtures containing ozone is not relevant, the DS decided to not propose SCL for target organ effects to avoid creating the impression that those values represent ambient air limits. For transparency reasons, and to underline that ozone has very low effect levels that normally would warrant the setting of lower SCL, the related calculations have been included in this dossier. It is at RACs discretion to include SCL for ozone in their opinion if they see fit.

5 IDENTIFIED USES

The substance is generated *in situ* as a biocidal active substance from oxygen and used to disinfect water and ambient air. According to the information available on ECHA´s dissemination website (https://echa.europa.eu/information-on-chemicals/registered-substances, accessed 08.07.2021) registration of EC 233-069-2 includes several non-biocidal uses by operation of an ozonation device utilising the oxidative

action of ozone e.g. (non exhaustive):

- Ozonation of mineral water and drinking water or water for swimming pools: removal of iron, manganese, arsenic and nitrite
- Pharmaceutical, medicine, cosmetics, and food industry: production of (ultra-)pure process water
- Pulp and paper bleaching
- Semiconductor industry: production of (ultra-)pure process water
- Off-gas treatment
- Laminating and coating
- Sludge reduction
- Soil and groundwater remediation
- Ozonation of wastewater

6 DATA SOURCES

Regulation (EU) No 528/2012: Draft risk assessment report (draft CAR for BPR) for "ozone generated from oxygen" by the evaluating Competent Authority: Federal Institute for Occupational Safety and Health (BAuA).

7 PHYSICOCHEMICAL PROPERTIES

Table 8: Summary of physicochemical properties

Property	**Value**	**Reference**	**Comment (e.g. measured or estimated)**
Physical state at 20°C and 101,3 kPa	Gas		
Melting/freezing point	ca. -193 °C	Lide (2005) (= CRC Handbook)	
Boiling point	ca. -111.35 °C	Lide (2005) (= CRC Handbook)	
Relative density	1.66 (air = 1.0)		Calculation by molecular weight and ideal gas law as follows: S = M / Mair, where S=gas specific gravity, M=gas molecular weight, Mair=28.96443 g/mole (molecular weight of standard air - CRC, 1983). S = 47.998 g/mole / 28.96443 g/mole = 1.66
Vapour pressure			
Surface tension	Liquid ozone: surface tension is 43.8 mN/m at -195.5 °C.	Hersh et al (1959)	Capillary rise method
Water solubility	Solubility ratio: 0.31 at 20 °C and pH 2.7 ((mg ozone/l H2O) / (mg ozone/l air))	Mizuno & Tsuno (2010)	Solubility in pure water depends on the ozone concentration in the feed gas (Henry's law), water temperature and pH. True saturation concentration of

Property	Value	Reference	Comment (e.g. measured or estimated)
			ozone in water remains a difficult concept because ozone self-decomposes continuously (see 3.11).
Partition coefficient n-octanol/water	Log Pow: - 0.87	SRC PhysProp Database	Estimation according to Meylan and Howard (1995). Title: Atom/fragment contribution method for estimating octanol–water partition coefficients.
Flash point			Not applicable to ozone (gas)
Flammability			
Explosive properties			
Self-ignition temperature			
Oxidising properties	Oxidising gas	European Standard EN ISO 10156:2010	documented
Granulometry			Not applicable to ozone (gas)
Dissociation constant			
Viscosity			

8 EVALUATION OF PHYSICAL HAZARDS

Ozone is a powerful oxidising agent, highly unstable and highly reactive, hence it is classified as an oxidising gas. Ozone cannot be stored or transported in vessels because it decomposes spontaneously in the presence of oxidisable impurities, humidity and solid surfaces. Nevertheless, ozone is explosive as pure substance, has a flammable range in mixture with air and would meet the criteria for the hazard class “flammable gases”. But this is not taken into account in this classification proposal. In consequence Ozone is not classified under the CLP Regulation as a chemically unstable gas, as unstable gases are included only in the hazard class for flammable gases. In addition, the supplemental hazard statement code EUH006 ‘Explosive with or without contact with air’ was deleted by Regulation (EU) 487/2013 (4th ATP to CLP), published on 1 June 2013.

8.1 Explosives

Hazard class not applicable. Gases are excluded per definition from the hazard class “Explosives” according to section 2.1 of Annex I to CLP.

8.2 Flammable gases (including chemically unstable gases)

Hazard class not applicable. Ozone is an oxidising gas.

8.3 Oxidising gases

Table 9: Summary table of studies on oxidising gases

Method	Results	Remarks	Reference
	Oxidising gas: Ci coefficient = 40	According to ISO 10156:2010 ozone is an oxidising gas. No testing needed.	European Standard EN ISO 10156:2010

8.3.1 Short summary and overall relevance of the provided information on oxidising gases

There are not many pure gases that are oxidising. Most oxidising gases are identified as such in the UN RTDG Model Regulations and in ISO 10156. Ozone is listed as an oxidising gas in ISO 10156.

8.3.2 Comparison with the CLP criteria

Any gas which may, generally by providing oxygen, cause or contribute to the combustion of other material more than air does, means pure gases or gas mixtures with an oxidising power greater than 23,5 % as determined by a method specified in ISO 10156 as amended, shall be classified as an oxidising gas of category 1.

8.3.3 Conclusion on classification and labelling for oxidising gases

The substance should be classified as oxidising gas, category 1 according to Annex I Part 2 of the CLP regulation.

8.4 Gases under pressure

Hazard class not applicable. Ozone gas is generated in situ and used immediately after its generation. Ozone gas does not get packaged or transported.

8.5 Flammable liquids

Hazard class not applicable. Ozone is a gas.

8.6 Flammable solids

Hazard class not applicable. Ozone is a gas.

8.7 Self-reactive substances

Hazard class not applicable. Ozone is a gas.

8.8 Pyrophoric liquids

Hazard class not applicable. Ozone is a gas.

8.9 Pyrophoric solids

Hazard class not applicable. Ozone is a gas.

8.10 Self-heating substances

Hazard class not applicable. Ozone is a gas.

8.11 Substances which in contact with water emit flammable gases

Hazard class not applicable. Ozone is a gas.

8.12 Oxidising liquids

Hazard class not applicable. Ozone is a gas.

8.13 Oxidising solids

Hazard class not applicable. Ozone is a gas.

8.14 Organic peroxides

Hazard class not applicable. The study does not need to be conducted because the substance an inorganic gas and not a peroxide.

8.15 Corrosive to metals

Hazard class not applicable. Gases are out of the scope of the corrosive to metal hazard class.

RAC evaluation of physical hazards

Summary of the Dossier Submitter's proposal

Ozone is identified as an oxidising gas in ISO 10156 which is also the calculation method to identify an oxidising gas (see CLP Regulation 2.4.4). Consequently, the Dossier Submitter (DS) proposed to classify ozone as Oxidising Gas Category 1.

Comments received during consultation

No comments were received during the consultation.

Assessment and comparison with the classification criteria

Based on the calculation method ISO 10156, ozone has an oxidising power of 40% which is above the value of 23.5% indicated in the CLP Regulation. Consequently RAC agrees with the DS to classify ozone as Oxidising Gas Category 1.

9 TOXICOKINETICS (ABSORPTION, METABOLISM, DISTRIBUTION AND ELIMINATION)

Table 10: Summary table of toxicokinetic studies

Summary table of toxicokinetic studies					
Method Guideline, GLP status, Reliability	**Species, Strain, Sex, No/Group**	**Test substance, Dose levels Duration of exposure**	**Results**	**Remarks**	**Reference**
Inhalatory absorption in humans at exercise, GLP: No Reliability: 2	healthy adult non-smokers, 5 male, 5 female, 18–35 y	200 ppb 30 min 400 ppb 30 min 200 ppb 60 min 400 ppb 60 min	Fractional absorption: Mean (concentrations, time) ± SD of 0.86 ± 0.06 for all 2,000 Breaths. Inhalatory Absorption: 90 %	Absorbed fraction ranged from 0.56 to 0.98 (56-98 %).	Rigas, M. L. 2000
Inhalatory absorption in humans at rest, GLP: No Reliability: 2	healthy adult non-smokers, 10 male, 19–32 y	300 ppb 10 min	Inhalatory Absorption: 76 ± 3 % (oral breathing) and 73 ± 3 % (nasal breathing)	-	Wiester, M. L. 1996
Inhalatory Absorption in rodents, GLP: No Reliability: 2	Guinea pigs Rats: Fisher 344 Sprague-Dawley Long Evans 6/group, male	300 ppb 60 min 600 ppb 60 min 300 ppb 60 min 300 ppb 60 min	Absorption: 53 ± 10.7 % Absorption: 45 ± 9.1 % Absorption: 43.5 ± 9.9 % Absorption: 47.6 ± 7.5 %	-	Wiester, M. L. 1988
Study on O_3 reactions with proteins and fatty acids GLP: No, Reliability: 2	Cells from human blood (type A, Rh positive); human RBC membranes	Highest level: 0.41 µmol	88% of ozone was estimated to react with proteins and lipids in the lung lining fluid layer at the air/ lung boundry. Oxidative damage to proteins causes significant decreases in the content of thiol groups, the fluorescence of protein-tryptophan residues, and the activity of membrane-bound acetylcholinesterase. Oxidative damage to lipids causes changes in	Product appearance of unsaturated fatty acids is a more sensitive measure ozonation than is substrate disappearance	Uppu, R. M. 1995

			some of the unsaturated fatty acids in the lipid fraction of RBC membranes. Significant amounts of hexanal, heptanal, and nonanal are formed.		
Inhalatory Absorption in rodents Similar to OECD 417 GLP: no Reliability 2 (reliable with restrictions)	Rat: Fisher F344 males (n=4-6)	$^{18}O_3$ (gas) 2 ppm for 6 hours, whole body, or 5 ppm for 2 hrs	53% of applied dose of $^{18}O_3$ was recovered in the urine as 18O within 4 days and 20% was recovered in bronchial lavage fluid as ^{18}O in rats exposed to 2 ppm $^{18}O_3$ For 6 hours. ^{18}O was detected in blood plasma 7 hours post-exposure in rats ad mistered 5 ppm $^{18}O_3$ for 2 hours. No detectable 18O was found in red blood cells. No detectable ^{18}O was found in blood plasma after exposure to 2 ppm $^{18}O_3$. Washing of the fur of animals exposed to 5 ppm $^{18}O_3$ for 2 hours had minimal impact on O_3 concentration in the urine – internal exposure was based on inhalation. Appearance of ^{18}O in blood plasma was only detected at dose at 5 ppm. No measure of ^{18}O in nasal or upper airway passages was conducted.	Males only	Hatch G., Slade R., McKee J. (2013)

9.1 Short summary and overall relevance of the provided toxicokinetic information on the proposed classification(s)

Following inhalation, ozone is effectively absorbed. In rodents, the absorbed fraction amounted to approx. 50 % at a concentration of 300 ppb applied for 60 min. Under similar exposure conditions, absorption in humans was between 56 and 98 % with data suggesting slightly higher absorption under exercise than at rest. The regional deposition pattern was not described.

Rigas et al. studied ozone absorption in humans at exercise and four conditions and reported absorption ranging up to 98 %. Though the study was not performed under GLP (as all studies on toxicokinetics), this report was ranked as a crucial study because it was performed in human species and at exercise. Supportive data comes from Wiester (1996) that also studied ozone absorption in humans but at rest not at exercise. Here, about 75% absorption was detected at oral or nasal breathing. A comparative study by Wiester (1988), however, showed that experiments with laboratory animals are of limited value for prediction of ozone absorption by human

beings. Inhalatory absorption was detected in guinea pigs and two rat strains and ranged from 44 % in Fisher 344 to 53 % in guinea pigs. Overall, absorption by rodents was lower than in human: 100 % according to study in humans at exercise (Rigas, M.L. 2000); rodents not predictive for humans (Wiester, M.L. 1988)

There is no study on dermal absorption available. A default value of 100 % according to EFSA guidance on dermal absorption (EFSA, 2017) is not applicable to ozone. Based on physicochemical properties of ozone, however, the substance is not likely to permeate through the skin to a large extent.

There is no study on oral absorption available. As ozone is a gas, oral exposure is not the main exposure path. If necessary, a default value of 100 % can be applied to assess absorption of dissolved ozone.

Based on the physicochemical properties of ozone, it is expected that the majority of the substance reacts with the tissue at the site of contact. Reaction products might be expected to distribute more widely. Elimination was assessed by studies of Uppu (1995) and Hatch (2013). Ozone oxidizes lung lipids and the reaction products excreted include malonaldehyde, ethane, and pentane. Ozone is totally consumed almost immediately upon reactions with antioxidants and unsaturated fatty acids. These reactions generate the actual ozone messengers represented by either hydrogen peroxide as a fast acting compound or a variety of lipid oxidation products as late effectors. Ozone may interact with many of the components in the ELF including phospholipids, neutral lipids like cholesterol, free fatty acids, proteins, and low molecular weight antioxidants as has been demonstrated in in vitro studies. It was estimated by Uppu et al. (1995) (and cited by US EPA, 2013) that 88% of the O_3 that does not come in contact with antioxidants will react with unsaturated fatty acids in the ELF (US EPA, 2013). The study of Hatch et al. (2013) reported excretion of ^{18}O in the urine after inhalation of 2 ppm $^{18}O3$ in rats. 53 % of the applied dose was recovered as ^{18}O in the urine of rats within 4 days and 20 % in the BAL fluid.

10 EVALUATION OF HEALTH HAZARDS

Acute toxicity

10.1 Acute toxicity - oral route

Data lacking. Ozone is a gas.

10.2 Acute toxicity - dermal route

Data lacking. Ozone is a gas. Based on physico-chemical properties of ozone, the substance is not likely to permeate through the skin to a large extent.

10.3 Acute toxicity - inhalation route

Table 11: Summary table of animal studies on acute inhalation toxicity

Method, Guideline, GLP status, Reliability	**Species, Strain, Sex, No/group**	**Test substance, form** *(gas, vapour, dust, mist)* **and particle size** *(MMAD)* **Actual and nominal concentration, Type of administration** *(nose only / whole body/ head only)*	**Value LC_{50}**	**Remarks** *(e.g. major deviations)*	**Reference**
Studies for LC50 derivation					
Method, **Guideline:** None **GLP:** No **Reliability:** 2	Mice, female Rats, male and female	**Ozone** **Exposure:** 4 h **No of dose groups:** 7 (rats), 4 (mice) **Dose range:** 3.4-14 (female rats) and 3.6-36 ppm (male rats), 9-24 ppm (mice) **Group size:** 3 (female rats), 4 (male rats), 5-7 (mice) **Mean bw:** 150-202 g (rats), 20 g (mice)	**LC_{50}:** No statistical determination of LC50 performed. 50 % mortality occurred at **9 ppm** (mice) Rats: none died at 3.4, 3.6 and 9 ppm, 25 % died at 8 ppm, 100 % died at 14 ppm and above Mice: 50 % died at 9ppm, 100 % died at 12.7 ppm and above **Effects:** laboured breathing (reversible) started at lowest dose (3.4 ppm) in rats. **Cause of death:** acute pulmonary oedema	Group size too small for rats, but acceptable for mice, strain not identified, no 14d post-exposure observation	Diggle W.M. and Gage J.C. (1955), British Journal of Industrial Medicine 12(1):60-64
Method, **Guideline:** None **GLP:** No **Reliability:** 4 supporting data	**Mice** (Swiss, adult): **10** per dose group **Rats** (Wistar, adult): **5** per dose group	**Ozone** generated from various precursors (scrubbed air, tank oxygen, tank oxygen and nitrogen, scrubbed air-furnace treated, unscrubbed air) using two different types of generators (plastic-type	LC_{50} as reported by authors: 1.4-6.6 ppm (mice) 2.4-8.2 ppm (rats)	Reporting deficiencies: Dose levels and % mortality at the different dose levels were not reported. Only LC_{50} and range (of mortalities?) of each precursor/generator combination was reported. This is because the study purpose was not	Svirbely J.L. and Saltzman B.E. (1957), AMA Arch. Ind. Health 15(2):111-118

Method, Guideline, GLP status, Reliability	**Species, Strain, Sex, No/group**	**Test substance, form** *(gas, vapour, dust, mist)* **and particle size** *(MMAD)* **Actual and nominal concentration, Type of administration** *(nose only / whole body/ head only)*	**Value LC_{50}**	**Remarks** *(e.g. major deviations)*	**Reference**
		ozonizer, mica-type ozonizer) **Dose levels:** not reported.		determination of LC50, but comparison of toxicity of ozone made from different precursors/generators.	
Method, **Guideline:** None **GLP:** No **Reliability:** 4 supporting data	**Rat,** Sprague-Dawley, male 7, 20, 23 per group, depending on experiment	**Ozone** generated from oxygen 8 ppm (7.5-10.6 ppm) Closed chamber	Rats exposed only to ozone (8 ppm) died within 210 min (mean)	Study was not designed to investigate ozone toxicity. Instead, ozone was used as a lethal agent in order to investigate the influence of PABA injection on survival time after ozone exposure Cause of death was not reported, but according to the authors it is known that lethal ozone levels cause pulmonary oedema	Goldstein B.D. and Balchum O.J. (1974), Toxicol. Appl. Pharmacol. 27: 330

10.3.1 Short summary and overall relevance of the provided information on acute inhalation toxicity

Ozone is of high toxicity in the mice and rat after inhalation exposure. No LC_{50} could be determined from the studies listed, as most of them were not designed for the determination of an LC_{50} and others were either not reliable or not suitable to derive an actual LC_{50} value. All studies were from open literature and not in accordance with OECD Test Guidelines (TG403). However, the studies indicate that the LC_{50} is clearly below the cut-off for classification for Acute Tox. 1; H330. In the study by Diggle & Gage (1955), 50 % mortality in mice after exposure to 9 ppm of ozone were reported. Cause of death was acute pulmonary oedema. However, these values likely underestimate the acute toxicity of ozone as no post-exposure observation was performed. In supporting studies, LC_{50} values of 1.4-6.6 ppm in mice, 2.4-8.2 ppm and 8 ppm in rats were reported. In summary, the studies allow an estimate of the LC50 in the range of 1-10 ppm. This range is by at least a factor of 10 below the cut-off for classification for acute inhalation toxicity (Acute Tox. 1; H330). Therefore, a classification as Acute Tox. 1; H330 is proposed.

10.3.2 Comparison with the CLP criteria

Toxicological result	CLP criteria
Inhalation LC_{50}, mice ≤ 10 ppm/4h *Diggle & Gage (1955):* LC_{50} = 9 ppm (mice) *Svirbely & Saltzman (1957):* LC_{50} = 1.4-6.6 ppm (mice) LC_{50} = 2.4-8.2 ppm (rats)	Gases (ppmV) (a) Cat. 4 (H332): 2500 < LC_{50} ≤ 20000 ppm (gas) Cat. 3 (H331): 500 < LC_{50} ≤ 2500 ppm (gas) Cat. 2 (H330): 100 < LC_{50} ≤ 500 ppm (gas) Cat. 1 (H330): LC_{50} ≤ 100 ppm (gas) (a) The acute toxicity estimate (ATE) for the classification of a substance is derived using the LD_{50}/LC_{50} where available.

10.3.3 Conclusion on classification and labelling for acute inhalation toxicity

In summary and based on the submitted data, ozone meets the criteria to be classified for Acute Toxicity Inhalation, Category 1, H330 according to the criteria in CLP regulation.

RAC evaluation of acute toxicity

Summary of the Dossier Submitter's proposal

No classification was proposed for acute toxicity via the oral and dermal route by the DS.

The DS proposed a classification for acute inhalation toxicity in category 1 (Acute Tox. 1; H330) based on results from inhalation studies conducted in mice and rats.

Comments received during consultation

One Member State Competent Authority (MSCA) agreed with the DS proposal and with the ATE value.

Assessment and comparison with the classification criteria

No classification is warranted for ozone via oral and dermal routes as ozone is a gas. Based on the physico-chemical properties of ozone, the substance is not likely to permeate through the skin to a large extent.

For acute inhalation toxicity, studies from open literature have been assessed and are summarised below:

***Table**: Summary of studies relevant for Acute Toxicity*

Method, Guideline, GLP, Reliability (Klimisch), Ref.	Species, Strain, Sex, No/group	Test substance and type of administration	Value LC_{50}
Guideline: None GLP: No Reliability: 2 Diggle, Gage, 1955.	Mice, female Rats, male and female	Ozone Exposure: 4 h No of dose groups: 7 (rats), 4 (mice) Dose range: 3.4-14 (female rats) and 3.6-36 ppm (male rats), 9-24 ppm (mice) Group size: 3 (female rats), 4 (male rats), 5-7 (mice) Mean bw: 150-202 g (rats), 20 g (mice)	LC_{50}: No statistical determination of LC_{50} performed. 50% mortality occurred between 8 - 10 ppm (rats) and at 9 ppm (mice) Rats: none died at 3.4, 3.6 and 9 ppm, 25% died at 8 ppm, 100% died at 14 ppm and above Mice: 50% died at 9 ppm, 100% died at 12.7 ppm and above Effects: laboured breathing (reversible) started at lowest dose (3.4 ppm) in rats. Cause of death: acute pulmonary oedema
Guideline: None GLP: No Reliability: 4 supporting data Svirbely and Saltzman, 1957	Mice (Swiss, adult): 10 per dose group Rats (Wistar, adult): 5 per dose group	Ozone generated from various precursors (scrubbed air, tank oxygen, tank oxygen and nitrogen, scrubbed air-furnace treated, unscrubbed air) using two different types of generators (plastic-type ozoniser, mica-type ozoniser) Dose levels: not reported.	LC_{50} as reported by authors: 1.4-6.6 ppm (mice) 2.4-8.2 ppm (rats)
Guideline: None GLP: No Reliability: 4 supporting data Goldstein, Balchum, 1974	Rat, Sprague-Dawley, male 7, 20, 23 per group, depending on experiment	Ozone generated from oxygen 8 ppm (7.5-10.6 ppm) Closed chamber	Rats exposed only to ozone (8 ppm) died within 210 min (mean)

Ozone is highly toxic in mice and rats following exposure by inhalation. No LC_{50} could be determined from the available studies, as most of them were not designed for determination of an LC_{50} value and others were either not reliable or not suitable to derive an LC_{50} value.

All studies were from open literature and not in accordance with OECD Test Guideline (TG) 403. However, the studies indicate that the LC_{50} is clearly below the cut-off (< 100 ppm) for classification for Acute Tox. 1; H330. In the study by Diggle and Gage (1955), 50% mortality in mice and rats after exposure to 9 ppm and 8-10 ppm of ozone were reported, respectively.

The cause of death was acute pulmonary oedema. However, these values likely underestimate the acute toxicity of ozone as no post-exposure observation was performed in this study.

In supporting studies, LC_{50} values of 1.4-6.6 ppm in mice, 2.4-8.2 ppm and 8 ppm in rats (Svirbely and Saltzman, 1957) were reported. In summary, the studies allow an estimation of the LC_{50} in the range of 1-10 ppm. This range is at least a factor of 10 below the cut-off value for classification for acute inhalation toxicity in category 1 (Acute Tox. Cat. 1; H330: $LC_{50} \leq$ 100 ppm (gas)).

Therefore, RAC concludes that a classification as Acute Tox. 1; H330 is warranted.

RAC agrees with an ATE of 10 ppm based on the converted acute toxicity point estimate for a classification in category 1 and on the available data. In addition, RAC notes that the LC_{50} is most likely lower than this value.

10.4 Skin corrosion/irritation

Table 12: Summary table of animal studies on skin corrosion/irritation

Method, Guideline, GLP status, Reliability	**Species, Strain, Sex, No/group**	**Test substance, Vehicle, Dose levels, Duration of exposure**	**Results** *Average score (24, 48, 72 h), observations and time point of onset, reversibility, other adverse local/systemic effects, histopathological findings*	**Remarks** *(e.g. major deviations)*	**Reference**
Method, Guideline: None GLP: No Reliability: 4	Hairless mice	0, 10 ppm 2 h	Decreased alpha-tocopherol and ascorbic acid in upper epidermis, but not in lower skin layers; 10-fold increase in Malondialdehyde (MDA, a lipid peroxidation product) in upper epidermis (suggesting reactivity of ozone) and 2-fold increase in lower epidermis, but unchanged in dermis	Study not applicable for this endpoint	Thiele J.J. et al. (1997a), Free Radic Biol Med. 23(3):385-91
Method, Guideline: None GLP: No Reliability: 4	SKH-1 Hairless mice 4 per group, except single exposure control group (n=12)	Single exposure: 0, 1, 5, 10 ppm 2 h Repeated exposure: 0, 1 ppm 6 days	Depletion of vitamin E; increase of MDA formation in Stratum corneum (SC)	Study not applicable for this endpoint	Thiele J.J. et al. (1997b), J Invest Dermatol 108(5): 753-757
Method, Guideline: None GLP: No Reliability: 4	Hairless mice	2 ppm 1 wk	No alteration of transepidermal water loss (an indicator of skin barrier integrity) up to 72 h after last exposure	Study not applicable for this endpoint	Thiele J.J. et al. (2003), Skin Pharmacol Appl Skin Physiol. 16(5):283-90

Method, Guideline, GLP status, Reliability	**Species, Strain, Sex, No/group**	**Test substance, Vehicle, Dose levels, Duration of exposure**	**Results** *Average score (24, 48, 72 h), observations and time point of onset, reversibility, other adverse local/systemic effects, histopathological findings*	**Remarks** *(e.g. major deviations)*	**Reference**
Method, Guideline: None GLP: No Reliability: 4	n/a	n/a	n/a	Study not applicable to this endpoint. Study designed to investigate macromolecular carbonyls in SC as biomarker for environmental oxidant exposure	Thiele J.J. et al. (1998), FEBS Lett. 22(3):403-6
Method, Guideline: None GLP: No Reliability: 4	SKH-1 hairless mice	0, 0.8, 1, 10 ppm 2 h	Depletion of vitamin C, glutathione and uric acid in stratum corneum at 1 ppm and above	Study not applicable to this endpoint.	Weber S.U. et al. (1999), J Invest Dermatol 13(6):1128-32

Table 13: Summary table of human studies on skin corrosion/irritation

Type of data/ report, Reliability	**Test substance**	**Relevant information about the study**	**Observations**	**Reference**
Method, Guideline: None GLP: No Reliability: 4	Ozone generated from oxygen 0.8 ppm 20 human subjects: one forearm was exposed in a chamber for 2 h	Effects on superficial stratum corneum: 70 % reduction of vitamin E; 2.3 fold increase in lipid hydroperoxides; 50 % reduction of microflora population; state of oxidative stress; no signs of skin dryness or erythema	Study not applicable for this endpoint	He Q.C. et al. (2006), Int J Cosmet Sci. 28(5):349-57

10.4.1 Short summary and overall relevance of the provided information on skin corrosion/irritation

In principle and in accordance with the CLP Guidance (chapter 3.2.2.1.2.1), strong oxidising properties in conjunction with highly exothermic reactions provide a reason for concern for skin irritation / corrosion. In fact, the available human and animal information demonstrated the formation of reactive oxygen species in exposed skin, as well as depletion of antioxidants after dermal exposure to ozone. However, exothermic reactions were not reported. This information should be taken into account when assessing risk from repeated skin exposure. According to Guidance on IR/CSA Section R.7.2.4.2 (4.1, 2015), human data on local skin effects may be obtained from existing data and corrosive reactions are typified by ulcers, bleeding and bloody scabs. In the human study submitted by the applicant, no signs of corrosion or erythema were reported.

Although the available studies demonstrate some effects on the skin, the studies are not applicable to determine skin irritation and corrosion and can only be used as supportive information. Based on the available information, the effects of ozone are limited to the upper layer of the epidermis. There are no publicly available studies evaluating the irritation potential of ozone. Existing studies evaluating ozone exposure at environmentally relevant concentrations do not demonstrate dermal irritation. However, as the environmental concentrations are low, they cannot be used for classification for skin irritation. In accordance with the CLP Guidance in a weight of evidence approach "*All information that is available should be considered and an overall determination made on the total weight of evidence. This is especially true when there is conflict in information available on some parameters. Expert judgment should be exercised prior to making such a determination. Negative results from applicable validated skin corrosion/irritation in vitro tests are considered in the total weight of evidence evaluation*.". Considering the physical properties of ozone as a strong oxidising agent, it is pointed out that highly exothermic reactions could not be demonstrated. The conditions of the CLP Guidance (chapter 3.2.2.1.2.1) are thus not fulfilled. There are indeed indications for induction of oxidative stress in the skin but these are considered insufficient to justify a classification for skin irritation. Due to the absence of robust experimental information that can be used for classification purposes for this endpoint, classification for skin irritation cannot be proposed.

10.4.2 Comparison with the CLP criteria

Toxicological result (animal)	CLP criteria
No erythema and oedema scores available	Category 1 (corrosion) Destruction of skin tissue, namely, visible necrosis through the epidermis and into the dermis, in at least one tested animal after exposure ≤ 4 h No such effects observed, category not applicable. Category 2 (Irritation) (1) Mean score of ≥ 2.3 and ≤ 4.0 for erythema/ eschar or for oedema in at least 2 of 3 tested animals from gradings at 24, 48 and 72 hours after patch removal or, if reactions are delayed, from grades on 3 consecutive days after the onset of skin reactions; or (2) Inflammation that persists to the end of the observation period normally 14 days in at least 2 animals, particularly taking into account alopecia (limited area), hyperkeratosis, hyperplasia, and scaling reactions; or (3) In some cases where there is pronounced variability of response among animals, with very definite positive effects related to chemical exposure in a single animal but less than the criteria above.
Strong oxidising agent, induction of oxidative stress, inflammatory responses observed in animals	Weight of evidence approach

10.4.3 Conclusion on classification and labelling for skin corrosion/irritation

In summary and based on the submitted data, the results of the abovementioned skin corrosion/irritation studies with ozone are considered inconclusive for classification and labelling.

RAC evaluation of skin corrosion/irritation

Summary of the Dossier Submitter's proposal

The DS proposed no classification based on inconclusive data.

Comments received during consultation

One MSCA commented that the data are inconclusive. However, another MSCA pointed out that according to the CLP guidance, substances with strong oxidising properties cause a concern for skin irritation / corrosion. The MSCA stated that the available information demonstrated the formation of reactive oxygen species in exposed skin, as well as depletion of antioxidants. Considering the physico-chemical properties of ozone as a strong oxidising agent, and the indications for induction of oxidative stress in the skin, the MSCA considered that a classification for skin irritation should be discussed.

A company – manufacturer suggested classification as Skin. Irrit. 2 based on publicly available human data.

An industry or trade association agreed with no classification based on the poor reliability of the available animal studies.

Assessment and comparison with the classification criteria

The DS assessed the studies summarised in the table below in the CLH report:

***Table**: Summary of the animal studies assessed for skin irritation.*

Method, Guideline, GLP status, Reliability Ref.	Test substance, Species	Results
Guideline: None GLP: No Reliability: 4 Thiele *et al.,* 1997a	0, 10 ppm 2 h Hairless mice	Decreased alpha-tocopherol and ascorbic acid in upper epidermis, but not in lower skin layers; 10-fold increase in MDA (a lipid peroxidation product) in upper epidermis (suggesting reactivity of ozone) and 2-fold increase in lower epidermis, but unchanged in dermis
Guideline: None GLP: No Reliability: 4 Thiele *et al.,* 1997b	Single exposure: 0, 1, 5, 10 ppm 2 h Repeated exposure: 0, 1 ppm 6 days SKH-1 Hairless mice 4 per group, except single exposure control group (n = 12)	Depletion of vitamin E; increase of MDA formation in SC

Guideline: None GLP: No Reliability: 4 Thiele *et al.,* 2003	2 ppm 1 week Hairless mice	No alteration of transepidermal water loss (an indicator of skin barrier integrity) up to 72 h after last exposure
Guideline: None GLP: No Reliability: 4 Weber *et al.,* 1999	0, 0.8, 1, 10 ppm 2 h SKH-1 hairless mice	Depletion of vitamin C, glutathione and uric acid in stratum corneum at 1 ppm and above

***Table**: Summary of assessed human data assessed for skin irritation*

Type of data/ report, Reliability	Test substance	Relevant information about the study
Method, Guideline: None GLP: No Reliability: 4 He *et al.,* 2006	Ozone generated from oxygen 0.8 ppm 20 human subjects: one forearm was exposed in a chamber for 2 h	Effects on superficial stratum corneum: 70% reduction of vitamin E; 2.3fold increase in lipid hydroperoxides; 50% reduction of microflora population; state of oxidative stress; no signs of skin dryness or erythema

Ozone is a strong oxidising agent and there is indication for induction of oxidative stress in the skin as well as depletion of antioxidants. However, although the available studies demonstrate some effects on the skin, the studies are not applicable to determine skin corrosion/irritation, and can only be used as supportive information.

Based on the available information, the effects of ozone are limited to the upper layer of the epidermis. There are no publicly available studies evaluating the irritation potential of ozone. Existing studies evaluating ozone exposure at environmentally relevant concentrations did not demonstrate dermal irritation. The available human data demonstrated some effects on the skin. However, RAC considers the study in a weight of evidence approach as not applicable or sufficient to warrant classification for skin corrosion/irritation according to the CLP criteria.

RAC agrees with the DS that, due to the absence of robust experimental evidence that can be used for classification purposes for this hazard class, **no classification for skin corrosion/irritation is warranted.**

10.5 Serious eye damage/eye irritation

Table 14: Summary table of animal studies on serious eye damage/eye irritation

Method, Guideline, GLP status, Reliability	**Species, Strain, Sex, No/group**	**Test substance Dose levels, Duration of exposure**	**Results** *Average score (24, 48, 72 h), observations and time point of onset, reversibility*	**Remarks** *(e.g. major deviations)*	**Reference**
Method, Guideline: None GLP: No Reliability: 2	rabbits, albino, male n=3 per group	group 1: 0 and 1.9-2.8 ppm single exposure of 4 h group 2: 0 and 2 ppm 25 days for a period of one hour	group 1: no difference to control regarding the level of chemosis, iritis, corneal swelling or rate of regeneration group 2: no effect on eyes	Another experiment was conducted with human subjects. Results are presented in table 15 below this one.	Hine C.H. et al. (1960a), J. Air Pollut. Control Assoc. 10:17–20
Method, Guideline: None GLP: No Reliability: 4	mice, ICR, male n=10 per group	0, 0.5, 2.0 ppm 3 h/d for 2 weeks in whole-body chamber	breakdown of corneal epithelial integrity decreased number of mucin-secreting cells production of inflammatory cytokines	Another experiment was conducted in vitro. Results are presented in table 16 below.	Lee H. et al. (2013a), Free Radic Biol Med 63:78-89

Table 15: Summary table of human data on serious eye damage/eye irritation

Type of data/ report, Reliability	**Test substance**	**Relevant information about the study**	**Observations**	**Reference**
Method, Guideline: None GLP: No Reliability: 2	Ozone	Subjects were mainly medical students and staff from the University of California School of Medicine. Groups consisted of 5 or 10 subjects. Number of groups not reported.	Degree of eye irritation was self-reported by subjects (absent (0), slight (1), moderate (2), severe (3), extreme (4)) and also examined by attending ophthalmologist. Mean scores (single values not reported) at different ozone exposures: 1.6 ppm: not reported 2.0 ppm: 0.9 3.7 ppm: 1.2	Hine C.H. et al. (1960b), J. Air Pollut. Control Assoc. 10:17–20

Type of data/ report, Reliability	Test substance	Relevant information about the study	Observations	Reference
			Authors reported a large variability in responses, ranging in most groups from slight to moderate	
Method, Guideline: None GLP: No Reliability: 4	Ozone, 40 ppb and 71 ppb	Humans, male n=8	Changes in eye blink frequency in response to a number of compounds, including ozone, was investigated. The effect of 40 ppb and 71 ppb ozone on blink frequency was negligible compared to control clean air. However, 4 out of 8 subjects reported irritation (data not shown).	Kleno J. and Wolkoff P. (2004), Int Arch Occup Environ Health 77:235–243
Method, Guideline: None GLP: No Reliability: Review	Not applicable	Not applicable	Excess ozone auto decomposes rapidly to produce oxygen and thus leaves no residues in foods from its decomposition.	Prabha V., Barma RD., Singh R., Madan A. (2015)

Table 16: Summary table of other studies relevant for serious eye damage/eye irritation

Method, Guideline, GLP status, Reliability	Test substance, Doses	Relevant information about the study	Results	Remarks *(e.g. major deviations)*	Reference
Method, Guideline: None GLP: No Reliability: 4	ozone	cells: human cultured conjunctival epithelial cells dose: 2.0 ppm exposure time: 0, 0.5, 1, 3, 5 or 8 h	increased NF-κB nuclear translocation, κB-dependent transcriptional activity, NF-κB inhibitor α proteolysis and expression of phosphorylated IκBα induced expression of inflammatory cytokines, Toll-like receptors and C-C chemokine receptors decreased expression of mucins no cytotoxicity or cellular apoptosis	Another experiment was conducted in vivo. Results are presented in table 14 above.	Lee H. et al. (2013b), Free Radic Biol Med 63:78-89

10.5.1 Short summary and overall relevance of the provided information on serious eye damage/eye irritation

Only few studies are available in which effects on the eyes were evaluated. No irritating effects on the eye were found in the rabbit at concentrations up to 1.9-2.8 ppm (Hine et al 1960a). In a not assignable study breakdown of corneal epithelial integrity was observed in mice (Lee 2013). No irritating effects on the eye were found in the rabbit.

However, eye irritation was reported in two studies conducted with human subjects. In one of these studies, slight to moderate irritation was self-reported/observed (Hine et al 1960b) and in the second study, half of the subjects self-reported irritating effects which were not further classified (Kleno and Wolkoff 2004). Kleno and Wolkoff also investigated blinking frequency but found only negligible effects of ozone comparable to control. Prabha et al. (2015), reported that at short-term exposure rates of 0.1–1.0 ppm, symptoms include headaches, nosebleeds, eye irritation, dry throat and respiratory irritation. Although these studies do demonstrate some effects to the eyes, these studies do not provide sufficient information to support classification for eye irritation as the studies are not directly applicable to this endpoint. According to Guidance on IR/CSA Section R.7.2.9.2 (6.0, 2017), the quality and relevance of existing human data studies should be critically reviewed. Reliable and relevant human data were not submitted.

In addition, in mice, the integrity of the corneal epithelium was compromised, the number of mucin-secreting cells was reduced and the production of inflammatory cytokines was induced by ozone exposure (Lee 2013a). An in vitro study (Lee 2013b) provided evidence for an inflammatory response by showing that ozone exposure induced several responses involving NF-κB, inflammatory cytokines, Toll-like receptors and C-C chemokine receptors. Mucin expression was also decreased in this study.

Overall, the severity of effects observed at the concentrations tested are not considered to trigger classification for eye irritation according to the CLP regulation.

However in accordance with the CLP Guidance in a weight of evidence approach “All information that is available on a substance should be considered and an overall determination made on the total weight of evidence. This is especially true when there is conflict in information available on some parameters. The weight of evidence including information on skin irritation may lead to classification for eye irritation. Negative results from applicable validated in vitro tests are considered in the total weight of evidence evaluation.”. Strong oxidising properties provide a reason for concern for eye irritation / corrosion and appropriate evidence must be provided in order to consider no classification of substances with oxidising properties. Thus, although no data is available on corneal opacity, conjunctival redness or chemosis at 24, 48 and 72 h of the test material to facilitate a comparison with the CLP criteria, taking into account the physico-chemical properties and the indicative information from available studies demonstrating irritating effects in human eyes as well as inflammatory responses observed in animals, classification with H319 could be proposed.

10.5.2 Comparison with the CLP criteria

Toxicological result	CLP criteria
No erythema and oedema scores available *Hine C.H. et al. (1960b):* Mild to moderate eye irritation at ≥ 2000 ppb in humans (CLP Regulation does not have criteria for classification based on human data)	Irritating to eyes (Category 2, H319): at least in 2/3 tested animal a positive response of: a) corneal opacity: ≥ 1 and/or b) iritis: ≥ 1 and/or c) conjunctival redness: ≥ 2 and/or d) conjunctival oedema (chemosis): ≥ 2
Strong oxidising agent, induction of oxidative stress, inflammatory responses observed in animals indicate that ozone is an eye irritant	Weight of evidence approach

10.5.3 Conclusion on classification and labelling for serious eye damage/eye irritation

In summary and based on the submitted data, the results of the abovementioned serious eye damage/eye irritation studies with ozone are considered inconclusive for classification and labelling.

RAC evaluation of serious eye damage/irritation

Summary of the Dossier Submitter's proposal

The DS proposed no classification for eye damage/irritation based on inconclusive data. No data was available on corneal opacity, conjunctival redness or chemosis. The DS concluded no classification as eye damage/irritation in category 2 also after assessing the impact of physico–chemical properties and the information from available studies demonstrating irritating effects in human eyes, as well as inflammatory responses observed in animals.

Comments received during consultation

One MSCA agreed that the data are inconclusive. However, a second MSCA pointed out that according to the CLP guidance, substances with strong oxidising properties cause concern for eye irritation / corrosion. The MSCA also noted that the human evidence can be regarded as supportive evidence for the eye irritation potential and that animal studies with higher concentration levels that may produce irritation/corrosion effects are not available. Considering the physico-chemical properties of ozone as a strong oxidising agent and the indications for induction of oxidative stress in the eye, the MSCA considered classification for eye irritation possible, and discussion needed.

A company – manufacturer suggested classification with Eye Irrit. 2 based on the available human data.

An Industry or trade association agreed with no classification based on the poor reliability of the available animal studies.

Assessment and comparison with the classification criteria

The DS assessed the studies summarised in the tables below in the CLH report.

***Table**: Summary of animal eye irritation/damage studies.*

Method, Guideline, GLP status, Reliability, Ref	**Species, Strain, Sex, No/group**	**Test substance** **Dose levels, Duration of exposure**	**Results**
Method, Guideline: None GLP: No Reliability: 2 Hine *et al.,* 1960	Rabbits, albino, male n = 3 per group	Group 1: 0 and 1.9-2.8 ppm single exposure of 4 h Group 2: 0 and 2 ppm 25 days for a period of one hour	Group 1: no difference to control regarding the level of chemosis, iritis, corneal swelling or rate of regeneration Group 2: no effect on eyes
Method, Guideline: None GLP: No Reliability: 4 Lee *et al.,* 2013a	Mice, ICR, male n = 10 per group	0, 0.5, 2.0 ppm 3 h/d for 2 weeks in whole-body chamber	Breakdown of corneal epithelial integrity Decreased number of mucin-secreting cells Production of inflammatory cytokines

***Table**: Summary of human data on eye irritation/damage.*

Type of data/ report, Reliability, Ref.	**Test substance**	**Relevant information about the study**	**Observations**
Method, Guideline: None GLP: No Reliability: 2 Hine *et al.,* 1960	Ozone	Subjects were mainly medical students and staff from the University of California School of Medicine. Groups consisted of 5 or 10 subjects. Number of groups not reported.	Degree of eye irritation was self-reported by subjects (absent (0), slight (1), moderate (2), severe (3), extreme (4)) and also examined by attending ophthalmologist. Mean scores (single values not reported) at different ozone exposures: 1.6 ppm: not reported 2.0 ppm: 0.9 3.7 ppm: 1.2 Authors reported a large variability in responses, ranging in most groups from slight to moderate.
Method, Guideline: None GLP: No Reliability: 4 Kleno and Wolkoff, 2004	Ozone, 40 ppb and 71 ppb	Humans, male n = 8	Changes in eye blink frequency in response to a number of compounds, including ozone, was investigated. The effect of 40 ppb and 71 ppb ozone on blink frequency was negligible compared to control clean air. However, 4 out of 8 subjects reported irritation (data not shown).

***Table**: Summary of a study relevant for eye irritation/damage.*

Method, Guideline, GLP status, Reliability	**Relevant information about the study**	**Results**
Method, Guideline: None GLP: No Reliability: 4 Lee *et al.,* 2013b	Cells: human cultured conjunctival epithelial cells Dose: 2.0 ppm Ozone exposure time: 0, 0.5, 1, 3, 5 or 8 h	Increased NF-κB nuclear translocation, κB-dependent transcriptional activity, NF-κB inhibitor α proteolysis and expression of phosphorylated IκBα Induced expression of inflammatory cytokines, Toll-like receptors and C-C chemokine receptors Decreased expression of mucins No cytotoxicity or cellular apoptosis

No irritating effects on the eye were found in the rabbit at concentrations up to 1.9-2.8 ppm (Hine *et al*., 1960a). In a not assignable study by Lee *et al*. (2013a), integrity of the corneal epithelium was compromised, the number of mucin-secreting cells was reduced, and the production of inflammatory cytokines was induced by ozone exposure.

An *in vitro* study by Lee *et al*. (2013b) provided evidence for an inflammatory response by showing that ozone exposure induced several responses involving NF-κB, inflammatory cytokines, Toll-like receptors and C-C chemokine receptors. Mucin expression was also decreased in this study.

Eye irritation was reported in two studies conducted with human subjects. In one of these studies, slight to moderate irritation was self-reported/observed (Hine *et al.,* 1960) and in the second study half of the subjects self-reported irritating effects which were not further classified (Kleno and Wolkoff, 2004). Kleno and Wolkoff also investigated blinking frequency but found only negligible effects of ozone comparable to control. A review by Prabha *et al.* (2015) reported that short-term exposure to concentrations of 0.1–1.0 ppm, symptoms included headaches, nosebleeds, eye irritation, dry throat and respiratory irritation. Although these studies demonstrated some effects to the eyes, they did not provide sufficient information to support classification for eye irritation according to CLP Regulation criteria.

Overall, the severity of effects observed at the concentrations tested are not considered sufficient to trigger classification for eye irritation according to the CLP Regulation.

In accordance with the CLP guidance: "*All information that is available on a substance should be considered and an overall determination made on the total weight of evidence. This is especially true when there is conflict in information available on some parameters. The weight of evidence including information on skin irritation may lead to classification for eye irritation. Negative results from applicable validated in vitro tests are considered in the total weight of evidence evaluation.*" Strong oxidising properties provide a reason for concern for eye damage/irritation and appropriate evidence must be provided in order to consider no classification of substances with oxidising properties.

RAC notes that although no data is available on corneal opacity, conjunctival redness or chemosis, taking into account the physico-chemical properties of ozone, and the indicative information from available studies demonstrating irritating effects in human eyes, as well as the inflammatory responses observed in animals, classification as Eye. Irrit. 2; H319 could be considered.

Overall, RAC concludes that **no classification of ozone for eye damage/irritation is warranted** because the severity of effects observed at the concentrations tested is not

considered sufficient to trigger classification for eye damage/irritation according to the CLP Regulation.

10.6 Respiratory sensitisation

Table 17: Summary table of animal studies on respiratory sensitisation, here: AHR (Airway Hyperresponsiveness)

Method, Guideline, GLP status, Reliability	**Species, Strain, Sex, No/group**	**Test substance** **Dose levels,** **Duration of exposure**	**Results**	**Remarks** *(e.g. major deviations)*	**Reference**
Method: AHR-model[1] to 5-hydroxytryptamine (HT) in rat. Measurement of Airway responsiveness and Bronchoalveolar lavage fluid (BALF). Guideline: None GLP: No Reliability: 2 [1] Well-established animal model for human asthma. In mice induced by ovalbumin and accompanied by features of AHR.	Rat, 9 different strains: Long-Evans, Sprague-Dawley, Fisher 344, Brown-Norway, BDII, BDE, DA, Lewis and Wistar; all male, n=10 per strain and exposure.	Ozone generated by high voltage electrostatic pulses in an Ozomat COM ozone generator (Anseros, Tübingen,Germany) 0.05 ppm 4 h Ozone measurement: Ozomat MP Ozone Analyser (Anseros)	Effect: Lung resistance (RL). Lung resistance (RL) continuously calculated from tidal volume, air flow and transpulmonary pressure. Lewis, BDII and Long-Evans rats developed airway hyperresponsiveness AHR 90 min after ozone detected by a leftward shift (ANOVA $p<0.05$) of the dose-response curve compared to control animals. Wistar, Sprague-Dawley, Fisher 344, Brown-Norway, BDE and DA rats did not develop AHR. In Long-Evans rats, AHR lasted up to 12 h post-exposure in the absence of an inflammatory cell influx or increase in lactate dehydrogenase, alkaline phosphatase or total protein.	Ozone concentrations measured in the exposure chamber varied around 50 ppb (range 40-57), Statistics by ANOVA; Student's t-test, Mann-Whitney U-test.	Depuydt, P. et al. (1999), Eur Respir J 14:125-131

Method, Guideline, GLP status, Reliability	**Species, Strain, Sex, No/group**	**Test substance** **Dose levels,** **Duration of exposure**	**Results**	**Remarks** *(e.g. major deviations)*	**Reference**
Method: AHR-model[1] in mice to ovalbumin (OVA) and methacholine (MCh) exposure. Airway responsiveness and Bronchoalveolar lavage fluid (BALF). Guideline: None GLP: No Reliability: 2	BALB/c mice, female, groups of n=8 mice.	OVA: 1 %, 20 min/day Day 1-10 Ozone 0, 100, 250, 500 ppb 3 h on day 11 Ozone generated using Sander Ozonizer model 25 (Erwin Sander Elektroapparatebau, Uetze-Eltze, Germany)	Ozone induced airway hyperresponsiveness (AHR) in mice previously exposed to OVA when compared to non-exposed (saline) control mice. After a 10-d exposure to OVA, a single exposure to a low (100 ppb) ozone concentration was sufficient to induce AHR. In mice challenged by 12.5 mg/ml MCh a significant increase in lung resistance (> 2.5 fold in OVA compared to saline at 24 hrs) and decrease in dynamic compliance (46 % in OVA compared to 27 % of the baseline in saline at 24 hrs) was detected 24 h after ozone exposure, a significantly higher number ($x10^{-3}$ per ml BALF) of epithelial cells was seen in the OVA-500 ppb group compared to the saline-500 group. Neutrophils were only slightly enhanced. AHR response was associated with goblet-cell metaplasia after exposure to 10 d of OVA followed by 3-h exposure to 100 or 250 ppb ozone. **LOAEC: 100 ppb** **NOAEC: n/a**	The actual ozone concentrations deviated less than 10 % from the target concentrations. Even the lowest concentration of ozone tested, 100 ppb, resulted in a significant increase in AHR.	Larsen, S.T. et al. (2010), Journal of Toxicology and Environmental Health, Part A, 73(11);738-747

Method, Guideline, GLP status, Reliability	**Species, Strain, Sex, No/group**	**Test substance** **Dose levels,** **Duration of exposure**	**Results**	**Remarks** *(e.g. major deviations)*	**Reference**
Method: AHR-model[1] in guinea pigs to ovalbumin (OVA) exposure, long-term repeated ozone exposures, specific airway conductance sGaw measured by constant volume plethysmography Guideline: None GLP: No Reliability: 2	Hartley guinea pigs, male and female	OVA: 1 % in pyrogen-free isotonic saline; inhalation challenge; 30 min/day for day 1-4 Ozone: 0, 100 or 300 ppb 4 h/day, 4 days/week for 24 weeks. Ozone generated using OREC model 03V1-0 Definition of PC50: provocation concentration that resulted in a decrease in specific airway conductance sGaw of 50 % from the PBS baseline.	Exacerbation of AHR by ozone to specific (OVA) and nonspecific (acetylcholine) bronchoprovocation in male and female. Effect persisted 4 weeks. Airway response to ozone exposure did not differ between the two groups. PC50 values for animals exposed to 100 ppb ozone were generally lower than values for air controls but were generally higher than values for animals exposed to 300 ppb ozone. Number of pulmonary eosinophils or any chronic pulmonary inflammatory response not increased. Levels of antigen-specific antibodies increased in sensitized animals, significant correlation between airway responsiveness and IgG levels.	Small groups of 5 animals.	Schlesinger, R.B. et al. (2002), Boston, MA: Health Effects Institute, research report no. 109

Table 18: Summary table of human data on respiratory sensitisation (Epidemiological Data)

Reference / study characteristics	**ozone exposure**			**Statistical Analysis**	**Effect**	**Results**	**Others/ Remarks**
	Conc. µg/m3	**Conc. ppb**	**Duration hours**				
Lin, S. et al. 2008, New York State (10 regions) birth cohort with 1,204.396 eligible births; data from 1995 until 1999. Follow up each individual until first asthma hospital	80.65 to 102.73	37.51 to 47.78 Range of mean ozone concentrations over the 10 New York Regions.	Chronic exposure/ long-term.	Two-stage Bayesian hierarchical model analysis	First asthma hospital admission	Significant positive associations between chronic ozone level and asthma hospital admissions for all exposure indicators after adjusting for potential confounding variables (ORs =1.16–1.68). Chronic exposure to ambient ozone in early life was significantly and positively associated	Impacts related to hospital admission investigated by "negative control" group of admissions due to gastroenteritis: No positive association with ozone as found for

Reference / study characteristics	ozone exposure			Statistical Analysis	Effect	Results	Others/ Remarks
	Conc. µg/m3	Conc. ppb	Duration hours				
admission or until 31.12.2000. Hourly ambient ozone data from the New York State Depart. Of Environmental Conservation (32 ozone monitoring sites), measured hourly for each day (8-hr maximum hourly value). Study included in U.S. EPA/ISA Report 2013.						with an increased risk of asthma hospital admissions among a birth cohort in New York State (lowest mean ozone level in New York City: 37.5 ppb). The risk of hospital admissions increased 22 % with a 1-ppb increase in mean ozone concentration during the ozone season. An OR of 1.69 (1.52–1.80) for high exposure ≥ 67 % was found. Indicators using the entire follow-up period weaker elevated risks for asthma admissions. By using the exceedance proportion, significant increase (OR = 1.68; 95 % CI, 1.64–1.73) in hospital admissions associated with an interquartile range (IQR =2.51 % increase in ozone was found.	admissions due to asthma. Reliable study, statistical method appropriate, birth, maternal confounders and geographic regions considered.
Moore, K. et al. 2008, ecologic study, California's South Coast Air Basin (195 spatial grids), children who ranged in age from birth to 19 years, from 1983 to 2000, measurements for 3-month periods along with demographic variables (U.S. Census Bureau's decadal surveys for years 1980, 1990 and 2000). Average concentrations of the 1-hr daily maximum ozone.	64.5 - >322.5	30 - >150 (quarterly 1-hr maximum ozone)	Chronic exposure/ long-term.	Regression model, history-restricted marginal structural models (HRMSMs)	First asthma hospital admission (parameter: discharge)	A linear relation was detected for asthma hospital discharges. High correlation between median 1-hr and 8-hr maximum average ozone levels (r =0.99). During 1980–2000, ozone concentrations showed moderate correlation with particulate matter with aerodynamic diameter ≤ 10 µm (PM_{10}) and little correlation with the pollutants NO_2, CO, SO_2. A 10-ppb increase above the median ozone concentration of 87.7 ppb is estimated to lead to a 4.6 % increase in the proportion of discharges (3.26 × 10–4).	Many areas included that consistently exceeded National Ambient Air Quality Standards for ozone during the 1980–2000 study period (U.S. EPA 2000). Reliable study, statistical method appropriate, confounders considered.

Reference / study characteristics	ozone exposure			Statistical Analysis	Effect	Results	Others/ Remarks
	Conc. µg/m3	Conc. ppb	Duration hours				
Study included in U.S. EPA/ISA Report 2013.							
Mortimer, K.M. et al. 2002, cohort of 846 asthmatic children (4-9 y) in 8 urban areas of the USA, data from the National Cooperative Inner-City Asthma Study (NCICAS), daily air pollution concentrations from the Aerometric Information Retrieval System database from US EPA.	103.2	48, daily ambient, across all urban areas	8-h average ozone (10:00–18:00 h)	Linear mixed effect models (SAS Proc Mixed)	Peak expiratory flow rate (PEFR) and symptoms (cough, chest tightness, wheeze)	A 15 ppb increase in 5-day moving average ozone was associated with a 0.59 % decline in morning PEFR (95 % CI 0.13–1.05) and with a sign. Increased incidence of a ≥10 % decline in morning PEFR (OR=1.14, 95 % CI 1.02–1.27).	This longitudinal analysis supports previous time-series findings that at levels below current USA air-quality standards, summer-air pollution is significantly related to symptoms and decreased pulmonary function among children with asthma. Reliable study, statistical method appropriate, confounders considered.
Silverman, R.A. and Ito, K (2010). Daily time-series analysis of 6008 asthma ICU admissions and 69,375 general (non-ICU) asthma admissions in 4 age groups (<6, 6-18, 19-49, 50+ y) in 74 New York City hospitals for the months April to August from 1999 to 2006. Ozone data from UC EPA`s Air Quality System.	< 172	< 80; daily ambient, NAAQS (the 3-year average of the fourth-highest daily concentrations should not exceed this value); exceeded on 46 days.	Risks for interquartile range increases in the a priori exposure time window of the average of 0-day and 1-day lagged pollutants.	Adjusted regression model	asthma hospitalisation, ICU: life threatening episodes requiring intensive care unit admission	Susceptibility to ozone is age-dependent, with children at highest risk for non-ICU hospitalizations and ICU admission. For each 22-ppb increase in ozone, there was a 19 % (95 % CI, 1 % to 40 %) increased risk for ICU admissions and a 20 % (95 % CI, 11 % to 29 %) increased risk for general hospitalizations.	There appear to be severe adverse health effects to exposures even below the currently accepted standard of 80 ppb. Reliable study, statistical method appropriate, confounders considered.

10.6.1 Short summary and overall relevance of the provided information on respiratory sensitisation

Ozone is not a causal factor for the development of allergic asthma in the sensitisation and the elicitation phases. However, exposure to ozone for atopic patients with bronchial asthma can result in so-called acute, unspecific hyperreactivity, exacerbation or AHR (airway hyperresponsiveness). AHR is a serious health impairment. Lin (2008) demonstrated that in the group that was exposed in the range of 38-48 ppb of mean ozone concentrations asthma cases in humans occurred. The risk of hospital admissions increased 22 % with a 1 ppb increase in mean ozone concentration during the ozone season. Moore, K. (2008) (reported that a 10 ppb increase above the median ozone concentration of 88 ppb is estimated to lead to a 4.6 % increase in the proportion of discharges. This is one of the reasons why STOT SE 3 is proposed for ozone in Section 10.11.

Results from animal testing studies supported the observations from human studies. It was shown that even an ozone concentration of 50 ppb can trigger AHR symptoms as reported by Depuydt (1999) in three rat strains. An important observation in the study by Depuydt is that AHR could be induced by a single, short exposure time of 90 min and to a low concentration of ozone, which is even below the current upper limit of the National Ambient Air Quality Standards. Interestingly, exposure to an ambient concentration of ozone induced AHR in the absence of airway inflammation.

The study by Schlesinger (2002), however, observed exacerbation of AHR at 100 ppb ozone in OVA-sensitised guinea pigs and thereby provides support for a role of ambient ozone exposure in exacerbation of airway dysfunction in persons with atopy. It is imperative that handlers of ozone with allergies be protected against this serious adverse effect.

10.6.2 Comparison with the CLP criteria

Toxicological result	**CLP criteria**
There is evidence in humans for worsening of respiratory allergy/asthma symptoms by induction of severe AHR effects such as bronchoconstriction and inflammation following ozone inhalation. There is no evidence in humans that ozone can lead to specific respiratory hypersensitivity - human data, risk of first asthma hospital admissions increased by ozone (Lin 2008, Moore 2008, Silverman 2010) - animal data for AHR symptoms in three rat strains (Depuydt et al 1999), but not for being a respiratory sensitizer - high frequency in occurrence of AHR in humans could not be deduced from a study	<u>Category 1 :</u> Substances shall be classified as respiratory sensitisers (Category 1) where data are not sufficient for sub-categorisation in accordance with the following criteria : – if there is evidence in humans that the substance can lead to specific respiratory hypersensitivity ; and/or – if there are positive results from an appropriate animal test <u>Sub-category 1A :</u> – Substances showing a high frequency of occurrence in humans; or a probability of occurrence of a high sensitisation rate in humans based on animal or other tests. Severity of reaction may also be considered. <u>Sub-category 1B :</u> – Substances showing a low to moderate frequency of occurrence in humans; or a probability of occurrence of a low to moderate sensitisation rate in humans based on animal or other tests. Severity of reaction may also be considered.

10.6.3 Conclusion on classification and labelling for respiratory sensitisation

Ozone is not a sensitiser itself, but unequivocally exacerbates existing asthma by worsening allergen-induced symptoms in humans and animals (First asthma admission; AHR symptoms in 3 rat strains, guinea pigs, mice)

by the inhalative route and after single exposure to concentrations ≤ 0.1ppm. Thereby, ozone can cause asthma symptoms and breathing difficulties as described in hazard sentence H334: "*May cause allergy or asthma symptoms or breathing difficulties if inhaled.*". The worsening of asthma symptoms by ozone is not covered by STOT SE 3, because for the occurrence of AHR symptoms an existing allergy is a prerequisite. But as ozone is not an allergen, it could according to GLP not be classified for respiratory sensitisation.

Existing classification: none

Proposal: None

RAC evaluation of respiratory sensitisation

Summary of the Dossier Submitter's proposal

The DS proposed no classification for respiratory sensitisation. Ozone is not a sensitiser itself but exacerbates existing asthma by worsening allergen-induced symptoms in humans and animals. However, as ozone is not an allergen, it could not be classified for respiratory sensitisation according to CLP.

Comments received during consultation

One commenting MSCA agreed with the DS proposal for no classification.

Assessment and comparison with the classification criteria

Only studies from the public domain were submitted.

Table: *Summary of animal studies on respiratory sensitisation:*

Method, Guideline, GLP status, Reliability, Reference	**Species, Strain, Sex, No/group Dosis**	**Results**
Method: Airway hyperresponsiveness (AHR)-model to 5-hydroxytryptamine (HT) in rat. Measurement of Airway responsiveness and Bronchoalveolar lavage fluid (BALF). Guideline: None GLP: No Reliability: 2 Depuydt *et al.,* 1999	Rat, 9 different strains 0.05 ppm 4 h	Effect: Lung resistance (RL) continuously calculated from tidal volume, air flow and transpulmonary pressure. Lewis, BDII and Long-Evans rats developed AHR 90 min after ozone detected by a leftward shift (ANOVA $p < 0.05$) of the dose-response curve compared to control animals. Wistar, Sprague-Dawley, Fisher 344, Brown-Norway, BDE and DA rats did not develop AHR. In Long-Evans rats, AHR lasted up to 12 h post-exposure in the absence of an inflammatory cell influx or increase in lactate dehydrogenase, alkaline phosphatase or total protein.

Method: AHR-model in mice to ovalbumin (OVA) and methacholine (MCh) exposure. Airway responsiveness and Bronchoalveolar lavage fluid (BALF). Guideline: None GLP: No Reliability: 2 Larsen *et al.,* 2010	BALB/c mice, female 0, 0.1, 0.25, 0.5 ppm 3 h on day 11	Ozone induced AHR in mice previously exposed to OVA when compared to non-exposed (saline) control mice. After a 10d exposure to OVA, a single exposure to a low (100 ppb) ozone concentration was sufficient to induce AHR. In mice challenged by 12.5 mg/mL MCh a significant increase in lung resistance (> 2.5 fold in OVA compared to saline at 24 h) and decrease in dynamic compliance (46% in OVA compared to 27% of the baseline in saline at 24 h) was detected 24 h after ozone exposure, a significantly higher number ($\times 10^{-3}$ per mL BALF) of epithelial cells was seen in the OVA-500 ppb group compared to the saline-500 group. Neutrophils were only slightly enhanced. AHR response was associated with goblet-cell metaplasia after exposure to 10 d of OVA followed by 3 h exposure to 100 or 250 ppb ozone. LOAEC: 100 ppb NOAEC: n/a
Method: AHR-model in guinea pigs to ovalbumin (OVA) exposure, long-term repeated ozone exposures, specific airway conductance Guideline: None GLP: No Reliability: 2 Schlesinger *et al.,* 2002	Hartley guinea pigs, male and female 0, 0.1 or 0.3 ppm 4 h/day, 4 days/week for 24 weeks.	Exacerbation of AHR by ozone to specific (OVA) and nonspecific (acetylcholine) bronchoprovocation in male and female. Effect persisted 4 weeks. Airway response to ozone exposure did not differ between the two groups. PC_{50} values for animals exposed to 100 ppb ozone were generally lower than values for air controls but were generally higher than values for animals exposed to 300 ppb ozone. Number of pulmonary eosinophils or any chronic pulmonary inflammatory response not increased. Levels of antigen-specific antibodies increased in sensitized animals, significant correlation between airway responsiveness and IgG levels.

Table*19: Summary of human data:*

Reference / study characteristics	**ozone exposure**			**Results**
	Conc. µg/m3	**Conc. ppb**	**Duration hours**	
Lin *et al.,* 2008, New York State (10 regions) birth cohort with 1 204 396 eligible births; data from 1995 until 1999. Follow up each individual until first asthma hospital admission or until 31.12.2000. Hourly ambient ozone data from the New York State Depart. Of Environmental Conservation (32 ozone monitoring sites), measured hourly for each day (8 h maximum hourly value). Study included in U.S. EPA/ISA Report 2013.	80.65 to 10273	37.51 to 47.78 Range of mean ozone concentrations over the 10 New York Regions.	Chronic exposure/ long-term	Significant positive associations between chronic ozone level and asthma hospital admissions for all exposure indicators after adjusting for potential confounding variables (Ors = 1.16–1.68). Chronic exposure to ambient ozone in early life was significantly and positively associated with an increased risk of asthma hospital admissions among a birth cohort in New York State (lowest mean ozone level in New York City: 37.5 ppb). The risk of hospital admissions increased 22% with a 1 ppb increase in mean ozone concentration during the ozone season. An OR of 1.69 (1.52–1.80) for high exposure ≥ 67% was found. Indicators using the entire follow-up period weaker elevated risks for asthma admissions. By using the exceedance proportion, significant increase (OR = 1.68; 95% CI, 1.64–1.73) in hospital admissions associated with an interquartile range (IQR = 2.51% increase in ozone was found.

Moore *et al.,* 2008, ecologic study, California's South Coast Air Basin (195 spatial grids), children who ranged in age from birth to 19 years, from 1983 to 2000, measurements for 3-month periods along with demographic variables (U.S. Census Bureau's decadal surveys for years 1980, 1990 and 2000). Average concentrations of the 1 h daily maximum ozone. Study included in U.S. EPA/ISA Report 2013.	64.5 -> 322.5	30 - > 150 (quarterly 1 h maximum ozone)	Chronic exposure/ long-term.	A linear relation was detected for asthma hospital discharges. High correlation between median 1 h and 8 h maximum average ozone levels (r = 0.99). During 1980–2000, ozone concentrations showed moderate correlation with particulate matter with aerodynamic diameter ≤ 10 μm (PM_{10}) and little correlation with the pollutants NO_2, CO, SO_2. A 10 ppb increase above the median ozone concentration of 87.7 ppb is estimated to lead to a 4.6% increase in the proportion of discharges (3.26×10^{-4}).
Mortimer *et al.,* 2002, cohort of 846 asthmatic children (4-9 y) in 8 urban areas of the USA, data from the National Cooperative Inner-City Asthma Study (NCICAS), daily air pollution concentrations from the Aerometric Information Retrieval System database from US EPA.	103.2	48, daily ambient, across all urban areas	8 h average ozone (10:00–18:00 h)	A 15 ppb increase in 5 days moving average ozone was associated with a 0.59% decline in morning PEFR (95% CI 0.13–1.05) and with a sign. Increased incidence of a ≥ 10% decline in morning PEFR (OR = 1.14, 95% CI, 1.02–1.27).
Silverman *et al.*, 2010. Daily time-series analysis of 6008 asthma ICU admissions and 69375 general (non-ICU) asthma admissions in 4 age groups (<6, 6-18, 19-49, 50+ y) in 74 New York City hospitals for the months April to August from 1999 to 2006. Ozone data from UC EPA's Air Quality System.	<172	< 80; daily ambient, NAAQS (the 3 years average of the fourth-highest daily concentrations should not exceed this value); exceeded on 46 days.	Risks for interquartile range increases in the a priori exposure time window of the average of 0 day and 1 day lagged pollutants.	Susceptibility to ozone is age-dependent, with children at highest risk for non-ICU hospitalizations and ICU admission. For each 22 ppb increase in ozone, there was a 19% (95% CI, 1–40) increased risk for ICU admissions and a 20% (95% CI, 11–29) increased risk for general hospitalizations.

Results from animal studies supported the observations from human studies. Depuydt *et al.* (1999) reported that even an ozone concentration of 0.05 ppm can trigger AHR symptoms in three rat strains. Furthermore, the authors observed that AHR could be induced by a single, short exposure time of 90 min and a low concentration of ozone (0.05 ppm), which is even lower than the current upper limit of the National Ambient Air Quality Standards. Interestingly, exposure to such ambient concentration of ozone induced AHR in the absence of airway inflammation. Genetic factors are likely to account for the observed variability in sensitivity of the airways to ozone.

However, Schlesinger *et al.* (2002) observed exacerbation of AHR at 0.1 ppm ozone in OVA-sensitised guinea pigs and thereby provided support for a role of ambient ozone exposure in exacerbation of airway dysfunction in persons with atopy.

Ozone is not a causal factor for the development of allergic asthma in the sensitisation and the elicitation phases. However, exposure to ozone for atopic patients with bronchial asthma can result in so-called acute, unspecific hyperreactivity, exacerbation or AHR. AHR is a serious

health impairment. Lin *et al.* (2008) demonstrated that in the group that was exposed in the range of 0.038-0.048 ppm of mean ozone concentrations, asthma cases in humans occurred. The risk of hospital admissions increased by 22% with a 0.001 ppm increase in mean ozone concentration during the ozone season (April–October). Moore *et al.* (2008) reported that a 0.01 ppm increase above the median ozone concentration of 0.088 ppm is estimated to lead to a 4.6% increase in the proportion of discharges (after first asthma hospital admission). This is one of the reasons why STOT SE 3 is proposed for ozone.

Ozone is not a respiratory sensitiser itself, but unequivocally exacerbates existing asthma by worsening allergen-induced symptoms in humans and animals (first asthma admission; AHR symptoms in 3 rat strains, guinea pigs, mice) by the inhalation route and after single exposure to concentrations ≤ 0.1 ppm. Thereby, ozone can cause asthma symptoms and breathing difficulties.

The worsening of asthma symptoms by ozone is not covered by STOT SE 3, because for the occurrence of AHR symptoms an existing allergy is a prerequisite. In addition, as ozone is not an allergen, it doesn't fulfil the CLP criteria for classification for respiratory sensitisation.

RAC therefore agrees with the DS that **no classification for respiratory sensitisation is warranted.**

10.7 Skin sensitisation

There are no studies available and there is no evidence from the scientific open literature that ozone is a dermal sensitiser. The LLNA and other in vivo tests are considered not applicable to ozone gas. Based on these results, ozone is not regarded as a skin sensitiser.

10.8 Germ cell mutagenicity

Table 20: Summary table of mutagenicity/genotoxicity tests in vitro

Method, Guideline, GLP status, Reliability	**Test substance, Doses**	**Relevant information about the test system** *(e.g. organism, strain)*	**Results**		**Remarks** *(e.g. major deviations and information on cytotoxicity)*	**Reference**
			-S9	**+S9**		
Ames test/ Sim. to OECD 471 GLP: no Rel. 2	Ozone (Production in ozone generator from oxygen) Purity oxygen: not given Purity ozone (residual oxygen): not given Vehicle: air Incubation time: 35 min Flow rate of oxygen: 5 or 7 L/min Exp. 1;+S9; flow 5 L/min: 0 (air or oxygen)-0.039-0.39-1.21-4.09-6.60-9.00 ppm Exp. 2;-S9; flow 5 L/min: 0 (air or oxygen)-0.033-0.30-1.14-3.99-6.38-8.70 ppm Exp. 3;+S9; flow 5 L/min: 0 (air or oxygen)-0.039-0.36-1.18-4.05-6.45-8.99 ppm Exp. 4;-S9; flow 5 L/min: 0 (air or oxygen)-0.033-0.37-1.14-3.94-6.57-8.75 ppm Exp. 5;+/-S9; flow 7 L/min (only TA102):	*S. typhimurium*: TA1535, TA98, TA100, TA102, TA104	positive (TA102, 0.019 ppm and above) Oxygen flow rate: 7 L/min Number of revertants doubled	positive (TA102, 0.019 ppm and above) Oxygen flow rate: 7 L/min Number of revertants doubled	<u>Statistically significant (p<0.01) increase of revertants from air controls (no doubling):</u> TA102 at 0.039 ppm (+S9) and 0.33 ppm (-S9) and above, flow rate of 5 L/min Therefore, a weak mutagenic effect by incomplete converted oxygen (7 L/min) cannot be ruled out. <u>Statistically significant (p<0.01) increase of revertants from air controls (no doubling, not reproducible):</u> TA104 at 0.039 ppm (+S9), flow rate of 5 L/min Cytotoxicity (decrease in revertant number) beginning at ~ 0.4 ppm (TA102) or 1-4 ppm (remaining strains) <u>Shortcomings:</u> - no purity or batch - no gaseous positive controls (but ozone itself can serve as positive control in TA102) - efficacy of S9 mix solely tested with 2-AA - strain TA1537 or TA97(a) not tested - number of plates/dose unclear - titer not given	Dillon D. et al. (1992), Environ. Mol. Mutagen. 19: 331-337

Method, Guideline, GLP status, Reliability	**Test substance, Doses**	**Relevant information about the test system** *(e.g. organism, strain)*	**Results**		**Remarks** *(e.g. major deviations and information on cytotoxicity)*	**Reference**
			-S9	**+S9**		
	0 (air or oxygen)-0.024-0.19-0.53-1.48-3.62-7.04 ppm Exp. 6;+/-S9; flow 7 L/min (only TA102): 0 (air or oxygen)-0.019-0.22-0.64-1.52-3.48-7.08 ppm				- no individual plate counts - no historical negative/positive control data	
Ames test/ Sim. to OECD 471 GLP: no Rel. 2 (for strain TA100) Rel. 3 for strain TA102 and TA104	Ozone (production by radiation of air with UV light) Purity air: not given Purity ozone (residual oxygen): not given Vehicle: air Incubation time: 6 h Flow rate of ozone: 0.25, 0.5 or 1 L/min Dose: 0.1 to 3.5 ppm (also control)	*S. typhimurium*: TA100, TA102, TA104	negative (TA100) unclear (TA102, TA104)	negative (TA100) unclear (TA102, TA104)	Negative results for strains TA102 and TA104 are of limited reliability (no positive controls and no cytotoxicity to confirm that ozone reached the target). - cytotoxicity for TA100 (decrease in revertant number) beginning at ~ 2 ppm <u>Shortcomings:</u> - no purity or batch - no positive control for TA102 and TA104 - data not shown for TA102 and TA104 - strains TA1537 (or TA97[a]), TA1535 and TA98 not tested - titer not given - no individual plate counts and no mean number of revertant colonies and SD - no historical negative/positive control data - not enough doses tested	Victorin K. and Stahlberg M. (1988), Environ. Mol. Mutagen. 11: 65-77
Chromosomal aberration/ Sim. to OECD TG 473 GLP: no	Ozone (production in ozone generator with UV radiation) Purity oxygen: not given Purity ozone (residual oxygen): not given	Human peripheral leukocytes	positive (7.23 and 7.95 ppm/h, ozone, 36 h after	Not tested	Increase in chromosomal aberration observed (no dose-response). <u>Shortcomings:</u>	Gooch P. C. et al. (1976), Environ. Res. 12:188-195

Method, Guideline, GLP status, Reliability	**Test substance, Doses**	**Relevant information about the test system** *(e.g. organism, strain)*	**Results**		**Remarks** *(e.g. major deviations and information on cytotoxicity)*	**Reference**
			-S9	**+S9**		
Rel. 2	Vehicle: air/oxygen Flow rate: not given Method 1: Incubation: 12 h following PHA Dose ozone: 0-1.3-2.4-2.6-4.8-7.5 ppm/h Incubation: 36 h following PHA Dose ozone: 0-1.65-2.5-4.06-5.2-5.73-7.0-7.23-7.95-14.2 ppm/h Method 2 (2 ppm wt/wt): Ozone-saturated phosphate-buffered saline D solution Incubation: 12 h after PHA for 30, 60, 90 min Incubation: 36 h after PHA for 5, 10, 15, 30, 60, 90 min		phytohaemagglutinin PHA)		- no purity or batch - no positive control (but ozone itself can serve as positive control) - actual ozone concentration in method 1 not given (no calculable from exposure time) - no cytotoxicity tested - number of cultures not given - at 7.23 and 14.2 ppm/h less than 200 metaphases tested - no data for individual cultures given - no information on ploidy - no historical negative/positive controls given - deviation from incubation and sampling time	
MN/ Sim. to OECD 487 GLP: no Rel. 2	Ozone (electrically generated from oxygen) Purity oxygen: 99.99 % Purity ozone (residual oxygen): not given Vehicle: air Incubation: 6h Flow rate air: 1L/min; ozone was fed into this flow Dose: 400 ppb (also control)	Rat alveolar type II cells, Male Wistar rats	positive (400 ppb)	Not tested	Statistically significant (2.5-fold) higher MN/1000 cells in ozone-exposed group Only one dose was tested, hence no conclusion on dose-response is possible. <u>Shortcomings:</u> - only short communication (lack of details in material and result section) - only one dose - no purity or batch - no cytotoxicity tested - number of cultures unclear	Chorvatovicova et al. (2000), Physiol. Res. 49: 733-736

Method, Guideline, GLP status, Reliability	**Test substance, Doses**	**Relevant information about the test system** *(e.g. organism, strain)*	**Results**		**Remarks** *(e.g. major deviations and information on cytotoxicity)*	**Reference**
			-S9	**+S9**		
					- no single data - no positive control (but ozone itself can serve as positive control) - cell line is not common for MN testing - negative control was not specified (air or oxygen) - unclear whether slides were independently coded - no historical negative/positive control data - no further test for aneuploidy vs. chromosome breakage	
SCE or chromosomal aberration /Sim. to OECD TG 479 and 473 GLP: no Rel. 2	Ozone (production with ozone generator) Purity oxygen: not given Purity ozone (residual oxygen): not given Vehicle: seems to be air Incubation: 1 h Flow rate: not given Dose: 0-0.25-0.50-0.75-1.00 ppm	WI-38 cells (human fetal lung cell line)	positive (for SCE 0.25 ppm and above) positive (for chromosomal aberration 0.5 ppm and above)	Not tested	Linear and statistically significant dose-related increase in SCEs per chromosome spread. Dose-related increase in percentage of cells with endoreduplications or chromatide deletions at 0.5 or 0.75 ppm and above, respectively. <u>Shortcomings:</u> - no description of ozone production method - no purity or batch - only one dose - cytotoxicity not tested - no common cell line - no positive control (but ozone itself can serve as positive control) - no single data for each culture - chromosomal aberrations were not reported	Guerrero et al. (1979), Environ. Res. 18:336-346

Method, Guideline, GLP status, Reliability	**Test substance, Doses**	**Relevant information about the test system** *(e.g. organism, strain)*	**Results**		**Remarks** *(e.g. major deviations and information on cytotoxicity)*	**Reference**
			-S9	**+S9**		
					in tabular form - less than 200 metaphases tested - no information on ploidy - no historical negative/positive controls given - deviation from incubation and sampling time	
Comet assay/ No OECD TG available GLP: no Rel. 2	Ozone (produced in a generator) Purity oxygen: not given Purity ozone (residual oxygen): not given Vehicle: seems to be air Incubation: 1 h Flow rate: not given Dose ozone: 0.875-1.75-3.5-5.25 mM Dose hydrogen peroxide: 4 and 40 mM (also controls)	Human peripheral blood leukocytes	positive (0.875 mM and above)	Not tested	Dose-dependent increase of damaged cells and comet length (statistically significant) after ozone or H_2O_2 treatment. Effects were reduced with catalase, hence DNA damage might be (at least partly) mediated by H_2O_2. Cytotoxicity: 80-98 % of the cells survived <u>Shortcomings:</u> - it seems that only 1 culture per donor was used - no purity or batch - no single data for each slide - no positive control (but ozone itself can serve as positive control) - only 50 cells per donor treatment evaluated - lack of details in material and result section (*e.g.* independent coding of slides)	Díaz-Llera et al. (2002), Mutat. Res. 517: 13-20
Comet assay **Guideline:** no guideline	Ozone, gas	Human primary fibroblast (three females and three	Negative	Not tested	Reliable with restriction Non-guideline, non-GLP study, only one dose level. No dedicated, guideline compliant positive	Akdeniz et al (2018), Clin Oral Invest 22, 867–873

Method, Guideline, GLP status, Reliability	**Test substance, Doses**	**Relevant information about the test system** *(e.g. organism, strain)*	**Results**		**Remarks** *(e.g. major deviations and information on cytotoxicity)*	**Reference**
			-S9	**+S9**		
available, but similar to OECD test guideline 489 (2016): In vivo mammalian alkaline comet assay **GLP status:** no GLP Reliability: 2	Purity ozone: not given Gas plasma application (gingival healing stimulator mode of applicator) Control: no treatment Dose: 60 µg/µl for 30 s of ozone gas plasma application after drug treatment at 24-h intervals as 3 s/cm2	males, aged 18–30)			control group was used in the study, individual results not available, only mean and standard deviation reported graphically and in a table and results reported as "AU", which was not defined.	(2018).
(1) Comet assay Guideline: no guideline, bit similar to OECD 489 (2016) In vivo mammalian alkaline comet assay (2) mammalian cell micronucleus test OECD 487 (2016) In vitro mammalian cell	Ozone, gas Purity ozone: not given Flow rate: 3.0 L/min. Dose: 120 ppb Ozone Positive control: colchicine (micronucleus assay) and hydrogen peroxide (Comet assay) Incubation: 48 and 72 hrs (micronucleus assay and Comet assay)	Adenocarcinoma human alveolar basal epithelial cells A549 Normal human fibroblasts Hs27 (Source: American Tissue Type Collection)	(1) Comet Assay: Positive (2) mammalian cell micronucleus test: Equivocal	Not tested	Comet assay: A549 cells showed a higher mean value for tail DNA % in respect to the control: 8.3% (48h), 7.3% (72h); Hs27 cells 2.88% (48 h) 3.7% (72 h). Mammalian cell micronucleus test: about 100% compared to the control at 48 h & 72 h exposure in A549 cells and at 48 h in Hs27 cells; significant changes in Hs27 cells at 72 h Only one dose level tested. <u>Shortcomings:</u> OECD 489: - hydrogen peroxide used as positive control but no data on results were presented or discussed for the positive control, - only one positive control animal (a minimum of 3 analysable animals of one sex is suggested by the guideline). OECD 487: - non-standard cell lines,	Poma et al. (2017), PLoS One 2017;12:e0184519.

Method, Guideline, GLP status, Reliability	**Test substance, Doses**	**Relevant information about the test system** *(e.g. organism, strain)*	**Results**		**Remarks** *(e.g. major deviations and information on cytotoxicity)*	**Reference**
			-S9	**+S9**		
micronucleus test GLP: no Reliability: 2					- no determination if the cell lines were capable of metabolism and no S9 treatment group, - colchicine used as positive control however no data on the results were included in report or supplemental data, - treatment duration was 48 and 72 hrs as opposed to 3-6 hours followed by removal to test chemical and a time equivalent to 1.5 - 2.0 normal cell cycle length after the beginning of treatment recommended in the guideline. - No justification was reported for the treatment duration. -Based on the data available in the report, the peak cell proliferation was at 48 hours and at 72 hours cell proliferation was declining.	

Table 21: Summary table of mutagenicity/genotoxicity tests in mammalian somatic or germ cells in vivo

Method, Guideline, GLP status, Reliability	**Test substance, Doses, route and frequency of application**	**Relevant information about the study** *(e.g. species and strain, sex, No/Group, duration of exposure, sampling time)*	**Observations give dose, sampling time, result**	**Remarks** *(e.g. major deviations)*	**Reference**
Chromosomal aberration / Sim. to OECD TG 475 GLP: no Rel. 2	Ozone (production in ozonizer) Purity oxygen: not given Purity ozone (residual oxygen): not given Vehicle: air Flow rate air/ozone mixture: 15 chamber air	Species/strain/sex: Rats F344/N, female Cells: pulmonary alveolar macrophages No/group:	0.12 and 0.27 ppm: dose-related increase in abnormal cells (gaps not included) 0.8 ppm: decrease in abnormal cells (gaps not included)	An ozone-mediated increase in the number of macrophages (and therefore dilution of macrophages responsible for under-estimation of cytogenic effects) was given as explanation for the decrease of abnormal cells at higher doses. This theory is supported with the dose-related increase of the mitotic index (MI) from 0.27 to 0.8 ppm.	Rithidech K. (1990), Mutat. Res. 241: 67-73

Method, Guideline, GLP status, Reliability	**Test substance, Doses, route and frequency of application**	**Relevant information about the study** *(e.g. species and strain, sex, No/Group, duration of exposure, sampling time)*	**Observations** **give dose, sampling time, result**	**Remarks** *(e.g. major deviations)*	**Reference**
LOCAL	changes/h Doses: 0 – 0.12 – 0.27 – 0.8 ppm Application: inhalative, whole-body, once for 6 h	5 animals/dose Exposure: 6h Sampling time: rats were sacrificed 28 h after exposure		MI was strongly decreased after exposure to 0.27 ppm ozone. <u>Shortcomings:</u> - no purity or batch - no positive control - only 50 instead of 200 cells per animal scored - no individual data for each animal - ploidy not tested - no historical positive/negative control data given - highest dose should lead to 50 % reduction of MI (but negligible in case of positive findings) - cell type not common for this test	
Comet assay/Sim. to OECD TG 489 GLP: no Rel. 2 **LOCAL**	Ozone (ozonator) Purity oxygen: "pure" Purity ozone (residual oxygen): not given Vehicle: air Flow rate air: 20 L/h Doses: 0-0.25-0.5 ppm Application: inhalative, whole-body	Species/strain/sex: Mice, 129/SV, male Cells: bronchoalveolar lavage cells (BAL cells, 95 % alveolar macrophages) No/group: 3 mice/dose 2 mice as controls Exposure: 3 h Sampling time: 3h	0.25 ppm: Statistically significant increase in DNA SSBs in comparison to control 0.5 ppm: Statistically significant increase in DNA SSBs in comparison to control; number of cells with high damage (31+ mm) 2-fold higher compared to 0.25 ppm No dose-response regarding number of	The viability of 129/SV BAL cells was not markedly changed in comparison to the control. <u>Shortcomings:</u> - no purity or batch - no positive control - only 2-3 animals/dose - no observation of clinical signs - only 50 cells/animal analysed instead of 150 - only 2 doses - no historical positive/negative control data given - no hedgehogs reported - DNA SSB (single strand breaks) evaluation: tail length was used (distance of DNA migration from the body of the nuclear core)with 4 categories: no damage (0 mm);	Haney J. T. and Connor T. H. (1999), Inhalation Toxicol. 11: 331-341

Method, Guideline, GLP status, Reliability	**Test substance, Doses, route and frequency of application**	**Relevant information about the study** *(e.g. species and strain, sex, No/Group, duration of exposure, sampling time)*	**Observations** **give dose, sampling time, result**	**Remarks** *(e.g. major deviations)*	**Reference**
		after exposure	damaged cells. According to the authors this could be attributed to the endpoint (Length of comet tail instead of DNA migration area or % of DNA in tail)	low damage (1–10 mm); medium damage (11–30 mm); high damage (31+mm).	
Comet assay/Sim. to OECD TG 489 Germ cell mutation Sim. to OECD TG 488 GLP. No Rel. 2 (SSBs) Rel. 3 (Muta™Mouse) **LOCAL**	Ozone (production photochemically from oxygen) Purity oxygen: 99.99 % Purity ozone (residual oxygen): not given Vehicle: air or air + oxygen Flow rate oxygen: 0.150 L/min Flow rate air: ozone mixed into flow of 24.5 L/min air Doses: BALB/c mice: 0-1-2 ppm (90 min) Muta™Mice: 0 or 2 ppm for 5 consecutive days Application: inhalative, whole-body	Species/strain/sex: Mice, BALB/c, female Muta™Mice129/SV, female Cells: BAL cells and lung cells No/group: Single exposure: 3-8/group Repeated exposure: 5/group Exposure: 90 min (BALB/c) or 90 min for 5 consecutive days (Muta™Mice) Sampling time: BALB/c: 20-1400 min after exposure Muta™Mouse: 14 days	<u>BALB/c mice</u> BAL cells: Statistically significant and linear dose-related increase in SSBs (reversible after a few hours; endpoint: tail moment). There was no additive effect of oxygen on strand breaks. Lung cells: No increase in SSBs. <u>Muta™Mouse</u> No difference in mutation frequency between exposed and control animals.	No positive control was used for the Muta™Mouse experiment. For this reason it remains unclear whether ozone reached the target. The study is therefore considered not acceptable. There were no changes in viability of exposed or unexposed BAL cells. <u>Further investigations with BALB/c mice:</u> - BAL fluid contains mainly macrophages (therefore no confounding by lymphocytes) - strong time- and dose-related increase in IL-6 mRNA in lung homogenate - DNA damage seems to peak before induction of pro-inflammatory cytokine IL-6 reaches maximum: indication that DNA damage is independent of inflammation - no difference in 8-oxo-dG/dG ratio or ERCC1 mRNA level in lung homogenate (indicator for DNA repair) between exposed and unexposed mice <u>Shortcomings:</u> - no purity or batch - viability of lung cells not investigated - deviations from treatment in OECD TG 488 (*e.g.*	Bornholdt J. et al. (2002), Mutat. Res. 520: 63-72

Method, Guideline, GLP status, Reliability	**Test substance, Doses, route and frequency of application**	**Relevant information about the study** *(e.g. species and strain, sex, No/Group, duration of exposure, sampling time)*	**Observations** **give dose, sampling time, result**	**Remarks** *(e.g. major deviations)*	**Reference**
		after exposure		treatment and sampling time) - no positive control - no observation of clinical signs - only 2 doses for SSBs, only 1 dose for Muta™Mouse - no individual data, different group size - no historical positive/negative control data given - no independent scoring mentioned - no hedgehogs reported - DNA SSB (single strand breaks) evaluation: tail moment was used (by Comet assay definition tail: area where the intensity lower than 10% of intensity in the head). Each tail moment was normalised by dividing the tail moment by the mean of tail moments of the untreated control mice for that day.	
Comet assay/Sim. to OECD TG 489 GLP: no Rel. 2 **LOCAL**	Ozone (ozone generator) Purity oxygen: "pure" Purity ozone (residual oxygen): not given Vehicle: air Flow rate: 28 L/min Doses: 0-0.4-1 ppm Application: inhalative, whole-body	Species/strain/sex: Hartley strain, guinea pigs, male Cells: tracheal epithelial cells, BAL cells (primarily macrophages) No/group: 5 guinea pigs Exposure: 2 h Sampling time: after	Endpoint: DNA migration distance, DNA migration area and DNA density Tracheal epithelial and BAL cells: 0.4 ppm and above: Statistically significant increase in DNA SSBs in comparison to control, also dose-related	Toxicity is indicated at 1 ppm by increased total protein and LDH as well as changes in cell differentiation in bronchoalveolar lavage. <u>Shortcomings:</u> - no purity or batch - no positive control - cytotoxicity in tracheal epithelial cells not investigated - no observation of clinical signs - no individual data (only figures) - only 2 doses - no historical positive/negative control data given - no hedgehogs reported - no independent scoring mentioned	Lee J.-G. et al. (1997a), Inhalation Toxicol. 9: 811-828

Method, Guideline, GLP status, Reliability	**Test substance, Doses, route and frequency of application**	**Relevant information about the study** *(e.g. species and strain, sex, No/Group, duration of exposure, sampling time)*	**Observations** **give dose, sampling time, result**	**Remarks** *(e.g. major deviations)*	**Reference**
		exposure		- DNA SSB (single strand breaks) evaluation: stained DNA length, area, and average fluorescence intensity was used (average DNA staining/area) <u>Further investigations in BAL cells:</u> - 1 ppm: increase in total protein and LDH content, increase in epithelial cells (and some other unidentified cells in cell differential), decrease in macrophages A study with 0.4 or 1 ppm ^{18}O-enriched ozone revealed a ~ 4.7 and 20.2 µg/g dry cell weight excess ^{18}O in lavage cells.	
DNA single strand breaks (FADU)/ No OECD TG GLP. No Rel. 2 **LOCAL**	Ozone (ozonizer) Purity oxygen: 100 % medical-grade Purity ozone (residual oxygen): not given Vehicle: air Flow rate oxygen 0.5 L/min, then dilution with air Doses: 0-0.45-1 ppm Application: inhalative, whole-body	Species/strain/sex: Dunkin-Hartley strain, guinea pigs, male Cells: tracheobronchial epithelial cells (TE cells) No/group: 4 guinea pigs Exposure: 72 h Sampling time: after exposure	Endpoint: % double-stranded DNA or DNA single strand breaks/TE cell 1 ppm: Statistically significant decrease in % double-stranded DNA and increase in DNA strand breaks/TE cell; also dose-related (but not statistically significant at 0.45 ppm)	Statistically significant % change in body weight change after exposure to 1 ppm. Statistically significant increase in protein content (possibly an indicator for inflammation) in the lavage fluid of trachea. However, no ozone-related impact on either TE cell yield or cell viability (trypan blue). <u>Shortcomings:</u> - no purity or batch - no positive control - no observation of clinical signs - only 2 doses for SSBs - no historical positive/negative control data given - no independent scoring mentioned - no individual data	Ferng S.-F. (2002), Inhalation Toxicol. 14:621-633

Method, Guideline, GLP status, Reliability	**Test substance, Doses, route and frequency of application**	**Relevant information about the study** *(e.g. species and strain, sex, No/Group, duration of exposure, sampling time)*	**Observations** **give dose, sampling time, result**	**Remarks** *(e.g. major deviations)*	**Reference**
				- only 4 animals per group	
Chromosomal aberration /Sim. to OECD TG 475 (bone marrow) and OECD TG 483 GLP: no Rel. 4 (mouse leucocytes), Rel. 3 for other test systems **SYSTEMIC**	Ozone (production in ozone generator with UV radiation) Purity oxygen: not given Purity ozone (residual oxygen): not given Vehicle: air/oxygen Flow rate air: 40 chamber air changes/h Doses: Hamster (bone marrow): 0-1.15-31.2 ppm/h corresponding to 5 h at 1.15 ppm/h and 6 h at 5.2 ppm Mouse (leucocytes): 0-0.75-1.05-1.98 ppm/h corresponding to 5 h at 0.15 ppm, 5 h at 0.21 ppm and 2 h at 0.99 ppm Mouse (spermatocyte): see exp. with leucocytes Application: inhalative, whole-body	Species/strain/sex: C3H mice, male; Chinese hamster, sex unclear Cells: Chinese hamster bone marrow, mouse peripheral leucocytes, mouse primary spermatocytes No/group: Not given Exposure: see left column Sampling time: Hamsters: 2, 6 or 12 h following termination of exposure Mice/blood: immediately or 2 weeks after exposure (further: 12 h and 1 week for lowest ozone conc. and 1 week for highest ozone conc.) Mice/spermatocytes: 8	Hamster: no effect Mice/leucocytes: small increase already at lowest dose, but not dose-related Mouse/spermatocyte: no translocations	As neither mutagenic potential was observed nor cytotoxicity was measured in hamster bone marrow cells or mouse spermatocytes it remains unclear whether ozone reached the targets. Therefore, the test is of limited reliability and considered not acceptable. <u>Shortcomings:</u> - no purity or batch - no positive control for bone marrow and spermatocytes (but ozone itself can serve as positive control for leucocytes) - no cytotoxicity - no historical negative/positive controls given - deviation from exposure and sampling time - unclear how many cells per animal were analysed - ploidy not tested - sex of hamsters not given - number of animals per sex/dose not given - unclear whether test protocol is also valid for leucocytes (but positive response in leucocytes) - colchichine treatment of hamster 2 h instead of 4-5 h prior sacrifice	Gooch P. C. et al. (1976), Environ. Res. 12:188-195

Method, Guideline, GLP status, Reliability	**Test substance, Doses, route and frequency of application**	**Relevant information about the study** *(e.g. species and strain, sex, No/Group, duration of exposure, sampling time)*	**Observations** **give dose, sampling time, result**	**Remarks** *(e.g. major deviations)*	**Reference**
		weeks after ozone exposure			
Chromosomal aberration and MN Sim. to OECD TG 474 and 475 GLP: no Rel. 2 **SYSTEMIC**	Ozone (production in ozonizer) Purity oxygen: "pure" Purity ozone (residual oxygen): not given Vehicle: air Flow rate air: 15 chamber air changes/h Doses: 0 or 0.5 ppm Application: inhalative, whole-body	Species/strain/sex: Mice B6C3F1 , male and female Cells: splenic lymphocytes (chromosomal aberrations) and reticulocytes (MN) No/group: 5 animals/dose/expo-sure time Exposure: 6 h/day and 5 days/week for 16, 32 and 52 weeks Sampling time: mice were sacrificed after 16, 32 and 52 weeks	0.5 ppm: time-dependent and statistically significant increase in aberrant cells in males and females (gaps not included) 0.5 ppm: time-dependent and statistically significant increase in micronucleated reticulocytes in males and females (no time-dependency: for males, 52 weeks) Only one dose was tested, hence no conclusion on dose-response is possible.	An increase in the number of chromosomal aberrations and MN vs. controls was observed. This increase was statistically significant and mainly related to exposure time. <u>Shortcomings:</u> - no purity or batch - no positive control (but ozone itself can serve as positive control) - cytotoxicity was not tested - only one dose tested - no individual data for each animal - ploidy not tested - unclear whether test protocol for chromosomal aberrations is also valid with its modifications for splenic lymphocytes (but positive response reported) - no historical positive/negative control data given - only 1000 erythrocytes/animal were examined - continuous treatment	Kim M. Y. et al. (2002), Mutagenesis 17: 331-336
MN / Chromosomal aberrations /HPRT Sim. to OECD TG 474, 475 and 476 (albeit *in vivo* test followed by 96-	Ozone (ozonator) Purity oxygen: "pure" Purity ozone (residual oxygen): not given Vehicle: air Flow rate air: 15 chamber air changes/h	Species/strain/sex: Mice, B6C3F1, male and female Cells: splenic lymphocytes (Chromosomal aberrations),	0.5 ppm: Statistically significant increase in chromosomal aberrations in comparison to control in male and female mice. Statistically significant	<u>Shortcomings:</u> - no purity or batch - no positive control (but ozone itself can serve as positive control) -no PCE/NCE ratio in the MN test (but positive response with ozone) - deviation from recommended treatment schedule in TG (*e.g.* colcemid treatment in chromosomal aberration assay	Kim M. Y. et al. (2001), J. Toxicol. Pub. Health 17: 1-6

Method, Guideline, GLP status, Reliability	**Test substance, Doses, route and frequency of application**	**Relevant information about the study** *(e.g. species and strain, sex, No/Group, duration of exposure, sampling time)*	**Observations give dose, sampling time, result**	**Remarks** *(e.g. major deviations)*	**Reference**
well microtiter plating) GLP: no Rel. 2 **SYSTEMIC**	Doses: 0 or 0.5 ppm **(same concentration as in NTP study, 1994)** Application: inhalative, whole-body	reticulocytes (MN), splenic cells (HPRT) No/group: 5 animals/sex/dose Exposure: 6 h/day and 5 days /week for 12 weeks Sampling time: after 12 weeks	increase in frequency of MN-reticulocytes in comparison to control in male and female mice. Mutation frequency of hprt gene in splenic cells was almost doubled in treated group in comparison to the control. Only one dose was tested, hence no conclusion on dose-response is possible.	*in vitro*, HPRT assay *in vitro* following inhalation experiment *in vivo* and continuous treatment of animals) - only 1000 erythrocytes per animal evaluated in the MN test - only one dose - no independent scoring mentioned - no cytotoxicity tested for MN/chromosomal aberrations - only 5 animals per dose/sex - no individual data for each animal - ploidy not tested - no historical positive/negative control data given - unclear whether cells used for HPRT assay are appropriate (but positive result)	
MN/ Sim. to OECD TG 474 GLP: no Rel. 2 **SYSTEMIC**	Ozone (electrical discharge of air) Purity oxygen: not given Purity ozone (residual oxygen): not given Vehicle: air Flow rate air: not given Doses: 0 or 3 ppm Application: inhalative, whole-body	Species/strain/sex: Rats, Wistar, male Cells: bone marrow erythrocytes No/group: 4 animals/dose Exposure: 6 h/day for 10 days Sampling time: immediately (treatment group 1) or 11 days after	3 ppm: statistically significant increase in frequency of MN in bone marrow erythrocytes in comparison to control in both groups (higher in treatment group 2) Only one dose was tested, hence no conclusion on dose-response is possible.	The small size of MN points to structural damage of chromosomes. PCE/NCE + PCE ratio was reduced statistically significantly after treatment pointing to bone marrow toxicity (reversibility: higher value in treatment group 2) <u>Shortcomings:</u> - no purity or batch - no positive control - only 4 animals/dose - females not tested - only one dose tested - no individual data for each animal	Haddad et al. (2009), Ferdowsi University International Journal of Biological Sciences (Volume unclear): 41-46

Method, Guideline, GLP status, Reliability	**Test substance, Doses, route and frequency of application**	**Relevant information about the study** *(e.g. species and strain, sex, No/Group, duration of exposure, sampling time)*	**Observations** **give dose, sampling time, result**	**Remarks** *(e.g. major deviations)*	**Reference**
		last ozone inhalation (treatment group 2)		- ploidy not tested - deviation from recommended treatment schedule in TG - no historical positive/negative control data given	
(1) In vivo Comet (blood) not performed according a specific guideline, but similar to OECD 489 (2016) In vivo mammalian alkaline comet assay (2) in vivo bone marrow micronucleus assays Similar to OECD 474 (2016) Mammalian erythrocyte micronucleus test **GLP:** no **Reliability:** 2	Ozone, gas Purity ozone: not given Application: inhalative, whole body Control: Dose: 0.05 ppm Ozone generated by air purifier	Species/strain/sex: Rat, Wistar Rat, male No/ group: 6 rats Exposure duration: 3 h/day and 24 h for 14 (acute study) and 28 days (sub-acute study).	(1) vivo Comet (blood) - negative (2) vivo bone marrow micronucleus assays - negative	only one dose level tested <u>Shortcomings:</u> OECD 489: only one positive control animal, individual results not available; only mean and standard deviation reported graphically, images of 100 randomly cells (50 cells from each of two replicated slides) were analyzed from each rat and OECD 489 guideline states 150 cells per animal. Comet assay conducted with whole blood and verification that test material reached the target tissue was not available.. OECD 474: no positive controls, approximately 2000 polychromatic erythrocytes (PCE's) scored per animal in the study (1000 cells x 2 replicates per animal) vs. the test guideline suggested 4000 per animal, individual results not available; only mean and standard deviation reported graphically, no verification of target tissue exposure, only proportion of immature erythrocytes among total erythrocytes reported, no data on the number of micronucleated immature erythrocytes for each animal or mean ± standard deviation of micronucleated immature erythrocytes per group; individual results not available. There are limitations in data reporting versus guideline recommendations.	Cestonaro et al. (2017), Environ Sci Pollut Res 24, 22673–22678

Table 22: Summary table of human data relevant for germ cell mutagenicity

Method, Guideline, Study design, GLP status, Reliability	**Test substance, Doses, route and frequency of application**	**Relevant information about the study** *(e.g., sex, No/Group, duration of exposure, sampling time, examinations, endpoints)*	**Observations** **give dose, sampling time, result** **Remarks** *(e.g. major deviations)*	**Reference**
Comet assay/Sim. to OECD TG 489 Study design: Longitudinal study design; subjects were exposed to air or ozone for 2 h GLP: no Rel. 2 **LOCAL**	Ozone (ozone generator) Purity oxygen: "pure" Purity ozone (residual oxygen): not given Vehicle: air Flow rate: not given Doses: 0 and 0.4 ppm Application: inhalative, whole-body, 2 h exposure	male and female (number of persons unclear) Each person was exposed to either air or 0.4 ppm ozone. (without exercise) on separate days; at least 4 weeks apart Examinations: BAL (1-2 h after exposure), bronchoscopy (after exposure), bronchial epithelial cells (time unclear) Relevant endpoints: DNA SSB Only one dose was tested, hence no conclusion on dose-response is possible.	<u>Bronchial epithelial cells and lavage cells:</u> No significant difference in DNA single strand breaks SSB in comparison to control (represented by change of DNA length). However, a moderate increase in mean values was measured. <u>Comment:</u> Healthy participants without asthma, allergic rhinitis or chronic respiratory disease were used. No purity or batch. Number of participants unclear. No individual data (only figures). No hedgehogs reported. No independent scoring mentioned. No cytotoxicity determined. - DNA SSB (single strand breaks) evaluation: stained DNA length, area, and average fluorescence intensity was used (average DNA staining/area). In a further experiment it was shown that pretreatment of humans with steroids before ozone exposure (under exercise) has no statistically significant impact on DNA SSBs (DNA length, DNA area and density). However, exposure of persons to ozone under exercise led to statistically significant increase of SSBs in epithelial cells relative to air control without exercise.	Lee J.-G. et al. (1997b), Inhalation Toxicol. 9: 811-828
Endpoint: MN/Sim. to OECD TG 474 Study design: Longitudinal study design; subjects performed intermittent moderate-	Ozone (production not specified) Purity oxygen: not given Purity ozone (residual oxygen): not given Vehicle: air	10 male and 12 female Each person was exposed to 0-0.1 or 0.2 ppm ozone for 4 h (including alternating 30-min exercise and rest periods); 3-week recovery	4 h exposure to doses of 100 and 200 ppb was "chosen to represent real world low and high ambient levels of ozone" in the US Lymphocytes: - significant and dose-dependent increase in MN frequency and number of micronucleated cells in all groups	Holland N. (2015), Environ. Mol. Mutagen. 56: 378-387

Method, Guideline, Study design, GLP status, Reliability	**Test substance, Doses, route and frequency of application**	**Relevant information about the study** *(e.g., sex, No/Group, duration of exposure, sampling time, examinations, endpoints)*	**Observations** **give dose, sampling time, result** **Remarks** *(e.g. major deviations)*	**Reference**
intensity exercise for 4-h in a chamber with air or ozone GLP: no Rel. 2 **SYSTEMIC**	Flow rate: not given Doses: 0-0.1-0.2 ppm Application: inhalative, in 2.5*2.5*2.4 m chambers, 4 h exposure	periods in between Endpoints were measured before and 24 h after exposure Examinations: BAL, bronchoscopy (only after 24 h), blood lymphocytes (cytogenetic damage) Relevant endpoints: MN, nucleoplasmic bridges, nuclear buds, cytotoxicity, airway neutrophilia	- effect in air control could be attributed to exercise-mediated oxidative stress - frequencies of nuclear buds and bridges also dose-dependent increased, but not statistically significant - significant and dose-dependent increase in apoptotic cells (post-exposure) - no differences in necrotic cells - cell proliferation was not statistically significant affected - MN, buds, bridges, apoptotic cells and necrotic cells differed between base line and air controls → possibly not all effects ozone-related (effects of neutrophilia and exercise may lead to oxidative stress in blood stream and chromosome damage) BAL: - concentration of neutrophils increased dose-related but not statistically significant Logistic regression model: Neutrophilia and ozone exposure were statistically significant associated with MN frequency (but independent) <u>Comment:</u> Healthy participants or subjects with mild asthma in remission were used. Only 22 participants used. No purity or batch. Only 1000 binucleated lymphocytes were analysed.	
SCE/Sim. to OECD TG 479 GLP: no Rel. 4	Ozone (production with ozone generator) Purity oxygen: not given Purity ozone (residual	31 volunteers (both sexes) Each person was exposed to either air or 0.5 ppm for 2 h	No increase in total number of SCEs or SCEs/chromosome after ozone exposure. <u>Shortcomings:</u>	Guerrero et al. (1979), Environ. Res. 18:336-346

Method, Guideline, Study design, GLP status, Reliability	**Test substance, Doses, route and frequency of application**	**Relevant information about the study** *(e.g., sex, No/Group, duration of exposure, sampling time, examinations, endpoints)*	**Observations** **give dose, sampling time, result** **Remarks** *(e.g. major deviations)*	**Reference**
SYSTEMIC	oxygen): not given Vehicle: air Flow rate: not given Dose: 0 and 0.5 ppm Application: inhalative, whole-body, 2 h exposure (including exercise)	ozone (with exercise). Examinations: blood (human peripheral lymphocytes) after exposure Relevant endpoints: SCE	No description of ozone production method. No purity or batch. Only one dose. Confounders like smoking habits or asthma were not queried No single data for each culture. Cytotoxicity not tested.	
DNA single-strand breaks (Fast Micromethod)/ No OECD TG Study design: Randomized parallel study; subjects were exposed to air or ozone for 2 h GLP: no Rel. 2 **SYSTEMIC**	Ozone (source unclear) Purity oxygen: "pure" Purity ozone (residual oxygen): not given Vehicle: air Flow rate: not given Doses: 0 and 0.21 ppm Application: inhalative, whole-body, 2 h exposure	19 male subjects (placebo group) 18 male subjects (ozone group) Persons were exposed to either air or 0.21 ppm ozone (with exercise). Examinations: blood (human peripheral lymphocytes), before exposure and 30 min, 4.5 h afterwards Relevant endpoints: SSBs Only one dose was tested, hence no conclusion on	No significant difference between strand scission factor values between the exposed and control group at both time points. <u>Comment:</u> Healthy participants without smoking habits were used. No purity or batch. No individual data (only figures). No independent scoring mentioned. No source for ozone given. No cytotoxicity measured.	Finkenwirth et al. (2013), Human and experimental Toxicology (volume unclear): 1-5

Method, Guideline, Study design, GLP status, Reliability	**Test substance, Doses, route and frequency of application**	**Relevant information about the study** *(e.g., sex, No/Group, duration of exposure, sampling time, examinations, endpoints)*	**Observations** **give dose, sampling time, result** **Remarks** *(e.g. major deviations)*	**Reference**
		dose-response is possible.		
Measurement of chromatin modification levels **Guideline:** no OECD TG available **GLP:** no **Reliability: 2**	Ozone, gas Purity ozone: not given Control: clean air Dose: 0.5 ppm Incubation: 2 hours	Primary human bronchial epithelial cells from a panel of 11 donors (from 17 healthy, non-smoking donors aged 18–40 (13 males and 4 females)) Immediately after exposure cells were removed from the chambers and total RNA was harvested	The authors demonstrate that baseline levels of specific chromatin modifications correlate with the interindividual variability in both basal and ozone-induced expression of proinflammatory stress genes. Shortcomings: only one dose level tested, no positive control	McCullough et a. (2016), Toxicological Sciences, 150(1), 216–224,

10.8.1 Short summary and overall relevance of the provided information on germ cell mutagenicity

In Vitro

Only studies from the public domain were submitted by the applicant for draft risk assessment report (draft CAR for BPR) for "ozone generated from oxygen" in accordance with Regulation (EU) No 528/2012. Therefore, the overall conclusion is based on open literature data. None of these studies fully complies with the relevant OECD TG recommendations. Accordingly, a weight of evidence approach was taken.

Under the conditions of the published studies and based on the information given therein, ozone is mutagenic in bacterial strains and mammalian cell lines. These findings are supported by positive indicator tests in vitro.

(1) Mutagenicity studies in bacteria

The mutagenic potency of ozone was investigated in different bacterial strains.

Dillon et al. exposed S. typhimurium strains TA100, TA98, TA1535, TA104 and TA102 for 35 min with several ozone concentrations (0.02-9 ppm) in the presence and absence of liver S9-mix. No doubling of revertant colonies was observed in strain TA100, TA98, TA1535 and TA104.

In contrast to this, ozone induced a dose-related 2-3 fold increase in revertant colonies in strain TA102 at 0.02 ppm and above. This increase was statistically significant from air control and independent of S9 mix.

A statistically significant increase in revertants/plate was also observed in strain TA104 (+S9-mix) at 0.04 ppm. However, this effect was not considered relevant as it was not reproducible in another experiment. Furthermore, no doubling of revertant colonies was reached at this dose.

Cytotoxicity was remarkable in all strains at around 0.4 ppm (TA102) or 1-4 ppm (remaining strains) reflected by a rapid decline in revertant colonies.

Victorin and Stahlberg examined the mutagenicity of ozone in a dose range between 0.1 and 3.5 ppm in bacterial strain TA100. The incubation time was set at 6 h. Ozone did not pose any mutagenic activity (no doubling of revertant colonies). Cytotoxicity in TA100 was observed at ~ 2 ppm. Experiments performed with TA102 and TA104 were negative but cannot be used for evaluation as no positive control, individual data or cytotoxicity testing was presented for both strains.

A further publication retrieved after literature search by the dossier submitter supports the assumption that TA100 is not involved in ozone-mediated mutagenicity (Shepson 1985). The authors incubated bacterial strain TA100 with 0.5 ppm ozone for 20 h. No mutagenic effects were reported by the Shepson et al. (1985). Information about cytotoxicity was not given in the publication.

Taken together, the mutagenic activity of ozone seems to depend on the bacterial strain used which in turn represents a certain mechanism of mutagenicity. Strain TA102 is sensitive to oxidative damage as mediated by peroxides and oxygen radical generators (Abu-Shakra and Zeiger 1990, Levin et al. 1982). In a similar way the cytotoxicity of ozone depends on the bacterial strain and increases with dose. Given that mutagenicity was independent of S9-mix, ozone seems to act as a direct mutagen.

(2) Mutagenicity studies in mammalian cells

Studies on mutagenic endpoints (as defined in CLP regulation) were conducted in different cell lines.

Gooch et al. (1976) incubated human peripheral lymphocytes with several ozone doses between 1.3 and 7.5 ppm/h (12 h following PHA) or 1.65 and 14.2 ppm/h (36 h following PHA) for different time intervals. The incubation was performed in the absence of S9-mix. The exact time of exposure was not reported, hence the actual ozone concentration remains unclear. In a further experiment leukocytes were added to ~ 2 ppm ozone-saturated phosphate-buffered saline D – again 12 h (incubation 30-90 min) or 36 h (incubation 5-90 min) after PHA stimulation. The percentage of cells with chromosomal or chromatide aberrations remained basically unchanged in comparison to controls after incubation with ozone-saturated solution or after ozone treatment

12 h following PHA stimulation. In contrast to this, ozone treatment 36 h after PHA stimulation resulted in a 3 to 4-fold or 1.4 to 3-fold increase in chromatide aberrations at 7.23 or 7.95 ppm/h in comparison to controls, respectively. This effect was not dose-related. Cytotoxicity was not reported by the authors.

Guerrero et al. (1979) incubated a fetal lung cell line (WI-38 cells) with ozone in the range between 0.25 and 1 ppm for 1 h without S9-mix. The authors reported a dose-related increase in the percentage of cells exhibiting endoreduplications (at 0.5 ppm and above) or chromatide deletions (at 0.75 ppm and above). Cytotoxicity was not tested by the authors.

A publication retrieved after literature search also addresses chromosomal aberration formation in mammalian cells.

The publication by Fetner et al. (1962) reports on the exposure of the KB human cell line at a dose of 8 ppm ozone. The incubation time was set at 5 or 10 min. S9-mix was not added to the incubation. Whereas no deletions were found in the negative control, 20 deletions per 4158 chromosomes or 23 deletions per 1283 chromosomes were observed in the treated groups after exposure for 5 or 10 min, respectively. The authors further mention that cells dislodge from the glass surface at higher doses or longer exposure time.

Another publication – also retrieved after literature search – investigated the impact of ozone on embryonic chick fibroblasts. Sachsenmaier et al. (1965) detected cytotoxic (e.g. lysed cells or shrunken nuclei) or genotoxic effects (anaphase and telophase bridge formation) in cells after exposure to 1-10 γ ozone/ml for 30 min (-S9-mix). According to Victorin (1992) this dose corresponds to a concentration of 700-7000 ppm.

Besides chromosomal aberration induction, the applicant also submitted a short communication by Chorvatovicova et al. (2000) focusing on MN formation in rat alveolar type II cells. The cells were exposed to 400 ppb ozone for 6 h in the absence of S9-mix. A statistically significant increase (2.5-fold) in MN formation/1000 cells in comparison to the negative control was measured. Only one dose was tested, hence no conclusion on dose-response is possible. Moreover, no comment on cytotoxicity was given by the authors.

To sum up, ozone resulted in chromosomal aberration and MN formation in all in vitro test systems reported. The absence of genotoxic effects after exposure to ozone-saturated phosphate-buffered saline D may be due to the high reactivity of ozone in buffer. Cytotoxicity tests were mostly not reported in the studies, hence a relationship between cytotoxicity and genotoxicity cannot be evaluated.

(3) Indicator tests in mammalian cells

Guerrero et al. (1979) incubated a human fetal lung cell line – namely WI-38 – with ozone at doses ranging from 0.25-1 ppm for 1 h. S9-mix was not added to the incubation mixture. It was found that ozone leads to a statistically significant and dose-related increase in SCEs/chromosome from negative control already at the lowest dose tested. Cytotoxicity tests were not reported in the study.

The test guideline for SCE has been deleted in 2014, hence further publications from literature search addressing this endpoint are not listed hereafter.

Díaz-Llera (2002) determined the potency of ozone to induce DNA strand breaks with the Comet assay. For this purpose human peripheral blood lymphocytes (obtained from 6 donors) were exposed for 1 h to different ozone concentrations ranging from 0.875-5.25 mM. The authors dispensed with the application of S9 mix. Beginning at 0.875 there was a dose-related increase in percentages of damaged cells as well as a statistically significant and dose-related increase of tail image length in comparison to the untreated control. A cell viability assay revealed that only minimal or no cytotoxic effects occur at the dose levels used.

After literature search performed by the dossier submitter 4 further publications focusing on DNA strand breaks were retrieved.

Lee et al. (1996) also investigated strands breaks by the Comet assay method. Either human bronchial cells (BEAS-2B) or SV-20 transformed human tracheobronchial epithelial cells (NHBE) were exposed to 0.1 ppm ozone for 60 or 120 min and 0.4 ppm for 20, 40 or 60 min or 0.4 ppm ozone for 60 min, respectively. No S9-mix was used. A time-dependent increase of the DNA migration area – which also resulted in statistically significant effects after 120 min (0.1 ppm) or 40 and 60 min (0.4 ppm) – was observed in BEAS-2B cells. These effects were accompanied by significant increases in DNA length and decrease in DNA density. The

DNA migration area was further statistically significantly increased in NHBE cells at 0.4 ppm in comparison to the air control. A decrease in cell viability determined with the LDH assay was measured in both cell types after 1h-exposure to 0.1-1 ppm ozone, hence an impact of ozone-mediated cytotoxicity on genotoxic events cannot be ruled out at longer incubation times.

Zee et al. (1987) determined DNA strand breaks with the alkaline elution or FADU method. No S9 mix was used. Murine L929 fibroblasts were cultured in ozone loaded medium, generated by bubbling with gas containing 61 % (vol/vol) of ozone, for 2 h (alkaline elution method) or up to 150 min (FADU technique). Whereas only minimal effects were observed in the alkaline-elution method, ozone led to a decrease in double-stranded DNA already after 30 min ozone exposure using the FADU method. This decrease was time-related. The authors did no comment on ozone-mediated cytotoxic effects. However, such effects should be taken into account at the dose level tested. Furthermore, indications for the involvement of ozone in the formation of interstrand crosslinks and DNA protein crosslinks were given in the publication. The authors explained the negative results obtained after alkaline elution with the presence of those crosslinks which to their opinion interfere with the sensitivity of this method.

Borek et al. (1988) used the sucrose gradient centrifugation or alkaline elution method for determining DNA strand breaks in human epidermal cells (RHEK line) after exposure to ozone. Cells were treated with 5 ppm ozone for 10 min. There was no obvious induction of ozone-mediated increase in DNA strand breaks with both methods applied. The authors argued that a reason for this finding could be the freezing and thawing procedure of cells (negative control or treatment group) which may contribute to the induction of DNA strand breaks thereby limiting the resolution of the method. This technical limitation became in particular apparent with the alkaline elution method – which is according to the authors more sensitive than the alkaline sucrose gradient technique. Cytotoxicity was not determined in this study.

The DNA alkaline elution assay was also applied by Kozumbo and Agarwal (1990). The aim of their study was to determine the DNA damaging potential of arylamines in CCD-18Lv human lung fibroblasts and A549 human lung type II epithelial cells after treatment with ozone. Ozone exposed buffer served as negative control. Human lung fibroblasts were exposed to ozonated buffer (1 ppm for 2h). The dose and duration of exposure was not given for the human lung type II epithelial cells. Kozumbo and Agarwal concluded that there was no increase in DNA strand breaks in comparison to the unexposed buffer. No cytotoxic effects were observed using the trypan blue dye exclusion assay.

Taking the results of the indicator tests into consideration, ozone induces SCE/DNA strand breaks in mammalian cell lines. Negative findings are rather due to technical weaknesses of the methods applied than true negatives. Zee et al. (1987), Borek et al. (1988) as well as Kozumbo and Agarwal (1990) made use of the alkaline elution method which may lower the sensitivity in the presence of interstrand or DNA protein crosslinks. Likewise freezing-thawing procedures may decrease the resolution by inducing artefacts. Another aspect leading to false-negative results is the exposure with ozone in combination with a buffer. Ozone is a very reactive gas that may react with a buffer before reaching the target system. Again, cytotoxicity studies in the publications are scarce. This hampers a clear correlation of genotoxicity with cytotoxicity. However, the study by Díaz-Llera (2002) indicates that clear genotoxic effects may occur independent of cytotoxicity.

In Vivo

Also for the in vivo situation only studies from the public domain were submitted by the applicant for draft risk assessment report (draft CAR for BPR) for “ozone generated from oxygen” in accordance with Regulation (EU) No 528/2012. No publication fully complies with the appropriate OECD TG criteria. Therefore, the overall conclusion is based on a weight of evidence approach.

Under the conditions of the published studies and based on the information given therein, ozone possesses mutagenic potency in animals and humans. This is supported by positive findings in indicator tests for genotoxicity. Negative findings seem to be the result of the chosen dose and time or combination of both in the experiments. Further evidence for ozone-mediated mutagenicity and genotoxicity in vivo is provided by epidemiological studies.

(1) Indications for germ cell mutagenicity or chromosome abnormalities in vitro and in vivo

After literature search performed by the dossier submitter a dominant lethal test in flies was retrieved. Erdman & Hernandez (1982) exposed male Drosophila virilis flies for 3 h to 30 ppm ozone. The number of pupaes that failed to develop from eggs was determined as a measure of dominant lethals in the offspring. The number of dominant lethals was elevated after ozone treatment.

In the review published by Victorin in 1992 a further study with flies - but Drosophila melanogaster - (Chigusa and Nakada, 1972 article in Japanese) is mentioned in which the genetic effects of ozone on fecundity, hatchability, emergence rate and longevity are presented. Victorin came to the conclusion that ozone exposure to females (1) induces dominant lethals, (2) is connected with a life-span shortening of male offspring and (3) decreases the hatchability of eggs after repeated exposure to 27 ppm for 1-2 h.

A further indication for ozone-mediated germ cell mutagenicity is given in the evaluation of the US-EPA (2013). It is reported that exposure to 0.2 ppm ozone during gestation leads to mutagenic effects in the offspring. The reference for this study was not cited in the text, but identified by literature search performed by the dossier submitter. In the referred study published by Brinkman et al. (1964)- that was further taken up in a study published by Veninga (1967) – the toxicity of ozone was compared with detrimental health effects mediated by ionizing radiation. In both publications only little information is given on the experimental study design (e.g. number of animals, number of litters affected by toxicological effects). Either Grey mice (inbred strain from University of Groningen) or inbred C57 black mice were exposed to air, 0.1 (only Grey mice) or 0.2 ppm ozone for 7 h/day and 5 days/week over 3 weeks. Ozone-mediated impact on litter size, number of litters, neonatal death and congenital abnormalities were investigated. Brinkman et al. (1964) report that litter size from couples of Grey mice was normal whereas the number of litters was almost halved after ozone exposure (0.2 ppm) in Grey mice or C57 black mice. The neonatal mortality in the first 3 weeks was 6.8 % (0.1 ppm ozone) and 7.5 % (0.2 ppm ozone) against 1.6 % in the control animals. The neonatal mortality was also increased to 34 % in C57 black mice treated with 0.2 ppm ozone against 9 % in the control animals. Besides neonatal death, a higher frequency of blepharophimosis (unilateral or occasionally bilateral) was observed in inbred Grey mice. The frequency increased from 0.6 % or 4.5 % in the controls to 9.6 % or 9.2 % in ozone treated Grey or C57 black mice, respectively. In the latter strain this finding was accompanied by increased jaw anomalies (unlimited growth of incisors) after exposure to 0.2 ppm ozone (5.4 %). Veninga (1967) stresses that this anomaly normally occurs in only 0.9 % of new-born mice. It could be assumed that the observed jaw anomalies are one explanation for the strong neonatal mortality observed in C57 black mice.

After literature search by the dossier submitter conducted a cross-sectional study performed in Poland by Jurewicz et al. (2015) was retrieved. In this study the relationship between human exposure to air pollutants (e.g. sulphur dioxide or ozone) and sperm disomy (hereinafter referred to as sperm aneuploidy) was investigated. Air quality data were taken from the AirBase database that in case of ozone includes the maximum 8-h average collected at 52 monitoring stations. The mean value was 45.09 µg/m³ (22.5 ppb). Sperm from a number of 212 men attending an infertility clinic was used for aneuploidy analysis. Sperm aneuploidy for chromosomes 13, 18, 21, X and Y was investigated by means of multicolour fluorescence in situ hybridization. According to the authors there was no association between ozone pollution and sperm aneuploidy either after multivariate analysis (adjustment for different confounders like smoking, age, alcohol consumption) or multivariate analysis with other air pollutants (i.e. further adjustment to other air pollutants like sulphur dioxide and PM10).

Taken together, there are indications for germ cell mutagenicity by ozone in both flies and mice. However, no study is in conformity with the actual OECD test guidelines for studying germ cell mutagenicity.

The mice study suffers additionally from a very poor data quality which hampers the transparency and validity of the effects presented. Therefore, it remains difficult to draw a clear conclusion on germ cell mutagenicity in mammals. In contrast to this, sperm aneuploidies were not associated with ozone burden in humans.

(2) Mutagenic studies in vivo

(a) local effects

Rithidech (1990) exposed female rats once for 6 h to several ozone doses between 0.1 and 0.6 ppm. Afterwards, pulmonary alveolar macrophages were isolated and chromosomal damage as an increase in abnormal cells was investigated. The number of abnormal cells was dose-related increased after exposure to 0.1 or 0.22 ppm

ozone. However, at the next higher dose (0.6 ppm) a decrease of abnormal cells was observed. The authors explained this finding with an increase in ozone-mediated influx and division stimulation of macrophages. They further argued that this dilution of macrophages could be the reason for an underestimation of cytogenetic effects. This theory is supported by a dose-related increase of the mitotic index from 0.22 to 0.6 ppm. In contrast to this, the mitotic index was not affected at the lowest dose applied (0.1 ppm), but strongly reduced at 0.22 ppm.

(b) systemic effects

Gooch et al. (1976) investigated the potency of ozone to induce genetic damages systemically. Male mice were once exposed for 5 h to 0.15 ppm/h or 0.21 ppm/h or for 2 h to 0.99 ppm/h corresponding to ozone doses of 0.75, 1.05 or 1.98 ppm. Chromosomal and chromatide aberrations were determined in leukocytes. For this purpose, blood was taken immediately or up to 2 weeks after exposure.

A slight increase in both chromosomal and chromatide aberrations was obtained after ozone treatment in comparison to the untreated controls. However, the effect was neither dose-related nor correlated with time of blood withdrawal after ozone exposure. Ozone-mediated cytotoxicity was not measured.

Kim et al. (2001) treated male and female mice for 6 h/day and 5 days/week with 0.5 ppm ozone for an overall exposure period of 12 weeks. The systemic DNA damage induced by ozone was measured in lymphocytes, reticulocytes and splenic cells by means of chromosomal aberration, MN formation or mutation frequency in hprt gene, respectively. Ozone treatment was connected with a statistically significant increase in chromosomal aberrations and MN formation in both genders. Furthermore, the mutation frequency in splenic cells from ozone-treated mice was almost doubled in comparison to the untreated control animals. Whereas no cytotoxicity was determined in lymphocytes and reticulocytes, no obvious toxicity was evident in splenic cells (clonal efficiency: 0.23 and 0.19 in control and treated animals, respectively).

Kim et al. (2002) repeated this study, but extended exposure time to 16, 32 or 52 weeks. Afterwards, splenic lymphocytes and reticulocytes were taken for analyses of chromosomal aberrations or MN formation, respectively. Ozone treatment resulted in both genders again in a time-related and statistically significant increase in chromosomal aberrations and MN. Also in this study cytotoxic effects were not reported.

Haddad et al. (2009) used male rats for their MN test in bone marrow erythrocytes. Animals were exposed to 3 ppm ozone for 6 h/day for 10 consecutive days. Animals were sacrificed immediately (treatment group 1) after ozone exposure or 11 days (treatment group 2) after the last ozone treatment. Independent from time point of sacrifice there was a statistically significant increase in MN frequency in comparison to the negative controls. Furthermore, the PCE/NCE + PCE ratio was reduced in both treatment groups (less pronounced in treatment group 2). This finding indicates on the one hand that the bone marrow was reached by the test substance (or its derivatives) and on the other hand that ozone-mediated cytotoxicity is reversible.

After literature search performed by the dossier submitter 3 further publications focusing on chromosomal/chromatide aberrations were retrieved.

Zelac et al. (1971a) and Tice et al. (1978) exposed male and female Chinese hamsters to 0.24 or 0.3 ppm and 0.43 ppm ozone for 5 h, respectively. Blood was taken immediately, 1 week or 2 weeks after ozone exposure. Afterwards, chromosomal aberrations were scored in lymphocytes. Zelac et al. (1971a) determined an increased frequency in chromosome breaks after ozone treatment in comparison to untreated controls that did not diminish with time. Tice et al. (1978) also detected ozone-mediated DNA damage reflected by a statistically significant increase in abnormal cells with chromatide aberrations. This effect became apparent at later sampling times (1 or 2 weeks). Besides lymphocytes, Tice et al. (1978) could also detect an increased frequency in abnormal cells with chromatide aberrations – even though not statistically significant – in bone-marrow cells. Whereas no indicators for cytotoxicity was measured by Zelac et al. (1971a), Tice et al. (1978). reported that replication rates after ozone treatment were not markedly affected.

In another study from Zelac et al. (1971b) the impact of ozone was investigated in Chinese hamsters that were additionally exposed to x-radiation. For this purpose, animals were exposed for 5 h to 0.2 ppm ozone. Blood was taken ~ 2 weeks after treatment and lymphocytes were scored for chromosomal aberrations. The authors reported on a slight increase in chromosomal aberrations after additional treatment with ozone. The authors did not show any results regarding possible cytotoxic effects of ozone.

Together with both other studies studying chromosomal aberrations in lymphocytes of Chinese hamsters after 5 h-exposure to ozone (Zelac et al 1971a and Tice et al. 1978) this points to a dose-related mutagenic effect.

In a review published by Victorin in 1992 a study written by Zhurkov et al. (1979, Russan article) is described as follows: Male rats were exposed to either 0.075 ppm ozone for 8 h and 7 days or 2.8 ppm ozone continuously for 5 days. According to the review article neither chromosomal nor chromatide aberrations were determined in bone marrow cells. The authors further report on a depressed mitotic activity in the bone marrow cells after exposure to 2.8 ppm ozone.

The applicant further submitted a paper addressing mutagenicity in human lymphocytes after short-term exposure to ozone.

This study published by Holland et al. (2015) addresses the MN formation in blood lymphocytes of humans after single exposure to 0.1 or 0.2 ppm ozone for 4 h. Each 11 male and female persons were allowed to exercise for 30 minutes during ozone treatment in order to increase ozone intake. Smoker or persons suffering from cardiovascular, pulmonary or hematologic diseases (other than mild asthma) were excluded from the study. The authors report on a dose-related and statistically significant increase in MN frequencies. Whereas cell proliferation was not affected by ozone treatment, the percentage of apoptotic cells increased statistically significantly after exposure. It was further concluded that also exercise has a detrimental impact on DNA integrity – most likely attributed to oxidative stress – as reflected by higher MN formation frequency following exercise in the untreated group. According to the authors another factor contributing (independent from ozone exposure) to MN formation could be recruitment of neutrophils as indicated in bronchoalveolar lavage.

Hereafter, human studies retrieved after literature by the dossier submitter focusing on mutagenic endpoints are summed up.

In a short communication Merz et al. (1975) describe the potency of ozone to induce chromatide or chromosome type aberrations in individuals. For this purpose, 2 persons were exposed to 0.5 ppm ozone for 6 h and 4 further persons for 10 h. The persons served as their own controls (pre-exposure and post-exposure). Aberrations were determined in lymphocytes. Blood was taken either immediately after exposure (6 h) or additionally 2 and 6 weeks after exposure (10 h). The authors detected an increased number of chromatide type aberrations after ozone exposure in both treatment groups. Shortcomings of this study are that (1) only 2 or 4 persons were used per group, (2) confounders like smoking habits were not queried and (3) no cytotoxic impact of ozone was reported.

McKenzie et al. (1977) studied chromosomal and chromatide aberration formation in blood lymphocytes after single exposure to 0.4 ppm ozone for 4 h in 26 healthy and non-smoking individuals. During the exposure period individuals were allowed to moderate exercise for 15 min. Blood samples were collected immediately after or 3 days, 2 weeks or 4 weeks after exposure. The results from all individuals were pooled. The frequency with cells showing chromosomal and/or chromatide aberrations in individuals after ozone treatment remained basically unchanged. Cytotoxic effects mediated by ozone - that could give evidence whether the test substance reached the target organ - were not investigated in this study.

The same author published 5 years later a study (McKenzie,1982) in which genotoxic effects of ozone were further investigated in lymphocytes of subjects prior and after single exposure to 0.6 ppm ozone for 2 h or 0.4 ppm ozone for 4 h (once or repeated exposure for 4 days). A number of 10-30 healthy, non-smoking adult males per exposure duration were enrolled in the study. During exposure subjects exercised on a bicycle ergometer for two 15-minute periods. Blood samples were taken prior to exposure, immediately post-exposure, at 3 days, at week 2 and week 4 post-exposure. Also in this study results from all individuals were pooled. According to the authors there were only non-significant differences in the frequency of numerical or structural aberrations. Again cytotoxic effects of ozone - in order to evaluate whether ozone reached the target – were not addressed in the study. Another weakness of the study is the poor reporting on experimental details.

In the review published by Victorin in 1992 a further study published by Sarto and Viola (Italian article) is mentioned. Sarto and Viola (1980) investigated a possible relationship between ozone exposure and cytogenetic damage in lymphocytes. For this purpose, DNA damage in lymphocytes from 10 workers exposed to 0.3 ppm ozone for 1-3 years were compared with lymphocytes from 10 unexposed workers. According to Victorin the frequency of chromatide gaps increased statistically significant in the lymphocytes of exposed

workers. No other chromosomal aberrations were observed. As a shortcoming of the study no confounders (e.g. smoking habits or other air pollutants in the workplace) were taken into account.

Ozone is a very reactive gas and is therefore predicted to induce mutagenic effects in cells of first contact. In agreement with this assumption chromosomal aberrations were detected in pulmonary alveolar macrophages after ozone exposure to rats. Furthermore, there is evidence from many studies that ozone also leads to mutagenic effects in cells distant from site of first contact as indicated by positive MN in murine reticulocytes and rat bone marrow cells. Chromosomal aberrations after ozone exposure were detected in murine leucocytes and splenic lymphocytes as well as Chinese hamster lymphocytes. MN and chromosomal aberrations were also detected in human lymphocytes after experimental short-term exposure of ozone to humans. The study published by Holland et al. (2015) shows statistically significant and dose-related MN formation in lymphocytes at the lowest ozone dose (0.1 or 0.2 ppm for 4 h) tested under controlled conditions in a human study with intermittent moderate intensity exercise. A similar dose and exposure time (0.2 ppm, 5 h) led to chromosomal aberrations in hamster lymphocytes (Zelac et al. 1971b). Besides positive mutagenic findings after ozone exposure, there are also 2 studies indicating rather no ozone-related impact on chromosomal aberrations in bone marrow of rats (Zhurkov et al. 1979) or lymphocytes of humans (both Mc Kenzie studies). One possible explanation for this contradiction could be a lower dose, shorter exposure time or combination of both in comparison to similar studies with rats and humans reporting positive results. In general, ozone studies involving cytotoxicity tests (or its endpoints) are scarce. However, in some studies mutagenic effects by ozone are reported in absence of cytotoxicity. Therefore, the possibility that mutagenicity is solely triggered by cytotoxicity as secondary effect should be neglected.

(3) Indicator tests in vivo

(a) local effects

Haney and Connor (1999) used the Comet assay method in order to detect DNA strand breaks following ozone exposure in vivo. For this purpose, male 129/SV mice were once exposed to either 0.25 or 0.5 ppm ozone for 3 h. After exposure BAL cells were taken. The DNA damage was evaluated on the basis of the Comet tail length. The authors determined at both doses statistically significant increases in the number of DNA damaged cells. At 0.5 ppm the number of cells showing high DNA damage (tail length 31+ mm) was 2-fold higher than in the lower dose group. However, the number of damaged cells in general was not dose-relatedly increased at 0.5 ppm. The authors explained this finding with the endpoint chosen for DNA damage (Comet tail length instead of DNA migration area or % of DNA in tail). At both doses the viability of BAL cells from 129/SV mice was not markedly changed in comparison with the control.

Bornholdt et al. (2002) also studied the genotoxic potency of ozone with the Comet assay. They exposed female mice once for 90 min to 1 or 2 ppm ozone. 20-1400 min following exposure DNA strand breaks were investigated in BAL or lung cells. The tail moment was chosen as indicator for genotoxicity. The authors found a statistically significant and linear dose-related increase in DNA strand breaks in BAL cells. However, no increase in DNA strand breaks were observed in lung cells. The viability of BAL cells was not affected by ozone. No viability assay was performed in lung cells. In the publication it was hypothesized that DNA strand breaks detected in BAL cells could also be representative for genotoxic effects in lung cells as lung epithelial cells are closely located to BAL cells. The authors further argued that the sensitivity for the detection of strand breaks in lungs cells could be reduced as a consequence of dilution effects when the whole lung is taken for analysis.

The third submitted study showing ozone-mediated DNA strand breaks in animals with the Comet assay was written by Lee et al. (1997a). Male guinea pigs were exposed for 2 h to 0.4 or 1 ppm ozone. After exposure tracheal epithelial and BAL cells were used for genotoxic investigation. At a dose of 0.4 ppm and above there was a statistically significant and dose-related increase in DNA single strand breaks in both cell types as indicated by an increased DNA migration area and DNA migration distance whereas DNA density was reduced. Cytotoxicity was indicated at 1 ppm by increased total protein and LDH content as well as changes in cell differentiation in bronchoalveolar lavage. In tracheal epithelial cells cytotoxicity was not reported.

Besides studies including Comet assays, the applicant further submitted a study investigating DNA single strand breaks after ozone exposure by fluorometric analysis of DNA unwinding (FADU). Ferng (2002) exposed male guinea pigs once to 0.45 or 1 ppm ozone for 72 h. Immediately after exposure tracheobronchial epithelial (TE) cells were sampled and analysed for DNA strand breaks. Ferng (2002) reported on a dose-related decrease in percentage of double-stranded DNA that is associated with an increase in DNA single strand breaks/TE cell. This effect was statistically significant at the higher dose level. In contrast to this, Lee et al. detected statistically significant increases in DNA single strand breaks in tracheal epithelial cells and BAL cells already after 2 h-exposure to 0.4 ppm. The authors explained this finding with the higher sensitivity when applying the single-cell gel electrophoresis. Whereas a statistically significant increase in the protein content was determined in the lavage fluid of trachea (possibly an indicator for inflammation), no ozone-related impact on either TE cell yield or cell viability was measured by the trypan blue method.

An experimental human study investigating ozone-mediated DNA strand breaks published by Lee et al. (1997b) was also submitted by the applicant. Non-smoking and healthy individuals (number not given) were exposed to air or 0.4 ppm ozone for 2 h. The persons served as their own controls. Bronchial epithelial cells and lavage cells were taken up to 2 h after the end of exposure. DNA breaks were determined with the Comet assay. There was no statistically significant difference in the DNA length between air-exposed and ozone-exposed persons. However, the mean values were slightly increased after ozone exposure. Cytotoxicity was not determined by the authors.

(b) systemic effects

Guerrero et al. (1979) investigated the formation of SCEs in lymphocytes from individuals after ozone exposure. For this purpose 31 male and female volunteers were once exposed to 0.5 ppm ozone for 2 h. During the exposure the individuals were allowed to exercise. The persons served as their own controls. Blood was taken before and after exposure to ozone. The authors did not detect an increase in the number of SCEs or SCEs/chromosome in comparison with the negative control. Cytotoxicity tests were not mentioned in the publication. One major weakness of the study is that confounders like smoking habits were not taken into account.

The test guideline for SCE has been deleted in 2014, hence further publications from literature search addressing this endpoint are not listed hereafter.

Finkenwirth et al. (2013) exposed 18 male subjects once to 0.21 ppm ozone for 2 h whereas a group of 19 male subjects served as placebo group. Unhealthy and smoking individuals were excluded from the study. During exposure subjects exercised to improve their ozone inhalation. Blood was taken before, 30 min or 4.5 h after exposure. DNA single-strand breaks were measured in lymphocytes using the Fast Micromethod. There was no major difference in the strand scission factors between the exposed and control group at both time points. According to the authors possible reasons for this outcome might be the low ozone concentration (compared to animal experiments) or a fast repair of single-strand breaks between end of exposure and blood sampling time. Ozone-mediated cytotoxicity was not mentioned in the publication.

Taken together, indicator tests for genotoxicity focusing on DNA strand breaks in animals support the mutagenic findings of ozone at first site of contact. DNA strand breaks were dose-related and also observed in the absence of ozone-mediated toxicity. Therefore genotoxic effects seem to be (at least at lower ozone doses) independent of cytotoxicity. DNA strand breaks were not detected at local site or systemically in humans after ozone intervention. No cytotoxicity tests were presented in these studies. Therefore, it remains unclear whether the test substance – at the low exposure durations applied – reached the target organ in order to have the ability to induce genetic damage.

Epidemiological studies

In the following section epidemiological studies retrieved by the dossier submitter in either one of the sources mentioned above or after literature search (2013 until 09/2016) are summed up.

(a) Studies focusing on mutagenic endpoints

In a cross-sectional study Huen et al. (2006) investigated the impact of regional ozone levels in Oakland (California) on cytogenic effects in lymphocytes or buccal cells from 65 African-American children and their mothers (n = 39). After statistical analyses the authors found a strong correlation between increased ozone

levels (monthly 8-h average ozone ranged from about 30 ppb in April to 14 ppb in November) and MN frequencies in both cell types from both donors. According to the authors a high association was also observed after adjusting for distance-weighted traffic density and smoking.

For a longitudinal cohort study Chen et al. (2006) recruited 2 groups of students (126 non-smoking students in total) from the University of California (Berkeley). Whereas one group spent the summer in Los Angeles (higher ozone burden), the other group spent summer in San Francisco (lower ozone burden).

Buccal cells were collected from students in spring and fall. In both groups the authors observed a higher MN frequency level of normal cells in fall than in spring whereas the increase was stronger in the group spending summer in Los Angeles. The authors attributed this seasonal effect to the higher ozone levels in summer. A similar effect was seen in degenerated buccal cells from persons spending summer in Los Angeles. In a sub-cohort the authors further exposed 15 students to 200 ppb for 4 h (intermitting exercise). MN frequencies in degenerated buccal cells and lymphocytes were increased post-exposure.

Demircigil et al. (2014) investigated in a cohort study the relationship between air pollutants, season and MN in buccal epithelial cells (BEC).

For this purpose, non-smoking children from 2 schools in Eskisehir (northwest of Central Anatolia in Turkey) - either suburban (school A) or urban-traffic (school B) located – were recruited. Ozone levels in the suburban site were higher than in the urban-traffic site. For school A ozone concentrations were ~ 120-124 and 87-93 µg/m³ in summer and winter, respectively. The ozone concentrations for school B were 81-77 and 34-38 µg/m³ in summer and winter, respectively. For buccal cell sampling in summer and winter a number of 50 or 46 children from school A and 51 or 47 children from school B was involved. Children included in the study were the same in both seasons. Either BEC-MN frequencies (mean frequency of MN per thousand BEC) or BEC frequency with MN (mean frequency of cells bearing at least one MN per 1000 BEC) were assessed as endpoint for mutagenicity. Both parameters were higher in children from school A – even though not statistically significant – in comparison with children from school B. Furthermore, there was no statistically significant difference between summer and winter period. However, BEC-MN frequencies and BEC frequency with MN was significant higher in summer in comparison to winter in children from school B. Cytotoxicity tests were not reported by the authors. Demircigil et al. concluded (2014) on the basis of this study that MN formation is independent from the location of school whereas seasonal variation in MN formation depends on higher ozone levels - connected with increased time spent outdoors - in summer. Three basic shortcomings of the study are that (1) personal sampling of ozone during summer is not presented, (2) no confounders (*e.g.* asthma, lung functions, other pollutants) are included in the statistical analysis and (3) cytotoxicity is not reported.

A cross-sectional study Fleck et al. (2014) investigated the relationship between air pollution and MN formation in Porto Alegre (capital of Rio Grande do Sul, southern Brazil). A number of 101 students participated in the study. Children with smoking habits or frequent alcohol consumption were excluded. Each 33, 34 and 34 children were assigned to 3 groups representing the degree of urbanization in different areas. Group A was associated with high, B with intermediate and C with low population density. In summer mean ozone concentrations amounted to 43.2, 44.5 and 34.3 µg/m³ for group A, B and C, respectively. In winter the corresponding values were 35.9, 34.9 and 23.7 µg/m³. BEC were sampled from June 2013 - March 2014. The authors observed the highest MN frequency in group A (4.57 MN per 1000 cells), followed by group B (4.30 MN per 1000 cells) and C (2.31 MN/1000 cells). MN frequency in group A and B differed statistically significant from group C. There was no statistically significant difference in confounding factors (age, gender, socioeconomic status and passive smoking) between the 3 groups. The cytotoxicity in BEC was not measured. In the conclusion the authors mention 2 limitations of the study: Individual exposure to ozone was not determined and further genotoxic air pollutions (*e.g*. sulphur dioxide or polycyclic aromatic hydrocarbons) were not measured.

(b) Studies focusing on indicator tests

In a cross-sectional study Tovalin et al. (2006) studied a possible association between exposure to ozone and the severity of DNA damage in blood lymphocytes from outdoor workers. For this purpose indoor (n = 27) and outdoor workers (n = 28) from México City and Puebla were included in the study. According to Tovalin et al. (2006) the estimated median ozone exposures amounted to 28.5 ppb and 5.1 ppb for outdoor and indoor workers in México City, respectively. In Puebla outdoor and indoor workers were exposed to 36.1 ppb and 19.5 ppb ozone, respectively. After performing statistical analyses the authors obtained a greater DNA damage

potency (Comet assay) and higher percentage of damaged cells in outdoor workers in comparison with indoor workers in México City. In contrast to this, DNA damage in outdoor and indoor workers in Puebla was similar. However, the authors noticed a higher tendency for alkali labile sites in outdoor workers. In general, the authors concluded that ozone exposure was positively correlated with the magnitude of DNA damage.

Another longitudinal study investigating a correlation between ozone burden and DNA damage (DNA strand breaks and oxidized purine bases) was published by Giovannelli et al. (2006). A number of 79 healthy subjects (almost all non-smokers) living in Florence (Italy) were exposed for 3, 7 or 30 days before blood sampling. The US EPA (2013) estimated the ozone concentrations during the study as follows: 4-40 ppb for 3-day averages, 5-35 ppb for 7-day averages, and 7.5-32.5 ppb for 30-day averages. Whereas ozone concentration was positively correlated with DNA strand breaks in lymphocytes at 7 days and 30 days, the authors did not report on a correlation between ozone exposure and oxidative DNA damage. According to the authors age and gender of the study participants do not have an impact on DNA breaks or oxidative DNA damage in the study.

Peluso *et al.* (2005) published results of the Gen-Air case control study nested in the EPIC (European Prospective investigation into Cancer and Nutrition) cohort with > 500,000 healthy volunteers. For the Gen-Air study only non-smokers were chosen. The authors investigated the relationship between DNA damage – reflected by DNA adducts in leucocytes – and ozone concentrations. After performing multivariate modelling (air pollutants, age, gender, education level, country and batch as independent variables) the authors found a statistically significant positive correlation between ozone concentrations (not specified) and DNA adduct levels in the time period 1990-1994, but not from 1995-1999. Also logistic regression reflected a statistically significant association between ozone levels and adduct formation after adjustment to the confounders mentioned above.

Palli *et al.* (2009) re-invited individuals enrolled in the EPIC study to investigate the relationship between the ozone exposure in Florence (Italy) and the severity of oxidative DNA damage (DNA strand breaks). For this purpose, 71 healthy adults (12 smokers and 59 non-smokers) were recruited. DNA strand breaks were determined in lymphocytes. Blood was taken after exposure for 10 different time windows. According to the authors especially at longer time periods there was a statistically significant positive correlation between means of ozone concentration and DNA strand breaks. This correlation was stronger among males, non-smokers and traffic-exposed workers. Multivariate regression analysis adjusted for age, gender, smoking, traffic pollution exposure, period of blood drawing and area of residence revealed that effects at average ozone concentrations in 0-60, 0-75 or 0-90 day time windows prior to blood draw are independent.

Valverde *et al*. (1997) recruited for a cross-sectional study 42 students (24 female, 18 male) among them 10 with smoking habits (< 10 cigarettes/day), 7 with history of bronchitis in childhood, 1 with allergic rhinitis and 2 with a history of asthma. Study participants lived either in the northern part or in the southern part of Mexico City. After determining atmospheric ozone exposure at 5 different days in both parts of Mexico City the authors concluded a 1.5-fold higher ozone burden in the South (1.46 h > 0.11 ppm per day vs. 0.93 h > 0.11 ppm per day). DNA damage was investigated using the single-cell gel electrophoresis (Comet assay). The authors noticed a statistically significant increase in DNA migration values for leucocytes and nasal cells sampled from individuals living in the southern part of Mexico City. DNA migration was also increased – even though not statistically significant – for buccal cells from persons living in the South. The cell viability between the north and south group did not differ for all 3 cell types. It amounted to 90 % for blood leucocytes and 60-70 % for nasal and buccal cells.

Calderón-Garcidueñas *et al.* (1996) investigated the relationship between DNA strand breaks in nasal respiratory epithelium from humans and ozone-polluted atmosphere. For this purpose, the single-cell gel electrophoresis (Comet assay) was performed and cell viability was determined. A number of 139 healthy non-smoking volunteers participated in the study among them 19 children and 13 adult males living in low-polluted Pacific Port (control group), 16 children and 69 males living in Southwest Metropolitan Mexico City (chronic exposure) and 22 young males who newly arrived to Southwest Metropolitan Mexico City (observation group, 12 weeks). The ozone burden was reported as follows: Volunteers from Southwest Metropolitan Mexico City were exposed to > 0.12 ppm ozone for 4.4 h per day (average maximum concentration of 0.269 ppm from November 1994 – May 1995) and newly arrived volunteers were exposed to an average maximum ozone concentration of 0.262 ppm. The number of cells with DNA damage was statistically significantly increased in chronically exposed children and adults in comparison with the control group. The percentage of DNA

strand breaks in newly arrived subjects increased from 39.8 ± 8.34 % in the first week to 67.29 ± 2.35 by week 2. Thereafter no further increase was observed. Cell viability in all groups did not fall below 60 %. Shortcomings of this study are that ozone burden was not presented in control group and other air pollutants (sulphur dioxide and nitrogen dioxide) were not involved in the interpretation of data.

Pacini *et al.* (2003) recruited a number of 106 persons living in the urban area of Florence (among them 51 females, 55 males and 18 smokers) and further 17 volunteers from Sassari (Sardinia) as controls (among them 10, 7 males and 5 smokers). The study was conducted from June 2001 - January 2002. The ozone concentration in Florence fluctuated between ~ 75 µg/m³ (June) and ~ 15 µg/m³ (January). Nasal cells of individuals were taken in order to determine the DNA damage (% tail, Comet assay) and to investigate their morphology. The authors noticed that the level of DNA damage was higher in residents from Florence than from Sassari. Furthermore they reported on a correlation between atmospheric ozone levels and DNA damage. According to the authors the prevalence of inflammational findings of the upper respiratory tract was also related to ozone burden. No difference in DNA damage level of smokers and non-smokers was reported. The authors further commented that it remains unclear whether ozone or other pollutants induced the observed effects in nasal mucosa.

Taken together, epidemiological studies may further point to a possible relationship between ozone exposure and mutagenic events at local or distant site of contact (MN in lymphocytes or buccal cells). In line with this findings also indicator tests for genotoxicity in blood or nasal cells may provide evidence for an association between ozone exposure and genotoxicity. Thus, negative results for mutagenicity or indicator tests in human experimental studies may be attributed to the short time of intervention.

10.8.2 Comparison with the CLP criteria

Toxicological result	**CLP criteria**
There was no evidence demonstrating heritable mutations in human germ cells in vivo.	The classification in Category 1A is based on positive evidence from human epidemiological studies. Substances to be regarded as if they induce heritable mutations in the germ cells of humans.
Dominant lethal studies with Drosophila (Erdman & Hernandez 1982, Victorin 1992)., and a mice study (Brinkman et al. 1964, Veninga 1967 both evaluated by US EPA 2013) give indications that ozone may reach the germ cells. In these studies ozone exposure was related to death (flies and mice), jaw anomalies and unilateral or occasionally bilateral blepharophimosis (mice). Blepharophimosis is considered as genetically heritable disease and could therefore indicate mutagenic damage of germ cells. However, the mouse study suffers from a very poor data quality which hampers the transparency and validity of the effects presented. In an epidemiological study it was concluded that ozone burden of humans is not associated with aneuploidies in sperm cells (Jurewicz et al., 2015). Given this negative finding together with the poor data quality of the mice study, the database is considered insufficient for classification into Muta. 1B category.	The classification in Category 1B is based on: - positive result(s) from in-vivo heritable germ cell mutagenicity tests in mammals; or - positive result(s) from in-vivo somatic cell mutagenicity tests in mammals, in combination with some evidence that the substance has potential to cause mutations to germ cells. It is possible to derive this supporting evidence from mutagenicity/genotoxicity tests in germ cells in vivo, or by demonstrating the ability of the substance or its metabolite(s) to interact with the genetic material of germ cells; or - positive results from tests showing mutagenic effects in the germ cells of humans, without demonstration of transmission to progeny; for example, an increase in the frequency of aneuploidy in sperm cells of exposed people.
Positive evidence for somatic cell mutagenicity and genotoxicity obtained from in vivo studies: Rithidech (1990), Haney & Conner (1999), Lee et al. (1997a), Ferng (2002), Kim et al. (2001), Kim et al. (2002) and Haddad et al. (2009) Supported by positive in vitro tests: Gooch et al. (1976), Guerrero et al. (1979), Fetner et al. (1962),	The classification in Category 2 is based on: - positive evidence obtained from experiments in mammals and/or in some cases from in vitro experiments, obtained from: - somatic cell mutagenicity tests in vivo, in mammals; or

Toxicological result	CLP criteria
Chorvatovicova et al. (2000) and Díaz-Llera et al. (2002) Further evidence for the genotoxic and mutagenic potency is provided by epidemiological studies.: Holland (2015), and Finkenwirth et al. (2013)	- other in vivo somatic cell genotoxicity tests which are supported by positive results from in vitro mutagenicity assays. Note: Substances which are positive in in vitro mammalian mutagenicity assays, and which also show chemical structure activity relationship to known germ cell mutagens, shall be considered for classification as Category 2 mutagens. [Please compare the results with the CLP classification criteria for the hazard class in question, i.e. germ cell mutagenicity.]

10.8.3 Conclusion on classification and labelling for germ cell mutagenicity

Based on the results listed above, harmonised classification and labelling for germ cell mutagenicity is proposed: Germ cell mutagen Category 2, H341: Suspected of causing genetic defects.

RAC evaluation of germ cell mutagenicity

Summary of the Dossier Submitter's proposal

The DS proposed to classify ozone as germ cell mutagenicity category 2; H341: suspected of causing genetic defects. This conclusion is based on positive evidence for somatic cell mutagenicity and genotoxicity obtained from *in vivo* studies. These findings are supported by results for mutagenicity in bacterial strains and mammalian cell lines together with positive indicator tests *in vitro*.

Comments received during consultation

One MSCA agreed with the DS proposal. One company-manufacturer provided critical observations for the studies provided. A recurring criticism on the different studies regarding the bioavailability, and on whether ozone is able to reach the tested target cells was received.

Assessment and comparison with the classification criteria

Ozone is a powerful oxidant and reactive to biomolecules. In aqueous solution, it decomposes to give hydrogen peroxide, superoxide, and hydroxy radicals which can take part in secondary reactions. Ozone is registered as a biocide and inactivates both viruses and bacteria. Although other reactions are primarily responsible for the inactivation, cellular DNA is also damaged.

As early as 1954, it was shown that bubbling of ozone through a solution of DNA causes a rapid change in the UV spectra, probably resulting from effects on the constituent purines and pyrimidines (Christensen and Giese, 1954). The nucleotide bases thymine and guanine have been found to be the most sensitive to ozonation (Prat *et al.,* 1968). The DNA damage proceeds both directly via ozone molecules and indirectly via hydroxyl radicals when solutions of nucleotides or DNA are treated with ozone.

The mutagenic potency of ozone investigated *in vitro:*

***Table**: Summary table of evaluated mutagenicity test in vitro*

Test organism	Endpoint	Ozone exposure	Results	Reference
Salmonella TA (1535, 98, 100, 104)	Reverse mutation	0.02-0.5 ppm, 35 min	-	Dillon *et al.,* 1992
Salmonella TA102	Reverse mutation	0.02-0.5 ppm, 35 min	+	Dillon *et al.,* 1992
Salmonella TA100	Reverse mutation	0.1-2.0 ppm, 6 h	-	Victorin *et al.,* 1988
Human lymphocytes	Chromatid-type aberrations	Bubbling through cell suspension (7.23 and 7.95 ppm, 36 h)	+	Gooch *et al.,* 1976
Human lymphocytes	Chromosome type aberrations	Bubbling through cell suspension (7.23 and 7.95 ppm, 12 h)	-	Gooch *et al.,* 1976
Human lymphocytes	Chromatid and chromosome type aberrations	Ozone-saturated buffer (~2 ppm)	-	Gooch *et al.,* 1976
Micronucleus, Rat aveolar type II cell	Micronuclei	Air flow 400 ppm, 6 h	+	Chorvatovicova *et al.,* 2000
WI-38 cells	SCE	0.25-1 ppm, 1 h	+	Guerrero *et al.,* 1979
WI-38 cells	Chromatid type aberrations	0.25-1 ppm, 1 h	-	Guerrero *et al.,* 1979
WI-38 cells	Chromosome aberrations	0.25-1 ppm, 1 h	-	Guerrero *et al.,* 1979
Human lymphocytes, Comet assay	DNA damage	0.875-5.25 mM, 1 h	+	Diaz-Llera *et al.,* 2002
Human fibroblast, Comet assay	DNA damage	60 µg/µL, 30s	-	Akdeniz *et al.,* 2018
Human alveolar cell Comet assay	DNA damage	120 ppb, 72 h	+	Poma *et al.,* 2017
Human alveolar cell, Micronucleus	Micronuclei	120 ppb, 48 h	(+)	Poma *et al.,* 2017
KB human cell line	Chromatid-type aberration	8 ppm, 5 and 10 min	+	Fetner, 1962

In vivo studies:

***Table**: Summary table of evaluated mutagenicity test in vivo*

Test organism	Endpoint	Ozone exposure	Results	Reference
Pulmonary macrophages	Chromatid-type aberration / local	0.12-0.8 ppm, 6 h	+	Rithidech *et al.,* 1990
BAL cell, Comet assay	DNA damage / local	0.25-0.5 ppm, 3 h	+	Haney *et al.,* 1999

BAL cell. Comet assay	DNA damage / local	1-2 ppm, 90 min	+	Bornholdt *et al.,* 2002
Lung cell. Comet assay	DNA damage / local	1-2 ppm, 90 min	-	Bornholdt *et al.,* 2002
BAL cell. Comet assay	DNA damage/ local	0.4-1 ppm, 2 h	+	Lee *et al.,* 1997
Tracheal epithelial cell. Comet assay	DNA damage / local	0.4-1 ppm, 2 h	+	Lee *et al.,* 1997
Tracheobronchial epithelial cell, FADU	DNA single strand breaks / local	0-45-1 ppm, 72 h	+	Ferng *et al.,* 2002
Mouse strain C3H	Chromatid and chromosome type aberration in lymphocytes / systemic	0.15 and 0.21 ppm, 5 h, 0.99 ppm, 2 h	-	Gooch *et al.,* 1976
Mouse strain C3H	Reciprocal translocations in spermatocytes / systemic	0.15 and 0.21 ppm, 5 h, 0.99 ppm, 2 h	-	Gooch *et al.,* 1976
Chinese hamster	Chromosome and chromatid aberrations in bone marrow cells / systemic	0.23 ppm, 5 h, 5.2 ppm, 6 h	-	Gooch *et al.,* 1976
Mouse, splenic lymphocytes, Chromosome aberration	Chromosome aberrations / systemic	0-0.5 ppm, 6 h/d, 5 days/week for 16, 32 and 52 weeks	+	Kim *et al.,* 2002
Mouse, reticulocytes, Micronucleus test	Micronuclei / systemic	0-0.5 ppm, 6 h/d, 5 days/week for 16, 32 and 52 weeks	+	Kim *et al.,* 2002
Mouse splenic lymphocytes	Chromosome aberrations / systemic	0-0.5 ppm, 6 h/d, 5 day/week for 12 weeks	+	Kim *et al.,* 2001
Mouse reticulocytes, Micronucleus test	Micronuclei / systemic	0-0.5 ppm, 6 h/d, 5 days/week for 12 weeks	+	Kim *et al.,* 2001
Mouse splenic cells. HPRT	Mutation frequency HPRT gene / systemic	0-0.5 ppm, 6 h/d, 5 days/week for 12 weeks	+	Kim *et al.,* 2001
Mouse bone marrow erythrocytes, Micronucleus test	Micronuclei / systemic	0, 3 ppm, 6h/d 10 days.	+	Haddad *et al.,* 2009
Rat blood, Comet assay	DNA damage / systemic	0.05 ppm, 3 h/d for 14, 28 days	-	Cestonaro *et al.,* 2017
Bone marrow, Micronucleus	Micronuclei / systemic	0.05 ppm, 3 h/d for 14, 28 days	-	Cestonaro *et al.,* 2017

Human *in vitro* studies:

***Table**: Summary of human studies describing mutagenicity*

Test organism	Endpoint	Ozone exposure	Results	Reference
BAL cells / Comet assay	DNA damage / local	0, 0.4 ppm, 2 h	-	Lee *et al.,* 1997
Lymphocytes / Micronucleus test	Micronuclei / systemic	0-0.2 ppm, 4 h	+	Holland *et al.,* 2015
BAL cells / Micronucleus test	Micronuclei / systemic	0-0.2 ppm, 4 h	-	Holland *et al.,* 2015
Lymphocytes /SCE test	SCE / systemic	0, 0.5 ppm, 2 h	-	Guerrero *et al.,* 1979
Lymphocytes / Fast micromethod	DNA damage / systemic	0, 0.21 ppm, 2 h	-	Finkenwirth *et al.,* 2013
BAL cells	Chromatin modification	0.5 ppm	(see interpretation of results below)	McCullough *et al.,* 2016

Summary of the submitted in vitro test

The gene mutation potential of ozone was investigated in various bacterial strains.

Dillon *et al.* (1992) exposed *S. typhimurium* strains TA100, TA98, TA1535, TA104 and TA102 in the presence and absence of liver S9-mix. No doubling of revertant colonies was observed and the substance was therefore negative in the strains TA100, TA98, TA1535 and TA104. In contrast, ozone induced a dose-related 2 to 3-fold increase in revertant colonies in strain TA102 at 0.02 ppm and above. The increase was statistically significant from air control and independent of S9 mix. Cytotoxicity was observed in one strain at around 0.4 ppm (TA102) or 1-4 ppm (remaining strains) reflected by a rapid decline in revertant colonies.

Victorin *et al.* (1988) examined the mutagenicity of bacterial strain TA100. Ozone did not pose any mutagenic activity.

To investigate the chromosomal aberration in mammalian cells, Gooch *et al.* (1976) incubated human peripheral lymphocytes with a range of ozone doses. The incubation was performed in the absence of S9-mix. In another experiment ~2 ppm ozone-saturated phosphate buffered saline D was added to leukocytes. The percentage of cells with chromosomal or chromatid aberrations remained unchanged. In contrast, ozone treatment 36 h after phytohaemagglutinin (PHA) stimulation resulted in a 3 to 4-fold or 1.4 to 3-fold increase in chromatid aberrations at 7.23 or 7.95 ppm/h ozone exposure in comparison to controls, respectively. This effect was not dose-related. Cytotoxicity was not reported by the authors.

The applicant also submitted a short communication by Chorvatovicova *et al.* (2000) focusing on micronucleus (MN) formation in rat alveolar type II cells. A statistically significant increase (2.5-fold) in MN formation/1 000 cells in comparison to the negative control was measured. Only one dose was tested, hence no conclusion on dose-response is possible.

Guerrero *et al.* (1979) incubated a foetal lung cell line (WI-38 cells) with ozone in the range between 0.25 and 1 ppm for 1 h without S9-mix. The authors reported a dose-related increase in the percentage of cells exhibiting endoreduplications (at 0.5 ppm and above) or chromatid deletions (at 0.75 ppm and above). Cytotoxicity was not tested by the authors.

Díaz-Llera (2002) determined the potential of ozone to induce DNA strand breaks with the Comet assay. For this purpose, human peripheral blood lymphocytes (obtained from 6 donors) were

exposed for 1 h to different ozone concentrations ranging from 0.75 to 5.25 mM. The study did not investigate conditions with S9 mix. Beginning at 0.875 mM there was a dose-related increase in percentages of damaged cells as well as a statistically significant and dose-related increase of tail image length in comparison to the untreated control. A cell viability assay revealed that only minimal or no cytotoxic effects occurred at the dose levels used.

Akdeniz *et al.* (2018) provided negative results with human primary fibroblast in a comet assay without S9 mix. A positive Comet assay provided by Poma *et al.* (2017) using 0.012 ppm ozone with adenocarcinoma human alveolar cells A549 and fibroblasts Hs27 reported a higher mean value for tail DNA% compared to the controls: A549: 8.3% (48 h), 7.3% (72 h) and the Hs27 cells 2.88% (48 h exposure) 3.7% (72 h exposure). The combined MN test showed an equivocal response. In A549 cells, significant and ~ 100% increase in MN frequency compared to the control after 48 h exposure was reported while no significant increase after 72 h exposure was reported. In Hs27 cells, significant increase in MN frequency was reported after 72 h exposure while no significant increase in MN frequency after 48 h exposure was reported.

The publication by Fetner *et al.* (1962) reported on the exposure of the KB human cell line at a dose of 8 ppm ozone. Whereas no deletions were found in the negative control, 20 deletions per 4158 chromosomes or 23 deletions per 1283 chromosomes were observed in the treated groups after exposure for 5 or 10 min, respectively. The authors further mentionned that cells dislodge from the glass surface at higher doses or longer exposure time.

Summary of the submitted in vivo data

Rithidech *et al.* (1990) exposed female rats to ozone for 6 h once. Several dosed groups were included in the study. Afterwards, pulmonary alveolar macrophages were isolated and chromosomal damage as an increase in abnormal cells was investigated. A dose-related increase in the number of abnormal cells after exposure to 0.12 or 0.27 ppm ozone was observed. However, at the next higher dose 0.8 ppm a decrease of abnormal cells was observed. The authors explained this finding with an increase in ozone-mediated influx and division stimulation of macrophages. They further argued that this dilution of macrophages could be the reason for underestimation of cytogenetic effects. This theory is supported by a dose-related increase of the mitotic index from 0.27 to 0.8 ppm. In contrast, the mitotic index was not affected at the lowest dose applied (0.12 ppm), but strongly reduced at 0.27 ppm.

Haney *et al.* (1999) used the Comet assay with male 129/SV mice exposed to ozone once. After exposure BAL cells were taken. Hanley determined at both doses statistically significant increases in the number of DNA damaged cells. At 0.5 ppm the number of cells showing high DNA damage (tail length 31+ mm) was 2-fold higher than in the lower dose group. At both doses the viability of BAL cells from 129/SV mice was not markedly changed in comparison with the control.

Bornholdt *et al.* (2002) also studied the genotoxic potency of ozone with the Comet assay. They exposed female mice once to ozone. Following exposure, DNA strand breaks were investigated in BAL and lung cells. The authors found a statistically significant and linear dose-related increase in DNA strand breaks in BAL cells. However, no increase in DNA strand breaks were observed in lung cells. The viability of BAL cells was not affected by ozone. No viability assay was performed in lung cells. In the publication, it was hypothesised that DNA strand breaks detected in BAL cells could also be representative for genotoxic effects in lung cells as lung epithelial cells are closely located to BAL cells. The authors further argued that the sensitivity for the detection of strand breaks in lungs cells could be reduced as a consequence of dilution effects when the whole lung is taken for analysis.

Ozone-mediated DNA strand breaks in animals with the Comet assay was also reported by Lee *et al.* (1997a). Male guinea pigs were exposed to ozone. After exposure, the tracheal epithelial (TE) and BAL cells were isolated for genotoxic investigation. At 0.4 ppm and above there was a statistically significant and dose-related increase in DNA single strand breaks in both cell types as indicated by an increased DNA migration area and DNA migration distance whereas DNA density was reduced. Cytotoxicity was indicated at 1 ppm by increased total protein and LDH content as well as changes in cell differentiation in BAL. In TE cells cytotoxicity was not reported.

Ferng *et al.* (2002) investigated DNA single strand breaks after ozone exposure by fluorometric analysis of DNA unwinding (FADU). Male guinea pigs were exposed to 0.45 or 1 ppm ozone for 72 h. Immediately after exposure, tracheobronchial epithelial cells were sampled and analysed for DNA strand breaks. A dose-related decrease in percentage of double-stranded DNA associated with an increase in DNA single strand breaks/tracheobronchial epithelial cell was reported. This effect was statistically significant at the higher dose level.

Gooch *et al.* (1976) investigated the potency of ozone to induce genetic damages systemically in male mice. Chromosomal and chromatid aberrations were determined in leukocytes. A slight increase in both chromosomal and chromatid aberrations was obtained after ozone treatment in comparison to the untreated controls. However, the effect was neither dose-related nor correlated with time of blood withdrawal after ozone exposure. Ozone-mediated cytotoxicity was not measured.

Kim *et al.* (2001) treated male and female mice for 6 h/d and 5 days/week with 0.5 ppm ozone for an overall exposure period of 12 weeks. The systemic DNA damage induced by ozone was measured in lymphocytes, reticulocytes and splenic cells by means of chromosomal aberration, MN formation or mutation frequency in HPRT gene, respectively. Ozone treatment was connected with a statistically significant increase in chromosomal aberrations and MN formation in males and females. Furthermore, the mutation frequency in splenic cells from ozone treated mice was almost doubled in comparison to the untreated control animals. Whereas no cytotoxicity was determined in lymphocytes and reticulocytes, no obvious toxicity was evident in splenic cells (clonal efficiency: 0.23 and 0.19 in control and treated animals, respectively).

Kim *et al.* (2002) repeated this study, but extended exposure time to 16, 32 or 52 weeks. Afterwards, splenic lymphocytes and reticulocytes were taken for analyses of chromosomal aberrations or MN formation, respectively. Ozone exposure resulted in a time-related and statistically significant increase in chromosomal aberrations and MN in males and females. Also in this study cytotoxic effects were not reported.

Haddad *et al.* (2009) used male rats for their MN test in bone marrow erythrocytes. Independent from time point of sacrifice (immediately or 11 days after the last exposure in groups 1 and 2) there was a statistically significant increase in MN frequency in comparison to the negative controls. Furthermore, the PCE/NCE + PCE ratio was reduced in both treatment groups (less pronounced in treatment group 2). This finding indicates on the one hand that the bone marrow was reached by the test substance (or its derivatives), and on the other hand that ozone mediated cytotoxicity is reversible.

A combined *in vivo* comet and micronucleus assay was performed by Cestonaro *et al.* (2017) in rats. Only one dose was tested. Both tests reported negative results.

Summary of the submitted human studies

An experimental human study investigating ozone-mediated DNA strand breaks published by Lee (1997b) where non-smoking and healthy individuals (number not given) were exposed to air or

0.4 ppm ozone for 2 h with exercises. The volunteers served as their own controls. Bronchial epithelial cells and lavage cells were taken 1 to 2 h after the end of exposure. DNA breaks were determined with the Comet assay. There was no statistically significant difference in the DNA length between air-exposed and ozone-exposed persons. However, the mean values were slightly increased after ozone exposure. Cytotoxicity was not determined by the authors.

A study published by Holland *et al.* (2015) addresses the MN formation in blood lymphocytes of 10 male and 12 female subjects after single exposure to 0.1 or 0.2 ppm ozone for 4 h including alternating 30 min exercise and rest periods. Smokers or persons suffering from cardiovascular, pulmonary or hematologic diseases (other than mild asthma) were excluded from the study. The authors reported a dose-related and statistically significant increase in MN frequencies. Whereas cell proliferation was not affected by ozone treatment, the percentage of apoptotic cells increased statistically significantly after exposure. It was further concluded that also exercise had a detrimental impact on DNA integrity, most likely attributed to oxidative stress, as reflected by higher MN formation frequency following exercise in the untreated group. According to the authors, another factor contributing (independent from ozone exposure) to MN formation could be recruitment of neutrophils as indicated in bronchoalveolar lavage.

Guerrero *et al.* (1979) investigated the formation of SCEs in lymphocytes from individuals after ozone exposure. 31 male and female volunteers were once exposed to 0.5 ppm ozone for 2 h. During the exposure the individuals were allowed to exercise. The persons served as their own controls. Blood was taken before and after exposure to ozone. The authors did not detect an increase in the number of SCEs or SCEs/chromosome in comparison with the negative control. Cytotoxicity tests were not mentioned in the publication. One major weakness of the study is that confounders like smoking habits or asthma were not taken into account.

Finkenwirth *et al.* (2013) exposed 18 male subjects once to 0.21 ppm ozone for 2 h whereas a group of 19 male subjects served as placebo group. Unhealthy and smoking individuals were excluded from the study. During exposure subjects exercised to improve their ozone inhalation. Blood was taken before, 30 min or 4.5 h after exposure. DNA single-strand breaks were measured in lymphocytes using the Fast Micromethod. There was no major difference in the strand scission factors between the exposed and control group at both time points. According to the authors possible reasons for this outcome might be the low ozone concentration (compared to animal experiments) or a fast repair of single-strand breaks between end of exposure and blood sampling time. Ozone-mediated cytotoxicity was not mentioned in the publication.

McCullough *et al.* (2016) studied chromatin modification levels in human BAL cells from 11 donors when exposed with 0.5 ppm for 2 h. Cells were removed from the chambers and total RNA was harvested immediately after exposure. The authors reported that baseline levels of specific chromatin modifications correlate with the interindividual variability in both basal and ozone-induced expression of proinflammatory stress genes.

<u>Germ cell mutagenicity data</u>

There are no reliable germ cell mutagenicity tests available. However, literature search performed by the DS retrieved a dominant lethal test in flies. Erdman *et al.* (1982) exposed male *Drosophila virilis* flies for 3 h to 30 ppm ozone. Longer exposure times were not tolerated by the flies. Ozone induced dominant lethal effects in the offspring, as calculated by the proportion of eggs that failed to develop into pupae. In general, post meiotic cell stages of spermatogenesis were more sensitive to ozone-induced dominant lethal than meiotic and premeiotic stages. For most mating periods, the control group had higher total number of eggs, than those treated with ozone.

In the review by Victorin (1992), another study with flies, Drosophila melanogaster by Chigusa *et al.* (1972) is mentioned in which the genetic effects of ozone on fecundity, hatchability, emergence rate and longevity are presented. Victorin (1992) concluded that ozone exposure induced dominant lethal effects in females, and it is connected with a life-span shortening of male offspring and decreases the hatchability of eggs after repeated exposure to 27 ppm for 1-2 h.

A further indication for ozone-mediated germ cell mutagenicity is given in the evaluation of the US-EPA ISA review (2013). It is reported that exposure to 0.2 ppm ozone during gestation leads to mutagenic effects in the offspring in mice. In the referred study published by Brinkman *et al.* (1964) - that was further taken up in a study published by Veninga (1967) – the toxicity of ozone was compared with detrimental health effects mediated by ionizing radiation. Either grey mice or black mice were exposed to air, 0.1 or 0.2 ppm ozone for 7 h/d, 5 d/week for 3 weeks. Brinkman *et al.* (1964) reported that litter size from couples of grey mice was normal whereas the number of litters was almost halved after ozone exposure (0.2 ppm) in grey mice or black mice. The neonatal mortality in the first 3 weeks was 6.8% (0.1 ppm ozone) and 7.5% (0.2 ppm ozone) against 1.6% in the control animals. The neonatal mortality was also increased to 34% in black mice treated with 0.2 ppm ozone against 9% in the control animals. Besides neonatal death, a higher frequency of blepharophimosis (unilateral or occasionally bilateral) was observed in grey mice. The frequency increased from 0.6% or 4.5% in the controls to 9.6% or 9.2% in ozone treated grey or black mice, respectively. In the latter strain, this finding was accompanied by increased jaw anomalies (unlimited growth of incisors) after exposure to 0.2 ppm ozone (5.4%). Veninga (1967) stressed that this anomaly normally occurred in only 0.9% of new-born mice. It could be assumed that the observed jaw anomalies are one explanation for the strong neonatal mortality observed in black mice.

After literature search by the DS in 4 different databases, a cross-sectional study performed in Poland by Jurewicz *et al.* (2015) was retrieved. In this study, the relationship between human exposure to air pollutants (e.g. sulphur dioxide or ozone) and sperm disomy (hereinafter referred to as sperm aneuploidy) was investigated. According to the authors, there was no association between ozone pollution and sperm aneuploidy either after multivariate analysis or multivariate analysis with other air pollutants.

Other reviews of genotoxicity data

Victorin (1992) prepared a comprehensive review on ozone genotoxicity comprising of some studies which were not assessed by the DS in the CLH report. The author concluded that ozone showed genotoxicity potential *in vitro*. Mutations and DNA strand breaks occurred in bacteria and yeast cells mainly in experiments in which ozone was bubbled through suspension cultures of cells, in which case hydroxyl radicals and hydrogen peroxide were also formed. The bacteriotoxicity of ozone complicates the demonstration of mutagenicity in *Salmonella*, but a positive response has been observed with ozone in air with strain TA102 in one study. In cell cultures, chromatid-type chromosome aberrations, SCE, and neoplastic transformation have been demonstrated. The results from *in vivo* cytogenetic studies with laboratory animals after inhalation exposure are contradictory. Chromosome aberrations in lymphocytes, but not SCE, have been found in Chinese hamsters, but not in mice. No cytogenetic effects were reported for bone marrow cells or spermatocytes.

Final conclusion for mutagenicity

The mutagenic activity of ozone seems to depend on the bacterial strain representing a certain mechanism of mutagenicity. Strain TA102 is sensitive to oxidative damage as mediated by peroxides and oxygen radical generators. In a similar way, the cytotoxicity of ozone depends on

the bacterial strain and increases with dose. Given that mutagenicity was independent of S9-mix, ozone seems to act as a direct mutagen. Ozone resulted in chromosomal aberrations and MN formation in a part of the *in vitro* test systems reported.

The comet assays focusing on DNA strand breaks in animals supported the mutagenic findings of ozone at site of contact. DNA strand breaks were dose-related and also observed in the absence of ozone mediated toxicity. Therefore, genotoxic effects seem to be independent of cytotoxicity- at least at lower ozone doses. DNA strand breaks were not detected at local site or systemically in humans after ozone intervention. No cytotoxicity tests were presented in these studies. Therefore, it remains unclear whether the test substance – at the low exposure durations applied – reaches the target organ in order to have the ability to induce genetic damage. Using the alkaline elution method may lower the sensitivity in the presence of interstrand or DNA protein crosslinks. Another aspect leading to false-negative results is the exposure to ozone in combination with a buffer. Ozone is a very reactive gas that may react with the buffer before reaching the target system.

Cytotoxicity studies in the publications are scarce. Therefore, there is a lack of a clear correlation of genotoxicity with cytotoxicity. However, the study by Diaz-Llera *et al.* (2002) indicates that clear genotoxicity effects may occur independent of cytotoxicity.

Regarding germ cell mutagenicity, there are indications for mutagenicity by ozone in both flies and mice. Dominant lethal studies with *Drosophila*, and a mice study gave indications that ozone may reach the germ cells. In these studies, ozone exposure was related to death (flies and mice), jaw anomalies and unilateral or occasionally bilateral blepharophimosis in mice. Blepharophimosis is considered as genetically heritable disease and could therefore indicate mutagenic damage of germ cells. However, no studies investigating mammalian germ cell mutagenicity are available (*Drosophila* studies cannot be used as basis for classification). The available studies were also evaluated in US EPA ISA review (2013) with the conclusion that the studies suffer from a very poor data quality which hampers the transparency and validity of the effects presented. Therefore, it remains difficult to draw a clear conclusion on germ cell mutagenicity in mammals.

From the *in vivo* studies, chromosomal aberrations were detected in pulmonary alveolar macrophages in rats after exposure to ozone. Furthermore, there is evidence from many studies that ozone also leads to mutagenic effects in cells distant from the site of contact as indicated by positive MN in murine reticulocytes and rat bone marrow cells. Chromosomal aberrations after ozone exposure were detected in murine leucocytes and splenic lymphocytes as well as Chinese hamster lymphocytes. MN and chromosomal aberrations were also detected in human lymphocytes after experimental short-term exposure of ozone to humans. The study published by Holland *et al.* (2015) reported statistically significant and dose-related MN formation in lymphocytes at the lowest ozone dose tested under controlled conditions in a human study with intermittent moderate intensity exercise. A similar dose and exposure time led to chromosomal aberrations in hamster lymphocytes by Zelac *et al*. (1971a). Besides positive mutagenic findings after ozone exposure, there are also 2 studies showing no ozone-related impact on chromosomal aberrations in bone marrow of rats (Zhurkov *et al.*, 1979) or lymphocytes of humans (Mc Kenzie 1977 and 1982). One possible explanation for this contradiction could be a lower dose, shorter exposure time or combination of both in comparison to similar studies with rats and humans reporting positive results. Again, cytotoxicity studies are scarce. However, in some studies, mutagenic effects by ozone were reported in absence of cytotoxicity.

Epidemiological studies may further point to a possible relationship between ozone exposure and local or distant to site of contact mutagenic events (MN in lymphocytes). Also tests for genotoxicity in blood or nasal cells may provide evidence for an association between ozone

exposure and genotoxicity. There were no evidence demonstrating heritable mutations in human germ cells.

Under the conditions of the published studies and based on the information given therein, ozone possesses mutagenic potency in animals.

According to the CLP Regulation:

The classification in Category 2 is based on:

- positive evidence obtained from experiments in mammals and/or in some cases from in vitro experiments, obtained from:

- somatic cell mutagenicity tests in vivo, in mammals; or other in vivo somatic cell genotoxicity tests which are supported by positive results from in vitro mutagenicity assays.

Positive somatic cell mutagenicity and genotoxicity *in vivo* studies: Rithidech (1990), Haney & Conner (1999), Lee *et al.* (1997a), Ferng (2002), Kim *et al.* (2001), Kim *et al.* (2002) and Haddad *et al.* (2009).

Supporting positive *in vitro* tests: Gooch *et al.* (1976), Guerrero *et al.* (1979), Fetner *et al.* (1962) Chorvatovicova *et al.* (2000) and Díaz-Llera *et al.* (2002).

Further evidence for the genotoxic and mutagenic potency is provided by the epidemiological study by Holland (2015).

RAC agrees with the DS that a **classification as Muta. 2 (H341) is warranted for ozone**.

10.9 Carcinogenicity

Table 23: Summary table of animal studies on carcinogenicity

Method, Guideline, GLP status, Reliability	**Species, Strain, Sex, No/ group**	**Test substance, Dose levels, Route of exposure, Duration of exposure**	**NOAEC, LOAEC**	**Results** *(Please indicate any results that might suggest carcinogenic effects, as well as other toxic effects)*	**Remarks** *(e.g. major deviations)*	**Reference**
Method: 18-week inhalation study **Guideline:** no **GLP:** no **Reliability:** 2	**Mice,** A/J, male **No/Group:** 31-37	Ozone 0 (filtered air), 0.4 ppm or 0.8 ppm 0.9 % sodium chloride vehicle 1 day prior exposure initiation 8h/day 7days/week for 18 weeks (whole body) Animals were sacrificed 4 months after start of treatment.	**Neoplastic** LOAEC: 0.8 ppm **Non-neoplastic** NOAEC: <0.4 ppm LOAEC: 0.4 ppm	**Neoplastic findings:** Statistically significant increased lung tumour incidence and multiplicity at 0.8 ppm ozone (χ^2 test, $p < 0.05$). Tumour incidence and multiplicity (mean±SE): <u>NaCl + air:</u> 4/33 (12 %); 0.13±0.06 <u>NaCl + 0.4 ppm ozone:</u> 2/23 (9 %); 0.09±0.06 <u>NaCl + 0.8 ppm ozone:</u> 12/32 (38 %)*; 0.55±0.15* **Non-neoplastic findings:** <u>0.4 ppm ozone</u> Diffuse mild-to-moderate bronchiolar epithelial hyperplasia with some infiltrates of macrophages and neutrophils in the affected epithelium, the tissue around the bronchioles and associated lymphoid aggregates. <u>0.8 ppm ozone</u> Lesions characteristic of mild-to-moderate chronic active bronchiolitis. Diffuse moderate-to-marked bronchiolar	11/34 deaths in A/J mice treated with saline and 0.4 ppm ozone. No individual data. No other organs beside lung, heart and mediastinum investigated. No haematology/ urine analysis/ clinical chemistry. Only 1 sex used.	Last J. A. *et al.* (1987), JNCI 78: 149-154

Method, Guideline, GLP status, Reliability	**Species, Strain, Sex, No/ group**	**Test substance, Dose levels, Route of exposure, Duration of exposure**	**NOAEC, LOAEC**	**Results** *(Please indicate any results that might suggest carcinogenic effects, as well as other toxic effects)*	**Remarks** *(e.g. major deviations)*	**Reference**
				epithelial hyperplasia and prominent peribronchiolar lymphoid nodules. Mild-to-moderate infiltrate of macrophages, often containing hemosiderin, and neutrophils in the bronchioles, lymphoid nodules, and surrounding tissue. Occasional bronchioles with mild mucopurulent exudate.		
Method: 5-month inhalation study followed by killing, 4-month recovery or 4 further months of ozone exposure **Guideline:** None **GLP:** No **Reliability:** 2	**Mice,** A/J, female **No/Group:** 29-35	**Ozone** 0 (Filtered air), 0.12, 0.5 and 1.01 ppm (mean measured concentration) whole body 6h/day on 5 days/week group A: 5 months, group B: 9 months; group C. for 5 months + 4 months filtered aiR	**Neoplastic** → derived from group A LOAEC: 1.01 ppm NOAEC: 0.5 ppm **Non-neoplastic** NOAEC/LOAEC not derived	- no ozone-related deaths - no ozone-related weight gain change **Neoplastic findings, group A:** Lung tumour incidence and multiplicity (mean±SEM): Control: 3/35 (9 %); 0.11±0.05 0.12 ppm: 3/35 (9 %); 0.09±0.05 0.50 ppm: 4/35 (11 %); 0.14±0.07 1 ppm: 8/35 (23 %); 0.23±0.07 - dose-dependent increase in tumour incidence by Cochran-Armitage trend test (p = 0.0234) - no statistically significant difference between groups : **Neoplastic findings, group B:** Lung tumour incidence and multiplicity (mean±SEM): Control: 15/30 (50 %); 0.83±0.19 0.12 ppm: 19/31 (61 %); 1.12±0.20	For 0.5 ppm no tissue volumes were determined. No individual data. No other organs beside lungs investigated. No haematology/ urine analysis/ clinical chemistry. Only one sex used. The reliability of results observed in exp. B and C is limited due to high spontaneous tumour incidence.	Witschi H. *et al.* (1999), Toxicological Sciences 52: 162-167

Method, Guideline, GLP status, Reliability	**Species, Strain, Sex, No/ group**	**Test substance, Dose levels, Route of exposure, Duration of exposure**	**NOAEC, LOAEC**	**Results** *(Please indicate any results that might suggest carcinogenic effects, as well as other toxic effects)*	**Remarks** *(e.g. major deviations)*	**Reference**
				0.50 ppm: 26/32 (81 %)*; 1.25±0.16 1 ppm: 20/35 (57 %); 0.97±0.19 - increase in lung tumour incidence, statistically significant ($p < 0.05$, Fisher's exact test) in mid-dose group; no statistically significant increase in tumour multiplicity **Neoplastic findings, group C:** Lung tumour incidence and multiplicity (mean±SEM): Control: 14/29 (48 %); 0.83±0.19 0.12 ppm: 26/29 (90 %)*; 1.93±0.25* 0.50 ppm: 20/30 (66 %); 1.2±0.27 1 ppm: 21/34 (62 %); 0.97±0.17 - increase in lung tumour incidence multiplicity, statistically significant in low dose group ($p < 0.05$, ANOVA and Fisher's exact test) : **Histology** - most tumours were alveolar/bronchiolar adenomas - alveolar/bronchiolar carcinomas arose within existing adenomas (focal areas manifesting a different growth pattern from adenoma) - occasionally papillary adenomas **Non-neoplastic changes**		

<table>
<tr><th>Method, Guideline, GLP status, Reliability</th><th>Species, Strain, Sex, No/ group</th><th>Test substance, Dose levels, Route of exposure, Duration of exposure</th><th>NOAEC, LOAEC</th><th>Results (Please indicate any results that might suggest carcinogenic effects, as well as other toxic effects)</th><th>Remarks (e.g. major deviations)</th><th>Reference</th></tr>
<tr><td></td><td></td><td></td><td></td><td><u>Tissue volumes per surface area (m^3/m^2)</u>
- no statistically significant changes due to large SDs; according to authors individual animals showed volume changes in septal tip tissues</td><td></td><td></td></tr>
<tr><td>Method: 6-month inhalation study for ozone
Guideline: no
GLP: no
Rel. 2</td><td>Mice, A/J, female

No/Group: 40</td><td>Ozone

0, 0.31 (Exp. 1) or 0.5 ppm (Exp. 2)

Route of exposure:
Inhalation
Duration of exposure:
Exp 1:103 h/week for 6 months; sacrifice 5 months after final ozone exposure
Exp. 2: 102 h/first week of each month for 6 months; sacrifice 3 months after final ozone dose
(whole body)</td><td>Neoplastic
LOAEC: 0.5 ppm (derived from Exp.2)
Non-neoplastic
NOAEC/LOAEC not available under the conditions of the study.</td><td>Clinical findings
- no statistically significant weight or mortality differences between groups at study termination
Neoplastic findings
Exp. 1 (0.31 ppm, age of animals at sacrifice: ~ 1 year):
- <u>control:</u>
No. of tumour-bearing animals: 16/40
% Mice with tumours: 40
Total no. of lung tumours: 24
Average no. of tumours/mouse: 0.60
- <u>ozone:</u>
No. of tumour-bearing animals: 21/40
% Mice with tumours: 53
Total no. of lung tumours: 34
Average no. of tumours/mouse: 0.85
- tumour distribution control vs. ozone
<table>
<tr><td rowspan="2">No. tumour/animal</td><td colspan="2">No. of animals</td></tr>
<tr><td>Control</td><td>Ozone</td></tr>
<tr><td>0</td><td>24</td><td>19</td></tr>
<tr><td>1</td><td>11</td><td>9</td></tr>
<tr><td>2</td><td>4</td><td>11</td></tr>
<tr><td>≥3</td><td>1</td><td>1</td></tr>
</table>
No. of lung tumours greater in ozone</td><td>No individual data.

No haematology/urine analysis/clinical chemistry.

No severity for spleen enlargement given.

Unclear which other organs beside lung and spleen were investigated.

Only 1 sex used.
The reliability of results observed in exp. 1 is limited due to high spontaneous tumour incidence.</td><td>Hasset C. et al. (1985), JNCI 75: 771-777</td></tr>
</table>

<table>
<tr><th>Method, Guideline, GLP status, Reliability</th><th>Species, Strain, Sex, No/ group</th><th>Test substance, Dose levels, Route of exposure, Duration of exposure</th><th>NOAEC, LOAEC</th><th>Results (Please indicate any results that might suggest carcinogenic effects, as well as other toxic effects)</th><th>Remarks (e.g. major deviations)</th><th>Reference</th></tr>
<tr><td></td><td></td><td></td><td></td><td>group vs. control (χ^2 test, $p < 0.005$)

Exp. 2 (0.5 ppm, age of animals at sacrifice: ~ 9 months):
- <u>control:</u>
No. of tumour-bearing animals: 8/45
% Mice with tumours: 18
Total no. of lung tumours: 9
Average no. of tumours/mouse: 0.20
- <u>ozone:</u>
No. of tumour-bearing animals: 17/45
% Mice with tumours: 38
Total no. of lung tumours: 29
Average no. of tumours/mouse: 0.64
- tumour distribution control vs. ozone
<table>
<tr><td rowspan="2">No. tumour/animal</td><td colspan="2">No. of animals</td></tr>
<tr><td>Control</td><td>Ozone</td></tr>
<tr><td>0</td><td>37</td><td>28</td></tr>
<tr><td>1</td><td>7</td><td>12</td></tr>
<tr><td>2</td><td>1</td><td>4</td></tr>
<tr><td>≥3</td><td>0</td><td>1</td></tr>
</table>
No. of lung tumours greater in ozone group vs. control (χ^2 test, $p < 0.005$)

Tumours
- bronchio-alveolar origin
- well circumscribed
- localized areas of increased prominence of alveolar lining cells (isolated changes and in continuity with established adenomas) → according to the authors</td><td></td><td></td></tr>
</table>

Method, Guideline, GLP status, Reliability	**Species, Strain, Sex, No/ group**	**Test substance, Dose levels, Route of exposure, Duration of exposure**	**NOAEC, LOAEC**	**Results** *(Please indicate any results that might suggest carcinogenic effects, as well as other toxic effects)*	**Remarks** *(e.g. major deviations)*	**Reference**
				this could be indicative of pathway from hyperplasia to neoplasia **Non-neoplastic lesions** - enlarged spleens		
Method: 1-year carcinogenicity study for ozone **Guideline:** None **GLP:** No **Reliability:** 3	**Mice,** B6C3F$_1$, female and male **No/Group:** 20 M + 20 F	**Ozone** 0 or 0.5 ppm **Route of exposure:** Inhalation (ozone) **Duration of exposure:** 6h/day 5 days/week for 1 year (whole body)	**Neoplastic** no effects observed at 0.5 ppm **Non-neoplastic** NOAEC: < 0.5 ppm LOAEC: 0.5 ppm	- no ozone-related deaths body weights not affected **Neoplastic findings** - no treatment related increase in tumour incidence in lung, oviduct and liver **Non-neoplastic findings** - relative organ weight of kidney in males statistically significantly increased (≥ 10 %); for kidney (left) and testis (right) organ weights statistically decreased (≥ 10 %); (analysis of variance and Student's *t*-test, p<0.05) - relative organ weight of lung and kidney (right) statistically significantly increased (but: < 10 %); relative organ weight of adrenal (right) and ovary (left and right) decreased (≥ 10 %) after 1 year; (analysis of variance and Student's *t*-test, p<0.05) - peribronchial mononuclear cell infiltration (10 % males treated with ozone) - focal bronchiolar alveolar hyperplasia (10 % males treated with ozone) - bronchiolar epithelium hyperplasia (10	Only one dose exposed for 1 year. No individual data. No haematology/urine analysis/ clinical chemistry. No severity grade for lesions given. It remains unclear which organs were examined histopathologically.	Kim M. Y. and Cho M. Y. (2009), Toxicology and industrial health 25: 189-195

Method, Guideline, GLP status, Reliability	**Species, Strain, Sex, No/ group**	**Test substance, Dose levels, Route of exposure, Duration of exposure**	**NOAEC, LOAEC**	**Results** *(Please indicate any results that might suggest carcinogenic effects, as well as other toxic effects)*	**Remarks** *(e.g. major deviations)*	**Reference**
				% males and 10 % females treated with ozone) - alveolar fibrosis (10 % males treated with ozone) - hepatocyte vacuolation (10 % females treated with ozone) - focal necrosis (10 % males treated with ozone) in liver - congestion in cerebrum (10 % males treated with ozone) - mild hyperplasia in adrenal gland (10 % males treated with ozone) - seminiferous disengagement in testis (10 % after ozone treatment)		
Method: 2-year inhalation study **Guideline:** similar to TG 451 **GLP:** in compliance with Food and Drug Administration (FDA) Good Laboratory Practice Regulations **Reliability:** 1	**Mice,** $B6C3F_1$ mice (Simonsen Laboratories (Gilroy, CA)), male and female **No/Group:** 50 per sex and group	**Ozone** 0 (filtered air), 0.12, 0.5 and 1.0 ppm 6h/day 5 days/week for 2 years (whole body)	**Neoplastic:** LOAEC: 0.5 ppm **Non-neoplastic:** NOAEC:< 0.12 ppm LOAEC: 0.12 ppm	**Survival, Body weight and clinical findings:** <u>Survival:</u> - no effect - males: 0: 30, 0.12: 34, 0.5: 25, 1.0: 27 animals - females: 0: 29, 0.12: 37, 0.5: 33, 1.0: 40 animals <u>Mean body weight</u> - males: slightly reduced at 1.0 ppm throughout the study (2-6 %) - females: lower at 0.12 and 1.0 ppm throughout the study and at week 53-104 (0.12: 8 %, 0.5: 5 %, 1.0:12 %) <u>Clinical:</u>	Different number of animals at study termination (20 % survivors per group) used for histopathology. No haematology, clinical biochemistry and urinalysis data presented. Severity grade of lesions only presented for selected lesions.	NTP, Toxicology and carcinogenesis studies of ozone and ozone/NKK in F344/N rats and B6C3F1 mice, National toxicology program, Technical report series 440 (1994)

Method, Guideline, GLP status, Reliability	**Species, Strain, Sex, No/ group**	**Test substance, Dose levels, Route of exposure, Duration of exposure**	**NOAEC, LOAEC**	**Results** *(Please indicate any results that might suggest carcinogenic effects, as well as other toxic effects)*	**Remarks** *(e.g. major deviations)*	**Reference**
				- hypoactivity, in particular at 1 ppm **Neoplastic lesions:** - Alveolar/bronchiolar combined adenoma or carcinoma increased in males and females (Tab. 27) positive trend: life table test, logistic regression test and for females Cochran-Armitage-test) and stat. significant increased in females at 1.0 ppm (logistic regression test, Fisher exact test) - alveolar/bronchiolar carcinoma increased in females (positive trend: logistic regression test, life table test and Cochran-Armitage test) - alveolar/bronchiolar adenoma stat. sign. at 0.5 ppm in males (life table test) - hepatocellular carcinoma positive trend (life table test) in males - hardarian gland combined adenoma or carcinoma stat. significant for pairwise comparison at 0.12 (life time table, logistic regression, Fisher exact test) and 0.5 ppm (life table test) (males) - stromal polyp in uterus positive trend (life table test, logistic regression test, Cochran-Armitage test) in females **Non-neoplastic lesions:** <u>0.12 ppm:</u> - nose: inflammation (only males), lateral wall hyaline degeneration (only females)		Boorman G. A. *et al.* (1995), Toxicol Lett. 82-83:301-6 Herbert R. A. *et al.* (1996), Toxicol. Pathol. 24: 539-548

Method, Guideline, GLP status, Reliability	**Species, Strain, Sex, No/ group**	**Test substance, Dose levels, Route of exposure, Duration of exposure**	**NOAEC, LOAEC**	**Results** *(Please indicate any results that might suggest carcinogenic effects, as well as other toxic effects)*	**Remarks** *(e.g. major deviations)*	**Reference**
				<u>additional 0.5 ppm and 1.0 ppm:</u> - nose: lateral wall hyperplasia, inflammation (only females), lateral wall fibrosis and lateral wall squamous metaplasia (only males), lateral wall hyaline degeneration (only males), olfactory epithelium atrophy (limited to females) - lung: alveolar epithelium metaplasia, histiocytic infiltration in alveolus <u>additional 1.0 ppm:</u> - epiglottis: hyperplasia (only females)		
Method: lifetime inhalation study **Guideline:** no **GLP:** in compliance with Food and Drug Administration (FDA) Good Laboratory Practice Regulations **Reliability:** 1	**Mice,** B6C3F$_1$ mice (Simonsen Laboratories (Gilroy, CA)), male and female **No/Group:** 50 per sex and group	**Ozone** 0 (filtered air), 0.5 and 1.0 ppm 6h/day 5 days/week for 130 weeks (whole body)	**Neoplastic:** LOAEC: 0.5 ppm **Non-neoplastic:** NOAEC: < 0.5 ppm LOAEC: 0,5 ppm	**Survival, Body weight and clinical findings:** <u>Survival:</u> - males: 0: 14, 0.5: 11, 1.0: 12 animals - females: 0: 9, 0.5: 12, 1.0: 10 animals <u>Mean body weight</u> - mean bw (51.9 g at 0 ppm, 45.5 g at 1 ppm for week 53-104 in females) were slightly lower than in controls <u>Clinical:</u> - hypoactivity, particularly at 1 ppm **Neoplastic lesions:** - alveolar/bronchiolar carcinoma in males	Different number of animals at study termination No haematology, clinical biochemistry and urinalysis data presented. Severity grade of lesions only presented for selected non-neoplastic lesions. Only 2 doses.	NTP, Toxicology and carcinogenesis studies of ozone and ozone/NKK in F344/N rats and B6C3F1 mice, National toxicology program, Technical report series 440 (1994)

Method, Guideline, GLP status, Reliability	**Species, Strain, Sex, No/ group**	**Test substance, Dose levels, Route of exposure, Duration of exposure**	**NOAEC, LOAEC**	**Results** *(Please indicate any results that might suggest carcinogenic effects, as well as other toxic effects)*	**Remarks** *(e.g. major deviations)*	**Reference**
				(pos. trend [Life table test, Logistic regression, Cochran-Armitage test], statistically significant for 0.5 ppm [Life table test and logistic regression test], statistically significant for 1 ppm [Life table test, logistic regression test and Fisher's exact test] incidences in Tab. 28) - alveolar/bronchiolar adenoma in females (pos. trend [Life table test, Logistic regression test and Cochran-Armitage test] and statistically significant for 1 ppm [Life table test, Logistic regression and Fisher's exact test], incidences in Tab. 28) **Non-neoplastic lesions:** 0.5 ppm: - nose: lateral wall, hyaline degeneration; lateral wall, fibrosis; lateral wall, hyperplasia; lateral wall, inflammation, suppurative; olfactory, epithelium, atrophy (only females) - lung: alveolar epithelial metaplasia; alveolar infiltration, histiocyte additional 1.0 ppm: - Larynx: hyperplasia; epiglottis, metaplasia, squamous - nose: lateral wall, metaplasia, squamous; olfactory, epithelium, atrophy (only males)		Boorman G. A. *et al.* (1995), Toxicology letters 82/83: 301-306 Herbert R. A. *et al.* (1996), Toxicol. Pathol. 24: 539-548
Method: 2-year inhalation study	**Rat,** F344/N rats (Simonsen	**Ozone**	**Neoplastic:** LOAEC: -	**Survival, Body weight and clinical findings:**	Different group size for histopathology.	NTP, Toxicology

Method, Guideline, GLP status, Reliability	**Species, Strain, Sex, No/ group**	**Test substance, Dose levels, Route of exposure, Duration of exposure**	**NOAEC, LOAEC**	**Results** *(Please indicate any results that might suggest carcinogenic effects, as well as other toxic effects)*	**Remarks** *(e.g. major deviations)*	**Reference**
Guideline: similar to TG 451 **GLP:** in compliance with Food and Drug Administration (FDA) Good Laboratory Practice Regulations **Reliability:** 2	Laboratories (Gilroy, CA)), male and female **No/Group:** 50 per sex and group	0 (Filtered air), 0.12, 0.5 and 1.0 ppm 6h/day 5 days/week for 2 years (whole body)	**Non-neoplastic:** NOAEC:< 0.12 ppm LOAEC: 0.12 ppm	<u>Survival:</u> - no differences between exposure groups and control - males: in every group high number of moribund animals 35-40 animals; animals surviving to study termination 0: 8, 0.12: 5, 0.5: 7, 1.0: 7 animals - females: animals surviving to study termination 0: 28, 0.12: 24, 0.5: 30, 1.0: 27 animals <u>Mean body weight</u> - no differences at 0.12 and 0.5 ppm - slightly reduced (male: 6 %; female: 8-6 %) at 1.0 ppm during exposure <u>Clinical:</u> - hypoactivity, in particular at 1 ppm **Neoplastic lesions:** (only in males) - skin: positive trend for keratoacanthoma (life table test, logistic regression test) and combined Squamous cell papilloma, keratoacanthoma, trichoepithelioma, basal cell adenoma, or squamous cell carcinoma (life table test, logistic regression test, Cochran-Armitage) **Non-neoplastic lesions:** <u>0.12 ppm:</u> - nose: inflammation (limited to males), lateral wall hyperplasia (limited to males), lateral wall metaplasia squamous (limited to females) - lung: alveolar epithelium metaplasia	More than 50 % male rats in 2-year study died before study termination. No haematology, clinical biochemistry and urinalysis data presented. Severity grade of lesions only presented for selected non-neoplastic lesions.	and carcinogenesis studies of ozone and ozone/NKK in F344/N rats and B6C3F1 mice, National toxicology program, Technical report series 440 (1994) Boorman G. A. *et al.* (1994), Toxicol Pathol. 22(5):545-54 Boorman G.A. *et al.* (1995), Toxicol Lett. 82-83:301-6

Method, Guideline, GLP status, Reliability	**Species, Strain, Sex, No/ group**	**Test substance, Dose levels, Route of exposure, Duration of exposure**	**NOAEC, LOAEC**	**Results** *(Please indicate any results that might suggest carcinogenic effects, as well as other toxic effects)*	**Remarks** *(e.g. major deviations)*	**Reference**
				(extension of bronchial epithelium into alveoli) <u>additional 0.5 ppm and 1.0 ppm:</u> - larynx: epiglottis squamous metaplasia - nose: goblet cell hyperplasia, lateral wall squamous metaplasia, lateral wall hyperplasia (limited to females) - lung: histiocytic infiltration in alveolus, interstitial fibrosis		
Method: lifetime inhalation study **Guideline:** no **GLP:** in compliance with Food and Drug Administration (FDA) Good Laboratory Practice Regulations **Reliability: 2**	**Rat,** F344/N rats (Simonsen Laboratories (Gilroy, CA)), male and female **No/Group:** 50 per sex and group	**Ozone** 0 (filtered air), 0.5 and 1.0 ppm 6h/day 5 days/week for 125 weeks (whole body)	**Neoplastic:** LOAEC: - **Non-neoplastic:** NOAEC: < 0.5 ppm LOAEC: 0.5 ppm	**Survival, Body weight and clinical findings:** <u>Survival:</u> - no differences between exposure groups and control - in every group high number of moribund animals (males: 42-47 ; females: 36-40) - males: animals surviving to study termination 0: 0, 0.5: 0, 1.0: 1 animals - females: animals surviving to study termination 0: 6, 0.5: 6, 1.0: 7 animals <u>Mean body weight</u> - mean bw and bw gains in females and males (1 ppm) were slightly (94 % and 93 %) lower than in controls - final mean bw similar to controls <u>Clinical:</u>	Different number of animals at study termination. No haematology, clinical biochemistry and urinalysis data presented. Severity grade of lesions only presented for selected lesions. Only 2 doses.	NTP, Toxicology and carcinogenesis studies of ozone and ozone/NKK in F344/N rats and B6C3F1 mice, National toxicology program, Technical report series 440 (1994) Boorman G. A. *et al.* (1995), Toxicology

Method, Guideline, GLP status, Reliability	**Species, Strain, Sex, No/ group**	**Test substance, Dose levels, Route of exposure, Duration of exposure**	**NOAEC, LOAEC**	**Results** *(Please indicate any results that might suggest carcinogenic effects, as well as other toxic effects)*	**Remarks** *(e.g. major deviations)*	**Reference**
				- hypoactivity, particularly at 1 ppm **Neoplastic lesions:** - Oral mucosa/males: Squamous cell papilloma or squamous cell carcinoma (pos. trend with Cochran-Armitage test) - clitoral gland/females: Adenoma or carcinoma (incidences: 8 adenoma at 1 ppm, 5 at 0 ppm; 1 carcinoma at 1 ppm, 0 at 0 ppm) pos. trend with Cochran-Armitage test) **Non-neoplastic lesions:** ≥0.5 ppm: - larynx: epiglottis, squamous metaplasia - nose: goblet cell, lateral wall, hyperplasia; lateral wall, hyperplasia; lateral wall, squamous metaplasia - lung: alveolar epithelial metaplasia; alveolar infiltration, histiocyte; interstitial fibrosis		letters 82/83: 301-306 Herbert R. A. *et al.* (1996), Toxicol. Pathol. 24: 539-548

Annotation: Lung tumours were not increased or did not occur in the following inhalation studies with ozone:

- Swiss Webster mice, 18 weeks, 0.4 and 0.8 ppm ozone (Last J.A. et al. 1987)

- B6C3F1 mice, 12 weeks, 0.5 ppm ozone (Kim et al. 2001)

- B6C3F1 mice, 16 and 32 weeks, 0.5 ppm ozone (Kim M.Y. and Cho M.Y. 2009)

- Golden Syrian hamster, 16 weeks (with and without 8 weeks of recovery), 0.8 ppm ozone (Witschi H. et al. 1993)

10.9.1 Short summary and overall relevance of the provided information on carcinogenicity

Studies focussing on the investigation of neoplasms following ozone exposure were performed in mice, rats and hamsters. Most of the studies are flawed by reporting deficiencies (e.g. no reporting of examined organs, no individual data, no severity of lesions presented). These quality shortcomings make it difficult to decide on (or exclude) carcinogenic activity in all organs, which is further exacerbated by the fact that many studies are not comparable with the appropriate OECD TG for carcinogenicity.

10.9.1.1 Incidences of non-neoplastic and neoplastic findings of the relevant studies (if not mentioned above in table 23)

Table 24: Lung tumour incidence and multiplicity in A/J mice (Last *et* al. 1987) after 18 weeks of exposure

Treatment	**Tumour incidence**	**No. of tumours/lung**
NaCl + air	4/33 (12 %)	0.13 ± 0.06
NaCl + 0.4 ppm O_3	2/23 (9 %)	0.09 ± 0.06
NaCl + 0.8 ppm O_3	12/32 (38 %)*	0.55 ± 0.15*

* P<0.05, compared to NaCl+air control (χ^2-test)

Table 25: Lung tumour data in strain A/J mice exposed for different time schedules (Witschi *et* al. 1999)

		Lung tumour multiplicity [a]		
Group	**Exposure**	**Lung tumour incidence [b]**	**All animals**	**Tumour-bearing animals only**
A 5 months exposure	filtered air	3/35 (9 %)	0.11 ± 0.05 (35)	1.00 ± 0.00 (3)
	0.12 ppm ozone	3/35 (9 %)	0.09 ± 0.05 (35)	1.00 ± 0.00 (3)
	0.50 ppm ozone	4/35 (11 %)	0.14 ± 0.07 (35)	1.3 ± 0.3 (4)
	1.00 ppm ozone	8/35 (23 %)	0.23 ± 0.07 (35)	1.00 ± 0.00 (8)
B 9 months exposure	filtered air	15/30 (50%)	0.83 ± 0.19 (29)	1.71 ± 0.22 (14)
	0.12 ppm ozone	19/31 (61 %)	1.12 ± 0.20 (31)	1.84 ± 0.18 (19)
	0.50 ppm ozone	26/32 (81 %)*	1.25 ± 0.16 (32)	1.54 ± 0.14 (26)
	1.00 ppm ozone	20/35 (57 %)	0.97 ± 0.19 (35)	1.70 ± 0.21 (20)
C 5 months exposure and 4 months recovery	filtered air (same animals as group B)	14/29 (48 %)	0.83 ± 0.19 (29)	1.71 ± 0.22 (14)
	0.12 ppm ozone	26/29 (90 %)[#]	1.93 ± 0.25 (29)[#]	2.15 ± 0.25 (26)
	0.50 ppm ozone	20/30 (66 %)	1.20 ± 0.27 (30)	1.80 ± 0.19 (20)
	1.00 ppm ozone	21/34 (62 %)	0.97 ± 0.17 (34)	1.57 ± 0.16 (21)

* Significantly different ($p < 0.05$) from control and 1.0 ppm groups (Fisher´s exact test)
[#] Significantly different ($p < 0.05$) from all other groups (ANOVA and Fisher´s exact test)
[a] Number of tumours per lung. All data given as mean ± SEM, number of animals in brackets
[b] Number of tumour bearing animals per total number of animals at risk, percentage in brackets

Table 26: Incidences of selected neoplasms and non-neoplastic lesions of the respiratory tract in rats in the 2-year inhalation study with ozone (NTP 1994)

Dose (ppm)	0	0.12	0.5	1.0
Male				
Larynx[a]	50	50	50	50
Epiglottis, Metaplasia, Squamous[b]	0	2 (2.5)[c]	16** (1.3)	43** (2.3)
Nose	50	50	50	50
Inflammation, Suppurative	3 (1.7)	10* (1.7)	12* (1.8)	20** (1.9)
Goblet Cell, Lateral Wall, Hyperplasia	1 (2.0)	4 (1.5)	41** (1.5)	48** (2.1)
Lateral Wall, Hyperplasia	0	8** (2.3)	50** (2.0)	49** (2.7)
Lateral Wall, Metaplasia, Squamous	2 (1.5)	6 (1.8)	36** (1.8)	46** (2.3)
Lung	50	50	50	50
Alveolar Epithelium, Metaplasia	0	9** (1.0)	46** (1.9)	47** (2.9)
Alveolus, Infiltration Cellular, Histiocyte	1 (2.0)	0	27** (1.2)	42** (1.9)
Interstitial, Fibrosis	0	2 (1.0)	40** (1.4)	44** (2.2)
Alveolar/bronchiolar Adenoma				
Overall rate[d]	1/50 (2%)	2/50 (4%)	2/50 (4%)	3/50 (6%)
Adjusted rate[e]	2.2%	16.4%	20.4%	25.4%
Terminal rate[f]	0/8 (0%)	0/5 (0%)	1/7 (14%)	1/7 (14%)
First incidence (days)	514	537	698	619
Logistic regression test[g]	P=0.246	P=0.500	P=0.501	P=0.309
Alveolar/bronchiolar Carcinoma				
Overall rate	1/50 (2%)	1/50 (2%)	1/50 (2%)	1/50 (2%)
Alveolar/bronchiolar Adenoma or Carcinoma[h]				
Overall rate	2/50 (4%)	3/50 (6%)	3/50 (6%)	4/50 (8%)
Adjusted rate	14.4%	18.6%	33.7%	30.1%
Terminal rate	1/8 (13%)	0/5 (0%)	2/7 (29%)	1/7 (14%)
First incidence (days)	514	537	698	619
Logistic regression test	P=0.284	P=0.500	P=0.515	P=0.341

(continued)

Table 27: Incidences of selected neoplasms and non-neoplastic lesions of the respiratory tract in rats in the 2-year inhalation study with ozone (NTP 1994) (cont.)

Dose (ppm)	0	0.12	0.5	1.0
Female				
Larynx	50	50	50	50
Epiglottis, Metaplasia, Squamous	4 (3.3)	5 (2.8)	9 (2.3)	43** (2.3)
Nose	50	50	50	50
Goblet Cell, Lateral Wall, Hyperplasia	1 (2.0)	2 (1.0)	45** (1.7)	50** (2.5)
Lateral Wall, Hyperplasia	2 (2.0)	8 (1.5)	48** (1.8)	50** (2.6)
Lateral Wall, Metaplasia, Squamous	2 (2.5)	11** (1.4)	21** (1.8)	45** (1.9)
Suppurative Inflammation	3 (1.0)	6 (1.5)	2 (1.0)	2 (2.0)
Lung	50	50	50	50
Alveolar Epithelium, Metaplasia	0	6** (1.0)	48** (1.7)	48** (2.8)
Alveolus, Infiltration Cellular, Histiocyte	0	0	31** (1.2)	43** (1.8)
Interstitial, Fibrosis	0	0	42** (1.4)	47** (2.0)
Alveolar/bronchiolar Adenoma[i]				
Overall rate	0/50 (0%)	0/50 (0%)	2/50 (4%)	0/50 (0%)
Adjusted rate	0.0%	0.0%	6.4%	0.0%
Terminal rate	0/28 (0%)	0/24 (0%)	1/30 (3%)	0/27 (0%)
First incidence (days)	–[j]	–	723	–
Logistic regression test	P=0.545	–	P=0.255	–

* Significantly different (P≤0.05) from the control group by the logistic regression test
** P≤0.01
[a] Number of animals with organ examined microscopically
[b] Number of animals with lesion
[c] Average severity grade of lesions in affected animals (1=minimal; 2=mild; 3=moderate; 4=marked).
[d] Number of animals with neoplasm per number of animals necropsied
[e] Kaplan-Meier estimated neoplasm incidence at the end of the study after adjustment for intercurrent mortality
[f] Observed incidence at terminal sacrifice
[g] Beneath the control incidence are the P values associated with the trend test. Beneath the exposed group incidence are the P values corresponding to pairwise comparisons between the controls and that exposed group. The logistic regression test regards these lesions as nonfatal.
[h] Historical incidence for 2-year inhalation studies with untreated control groups (mean ± standard deviation): 17/398 (4.3% ± 4.5%); range, 0%-10%
[i] Historical incidence: 4/398 (1.0% ± 1.5%); range, 0%-4%
[j] Not applicable; no neoplasms in animal group

Table 28: Incidences of selected neoplasms and non-neoplastic lesions of the respiratory tract in rats in the lifetime inhalation study of ozone (NTP 1994)

Dose (ppm)	0	0.5	1.0
Male			
Larynx[a]	50	48	47
Epiglottis, Squamous Metaplasia[b]	0	20** (1.3)[c]	43** (1.8)
Nose	50	49	49
Goblet Cell, Lateral Wall, Hyperplasia	1 (1.0)	46** (1.5)	48** (2.6)
Lateral Wall, Hyperplasia	10 (1.5)	48** (1.9)	47** (2.8)
Lateral Wall, Squamous Metaplasia	10 (2.5)	23** (1.6)	40** (2.3)
Lung	50	50	50
Alveolar Epithelial Metaplasia	0	45** (1.9)	50** (2.9)
Alveolar Cellular Infiltration, Histiocyte	0	38** (1.2)	49** (1.9)
Interstitial Fibrosis	0	44** (1.7)	50** (2.4)
Alveolar/bronchiolar Adenoma			
Overall rate[d]	2/50 (4%)	3/50 (6%)	0/50 (0%)
Adjusted rate[e]	25.9%	22.3%	0.0%
Terminal rate[f]	0/0	0/0	0/1 (0%)
First incidence (days)	708	581	–[h]
Logistic regression test[g]	P=0.161N	P=0.427	P=0.169N
Alveolar/bronchiolar Carcinoma			
Overall rate	0/50 (0%)	1/50 (2%)	0/50 (0%)
Alveolar/bronchiolar Adenoma or Carcinoma			
Overall rate	2/50 (4%)	4/50 (8%)	0/50 (0%)
Adjusted rate	25.9%	26.2%	0.0%
Terminal rate	0/0	0/0	0/1 (0%)
First incidence (days)	708	581	–
Logistic regression test	P=0.182N	P=0.266	P=0.169N

(continued)

Table 29: Incidences of selected neoplasms and non-neoplastic lesions of the respiratory tract in rats in the lifetime inhalation study of ozone (NTP 1994) (cont.)

Dose (ppm)	0	0.5	1.0
Female			
Larynx	49	47	50
Epiglottis, Squamous Metaplasia	2 (2.0)	16** (1.1)	48** (2.0)
Nose	50	49	50
Goblet Cell, Lateral Wall, Hyperplasia	0	47** (1.8)	50** (2.4)
Lateral Wall, Hyperplasia	4 (1.8)	49** (1.9)	50** (2.8)
Lateral Wall, Squamous Metaplasia	5 (2.4)	25** (1.3)	35** (1.6)
Lung	50	50	50
Alveolar Epithelial Metaplasia	0	44** (1.7)	50** (2.9)
Alveolar Cellular Infiltration, Histiocyte	0	38** (1.1)	49** (2.0)
Interstitial Fibrosis	0	41** (1.2)	50** (2.5)
Alveolar/bronchiolar Adenoma			
Overall rate	0/50 (0%)	1/50 (2%)	1/50 (2%)
Adjusted rate	0.0%	3.0%	3.3%
Terminal rate	0/6 (0%)	0/6 (0%)	0/7 (0%)
First incidence (days)	–	710	685
Logistic regression test	P=0.330	P=0.507	P=0.500
Alveolar/bronchiolar Carcinoma			
Overall rate	1/50 (2%)	1/50 (2%)	0/50 (0%)
Alveolar/bronchiolar Adenoma or Carcinoma			
Overall rate	1/50 (2%)	2/50 (4%)	1/50 (2%)
Adjusted rate	12.5%	8.7%	3.3%
Terminal rate	0/6 (0%)	0/6 (0%)	0/7 (0%)
First incidence (days)	827	710	685
Logistic regression test	P=0.594N	P=0.598	P=0.738N

** Significantly different (P≤0.01) than the control group by the logistic regression test
[a] Number of animals with organ examined microscopically
[b] Number of animals with lesion
[c] Average severity grade of lesions in affected animals (1=minimal; 2=mild; 3=moderate; 4=marked).
[d] Number of animals with neoplasm per number of animals necropsied
[e] Kaplan-Meier estimated neoplasm incidence at the end of the study after adjustment for intercurrent mortality
[f] Observed incidence at terminal sacrifice
[g] Beneath the control incidence are the P values associated with the trend test. Beneath the exposed group incidence are the P values corresponding to pairwise comparisons between the controls and that exposed group. The logistic regression test regards these lesions as nonfatal. A negative trend or a lower incidence in an exposure group is indicated by N.
[h] Not applicable; no neoplasms in animal group

Table 30: Incidences of neoplasms and non-neoplastic lesions of the respiratory tract in mice in the 2-year inhalation study of ozone (NTP 1994)

Dose (ppm)	0	0.12	0.5	1.0
Male				
Larynx[a]	50	50	50	50
Epiglottis, Hyperplasia[b]	1 (1.0)[c]	0	0	6 (1.0)
Nose	50	50	50	50
Lateral Wall, Hyaline Degeneration	2 (1.0)	1 (2.0)	49** (2.0)	50** (3.7)
Lateral Wall, Fibrosis	0	0	47** (1.6)	49** (2.7)
Lateral Wall, Hyperplasia	0	0	42** (1.6)	50** (2.3)
Lateral Wall, Inflammation, Suppurative	0	8**(1.0)	42** (1.5)	50** (2.1)
Lateral Wall, Metaplasia, Squamous	0	3 (1.7)	3 (1.0)	36** (1.7)
Lung	50	50	50	50
Alveolar Epithelium, Metaplasia	0	0	48** (1.6)	50** (2.6)
Alveolus, Infiltration Cellular, Histiocyte	0	0	18** (1.1)	31** (1.8)
Alveolar Epithelium, Hyperplasia	4 (1.5)	6 (2.3)	2 (2.0)	3 (3.3)
Alveolar/bronchiolar Adenoma				
Overall rate[d]	6/50 (12%)	9/50 (18%)	12/50 (24%)	11/50 (22%)
Adjusted rate[e]	18.8%	25.1%	40.9%	34.7%
Terminal rate[f]	5/30 (17%)	8/34 (24%)	9/25 (36%)	8/27 (30%)
First incidence (days)	611	440	464	484
Logistic regression test[g]	P=0.079	P=0.318	P=0.061	P=0.110
Alveolar/bronchiolar Carcinoma				
Overall rate	8/50 (16%)	4/50 (8%)	8/50 (16%)	10/50 (20%)
Adjusted rate	25.5%	10.3%	30.7%	35.4%
Terminal rate	7/30 (23%)	1/34 (3%)	7/25 (28%)	9/27 (33%)
First incidence (days)	653	612	701	630
Logistic regression test	P=0.062	P=0.154N	P=0.449	P=0.270
Alveolar/bronchiolar Adenoma or Carcinoma[h]				
Overall rate	14/50 (28%)	13/50 (26%)	18/50 (36%)	19/50 (38%)
Adjusted rate	43.1%	33.4%	60.9%	60.0%
Terminal rate	12/30 (40%)	9/34 (26%)	14/25 (56%)	15/27 (56%)
First incidence (days)	611	440	464	484
Logistic regression test	P=0.030	P=0.445N	P=0.124	P=0.103

(continued)

Table 31: Incidences of neoplasms and non-neoplastic lesions of the respiratory tract in mice in the 2-year inhalation study of ozone (NTP 1994) (cont.)

Dose (ppm)	0	0.12	0.5	1.0
Female				
Larynx	50	50	49	50
Epiglottis, Hyperplasia	0	0	0	7** (1.0)
Nose	50	50	48	50
Lateral Wall, Hyaline Degeneration	5 (1.0)	18* (1.0)	48** (2.6)	50** (3.5)
Lateral Wall, Fibrosis	0	3 (1.8)	46** (1.8)	50** (2.7)
Lateral Wall, Hyperplasia	0	0	42** (1.9)	50** (2.5)
Lateral Wall, Inflammation, Suppurative	0	5 (1.0)	46** (1.7)	50** (2.1)
Lateral Wall, Metaplasia, Squamous	1 (1.0)	1 (1.0)	11** (1.5)	36** (2.2)
Olfactory Epithelium, Atrophy	4 (1.8)	1 (1.0)	14* (1.5)	41** (1.8)
Lung	50	50	49	50
Alveolar Epithelium, Metaplasia	0	0	43** (1.5)	49** (2.6)
Alveolus, Infiltration Cellular, Histiocyte	0	0	11** (1.0)	42** (1.8)
Alveolar Epithelium, Hyperplasia	2 (2.0)	1 (4.0)	1 (1.0)	2 (2.0)
Alveolar/bronchiolar Adenoma				
Overall rate	4/50 (8%)	5/50 (10%)	5/49 (10%)	8/50 (16%)
Adjusted rate	12.5%	12.9%	13.4%	20.0%
Terminal rate	3/29 (10%)	4/37 (11%)	2/33 (6%)	8/40 (20%)
First incidence (days)	636	681	667	735 (T)
Logistic regression test	P=0.153	P=0.549	P=0.515	P=0.239
Alveolar/bronchiolar Carcinoma				
Overall rate	2/50 (4%)	2/50 (4%)	5/49 (10%)	8/50 (16%)
Adjusted rate	6.9%	5.2%	14.1%	19.2%
Terminal rate	2/29 (7%)	1/37 (3%)	3/33 (9%)	7/40 (18%)
First incidence (days)	735 (T)	703	709	488
Logistic regression test	P=0.011	P=0.649N	P=0.259	P=0.053
Alveolar/bronchiolar Adenoma or Carcinoma[i]				
Overall rate	6/50 (12%)	7/50 (14%)	9/49 (18%)	16/50 (32%)
Adjusted rate	19.2%	17.7%	24.0%	38.8%
Terminal rate	5/29 (17%)	5/37 (14%)	5/33 (15%)	15/40 (38%)
First incidence (days)	636	681	667	488
Logistic regression test	P=0.005	P=0.571	P=0.326	P=0.022

* Significantly different (P≤0.05) than the control group by the logistic regression test
** P≤0.01
(T) Terminal sacrifice
[a] Number of animals with organ examined microscopically
[b] Number of animals with lesion
[c] Average severity grade of lesions in affected animals (1=minimal; 2=mild; 3=moderate; 4=marked).
[d] Number of animals with neoplasm per number of animals necropsied
[e] Kaplan-Meier estimated neoplasm incidence at the end of the study after adjustment for intercurrent mortality
[f] Observed incidence at terminal sacrifice
[g] Beneath the control incidence are the P values associated with the trend test. Beneath the exposed group incidence are the P values corresponding to pairwise comparisons between the controls and that exposed group. The logistic regression tests regard these lesions as nonfatal. A lower incidence in an exposure group is indicated by N.
[h] Historical incidence for 2-year inhalation studies with untreated control groups (mean ± standard deviation): 150/673 (22.3% ± 9.0); range, 10%-42%
[i] Historical incidence: 58/659 (8.8 ± 3.5); range, 0%-15%

Table 32: Incidences of neoplasms and non-neoplastic lesions of the respiratory tract in mice in the lifetime inhalation study of ozone (NTP 1994)

Dose (ppm)	0	0.5	1.0
Male			
Larynx[a]	49	49	50
Hyperplasia[b]	4 (1.0)[c]	7 (1.3)	15**(1.1)
Epiglottis, Metaplasia, Squamous	2 (1.0)	1 (1.0)	10** (1.1)
Nose	49	48	49
Lateral Wall, Hyaline Degeneration	2 (1.5)	48** (1.1)	49** (2.5)
Lateral Wall, Fibrosis	0	8** (1.0)	43** (1.3)
Lateral Wall, Hyperplasia	2 (1.0)	33** (1.1)	45** (1.8)
Lateral Wall, Inflammation, Suppurative	1 (1.0)	38** (1.0)	46** (1.3)
Lateral Wall, Metaplasia, Squamous	1 (1.0)	2 (1.5)	20**(1.2)
Olfactory, Epithelium, Atrophy	4 (1.8)	4 (2.3)	18**(1.7)
Lung	49	49	50
Alveolar Epithelium, Metaplasia	0	48** (1.5)	47** (2.2)
Alveolus, Infiltration Cellular, Histiocyte	3 (3.0)	40** (1.8)	41** (1.7)
Alveolar Epithelium, Hyperplasia	10 (2.8)	8 (3.3)	1** (4.0)
Alveolar/bronchiolar Adenoma			
Overall rate[d]	8/49 (16%)	8/49 (16%)	9/50 (18%)
Adjusted rate[e]	33.9%	32.8%	50.6%
Terminal rate[f]	3/14 (21%)	2/11 (18%)	5/12 (42%)
First incidence (days)	391	678	620
Logistic regression test[g]	P=0.427	P=0.606N	P=0.473
Alveolar/bronchiolar Carcinoma			
Overall rate	8/49 (16%)	15/49 (31%)	18/50 (36%)
Adjusted rate	42.3%	65.3%	70.9%
Terminal rate	4/14 (29%)	5/11 (45%)	6/12 (50%)
First incidence (days)	805	693	609
Logistic regression test	P=0.005	P=0.050	P=0.007
Alveolar/bronchiolar Adenoma or Carcinoma			
Overall rate	16/49 (33%)	22/49 (45%)	21/50 (42%)
Adjusted rate	66.0%	76.3%	77.0%
Terminal rate	7/14 (50%)	6/11 (55%)	7/12 (58%)
First incidence (days)	391	678	609
Logistic regression test	P=0.127	P=0.140	P=0.149

(continued)

Table 33: Incidences of neoplasms and non-neoplastic lesions of the respiratory tract in mice in the lifetime inhalation study of ozone (NTP 1994) (cont.)

Dose (ppm)	0	0.5	1.0
Female			
Larynx	50	49	50
Hyperplasia	13 (1.2)	11 (1.3)	24* (1.3)
Epiglottis, Metaplasia, Squamous	2 (1.5)	2 (1.0)	19** (1.1)
Nose	50	49	50
Lateral Wall, Hyaline Degeneration	0	49** (2.0)	50** (2.4)
Lateral Wall, Fibrosis	1 (1.0)	23** (1.1)	48** (1.2)
Lateral Wall, Hyperplasia	1 (1.0)	42** (1.9)	47** (2.0)
Lateral Wall, Inflammation, Suppurative	3 (1.0)	44** (1.0)	50** (1.3)
Lateral Wall, Metaplasia, Squamous	2 (1.0)	3 (1.0)	28** (1.4)
Olfactory Epithelium, Atrophy	9 (1.4)	23* (1.9)	40** (2.2)
Lung	50	49	50
Alveolar Epithelium, Metaplasia	0	43** (1.0)	50** (2.1)
Alveolus, Infiltration Cellular, Histiocyte	5 (2.2)	39** (1.3)	45** (1.8)
Alveolar Epithelium, Hyperplasia	3 (1.7)	1 (2.0)	3 (3.0)
Alveolar/bronchiolar Adenoma			
Overall rate	3/50 (6%)	3/49 (6%)	11/50 (22%)
Adjusted rate	15.7%	8.9%	56.1%
Terminal rate	1/9 (11%)	0/12 (0%)	4/10 (40%)
First incidence (days)	721	616	455
Logistic regression test	P=0.009	P=0.633	P=0.020
Alveolar/bronchiolar Carcinoma			
Overall rate	3/50 (6%)	5/49 (10%)	2/50 (4%)
Adjusted rate	12.2%	26.4%	13.9%
Terminal rate	0/9 (0%)	2/12 (17%)	1/10 (10%)
First incidence (days)	521	721	833
Logistic regression test	P=0.423N	P=0.328	P=0.496N
Alveolar/bronchiolar Adenoma or Carcinoma			
Overall rate	6/50 (12%)	8/49 (16%)	12/50 (24%)
Adjusted rate	26.0%	33.1%	58.0%
Terminal rate	1/9 (11%)	2/12 (17%)	4/10 (40%)
First incidence (days)	521	616	455
Logistic regression test	P=0.072	P=0.341	P=0.096

* Significantly different (P≤0.05) from the control group by the logistic regression test
** P≤0.01
[a] Number of animals with organ examined microscopically
[b] Number of animals with lesion
[c] Average severity grade of lesions in affected animals (1=minimal; 2=mild; 3=moderate; 4=marked).
[d] Number of animals with neoplasm per number of animals necropsied
[e] Kaplan-Meier estimated neoplasm incidence at the end of the study after adjustment for intercurrent mortality
[f] Observed incidence at terminal sacrifice
[g] Beneath the control incidence are the P values associated with the trend test. Beneath the exposed group incidence are the P values corresponding to pairwise comparisons between the controls and that exposed group. The logistic regression test regards these lesions as nonfatal. A negative trend or a lower incidence in an exposure group is indicated by N.

(1) Studies with hamsters

Witschi et al. exposed male Syrian hamsters for 16 weeks to filtered air or 0.8 ppm ozone. According to the authors no lung tumours developed. Pathology was performed for larynx, trachea, mediastinum, heart, lungs, kidneys, adrenals, liver, testes, brain, nasal cavities, pancreas, and femur. Statistically significant lung lesions including bronchiolar hyperplasia were observed. Due to the limited exposure time, this study cannot be

considered as a fully reliable carcinogenicity study.

(2) Studies with A/J mice

Last *et al*. exposed male A/J mice to filtered air, 0.4 or 0.8 ppm ozone for 18 weeks. A/J mice showed a statistically significant increased lung tumour incidence and multiplicity at 0.8 ppm (**Table 24**). At 0.4 ppm diffuse mild-to-moderate bronchiolar alveolar epithelial hyperplasia was observed. Witschi *et al*. exposed female A/J mice to filtered air, 0.12, 0.5 or 1.01 ppm ozone for 5 months. Afterwards animals were killed (group A), allowed to recover for 4 months (group B) or exposed to 4 further months to ozone (group C). Group A animals showed no statistically significant increase in lung tumour incidence or multiplicity (**Table 25**) compared to concurrent controls. However, the Cochran-Armitage trend test performed by the dossier submitter demonstrated a dose-related increase of lung tumour (most tumours were alveolar/bronchiolar adenomas) incidence (p= 0.0234). The Fisher's exact test revealed a statistically significant increased lung tumour incidence at 0.5 ppm (group B) or 0.12 ppm (group C). Furthermore, tumour multiplicity was statistically significantly increased at 0.12 ppm in Group B animals.

Hasset *et al*. exposed female A/J mice to 0.31 ozone for 6 months (103 h/week) or 0.5 ppm ozone for 1 week/month (102 h) over 6 months. The numbers of tumour-bearing animals, % mice with tumours, total number of lung tumours (isolated changes and in continuity with established adenomas) and average number of tumours/mouse were increased by ozone in both experiments. The controls in the study exposing animals to 0.31 ppm ozone and scarified 5 months after the final ozone exposure showed high background lung tumour incidences of 40 % and an incidence of 53 % in the exposed group. However, the other study part with a sacrifice 3 months after 6 months intermittent exposure resulted in a lower control tumour incidence of 18 % and an increase of lung tumours in ozone exposed mice to 38 %.

(3) Studies with B6C3F1 mice

In a study by Kim *et al.* in 2009 male and female mice were exposed for 1 year to air or 0.5 ppm ozone. No neoplasms were detected in lung, oviduct and liver. Non-neoplastic changes comprised organ weight changes as well as liver, lung, brain and adrenal lesions (e.g. focal bronchiolar alveolar hyperplasia, alveolar fibrosis, congestion in cerebrum). Therefore, the non-neoplastic LOAEC was set at 0.5 ppm. Due to the limited exposure time, this study cannot be considered as a fully reliable carcinogenicity study for this strain.

(4) Studies with B6C3F1 mice and F344/N rats

(a) 2-year studies

In line with the National toxicology program (NTP) male and female rats or mice were exposed to filtered air, 0.12, 0.5 or 1 ppm ozone for 2 years.

For rats, no statistically significant increases in neoplastic effects were reported by pairwise comparison to concurrent controls (**Table 26**). However, a positive trend for keratoacanthoma and combined squamous cell papilloma, keratoacanthoma, trichoepithelioma, basal cell adenoma or squamous cell carcinoma papilloma was evident in males. As more than 50 % of male rats died before study termination, a carcinogenic potency of ozone cannot be ruled out for this sex. Non-neoplastic lesions were statistically significantly increased at and above 0.12 ppm and comprised nose and lung effects (e.g. nose: suppurative inflammation, lateral wall hyperplasia; lung: alveolar epithelium metaplasia).

For mice, statistically significant increases in neoplastic effects comprised alveolar/bronchiolar combined adenoma or carcinoma at 1 ppm (females), alveolar/bronchiolar adenoma at 0.5 ppm (males) and hardarian gland combined adenoma or carcinoma at 0.12 and 0.5 ppm (males) (**Table 30**). According to the CLP regulation tumours in the hardarian gland have […] "no human equivalent" […]. Therefore, these tumours were neglected from the carcinogenicity evaluation. Furthermore, a positive trend was calculated for alveolar/bronchiolar combined adenoma or carcinoma (males, females), alveolar/bronchiolar carcinoma (females), hepatocellular carcinoma (males) and stromal polyp in uterus (females). Non-neoplastic lesions were already statistically significantly increased at 0.12 ppm and comprised nose effects (suppurative lateral wall inflammation and lateral wall hyaline degeneration).

(b) lifetime studies

Two lifetime studies – one with rats and one with mice – are included in the NTP document. Male and female rats and mice were exposed to filtered air, 0.5 or 1 ppm ozone for 125 weeks or 130 weeks, respectively.

For rats, no statistically significant increases in neoplastic effects were reported based on pairwise comparison of treatment groups (**Table 28**). However, a positive correlation with dose was determined for oral mucosa squamous cell papilloma or carcinoma (males) and clitoral gland adenoma or carcinoma (females) by trend test. Non-neoplastic lesions were statistically significant from 0.5 ppm and involved nose, larynx, and lung effects (e.g. goblet cell lateral wall hyperplasia, interstitial fibrosis in lung and epiglottis squamous metaplasia in larynx).

For mice statistically significant increases in neoplastic lesions comprised alveolar/bronchiolar carcinoma at 0.5 ppm and 1 ppm (males) as well as alveolar/bronchiolar adenoma at 1 ppm (females) (**Table 32**). Furthermore, a positive trend was determined for alveolar/bronchiolar carcinoma (males) and alveolar/bronchiolar adenoma (females). Non-neoplastic lesions were statistically significantly increased at 0.5 ppm and related to nose and lung (e.g. lateral wall hyaline degeneration, lateral wall fibrosis, alveolar epithelial metaplasia).

(5) Epidemiological studies

Evidence for carcinogenic potential in humans: Two epidemiological studies by Beeson et al. (1998) and Gharibvand et al. (2017) analysed associations between selected ambient air pollutants (including ozone) and incident lung cancer in Seventh-day Adventists.
In the Adventist Health Study on Smog (AHSMOG) by Beeson et al. (1998) 6,338 Californian non-smoking adults participated in a prospective cohort study. These participants were part of a greater prospective cohort study – the Adventist Health Study (AHS) – which included more than 34,000 Seventh-day Adventists residing in California (Beeson et al. 1989). In the AHSMOG study, the participants were followed for newly diagnosed lung cancers from 1977 to 1992. A computer-assisted record linkage with local and state-wide cancer registries as well as medical records from self-reported hospitalisations were used to ascertain these lung cancer incidences. In order to generate estimates of monthly ambient mean concentrations, exceedance frequencies (i.e. sum of hours above a specified cut-off) and excess concentrations (i.e. sum of concentrations above a specified cut-off), ozone exposure data from fixed-site monitoring stations maintained by the California Air Resources Board (CARB) from 1966-1992 was analysed. PM_{10}, SO_2 and NO_2 were also studied in the AHSMOG study. Within the 15-year observation period there was a total of 36 incident cases of lung cancer (20 in females, 16 in males), most of them carcinomas and adenocarcinomas. Cox proportional hazards regression models stratified by sex and adjusted for the potential confounding effects of current alcohol use, pack-years of past cigarette smoking and educational level were used to analyse the association between the selected air pollutants and the incidence of lung cancer. With regard to exceedance frequencies and based on derived interquartile ranges of the population exposed, the data suggests for males an association between ozone and an elevated lung cancer risk:

60 ppb (935 hr/y; relative risk (RR) = 2.14, 95 % confidence interval (CI) 0.82-5.62);
80 ppb (756 hr/y; RR = 2.96, CI 1.09-8.04);
100 ppb (556 hr/y; RR = 3.56, CI 1.35-9.42);
120 ppb (367 hr/y; RR = 3.75, CI 1.55-9.09);
150 ppb (185 hr/y; RR = 3.61, CI 1.78-7.35).

However, the association was only observed in males and for an 8-hour mean concentration of ozone the RR was only 1.65 and not statistically significant (CI 0.72-3.8). In contrast, mean concentrations of PM_{10} showed per 24µg/m³ increment a significant increased risk of incident lung cancer in males (RR = 5.21, CI 1.94-13.99). Moreover, a high correlation between ozone concentration and PM_{10} concentration was found and the authors describe the ozone effect as being not as stable or strong as the PM_{10} and SO_2 effects in multipollutant analyses.

In the AHSMOG-2 study, Gharibvand et al. (2017) assessed in 80,285 Seventh-day Adventists the association between $PM_{2.5}$ and lung cancer incidence using ozone as a covariate. The participants were a subpopulation of the Adventist Health Study-2 (AHS-2) which included about 96,000 Seventh-day Adventists from all 50 U.S. states and 5 provinces of Canada (Butler et al. 2008). In the AHSMOG-2 study, the participants were followed for an average of 7.5 years. For the purposes of cancer incidence ascertainment, a computer-assisted record linkage of each study participant with state cancer registries (2002-2011) as well medical records were used.

Ambient air pollution data for ozone were retrieved from the U.S. Environmental Protection Agency Air Quality System and monthly exposure averages were based on 24-hour measurements. A total of 250 incident lung cancer cases were registered during the observation time, most of them adenocarcinomas. Analyses of the study demonstrate a non-significant association with lung cancer for each 10 ppb increment in 24-hour ozone concentration in a two-pollutant ($PM_{2.5}$ and O_3) multivariable (sex, education level, race, and smoking) model: HR (hazard ratio) = 1.07, CI 0.78-1.48. In contrast, the same model calculated for incident lung cancer per 10 µg/m³ increment in mean monthly ambient $PM_{2.5}$ a significant association (HR = 1.43, CI 1.03-2.00). The authors concluded that there was no independent association between incident lung cancer and ambient 24-hour ozone concentrations.

As both AHSMOG studies are not able to show an independent and clear association between ozone and lung cancer, the data is not sufficient to infer a causal relationship between chronic ozone exposure and an increased risk of lung cancer.

10.9.1.2 Quantitative summary regarding increase and decrease of all cytotoxic and genotoxic findings (dose-response) observed in the key studies for mutagenicity

Summary of key studies *in vitro*

Ozone induced a dose-related 2-3 fold increase in revertant colonies in strain TA102 at 0.02 ppm and above (Dillon et al., 1992). This increase was statistically significant from air control and independent of S9 mix. Therefore, ozone seems to act as a direct mutagen. The mutagenic activity of ozone seems to depend on the bacterial strain used which in turn represents a certain mechanism of mutagenicity. Strain TA102 is sensitive to oxidative damage as mediated by peroxides and oxygen radical generators (Abu-Shakra and Zeiger 1990, Levin *et al.* 1982). Cytotoxicity was remarkable at around 0.4 ppm (TA102) reflected by a rapid decline in revertant colonies. Mutagenicity was observed before cytotoxicity became apparent (Table 20).

In the study published by Gooch et al. (1976), ozone treatment 36 h after PHA stimulation resulted in a 3 to 4-fold or 1.4 to 3-fold increase in chromatide aberrations at 7.23 or 7.95 ppm/h in comparison to controls, respectively. This effect was not dose-related. Cytotoxicity was not reported by the authors (Table 20).

A short communication by Chorvatovicova et al. (2000) reported a statistically significant increase (2.5-fold) in MN formation/1000 cells in comparison to the negative control. Only one dose was tested, hence no conclusion on dose-response is possible. Moreover, no comment on cytotoxicity was given by the authors (Table 20).

Summary of key studies *in vivo*

Kim et al. (2001) treated mice with 0.5 ppm ozone for an overall exposure period of 12 weeks. Ozone treatment was connected with a statistically significant increase in chromosomal aberrations and MN formation in both genders. Furthermore, the mutation frequency in splenic cells from ozone-treated mice was almost doubled in comparison to the untreated control animals. Whereas no cytotoxicity was determined in lymphocytes and reticulocytes, no obvious toxicity was evident in splenic cells (Table 21).

Kim et al. (2002) repeated this study, but extended exposure time to 16, 32 or 52 weeks. Ozone treatment resulted in both genders again in a time-related and statistically significant increase in chromosomal aberrations and MN. As a deficiency cytotoxic effects were not reported (Table 21).

Haddad et al. (2009) exposed rats to 3 ppm ozone for 10 consecutive days. Animals were sacrificed immediately (treatment group 1) after ozone exposure or 11 days (treatment group 2) after the last ozone treatment. Independent from time point of sacrifice there was a statistically significant increase in MN frequency in comparison to the negative controls. Furthermore, the PCE/NCE + PCE ratio was reduced in both treatment groups (less pronounced in treatment group 2). This finding indicates on the one hand that the bone marrow was reached by the test substance (or its derivatives) and on the other hand that ozone-mediated cytotoxicity is reversible (Table 21).

Summary of key studies in humans

The study published by Holland (2015) shows - in the absence of obvious cytotoxicity - statistically significant and dose-related MN formation in lymphocytes at the lowest ozone dose (0.1 or 0.2 ppm for 4 h, not effective dose) tested under controlled conditions in a human study (Table 22).

Summary regarding the relationship between ozone-mediated cytotoxicity and genotoxicity

In general, ozone studies involving cytotoxicity tests (or its endpoints) are scarce. However, in some studies mutagenic effects by ozone are reported in the absence of cytotoxicity. Therefore, direct interaction of ozone with DNA molecules could not be excluded as a further relevant mechanism. Owing to the positive evidence for somatic cell mutagenicity and genotoxicity obtained from *in vivo* studies - which are further supported by positive *in vitro* tests - a classification in Muta. 2 category is proposed. Furthermore, systemic mutagenicity indicates that mutagenicity mediated by ozone (or its oxidation products) is not only limited to first site of contact. For further information it is referred to the mutagenicity section of this dossier.

10.9.1.3 Comparison of NOAEC and LOAEC of (pre)neoplastic and non-neoplastic lesions

Table 34: Summary of toxicological findings in submitted carcinogenicity studies with mice[1)]

Dose level in ppm	Species, Strain, No/group	Exposure	Non-neoplastic effects (NOAEC/LOAEC)	Pre-neoplastic lesions (NOAEC/LOAEC)	Neoplastic lesions (NOAEC/LOAEC)	Remarks (tumours in other organs, not investigated tissue)	Reference
0.4	Swiss webster or A/J mice (male) No/group : 31-37	18-wk	Both strains: - some infiltrates of macrophages and neutrophils in the affected epithelium, the tissue around the bronchioles and associated lymphoid aggregates	Both strains: - diffuse mild-to-moderate bronchiolar epithelial hyperplasia	Swiss webster and A/J mice: - no	11/34 deaths (A/J mice)	Last J. A. et al. (1987), JNCI 78: 149-154
0.8			Both strains: - mild-to-moderate chronic active bronchiolitis - mild-to-moderate infiltrate of macrophages and neutrophils in the bronchioles, lymphoid nodules, and surrounding tissue - bronchioles with mucopurulent exudate	Both strains: - diffuse moderate-to-marked bronchiolar epithelial hyperplasia - prominent peribronchiolar lymphoid nodules	Swiss webster: - no A/J mice: - statist. sign. increased lung tumour incidence and multiplicity	1/33 deaths (A/J mice)	
Dose-response			both strains: NOAEC < 0.4 ppm LOAEC 0.4 ppm Incidence: No evaluation of dose-response possible Severity: Yes, stronger with dose	both strains: NOAEC < 0.4 ppm LOAEC 0.4 ppm Incidence: No evaluation of dose-response possible Severity: Yes, stronger with dose	NOAEC 0.4 /0.8 ppm (AJ/SW) LOAEC 0.8 /> 0.8 ppm (AJ/SW) A/J mice Incidence: 12 %-9 %-38 %* Multiplicity: 0.13-0.09-0.55* Dose-response: Likely (limited to top dose)	no deaths in control Other investigated organs: Heart, mediastinum	
0.12	A/J mice (female)	A) 5-mo	Not assessed	Not assessed	A) no stat. sign. (trend test positive) B) no stat. sign.		Witschi H. et al. (1999),

Dose level in ppm	**Species, Strain, No/group**	**Exposure**	**Non-neoplastic effects (NOAEC/LOAEC)**	**Pre-neoplastic lesions (NOAEC/LOAEC)**	**Neoplastic lesions (NOAEC/LOAEC)**	**Remarks (tumours in other organs, not investigated tissue)**	**Reference**
	No/group: 29-35	B) 5-mo+4-mo recovery C) 9-mo			C) stat. sign. increase in lung tumour incidence and multiplicity	no ozone related deaths during entire experimental period	Toxicological Sciences 52: 162-167
0.5			Not assessed	Not assessed	A) no stat. sign. (trend test positive) B) stat. sign. increase in lung tumour incidence C) not stat. sign.		
1.01			individual animals showed volume changes in septal tip tissues	Not assessed	A) no stat. sign. (trend test positive) B) not stat. sign. C) not stat. sign.		
Dose-response			NOAEC/LOAEC: not possible Incidence/Severity: No evaluation of dose-response possible	NOAEC/LOAEC: not possible Incidence/Severity: No evaluation of dose-response possible	A) LOAEC/NOAEC: 1.01/0.5 ppm B) LOAEC/NOAEC: 0.5/0.12 ppm C) LOAEC/NOAEC: 0.12/ < 0.12 ppm A) Incidence: 9 %-9 %-11 %-23 % Multiplicity: 0.11-0.09-0.14-0.23 Dose-response: A positive trend was determined by Cochran-Armitage-Test performed by the eCA (p = 0.0234) B) Incidence: 48 %-61 %-81 %*-57 % Multiplicity: 0.83-1.12-1.25-0.97 Dose-response: No	No other invest. organs. Most tumours were alveolar/bronchiolar adenomas. Alveolar/bronchiolar carcinomas arose within existing adenomas. Light microscope evaluation revealed striking absence of inflammatory changes (airways and lung parenchyma). High background incidence.	

Dose level in ppm	Species, Strain, No/group	Exposure	Non-neoplastic effects (NOAEC/LOAEC)	Pre-neoplastic lesions (NOAEC/LOAEC)	Neoplastic lesions (NOAEC/LOAEC)	Remarks (tumours in other organs, not investigated tissue)	Reference
					C) Incidence: 48 %-90 %*-66 %-62 % P < 0.05 Multiplicity: 0.83-1.93*-1.2-0.97 Dose-response: No		
0.31	A/J mice (female) No/group: 48	A) 6-mo (103 h/week for 6 months; sacrifice 5 months after final ozone exposure)	- enlarged spleens	- localized areas of increased prominence of alveolar lining cells (isolated changes and in continuity with established adenomas) → according to the authors this could be indicative of pathway from hyperplasia to neoplasia	- lung tumours of bronchiolo-alveolar origin (adenomas)	Animal mortality due to experimental treatment was low, in most cases, did not exceed that of the untreated controls.	Hasset C. et al. (1985), JNCI 75: 771-777
0.5		B) 6-mo (102 h/first week of each month for 6 months; sacrifice 3 months after final ozone dose)					
Dose-response			NOAEC/LOAEC: not possible Incidence/Severity: No further information to what extent spleens were enlarged	NOAEC/LOAEC: 0.31 ppm Incidence/Severity: No evaluation possible.	A) LOAEC/NOAEC: 0.31/< 0.31 ppm lung tumour incidence: 40 %-53 % tumours/mouse: 0.6-0.85 No. of lung tumours greater in ozone group vs. control (χ^2 test, $p < 0.005$)	Unclear which other organs beside lung and spleen were investigated	

Dose level in ppm	**Species, Strain, No/group**	**Exposure**	**Non-neoplastic effects (NOAEC/LOAEC)**	**Pre-neoplastic lesions (NOAEC/LOAEC)**	**Neoplastic lesions (NOAEC/LOAEC)**	**Remarks (tumours in other organs, not investigated tissue)**	**Reference**
					B) LOAEC/NOAEC: 0.5/<0.5 ppm lung tumour incidence: 18 %-38 % tumours/mouse: 0.2-0.64 No. of lung tumours greater in ozone group vs. control (χ^2 test, $p < 0.005$)		
0.5	$B6C3F_1$ mice (female+ male) No/group: 20f +20m	1-y	- peribronchial mononuclear cell infiltration (males) - alveolar fibrosis (males) - seminiferous disengagement in testis - hepatocyte vacuolation (females) - focal necrosis in liver (males) - congestion in cerebrum (males)	- bronchiolar alveolar hyperplasia (males) - bronchiolar epithelium hyperplasia - hyperplasia in adrenal gland (males)	- no incidence of tumour formation in lung, liver, but oviductal carcinoma	- restricted reliability due to insufficient study duration - no treatment related deaths - relative weight of some organs changed >10 %	Kim M. Y. and Cho M. Y. (2009), Toxicology and industrial health 25: 189-195
Dose-response			NOAEC < 0.5 ppm LOAEC: 0.5 ppm Incidence/Severity: not possible	NOAEC < 0.5 ppm LOAEC: 0.5 ppm Incidence/Severity: not possible	NOAEC: 0.5 ppm LOAEC: >0.5 ppm Incidence/Severity: not possible	- No severity grade for lesions given. Other organs	
0.12	$B6C3F_1$ mice (female+ male) No/group: 50	2-y	-nose: inflammation**			- Uterus: Polyp stromal pos. trend (females) - hepatocellular carcinoma positive trend (males) - hardarian gland combined adenoma or carcinoma stat. significant for	NTP, Toxicology and carcinogenesis studies of ozone and ozone/NKK in F344/N rats and
0.5		2-y	- lung: histiocytic infiltration** -nose: hyaline degeneration**, fibrosis**, inflammation**	-nose: hyperplasia** -lung: metaplasia**	- alveolar/bronchiolar adenoma* (males)		
1.0		2-y	- lung: histiocytic infiltration** -nose: hyaline degeneration**, fibrosis**, inflammation**	-nose: hyperplasia**, metaplasia** -lung: metaplasia**	- alveolar/bronchiolar adenoma or carcinoma* (females)		

Dose level in ppm	Species, Strain, No/group	Exposure	Non-neoplastic effects (NOAEC/LOAEC)	Pre-neoplastic lesions (NOAEC/LOAEC)	Neoplastic lesions (NOAEC/LOAEC)	Remarks (tumours in other organs, not investigated tissue)	Reference
						pairwise comparison at 0.12 and 0.5 ppm (males)	B6C3F1 mice, National toxicology program, Technical report series 440 (1994)
Dose-response			LOAEC/NOAEC: 0.12/<0.12 ppm all effects showed dose-response	LOAEC/NOAEC: 0.5/0.12 ppm all effects showed dose-response	- alveolar/bronchiolar carcinoma pos. trend incidences: 4 %-4 %-10 %-16 % (females) - alveolar/bronchiolar carcinoma, incidences: 1 6%-8 %-16 %-20 % (males) - alveolar/bronchiolar adenoma or carcinoma pos. trend incidences: 28 %-26 %-36 %-38%(males) 12 %-14 %-18 %-32 %* (females) - alveolar/bronchiolar adenoma, incidences: 8 %-10 %-10 %-16 % (females), 12 %-18 %-24 %-22 % (males) LOAEC/NOAEC: 0.5/0.12 ppm		
0.5	$B6C3F_1$ mice (female+ male) No/group: 50	lifetime	-nose: hyaline degeneration**, fibrosis**, inflammation** - lung: histiocyte infiltration**	- nose: hyperplasia** - lung: metaplasia**	- alveolar/bronchiolar carcinoma* (males)		
1.0		lifetime	-nose: hyaline degeneration**, fibrosis**, inflammation**, olfactory atrophy** - lung: histiocyte infiltration**	-larynx: hyperplasia**, metaplasia** - nose: hyperplasia**, metaplasia** - lung: metaplasia**, hyperplasia** (males)	- alveolar/bronchiolar carcinoma* (males) alveolar/bronchiolar adenoma* (females)		

Dose level in ppm	Species, Strain, No/group	Exposure	Non-neoplastic effects (NOAEC/LOAEC)	Pre-neoplastic lesions (NOAEC/LOAEC)	Neoplastic lesions (NOAEC/LOAEC)	Remarks (tumours in other organs, not investigated tissue)	Reference
Dose-response			LOAEC/NOAEC: 0.5/ < 0.5 ppm all effects showed dose-response	LOAEC/NOAEC: 0.5/<0.5 ppm all effects showed dose-response	- alveolar/bronchiolar carcinoma pos. trend incidences: 16 %-31 %*-36 %* (males) - alveolar/bronchiolar carcinoma, incidences: 6 %-10 %-4 % (females) - alveolar/bronchiolar adenoma pos. trend incidences: 6 %-6 %-22 %* (females) - alveolar/bronchiolar adenoma, incidences: 16 %-16 %-18 % (males) - alveolar/bronchiolar adenoma or carcinoma, incidences: 12 %-16 %-24 % (females), 33 %-45 %-42 % (males), LOAEC/NOAEC: 0.5 ppm/ < 0.5 ppm		

[1)] A/J mice represent a susceptible strain with a high incidence of spontaneous lung tumours. The relevance of results obtained with this strain is discussed in chapter 10.9.2.

Table 35: Summary table of epidemiological studies on carcinogenicity

Reference / study characteristics	ozone **exposure**			**Statistical Analysis**	**Effect**	**Results**	**Others/ Remarks**
	Conc. µg/m3	**Conc. ppb**	**Duration hours**				
Beeson, W.L. et al. 1998 Environmental Health Perspectives, 106: 813-23. 6,338 subjects, cohort of the Adventist Health and Smog Study (AHSMOG) of Californian adults. Data from 1977-1992. Pollution data from California Air Resources Board (CARB) from 1966-1992.	2-86	0-40	Average annual mean concentration	Cox proportional hazards regression models adjusting for potential confounding effects of other covariates	Incidences of lung cancer	Increased moderate risks of incident lung cancer were associated with elevated long-term ambient concentrations of ozone in males.	Associations were significant for hours per year exceedance frequencies of ozone thresholds as low as 80 ppb.
Gharibvand, L. et al. 2017, Environmental Health Perspectives, 125: 378-84. 80,285 subjects, cohort of the Adventist Health and Smog Study-2 (AHSMOG-2) of 50 US states and 5 provinces of Canada. Data from 2002-2010. Pollution data from U.S. EPA/AQS from 2001-2002.	36.6-86	17-40	Monthly mean concentration of 24-hr ozone	Cox proportional-hazard models and sandwich variance estimate	Incidences of lung cancer	No independent association between incident lung cancer and ambient 24-hr ozone concentrations	Not ozone directly but independent relationship with ambient ozone in two pollutant models with $PM_{2.5}$ and the association with lung cancer incidence was assessed.

10.9.2 Comparison with the CLP criteria

CLP regulation
A substance is classified in Category 1 (known or presumed human carcinogens) for carcinogenicity on the basis of epidemiological and/or animal data. A substance may be further distinguished as: Category 1A, known to have carcinogenic potential for humans, classification is largely based on human evidence, or Category 1B, presumed to have carcinogenic potential for humans, classification is largely based on animal evidence. The classification in Category 1A and 1B is based on strength of evidence together with additional considerations (see section 3.6.2.2). Such evidence may be derived from: — human studies that establish a causal relationship between human exposure to a substance and the development of cancer (known human carcinogen); or — animal experiments for which there is sufficient evidence to demonstrate animal carcinogenicity (presumed human carcinogen). In addition, on a case-by-case basis, scientific judgement may warrant a decision of presumed human carcinogenicity derived from studies showing limited evidence of carcinogenicity in humans together with limited evidence of carcinogenicity in experimental animals.
The placing of a substance in Category 2 (suspected human carcinogens) is done on the basis of evidence obtained from human and/or animal studies, but which is not sufficiently convincing to place the substance in Category 1A or 1B, based on strength of evidence together with additional considerations (see section 3.6.2.2). Such evidence may be derived either from limited evidence of carcinogenicity in human studies or from limited evidence of carcinogenicity in animal studies.

Table 36: Summary of specific and additional considerations for classification on carcinogenicity

	Category	**Considerations for classification**	**Yes/ No**	**Results**	**Increased/ decreased concern**
Specific considerations CLP Annex I 3.6.2.2.3	Carc 1A: Sufficient evidence in humans	Causal relationship between agent exposure and human cancer	No	No sufficient data for a causal relationship of ozone and lung cancer from epidemiological studies. Data by Beeson, W.L. et al. 1998 suggests an association which is however distorted by a high correlation between ozone and $PM_{10.}$ Data by Gharibvand, L. et al. 2017 suggests no independent association of 24-h ozone exposure with incident lung cancer in humans.	n. a.
	Carc 1 B: Sufficient evidence in animals	Benign and malignant neoplasms in two or more species of animals	No	Only in mice; in rat study (NTP,1994) poor survival	n. a.
		Two or more independent studies in one species at different times or in different laboratories or under different protocols	No	B6C3F1 mice (NTP, 1994); not supported by A/J mice (Last et al 1987, Witschi t al. 1999, Hasset et al. 1985)	n. a.
		Increased incidence of tumours in both sexes of a single species in a well-conducted study, ideally under GLP	Yes	2-y study in B6C3F1 mice (NTP, 1994), lung carcinomas dose-dependent and above historical controls	n. a.
Additional considerations CLP		Relevance for humans based on tumour type and background incidence?	Yes	Alveolar/bronchiolar carcinomas	↑
		Multi-site responses?	No	Only lung; skin data inconclusive	→

	Category	Considerations for classification	Yes/ No	Results	Increased/ decreased concern
		Progression of lesions to malignancy?	Yes	Alveolar/bronchiolar carcinomas in F and M (2-y & lifetime NTP studies in B6C3F1 mice)	↑
		Reduced tumour latency?	Yes	B6C3F1 mice (lifetime study, NTP, 1994, M), first incidence day 805 (0 ppm, 42 %), 693 (0.5 ppm, 65 %) and 609 (1 ppm, 71 %) for alveolar/bronchiolar carcinomas	↑
		Responses in both sexes?	Equivocal	B6C3F1 mice, lung (F clear response, M only trend for combined carcinomas and adenomas, 2-y NTP study)	→
		Responses in several species?	No	Only in mice; in rat study (NTP,1994) poor survival	↓
		Structural similarity to a substance(s) for which there is good evidence of carcinogenicity?	-	No data on carcinogenicity for structural related substances available	→
		Route specific effect?	Yes	Effect via inhalation on respiratory system	→
		Similar ADME in test animals and humans?	-	No data, but very likely	→
		Possibility of confounding effect of excessive toxicity at test doses?	n. a.	In rat study (NTP, 1994, inhalation, lifetime and 2-year) pos. trend for squamous cell carcinoma in skin of M, lethality potentially disguises long-term effects, carcinogenicity; no confounder in B6C3F1 mice study	→
		Relevance for humans of mode of action?	Yes	ROS, mutagenicity, cytotoxicity and regenerative hyperplasia in mice	→

Toxicological results and CLP classification in detail:

Lung tumours were observed in male and female A/J mice exposed to ozone. Adenomas and carcinomas were reported from a dose of 0.5 ppm ozone by Witschi et al. (1999) in female A/J mice after exposure for 5 months (killing after recovery period of 4 months) or continuous exposure to 0.12 ppm ozone for 9 months. A positive trend was observed after exposure of animals for 5 months to 0.12, 0.5 or 1.01 ppm ozone followed by immediate killing. Last et al. (1987) observed a statistically significant increase in lung tumours (adenomas) in male A/J mice after exposure to 0.8 ppm ozone for 18 weeks. Hasset *et al.* reported an increase in lung tumour (adenomas) incidence in mice exposed to 0.31 ppm or 0.5 ppm ozone for two different intermittent exposure regimes for 6 months, respectively. The controls in the study exposing animals to 0.31 ppm ozone and scarified 5 months after the final ozone exposure showed high background tumour incidences (40 %) that limits the reliability of this study part. However, the other study part with sacrifice 3 months after 6 months intermittent exposure resulted in a reliable control tumour incidence and an increase of lung tumours in ozone exposed female mice. However, the background incidence was moderate to high in all studies listed even if incidences were constantly higher in treated animals than in air controls. Accordingly, because of the high frequency of spontaneous tumour incidences in the strain A/J mice, the studies by Last et al. (1987) and Witschi et al. (1999) are not regarded as supportive for Carc 1B.

In contrast to this, sufficient evidence for a carcinogenic potential of ozone was provided by studies using B6C3F1 mice. Kim *et al.* (2009) reported pre-neoplastic lesions in the lung like bronchiolar alveolar hyperplasia and bronchiolar epithelium hyperplasia after a one-year-long exposure duration to 0.5 ppm ozone. This study time seems to be too short for tumour development in B6C3F1 mice. However, in the 2-year NTP study a statistically significant increase in alveolar/bronchiolar combined adenoma and carcinoma was obtained at 1 ppm in female B6C3F1 mice as well as alveolar/bronchiolar adenoma at 0.5 ppm in male B6C3F1 mice. The incidence of alveolar/bronchiolar adenoma or carcinoma combined exceeded the NTP historical control range for this neoplasm in 0.5 and 1 ppm exposed females (58/659; 0-15 %). Furthermore, alveolar/bronchiolar combined adenoma or carcinoma in male and female mice as well as alveolar/bronchiolar carcinoma in female mice followed a positive trend. The lung tumours observed from 0.5 ppm were accompanied by non-neoplastic and pre-neoplastic lesions (*e.g.* histiocytic infiltration and metaplasia) in the lung. In the lifetime study a statistically significant increase in alveolar/bronchiolar carcinoma at 0.5 ppm and 1 ppm in male B6C3F1 mice as well as in alveolar/bronchiolar adenoma at 1 ppm in female B6C3F1 mice was observed. A comparison with historical control data of the NTP is not possible as there are no data for lifetime studies. Furthermore, alveolar/bronchiolar carcinoma (males) and alveolar/bronchiolar adenoma (females) followed a positive trend. Also in this study, tumours observed from 0.5 ppm were accompanied by non-neoplastic and pre-neoplastic effects (same findings in lung as already observed in the 2-year study). As a result of the B6C3F1 mice studies, adenoma and carcinoma were found dose-dependent and above historical controls and findings are therefore regarded as relevant for classification.

Taken together, tumour development in B6C3F1 mice seems to take longer time than in A/J mice. This is in line with the phenotype of this mouse strain. A/J mice are susceptible to lung tumour development in response to carcinogens, as seen in the aforementioned studies with terminal sacrifice after 9 months study duration or longer. No lung tumours were detected in Syrian hamsters after 16-week exposure to 0.8 ppm ozone (Witschi *et al.* 1993).

In the 2-year NTP (1994) study with B6C3F1 mice a positive trend was calculated for stromal polyps in uterus. These findings may point to other organ targets for ozone-mediated carcinogenesis. The oviduct or uterus were not evaluated for neoplastic effects in A/J mice, hence carcinogenic effects in these organs cannot be ruled out.

Furthermore, a positive trend for keratoacanthoma and combined squamous cell papilloma, keratoacanthoma, trichoepithelioma, basal cell adenoma, or squamous cell carcinoma was determined in male rats after 2-year ozone exposure. In the NTP lifetime study with rats a positive trend for squamous cell papilloma or squamous cell carcinoma of the oral mucosa (males) and adenoma or carcinoma of the clitoral gland was calculated. Furthermore, a positive trend for hepatocellular carcinoma (males) was calculated in mice in the 2-year NTP study. However, no neoplasms in liver were detected by Kim *et al.* (2001, 2009) in B6C3F1 mice after exposure to 0.5 ppm ozone for 12-, 16- 32 weeks or 1 year. This could be explained by the shorter exposure time in comparison to the NTP study. With respect to multi-site responses, carcinoma seem to be restricted to the lung. As observations in uterus, skin and liver were inconclusive and did not reach a higher level of significance, this specific criterion is not fulfilled.

Evidence for carcinogenic potential in humans: Two epidemiological studies by Beeson et al. (1998) and Gharibvand et al. (2017) analysed associations between selected ambient air pollutants (including ozone) and incident lung cancer in Seventh-day Adventists.
In the Adventist Health Study on Smog (AHSMOG) by Beeson et al. (1998) 6,338 Californian non-smoking adults participated in a prospective cohort study. The participants were followed for newly diagnosed lung cancers from 1977 to 1992. Ozone exposure data from fixed-site monitoring stations maintained by the California Air Resources Board (CARB) from 1966-1992 was analysed. PM_{10}, SO_2 and NO_2 were also studied in the AHSMOG study. Within the 15-year observation period there was a total of 36 incident cases of lung cancer (20 in females, 16 in males), most of them carcinomas and adenocarcinomas. With regard to exceedance frequencies and based on derived interquartile ranges of the population exposed, the data suggests for males an association between ozone and an elevated lung cancer risk. However, the association was only observed in males and for an 8-hour mean concentration of ozone the RR was only 1.65 and not statistically significant (CI 0.72-3.8). In contrast, mean concentrations of PM_{10} showed per 24µg/m³ increment a significant increased risk of incident lung cancer in males (RR = 5.21, CI 1.94-13.99). Moreover, a high correlation between ozone concentration and PM_{10} concentration was found and the authors describe the ozone effect as being not as stable or strong as the PM_{10} and SO_2 effects in multipollutant analyses.

In the AHSMOG-2 study, Gharibvand et al. (2017) assessed in 80,285 Seventh-day Adventists the association between $PM_{2.5}$ and lung cancer incidence using ozone as a covariate. Analyses of the study demonstrate a non-significant association with lung cancer for each 10 ppb increment in 24-hour ozone concentration in a two-pollutant ($PM_{2.5}$ and O_3) multivariable (sex, education level, race, and smoking) model: HR (hazard ratio) = 1.07, CI 0.78-1.48. In contrast, the same model calculated for incident lung cancer per 10 µg/m³ increment in mean monthly ambient $PM_{2.5}$ a significant association (HR = 1.43, CI 1.03-2.00). The authors concluded that there was no independent association between incident lung cancer and ambient 24-hour ozone concentrations.

As both AHSMOG studies are not able to show an independent and clear association between ozone and lung cancer, the data is not sufficient to infer a causal relationship between chronic ozone exposure and an increased risk of lung cancer.

Genotoxicity: Evidence for genotoxicity of ozone in vivo in rodents and humans indicates that the substance has a potential for carcinogenic effects.

As there is no sufficient human evidence of carcinogenicity, ozone does not fall into Category 1A for carcinogenicity based on the available data.

According to the CLP regulation Category 1B is justified for substances for which animal experiments give […] "sufficient evidence to demonstrate animal carcinogenicity". As laid down in CLP regulation sufficient evidence means […] "a causal relationship […] between the agent and an increased incidence of malignant neoplasms or of an appropriate combination of benign and malignant neoplasms in

(a) two or more species of animals or

(b) two or more independent studies in one species carried out at different times or in different laboratories or under different protocols."

It is further stated: "An increased incidence of tumours in both sexes of a single species in a well-conducted study, ideally conducted under Good Laboratory practices, can also provide sufficient evidence. A single study in one species and sex might be considered to provide sufficient evidence of carcinogenicity when malignant neoplasms occur to an unusual degree with regard to incidence, site, type of tumour or age at onset, or when there are strong findings of tumours at multiple sites […].

Ozone leads to lung tumour formation in B6C3F1 mice, but not in rats and hamsters. Therefore, requirement (a) is not fulfilled. In this context, it should be noted that the studies in rat (NTP / Boormann et al., Herbert et al.) are not acceptable as negative evidence due to insufficient survival.

Lung tumour formation by ozone was found in many different and independent studies in mice (Last et al. 1987, Witschi et al. 1999, Hasset et al. 1985 and 2-year or lifetime NTP study conducted in 1994). All studies used different times and protocols. Lung tumours were not only observed in B6C3F1 mice but also in A/J mice. A/J mice show a high background of spontaneous incidence. As this strain is more susceptible to lung tumour formations following inhalation, tumours were observed after shorter time frames in these studies (starting from 18-weeks). But as studies in A/J mice are regarded as limited evidence, requirement (b) would not be fulfilled for classification, even if the lung effects were above the historical control data and followed a positive trend (NTP study).

Both sexes of B6C3F1 mice developed tumours in a 2-year NTP study which is in compliance with Food and Drug Administration Good Laboratory Practice Regulations. However in males, the formation of lung carcinomas showed only in combination with lung adenomas a positive trend. Hence, this study on its own is not appropriate to provide sufficient evidence of carcinogenicity according to the CLP criteria (i.e. based on an increased incidence of tumours in both sexes of a single species in a well-conducted study).

In the NTP studies with rats and mice, evidence for tumour formation at multiple sites was also not strong enough to support classification of ozone in category 1B.

The carcinogenic effects in the lungs - the first site of contact after inhalation exposure – are mechanistically plausible taking into account the genotoxic effects of ozone or its oxidation products in the lung (for further information see genotoxicity chapter). Therefore, lung carcinogenicity may be attributed to genotoxic effects (initiation events) potentially in combination with further oxidative stress and regenerative mitogenesis (initiation and tumour promoting events). However, genotoxicity was also observed systemically. This could

– in combination with extrapulmonary neoplastic effects observed for example in the NTP study – indicate genotoxic-mediated carcinogenicity in further organs.

As there is no human data available providing adequate evidence for a causal relationship between long-term exposure to ozone and an increased risk of lung cancer and as animal data do not fulfil the Category 1 criteria according to the CLP regulation, ozone falls into Category 2 for carcinogenicity.

Suspected human carcinogens

The placing of a substance in Category 2 is done on the basis of evidence obtained from human and/or animal studies, but which is not sufficiently convincing to place the substance in Category 1A or 1B, based on strength of evidence together with additional considerations. Such evidence may be derived either from limited evidence of carcinogenicity in human studies or from limited evidence of carcinogenicity in animal studies.

10.9.3 Conclusion on classification and labelling for carcinogenicity

Based on the results listed above, harmonised classification and labelling for carcinogenicity is proposed: Carcinogenic Category 2; H351 (Suspected of causing cancer).

RAC evaluation of carcinogenicity

Summary of the Dossier Submitter's proposal

The DS proposed a classification of ozone for carcinogenicity in category 2. Witschi *et al.* (1999) reported lung tumours in male and female A/J mice exposed to ozone (adenomas and carcinomas after doses of 0.5 ppm for 5 months or 0.12 ppm for 9 months). These findings were supported by an observed positive trend after exposure of animals for 5 months to 0.12, 0.5 or 1.01 ppm ozone.

Last *et al.* (1987) reported a statistically significant increase in lung tumours (adenomas) in male A/J mice after exposure to 0.8 ppm for 18 weeks. Furthermore, Hasset el al. (1985) reported an increase in lung tumour (adenomas) incidence in mice exposed to 0.31 or 0.5 ppm for two different intermittent exposure regimes for 6 months, respectively.

As the background incidence was moderate to high in all studies, and a high incidence of spontaneous tumour incidences in the A/J mice strain, the studies by Last *et al.* (1987) and Witschi *et al.* (1999) are not regarded as sufficient to classify for carcinogenicity in category 1B.

Comments received during consultation

One MSCA agreed with the conclusion presented in the CLH report based on the observation from the 2 years NTP study (Boorman *et al.*, 1995; Herbert *et al.*, 1996) conducted in B6C3F1 mice exposed to ozone showing an increase of alveolar/bronchiolar combined adenoma and carcinoma in males and females (statistically significant for females), and alveolar/bronchiolar carcinoma in females and alveolar/bronchiolar adenoma in males (statistically significant). In the NTP lifetime inhalation study conducted in B6C3F1 mice, an increase in alveolar/bronchiolar carcinoma in males (statistically significant) and alveolar/bronchiolar adenoma in females was reported (statistically significant).
These findings are supported by the studies conducted in A/J mice that showed development of lung tumours in both sexes (Witschi *et al.*, 1999; Last *et al.*, 1987; Hasset *et al.*, 1985). The MSCA agreed that due to high incidence of spontaneous tumours in controls, these studies cannot be used to

support Carc. 1B classification. However, the MSCA considered that the effects demonstrated in the NTP studies to be borderline for a classification as Carc. 1B.

Comments from the Company-Manufacture were related to the studies:

- With regard to the study by Last *et al.* (1987), this is a dual exposure to sodium chloride and ozone and as such should not be included in the overall weight of the evidence.
- With regard to the study by Witschi *et al.* (1999), this study did not demonstrate a carcinogenic effect; the effect was non-statistically significant, was not reproducible in at 9 months and the effect was not seen in the reversibility group (Group C). Additionally, there were no corresponding histopathological changes indicative of ozone toxicity.
- With regard to the study by Hasset *et al.* (1985), this is a common tumour in mice with a high spontaneous background; the occurrence in controls should not be a reason for limited reliability. No historical control data were provided to determine if the effects were within background

Comments related to the toxicokinetic issues and ozone bioavailability (genotoxicity section) and other comments related to the studies will be taking into account when assessing the studies in the next section.

Assessment and comparison with the classification criteria

Studies focussing on the investigation of neoplasms following ozone exposure were performed in mice and rats. One 16-week study in hamsters dosed at 0 and 0.8 ppm was submitted, however, due to the short exposure time, this study cannot be considered as a fully reliable carcinogenicity study. Most of the other studies are flawed by reporting deficiencies (e.g. no reporting of examined organs, no individual data, no severity of lesions presented) and the quality shortcomings make it difficult to decide on (or exclude) carcinogenic activity in all organs. Many studies also do not comply with the appropriate OECD TG for carcinogenicity.

***Table**: Summary of carcinogenicity studies*

Method, Guideline, GLP status, Reliability	**Species, Strain, Sex, No/ group NOAEC/ LOAEC**	**Ozone, Dose levels, Duration of exposure**	**Results**
Last *et al.*, 1987 Method: 18-week inhalation study Guideline: no GLP: no Reliability: 2	Mice, A/J, male No/Group: 31-37 Neoplastic LOAEC: 0.8 ppm Non-neoplastic NOAEC: < 0.4 ppm LOAEC: 0.4 ppm	0 (filtered air), 0.4 ppm or 0.8 ppm 0.9% sodium chloride vehicle 1 day prior exposure initiation 8h/day 7days/week for 18 weeks (whole body) Animals were sacrificed 4 months after start of	Neoplastic findings: Statistically significant increased lung tumour incidence and multiplicity at 0.8 ppm ozone (χ^2 test, $p < 0.05$). Tumour incidence and multiplicity (mean ± SE): <u>NaCl + air:</u> 4/33 (12%); 0.13 ± 0.06 <u>NaCl + 0.4 ppm ozone:</u> 2/23 (9%); 0.09 ± 0.06 <u>NaCl + 0.8 ppm ozone:</u> 12/32 (38%)*; 0.55 ± 0.15* Non-neoplastic findings: <u>0.4 ppm ozone</u> Diffuse mild-to-moderate bronchiolar epithelial hyperplasia with some infiltrates of macrophages and neutrophils in the affected epithelium, the tissue around the bronchioles and associated

		treatment.	lymphoid aggregates. 0.8 ppm ozone Lesions characteristic of mild-to-moderate chronic active bronchiolitis. Diffuse moderate-to-marked bronchiolar epithelial hyperplasia and prominent peribronchiolar lymphoid nodules. Mild-to-moderate infiltrate of macrophages, often containing hemosiderin, and neutrophils in the bronchioles, lymphoid nodules, and surrounding tissue. Occasional bronchioles with mild mucopurulent exudate.
Witschi *et al.,* 1999 Method: 5-month inhalation study followed by killing, 4-month recovery or 4 further months of ozone exposure Guideline: None GLP: No Reliability: 2	Mice, A/J, female No/Group: 29-35 Neoplastic → derived from group A LOAEC: 1.01 ppm NOAEC: 0.5 ppm Non-neoplastic NOAEC/LOAEC not derived	0 (Filtered air), 0.12, 0.5 and 1.01 ppm (mean measured concentration) whole body 6h/day on 5 days/week group A: 5 months exposure, group B: 9 months exposure, group C: 5 months exposure + 4 months filtered air	Neoplastic findings, group A: Lung tumour incidence and multiplicity (mean ± SEM): Control: 3/35 (9%); 0.11 ± 0.05 0.12 ppm: 3/35 (9%); 0.09 ± 0.05 0.50 ppm: 4/35 (11%); 0.14 ± 0.07 1 ppm: 8/35 (23%); 0.23 ± 0.07 - Dose-dependent increase in tumour incidence by Cochran-Armitage trend test (p = 0.0234) - No statistically significant difference between groups: Neoplastic findings, group B: Lung tumour incidence and multiplicity (mean ± SEM): Control: 15/30 (50%); 0.83 ± 0.19 0.12 ppm: 19/31 (61%); 1.12 ± 0.20 0.50 ppm: 26/32 (81%)*; 1.25 ± 0.16 1 ppm: 20/35 (57%); 0.97 ± 0.19 - Increase in lung tumour incidence, statistically significant (p < 0.05, Fisher's exact test) in mid-dose group; no statistically significant increase in tumour multiplicity Neoplastic findings, group C: Lung tumour incidence and multiplicity (mean ± SEM): Control: 14/29 (48%); 0.83 ± 0.19 0.12 ppm: 26/29 (90%)*; 1.93 ± 0.25* 0.50 ppm: 20/30 (66%); 1.2 ± 0.27 1 ppm: 21/34 (62%); 0.97 ± 0.17 - Increase in lung tumour incidence multiplicity, statistically significant in low dose group (p < 0.05, ANOVA and Fisher's exact test): Histology - Most tumours were alveolar/bronchiolar adenomas - Alveolar/bronchiolar carcinomas arose within existing adenomas (focal areas manifesting a different growth pattern from adenoma) - Occasionally papillary adenomas Non-neoplastic changes Tissue volumes per surface area (m^3/m^2) - No statistically significant changes due to large SDs; according to authors individual animals showed volume changes in septal tip tissues
Hasset *et al.,* 1985 Method: 6-month inhalation study for ozone Guideline: no	Mice, A/J, female No/Group: 40 Neoplastic LOAEC: 0.5 ppm (derived	0, 0.31 (Exp. 1) or 0.5 ppm (Exp. 2) Route of exposure: Inhalation Duration of	Neoplastic findings Exp. 1 (0.31 ppm, age of animals at sacrifice: ~ 1 year): - Control: No. of tumour-bearing animals: 16/40 % Mice with tumours: 40 Total no. of lung tumours: 24 Average no. of tumours/mouse: 0.60

<table>
<tr>
<td>GLP: no
Rel. 2</td>
<td>from Exp.2)
Non-neoplastic NOAEC/LOAEC not available under the conditions of the study.</td>
<td>exposure:
Exp. 1: 103 h/week for 6 months; sacrifice 5 months after final ozone exposure
Exp. 2: 102 h/first week of each month for 6 months; sacrifice 3 months after final ozone dose (whole body)</td>
<td>- <u>Ozone:</u>
No. of tumour-bearing animals: 21/40
% Mice with tumours: 53
Total no. of lung tumours: 34
Average no. of tumours/mouse: 0.85
- Tumour distribution control vs. ozone
<table>
<tr><td rowspan="2">No. tumour/animal</td><td colspan="2">No. of animals</td></tr>
<tr><td>Control</td><td>Ozone</td></tr>
<tr><td>0</td><td>24</td><td>19</td></tr>
<tr><td>1</td><td>11</td><td>9</td></tr>
<tr><td>2</td><td>4</td><td>11</td></tr>
<tr><td>≥3</td><td>1</td><td>1</td></tr>
</table>
No. of lung tumours greater in ozone group vs. control (χ^2 test, $p < 0.005$)

<u>Exp. 2 (0.5 ppm, age of animals at sacrifice: ~ 9 months):</u>

- <u>Control:</u>
No. of tumour-bearing animals: 8/45
% Mice with tumours: 18
Total no. of lung tumours: 9
Average no. of tumours/mouse: 0.20
- <u>Ozone:</u>
No. of tumour-bearing animals: 17/45
% Mice with tumours: 38
Total no. of lung tumours: 29
Average no. of tumours/mouse: 0.64
- Tumour distribution control vs. ozone
<table>
<tr><td rowspan="2">No. tumour/animal</td><td colspan="2">No. of animals</td></tr>
<tr><td>Control</td><td>Ozone</td></tr>
<tr><td>0</td><td>37</td><td>28</td></tr>
<tr><td>1</td><td>7</td><td>12</td></tr>
<tr><td>2</td><td>1</td><td>4</td></tr>
<tr><td>≥3</td><td>0</td><td>1</td></tr>
</table>
No. of lung tumours greater in ozone group vs. control (χ^2 test, $p < 0.005$)

Tumours
- Bronchio-alveolar origin
- Well circumscribed
- Localized areas of increased prominence of alveolar lining cells (isolated changes and in continuity with established adenomas) → according to the authors this could be indicative of pathway from hyperplasia to neoplasia

Non-neoplastic lesions
- Enlarged spleens</td>
</tr>
<tr>
<td>Kim and Cho, 2009
Method: 1-year carcinogenicity study for ozone
Guideline: None
GLP: No
Reliability: 3</td>
<td>Mice, $B6C3F_1$, female and male
No/Group: 20 M + 20 F
Neoplastic no effects observed at 0.5 ppm
Non-neoplastic NOAEC: < 0.5 ppm
LOAEC: 0.5 ppm</td>
<td>0 or 0.5 ppm
Route of exposure: Inhalation (ozone)
Duration of exposure: 6h/day
5 days/week for 1 year (whole body)</td>
<td>Neoplastic findings
- No treatment related increase in tumour incidence in lung, oviduct and liver

Non-neoplastic findings
- Relative organ weight of kidney in males statistically significantly increased (≥ 10%); for kidney (left) and testis (right) organ weights statistically decreased (≥ 10%); (analysis of variance and Student’s t-test, $p < 0.05$)
- Relative organ weight of lung and kidney (right) statistically significantly increased (but: < 10%); relative organ weight of adrenal (right) and ovary (left and right) decreased (≥ 10%) after 1 year; (analysis of variance and Student’s t-test, $p < 0.05$)
- Peribronchial mononuclear cell infiltration (10% males treated with ozone)</td>
</tr>
</table>

			- Focal bronchiolar alveolar hyperplasia (10% males treated with ozone) - Bronchiolar epithelium hyperplasia (10% males and 10% females treated with ozone) - Alveolar fibrosis (10% males treated with ozone) - Hepatocyte vacuolation (10% females treated with ozone) - Focal necrosis (10% males treated with ozone) in liver - Congestion in cerebrum (10% males treated with ozone) - Mild hyperplasia in adrenal gland (10% males treated with ozone) - Seminiferous disengagement in testis (10% after ozone treatment)
NTP-1, Toxicology and carcinogenesis studies of ozone and ozone/NKK in F344/N rats and B6C3F1 mice Boorman *et al.,* 1995 Herbert *et al.,* 1996 Method: 2-year inhalation study Guideline: similar to TG 451 GLP: in compliance with Food and Drug Administration (FDA) Reliability: 1	Mice, $B6C3F_1$ mice (Simonsen Laboratories (Gilroy, CA)), male and female No/Group: 50 per sex and group Neoplastic: LOAEC: 0.5 ppm Non-neoplastic: NOAEC:< 0.12 ppm LOAEC: 0.12 ppm	0 (filtered air), 0.12, 0.5 and 1.0 ppm 6h/d 5 days/week for 2 years (whole body)	Neoplastic lesions: - Alveolar/bronchiolar combined adenoma or carcinoma increased in males and females positive trend: life table test, logistic regression test and for females Cochran-Armitage-test) and stat. significant increase in females at 1.0 ppm (logistic regression test, Fisher exact test) - Alveolar/bronchiolar carcinoma increased in females (positive trend: logistic regression test, life table test and Cochran-Armitage test) - Alveolar/bronchiolar adenoma stat. sign. at 0.5 ppm in males (life table test) - Hepatocellular carcinoma positive trend (life table test) in males - Harderian gland combined adenoma or carcinoma stat. significant for pairwise comparison at 0.12 (life time table, logistic regression, Fisher exact test) and 0.5 ppm (life table test) (males) - Stromal polyp in uterus positive trend (life table test, logistic regression test, Cochran-Armitage test) in females Non-neoplastic lesions: <u>0.12 ppm:</u> - Nose: inflammation (only males), lateral wall hyaline degeneration (only females) <u>Additional 0.5 ppm and 1.0 ppm:</u> - Nose: lateral wall hyperplasia, inflammation (only females), lateral wall fibrosis and lateral wall squamous metaplasia (only males), lateral wall hyaline degeneration (only males), olfactory epithelium atrophy (limited to females) - Lung: alveolar epithelium metaplasia, histiocytic infiltration in alveolus <u>Additional 1.0 ppm:</u> - Epiglottis: hyperplasia (only females)
NTP-2, Toxicology and carcinogenesis studies of ozone and ozone/NKK in F344/N rats and B6C3F1 mice Boorman *et al.,* 1995 Herbert *et al.,* 1996 Method: lifetime	Mice, $B6C3F_1$ mice (Simonsen Laboratories (Gilroy, CA)), male and female No/Group: 50 per sex and group Neoplastic: LOAEC: 0.5 ppm Non-neoplastic:	0 (filtered air), 0.5 and 1.0 ppm 6 h/d 5 days/week for 130 weeks (whole body)	Neoplastic lesions: - Alveolar/bronchiolar carcinoma in males (pos. trend [Life table test, Logistic regression, Cochran-Armitage test], statistically significant for 0.5 ppm [Life table test and logistic regression test], statistically significant for 1 ppm [Life table test, logistic regression test and Fisher's exact test]) - Alveolar/bronchiolar adenoma in females (pos. trend [Life table test, Logistic regression test and Cochran-Armitage test] and statistically significant for 1 ppm [Life table test, Logistic regression and Fisher's exact test) Non-neoplastic lesions: 0.5 ppm: - Nose: lateral wall, hyaline degeneration; lateral wall, fibrosis; lateral wall, hyperplasia; lateral wall, inflammation, suppurative;

inhalation study Guideline: no GLP: in compliance with Food and Drug Administration (FDA) Good Laboratory Practice Regulations Reliability: 1	NOAEC: < 0.5 ppm LOAEC: 0.5 ppm		olfactory, epithelium, atrophy (only females) - Lung: alveolar epithelial metaplasia; alveolar infiltration, histiocyte Additional 1.0 ppm: - Larynx: hyperplasia; epiglottis, metaplasia, squamous - Nose: lateral wall, metaplasia, squamous; olfactory, epithelium, atrophy (only males)
NTP-3, Toxicology and carcinogenesis studies of ozone and ozone/NKK in F344/N rats and B6C3F1 mice. Boorman *et al.,* 1994 Boorman *et al.,* 1995 Method: 2-year inhalation study Guideline: similar to TG 451 GLP: in compliance with Food and Drug Administration (FDA) Reliability: 2	Rat, F344/N rats (Simonsen Laboratories (Gilroy, CA)), male and female No/Group: 50 per sex and group Non-neoplastic: NOAEC:< 0.12 ppm LOAEC: 0.12 ppm	0 (Filtered air), 0.12, 0.5 and 1.0 ppm 6 h/d 5 days/week for 2 years (whole body)	Neoplastic lesions: (only in males) - Skin: positive trend for keratoacanthoma (life table test, logistic regression test) and combined Squamous cell papilloma, keratoacanthoma, trichoepithelioma, basal cell adenoma, or squamous cell carcinoma (life table test, logistic regression test, Cochran-Armitage) Non-neoplastic lesions: <u>0.12 ppm:</u> - Nose: inflammation (limited to males), lateral wall hyperplasia (limited to males), lateral wall metaplasia squamous (limited to females) - Lung: alveolar epithelium metaplasia (extension of bronchial epithelium into alveoli) <u>0.5 ppm and 1.0 ppm:</u> - Larynx: epiglottis squamous metaplasia - Nose: goblet cell hyperplasia, lateral wall squamous metaplasia, lateral wall hyperplasia (limited to females) - Lung: histiocytic infiltration in alveolus, interstitial fibrosis
NTP-4, Toxicology and carcinogenesis studies of ozone and ozone/NKK in F344/N rats and B6C3F1 mice. Boorman *et al.,* 1995 Herbert *et al.,* 1996 Method: lifetime inhalation study Guideline: no GLP: in compliance with Food and Drug Administration (FDA) Reliability: 2	Rat, F344/N rats (Simonsen Laboratories (Gilroy, CA)), male and female No/Group: 50 per sex and group Neoplastic: LOAEC: - Non-neoplastic: NOAEC: < 0.5 ppm LOAEC: 0.5 ppm	0 (filtered air), 0.5 and 1.0 ppm 6 h/d 5 days/week for 125 weeks (whole body)	Neoplastic lesions: - Oral mucosa/males: Squamous cell papilloma or squamous cell carcinoma (pos. trend with Cochran-Armitage test) - Clitoral gland/females: Adenoma or carcinoma (incidences: 8 adenomas at 1 ppm, 5 at 0 ppm; 1 carcinoma at 1 ppm, 0 at 0 ppm) pos. trend with Cochran-Armitage test) Non-neoplastic lesions: ≥0.5 ppm: - Larynx: epiglottis, squamous metaplasia - Nose: goblet cell, lateral wall, hyperplasia; lateral wall, hyperplasia; lateral wall, squamous metaplasia - Lung: alveolar epithelial metaplasia; Alveolar infiltration, histiocyte; Interstitial fibrosis

Last *et al.* (1987) observed a statistically significant increased incidences in lung tumours (adenomas) in male A/J mice in the high dose group in a study with doses of filtered air, 0.4 and 0.8 ppm ozone for 18 weeks. A/J mice showed a statistically significant increased lung tumour

incidence and multiplicity at 0.8 ppm dose level. At 0.4 ppm dose level diffuse mild-to-moderate bronchiolar alveolar epithelial hyperplasia was observed.

The incidences are described below.

Table: *Last et al., 1987. Tumour incidences*

Treatment	Tumour incidence	No. of tumours/lung
NaCl + air	4/33 (12%)	0.13 ± 0.06
NaCl + 0.4 ppm O_3	2/23 (9%)	0.09 ± 0.06
NaCl + 0.8 ppm O_3	12/32 (38%)*	0.55 ± 0.15*

*p < 0.05 compared to NaCl + air (control) group.

Witschi *et al.* (1999) exposed female A/J mice to filtered air, 0.12, 0.5 or 1.01 ppm ozone for 5 months. The incidences are described below.

Table: Witschi *et al*., *1999*

		Lung tumour multiplicity [a]		
Group	**Exposure**	**Lung tumour incidence [b]**	**All animals**	**Tumour-bearing animals only**
A 5 months exposure	Filtered air	3/35 (9%)	0.11 ± 0.05 (35)	1.00 ± 0.00 (3)
	0.12 ppm ozone	3/35 (9%)	0.09 ± 0.05 (35)	1.00 ± 0.00 (3)
	0.50 ppm ozone	4/35 (11%)	0.14 ± 0.07 (35)	1.3 ± 0.3 (4)
	1.00 ppm ozone	8/35 (23%)	0.23 ± 0.07 (35)	1.00 ± 0.00 (8)
B 9 (5 + 4) months exposure	Filtered air	15/30 (50%)	0.83 ± 0.19 (29)	1.71 ± 0.22 (14)
	0.12 ppm ozone	19/31 (61%)	1.12 ± 0.20 (31)	1.84 ± 0.18 (19)
	0.50 ppm ozone	26/32 (81%)*	1.25 ± 0.16 (32)	1.54 ± 0.14 (26)
	1.00 ppm ozone	20/35 (57%)	0.97 ± 0.19 (35)	1.70 ± 0.21 (20)
C 5 months exposure + 4 months (filtered air) recovery	Filtered air (same animals as group B)	14/29 (48%)	0.83 ± 0.19 (29)	1.71 ± 0.22 (14)
	0.12 ppm ozone	26/29 (90%)[#]	1.93 ± 0.25 (29)[#]	2.15 ± 0.25 (26)
	0.50 ppm ozone	20/30 (66%)	1.20 ± 0.27 (30)	1.80 ± 0.19 (20)
	1.00 ppm ozone	21/34 (62%)	0.97 ± 0.17 (34)	1.57 ± 0.16 (21)

* Significantly different (p < 0.05) from control and 1.0 ppm groups (Fisher´s exact test)
[#] Significantly different (p < 0.05) from all other groups (ANOVA and Fisher´s exact test)
[a] Number of tumours per lung. All data given as mean ± SEM, number of animals in brackets
[b] Number of tumour bearing animals per total number of animals at risk, percentage in brackets

Group A animals showed no statistically significant increase in lung tumour incidence or multiplicity compared to concurrent controls. However, the Cochran-Armitage trend test performed by the DS demonstrated a dose-related increase of lung tumour (most tumours were alveolar/bronchiolar adenomas) incidence (p = 0.0234). The Fisher's exact test revealed a statistically significant increased lung tumour incidence at 0.5 ppm (group B) or 0.12 ppm (group C). Furthermore, tumour multiplicity was statistically significantly increased at 0.12 ppm in Group B animals.

Hasset *et al.* (1985) exposed female A/J mice to 0.31 ppm ozone for 6 months (103 h/week) or 0.5 ppm ozone over 6 months for 1 week/month (102 h/week). The numbers of tumour-bearing animals, % of mice with tumours, total number of lung tumours (isolated changes and in continuity

with established adenomas) and average number of tumours/mouse were increased by ozone in both experiments. In the experiment 1, animals were exposed to 0.31 ppm ozone and scarified 5 months, the control animal showed high background lung tumour incidences of 40% and an incidence of 53% in the exposed group. However, in the experiment 2 animals were sacrificed 3 months after 6 months intermittent exposure a lower control tumour incidence of 18% and an increase of lung tumours in ozone exposed mice to 38% were reported.

In a study by Kim *et al.* (2009) male and female B6C3F1 mice were exposed for 1 year to air or 0.5 ppm ozone. No neoplasms were detected in lung, oviduct and liver. Non-neoplastic changes comprised organ weight changes as well as liver, lung, brain and adrenal lesions (e.g. focal bronchiolar alveolar hyperplasia, alveolar fibrosis, congestion in cerebrum). Therefore, the non-neoplastic LOAEC was set at 0.5 ppm.

Boorman *et al.* (1995) and Herbert *et al.* (1996) conducted 2-year carcinogenicity NTP studies in rats and mice where male and female animals were exposed to filtered air, 0.12, 0.5 or 1 ppm ozone.

For rats, no statistically significant increases in neoplastic findings were reported by pairwise comparison to concurrent controls. However, a positive trend for keratoacanthoma and combined squamous cell papilloma, keratoacanthoma, trichoepithelioma, basal cell adenoma or squamous cell carcinoma papilloma was evident in males. Carcinogenic potency of ozone cannot be ruled out as more than 50% of male rats died before study termination. Non-neoplastic lesions were statistically significantly increased at and above 0.12 ppm and comprised nose and lung effects (e.g. nose: suppurative inflammation, lateral wall hyperplasia; lung: alveolar epithelium metaplasia).

***Table**: NTP – 2 years study in rats*

Dose (ppm)	0	0.12	0.5	1.0
Male				
Larynx[a]	50	50	50	50
Epiglottis, Metaplasia, Squamous[b]	0	2 (2.5)[c]	16** (1.3)	43** (2.3)
Nose	50	50	50	50
Inflammation, Suppurative	3 (1.7)	10* (1.7)	12* (1.8)	20** (1.9)
Goblet Cell, Lateral Wall, Hyperplasia	1 (2.0)	4 (1.5)	41** (1.5)	48** (2.1)
Lateral Wall, Hyperplasia	0	8** (2.3)	50** (2.0)	49** (2.7)
Lateral Wall, Metaplasia, Squamous	2 (1.5)	6 (1.8)	36** (1.8)	46** (2.3)
Lung	50	50	50	50
Alveolar Epithelium, Metaplasia	0	9** (1.0)	46** (1.9)	47** (2.9)
Alveolus, Infiltration Cellular, Histiocyte	1 (2.0)	0	27** (1.2)	42** (1.9)
Interstitial, Fibrosis	0	2 (1.0)	40** (1.4)	44** (2.2)
Alveolar/bronchiolar Adenoma				
Overall rate[d]	1/50 (2%)	2/50 (4%)	2/50 (4%)	3/50 (6%)
Adjusted rate[e]	2.2%	16.4%	20.4%	25.4%
Terminal rate[f]	0/8 (0%)	0/5 (0%)	1/7 (14%)	1/7 (14%)
First incidence (days)	514	537	698	619
Logistic regression test[g]	P=0.246	P=0.500	P=0.501	P=0.309
Alveolar/bronchiolar Carcinoma				
Overall rate	1/50 (2%)	1/50 (2%)	1/50 (2%)	1/50 (2%)
Alveolar/bronchiolar Adenoma or Carcinoma[h]				
Overall rate	2/50 (4%)	3/50 (6%)	3/50 (6%)	4/50 (8%)
Adjusted rate	14.4%	18.6%	33.7%	30.1%
Terminal rate	1/8 (13%)	0/5 (0%)	2/7 (29%)	1/7 (14%)
First incidence (days)	514	537	698	619
Logistic regression test	P=0.284	P=0.500	P=0.515	P=0.341

(continued)

Dose (ppm)	0	0.12	0.5	1.0
Female				
Larynx	50	50	50	50
Epiglottis, Metaplasia, Squamous	4 (3.3)	5 (2.8)	9 (2.3)	43** (2.3)
Nose	50	50	50	50
Goblet Cell, Lateral Wall, Hyperplasia	1 (2.0)	2 (1.0)	45** (1.7)	50** (2.5)
Lateral Wall, Hyperplasia	2 (2.0)	8 (1.5)	48** (1.8)	50** (2.6)
Lateral Wall, Metaplasia, Squamous	2 (2.5)	11** (1.4)	21** (1.8)	45** (1.9)
Suppurative Inflammation	3 (1.0)	6 (1.5)	2 (1.0)	2 (2.0)
Lung	50	50	50	50
Alveolar Epithelium, Metaplasia	0	6** (1.0)	48** (1.7)	48** (2.8)
Alveolus, Infiltration Cellular, Histiocyte	0	0	31** (1.2)	43** (1.8)
Interstitial, Fibrosis	0	0	42** (1.4)	47** (2.0)
Alveolar/bronchiolar Adenoma[i]				
Overall rate	0/50 (0%)	0/50 (0%)	2/50 (4%)	0/50 (0%)
Adjusted rate	0.0%	0.0%	6.4%	0.0%
Terminal rate	0/28 (0%)	0/24 (0%)	1/30 (3%)	0/27 (0%)
First incidence (days)	–[j]	–	723	–
Logistic regression test	P=0.545	–	P=0.255	–

* Significantly different (P≤0.05) from the control group by the logistic regression test
** P≤0.01
[a] Number of animals with organ examined microscopically
[b] Number of animals with lesion
[c] Average severity grade of lesions in affected animals (1=minimal; 2=mild; 3=moderate; 4=marked).
[d] Number of animals with neoplasm per number of animals necropsied
[e] Kaplan-Meier estimated neoplasm incidence at the end of the study after adjustment for intercurrent mortality
[f] Observed incidence at terminal sacrifice
[g] Beneath the control incidence are the P values associated with the trend test. Beneath the exposed group incidence are the P values corresponding to pairwise comparisons between the controls and that exposed group. The logistic regression test regards these lesions as nonfatal.
[h] Historical incidence for 2-year inhalation studies with untreated control groups (mean ± standard deviation): 17/398 (4.3% ± 4.5%); range, 0%-10%
[i] Historical incidence: 4/398 (1.0% ± 1.5%); range, 0%-4%
[j] Not applicable; no neoplasms in animal group

For mice, Boorman *et al.* (1995) and Herbert *et al.* (1996) reported statistically significant increases in neoplastic effects including alveolar/bronchiolar combined adenoma or carcinoma at 1 ppm (females), and harderian gland combined adenoma or carcinoma at 0.12 and 0.5 ppm (males). According to the CLP guidance, tumours in the harderian gland have no human equivalent. Therefore, these tumours were excluded from the carcinogenicity evaluation. Furthermore, a positive trend was calculated for alveolar/bronchiolar combined adenoma or carcinoma (males, females), alveolar/bronchiolar carcinoma (females), hepatocellular carcinoma (males) and stromal polyp in uterus (females). Non-neoplastic lesions were already statistically significantly increased at 0.12 ppm and comprised nose effects (suppurative lateral wall inflammation and lateral wall hyaline degeneration).

***Table**: NTP 2 years study in mice*

Dose (ppm)	0	0.12	0.5	1.0
Male				
Larynx[a]	50	50	50	50
Epiglottis, Hyperplasia[b]	1 (1.0)[c]	0	0	6 (1.0)
Nose	50	50	50	50
Lateral Wall, Hyaline Degeneration	2 (1.0)	1 (2.0)	49** (2.0)	50** (3.7)
Lateral Wall, Fibrosis	0	0	47** (1.6)	49** (2.7)
Lateral Wall, Hyperplasia	0	0	42** (1.6)	50** (2.3)
Lateral Wall, Inflammation, Suppurative	0	8**(1.0)	42** (1.5)	50** (2.1)
Lateral Wall, Metaplasia, Squamous	0	3 (1.7)	3 (1.0)	36** (1.7)
Lung	50	50	50	50
Alveolar Epithelium, Metaplasia	0	0	48** (1.6)	50** (2.6)
Alveolus, Infiltration Cellular, Histiocyte	0	0	18** (1.1)	31** (1.8)
Alveolar Epithelium, Hyperplasia	4 (1.5)	6 (2.3)	2 (2.0)	3 (3.3)
Alveolar/bronchiolar Adenoma				
Overall rate[d]	6/50 (12%)	9/50 (18%)	12/50 (24%)	11/50 (22%)
Adjusted rate[e]	18.8%	25.1%	40.9%	34.7%
Terminal rate[f]	5/30 (17%)	8/34 (24%)	9/25 (36%)	8/27 (30%)
First incidence (days)	611	440	464	484
Logistic regression test[g]	P=0.079	P=0.318	P=0.061	P=0.110
Alveolar/bronchiolar Carcinoma				
Overall rate	8/50 (16%)	4/50 (8%)	8/50 (16%)	10/50 (20%)
Adjusted rate	25.5%	10.3%	30.7%	35.4%
Terminal rate	7/30 (23%)	1/34 (3%)	7/25 (28%)	9/27 (33%)
First incidence (days)	653	612	701	630
Logistic regression test	P=0.062	P=0.154N	P=0.449	P=0.270
Alveolar/bronchiolar Adenoma or Carcinoma[h]				
Overall rate	14/50 (28%)	13/50 (26%)	18/50 (36%)	19/50 (38%)
Adjusted rate	43.1%	33.4%	60.9%	60.0%
Terminal rate	12/30 (40%)	9/34 (26%)	14/25 (56%)	15/27 (56%)
First incidence (days)	611	440	464	484
Logistic regression test	P=0.030	P=0.445N	P=0.124	P=0.103

(continued)

Dose (ppm)	0	0.12	0.5	1.0
Female				
Larynx	50	50	49	50
Epiglottis, Hyperplasia	0	0	0	7** (1.0)
Nose	50	50	48	50
Lateral Wall, Hyaline Degeneration	5 (1.0)	18* (1.0)	48** (2.6)	50** (3.5)
Lateral Wall, Fibrosis	0	3 (1.8)	46** (1.8)	50** (2.7)
Lateral Wall, Hyperplasia	0	0	42** (1.9)	50** (2.5)
Lateral Wall, Inflammation, Suppurative	0	5 (1.0)	46** (1.7)	50** (2.1)
Lateral Wall, Metaplasia, Squamous	1 (1.0)	1 (1.0)	11** (1.5)	36** (2.2)
Olfactory Epithelium, Atrophy	4 (1.8)	1 (1.0)	14* (1.5)	41** (1.8)
Lung	50	50	49	50
Alveolar Epithelium, Metaplasia	0	0	43** (1.5)	49** (2.6)
Alveolus, Infiltration Cellular, Histiocyte	0	0	11** (1.0)	42** (1.8)
Alveolar Epithelium, Hyperplasia	2 (2.0)	1 (4.0)	1 (1.0)	2 (2.0)
Alveolar/bronchiolar Adenoma				
Overall rate	4/50 (8%)	5/50 (10%)	5/49 (10%)	8/50 (16%)
Adjusted rate	12.5%	12.9%	13.4%	20.0%
Terminal rate	3/29 (10%)	4/37 (11%)	2/33 (6%)	8/40 (20%)
First incidence (days)	636	681	667	735 (T)
Logistic regression test	P=0.153	P=0.549	P=0.515	P=0.239
Alveolar/bronchiolar Carcinoma				
Overall rate	2/50 (4%)	2/50 (4%)	5/49 (10%)	8/50 (16%)
Adjusted rate	6.9%	5.2%	14.1%	19.2%
Terminal rate	2/29 (7%)	1/37 (3%)	3/33 (9%)	7/40 (18%)
First incidence (days)	735 (T)	703	709	488
Logistic regression test	P=0.011	P=0.649N	P=0.259	P=0.053
Alveolar/bronchiolar Adenoma or Carcinoma[i]				
Overall rate	6/50 (12%)	7/50 (14%)	9/49 (18%)	16/50 (32%)
Adjusted rate	19.2%	17.7%	24.0%	38.8%
Terminal rate	5/29 (17%)	5/37 (14%)	5/33 (15%)	15/40 (38%)
First incidence (days)	636	681	667	488
Logistic regression test	P=0.005	P=0.571	P=0.326	P=0.022

* Significantly different (P≤0.05) than the control group by the logistic regression test
** P≤0.01
(T) Terminal sacrifice
[a] Number of animals with organ examined microscopically
[b] Number of animals with lesion
[c] Average severity grade of lesions in affected animals (1=minimal; 2=mild; 3=moderate; 4=marked).
[d] Number of animals with neoplasm per number of animals necropsied
[e] Kaplan-Meier estimated neoplasm incidence at the end of the study after adjustment for intercurrent mortality
[f] Observed incidence at terminal sacrifice
[g] Beneath the control incidence are the P values associated with the trend test. Beneath the exposed group incidence are the P values corresponding to pairwise comparisons between the controls and that exposed group. The logistic regression tests regard these lesions as nonfatal. A lower incidence in an exposure group is indicated by N.
[h] Historical incidence for 2-year inhalation studies with untreated control groups (mean ± standard deviation): 150/673 (22.3% ± 9.0); range, 10%-42%
[i] Historical incidence: 58/659 (8.8 ± 3.5); range, 0%-15%

Boorman *et al.* (1995) and Herbert *et al.* (1996) also conducted two lifetime NTP carcinogenicity studies with rats and mice. Male and female rats and mice were exposed to filtered air, 0.5 or 1 ppm ozone for 125 weeks or 130 weeks, respectively.

For rats, no statistically significant increase in neoplastic findings were reported based on pairwise comparison of treatment groups.

However, a positive correlation with dose was determined for oral mucosa squamous cell papilloma or carcinoma (males) and clitoral gland adenoma or carcinoma (females) by trend test made by the DS. Non-neoplastic lesions were statistically significant from 0.5 ppm and involved nose, larynx, and lung effects (e.g. goblet cell lateral wall hyperplasia, interstitial fibrosis in lung and epiglottis squamous metaplasia in larynx).

***Table**: NTP lifetime study in rats*

Dose (ppm)	0	0.5	1.0
Male			
Larynx[a]	50	48	47
Epiglottis, Squamous Metaplasia[b]	0	20** (1.3)[c]	43** (1.8)
Nose	50	49	49
Goblet Cell, Lateral Wall, Hyperplasia	1 (1.0)	46** (1.5)	48** (2.6)
Lateral Wall, Hyperplasia	10 (1.5)	48** (1.9)	47** (2.8)
Lateral Wall, Squamous Metaplasia	10 (2.5)	23** (1.6)	40** (2.3)
Lung	50	50	50
Alveolar Epithelial Metaplasia	0	45** (1.9)	50** (2.9)
Alveolar Cellular Infiltration, Histiocyte	0	38** (1.2)	49** (1.9)
Interstitial Fibrosis	0	44** (1.7)	50** (2.4)
Alveolar/bronchiolar Adenoma			
Overall rate[d]	2/50 (4%)	3/50 (6%)	0/50 (0%)
Adjusted rate[e]	25.9%	22.3%	0.0%
Terminal rate[f]	0/0	0/0	0/1 (0%)
First incidence (days)	708	581	–[h]
Logistic regression test[g]	P=0.161N	P=0.427	P=0.169N
Alveolar/bronchiolar Carcinoma			
Overall rate	0/50 (0%)	1/50 (2%)	0/50 (0%)
Alveolar/bronchiolar Adenoma or Carcinoma			
Overall rate	2/50 (4%)	4/50 (8%)	0/50 (0%)
Adjusted rate	25.9%	26.2%	0.0%
Terminal rate	0/0	0/0	0/1 (0%)
First incidence (days)	708	581	–
Logistic regression test	P=0.182N	P=0.266	P=0.169N

(continued)

Dose (ppm)	0	0.5	1.0
Female			
Larynx	49	47	50
Epiglottis, Squamous Metaplasia	2 (2.0)	16** (1.1)	48** (2.0)
Nose	50	49	50
Goblet Cell, Lateral Wall, Hyperplasia	0	47** (1.8)	50** (2.4)
Lateral Wall, Hyperplasia	4 (1.8)	49** (1.9)	50** (2.8)
Lateral Wall, Squamous Metaplasia	5 (2.4)	25** (1.3)	35** (1.6)
Lung	50	50	50
Alveolar Epithelial Metaplasia	0	44** (1.7)	50** (2.9)
Alveolar Cellular Infiltration, Histiocyte	0	38** (1.1)	49** (2.0)
Interstitial Fibrosis	0	41** (1.2)	50** (2.5)
Alveolar/bronchiolar Adenoma			
Overall rate	0/50 (0%)	1/50 (2%)	1/50 (2%)
Adjusted rate	0.0%	3.0%	3.3%
Terminal rate	0/6 (0%)	0/6 (0%)	0/7 (0%)
First incidence (days)	–	710	685
Logistic regression test	P=0.330	P=0.507	P=0.500
Alveolar/bronchiolar Carcinoma			
Overall rate	1/50 (2%)	1/50 (2%)	0/50 (0%)
Alveolar/bronchiolar Adenoma or Carcinoma			
Overall rate	1/50 (2%)	2/50 (4%)	1/50 (2%)
Adjusted rate	12.5%	8.7%	3.3%
Terminal rate	0/6 (0%)	0/6 (0%)	0/7 (0%)
First incidence (days)	827	710	685
Logistic regression test	P=0.594N	P=0.598	P=0.738N

** Significantly different (P≤0.01) than the control group by the logistic regression test
[a] Number of animals with organ examined microscopically
[b] Number of animals with lesion
[c] Average severity grade of lesions in affected animals (1=minimal; 2=mild; 3=moderate; 4=marked).
[d] Number of animals with neoplasm per number of animals necropsied
[e] Kaplan-Meier estimated neoplasm incidence at the end of the study after adjustment for intercurrent mortality
[f] Observed incidence at terminal sacrifice
[g] Beneath the control incidence are the P values associated with the trend test. Beneath the exposed group incidence are the P values corresponding to pairwise comparisons between the controls and that exposed group. The logistic regression test regards these lesions as nonfatal. A negative trend or a lower incidence in an exposure group is indicated by N.
[h] Not applicable; no neoplasms in animal group

For mice, statistically significant increase in neoplastic lesions included alveolar/bronchiolar carcinoma at 0.5 ppm and 1 ppm (males) as well as alveolar/bronchiolar adenoma at 1 ppm (females). Furthermore, a positive trend was determined for alveolar/bronchiolar carcinoma (males) and alveolar/bronchiolar adenoma (females). Non-neoplastic lesions were statistically significantly increased at 0.5 ppm and related to nose and lungs (e.g. lateral wall hyaline degeneration, lateral wall fibrosis, alveolar epithelial metaplasia).

***Table**: NTP lifetime study in mice*

Dose (ppm)	0	0.5	1.0
Male			
Larynx[a]	49	49	50
Hyperplasia[b]	4 (1.0)[c]	7 (1.3)	15**(1.1)
Epiglottis, Metaplasia, Squamous	2 (1.0)	1 (1.0)	10** (1.1)
Nose	49	48	49
Lateral Wall, Hyaline Degeneration	2 (1.5)	48** (1.1)	49** (2.5)
Lateral Wall, Fibrosis	0	8** (1.0)	43** (1.3)
Lateral Wall, Hyperplasia	2 (1.0)	33** (1.1)	45** (1.8)
Lateral Wall, Inflammation, Suppurative	1 (1.0)	38** (1.0)	46** (1.3)
Lateral Wall, Metaplasia, Squamous	1 (1.0)	2 (1.5)	20**(1.2)
Olfactory, Epithelium, Atrophy	4 (1.8)	4 (2.3)	18**(1.7)
Lung	49	49	50
Alveolar Epithelium, Metaplasia	0	48** (1.5)	47** (2.2)
Alveolus, Infiltration Cellular, Histiocyte	3 (3.0)	40** (1.8)	41** (1.7)
Alveolar Epithelium, Hyperplasia	10 (2.8)	8 (3.3)	1** (4.0)
Alveolar/bronchiolar Adenoma			
Overall rate[d]	8/49 (16%)	8/49 (16%)	9/50 (18%)
Adjusted rate[e]	33.9%	32.8%	50.6%
Terminal rate[f]	3/14 (21%)	2/11 (18%)	5/12 (42%)
First incidence (days)	391	678	620
Logistic regression test[g]	P=0.427	P=0.606N	P=0.473
Alveolar/bronchiolar Carcinoma			
Overall rate	8/49 (16%)	15/49 (31%)	18/50 (36%)
Adjusted rate	42.3%	65.3%	70.9%
Terminal rate	4/14 (29%)	5/11 (45%)	6/12 (50%)
First incidence (days)	805	693	609
Logistic regression test	P=0.005	P=0.050	P=0.007
Alveolar/bronchiolar Adenoma or Carcinoma			
Overall rate	16/49 (33%)	22/49 (45%)	21/50 (42%)
Adjusted rate	66.0%	76.3%	77.0%
Terminal rate	7/14 (50%)	6/11 (55%)	7/12 (58%)
First incidence (days)	391	678	609
Logistic regression test	P=0.127	P=0.140	P=0.149

(continued)

Dose (ppm)	0	0.5	1.0
Female			
Larynx	50	49	50
Hyperplasia	13 (1.2)	11 (1.3)	24* (1.3)
Epiglottis, Metaplasia, Squamous	2 (1.5)	2 (1.0)	19** (1.1)
Nose	50	49	50
Lateral Wall, Hyaline Degeneration	0	49** (2.0)	50** (2.4)
Lateral Wall, Fibrosis	1 (1.0)	23** (1.1)	48** (1.2)
Lateral Wall, Hyperplasia	1 (1.0)	42** (1.9)	47** (2.0)
Lateral Wall, Inflammation, Suppurative	3 (1.0)	44** (1.0)	50** (1.3)
Lateral Wall, Metaplasia, Squamous	2 (1.0)	3 (1.0)	28** (1.4)
Olfactory Epithelium, Atrophy	9 (1.4)	23* (1.9)	40** (2.2)
Lung	50	49	50
Alveolar Epithelium, Metaplasia	0	43** (1.0)	50** (2.1)
Alveolus, Infiltration Cellular, Histiocyte	5 (2.2)	39** (1.3)	45** (1.8)
Alveolar Epithelium, Hyperplasia	3 (1.7)	1 (2.0)	3 (3.0)
Alveolar/bronchiolar Adenoma			
Overall rate	3/50 (6%)	3/49 (6%)	11/50 (22%)
Adjusted rate	15.7%	8.9%	56.1%
Terminal rate	1/9 (11%)	0/12 (0%)	4/10 (40%)
First incidence (days)	721	616	455
Logistic regression test	P=0.009	P=0.633	P=0.020
Alveolar/bronchiolar Carcinoma			
Overall rate	3/50 (6%)	5/49 (10%)	2/50 (4%)
Adjusted rate	12.2%	26.4%	13.9%
Terminal rate	0/9 (0%)	2/12 (17%)	1/10 (10%)
First incidence (days)	521	721	833
Logistic regression test	P=0.423N	P=0.328	P=0.496N
Alveolar/bronchiolar Adenoma or Carcinoma			
Overall rate	6/50 (12%)	8/49 (16%)	12/50 (24%)
Adjusted rate	26.0%	33.1%	58.0%
Terminal rate	1/9 (11%)	2/12 (17%)	4/10 (40%)
First incidence (days)	521	616	455
Logistic regression test	P=0.072	P=0.341	P=0.096

* Significantly different (P≤0.05) from the control group by the logistic regression test
** P≤0.01
[a] Number of animals with organ examined microscopically
[b] Number of animals with lesion
[c] Average severity grade of lesions in affected animals (1=minimal; 2=mild; 3=moderate; 4=marked).
[d] Number of animals with neoplasm per number of animals necropsied
[e] Kaplan-Meier estimated neoplasm incidence at the end of the study after adjustment for intercurrent mortality
[f] Observed incidence at terminal sacrifice
[g] Beneath the control incidence are the P values associated with the trend test. Beneath the exposed group incidence are the P values corresponding to pairwise comparisons between the controls and that exposed group. The logistic regression test regards these lesions as nonfatal. A negative trend or a lower incidence in an exposure group is indicated by N.

In the study by Witschi *et al.* (1993), male Syrian hamsters were exposed for 16 weeks to filtered air or 0.8 ppm ozone. According to the authors no lung tumours developed. Statistically significant lung lesions including bronchiolar hyperplasia were observed. However, due to the short exposure time and quality, the study has relatively low reliability and cannot be used to rule out a possible carcinogenic effect in hamsters.

Evidence for carcinogenic potential in humans

Two epidemiological studies by Beeson *et al.* (1988) and Gharibvand *et al.* (2017) analysed associations between selected ambient air pollutants (including ozone) and incident lung cancer in Seventh-day Adventists.

In the Adventist Health Study on Smog (AHSMOG) by Beeson *et al.* (1998) 6 338 Californian non-smoking adults participated in a prospective cohort study. These participants were part of a greater prospective cohort study – the Adventist Health Study (AHS) – which included more than 34 000 Seventh-day Adventists residing in California (Beeson *et al.* 1989). In the AHSMOG study, the participants were followed for newly diagnosed lung cancers from 1977 to 1992. A computer-assisted record linkage with local and state-wide cancer registries as well as medical records from self-reported hospitalisations were used to ascertain these lung cancer incidences. In order to generate estimates of monthly ambient mean concentrations, exceedance frequencies (i.e. sum of hours above a specified cut-off) and excess concentrations (i.e. sum of concentrations above a specified cut-off), ozone exposure data from fixed-site monitoring stations maintained by the California Air Resources Board (CARB) from 1966-1992 was analysed. PM_{10}, SO_2 and NO_2 were also studied in the AHSMOG study. Within the 15-year observation period there was a total of 36 incident cases of lung cancer (20 in females, 16 in males), most of them carcinomas and adenocarcinomas. Cox proportional hazards regression models stratified by sex and adjusted for the potential confounding effects of current alcohol use, pack-years of past cigarette smoking and educational level were used to analyse the association between the selected air pollutants and the incidence of lung cancer. With regard to exceedance frequencies and based on derived interquartile ranges of the population exposed, the data suggests for males an association between ozone and an elevated lung cancer risk:

Concentration	Hr/y	Relative risk (95%)	Confidence interval (CI)
0.06 ppm	935	2.14	0.82-5.62
0.08 ppm	756	2.96	1.09-8.04
0.10 ppm	556	3.56	1.35-9.42
0.12 ppm	367	3.75	1.55-9.09
0.15 ppm	185	3.61	1.78-7.35

However, the association was only observed in males and for an 8-hour mean concentration of ozone the relative risk (RR) was only 1.65 and not statistically significant (CI, 0.72-3.8). In contrast, mean concentrations of PM_{10} showed per 24 µg/m³ increment a significant increased risk of incident lung cancer in males (RR = 5.21, CI, 1.94-13.99). Moreover, a high correlation between ozone concentration and PM_{10} concentration was found and the authors describe the ozone effect as being not as stable or strong as the PM_{10} and SO_2 effects in multipollutant analyses.

In the AHSMOG-2 study, Gharibvand *et al.* (2017) assessed in 80285 Seventh-day Adventists the association between $PM_{2.5}$ and lung cancer incidence using ozone as a covariate. The participants were a subpopulation of the Adventist Health Study-2 (AHS-2) which included about 96 000 Seventh-day Adventists from all 50 U.S. states and 5 provinces of Canada (Butler *et al.*, 2008). In the AHSMOG-2 study, the participants were followed for an average of 7.5 years. For the purposes of cancer incidence ascertainment, a computer-assisted record linkage of each study participant with state cancer registries (2002-2011) as well medical records were used. Ambient air pollution data for ozone were retrieved from the U.S. Environmental Protection Agency Air Quality System and monthly exposure averages were based on 24-hour measurements. A total of 250 incident lung cancer cases were registered during the observation time, most of them

adenocarcinomas. Analyses of the study demonstrate a non-significant association with lung cancer for each 0.01 ppm increment in 24-hour ozone concentration in a two-pollutant ($PM_{2.5}$ and O_3) multivariable (sex, education level, race, and smoking) model: HR (hazard ratio) = 1.07, CI, 0.78-1.48. In contrast, the same model calculated for incident lung cancer per 10 µg/m³ increment in mean monthly ambient $PM_{2.5}$ a significant association (HR = 1.43, CI, 1.03-2.00). The authors concluded that there was no independent association between incident lung cancer and ambient 24-hour ozone concentrations.

As both AHSMOG studies were not able to show an independent and clear association between ozone and lung cancer, the data is not sufficient to infer a causal relationship between chronic ozone exposure and an increased risk of lung cancer.

RAC conclusion for carcinogenicity

Lung tumours were observed in male and female A/J mice exposed to ozone. Adenomas and carcinomas were reported from a dose of 0.5 ppm ozone by Witschi *et al.* (1999) in female A/J mice after exposure for 5 months (killing after recovery period of 4 months) or continuous exposure to 0.12 ppm ozone for 9 months. Last *et al.* (1987) reported a statistically significant increase in lung tumours (adenomas) in male A/J mice after exposure to 0.8 ppm ozone for 18 weeks. Hasset *et al.* (1985) reported an increase in lung tumour (adenomas) incidence in A/J mice exposed to 0.31 ppm or 0.5 ppm ozone for two different intermittent exposure regimes for 6 months, respectively. The control animals of experiment 1, where animals exposed to 0.31 ppm ozone and scarified 5 months after the final ozone exposure, showed high background tumour incidences (40%). However, experiment 2 with sacrifice 3 months after 6 months intermittent exposure resulted in a lower control tumour incidence and an increase of lung tumours in ozone exposed female A/J mice. However, the background incidence was moderate to high in all studies listed even if incidences were constantly higher in treated animals than in air controls. Accordingly, because of the high frequency of spontaneous tumour incidences in the strain A/J mice, the studies by Last and Witschi are not regarded as supportive for a classification for carcinogenicity in category 1B.

In contrast, sufficient evidence for a carcinogenic potential of ozone was provided by studies using B6C3F1 mice. Kim *et al.* (2009) reported pre-neoplastic lesions in the lung like bronchiolar alveolar hyperplasia and bronchiolar epithelium hyperplasia after a one-year exposure duration to 0.5 ppm ozone. The study duration seems to be too short for tumour development in B6C3F1 mice. In the 2-year NTP study by Boorman *et al.* (1995) and Herbert *et al.* (1996) a statistically significant increase in alveolar/bronchiolar combined adenoma and carcinoma was obtained at 1 ppm in female B6C3F1 mice. The incidence of alveolar/bronchiolar adenoma or carcinoma combined exceeded the NTP historical control range for this neoplasm (58/659; 0-15%) in 0.5 and 1 ppm exposed females. Furthermore, alveolar/bronchiolar combined adenoma or carcinoma in male and female mice as well as alveolar/bronchiolar carcinoma in female mice followed a positive trend. The lung tumours observed at 0.5 ppm dose group were accompanied by non-neoplastic and pre-neoplastic lesions (e.g. histiocytic infiltration and metaplasia) in the lung. In the lifetime study by Boorman *et al.* (1995) and Herbert *et al.* (1996) a statistically significant increase in alveolar/bronchiolar carcinoma at 0.5 ppm and 1 ppm in male B6C3F1 mice as well as in alveolar/bronchiolar adenoma at 1 ppm in female B6C3F1 mice was observed. A comparison with historical control data of the NTP is not possible as there are no data for lifetime studies. Additionally in this study, tumours observed at 0.5 ppm were accompanied by non-neoplastic and pre-neoplastic effects (same findings in lung as already observed in the 2-year study). As a result of the B6C3F1 mice studies, adenoma and carcinoma were found dose-dependent and above historical controls, and findings are therefore regarded as relevant for classification.

Taken together, tumour development in B6C3F1 mice seems to take longer time than in A/J mice. This is in line with the phenotype of this mouse strain. A/J mice are susceptible to lung tumour development in response to carcinogens, as seen in the studies with terminal sacrifice after 9 months study duration or longer. No lung tumours were detected in Syrian hamsters after 16-week exposure to 0.8 ppm ozone (Witschi *et al.* 1993), however the study duration is not sufficient long to study carcinogenicity effects.

According to the CLP Regulation category 1B is justified for substances for which animal experiments give "sufficient evidence to demonstrate animal carcinogenicity". As laid down in CLP regulation sufficient evidence means "*a causal relationship […] between the agent and an increased incidence of malignant neoplasms or of an appropriate combination of benign and malignant neoplasms in*

(a) two or more species of animals or

(b) two or more independent studies in one species carried out at different times or in different laboratories or under different protocols."

It is further stated that: "An increased incidence of tumours in both sexes of a single species in a well-conducted study, ideally conducted under Good Laboratory practices, can also provide sufficient evidence. A single study in one species and sex might be considered to provide sufficient evidence of carcinogenicity when malignant neoplasms occur to an unusual degree with regard to incidence, site, type of tumour or age at onset, or when there are strong findings of tumours at multiple sites […]."

Ozone leads to lung tumour formation in B6C3F1 mice, but not in rats. Therefore, requirement (a) is not fulfilled. In this context, it should be noted that the NTP studies in rats (Boormann *et al.*, 1995 and Herbert *et al.*, 1996) are not acceptable as negative evidence due to insufficient survival.

Lung tumour formation by ozone was found in many different and independent studies in mice (Last *et al.*, 1987; Witschi *et al.*, 1999; Hasset *et al.*, 1985 and 2-year or lifetime NTP studies conducted in 1994). All studies used different times and protocols. Lung tumours were not only observed in B6C3F1 mice but also in A/J mice. A/J mice showed a high background of spontaneous incidence. As this strain is more susceptible to lung tumour formations following inhalation, tumours were observed after shorter time frames in these studies (starting from 18-weeks). But as studies in A/J mice are regarded as limited evidence, requirement (b) would not be fulfilled for classification, even if the lung effects reported in the NTP studies were above the historical control data and followed a positive trend.

In the NTP studies with rats and mice, evidence for tumour formation at multiple sites was also not strong enough to support classification of ozone in category 1B for carcinogenicity.

The carcinogenic effects in the lungs, which is the site of contact after inhalation exposure, are mechanistically plausible taking into account the genotoxic effects of ozone or its oxidation products in the lung. Therefore, lung carcinogenicity may be attributed to genotoxic effects (initiation events) potentially in combination with further oxidative stress and regenerative mitogenesis (initiation and tumour promoting events).

Overall, as there is no human data available providing adequate evidence for a causal relationship between long-term exposure to ozone and an increased risk of lung cancer and as the animal data do not fulfil the criteria for classification in category 1, RAC agrees with the DS that a classification for **carcinogenicity for ozone in category 2 is warranted**.

10.10 Reproductive toxicity

10.10.1 Adverse effects on sexual function and fertility

Table 37: Summary table of animal studies on adverse effects on sexual function and fertility

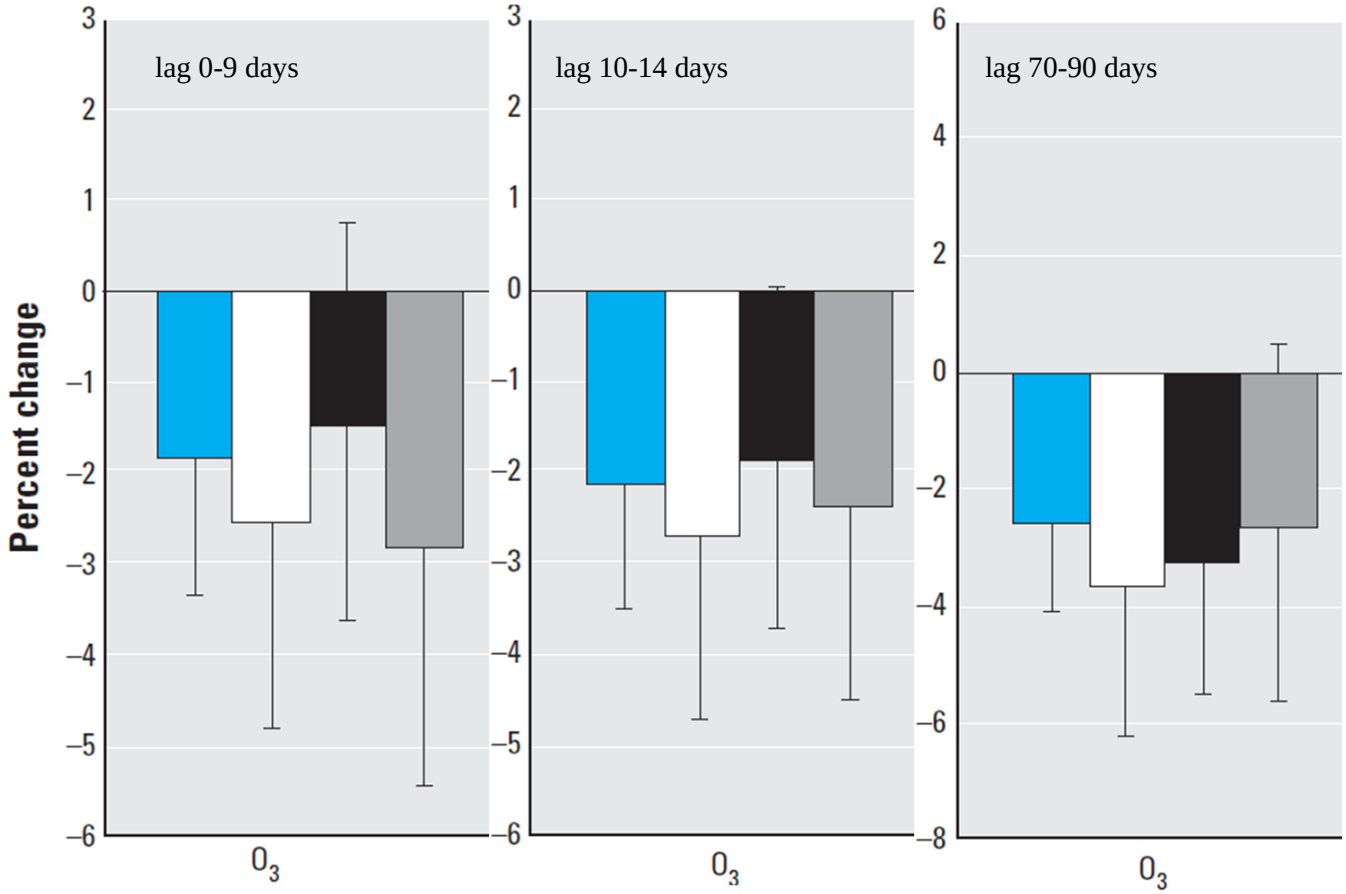
Method, Guideline, GLP status, Reliability	**Species, Strain, Sex, No/ group**	**Test substance Dose levels, Duration of exposure**	**NOAELs, LOAELs**	**Results**	**Remarks** *(e.g. major deviations)*	**Reference**
Reliability: 4	Female Wistar rats, nonpregnant and pregnant (5, 10, 18 days of gestation) group size: n=5-9	Ozone generated from air **0, 3±0.2ppm** 1 h exposure in chamber Uterine contractile response to oxytocin and acetylcholine was examined in uterine tissue strips 16-18 h post-exposure to ozone	n/a – only one dose tested	Influence of ozone on uterine contractile response to oxytocin and acetylcholine: <u>Oxytocin:</u> Area under the curve was increased in non-pregnant and pregnant rats on gestational day 5 (stat. sign.), but not different on gestational days 10 and 18 Amplitude was increased in non-pregnant and pregnant rats at gestational day 5 (stat. sign.), and decreased at gestational days 10 and 18 Frequency was increased in non-pregnant and pregnant rats at gestational days 5 (stat. sign.) and 10 (small effect), and decreased at gestational day 18 <u>Acetylcholine:</u> Area under the curve was increased in non-pregnant and pregnant rats on gestational days 5 and 10 (both stat. sign.) Amplitude was increased in non-pregnant and pregnant rats on gestational days 5 and 10, but decreased on GD 18		Campos-Bedolla P. et al. (2002), Reprod Toxicol. 16(3):269-73

				Frequency was decreased in non-pregnant and pregnant rats at GD 18, but increased at GD 5 and 10		
Method **Guideline** **GLP** **Reliability:** 2	**Species** rat **Strain** Wistar/Hannover **Sex** male (5 month old) **No/group** 8/control group 10/exposure group After 42 days of exposure males stayed 8 days with unexposed females for mating	Ozone Air control Ozone generation from compressed air in IMPOZ-4 ozonizer (Institute of Precision Mechanics, Warsaw, Poland) **0.5 ± 0.2 ppm** Exposure: 50 days, 5h/d Whole body inhalative exposure Sacrifice (males): immediately after exposure	**Male fertility:** NOAEL: **0.5 ppm**	Morphology of spermatozoa: no significant differences between ozone group and control, reduced in exposed rats: abnormal head, hookles, banana shaped, double headed, loose head, increased in exposed rats: folded around the head, coiled tail Sperm motility (by CASA): no significant differences, reduced in exposed rats: Curvilinear velocity (VCL, 3 %), increased in exposed rats: % motility (MOT, 7 %), straight-line velocity (VSL, 18 %), linearity (LIN, 20 %), beat cross frequency (BCF, 38 %), amplitude of lateral head displacement (ALH, 3 %) Sperm concentration: ~17 % lower in exposed rats (not stat. significant) Morphometric measurements: no differences shown in size and weight of testes and vesicular glands Fertilisation: successful matings, average number of pups and new-born mortality were similar to control	No guideline study Study purpose: Determine if ozone can disturb reproductive processes in rat In middle of exposure time, air in chamber was exchanged to avoid accumulation of CO_2 During exposure no access to food (pellets removed due to oxidising effect of ozone) In mating season, males still exposed to ozone	Jedlińska-Krakowska M. et al. (2006), Pol. J. Vet. Sci. 9(1):11-16

Table 38: Summary table of human data (epidemiological studies) on adverse effects on sexual function and fertility

Reference / study characteristics	ozone exposure			Statistical Analysis	Effect	Results	Others/ Remarks
	Conc. µg/m3	Conc. ppb	Duration hours				
Sokol, R.Z. et al. 2006 retrospective cohort study on sperm quality Forty-eight donors from Los Angeles, donors provided repeated semen samples over a 12-month period between January 1996 and December 1998. Environ Health Perspect. 2006 Mar; 114(3): 360–365.		Mean ± SD: 21.68 ± 9.43 Range:1.69 –47.51 Number measurements: 1,096	Chronic exposure/ long-term	Linear mixed-effects model to model linear relationships between transformed semen analysis data and air quality measurements	Average sperm concentration	Significant negative correlation between ozone levels at 0–9, 10–14, and 70–90 days before donation and average sperm concentration, which was maintained after correction for donor's birth date, age at donation, temperature, and seasonality ($p < 0.01$). **Result: sperm toxicant^**	Reliable study, statistical method appropriate
Tian, X.J. 2017 retrospective cohort study on sperm quality 1780 subjects, aged 20 to 40 years, study at Reproductive Medicine Center in Renmin Hospital of Wuhan University, 4/2013 - 6/2015. Semen quality measured according to standardized protocols. Chinese Journal of Preventive Medicine 51(3):197-202.		Mean ± SD: 114.20±74.88) µg/m^3	Chronic exposure/ long-term	Not stated in abstract (see remarks)	Sperm quality (during different stages of spermatogenesis)	Decreasing sperm concentration and count Mean sperm concentration: 76.32±50.17x 10^6/ml Count: 164.77 ± 133.05) x10^6/sample For every 1 µg/m^3 increase of O_3, the decrease of sperm concentration during lag 10, lag 0-9 and lag 10-14 days exposure: - 0.040 (95%CI: 0.004-0.077) x10^6/ml - 0.081 (95% CI: 0.003-0.158) x10^6/ml - 0.059 (95% CI: 0.001-0.116) x10^6/ml	Only abstract in english available, total study in Chinese Confounders: age, BMI, education level and other Reliability could not be approved, statistical method not approved

Figure 1: Percent change in sperm concentration for a 1 SD increase in air quality measure (lag 0-9 days, 10-14 days, and 70-90 days, respectively). Error bars indicate 95% confidence intervals. Base, blue; base + season, white; base + temperature, black; base + season + temperature, grey. Data from the study by Sokol 2006.



10.10.2 Short summary and overall relevance of the provided information on adverse effects on sexual function and fertility

Campos-Bedolla (2002) found that ozone influenced the effects of oxytocin and acetylcholine on uterine contractions in non-pregnant and pregnant rats exposed to 3 ppm ozone for one hour at different gestational stages. Different measures of the contractile response to oxytocin was increased in non-pregnant and pregnant rats on GD 5 and was decreased or close to unchanged on later GD (10 and 18). The effect of acetylcholine was overall increased in non-pregnant and pregnant rats on GD 5 and 10, but decreased on GD 18. The relevance of this study for the proposed endpoint of female fertility is not given, because of the lacking focus on female reproductive functions or capacity.

In addition Jedlińska-Krakowska (2006) performed a study on male rats exposed to 0.5 ppm ozone for 50 days to assess reproductive endpoints. The findings included no significant differences of spermatozoa morphology, sperm mobility and size/weight of testes and vesicular glands. The collected values do not deviate from values considered to be normal in male rats. Even if sperm concentration was 17 % lower in exposed rats, this did not affect the reproductive capacity. Fertilisation of exposed males was not impaired, because number of successful matings and average number of pups were the same as in control animals. The study of Jedlińska-Krakowska (2006) performed analyses, which were described for sperm parameters in OECD TG 416 and TG 443. In an epidemiological, retrospective cohort study on sperm quality, Sokol, R.Z. et al. 2006 observed a significant negative correlation between ozone levels and sperm concentration. Percent change were below 4 % under different conditions (Table 33). Results are supported by a study of Tian, X.J. 2017, reporting on a decrease of sperm concentration and count in young people in Wuhan. In a third human study, Slama 2013analyzed a birth cohort study conducted in Teplice district of Czech Republic and assessed short-term impact of PM2.5, PAH, NO_2 and ozone on fecundability. As a result, the levels of ozone, at lags 1 and 2 were not clearly associated with decreased fecundability in fully adjusted models.

Reference: Slama, Rémy, et al. "Short-term impact of atmospheric pollution on fecundability." *Epidemiology* (2013): 871-879

Further studies addressing developmental toxicity (see Chapter 10.10.1) should also be considered for reproductive performance, if female or male animals were exposed to ozone prior to mating. Sorace (2001) reported that ozone did not significantly affect the number of successful pregnancies, however there was a reduction after exposure to 0.6 ppm ozone. In this regime, female mice were exposed 30 days before the formation of breeding pairs with non-exposed males. In another study by Santucci (2006) with the same exposure regime, the data about successful pregnancies is unfortunately not reported. Moreover, the reproductive performance of exposed females and males 6 days before the formation of breeding pairs to concentrations of 0.2 – 0.9 ppm ozone was not affected, resulting in similar numbers of pregnancies in exposed and non-exposed control mice (Petruzzi, 1995 and 1999). Dell'Omo also reported that successful pregnancies were not affected, if female mice were exposed 6 days prior the formation of breeding pairs to weaning.

The U.S. EPA concluded that there is very little evidence for effects towards sperms and reproductive success for ozone exposure in epidemiology. Furthermore the reproductive success, by taking a few toxicological studies with rodents into account, seems to be unaffected after short-term exposure.

Reference: U.S. EPA (2013). Integrated science assessment for ozone and related photochemical oxidants. EPA600/R-10/076F

Ozone does not impair the fertility of male rats, therefore the **NOAEC** for male fertility is considered to be **0.5** ppm (Jedlińska-Krakowska, 2006). The **NOAEC** for female sexual function and fertility is based on pregnancy outcome and stated at 0.6 ppm (Petruzzi, 1995).

10.10.3 Comparison with the CLP criteria for adverse effects on sexual function and fertility

Toxicological results	**CLP criteria**
No study was performed according to OECD test guidelines for reproductive toxicity TG 415, 416, 421, 422 and 443 and partly they	Category 1A: Known human reproductive toxicant

Toxicological results	CLP criteria
were also not fully compliant with generic OECD test guideline criteria. All studies were reported in the public literature and the available information was regarded as of "limited reliability" for hazard evaluation and risk assessment. However, according to Regulation (EC) No. 528/2012, the study needs not be conducted if the substance is known to be a genotoxic carcinogen and appropriate risk management measures are implemented including measures related to reproductive toxicity. Classification not possible because of the deviations between open literature studies and OECD TG studies.	Category 1B: Presumed human reproductive toxicant largely based on data from animal studies - clear evidence of an adverse effect on sexual function and fertility in the absence of other toxic effects, or - the adverse effect on reproduction is considered not to be a secondary non-specific consequence of other toxic effects Category 2: Suspected human reproductive toxicant - some evidence from humans or experimental animals, possibly supplemented with other information, of an adverse effect on sexual function and fertility - where the evidence is not sufficiently convincing to place the substance in Category 1 (deficiencies in the study). - the adverse effect on reproduction is considered not to be a secondary non-specific consequence of the other toxic effects

Conclusion on classification and labelling for Reproductive toxicity, adverse effects on sexual function and fertility:

In summary and based on the submitted data, Ozone does not meet the criteria to be classified for Reproductive toxicity, for adverse effects on sexual function and fertility, according to the criteria in CLP regulation.

10.10.4 Adverse effects on development

Table 39: Summary table of animal studies on adverse effects on development

Method, Guideline, GLP status, Reliability	**Species, Strain, Sex, No/ group**	**Test substance Dose levels, Duration of exposure**	**NOAELs, LOAELs** *(also for maternal effects)*	**Results**	**Remarks** *(e.g. major deviations)*	**Reference**
Developmental Neurotoxicity Study Sim. to OECD 426 Reliability: 2	Charles River CD-1 mice **Group size:** Maternal: n=11 Offspring: 9-10 litters per dose group (37 total), divided into three experiments: -Somatic and neurobehavioral development tests (postnatal days 2-18; n=2m/2f) -Ultrasonic vocalisation test (postnatal days 3, 7 and 11; n=1m/1f) -Activity/ exploration tests, also in combination with response to d-amphetamine-induced activity increase (postnatal days 60-61; n=2m)	Ozone Whole-body exposure during pregnancy days 7-17 to **0, 0.4, 0.8 or 1.2 ppm**	**Maternal:** LOAEL: **0.8 ppm** (reduced body weight (stat. sign.)) NOAEL: **0.4 ppm** **Offspring:** LOAEL: **0.8 ppm** (bw) NOAEL: **0.4 ppm**	**Maternal:** Food consumption: stat. sign. lower food and water consumption during gestational days 7-10 in all dose groups compared to control, followed by an increase so that effect was no longer seen during gestational days 14-17 Body weight: stat. sign. decreased bw in mid and high dose groups on day 10 and a trend towards dose-related reduced bw-gain and reduced bw in all dose groups throughout gestation Pregnancy duration: slightly increased in two highest dose groups **Offspring:** Body weight: reduced bw gain in mid and high dose groups (stat. sign. only in high dose group), but slightly increased in low dose group Physical development: delayed (2-d delay) eye opening (stat. sign. only in low dose group) Not affected: proportion of successful pregnancies, litter size, sex ratio, frequency of stillbirth, neonatal mortality, ear opening, incisor eruption, hair growth, body/tail length, ultrasonic vocalisations (data not reported); all reflexes and responses assessed by the modified Fox battery (except eye opening); motor activity and habituation (within-session response reduction) in activity test; latency of approach to and number of approaches to novel stimulus object (data not reported)	Several study conditions not in line with OECD GL 426 (e.g. species; exposure period does not include lactation; type, number and timing of assessment tests) Offspring was reared by non-exposed foster mothers. Activity was retested after d-amphetamine injection. Results not reported here.	Bignami G. et al. (1994), Toxicol Appl Pharmacol. 129(2):264-71

Method, Guideline, GLP status, Reliability	**Species, Strain, Sex, No/ group**	**Test substance Dose levels, Duration of exposure**	**NOAELs, LOAELs** *(also for maternal effects)*	**Results**	**Remarks** *(e.g. major deviations)*	**Reference**
Prenatal Developmental Toxicity Study Sim. to OECD 414 Reliability: 4	Long-Evans female pregnant rats **Group size:** Maternal: n=14-37 Offspring: Animals: n=38-102 Litters: n=8-18	Ozone generated from air Whole-body chamber Treatment groups: **Early-term** (gestation days 6-9): continuous exposure to **0 or 1.04 ppm** **Mid-term, experiment 1:** (gestation days 9-12): continuous exposure to **0, 1.0, 1.26 or 1.49 ppm** **Mid-term, experiment 2:** (gestation days 9-12): continuous exposure to **0, 0.64, 0.93 and 1.97 ppm** **Organogenesis** (gestation days 6-15): 8 h/d exposure to **0 or 0.44 ppm**	**Maternal:** LOAEL: **0.44 ppm (bw)** NOAEL: not determinable, because LOAEL set at lowest dose **Offspring:** LOAEL: **1.49 ppm**(bw, mid-term 1) **1.26 ppm** (resorption) **1.0 ppm** (skeletal, supraoccipital, mid-term 1) NOAEL: **1.0 ppm** (resorption) **1.0 ppm** (skeletal)	**Maternal** bw gain: reduced in early-term group, in all dose groups of mid-term group 1 (stat. sign. at mid and high dose) and in organogenesis group (stat. sign); increased in mid and high dose of midterm 2 group Food/water intake: Dose-related decreases in food/water intake in all gestational/dose groups (stat. sign. only in both midterm dose groups and – for food only – in organogenesis group) Implants: fewer implants in early-term group (stat. sign) and in high dose of mid-term 2 groups; more implants in all other groups **Offspring** **Body weight:** ~11 % higher fetal weight in treated early-term group compared to control (stat. sign.); dose-related decreased fetal weight in mid-term 1 group (stat. sign.); slightly lower fetal weights compared to control at all doses in mid-term 2 and organogenesis (~5-6.5 %) groups, but no stat. sign. and no dose-response **Resorption:** In both mid-term groups, statistically significant dose-related **increase** in percentage resorptions with a statistically significant difference between the control and the highest dose group (8.9±9.9 vs 50.4±42.9 and 11.1±9.2 vs 58.8±45.8). Statistically non-significant increase in percentage resorptions in the early-term and organogenesis groups (8.1±8.8 vs 18.5±24.9 and 7.3±10.8 vs 9.0±16.9). **Visceral anomalies:** enlarged renal pelvis in 5.8 % of foetuses in treated early-term group (none in control) and at low dose of mid-term group 1 (2.2 %	Several study conditions not in line with OECD GL 414 (maternal group size too small in some groups, exposure period too short, dose spacing too close, no dose without toxic effects). The group exposed on day 9-12 additionally received a subteratogenic dose of sodium salicylate on day 10 to test synergism. The results are not reported in this table. Statistical significance was determined by authors and could not be double-checked by the dossier submitter because raw data	Kavlock R. et al. (1979), Toxicol Appl Pharmacol. 48(1Pt 1):19-28 (Experiment 1 – effect on skeletal ossifications)

Method, Guideline, GLP status, Reliability	**Species, Strain, Sex, No/ group**	**Test substance Dose levels, Duration of exposure**	**NOAELs, LOAELs** *(also for maternal effects)*	**Results**	**Remarks** *(e.g. major deviations)*	**Reference**
				vs 6.1 % in control; none at mid and high dose); enlarged lateral ventricles at low and high dose in mid-term group 2 (1.1 % and 2.6 % vs 2.4 % in control); no v.a. in organogenesis group <u>Skeletal ossification and malformations:</u> Unchanged or slightly better scores for **supraoccipital ossification** (scores from 1-4 with 1 indicating fusion of centres and 4 indicating no ossification) in early-term and organogenesis groups. Mid-term group 1 shows a significant dose-related increase in poorly ossified supraoccipitals, however a different scoring system with unclear units was used. Mid-term group 2 showed unchanged or slightly better scores at low and mid doses, but a worse score at the high dose compared to control. Stat. sign. higher **average number of sternebrae** in early-term group; stat. sign. dose-related decrease in mid-term group 1 (~93 % lower at high dose); decreased at all doses in mid-term group 2 (44 % at low dose), but no dose-response; ~42 % lower in organogenesis group ~7 % increased number of **post-thoracic vertebrae centrums** in early-term group; dose-related decrease in mid-term group 1 (~10 % decreased at high dose compared to control); ~5 % decreased at low and high dose in mid-term group 2; ~2 % decrease in organogenesis group ~35 and 44 % higher %age foetuses with **ossified Meckel's cartilage and ossified pubis, respectively** in treated early-term group than in control, but with high variability. In treated organogenesis group, 11 and 9 % lower %age foetuses with ossified Meckel's cartilage and ossified pubis, respectively than in control, but also with high variability. In mid-term	were not available.	

Method, Guideline, GLP status, Reliability	**Species, Strain, Sex, No/ group**	**Test substance Dose levels, Duration of exposure**	**NOAELs, LOAELs** *(also for maternal effects)*	**Results**	**Remarks** *(e.g. major deviations)*	**Reference**
				group 2 (effect not measured in group 1), 32 and 17 % lower %age foetuses with ossified Meckel's cartilage and ossified pubis, respectively at high dose than at other doses (incl. control), but higher % than control at low and mid dose, no dose-response, and high variability. Fewer foetuses with **supernumerary ribs** in treated vs control in early-term group. In treated organogenesis group, no foetuses with s.r. In mid-term groups, up to 5.9 % of foetuses with s.r., but no dose-response and no foetuses with s.r. at high doses in both mid-term groups. **Rib malformations** only found at the low and mid dose of mid-term group 1 in 1-4.4 % of the foetuses, but none at similar doses in mid-term group 2. Also no rib malformations in early-term and organogenesis groups.		
Prenatal Developmental Toxicity Study Sim. to OECD 414 Reliability: 4	Long-Evans female pregnant rats Maternal group size: not reported Fetal group size: 8 litters	Ozone generated from air Whole-body chamber <u>Treatment groups:</u> -no exposure -exposure on gestational days 9-12 (midterm) to **1.04 ppm** -exposure on gestational days 17-20 (late gestation) to **1.19 ppm**		Decreased heart rate in foetuses on gestational day 20 (other days not tested) in highest dose group (HR in control, 1.04 and 1.19 ppm groups: 157, 159, 149 beats/min, respectively) No changes in P-Q, PRS, Q-T intervals in any group (acc. to authors).	- No information on statistical nature of heart rate values (mean, median…) and statistical significance was reported. - Values of the other ECG parameters were not reported. Only interpretation was reported.	Kavlock R. et al. (1979), Toxicol Appl Pharmacol. 48(1Pt 1):19-28 (Experiment 2 – effect on ECG parameters)

Method, Guideline, GLP status, Reliability	**Species, Strain, Sex, No/ group**	**Test substance Dose levels, Duration of exposure**	**NOAELs, LOAELs** *(also for maternal effects)*	**Results**	**Remarks** *(e.g. major deviations)*	**Reference**
Prenatal Developmental Toxicity Study Sim. to OECD 414 Reliability: 4	Long-Evans female pregnant rats Maternal group size: not reported Fetal group size: 8 litters	Ozone generated from air Whole-body chamber exposure on gestational days 17-20 to **0 or 1.0 ppm**		Plasma electrolytes were measured on gestational day 20. No effects on fetal weight, haematocrit, plasma sodium and potassium (acc. to authors).	Values were not reported, only interpretation	Kavlock R. et al. (1979), Toxicol Appl Pharmacol. 48(1Pt 1):19-28 (Experiment 3 – effect on plasma electrolytes)
Study on the effect of ozone on noradrenaline in offspring brain **Guideline:** None Reliability: 4	Mothers: Female Wistar rats Offspring: Groups of 10, 20 or 30 day old pups from exposed and non-exposed mothers 8 per group	Ozone **0 or 1.0 ppm** during 12-h darkness phase for first 20 days of gestation Exposure chamber		Mothers: no differences in body weight gain and litter size Offspring: -Decreased body weight, but no differences in brain weight -Decreased noradrenaline compared to control in cerebellum in all age groups, in cerebral cortex only in 10 day old pups, in pons only in 30 day old pups Authors' interpretation: No clear conclusion presented, but authors indicate in the introduction that noradrenaline plays a role in proliferation, cell maturity and neural cytoarchitectural configuration during the brain's gestational period and during neonatal period.	-Precursor of ozone not reported -Analytical dose level not reported, but dose level was monitored throughout exposure period	Custodio V. et al. (2010), Environ Toxicol Pharmacol. 30(1):92-4
Developmental Neurotoxicity Study **Guideline:**	Charles River CD-1 mice **Group size:** Maternal: n=10	Ozone generated from air Whole-body exposure Continuous exposure from 6 days prior to	**Maternal:** Not analysed **Offspring:** LOAEL: **0.6 ppm** (bw)	**General effects:** Stat. sign: retarded body weight gain in offspring Not affected: number of successful pregnancies, litter size, sex ratio, neonatal mortality	No information on maternal toxicity reported. Tests were also conducted with additional	Dell'Omo G. et al. (1995), Arch Toxicol. 69(9):608-16

Method, Guideline, GLP status, Reliability	**Species, Strain, Sex, No/ group**	**Test substance Dose levels, Duration of exposure**	**NOAELs, LOAELs** *(also for maternal effects)*	**Results**	**Remarks** *(e.g. major deviations)*	**Reference**
None Reliability: 2	<u>Offspring:</u> 8 litters per dose group, divided into two groups for four experiments: - <u>open-field tests</u> with scopolamine hydrobromide or saline injection (postnatal day 24: n=16m/16f). The saline group was later used for response to novel environment test with d-amphetamine injection (postnatal day 29) - <u>conditioned place preference tests</u> with d-amphetamine sulphate or saline injection (postnatal days 28-31: n=16m/16f). The saline group was later used for passive avoidance acquisition and retention tests (postnatal day 59	formation of breeding pairs to weaning (postnatal day 22 or 26) to **0 or 0.6 ppm**	<u>NOAEL:</u> **0.6 ppm** (behaviour)	**Behavioural effects:** <u>Open-field tests (half of the group additionally injected with scopolamine hydrobromide):</u> Apart from the elimination of sex differences, no major effects on crossing, rearing, jumping, sniffing, grooming, freezing (data not reported) and response to stimulus object (latency to first approach, number of contacts – data not reported) were observed in ozone-exposed and ozone/scopolamine-exposed offspring. <u>Conditioned place preference tests (half of the group injected with d-amphetamine):</u> Mice were acclimated to the test apparatus consisting of a middle, a white-surface and a black-surface compartment by being allowed to freely explore it. Then they were pre-conditioned by being confined first in a white-surface compartment (half under amphetamine exposure) and then in a black-surface compartment (no amphetamine exposure). On the second day, they were allowed free access to all compartments of the apparatus after being placed in the middle compartment. Ozone-exposed offspring <u>not</u> previously exposed to amphetamine spent less time in the white and black compartments and more time in the middle compartment (not stat. sign.). On the other hand, ozone-exposed offspring previously exposed to amphetamine spent more time in the white and black compartments and less time in the middle (stat. sign). The interpretation of this test regarding developmental effects is not clear. <u>Response to novel environment:</u>	exposure to scopolamine hydrobromide and d-amphetamine. These results are not reported here.	

Method, Guideline, GLP status, Reliability	**Species, Strain, Sex, No/ group**	**Test substance Dose levels, Duration of exposure**	**NOAELs, LOAELs** *(also for maternal effects)*	**Results**	**Remarks** *(e.g. major deviations)*	**Reference**
	or 60)			Reduced grooming duration in ozone-exposed mice Other effects measured (rearing, sniffing, face-washing, gnawing) were not affected by ozone exposure Passive avoidance acquisition and retention: Transient retardation of passive avoidance acquisition		
Developmental Neurotoxicity Study **Guideline:** None Reliability: 4	rats dams: group size not reported offspring: n=6 per dose group	ozone (from Triozon P15 generator) exposed for 12 h/d to **1 ppm** throughout gestation control group exposed to air sleep recordings performed at postnatal days 30, 60 and 90 for 24 h each		**offspring:** body weight: decreased at birth and during the 90-d observation period after birth (data not reported) physical development: abnormal incisor growth in 2 of 6 animals (data not reported) sleep: inversion of the sleep-wake pattern as indicated by the following observations: *during light hours:* increased time spent in wakefulness (stat. sign) and decreased time spent in slow wave sleep (not stat. sign.) and paradoxical sleep (stat. sign.) on all test days *during dark hours:* decreased time spent in wakefulness and paradoxical sleep and increased time spent in slow wave sleep (all stat. sign.) on all test days not affected (data not reported): litter size	maternal group size not reported effects on dams not reported small group size	Haro R. and Paz C. (1993), Neuroscience Letters, 164:67-70
Developmental Toxicity Study **Guideline:** None **GLP:** No	**Species** rat **Strain** Long-Evans rats (Blue Spruce Farms, Altamont, N.Y.) **Sex** female **No/group** Number of litters:	Ozone Control: ozone generator turned off Ozone generation from air by UV irradiation in 0400M	**Maternal:** Not analysed **Offspring:** NOAEL: not determinable because	Dose related **growth** retardation of offspring: PND6 for I.+II.; both sexes I. (mid gestation): female weight reduction: 1.0:6 %, 1.5:8 %; male weight reduction: 1.0:6 %, 1.5:9 % II. (late gestation) female weight reduction: 1.0:12 %, 1.5:20 %; male weight reduction: 1.0:11 %, 1.5:19 % PND15 for II. (late gestation): female weight	No guideline study Study purpose: evaluate potential of ozone to produce effects in postnatal life following prenatal	Kavlock R.J. et al. (1980), Toxicol Lett. 5(1):3-9

Method, Guideline, GLP status, Reliability	**Species, Strain, Sex, No/ group**	**Test substance Dose levels, Duration of exposure**	**NOAELs, LOAELs** *(also for maternal effects)*	**Results**	**Remarks** *(e.g. major deviations)*	**Reference**
Reliability: 2	Control: 15 I. 1.0 ppm: 6 I. 1.5 ppm: 4 II. 1.0 ppm: 6 II. 1.5 ppm: 6 Litters reduced to 8 pups	ozone test chamber (Ozone Research and Equipment Corp., Phoenix, AZ) I. GD9-12 (mid gestation exposure) Continuously **1.0, 1.5 ppm** II. GD17-20 (late gestation) Continuously **1.0, 1.5 ppm** Weight observation: PND6,15,60 Behaviour: PND9-16	LOAEL set at lowest dose LOAEL: **1.0 ppm** (retardation of weight gain during late gestation) **1.5 ppm** (behaviour: reflexes)	reduction: 1.0:7 %, 1.5:12 %; male weight reduction: 1.0: 8 %, 1.5:12 % PND60 for II. (late gestation): male weight reduction: 1.0:8 %, 1.5:10 % II. (late gestation):14.3 % of male offspring at 1.5 ppm (3 males from 3 different litters) were permanently stunted (1 died at PND50, necropsy of stunted males on PND60, according to author: no obvious differences in size and appearance of major organs) Behavioural testing: I. (mid gestation): no significant effects on the appearance of early reflexes and activity II. (late gestation): 1.0, 1.5 ppm: dose related retardation of **early reflexes** (righting (1.5ppm: +1day), eye opening (1.5ppm: +1day), horizontal movement in open field test (1.5ppm: +0.5day)) Open field tests: I. (mid gestation): no significant effects on grooming, rearing II. (late gestation): delay in grooming and rearing behaviours; dose related decrease in grooming and rearing responses at all time points grooming (II.): day1 of testing in 1.5 ppm dose group 56 % less positive response, day4 of testing in 1.5 ppm dose group still 30 % less positive response rearing (II.): day1 of testing in 1.5 ppm dose group 77 % less positive response, day4 of testing in 1.5 ppm dose group still 18 % less positive response activity in open field unaffected	exposure No information about No/group exposed (42 litters in total, but only 37 litters listed) No simultaneous examination of more than 1 treatment group →randomised sequence of exposures	
Developmental Toxicity	**Species** rat **Strain** Wistar	Ozone	Offspring:	I. GD18 (glandular phase of rat lung development): swollen mitochondria, cytoplasmic vacuolisation in	No guideline study	López I. et al. (2008), J Electron

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Study **Guideline:** None **GLP:** No **Reliability:** 2	(Blue Spruce Farms, Altamont, N.Y.) **Sex** female **No/group** 6 animals/group (3 foetuses/group analysed for lung effects)	Filtered air control P15 Triozone generator Exposure: **1 ppm** ozone, 12 h/d I. GD0-GD18, II. GD0-GD20, III. GD0-GD21 GD18, 20, 21(=time points)	LOAEL: **1 ppm** (lung development)	bronchiolar epithelium cells and structural disarrangement →oxidative damage →cellular permeability II. GD20 (canalicular phase of lung development): increased amounts of glycogen in secretory cells, flake-off epithelial cells →epithelial damage of membrane, delayed maturation III. GD21 (sacular phase): swollen mitochondria deprived of cristae, granules in non-ciliated bronchiolar cells →alterations during rat fetal lung development (damage in fetal bronchiolar epithelium), rupture of membrane proteins and lipids ozone generates radicals which cross the hematoplacentaria barrier, distributed to fetal organs	Study purpose: identify alterations caused in bronchioles during intrauterine lung development in ozone exposed pregnant rats	Microsc (Tokyo). 57(1):19-23
Developmental Toxicity Study **Guideline:** None **GLP:** No **Reliability:** 2	**Species** mice **Strain** CD-1 (Charles River, Calco, Italy) **Sex** male and female **No/group** each 10/ group Litters reduced to 7 and culled to 8 pups (4 male and 4 female)	Ozone Not exposed control group Electric arc discharge ozone generator (ozonator) **0.3, 0.6, 0.9** ppm Exposure: 6 days before formation of breeding pairs until	**Maternal:** Not analysed (no sufficient data) **Offspring:** NOAEL: **0.6 ppm** (bw) LOAEL: **0.9 ppm** (bw)	No effect on pregnancies, litter size, sex, neonatal mortality (data not shown) 0: 8/10, 0.3: 10/10, 0.6: 10/10, 0.9: 7/10 pregnancies Retardation of postnatal **body weight** gain PND2-40, PND100 significant reduction of body weight gain for 0.9 ppm group (specific values not shown) Paw preference test PND70: 0.6 ppm: sex-dependent paw preferences (male: 30.33 ± 2.25; female: 19.33 ± 2.44 right paw entries) right paw: males, left paw: females Hot plate response test PND100 (injection of morphine or a saline):	No guideline study Study purpose: behavioural changes upon pre- and postnatal exposure to ozone ozone concentration deviated less than 15 % from stated value	Petruzzi S. et al. (1999), Acta Neurobiol Exp 59(2):115-22

Method, Guideline, GLP status, Reliability	**Species, Strain, Sex, No/ group**	**Test substance Dose levels, Duration of exposure**	**NOAELs, LOAELs** *(also for maternal effects)*	**Results**	**Remarks** *(e.g. major deviations)*	**Reference**
	On PND21 reduction to 3 pups/litter	PND26 (males and females exposed) Continuous		Reduced drug sensitivity (morphine) after 0.9 ppm ozone exposure: shorter latency + higher frequency in hind limp withdrawal and shorter latency + higher frequency (limited to males) of wall rearing of morphine injected mice compared to saline control		
Developmental Toxicity Study **Guideline:** None **GLP:** No **Reliability:** 2	**Species** mice **Strain** CD-1 (Charles River, Calco, Italy) **Sex** male and female **No/group** each 16/ exposure group Each 20/ control group Litters reduced to 4 males and 4 females and fostered to untreated dams Functional tests, Fox battery: 2 m + 2 f Social interaction: 4 m + 4 f Locomotor activity: 12 m/ treatment Maze:	Ozone generated from air Control: clean air Exposure (whole-body chamber) Electric arc discharge ozone generator (ozonator) **0.2, 0.4, 0.6** ppm Exposure: 6 days before formation of breeding pairs (7-10 days before start of gestation) until the morning of pregnancy day 17	**Maternal** NOAEL: **0.6 ppm** (bw and pregnancies) LOAEL: not determinable, because NOAEL was set at highest dose **Offspring:** NOAEL: **0.6 ppm** (bw, somatic and neurobehavioural development) LOEL: **0.6 ppm** (social interaction(grooming, exploring))	**Maternal** **body weight**: initially lower than control in mid and high dose groups, but by the end of exposure higher in low and mid dose groups and same as control in high dose group; throughout exposure low dose group had higher bw than mid and high groups food intake: overall increase throughout exposure; lower throughout most of exposure period (especially before gestation) in mid and high dose groups, but no real pattern could be observed because there was no consistent development in any of the groups water intake: overall increase throughout exposure; initially (pre-pregnancy) lower than control in all dose groups, then no real pattern because there was no consistent development in any of the groups No effect on successful pregnancies: 0: 14/20, 0.2: 16/16, 0.4: 14/16, 0.6: 13/16 pregnancies **Offspring** No effect on body weight gain of offspring but significantly higher weight of males than females (values not shown) No effect on litter size, sex ratio, neonatal mortality	No guideline study Study purpose: effects of pregestational and gestational ozone exposure on development ozone concentration deviated less than 10 % from stated value	Petruzzi S. et al. (1995), Neurotoxicol Teratol. 17(4):463-70

Method, Guideline, GLP status, Reliability	**Species, Strain, Sex, No/ group**	**Test substance Dose levels, Duration of exposure**	**NOAELs, LOAELs** *(also for maternal effects)*	**Results**	**Remarks** *(e.g. major deviations)*	**Reference**
	8 m/treatment			No effect on somatic and neurobehavioral development (data not shown) (as measured by modified Fox battery: body and tail length, day of eyelid and ear opening and of incisor eruption, righting reflex, cliff aversion, forelimb/hind limb placing and stick grasp reflexes, vibrissae placing reflex, level and vertical screen tests, screen climbing test, pole grasping, auditory startle response, tactile stimulation) Social interaction: sniffing of other mice: 0.2 - 0.6 ppm O_3 increased at PND23-25 (70 % in 0.6 ppm dose group) and PND43-45 (22 % in 0.2 ppm dose group) mutual circle response: 0.2 ppm elevated at PND23-25 (90 %) and PND43-45 (>100 %) digging: more frequent in males (data not shown); increased in 0.2 ppm dose group (PND23-25 (39 %) and PND43-45 (13 %)) and increased in 0.4 ppm dose group PND23-25 (20 %), but decreased in all other groups follow, squire, mutual circle: more frequent in females (data not shown) **exploring**: increased exploring frequency in PND23-25 at all doses, but not in PND43-45; decreased exploring duration in both age groups at all doses (stat. sign. at high dose) **self-grooming:** stat. sign. increased self-grooming frequency in PND23-25 (0.4 and 0.6 ppm dose group), but not in older age group; increased self-grooming duration only in younger age group at high dose; increased jumping in young age group at all doses, but only at high dose in older age group (low and mid dose decreased)		

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				Locomotor activity: no significant effects Eight-arm radial maze learning: reduction of rewarded trials in training phase (0.2 ppm significantly different), which increased to above control levels in subsequent phases, increased total time for first visit of all maze arms in high dose, but decreased in low and mid dose-groups		
Developmental Toxicity Study **Guideline:** None **GLP:** No **Reliability:** 2	**Species** rat **Strain** Wistar **Sex** female **No/group** 4 pregnant females/group Litters culled to 8 pups (4m+4f) Morphological analysis: 8 male born rats/group	Ozone Pollution-free control P15 Triozone generator **1 ppm** ozone for 12 h/d Exposure: during entire gestation (GD0 until PND0) Time point: PND90	**Maternal:** Not analysed **Offspring:** LOAEL: **1.0 ppm** (morphologic)	Abnormal structures in molecular layer of cerebellum of rats born to exposed dams Decrease of total area and number of Purkinje cells 0: 10.6±0.3 mm²; 1 ppm: 4.8±0.3 mm² 0: 832±31 cells; 1 ppm: 712±34 cells →Depopulation of Purkinje cells and also degenerating Purkinje cells and cell debris Circular bodies in molecular layer Incomplete folding pattern of some lobes	No guideline study Study purpose: morphology of the cerebellum of rats with prenatal exposure to ozone	Romero-Velázquez R.M. et al. (2002), Proc West Pharmacol Soc. 45:65-7
Developmental Toxicity Study **Guideline:** None **GLP:** No **Reliability:** 2	**Species** mice **Strain** CD-1 (Charles River, Calco, Italy) **Sex** females **No/group** 8/group Litters reduced to 4 males and 4 females and	Ozone Pollution-free control (dilution with clean air) P15 Triozone generator **0.3, 0.6 ppm** ozone	**Maternal:** Not analysed **Offspring:** LOEL: **0.3 ppm** (social interaction: nose sniff and	Aggressive behaviour test (>PND130): 0.3 and 0.6 ppm: significantly higher duration of **freezing** (day1 and day 3: circa 2-fold increased freezing), increased tail rattling and decreased submissive upright posture Non-agonistic behaviour: Reduction of sniffing: body sniff, anogenital, **nose sniff** showed dose related decrease (0: 39.1±6.3; 0.3: 23.5±6.0; 0.6: 18.4±3.1)	No guideline study Study purpose: effect of ozone exposure on aggressive behaviour and changes in CNS levels of neurotrophins NGF and BDNF	Santucci D. et al. (2006), Behav Brain Res. 166(1):124-30

Method, Guideline, GLP status, Reliability	**Species, Strain, Sex, No/ group**	**Test substance Dose levels, Duration of exposure**	**NOAELs, LOAELs** *(also for maternal effects)*	**Results**	**Remarks** *(e.g. major deviations)*	**Reference**
	fostered to untreated dams Behaviour test: 2 males of each litter (N=6) NGF/BDNF: 6 males/group	continuous exposure Exposure: 30 days before breeding pairs until GD17 Whole body	freezing)	Allogroom: increased at 0.3 ppm (21 %), reduced at 0.6 ppm (64 %) Push under: increased at 0.6 ppm (34 %) Social resting: increased at 0.6 ppm (70 %) →Impairment in investigative profile <u>NGF (nerve growth factor) and BDNF (brain derived neurotrophic factor) level:</u> Significant decrease of NGF level in hippocampus (0.3: 16 %; 0.6: 20 %) and increase of BDNF in striatum (0.3 and 0.6: 2.5-fold) vice versa not affected (→Functional significance of these changes not known)	ozone concentration deviated less than 15 % from stated value	
Developmental Toxicity Study **Guideline:** None **GLP:** No **Reliability:** 2	**Species** mice **Strain** Balb/c (Charles River Laboratories, Raleigh, NC) **Sex** female **No/group** 20 pregnants/group exposed (Experiment was performed 3 times) Analysis of offspring: BALF: 3-6/sex LDH/prot.: 3-7/sex DTH: 6-8/sex Sensitised offspring: BALF: 3-6/sex	Ozone HEPA-filtered room air as control Ozone generation from O_2 by a silent arc discharge generator (OREC, Phoenix, AZ) **0.4, 0.8, 1.2** ppm ozone for 4h/d at GD9-GD18 Whole body inhalation	**Maternal** <u>NOAEL:</u> **0.8 ppm** (pregnancies) <u>LOAEL:</u> **1.2 ppm** (pregnancies) **Offspring:** <u>NOAEL:</u> **0.8 ppm** (bw) <u>LOAEL:</u> **1.2 ppm** (bw)	0.4-1.2 ppm: decreased percentage of viable <u>pregnancies</u> 0: 58 %; 0.4: 45 %; 0.8: 45 %; 1.2: 33 % successful pregnancies (1.2 ppm significant at this concentration: 25 % less productive dams compared to control) No effect on litter size and sex ratio 1.2 ppm: reduced <u>weight gain in offspring</u> PND1: 13 %; PND3: 22 %; PND7: 15 % and in male still at PND42: 9 % lower weight <u>Inflammation:</u> No differences in number of Macrophages, lymphocytes, neutrophils, eosinophils and immunomodulatory cytokines (IL-4, IFN-γ, IL-17) in BALF at PND42 (no data for immune cells) No differences in percentage of splenic $CD4^+$, $CD8^+$, $CD25^+$ and $TCR\beta^+CD1d^+$ T-cells 1.2 ppm: increased LDH activity in BALF at PND42 in female offspring and same trend for protein	No guideline study Study purpose: Effect of maternal ozone exposure on immune responses in offspring Pregnant mice were purchased and delivered to facility on GD3	Sharkhuu T. et al. (2011), J Immunotoxicol. 8(2):183-94

Method, Guideline, GLP status, Reliability	**Species, Strain, Sex, No/ group**	**Test substance Dose levels, Duration of exposure**	**NOAELs, LOAELs** *(also for maternal effects)*	**Results**	**Remarks** *(e.g. major deviations)*	**Reference**
				→lung injury, normal lung development altered Delayed-type hypersensitivity (DTH) responses supressed in females at 0.8 and 1.2 ppm No effect on specific IgM and IgG titer in sheep red blood cell-specific antibody response testing In <u>OVA-sensitised female offspring</u> early sensitisation <PND3: at 1.2 ppm decrease in total cells (~47 %) in BALF (macrophages (~42 %), eosinophils (~95 %), lymphocytes (~82 %)) of females OVA-specific IgE antibodies decreased in both sexes late sensitisation >PND42: 0.8, 1.2 ppm: reduction of neutrophils (~65 %) OVA-specific IgE antibodies decreases no differences in pulmonary responsiveness to methacholinein after ozone exposure		
Developmental Neurotoxicity Study **Guideline:** None **GLP:** No **Reliability:** 2	**Species** mice **Strain** CD-1 (Charles River, Calco, Italy) **Sex** female **No/group** total: 30 females exposed, 15 males non-exposed (2f +1m per box) 10 females/group Litters culled at birth to 8 pups	Ozone Non-exposed control group Ozone generation with an electric arc discharge ozone generator (ozonator) **0.3, 0.6** ppm ozone Exposure: 30 days before formation of breeding pairs until GD17	**Maternal** <u>NOAEL:</u> **0.3 ppm** (pregnancies) <u>LOAEL:</u> **0.6 ppm** (pregnancies) **Offspring:** <u>NOAEL:</u> **0.6 ppm** <u>LOAEL:</u> **≥0.6 ppm**	Exposed dams: no differences in placental scars 0.6 ppm: reduction of successful <u>pregnancies</u> 0 and 0.3: 9/10; 0.6: 6/10 (not significant) No effect on <u>body weight</u> of pups (no data given) <u>Somatic and neurobehavioral development PND2-20:</u> Only <u>forelimb stick grasp reflex</u> affected (0.3 ppm: slight delay (values not shown)) of all tests in modified Fox battery retardation in <u>homing</u> PND12(0.3 ppm) (0: 69.0±3.1; 0.3: 104.7±3.2; 0.6: 65.4±5.8) <u>Passive avoidance test PND22-23:</u> initial phase of learning increased step-through latency (0.3 ppm) <u>Locomotor activity PND21:</u>	No guideline study Study purpose: Effect of maternal ozone exposure on neurobehavioral development of the offspring somatic and neurobehavioral development: 2m+2f per each litter Locomotor	Sorace A. et al. (2001), Environ Res. 85(2):122-34 Experiment 2: Prenatal Exposure to ozone

Method, Guideline, GLP status, Reliability	**Species, Strain, Sex, No/ group**	**Test substance Dose levels, Duration of exposure**	**NOAELs, LOAELs** *(also for maternal effects)*	**Results**	**Remarks** *(e.g. major deviations)*	**Reference**
	(4m+4f) and fostered to untreated dams on PND2			decrease (0.3 ppm) 0: 43.9±0.9; 0.3: 38.1±1.4; 0.6: 41.6±1.9 <u>Water maze test PND70-74:</u> decreased speed, longer swimming path and latency at platform reversal (0.3 ppm) <u>Hot plate test PND100:</u> lower frequency in wall rearing (0.3 and 0.6 ppm) 0: 13.2±1.7; 0.3: 6.3±1.6; 0.6: 10.1±1.3 higher latency in wall rearing (0.3 and 0.6 ppm) 0: 10.7±1.7; 0.3: 22.0±3.7; 0.6: 15.7±2.2 →no concentration dependent effects, only effects in 0.3 ppm group	activity: 2m+2f Passive avoidance: 1m+1f Water Maze: 1m Hot-plate: 2m	
Developmental Neurotoxicity Study **Guideline:** None **GLP:** No **Reliability:** 4	**Species** mice **Strain** CD-1 (Charles River, Calco, Italy) **Sex** male **No/group** total: 20 males exposed	Ozone Non-exposed control group Ozone generation with an electric arc discharge ozone generator (ozonator) **0.3, 0.6 ppm** ozone Exposure: 30 days Time points: Open field test: day4, 19 and 3 day after exposure Water maze test: day 24-28		Crossing and sniffing increased in open field test (0.6 ppm, day4) Water maze: Increased swimming sinuosity (0.3 ppm, day3) longer latency in reversal phase and swimming path length (0.3 ppm)		Sorace A. et al. (2001), Environ Res. 85(2):122-34 Experiment 1: Prolonged Exposure to ozone in adult males

Method, Guideline, GLP status, Reliability	**Species, Strain, Sex, No/ group**	**Test substance Dose levels, Duration of exposure**	**NOAELs, LOAELs** *(also for maternal effects)*	**Results**	**Remarks** *(e.g. major deviations)*	**Reference**
Developmental Neurotoxicity Study **Guideline:** None **GLP:**No **Reliability:** 4	**Species** Rat **Strain** Wistar **Sex** female (in estrus) **No/group 3 total: 6** 6 females, 3 One group exposed to O3 One free pollution air group	Ozone 1.0 ppm for 12 h/day (20:00±08:00 h) P15 Triozon generatorTime points: birth (P-0), immediately after prenatal O3 Exposure - 12 days of age (growth of the dendritic arborization of Purkinje cells) - 60 days of age (adulthood)	NOAEL: **0 ppm** LOAEL: **1 ppm**	Study of morphological aspects of the anterior cerebellar lobe of rats exposed to O3 during the gestation period. Analyses of sagittal sections of the anterior cerebellar lobe at postnatal days 0, 12 and 60: - cerebellar necrotic signs at age 0, - diminished area of the molecular layer with Purkinje cells with pale nucleoli and perinucleolar bodies at age 12 Purkinje cells showing nuclei with unusual clumps of chromatin in the periphery at age 60 Conclusion: 1 ppm ozone during gestation induces permanent cerebellar damage in rats Result: Adverse Effect on CNS development	- Number of animals/group too low for statistical analyses. However, ANOVA, P , 0:02) and Tukey (P , 0:05) test performed - Only one dose tested - double blind histological and planimetric analysis	Rivas-Manzano, P.R. et al. 1999 Neurosci. Lett. 276, 1: 37-40.
Developmental Neurotoxicity Study **Guideline:** None **GLP:** No **Reliability:** 4	Species Rats Strain: Sprague-Dawley Sex: female (pregnant) No/group: 4 total: 8 One group exposedto O3 One free pollution air group	Ozone 0.5 ppm 12 h/day from embryonic day E5 to E20) Ozone generator (UV-light) Duration from embryonic day E5 to E20	NOAEL: **0 ppm** LOAEL: **0.5 ppm**	Prenatal O3 increased baseline TH gray level per cell ($p < 0.001$). Number of Fos-IR cells, Fos-IR/TH-IR colabeled cells and proportion of TH double-labeled with Fos unchanged. After stress, the TH gray level ($p < 0.001$), number of Fos-IR cells ($p < 0.001$) and of colabeled Fos-IR/TH-IR cells ($p < 0.05$) and percentage of colabeled Fos-IR/TH-IR neurons against TH-IR cells ($p < 0.05$) increased in the control group. In prenatal-O3 rats, immobilization stress abolished these increases and reduced the TH gray level ($p < 0.05$), indicating that prenatal O_3 led to loss of adult NTS reactivity to stress.	- Number of animals/group too low for statistical analyses. However, two-way ANOVA and Newman–Keuls correction or tailed t-test ($p< 0.05$). - Only one dose tested	Boussouar, A. et al. 2009 Neurosci. Lett. 452:75-78.

Method, Guideline, GLP status, Reliability	**Species, Strain, Sex, No/ group**	**Test substance Dose levels, Duration of exposure**	**NOAELs, LOAELs** *(also for maternal effects)*	**Results**	**Remarks** *(e.g. major deviations)*	**Reference**
				Method: immunolabeling by confocal microscopy, adult offspring. Evaluation of adult nucleus tractus solitarius (NTS) regulating respiratory control. Fos protein immunolabeling (Fos-IR) Conclusion: long-lasting sequelae detected in the offspring beyond the prenatal O3 exposure. Prenatal O3 left a print on the NTS, revealed by stress		

10.10.5 Short summary and overall relevance of the provided information on adverse effects on development

(1) Effects on dams:

Custodio (2010) found no differences in body weight gain after exposure of rat dams 1.0 ppm during the first 20 days of gestation. Kavlock (1979) reported reduced body weight gain in rats exposed during different gestational stages to different doses of ozone (starting at 0.44 ppm). However, the effect was not consistent throughout all groups and did not reach statistical significance in all groups. This effect was somewhat consistent with a dose-related decrease in food and water consumption in all gestational and all dose groups, which was statistically significant in most exposure groups. Bignami (1994) saw dose-related reduced body weight gain in mice dams exposed to 0.4, 0.8 and 1.2 ppm, but the effect was not statistically significant. Statistically significant decreased body weight was observed only on pregnancy day 10 starting from 0.8 ppm. In mice dams exposed to 0.2, 0.4 and 0.6 ppm from 6 days prior to breeding pair formation until pregnancy day 17, Petruzzi (1995) saw similar body weight gain development in all dose groups with the mid and high dose groups showing lower body weight compared to control and the low dose group throughout exposure. Body weights were initially lower than in the control group, but by the end of exposure higher in the low and mid dose group compared to control. Regarding food and water consumption there was an effect only at the initial exposure period correlating with the body weight gain of dams until the start of the pairing for breeding. Although consumption was lower than control prior to gestation, no real pattern could be observed afterwards, because although consumption increased throughout exposure in all groups, there was no consistent development in any of the groups.

(2) Effects on implants, number of litters, litter size, stillbirths, neonatal mortality and offspring body weight:

Brinkman (1967) observed a reduced number of litters and a large increase in neonatal mortality in two strains of mice after pre-natal exposure to 0.1 and 0.2 ppm ozone. However, group size of exposed dams and toxicity of dams was not reported in the study.

Kavlock (1979) saw an increased number of resorptions in all groups of rat dams exposed at different gestational stages to different doses (0.44-1.97 ppm depending on gestational stage), with more than 50 % resorptions in the high dose groups of two sets of dams exposed during gestational days 9-12. Sorace (2001) reported a reduction of successful pregnancies of CD-1 mice in the highest dose group (0.6 ppm). Even if the reduced number of pregnancies reached no statistical significance, due to the small group size, this observation should be considered. Moreover the study of Sharkhuu (2011) showed a decrease in the percentage of delivered pregnancies in all exposure groups from 0.4 to 1.2 ppm ozone in mice exposed during gestational days 9-18 for 4 h/day. In the highest dose group the exposure significantly led to 25 % less productive dams.

Bignami (1994) and Dell'Omo (1995) found litter size and neonatal mortality in mice not affected after exposure of dams up to 1.2 ppm during gestational days 7-17 (Bignami) and 0.6 ppm during pregnancy and lactation (Dell'Omo). Haro and Paz (1993) also saw no effects on litter size in an unreported number of rats exposed to 1 ppm throughout gestation. Other parameters such as proportion of successful pregnancies, sex ratio (Bignami and Dell'Omo) and frequency of stillbirths (Bignami) were also not different from control animals. Custodio (2010) also did not observe changes in litter size in rats after exposure of dams to 1.0 ppm during the first 20 days of gestation. Neither Petruzzi (1995) showed any effect on successful pregnancies, litter size, sex ratio and neonatal mortality after the exposure of female mice 6 days prior the formation of breeding pairs until gestational day 17, nor Petruzzi (1999) after a prolonged exposure period until postnatal day 26.

The U.S. EPA found no association between prenatal ozone exposure and infant mortality in their review of epidemiological studies. Similarly, they found no effect on stillbirths in one epidemiological study. They further concluded that, based on epidemiological studies, preterm birth was not affected after short-term ozone exposure during late pregnancy, but that the evidence is inconsistent regarding long-term exposure in early pregnancy.

Bignami (1994) did find a reduction in body weight gain in offspring of the mouse dams exposed to 0.8 and 1.2 ppm ozone. This effect is supported by findings by Dell'Omo (1995), where exposure of mouse dams to 0.6 ppm ozone during pregnancy and lactation led to a reduction in body weight gain in offspring. Kavlock

(1979) reported a dose-related decreased body weight in foetuses from dams exposed during gestational days 9-12 to doses ranging from 1.0-1.49 ppm. Decreased fetal weights were also seen in a second group exposed during gestational days 9-12 (0.64-1.97 ppm) and in the group exposed during organogenesis, but without stat. sign and without dose-response. In the group exposed during early gestation (days 6-9), foetuses showed increased body weight. The dams in this study showed reduced body weight gain (although not consistent over all groups) and dose-related decreases in food/water intake. Kavlock (1980) reported a dose related postnatal growth retardation of rat offspring when dams exposed in midterm or late gestation to 1.0 ppm ozone. In fact in this study, exposure to 1.5 ppm ozone during late gestation showed, that 14.3 % of male offspring were permanently stunted. These results are further supported by Haro and Paz (1993), who observed reduced body weight from birth to PND 90 in rat offspring exposed prenatally to 1 ppm ozone. This study had, however, several limitations (see table). Sharkhuu (2011) also demonstrated reduced offspring body weight gain in the first postnatal week for both sex and also persisting reduced body weight in males until postnatal day 42 after exposure to 1.2 ppm ozone. Offspring body weight gain is reduced on postnatal days 19 until 100 after exposure to 0.9 ppm ozone, as also written by Petruzzi (1999). Petruzzi (1995), on the other hand, reported no effect on birth weight and postnatal body weight gain in offspring of mice exposed to 0.2, 0.4 and 0.6 ppm from 6 days prior to breeding pair formation until pregnancy day 17 (data not reported).

The U.S. EPA found inconsistent evidence for an effect of ozone exposure on fetal growth and birth weight in their review of epidemiological studies. Only one epidemiological study (Salam et al., 2005) provided strong evidence for reduced birth weight after prenatal ozone exposure, while other studies provide weak or inconsistent evidence. Some of the toxicological studies reviewed in this report were also reviewed by the U.S. EPA with the same conclusion (Sharkhuu, 2011: reduced birth weight in highest dose group, decreased postnatal growth); Bignami, 1994: decreased body weight gain; Haro and Paz, 1993: decreased birth weight and postnatal body weight gain; Kavlock, 1980: reduced body weight gain). Overall, the U.S. EPA concludes that the data concerning the effect of ozone on fetal growth, birth weight and postnatal growth is inconsistent.

(3) Effects on ossification and other physical development parameters in offspring:

Ear opening, incisor eruption, hair growth and body/tail length were not affected in offspring of mice exposed to ozone at 0.4, 0.8 and 1.2 ppm during pregnancy days 7-17 (Bignami, 1994) and at 0.2, 0.4 and 0.6 ppm from 6 days prior to breeding pair formation until pregnancy day 17 (Petruzzi, 1995; hair growth not measured). Eyelid opening was delayed by two days in all dose groups of the Bignami (1994) study (reaching stat. sign. only in the low dose group), but was not affected in the Petruzzi (1995) study. Ear opening, incisor eruption, hair growth and body/tail length were not affected in offspring of mice exposed to ozone at 0.4, 0.8 and 1.2 ppm during pregnancy days 7-17 (Bignami, 1994). However, eyelid opening was delayed by two days in all dose groups, reaching stat. sign. only in the low dose group. Brinkman (1964, as reported by Veninga, 1967) observed a 2-fold and 16-fold increased frequency of blepharophimosis in offspring of c57 black mice and inbred grey mice dams exposed to 0.2 ppm. In c57 black mice, the frequency of unlimited growth of the incisors was also increased 6-fold in this dose group. While these results appear substantial, however it should be noted that this study was poorly reported and, in particular, no information on maternal toxicity was provided. Findings regarding the number of litters (greatly reduced in both strains by ~40 and ~44 %) and neonatal mortality (greatly increased in both strains by 260-470 %) suggest that there may have been significant maternal toxicity that may have led to the described malformations. Haro and Paz (1993) also observed abnormal incisor growth in ~33 % of offspring. However, the study was not designed to examine effects on physical development, but on sleep patterns. In addition, the total offspring group size was only 6 and possible toxic effects on dams were not reported, although litter size was reported to be normal.

López (2008) found alterations caused in bronchioles during intrauterine lung development in ozone exposed pregnant rats to 1 ppm, focussing on the glandular, canalicular and sacular phase of rat lung development. Swollen mitochondria, cytoplasmic vacuolisation, structural disarrangement and flake-off epithelial cells were identified as indicators of delayed maturation and further alterations during rat lung development. In addition Romero-Velázquez (2002) observed abnormal structures in the molecular layer of cerebellum in the offspring of rat dams exposed to 1 ppm ozone during entire gestation. The study did find altered morphology of pup cerebellum, confirmed with a decrease of total area and number of Purkinje cells, because of depopulation of and degenerating Purkinje cells in the cerebellum, accompanied by incomplete folding pattern of some lobes, caused by ozone. Kavlock (1979) reported no notable effects regarding visceral anomalies, supernumerary ribs and rib malformations. However, supraoccipitals were poorly ossified in the first group of foetuses exposed

during gestational days 9-12 (1-1.49 ppm) in a dose-related manner. Resorptions were also increasing in this group. In a second group exposed during the same gestational period, but to different doses (0.64-1.97 ppm), a change in supraoccipital ossification (poorer compared to control) was only seen at the high dose where resorption was also above 50 %. Foetuses exposed during early gestation (days 6-9) and organogenesis had slightly more advanced or unchanged supraoccipital ossification compared to control. The number of sternebrae was stat. sign. higher in foetuses exposed during early gestation (1.04 ppm) compared to control. One of the groups exposed during mid-gestation showed a dose-related decrease in the number of sternebrae (~93 % lower at high dose compared to control), while the effect was not quite as pronounced (no dose-response), but also present in the second mid-term group (44 % lower at low dose compared to control) and in the group exposed during organogenesis (~42 % lower). Similar effects were observed regarding the number of post-thoracic vertebrae centrums, but the differences to control were not as large. A higher percentage of foetuses had ossified pubis and Meckel's cartilage in the early-term exposure group (~35 and 44 % compared to control) and in the mid-term group at low and mid doses, while this percentage was lower in the organogenesis group (~11 and 9 % compared to control) and in the mid-term group at the high dose (32 and 17 % compared to control). Variability was quite high in all groups.

The U.S. EPA did not include skeletal ossification endpoints in its review, but in their report they address cardiac and oral cleft defects in epidemiologic studies. The studies showed no clear association between ozone exposure and birth defects. Nevertheless a meta-analysis by Vrijheid et al. (2011), mentioned in the U.S. EPA report, claimed that there was no increase in risk of congenital abnormalities with ozone exposure.

(4) Effects on neurobehavioral parameters in offspring:

Bignami (1994) looked at reflexes, vocalisation and exploratory behaviour in offspring of mice dams exposed to ozone at 0.4, 0.8 and 1.2 ppm during pregnancy days 7-17. No effects were observed regarding these parameters. Reflexes and locomotor behaviour were also not affected in offspring of mice exposed to ozone at 0.2, 0.4 and 0.6 ppm from 6 days prior to breeding pair formation until gestational day 17 (Petruzzi, 1995). However, exploring duration was decreased while exploring frequency was increased in the youngest of the age groups tested. In addition, the younger age group tested engaged in self-grooming behaviour more frequently (stat. sign. at all doses) and for a longer duration (only at high dose). The young age group also engaged in jumping more frequently at all doses, an effect which was seen in the older age group only at the high dose (while low and mid dose exhibited a reduced frequency). In social interactions, both age groups engaged more frequently in sniffing other mice at all doses, but without a dose-response relationship. Maze learning tests including a reward showed an initially somewhat impaired learning. In another study by Petruzzi 1999 sex-dependent handedness in a paw preference test, female offspring exposed to 0.6 ppm showed an increased preference for the left paw. A modified hot plate test was performed in combination with the injection of morphine or saline as control after prenatal and postnatal exposure and pointed out reduced drug sensitivity. Dell'Omo (1995) found no effects of ozone on crossing, rearing, jumping, sniffing, grooming, freezing and response to a stimulus object in offspring of CD-1 mice exposed to 0.6 ppm ozone. The authors did, however, observe a reduced grooming duration when ozone-exposed offspring was placed in a novel environment. In addition, there was a retardation of passive avoidance acquisition. The study of Sorace (2001) analysed the effects of maternal exposure to 0.3 or 0.6 ppm ozone on neurobehavioral development in the CD-1 mice offspring. They reported a slight delay in forelimb stick grasp reflex, retardation in homing, a slight decrease in locomotor activity, increased step-through latency in passive avoidance test and impairment in platform reversal in water maze performance. Whereas these divergent responses were more pronounced at 0.3 ppm, a decrease in wall rearing in the hot plate test was observed for both exposure groups.

Kavlock (1980) reported behavioural changes of rats born to dams exposed to 1.0 and 1.5 ppm during late gestation, namely a dose-related retardation of early reflexes (righting, eye opening, horizontal movement in open field) and delay in grooming and rearing responses in the highest dose group after late gestational exposure. In contrast mid gestation exposure to the same concentrations was unaffected.

The outcome of an aggressive behaviour test with male offspring, where pregnant rats were exposed to 0.3 and 0.6 ppm ozone 30 days before mating until gestational day 17, was described by Santucci (2006). Both concentrations led to significantly higher duration of freezing, increased tail rattling and decreased submissive upright posture compared to the corresponding untreated control in daily encounters. Moreover, non-agonistic behaviour was also affected in ozone groups. Sniffing (body, anogenital and nose) showed a dose-related reduction for treatment groups while other behavioural characteristics like push under and social resting was

increased in the highest does group. In this study allogroom followed no clear pattern, because it was slightly increased for 0.3 ppm and reduced for 0.6 ppm. They also analysed changes of neurotrophins in CNS with an significant decrease of NGF level in hippocampus and increase of BDNF in striatum in both ozone groups, but the significance of these findings is not known. Haro and Paz (1993) observed an inversion of the sleep-wake pattern (light hours vs. dark hours) in rats exposed prenatally to 1 ppm ozone. However, the studied offspring group consisted of only 6 animals and group size and possible toxic effects on dams were not reported.

The U.S. EPA based their review of this endpoint on a subset of the toxicological studies also reviewed in this part. They conclude that the studies provide limited evidence for effects of ozone on the development of the CNS.

(5) Other effects on offspring:

Kavlock (1979) reported no effects on plasma electrolytes and no changes in several ECG parameters, except for a decrease in heart rate of offspring of dams exposed during late gestation to 1.19 ppm. Following a 10-day exposure during pregnancy to 0.4, 0.8 and 1.2 ppm ozone, Sharkhuu (2011) reported no differences of immune modulating cells and cytokines in BALF collected from the offspring. Only an increase of LDH activity in BALF and a suppression of delayed-type-hypersensitivity response to bovine serum albumin, restricted to female mouse offspring, were shown for the highest dose group. Additionally prenatal ozone exposure did not affect development of allergic airway inflammation but the highest ozone concentration attenuated the markers of allergic lung disease in late sensitised offspring.

10.10.6 Comparison with the CLP criteria on adverse effects on development

Toxicological results	CLP criteria
None of the submitted studies was performed according to OECD test guideline for reproductive toxicity TG 414, 415, 416, 421, 422, 426, 443 and partly they were also not compliant with generic OECD test guideline criteria. Classification not possible because of the deviations open literature studies and OECD TG studies.	Category 1A: Known human reproductive toxicant Category 1B: Presumed human reproductive toxicant largely based on data from animal studies - clear evidence of an adverse effect on development in the absence of other toxic effects, or - the adverse effect on reproduction is considered not to be a secondary non-specific consequence of other toxic effects Category 2: Suspected human reproductive toxicant - some evidence from humans or experimental animals, possibly supplemented with other information, of an adverse effect on development and - the evidence is not sufficiently convincing to place the substance in Category 1 (deficiencies in the study). - the adverse effect on reproduction is considered not to be a secondary non-specific consequence of the other toxic effects

Conclusion on classification and labelling for Reproductive toxicity, adverse effects on development:

In summary and based on the submitted data, Ozone does not meet the criteria to be classified for Reproductive toxicity, for adverse effects on development, according to the criteria in CLP regulation.

10.10.7 Conclusion on classification and labelling for reproductive toxicity

Based on the results listed above, no harmonised classification and labelling for reproductive toxicity, regarding adverse effects on sexual function and fertility or on development of the offspring and also for effects on or via lactation, is proposed: data inconclusive for classification and labelling.

RAC evaluation of reproductive toxicity

Summary of the Dossier Submitter's proposal

The DS proposed no classification for sexual function and fertility, developmental effects, or effects on/via lactation.

Comments received during consultation

Support was received from one MSCA for no classification based on available data.

Assessment and comparison with the classification criteria

Sexual function and fertility

The following studies were assessed.

***Table**: Summary of animal studies on sexual function and fertility*

Method, Guideline, GLP status, Reliability, reference	Species, Strain, Sex, No/ group	Test substance Dose levels, Duration of exposure	Results
Campos-Bedolla *et al.,* 2002 Reliability: 4	Female Wistar rats, nonpregnant and pregnant (5, 10, 18 days of gestation) Group size: n = 5-9	0 and 3 ppm 1 h exposure in chamber Uterine contractile response to oxytocin and acetylcholine was examined in uterine tissue strips 16-18 h post-exposure to ozone	Influence of ozone on uterine contractile response to oxytocin and acetylcholine: Oxytocin: Area under the curve was increased in non-pregnant and pregnant rats on gestational day 5 (stat. sign.), but not different on gestational days 10 and 18 Amplitude was increased in non-pregnant and pregnant rats at gestational day 5 (stat. sign.), and decreased at gestational days 10 and 18 Frequency was increased in non-pregnant and pregnant rats at gestational days 5 (stat. sign.) and 10 (small effect), and decreased at gestational day 18 Acetylcholine: Area under the curve was increased in non-pregnant and pregnant rats on gestational days 5 and 10 (both stat. sign.) Amplitude was increased in non-pregnant and pregnant rats on gestational days 5 and 10, but decreased on GD 18 Frequency was decreased in non-pregnant and pregnant rats at GD 18, but increased at GD 5 and 10
Jedlińska-Krakowska *et al.,* 2006 Reliability: 2	Rat Wistar/Hannover male (5 month old) 8/control group 10/exposure group	0.5 ± 0.2 ppm	Morphology of spermatozoa: no significant differences between ozone group and control, reduced in exposed rats: abnormal head, hookless, banana shaped, double headed, loose head, increased in exposed rats: folded around the head, coiled tail Sperm motility (by CASA): no significant differences, reduced in exposed rats: VCL (3%), increased in exposed rats: MOT (7%), VSL (18%), LIN (20%), BCF

	After 42 days of exposure males stayed 8 days with unexposed females for mating		(38%), ALH (3%) Sperm concentration: ~17% lower in exposed rats (not stat. significant) Morphometric measurements: no differences shown in size and weight of testes and vesicular glands Fertilisation: successful matings, average number of pups and new-born mortality were similar to control.

Campos-Bedolla (2002) reported that ozone influenced the effects of oxytocin and acetylcholine on uterine contractions in non-pregnant and pregnant rats when exposed to 3 ppm ozone for 1 h at different gestational stages. Different measures of the contractile response to oxytocin were increased in non-pregnant and pregnant rats on gestation day (GD) 5 and was decreased or close to unchanged later on GD 10 and 18. The effect of acetylcholine was overall increased in non-pregnant and pregnant rats on GD 5 and 10, but decreased on GD 18. The relevance of this study for the reproductive toxicity hazard class or female fertility is not clear because of the lacking investigations on female reproductive functions or capacity.

Jedlińska-Krakowska (2006) performed a study exposing male rats to 0.5 ppm ozone for 50 days. The findings included no significant differences of spermatozoa morphology, sperm mobility and size/weight of testes and vesicular glands. Sperm concentration was 17% lower in exposed rats. However, the reproductive capacity was not affected as fertilisation of exposed males was not impaired, and because number of successful mattings and average number of pups were the same as in control animals.

There are also human studies available assessing sperm parameters.

***Table**: Summary of submitted human studies assessing sperm parameters.*

Method, Guideline, GLP status, Reliability, reference	Species, Strain, Sex, No/ group	Test substance Dose levels, Duration of exposure	Results
Sokol *et al.,* 2006.	Retrospective cohort study on sperm quality Forty-eight donors from Los Angeles, donors provided repeated semen samples over a 12-month period between January 1996 and December 1998.	Mean ± SD: 21.68 ± 9.43	Negative correlation between ozone levels at 0–9, 10–14, and 70–90 days before donation and average sperm concentration, which was maintained after correction for donor's birth date, age at donation, temperature, and seasonality ($p < 0.01$). Result: sperm toxicant
Tian *et al.*, 2017.	Retrospective cohort study on sperm quality 1780 subjects, aged 20 to 40 years, study at Reproductive Medicine Center in Renmin Hospital of Wuhan University, 4/2013 - 6/2015.	Mean ± SD: 114.20 ± 74. 88) μg/m	Decreasing sperm concentration and count Mean sperm concentration: 76.32 ± 50.17 × 10^6/mL Count: 164.77 ± 133.05) × 10^6 /sample For every 1 μg/m3 increase of O_3, the decrease of sperm concentration during lag 0-9, lag 10 and lag 10-14 days exposure: - 0.081 (95% CI: 0.003-0.158) × 10^6/mL - 0.040 (95% CI: 0.004-0.077) × 10^6/mL - 0.059 (95% CI: 0.001-0.116) × 10^6/mL

Sokol *et al.* (2006) observed a significant negative correlation between ozone levels and sperm concentration. Percent change were below 4% under different conditions. Results are supported by the study of Tian *et al.* (2017), reporting a decrease of sperm concentration and count in young people in Wuhan, China. However, only the abstract in English was available.

Studies addressing developmental toxicity should also be considered for reproductive performance, if female or male animals were exposed to ozone prior to mating. The available studies reported that ozone did not significantly affect the number of successful pregnancies.

The U.S. EPA ISA review (2013) concluded that there is very little evidence for effects towards sperms and reproductive success for ozone exposure in epidemiology.

Based on available data for sexual function and fertility, RAC agrees with the DS that no classification is warranted.

Developmental toxicity

The following studies were submitted.

***Table**: Summary of submitted studies assessing developmental toxicity*

Method, Guideline, GLP status, Reliability	Species, Strain, Sex, No/ group	Ds	Results
Reliability: 2 Bignami *et al.*, 1994	Charles River CD-1 mice Group size: Maternal: n = 11 Offspring: 9-10 litters per dose group (37 total), divided into three experiments: -Somatic and neurobehavioral development tests -Ultrasonic vocalisation test -Activity/ exploration tests	Ozone during GD 7-17 to 0, 0.4, 0.8 or 1.2 ppm	Maternal: Food consumption: stat. sign. lower food and water consumption during gestational days 7-10 Body weight: stat. sign. decreased bw in mid and high dose groups on day 10 and a trend towards dose-related reduced bw-gain and reduced bw in all dose groups throughout gestation Pregnancy duration: slightly increased in two highest dose groups Offspring: Body weight: reduced bw gain in mid and high dose groups (stat. sign. only in high dose group), but slightly increased in low dose group Physical development: delayed (2 d delay) eye opening (stat. sign. only in low dose group) Maternal: LOAEL: 0.8 ppm (reduced body weight, stat. sign.) NOAEL: 0.4 ppm Offspring: LOAEL: 0.8 ppm (bw) NOAEL: 0.4 ppm
Reliability: 4 Kavlock *et al.*, 1979 (Experiment 1 – effect on skeletal	Long-Evans female pregnant rats Group size: Maternal: n =	Ozone gestation days 6-9: continuous exposure to 0 or 1.04 ppm	Maternal: Body weight gain: reduced in early-term group, in all dose groups of mid-term group 1 (stat. sign. at mid and high doses) and in organogenesis group (stat. sign); increased in mid and high doses of midterm 2 group Food/water intake: dose-related decreases in food/water intake in all gestational/dose groups (stat. sign. only in

ossifications)	14-37 <u>Offspring:</u> Animals: n = 38-102 Litters: n = 8-18	gestation days 9-12: continuous exposure to 0, 1.0, 1.26 or 1.49 ppm experiment 2: (gestation days 9-12): continuous exposure to 0, 0.64, 0.93 and 1.97 ppm Organogenesis (gestation days 6-15): 8 h/d exposure to 0 or 0.44 ppm	both midterm dose groups and – for food only – in organogenesis group) <u>Implants:</u> fewer implants in early-term group (stat. sign) and in high dose of mid-term 2 groups; more implants in all other groups Offspring: <u>Body weight:</u> ~11% higher foetal weight in treated early-term group compared to control (stat. sign.); dose-related decreased foetal weight in mid-term 1 group (stat. sign.); slightly lower foetal weights compared to control at all doses in mid-term 2 and organogenesis (~5-6.5%) groups, but no stat. sign. and no dose-response <u>Resorption:</u> in both mid-term groups, stat. sign. dose-related increase in percentage resorptions with a stat sign. difference between the control and the highest dose group (8.9 ± 9.9 vs 50.4 ± 42.9 and 11.1 ± 9.2 vs 58.8 ± 45.8). <u>Visceral anomalies:</u> enlarged renal pelvis in 5.8% of foetuses in treated early-term group (none in control) and at low dose of mid-term group 1 (2.2% vs 6.1% in control; none at mid and high dose); enlarged lateral ventricles at low and high dose in mid-term group 2 (1.1% and 2.6% vs 2.4% in control); no visceral anomalies in organogenesis group <u>Skeletal ossification and malformations:</u> Mid-term group 1 shows a significant dose-related increase in poorly ossified supraoccipitals. Stat. sign. higher average number of sternebrae in early-term group; stat. sign. dose-related decrease in mid-term group 1 (~93% lower at high dose); decreased at all doses in mid-term group 2 (44% at low dose), but no dose-response; ~42% lower in organogenesis group ~7% increased number of post-thoracic vertebrae centrums in early-term group; dose-related decrease in mid-term group 1 (~10% decreased at high dose compared to control); ~5% decreased at low and high dose in mid-term group 2; ~2% decrease in organogenesis group ~35 and 44% higher foetuses with ossified Meckel's cartilage and ossified pubis, respectively in treated early-term group than in control, but with high variability and no dose-response. Offspring: <u>LOAEL:</u> 1.49 ppm(bw, mid-term 1) 1.26 ppm (resorption) 1.0 ppm (skeletal, supraoccipital, mid-term 1) <u>NOAEL:</u> 1.0 ppm (resorption) 1.0 ppm (skeletal)
Reliability: 4 Kavlock *et al.,* 1979 (Experiment 2 – effect on ECG parameters)	Long-Evans female pregnant rats Maternal group size: not reported Foetal group size: 8 litters	<u>Treatment groups:</u> -no exposure -exposure on GD 9-12 (midterm) to 1.04 ppm -exposure on GD 17-20 (late gestation) to 1.19 ppm	Decreased heart rate in foetuses on gestational day 20 (other days not tested) in highest dose group (HR in control, 1.04 and 1.19 ppm groups: 157, 159, 149 beats/min, respectively) No changes in P-Q, PRS, Q-T intervals in any group (acc. to authors).

Reliability: 4 Kavlock *et al.,* 1979 (Experiment 3 – effect on plasma electrolytes)	Long-Evans female pregnant rats Maternal group size: not reported Foetal group size: 8 litters	Ozone generated from air Whole-body chamber exposure on gestational days 17-20 to 0 or 1.0 ppm	Plasma electrolytes were measured on gestational day 20. No effects on foetal weight, haematocrit, plasma sodium and potassium.
Reliability: 4 Custodio *et al.,* 2010	Mothers: female Wistar rats Offspring: groups of 10, 20 or 30 day old pups from exposed and non-exposed mothers 8 per group	Ozone 0 or 1.0 ppm during 12 h darkness phase for first 20 days of gestation Exposure chamber	Mothers: no differences in body weight gain and litter size Offspring: -decreased body weight, but no differences in brain weight -decreased noradrenaline compared to control in cerebellum in all age groups, in cerebral cortex only in 10 day old pups, in pons only in 30 day old pups Authors' interpretation: No clear conclusion presented, but authors indicated in the introduction that noradrenaline plays a role in proliferation, cell maturity and neural cytoarchitectural configuration during the brain's gestational period and during neonatal period.
Reliability: 2 Dell'Omo *et al.,* 1995	Charles River CD-1 mice Group size: Maternal: n=10 Offspring: 8 litters per dose group, divided into two groups for four experiments: - open-field tests with scopolamine hydrobromide or saline injection (postnatal day 24: n = 16m/16f). conditioned place preference tests with d-amphetamine sulphate or saline injection (postnatal days 28-31: n = 16m/16f).	Ozone Continuous exposure from 6 days prior to formation of breeding pairs to weaning (postnatal day 22 or 26) to 0 or 0.6 ppm	General effects: Stat. sign: retarded body weight gain in offspring Behavioural effects: Open-field tests (half of the group additionally injected with scopolamine hydrobromide): Apart from the elimination of sex differences, no major were observed in ozone-exposed and ozone/scopolamine-exposed offspring. Conditioned place preference tests (half of the group injected with d-amphetamine): Ozone-exposed offspring not previously exposed to amphetamine spent less time in the white and black compartments and more time in the middle compartment (not stat. sign.). On the other hand, ozone-exposed offspring previously exposed to amphetamine spent more time in the white and black compartments and less time in the middle (stat. sign). The interpretation of this test regarding developmental effects is not clear. Response to novel environment: Reduced grooming duration in ozone-exposed mice Passive avoidance acquisition and retention: Transient retardation of passive avoidance acquisition. Maternal: Not analysed Offspring: LOAEL: 0.6 ppm (bw) NOAEL: 0.6 ppm (behaviour)
Reliability: 4 Haro and Paz, 1993	Rats dams: group size not reported offspring: n = 6 per dose group	Exposed for 12 h/d to 1 ppm throughout gestation control group exposed to air sleep	Offspring: body weight: decreased at birth and during the 90 d observation period after birth (data not reported) physical development: abnormal incisor growth in 2 of 6 animals (data not reported) sleep: inversion of the sleep-wake pattern as indicated by the following observations: *during light hours:* increased time spent in wakefulness

		recordings performed at postnatal days 30, 60 and 90 for 24 h each	(stat. sign) and decreased time spent in slow wave sleep (not stat. sign.) and paradoxical sleep (stat. sign.) on all test days *during dark hours:* decreased time spent in wakefulness and paradoxical sleep and increased time spent in slow wave sleep (all stat. sign.) on all test days not affected (data not reported): litter size
Guideline: None GLP: No Reliability: 2 Kavlock *et al.,* 1980	Species rat Strain Long-Evans rats Number of litters: Control: 15 I. 1.0 ppm: 6 I. 1.5 ppm: 4 II. 1.0 ppm: 6 II. 1.5 ppm: 6 Litters reduced to 8 pups	I. GD 9-12 (mid gestation exposure) Continuously 1.0, 1.5 ppm II. GD 17-20 (late gestation) Continuously 1.0, 1.5 ppm	Dose related growth retardation of offspring: PND6 for I.+II.; both sexes I. (mid gestation): female weight reduction: 1.0:6%, 1.5:8%; male weight reduction: 1.0:6%, 1.5:9% II. (late gestation) female weight reduction: 1.0:12%, 1.5:20%; male weight reduction: 1.0:11%, 1.5:19% PND15 for II. (late gestation): female weight reduction: 1.0:7%, 1.5:12%; male weight reduction: 1.0: 8%, 1.5:12% PND60 for II. (late gestation): male weight reduction: 1.0:8%, 1.5:10% II. (late gestation):14.3% of male offspring at 1.5 ppm were permanently stunted. Behavioural testing: I. (mid gestation): no significant effects II. (late gestation): 1.0, 1.5 ppm: dose related retardation of early reflexes (righting (1.5ppm: +1day), eye opening (1.5 ppm: + 1 d), horizontal movement in open field test (1.5 ppm: + 0.5 d)) Open field tests: I. (mid gestation): no significant effects II. (late gestation): delay in grooming and rearing behaviours; dose related decrease in grooming and rearing responses at all time points grooming (II.): day 1 of testing in 1.5 ppm dose group 56% less positive response, day 4 of testing in 1.5 ppm dose group still 30% less positive response rearing (II.): day1 of testing in 1.5 ppm dose group 77% less positive response, day 4 of testing in 1.5 ppm dose group still 18% less positive response activity in open field unaffected. Maternal: Not analysed Offspring: NOAEL: not determinable because LOAEL set at lowest dose LOAEL: 1.0 ppm (retardation of weight gain during late gestation) 1.5 ppm (behaviour: reflexes)
Guideline: None GLP: No Reliability: 2 López *et al.,* 2008	Species rat Strain Wistar Sex female No/group 6 animals/group (3 foetuses/group analysed for lung effects)	Ozone Filtered air control P15 Triozone generator Exposure: 1 ppm ozone, 12 h/d I. GD 0-18, II. GD 0-20, III. GD 0-21 GD 18, 20, 21 (= time points)	I. GD 18 (glandular phase of rat lung development): swollen mitochondria, cytoplasmic vacuolisation in bronchiolar epithelium cells and structural disarrangement →oxidative damage →cellular permeability II. GD 20 (canalicular phase of lung development): increased amounts of glycogen in secretory cells, flake-off epithelial cells →epithelial damage of membrane, delayed maturation III. GD 21 (sacular phase): swollen mitochondria deprived of cristae, granules in non-ciliated bronchiolar cells →alterations during rat foetal lung development (damage in foetal bronchiolar epithelium), rupture of membrane proteins and lipids ozone generates radicals which cross the haematoplacentaria barrier, distributed to foetal organs.

			LOAEL offspring: 1 ppm (lung development)
Guideline: None GLP: No Reliability: 2 Petruzzi *et al.*, 1999	Species mice Strain CD-1 (Charles River, Calco, Italy) Sex male and female No/group each 10/ group Litters reduced to 7 and culled to 8 pups (4 male and 4 female) On PND21 reduction to 3 pups/litter	0.3, 0.6, 0.9 ppm Exposure: 6 days before formation of breeding pairs until PND26 (males and females exposed) Continuous	Retardation of postnatal body weight gain PND2-40, PND100 significant reduction of body weight gain for 0.9 ppm group (specific values not shown) Paw preference test PND70: 0.6 ppm: sex-dependent paw preferences (male: 30.33 ± 2.25; female: 19.33 ± 2.44 right paw entries) right paw: males, left paw: females Hot plate response test PND100 (injection of morphine or a saline): Reduced drug sensitivity (morphine) after 0.9 ppm ozone exposure: Shorter latency + higher frequency in hind limp withdrawal and shorter latency + higher frequency (limited to males) of wall rearing of morphine injected mice compared to saline control. Maternal: Not analysed (no sufficient data) Offspring: NOAEL: 0.6 ppm (bw) LOAEL: 0.9 ppm (bw)
Guideline: None GLP: No Reliability: 2 Petruzzi *et al.*, 1995	Species mice Strain CD-1 (Charles River, Calco, Italy) Sex male and female No/group each 16/ exposure group Each 20/ control group Functional tests, Fox battery: 2 m + 2 f Social interaction: 4 m + 4 f Locomotor activity: 12 m/ treatment Maze: 8 m/treatment	0.2, 0.4, 0.6 ppm Exposure: 6 days before formation of breeding pairs (7-10 days before start of gestation) until the morning of GD 17	Maternal: Body weight: initially lower than control in mid and high dose groups, but by the end of exposure higher in low and mid dose groups and same as control in high dose group; throughout exposure low dose group had higher bw than mid and high groups Food intake: overall increase throughout exposure; lower throughout most of exposure period (especially before gestation) in mid and high dose groups, but no real pattern could be observed because there was no consistent development in any of the groups Water intake: overall increase throughout exposure; initially (pre-pregnancy) lower than control in all dose groups, then no real pattern because there was no consistent development in any of the groups No effect on successful pregnancies: 0: 14/20, 0.2: 16/16, 0.4: 14/16, 0.6: 13/16 pregnancies Offspring No effect on somatic and neurobehavioural development (data not shown) Social interaction: sniffing of other mice: 0.2 - 0.6 ppm O_3 increased at PND23-25 (70% in 0.6 ppm dose group) and PND43-45 (22% in 0.2 ppm dose group) Mutual circle response: 0.2 ppm elevated at PND23-25 (90%) and PND43-45 (> 100%) Digging: more frequent in males (data not shown); increased in 0.2 ppm dose group (PND23-25 (39%) and PND43-45 (13%)) and increased in 0.4 ppm dose group PND23-25 (20%), but decreased in all other groups follow, squire, mutual circle: more frequent in females (data not shown) Exploring: increased exploring frequency in PND23-25 at all doses, but not in PND43-45; decreased exploring duration in both age groups at all doses (stat. sign. at high dose) Self-grooming: stat. sign. increased self-grooming frequency in PND23-25 (0.4 and 0.6 ppm dose group), but not in older age group; increased self-grooming duration

			only in younger age group at high dose; increased jumping in young age group at all doses, but only at high dose in older age group (low and mid dose decreased) Eight-arm radial maze learning: reduction of rewarded trials in training phase (0.2 ppm significantly different), which increased to above control levels in subsequent phases, increased total time for first visit of all maze arms in high dose, but decreased in low and mid dose-groups Maternal: NOAEL: 0.6 ppm (bw and pregnancies) LOAEL: not determinable, because NOAEL was set at highest dose Offspring: NOAEL: 0.6 ppm (bw, somatic and neurobehavioural development) LOEL: 0.6 ppm (social interaction (grooming, exploring))
Method Guideline: None GLP: No Reliability: 2 Romero-Velázquez *et al.,* 2002	Species rat Strain Wistar Sex female No/group 4 pregnant females/group Litters culled to 8 pups (4 m + 4 f) Morphological analysis: 8 male born rats/group	Ozone Pollution-free control P15 Triozone generator 1 ppm ozone for 12 h/d Exposure: during entire gestation (GD 0 until PND0) Time point: PND90	Abnormal structures in molecular layer of cerebellum of rats born to exposed dams Decrease of total area and number of Purkinje cells 0: 10.6 ± 0.3 mm²; 1 ppm: 4.8 ± 0.3 mm² 0: 832 ± 31 cells; 1 ppm: 712 ± 34 cells →Depopulation of Purkinje cells and also degenerating Purkinje cells and cell debris Circular bodies in molecular layer Incomplete folding pattern of some lobes Maternal: Not analysed Offspring: LOAEL: 1.0 ppm (morphologic)
Guideline: None GLP: No Reliability: 2 Santucci *et al.,* 2006	Species mice Strain CD-1 (Charles River, Calco, Italy) Sex females No/group 8/group Behaviour test: 2 males of each litter (n = 6) NGF/BDNF: 6 males/group	Ozone 0.3, 0.6 ppm ozone continuous exposure Exposure: 30 days before breeding pairs until GD 17	Aggressive behaviour test (> PND130): 0.3 and 0.6 ppm: significantly higher duration of freezing (day1 and day 3: circa 2-fold increased freezing), increased tail rattling and decreased submissive upright posture Non-agonistic behaviour: Reduction of sniffing: body sniff, anogenital, nose sniff showed dose related decrease (0: 39.1 ± 6.3; 0.3: 23.5 ± 6.0; 0.6: 18.4 ± 3.1) Allogroom: increased at 0.3 ppm (21%), reduced at 0.6 ppm (64%) Push under: increased at 0.6 ppm (34%) Social resting: increased at 0.6 ppm (70%) →Impairment in investigative profile NGF (nerve growth factor) and BDNF (brain derived neurotrophic factor) level: Significant decrease of NGF level in hippocampus (0.3: 16%; 0.6: 20%) and increase of BDNF in striatum (0.3 and 0.6: 2.5-fold) vice versa not affected (→Functional significance of these changes not known) Maternal: Not analysed Offspring: LOEL: 0.3 ppm (social interaction: nose sniff and freezing)
Guideline: None	Species mice Strain Balb/c No/group 20	Ozone 0.4, 0.8, 1.2 ppm	0.4-1.2 ppm: decreased percentage of viable pregnancies 0: 58%; 0.4: 45%; 0.8: 45%; 1.2: 33% successful pregnancies (1.2 ppm significant at this concentration:

GLP: No Reliability: 2 Sharkhuu *et al.,* 2011	pregnants/group exposed (Experiment was performed 3 times) Analysis of offspring: BALF: 3-6/sex LDH/prot.: 3-7/sex DTH: 6-8/sex Sensitised offspring: BALF: 3-6/sex	ozone for 4h/d at GD 9-18	25% less productive dams compared to control) No effect on litter size and sex ratio 1.2 ppm: reduced <u>weight gain in offspring</u> PND1: 13%; PND3: 22%; PND7: 15% and in male still at PND42: 9% lower weight <u>Inflammation:</u> 1.2 ppm: increased LDH activity in BALF at PND42 in female offspring and same trend for protein →Lung injury, normal lung development altered Delayed-type hypersensitivity (DTH) responses supressed in females at 0.8 and 1.2 ppm No effect on specific IgM and IgG titer in sheep red blood cell-specific antibody response testing In <u>OVA-sensitised female offspring</u> early sensitisation < PND3: at 1.2 ppm decrease in total cells (~47%) in BALF (macrophages (~42%), eosinophils (~95%), lymphocytes (~82%)) of females OVA-specific IgE antibodies decreased in both sexes late sensitisation > PND42: 0.8, 1.2 ppm: reduction of neutrophils (~65%) OVA-specific IgE antibodies decreases No differences in pulmonary responsiveness to methacholinein after ozone exposure Maternal: <u>NOAEL:</u> 0.8 ppm (pregnancies) <u>LOAEL:</u> 1.2 ppm (pregnancies) Offspring: <u>NOAEL:</u> 0.8 ppm (bw) <u>LOAEL:</u> 1.2 ppm (bw)
Guideline: None GLP: No Reliability: 2 Sorace *et al.,* 2001 Experiment 2: Prenatal Exposure to ozone	Species mice Strain CD-1 (Charles River, Calco, Italy) Sex female No/group total: 30 females exposed, 15 males non-exposed (2f +1m per box) 10 females/group	0.3, 0.6 ppm ozone Exposure: 30 days before formation of breeding pairs until GD 17	Exposed dams: no differences in placental scars 0.6 ppm: reduction of successful <u>pregnancies</u> 0 and 0.3: 9/10; 0.6: 6/10 (not significant) No effect on <u>body weight</u> of pups (no data given) <u>Somatic and neurobehavioural development PND2-20:</u> →No concentration dependent effects, only effects in 0.3 ppm group Maternal: <u>NOAEL:</u> 0.3 ppm (pregnancies) <u>LOAEL:</u> 0.6 ppm (pregnancies) Offspring: <u>NOAEL:</u> 0.6 ppm <u>LOAEL:</u> ≥ 0.6 ppm
Guideline GLP Reliability: 4 Sorace *et al.,* 2001 Experiment 1: Prolonged Exposure to ozone in adult males	Species mice Strain CD-1 (Charles River, Calco, Italy) Sex male No/group total: 20 males exposed	Ozone Non-exposed control group 0.3, 0.6 ppm ozone Exposure: 30 days	Crossing and sniffing increased in open field test (0.6 ppm, day 4) Water maze: Increased swimming sinuosity (0.3 ppm, day 3) longer latency in reversal phase and swimming path length (0.3 ppm)

Developmental Neurotoxicity Study Guideline: None GLP: No Reliability: 4 Rivas-Manzano *et al.,* 1999	Species Rat Strain Wistar Sex female (in oestrus) No/group 3 total: 6 6 females, 3 One group exposed to O_3 One free pollution air group	Ozone 1.0 ppm for 12 h/day	Study of morphological aspects of the anterior cerebellar lobe of rats exposed to O_3 during the gestation period. Analyses of sagittal sections of the anterior cerebellar lobe at postnatal days 0, 12 and 60: - cerebellar necrotic signs at age 0, - diminished area of the molecular layer with Purkinje cells with pale nucleoli and perinucleolar bodies at age 12 Purkinje cells showing nuclei with unusual clumps of chromatin in the periphery at age 60 Conclusion: 1 ppm ozone during gestation induces permanent cerebellar damage in rats Result: Adverse Effect on CNS development NOAEL: 0 ppm LOAEL: 1 ppm
Developmental Neurotoxicity Study Guideline: None GLP: No Reliability: 4 Boussouar *et al.,* 2009	Species Rats Strain: Sprague Dawley Sex: female (pregnant) No/group: 4 total: 8 One group exposed to O_3 One free pollution air group	Ozone 0.5 ppm 12 h/day from embryonic day E 5 to E20) Ozone generator (UV-light) Duration from embryonic day E5 to E20	Prenatal ozone increased baseline TH grey level per cell ($p < 0.001$). Conclusion: long-lasting sequelae detected in the offspring beyond the prenatal O_3 exposure. Prenatal O_3 left a print on the NTS, revealed by stress. NOAEL: 0 ppm LOAEL: 0.5 ppm

<u>Effects on dams</u>

Reduced body weight gain in rats exposed during different gestational stages to doses of ozone starting around 0.44 ppm were reported by Kavlock *et al.* (1979). However, the effects were not consistent throughout all groups and did not reach statistical significance in all groups. Also reduced weight gain was seen in mice by Bignami *et al.* (1994), where dams exposed from 0.4 ppm but the effect was not statistically significant. Statistically significant decrease in body weight was observed only on GD 10 starting from 0.8 ppm.

In study by Petruzzi *et al.* (1995) test group body weights were initially lower than in the control group, but by the end of exposure higher in the low and mid dose group compared to control. Regarding food and water consumption, there was an effect only at the initial exposure period correlating with the body weight gain of dams until the start of the pairing for breeding. Although consumption was lower than control prior to gestation, no real pattern could be observed afterwards because, although consumption increased throughout exposure in all groups, there was no consistent development in any of the groups.

<u>Effects on implants, number of litters, litter size, stillbirths, neonatal mortality and offspring body weight</u>

Kavlock *et al.* (1979) reported an increased number of resorptions in all groups of rats exposed at different gestational stages to different ozone doses (0.44 - 1.97 ppm depending on gestational stage), with more than 50% resorptions in the high dose groups of two sets of dams exposed during GD 9-12. Sorace *et al.* (2001) reported a reduction of successful pregnancies of CD-1 mice in the highest dose group (0.6 ppm). Even if the reduced number of pregnancies reached no statistical significance due to the small group size, this observation should be considered. Moreover, the study by Sharkhuu *et al.* (2011) reported a decrease in the percentage of delivered pregnancies in all exposure groups from 0.4 to 1.2 ppm ozone in mice exposed during GD 9-18 for 4 h/day. In the highest dose group the exposure led to 25% less productive dams (statistically significant).

Bignami *et al.* (1994) and Dell'Omo *et al.* (1995) reported that litter size and neonatal mortality in mice were not affected after exposure and during pregnancy and lactation (Dell'Omo *et al.*, 1995). Haro and Paz (1993) also reported no effects on litter size in an unreported number of rats exposed to 1 ppm throughout gestation. Other parameters such as proportion of successful pregnancies, sex ratio (Bignami *et al.,* 1994 and Dell'Omo *et al.,* 1995) and frequency of stillbirths (Bignami *et al.,* 1994) were also not different from control animals. Custodio *et al.* (2010) also did not report changes in litter size in rats after exposure of dams to 1.0 ppm during the first 20 days of gestation. Petruzzi *et al.* (1995) reported no effect on successful pregnancies, litter size, sex ratio and neonatal mortality after the exposure of female mice 6 days prior to the formation of breeding pairs until GD 17. Moreover, Petruzzi *et al.* (1999) reported no effects on these parameters after a prolonged exposure period until postnatal day (PND) 26.

Bignami *et al.* (1994) reported a reduction in body weight gain in offspring of the mouse dams exposed to 0.8 and 1.2 ppm ozone. This effect is supported by findings by Dell'Omo *et al.* (1995), where exposure of mouse dams to 0.6 ppm ozone during pregnancy and lactation led to a reduction in body weight gain in offspring. Kavlock *et al.* (1979) reported a dose-related decrease in body weight in foetuses from dams exposed during GD 9-12 to doses ranging from 1.0 - 1.49 ppm. Decreased foetal weights were also reported in a second group exposed during GD 9-12 (0.64 - 1.97 ppm) and in the group exposed during organogenesis, but without statistical significance, and without dose-response. In the group exposed during GD 6-9, foetuses showed increased body weight. The dams in this study showed reduced body weight gain (although not consistent over all groups) and dose-related decreases in food/water intake. Kavlock *et al.* (1980) reported a dose related postnatal growth retardation of rat offsprings when dams exposed in midterm or late gestation to 1.0 ppm ozone, and that exposure to 1.5 ppm ozone during late gestation reported 14.3% of male offspring permanently stunted. These results are further supported by Haro and Paz (1993), who reported reduced body weight from birth to PND 90 in rat offspring exposed prenatally to 1 ppm ozone. This study had, however, several limitations (see the above table). Sharkhuu *et al.* (2011) also demonstrated reduced offspring body weight gain in the first postnatal week for both sex and also persisting reduced body weight in males until PND 42 after exposure to 1.2 ppm ozone. Offspring body weight gain is reduced on PND 19 until 100 days after exposure to 0.9 ppm ozone, as also written by Petruzzi *et al.* (1999). Petruzzi *et al.* (1995), on the other hand, reported no effect on birth weight and postnatal body weight gain in mice offsprings exposed to 0.2, 0.4 and 0.6 ppm from 6 days prior to breeding pair formation until GD 17 (data not reported).

The U.S. EPA ISA review (2013) found inconsistent evidence for an effect of ozone exposure on foetal growth and birth weight in their review of epidemiological studies. Some of the toxicological studies reviewed by the DS were also reviewed by the U.S. EPA with the same conclusion (Sharkhuu *et al.*, 2011: reduced birth weight in highest dose group, decreased postnatal growth; Bignami *et al.*, 1994: decreased body weight gain; Haro and Paz, 1993: decreased birth weight and postnatal body weight gain; Kavlock *et al.*, 1980: reduced body weight gain). Overall, the U.S. EPA concluded that the data concerning the effect of ozone on foetal growth, birth weight and postnatal growth is inconsistent.

<u>Effects on ossification and other physical development parameters in offspring</u>

Ear opening, incisor eruption, hair growth and body/tail length were not affected in offspring of mice (Bignami *et al.*, 1994 and Petruzzi *et al.*, 1995). Eyelid opening was delayed by two days in all dose groups of the Bignami *et al.* (1994) study (reaching statistical significance

only in the low dose group), but was not affected in the Petruzzi *et al.* (1995) study. Ear opening, incisor eruption, hair growth and body/tail length were not affected in mice offsprings (Bignami *et al.*, 1994). However, eyelid opening was delayed by two days in all dose groups, reaching statistical significance only in the low dose group. Brinkman *et al.* (1964) observed a 2-fold and 16-fold increased frequency of blepharophimosis in offspring of black mice and inbred grey mice dams exposed to 0.2 ppm ozone. In black mice, the frequency of unlimited growth of the incisors was also increased 6-fold in this dose group. While the results reported by Brinkman *et al.* (1964) appear substantial, it should be noted that this study was poorly reported and no information on maternal toxicity was provided. Brinkman *et al.* (1964) reported findings regarding the number of litters (greatly reduced in both strains by ~40 and ~44%) and neonatal mortality (greatly increased in both strains by 260-470%) suggesting that there may have been significant maternal toxicity that may have led to the described malformations.

Haro and Paz (1993) also observed abnormal incisor growth in ~33% of offsprings. However, the study was not designed to examine effects on physical development, but on sleep patterns. In addition, the total offspring group size was only 6 animals and possible toxic effects on dams were not reported, although litter size was reported to be normal.

López *et al.* (2008) found alterations in bronchioles during intrauterine lung development in ozone exposed pregnant rats to 1 ppm, focussing on the glandular, canalicular and saccular phase of rat lung development. Swollen mitochondria, cytoplasmic vacuolisation, structural disarrangement and flake-off epithelial cells were identified as indicators of delayed maturation and further alterations during rat lung development.

Romero-Velázquez *et al.* (2002) observed abnormal structures in the molecular layer of cerebellum in the offspring of rat dams exposed to 1 ppm ozone during entire gestation. The study reported altered morphology of pup cerebellum, confirmed with a decrease of total area and number of Purkinje cells, because of depopulation of and degenerating Purkinje cells in the cerebellum, accompanied by incomplete folding pattern of some lobes, caused by ozone.

Kavlock *et al.* (1979) reported no notable effects regarding visceral anomalies, supernumerary ribs and rib malformations. Supraoccipitals were poorly ossified in the group of foetuses exposed during GD 9-12 (1 - 1.49 ppm) in a dose-related manner. Resorptions were also increasing in this group. In another group exposed during the same gestational period, but to different doses (0.64 - 1.97 ppm), a change in supraoccipital ossification (poorer compared to control) was only seen at the high dose where resorption was also above 50%. Foetuses in the groups exposed during early gestation GD 6-9 and during organogenesis GD 6-15 had slightly more advanced or unchanged supraoccipital ossification compared to control. The number of sternebrae was statistically significantly higher in foetuses exposed during early gestation GD 6-9 (1.04 ppm) compared to control. One of the groups exposed during mid-gestation GD 9-12 showed a dose-related decrease in the number of sternebrae (~93% lower at high dose compared to control), while the effect was not quite as pronounced (no dose-response) but also present in another group exposed during the same gestational period (44% lower at low dose compared to control), and in the group exposed GD 6-15 during organogenesis (~42% lower). Similar effects were observed regarding the number of post-thoracic vertebrae centrums, but the differences compared to controls were not as clear. A higher percentage of foetuses had ossified pubis and Meckel's cartilage in the early-gestation GD 6-9 exposure group (~35 and 44% compared to control) and in the mid-gestation exposure GD 9-12 group at low and mid doses, while this percentage was lower in the organogenesis exposure GD 6-15 group (~11 and 9% compared to control) and in the mid-gestation exposure

GD 9-12 group at the high dose (32 and 17% compared to control). Variability was quite high in all groups.

The U.S. EPA ISA review (2013) did not include skeletal ossification endpoints, but the review addressed cardiac and oral cleft defects in epidemiologic studies. The studies reported no clear association between ozone exposure and birth defects. A meta-analysis by Vrijheid *et al.* (2011), mentioned in the U.S. EPA review, claimed that there was no increase in risk of congenital abnormalities with ozone exposure.

Effects on neurobehavioural parameters in offspring

Bignami *et al.* (1994) investigated reflexes, vocalisation and exploratory behaviour in mice offsprings. No effects were observed regarding these parameters. Reflexes and locomotor behaviour were also not affected in mice offsprings (Petruzzi *et al.*, 1995). However, exploring duration was decreased while exploring frequency was increased in the youngest of the age groups tested PND 23-25. In addition, the same age group tested engaged in self-grooming behaviour more frequently (statistically significant at all doses) and for a longer duration (only at high dose). The young age group also engaged in jumping more frequently at all doses, an effect which was seen in the older age group only at the high dose (while low and mid dose exhibited a reduced frequency). In social interactions, both age groups, PND 23-25 and PND 43-45, engaged more frequently in sniffing other mice at all doses, but without a dose-response relationship. Maze learning tests including a reward showed initially somewhat impaired learning.

In another study by Petruzzi *et al.* (1999) sex-dependent handedness in a paw preference test, female offspring exposed to 0.6 ppm ozone showed an increased preference for the left paw. A modified hot plate test was performed in combination with the injection of morphine or saline as control after prenatal and postnatal exposure and pointed out reduced drug sensitivity. Dell'Omo *et al.* (1995) reported no effects of ozone on crossing, rearing, jumping, sniffing, grooming, freezing and response to a stimulus object in offspring of CD-1 mice exposed to 0.6 ppm ozone. The authors did, however, observe a reduced grooming duration when ozone-exposed offsprings were placed in a novel environment. In addition, there was a retardation of passive avoidance acquisition.

The study by Sorace *et al.* (2001) analysed the effects of maternal exposure to 0.3 or 0.6 ppm ozone on neurobehavioural development in the CD-1 mice offsprings. They reported a slight delay in forelimb stick grasp reflex, retardation in homing, a slight decrease in locomotor activity, increased step-through latency in passive avoidance test and impairment in platform reversal in water maze performance. Whereas these divergent responses were more pronounced at 0.3 ppm, a decrease in wall rearing in the hot plate test was observed for both exposure groups.

Kavlock *et al.* (1980) reported behavioural changes of rats born to dams exposed to 1.0 and 1.5 ppm during late gestation, namely a dose-related retardation of early reflexes (righting, eye opening, horizontal movement in open field) and delay in grooming and rearing responses in the highest dose group after late gestational exposure. In contrast mid-gestation exposure to the same concentrations behavioural testing was unaffected.

The outcome of an aggressive behaviour test with male offspring was described by Santucci *et al.* (2006). 0.3 and 0.6 ppm concentrations led to significantly higher duration of freezing, increased tail rattling and decreased submissive upright posture compared to the corresponding untreated control in daily encounters. Non-agonistic behaviour was also affected in ozone groups. Sniffing (body, anogenital and nose) showed a dose-related

reduction for treatment groups while other behavioural characteristics, like push under and social resting, was increased in the highest does group. In this study allogroom followed no clear pattern because it was slightly increased at 0.3 ppm and reduced at 0.6 ppm ozone. They also analysed changes of neurotrophins in CNS with a significant decrease of nerve growth factor (NGF) level in hippocampus and increase of brain derived neurotrophic factor (BDNF) in striatum in both ozone groups, but the significance of these findings is not known.

Haro and Paz (1993) observed an inversion of the sleep-wake pattern (light hours vs. dark hours) in rats exposed prenatally to 1 ppm ozone. However, the studied offspring group consisted of only 6 animals and group size and possible toxic effects on dams were not reported.

The U.S. EPA ISA review (2013) concluded that the studies provide limited evidence for effects of ozone on the development of the CNS.

Other effects on offspring

Kavlock *et al.* (1979) reported no effects on plasma electrolytes and no changes in several ECG parameters, except for a decrease in heart rate of offspring of dams exposed during late gestation to 1.19 ppm. Sharkhuu *et al.* (2011) reported no differences of immune modulating cells and cytokines in BALF collected from the offspring. Only an increase of LDH activity in BALF and a suppression of delayed-type-hypersensitivity response to bovine serum albumin, restricted to female mouse offspring, were shown for the highest dose group. Additionally, prenatal ozone exposure did not affect development of allergic airway inflammation but the highest ozone concentration attenuated the markers of allergic lung disease in late sensitised offspring.

Based on available data for developmental toxicity, RAC agrees with the DS that no classification is warranted.

Lactation

The DNT study by Bignami *et al.*(1994) in CD mice with dose during GD 7-17 with doses 0 - 1.2 ppm. The exposure period not including lactation and the offsprings were reared by non-exposed foster mothers.

The study reported effects on development. Delayed (2d) eye opening (statistically significant in the low dose group) and reduction in body weight gain in offspring in the high dose group were reported.

In another DNT study by Dell'Omo *et al.* (1995) in CD mice with continued ozone exposure from 6 days prior to formation of breeding pairs to weaning (PND 22 or 26) with 0 or 0.6 ppm, a reduction in body weight gain in offspring from exposed dams were reported. No information on maternal toxicity was reported.

Based on available data, RAC agrees with the DS that no classification is warranted for effects on or via lactation.

Conclusion

Because of high variability and the lack of consistency among reported effects, RAC agrees with the proposal by the DS that **no classification for reproductive toxicity is justified**.

10.11 Specific target organ toxicity-single exposure

Table 40: Summary table of animal studies on STOT SE

Method, Guideline, GLP status, Reliability	**Species, Strain, Sex, No/group**	**Test substance, form** *(gas, vapour, dust, mist)* **and particle size** *(MMAD)* **Actual and nominal concentration, Type of administration** *(nose only / whole body/ head only)*	**Value LC_{50}**	**Remarks** *(e.g. major deviations)*	**Reference**
Studies suitable for STOT SE classification					
Cardiovascular effects					
Acute Toxicity Study Guideline: None GLP: No Reliability: 2	**Rat**, Charles River, spontaneously hypertensive 12-wk-old number per dose group unclear	Ozone Single doses of 0, **0.2** and **0.8 ppm** 4 h exposure in whole-body chamber One cohort in each dose group was challenged with aconitine	**0.2 ppm:** increased sensitivity to aconitine-induced arrhythmia formation (compared to control) **0.8 ppm:** <u>HR and ECG:</u> decreased HR and QTc, increased PR and RR intervals, ST depression (compared to baseline); no post-exposure effects <u>Arrhythmia:</u> increased number of atrial premature beats, sinoatrial block, atrioventricular block during exposure (compared to baseline); little to no post-exposure effects <u>HR variability:</u> increased SDNN, RMSSD, LF, HF, LF:HF (compared to baseline); no post-exposure effects <u>Other:</u> decreased core body temperature, decreased serum	Increased sensitivity to arrhythmia persisted for 18h following exposure, all other cardiovascular effects observed during exposure only	Farraj A.K. et al. (2012), Environmental Health Perspectives" 2012, 120(3):348-354

Method, Guideline, GLP status, Reliability	**Species, Strain, Sex, No/group**	**Test substance, form** *(gas, vapour, dust, mist)* **and particle size** *(MMAD)* **Actual and nominal concentration, Type of administration** *(nose only / whole body/ head only)*	**Value LC_{50}**	**Remarks** *(e.g. major deviations)*	**Reference**
			HDL and creatinine, increased serum sorbitol dehydrogenase, increased sensitivity to aconitine-induced arrhythmia formation (compared to control)		
Review Guideline: Weight-of-evidence evaluation of short-term ozone exposure and cardiovascular effects; higher total scores indicate higher quality of the study. Only Tier I studies (quality score >0) considered in this dossier.	Morbidity 9 studies, in rats (8) and mice (1) including Farraj A.K. (total score = 6)	Different ways of administration, concentrations and exposure time of ozone	Tier I studies (quality score >0) *Heart rate* Increased: Chuang et al. (2009) Reduced: Farraj et al (2012) Arito et al. (1997) Wang et al. (2013) → inconsistent *Heart rate variability* Increased: Farraj et al (2012) Wang et al. (2013) → consistent (Tier I) *Arrhythmia* Increased: Farraj et al (2012) No changes:	Standardized evaluation by an in-house developed score system (Goodman W-o-E framework). Scoring for design, bias, size, statistics and confounders ranging from -1 over 0 to 1 is a pragmatic approach. Evaluation of original data could not be approved in this CLH-dossier. However, conflict of interest for support by American Petroleum Institute (API). Ozone is a secondary pollutant and is formed apart from different sources during the combustion process of oil and gas.	Goodman, JE et al. (2014), Critical Reviews in Toxicology 44:9, 725-790

Method, Guideline, GLP status, Reliability	**Species, Strain, Sex, No/group**	**Test substance, form** *(gas, vapour, dust, mist)* **and particle size** *(MMAD)* **Actual and nominal concentration, Type of administration** *(nose only / whole body/ head only)*	**Value LC_{50}**	**Remarks** *(e.g. major deviations)*	**Reference**
			Wang et al. (2013) → inconsistent *Blood pressure* Chuang et al. (2009) No changes (8 h) Changes (5 days) → inconsistent For more study details refer to Tab. 18 of the evaluation study by Goodman et al.		
Neurological effects					
Neurotoxicity study Guideline: None GLP: No Reliability: 2	**Rat**, Wistar, male n=24 animals (unclear whether per group or total)	Ozone Single doses of 0 and **1 ppm** 4 h exposure, closed chamber	Long-term (24 h) memory alteration: decreased time animal remained in safety compartment before entering shock compartment (with 2.5 mA footshock) Reduction in number of dendritic spines in hippocampus	Deficiency: no investigation of possible reversibility of effects	Avila-Costa M.R. et al. (1999), Neurosci Lett 270:107–9
Neurotoxicity study Guideline: None GLP: No Reliability: 2	**Rat**, Wistar, male n=24 animals (unclear whether per group or total)	Ozone (source not mentioned) Single doses of 0 and **1 ppm** 4 h exposure, closed chamber	Altered motor behaviour: decreased exploratory and increased freezing behaviour (measured for 10 minutes, 24h post-exposure);	Deficiency: no investigation of possible reversibility of effects	Avila-Costa M.R. et al. (2001), Int J Neurosci. 108(3-4):193- 200

Method, Guideline, GLP status, Reliability	**Species, Strain, Sex, No/group**	**Test substance, form** *(gas, vapour, dust, mist)* **and particle size** *(MMAD)* **Actual and nominal concentration, Type of administration** *(nose only / whole body/ head only)*	**Value LC_{50}**	**Remarks** *(e.g. major deviations)*	**Reference**
			reduction in number of dendritic spines in striatum and prefrontal cortex		
Neurotoxicity study Guideline: None GLP: No Reliability: 2	**Rat**, Wistar, male n=25 per dose group, divided into subgroups of 10, 10 and 5 to investigate different endpoints	Ozone generated from 98 % O_2 and 5 % CO_2 Single doses of 0, **0.1, 0.2, 0.5, 1 ppm** 4 h exposure, closed chamber	Short-term memory: no effects Long-term (24 h) memory: **0.2 and 0.5 ppm:** decreased time animal remained in safety compartment before entering shock compartment (with 2 mA footshock), compared to control **All treated groups:** decreased time animal remained in safety compartment before entering shock compartment (with 4 mA footshock) compared to control, but no dose-response Motor activity (measured for 10 min, 1 and 24 h post-exposure): 0.1, 0.2, 1ppm, but not 0.5 ppm: decreased motor activity 1 h post-exposure, reversible after 24 h Antioxidant enzyme levels: Continuous increase in pulmonary and brain Cu/Zn SOD levels up to 0.2 ppm dose group, continuous decrease higher dose		Rivas-Arancibia S. et al. (1998), Environ Res. 76(1):33-9

Method, Guideline, GLP status, Reliability	**Species, Strain, Sex, No/group**	**Test substance, form** *(gas, vapour, dust, mist)* **and particle size** *(MMAD)* **Actual and nominal concentration, Type of administration** *(nose only / whole body/ head only)*	**Value LC_{50}**	**Remarks** *(e.g. major deviations)*	**Reference**
Neurotoxicity study Guideline: None GLP: No Reliability: 2	**Rat**, Wistar, male Experiment 1: n=10 per dose group Experiment 2: n=6 per dose group Experiment 3: n=6 in ozone group, n=5 in control Experiment 4: not reported	Ozone generated from oxygen **0 and 1 ppm** 4 h exposure, closed chamber	Experiment 1: decreased exploratory behaviour and increased freezing behaviour 3 h post-exposure; reversible within 3 days Experiment 2: increased striatal lipoperoxidation levels 3 h post-exposure; reversible within 5d Experiment 3: increased basal dopamine, glutamate and nitric oxide levels; decreased 5-HT; GABA initially decreased (3 h post exposure), then increased (3 and 5 days post exposure) Experiment 4: increased lipofucsine, neuronal cytoplasm and dendrite vacuolation, dilation of rough endoplasmic reticulum cisterns and dark cells in striatal medium spiny neurons	Study was not designed to investigate ozone toxicity. Instead, ozone was used as a model of oxidative stress.	Rivas-Arancibia S. et al. (2003), Pharmacol Biochem Behav.74(4):891-900
Neurotoxicity study Guideline: None GLP: No Reliability: 2	**Rat**, Wistar, male group size not reported	Ozone **0 ppm**, 6 h **0.5 ppm**, 6 h **1.0 ppm**, 3 h Exposure chamber	Reduced amounts of wakefulness and paradoxical sleep, increased slow-wave sleep; lower EEG amplitude; lower HR	All effects were reversible. Administration of atropine sulfate blocked some of the described effects.	Arito H. et al. (1992), Industr. Health, 30: 23-34
Neurotoxicity study Guideline: None	**Rat**, Wistar, male n=10 per dose group	Ozone **0, 0.35, 0.75, 1.5 ppm**	Dose-dependent decrease in paradoxical sleep and increase in slow wave sleep;		Paz C. and Huitron-Resendiz S. (1996),

Method, Guideline, GLP status, Reliability	**Species, Strain, Sex, No/group**	**Test substance, form** *(gas, vapour, dust, mist)* **and particle size** *(MMAD)* **Actual and nominal concentration, Type of administration** *(nose only / whole body/ head only)*	**Value LC_{50}**	**Remarks** *(e.g. major deviations)*	**Reference**
GLP: No Reliability: 2		24 h exposure, closed chamber	wakefulness decrease at highest dose (1.5 ppm); all during exposure Dose-dependent increase in 5-HT concentration in rat pons, however significant only at highest dose group		Neurosci Lett. 204(1-2):49-52
Studies on pulmonary effects					
Acute Toxicity Study Guideline: None GLP: No Reliability: 2	**Rat,** Wistar (Harlan-Sprague Dawley, Indianapolis, IN) **Sex** male **No/group** 2-4 animals	Ozone, generated by ultraviolet light generator (Orec Corp., Phoenix, AR) **0, 1.8 ppm** Single **2h** or **4h** exposure Observations: day 0, 1, 3, 8	<u>Lung Pathology</u> **4hr** exposure effects: Infiltration of neutrophils followed by necrosis, bronchiolar walls were thickened, edema in proximal alveoli, fibrin deposition (lesions restricted to proximal alveolar regions) day3: thickened bronchiolar wall, proliferation of typeII cells, cell debris and foam cells in proximal alveoli day8: lesions resolved <u>BAL parameters:</u> LDH increased,	Study not designed to determine LC50 Study purpose: effects of ozone on inflammatory responses in rat lungs Lung Pathology: (2 animals/time point and concentration) BAL parameters: (4 animals/time point and concentration)	Bassett D. et al. (1988), Lung 166(1):355

Method, Guideline, GLP status, Reliability	**Species, Strain, Sex, No/group**	**Test substance, form** *(gas, vapour, dust, mist)* **and particle size** *(MMAD)* **Actual and nominal concentration, Type of administration** *(nose only / whole body/ head only)*	**Value LC_{50}**	**Remarks** *(e.g. major deviations)*	**Reference**
			Lymphocytes and neutrophils increased, followed by macrophages Bw: weight loss (for 2 and 4 h exposures)		
Acute Toxicity Study Guideline: None GLP: No Reliability: 2	**Rat,** Fischer 344 rats (from Charles River, St. Constant, Québec, Canada) **Sex** male **No/group** 6 animals per treatment group	Ozone, produced from pure oxygen in a silent arc generator (model 200, Sanders, Uetze, Germany) **0.8 ppm** **4h** nose-only exposure Single exposure (1-day exposure) and three consecutive days (3-day exposure) 20h recovery (Time point:24 h)	Increased neutrophils and protein(TNF-α, ET-1) in lavage fluid Decreased phagocytosis and NO production Histology: 4 h exposure resulted in a centriacinar injury, some edema, fibrin deposition in alveolar duct lumen, limited intra-alveolar + interstitial infiltration by neutrophils Morphometry: no significant shifts Higher recoveries of protein, fibronectin, neutrophils for 1-day Reduced yield of macrophages	Study not designed to determine LC50 Study purpose: effects of ozone on lung parameters Cell counting n=30 animals Nitrite n=28 animals TNF-α n= 18 animals MIP-2 n= 20 animals ET-1 n= 16 animals Lung morphometry n= 6 animals Integrity of macrophages n= 12-18 animals 3-day exposure: not reported here (does not belong to acute endpoint)	Bouthillier L. et al. (1998), Am J Pathol. 153(6):1873-84

Method, Guideline, GLP status, Reliability	**Species, Strain, Sex, No/group**	**Test substance, form** *(gas, vapour, dust, mist)* **and particle size** *(MMAD)* **Actual and nominal concentration, Type of administration** *(nose only / whole body/ head only)*	**Value LC_{50}**	**Remarks** *(e.g. major deviations)*	**Reference**
			Phagocytic activity of macrophages depressed, NO-production reduced		
Acute Toxicity Study Guideline: None GLP: No Reliability: 2	**Rat,** Fischer 344 rats (from Charles River Breeding Labs, Raleigh, NC) **Sex** male **No/group** 6 rats/group	Ozone, generated from O_2 using a silent-arc-discharge ozone generator (OREC, Phoenix, AZ) Mixed with filtered room air for dilution Experiment I: **0.1, 0.2, 0.4, 0.8 ppm** ozone Exposure: **2, 4, 8 h** Experiment II: **0.5, 0.8 ppm** ozone and intermittent CO_2 exposure Exposure: **2, 7 h** 1 h post exposure lung function testing	**0.8 ppm**: at all-time points increases in BAL protein (Impact of time on protein permeability as concentration increases) → concentration dependent Minor alterations in dynamic lung function (reduced lung function)	Study not designed to determine LC50 Study purpose: effects of ozone on pulmonary function CO_2 was superimposed upon ozone exposure to stimulate breathing and induce periodic hyperventilation (8 % CO_2+O_3) 7h: 7 x 45 min CO_2+O_3 and 15 min O_3 2h: alternating 15 min Normalization to 2 h exposure group for lung function	Costa D.L. et al. (1989) In: Schneider et al, Eds. Atmospheric ozone research and its policy implications: proceedings of the 3rd US-Dutch international symposium, May 1988. Nijmegen (The Netherlands): Elsevier, 1989:733-743. Studies in environmental sciences, 35.
Acute Toxicity Study Guideline: None GLP: No Reliability: 2	**Rat,** Wistar rats (Hilltop, Scottsdale, PA) **Sex** male **No/group** 3-6 rats/group	Ozone, generated with UV light generator (Orec Corp., model O_3V1-0, Phoenix, AZ) **2 ppm** Exposure: **4h** Time points: 0, 3, 24 h post-exposure	increase of neutrophils in BAL and lung tissue immediately after exposure and maximum at 3h post-exposure, but recovery Neutrophil elevation corresponds with airway hyperresponsiveness	Study not designed to determine LC50 Study purpose: effects of ozone on lung inflammatory parameters	DeLorme M.P. et al. (2002), Journal of Toxicology and Environmental Health, Part A, 65:1453–1470

Method, Guideline, GLP status, Reliability	**Species, Strain, Sex, No/group**	**Test substance, form** *(gas, vapour, dust, mist)* **and particle size** *(MMAD)* **Actual and nominal concentration, Type of administration** *(nose only / whole body/ head only)*	**Value LC_{50}**	**Remarks** *(e.g. major deviations)*	**Reference**
		Single, whole body, exposure	(challenge with methacholine) →nonspecific hyperresponsiveness to methacholine Macrophages in ozone exposed rats 3 h post-exposure lost their membrane integrity (PI-positive stain)		
Acute Toxicity Study Guideline: None GLP: No Reliability: 2	**Rat,** Wistar rats **Sex** male **No/group** 9 rats/group	Ozone, generated with P15 TRIOZON generator (TRIOZON, Tlalnepantla, MX) **1 ppm** Exposure: **1, 3, 6 h** Single, whole body, exposure	TNF-α, IL-6, NF-κB p50 and GFAP are elevated in lung and cerebral cortex Systemic inflammatory response Effects in lung after 3 and 6 h exposure Effects in brain after 6 h exposure	Study not designed to determine LC50 Study purpose: characterisation of inflammatory mechanism in lungs after ozone exposure	Gonzalez-Guevara E. et al. (2014), Inhal Toxicol, 26(8): 485–491
Acute Toxicity Study Guideline: None GLP: No Reliability: 2	**Rat,** Sprague-Dawley rats (from Kingston, New York facility of Charles River Laboratories, Wilmington, MA) **Sex** male/female **No/group** not explicitly mentioned	Ozone, generated by passing O2, in argon, through ozone generator OREC Model 03VI (Ozone Research & Equipment Corp., Phoenix, AZ) **1 ppm**	<u>BALF:</u> PGE_2 production: increase in (as indicator for ozone response) PMN and protein content increased in exposed rats and increases with exposure time	Study not designed to determine LC50 Study purpose: influence of animal age on pulmonary arachidonic acid metabolism after ozone exposure	Gunnison A.F. et al. (1992), Fundam Appl Toxicol. 18(3):360-9

Method, Guideline, GLP status, Reliability	**Species, Strain, Sex, No/group**	**Test substance, form** *(gas, vapour, dust, mist)* **and particle size** *(MMAD)* **Actual and nominal concentration, Type of administration** *(nose only / whole body/ head only)*	**Value LC_{50}**	**Remarks** *(e.g. major deviations)*	**Reference**
	Rats at different ages: 13 days, 18 days, 27 days, 8 weeks, and 16 weeks (13d – 16w: male) (13d + 18 d: male+ female)	Exposure: **2 h** Observations: immediately after exposure (18d old rats) 0, 2, 4 h (all ages) Single, whole body, exposure	16w old males exposed little evidence of damage to cells of the respiratory tract → age-dependent sensitivity to ozone-induced cellular damage young neonates may be at increased risk relative to adults to some consequences of ozone exposure		
Acute Toxicity Study Guideline: None GLP: No Reliability: 2	**Rat,** F344/N (from Inhalation Toxicology Research Institute) **Sex** female **No/group** 6 rats/group	Ozone, generated by OREC Model 03VI-O Ozonizer (Ozone Research & Equipment Corp., Phoenix, AZ) Exposure: **6 h** **0.10, 0.66, 1.23, 1.5 ppm** Time points: 0, 3, 18, 42, 66 h post exposure Single, whole body exposure	**0.66 ppm:** immediately after exposure elevated neutrophils in nasal lavage, then decline →Acute inflammatory response within nasal cavity, restricted to anterior portion (0.66 ppm) Epithelial changes of nasal cavity **1.5 ppm**: number of neutrophils in BAL increasing until 18 h post-exposure, then decline **0.66, 1.23 ppm** ozone**:** bronchiolitis and peribronchiolar alveolitis with inflammatory cell infiltrate (18,42,66 h)	Study not designed to determine LC50 Study purpose: effects of ozone on upper airways Weight varying between 280-400 g Conversion of ozone exposure concentrations to sea-level (Institute location New Mexico 1728 m): 0.12=0.1, 0.8=0.66, 1.5=1.23 ppm	Hotchkiss J.A. et al. (1989a), Exp Lung Res 15:1-16

Method, Guideline, GLP status, Reliability	**Species, Strain, Sex, No/group**	**Test substance, form** *(gas, vapour, dust, mist)* **and particle size** *(MMAD)* **Actual and nominal concentration, Type of administration** *(nose only / whole body/ head only)*	**Value LC_{50}**	**Remarks** *(e.g. major deviations)*	**Reference**
			1.23 ppm O_3: thickening of cell walls of alveoli Acute lung inflammation immediately after exposure to **0.66 and 1.23 ppm** O_3 at the lung, declining Simultaneous competing inflammatory stimuli in nasal cavity and lung		
Acute Toxicity Study Guideline: None GLP: No Reliability: 2	**Rat,** F344/N (from Inhalation Toxicology Research Institute) **Sex** male **No/group** 6 rats/group	Ozone, generated by OREC Model 03VI-O Ozonizer (Ozone Research & Equipment Corp., Phoenix, AZ) Exposure: **6 h** **0.12, 0.8, 1.5 ppm** Observations: 0, 3, 18, 42, 66 h post exposure Single, whole body inhalation	From 18 h post-exposure (0.8 and 1.5 ppm): mild bronchiolitis and peribronchiolar alveolitis, progressive thickening of the walls of terminal bronchioles and proximal alveoli 0.8 and 1.5 ppm (time-dependent): increase of alveolar macrophages **1.5 ppm**: transient influx of neutrophils →inflammatory response **0.12 ppm**: no histologic alterations at any time	Study not designed to determine LC50 Study purpose: effects of ozone on rat pulmonary alveolar macrophages Weight varying between 280-400 g (12-18 weeks old)	Hotchkiss J.A. et al. (1989b), Toxicol Appl Pharmacol 98:289- 302
Acute Toxicity Study Guideline: None	**Monkey**, rhesus, male **No/group:** 2-6	Ozone, generated by passing O_2 through Sanders model 25 ozonizer (Eltze, Germany)	Inhibition of neutrophil emigration and accumulation of necrotic airway epithelial cells	Study not designed to determine LC50	Hyde D.M. et al. (1999), Am. J. Physiol. 277: L1190-L1198

Method, Guideline, GLP status, Reliability	**Species, Strain, Sex, No/group**	**Test substance, form** *(gas, vapour, dust, mist)* **and particle size** *(MMAD)* **Actual and nominal concentration, Type of administration** *(nose only / whole body/ head only)*	**Value LC_{50}**	**Remarks** *(e.g. major deviations)*	**Reference**
GLP: No Reliability: 2		**0.8 ppm** ozone Exposure: **8 h** Observations: 24, 48 h post-exposure	(α-CD18 MAb treated, ozone exposed animals) Increase of PMNs (24 h post-exposure), none observed 48 h, therefor Macrophages increased 48 h thickness of the respiratory bronchiolar epithelium was significantly increased in ozone-exposed monkeys at 24 h (recovery) no necrotic epithelial cells beyond 24 h	Study purpose: effect of neutrophil influx on necrotic airway epithelial cells following ozone exposure	
Acute Toxicity Study Guideline: None GLP: No Reliability: 2	**Rat,** Sprague-Dawley rats (from Bantin & Kingman, Inc., Freemont, CA) **Sex** male **No/group** 5-7 animals/group ANS (rabbit-anti-rat neutrophil serum) and NRS (normal rabbit serum) treated animals	Ozone **0.94± 0.03 to 1.03 ± 0.03 ppm** Exposure: **8 h** Observations: 0, 4, 16 h post-exposure	Mild interstitial edema and fibroblast swelling in bronchiolar walls Necrosis of type I pneumocytes in alveoli Neutrophils peak at 4 h (in BALF and morphometry) Decrease in ciliated/ necrotic cells in bronchiole	Study not designed to determine LC50 Study purpose: effect of neutrophils on ozone-induced epithelial damage in lung all animals treated with ANS or NRS and exposed to ozone → no exposure only with ozone	Pino M.V. et al. (1992), Toxicol. Appl. Pharmacol. 114: 268-276

Method, Guideline, GLP status, Reliability	**Species, Strain, Sex, No/group**	**Test substance, form** *(gas, vapour, dust, mist)* **and particle size** *(MMAD)* **Actual and nominal concentration, Type of administration** *(nose only / whole body/ head only)*	**Value LC_{50}**	**Remarks** *(e.g. major deviations)*	**Reference**
Acute Toxicity Study Guideline: None GLP: No Reliability: 2	**Rat,** Sprague-Dawley rats (from Hiltop Lab Animals, Inc., Scottdale, PA) **Sex** male **No/group** 6 /exposure group for every post-exposure observation 2/ control group for every post-exposure observation	Ozone, generated by passing medical-grade O2 through Sanders ozonizer (Type III, Osterberg, Germany) **0.8 ppm** Exposure: **3 h** Observations: 0, 4, 8, 12, 16, 20, 24 h post-exposure Nose-only exposure	Short lived inflammatory response PMN elevated at 16 h post-exposure, but decline to control level PMNs migration from blood to the interstitium after ozone exposure Tracheal permeability increased immediately following ozone exposure (max. 8 h), then decline to control	Study not designed to determine LC50 Study purpose: effects of ozone on tracheal epithelial permeability and PMN populations	Young C. and Bhalla D.K. (1992), Fundamental and Applied Toxicology 18: 175-180
Acute Toxicity Study Guideline: None GLP: No Reliability: 2	**Rat,** Wistar, 7 wk old **Sex** male **No/group** 3 animals/group	Ozone, generated by irradiation of oxygen with UV-light Experiment 1: sedentary rats (day-time exposure) 0.75, 1.5, 2.5 or 4.0 mg/m³ (0.375, 0.75, 1.25, 2.0 ppm) 0, 1, 2, 4, or 8 h recovery until 54h Experiment 2: active rats (night-time exposure) 0.25, 0.50 or 0.75 mg/m³ (0.125,	Experiment 1 BALF: protein influx after acute exposure, fast increase followed by a gradual decrease of the protein concentration with a maximum response at 22 h 4h + 8h exposure (0.75 ppm): significant increase of protein in BALF at all-time points Experiment 2:	Study not designed to determine LC50 Study purpose: correlation of ozone exposure concentration and exposure time	Rombout P.J.A. et al. (1989), In: Schneider et al, Eds. Atmospheric ozone research and its policy implications: proceedings of the 3rd US-Dutch international symposium, May 1988. Nijmegen (The Netherlands): Elsevier, 1989:701-10. Studies in environmental sciences, 35.

Method, Guideline, GLP status, Reliability	**Species, Strain, Sex, No/group**	**Test substance, form** *(gas, vapour, dust, mist)* **and particle size** *(MMAD)* **Actual and nominal concentration, Type of administration** *(nose only / whole body/ head only)*	**Value LC_{50}**	**Remarks** *(e.g. major deviations)*	**Reference**
		0.25, 0.375 ppm) 0, 4, 8, or 12 h whole body exposure	BALF: protein still elevated after 8h or 12h exposure and recovery → strong influence of time to response of protein influx in BALF increases with concentration		
Acute Toxicity Study Guideline: None GLP: No Reliability: 2	**Rat,** Sprague-Dawley **Sex** male, young	**Test substance:** ozone **0.5 ppm** **2, 4 or 6 h**	Type II cells: resistant to damage by ozone Injured type I cells by ozone Mild swelling of mitochondria (earliest alterations: type I cells, 2h), epithelium peeling away from basement lamina predominantly at alveoli beyond terminal bronchiole Type II cells spreading over the injured area (after 4 and 6h) →recovery	Study not designed to determine LC50 Study purpose: effects of ozone on alveolar cell response	Stephens R.J. et al. (1974), Exp. Mol. Pathol. 20: 11
Acute Toxicity Study Guideline: None GLP: No Reliability: 2	**Rat,** Sprague-Dawley rats (from Hiltop Lab Animals, Inc., Scottdale, PA) **Sex** male **No/group** 35 in total	**Test substance:** Ozone 0.5 ppm: 0.54 ± 0.08 ppm 0.9 ppm: 0.88 ± 0.08 ppm ozone	2h (0.9 ppm): severe loss of cilia at terminal bronchiole (minor effect at 0.5 ppm), and damaged type I cells 6-9h (0.9 ppm): necrotic ciliated cells in epithelium and free in the lumen	Study not designed to determine LC50 Study purpose: early response of lung to low levels of ozone	Stephens R.J. et al. (1973), Amer. J. Pathol. 74:31

Method, Guideline, GLP status, Reliability	**Species, Strain, Sex, No/group**	**Test substance, form** *(gas, vapour, dust, mist)* **and particle size** *(MMAD)* **Actual and nominal concentration, Type of administration** *(nose only / whole body/ head only)*	**Value LC_{50}**	**Remarks** *(e.g. major deviations)*	**Reference**
		Experiment 1: exposure up to 48h (0.5 and 0.9 ppm) Experiment 2: 2, 4, 8, 12 h exposure and recover post-exposure for the remainder of 48-h period (0.9 ppm) Other experiments: exposed continuously for as long as 6 months and were sacrificed at various intervals beginning at 72 hours (not reported here, because does not fit to acute exposure)	24h (0.9 ppm): further cell damage and loss (minimal at 0.5 ppm) mucous layer present after 10-12h exposure beyond 48 h: epithelial response reverted towards normal state After recovery period, macrophages seem to accumulate within the lumen of terminal bronchiole Authors description: *initial injurious phase that reaches significant proportions within 2 to 4 h and continues to increase in severity for the next several hours, little change in the extent of the damage after 8 to 10 h of exposure*		
Acute Toxicity Study Guideline: None GLP: No Reliability: 2	**Rat,** Sprague-Dawley rats (from Kingston, New York facility of Charles River Laboratories, Wilmington, MA)	Ozone, Exposure: **1-4 h** 1. virgin rats: **0.5, 0.8, or 1.1 ppm**	assessment of dose (isotope: $^{18}O_3$ for 3h) and inflammatory responses (isotope: $^{16}O_3$ for 4h) BALF (20h after exposure): PMN: stat. sign. increased in	Study not designed to determine LC50 Study purpose: inflammatory effects of	Gunnison A.F. and Hatch G.E. (1999), Am J Physiol. 276(2 Pt 1):L332-40

Method, Guideline, GLP status, Reliability	**Species, Strain, Sex, No/group**	**Test substance, form** *(gas, vapour, dust, mist)* **and particle size** *(MMAD)* **Actual and nominal concentration, Type of administration** *(nose only / whole body/ head only)*	**Value LC_{50}**	**Remarks** *(e.g. major deviations)*	**Reference**
	Sex female **No/group** 5-7	2. pregnant rats at gestational day (GD)17: **0.5 or 0.8 ppm** 3. lactating rats, 13 days postpartum: **0.5 or 0.8 ppm** Single, nose-only, inhalative exposure	pregnant and lactating rats compared to virgin rats at same concentration; increase with concentration Protein: increase with concentration; lactating rats stat. sign. from virgin rats → direct proportionality of PMN inflammation with the estimate of relative dose to the lower lung regardless of physiological status (pregnancy)	ozone in pregnant and lactating rats Study submitted for chapter 3.10 (reproductive toxicity) but re-allocated	
Acute Toxicity Study Guideline: None GLP: No Reliability: 2	**Rat,** Sprague-Dawley, pregnant and virgin females 6-16 per exposure group <u>Exposure groups:</u> pregnant rats at days 10-12 and day 17 of pregnancy; rats 3, 13 and 20 days postpartum; rats 14 days after termination of lactation 8-9 wk-old virgin and 13-17 wk-old virgin rats <u>Control groups:</u> pregnant rats 17 days after conception; lactating rats	Ozone generated from oxygen 1 ppm 6 h exposure in chambers	n/a – only one dose tested Indicators of pulmonary inflammation (total leukocytes, total PMNs, protein, β-glucuronidase activity, LDH activity) were analysed in bronchoalveolar lavage. Exposed rats at 17 days of pregnancy and exposed lactating rats showed increases in the inflammatory indicators compared to exposed virgin rats. No difference was seen during early pregnancy. No differences between virgin and pregnant rats were observed at	Study designed to investigate differences in sensitivity to oxidants (ozone) between pregnant and non-pregnant rats It was not clearly reported whether rats were exposed several times during different pregnancy/lactation stages or only once Study submitted for chapter 3.10 (reproductive toxicity) but re-allocated	Gunnison A.F. et al. (1992a), Fundam Appl Toxicol. 18(3):360-9

Method, Guideline, GLP status, Reliability	**Species, Strain, Sex, No/group**	**Test substance, form** *(gas, vapour, dust, mist)* **and particle size** *(MMAD)* **Actual and nominal concentration, Type of administration** *(nose only / whole body/ head only)*	**Value LC_{50}**	**Remarks** *(e.g. major deviations)*	**Reference**
	13 and 20 days postpartum; 8-9 wk-old virgin rats		14 days after termination of lactation.		

Table **41**: Setting of specific concentration limits for STOT SE (nervous system)

Study references	**Effective dose (ppm)**	**Species, Length of exposure**	**SCL Cat.1**	**SCL Cat.2**
Avila-Costa M.R. et al. (1999), Neurosci Lett 270:107–9 Avila-Costa M.R. et al. (2001), Int J Neurosci. 108(3-4):193-200 Rivas-Arancibia S. et al. (1998), Environ Res. 76(1):33-9 Rivas-Arancibia S. et al. (2003), Pharmacol Biochem Behav.74(4):891-900	1.0 Long-term memory alteration, reduction in number of dendritic spines in hippocampus, altered motor behaviour and reduction in number of dendritic spines in striatum and prefrontal cortex	Rat 4h	SCL Cat.1= (1ppm/2500 ppm)x100 % = 0.04 % → 0.02 %	SCL Cat.2 = (1ppm/20000 ppm)x100 % = 0.01 %

Table 42: Summary table of human data on STOT SE

Reference / study characteristics	**ozone exposure**			**(Lung) function parameters**	**Venti-lation rate**	**Results**	**Others/ Remarks**
	Conc. mg/m3	**Conc. ppm**	**Duration hours**				
Adams, W. C. 2002, 30 healthy non-smoking, non-asthmatic individuals, male and female subjects, young adults, normal lung function, chamber or	0 0.09 0.16 0.26	0 0.04 0.08 0.12	6.6 including six 50-min periods of exercise	Lung function, subjective symptoms effect FVC, $FEV_{1.0}$, $\%FEV_{1.0}$/ FVC	normalised to body surface: 20 $L/m^2/min$	Post-exposure % change in FVC and $FEV_{1.0}$ was significantly greater at 0.08 ppm than at 0.04 ppm or in free air. TSS (Total symptoms score by throat tickle, cough, shortness of breath and pain on deep inspiration) and PDI (Pain	Chamber 1328-M: 2.45x2.45x2.39 m Air volume: 14.3 m^3 Silicone rubber face mask: 97 ml dead space

Reference / study characteristics	ozone exposure			(Lung) function parameters	Venti-lation rate	Results	Others/ Remarks
	Conc. mg/m3	Conc. ppm	Duration hours				
face-mask exposure; 6 subjects /group				Protocols: 1. Chamber 0.12 ppm 2. Free air 3. Mask 0.12 ppm 4. Mask 0.08 ppm 5. Mask 0.04 ppm		on deep inspiration) significantly greater at 0.12 ppm using chamber and mask, TSS at 0.08 ppm significantly greater than free air. No significant differences in changes of FVC, FEV and % $FEV_{1.0}$/FVC after chamber compared to face-mask exposure. **LOAEC: 0.08 ppm (FVC, $FEV_{1.0}$ and TSS)** **NOAEC: 0.04 ppm**	
Adams, W. C. 2006, 30 healthy non-smoking, non-asthmatic individuals, male and female subjects, young adults, normal lung function	0 0.09 0.13 0.13 0.17 0.17	0 0.04 tri 0.06 0.06 tri 0.08 0.08 tri	6.6 including six 50-min periods of exercise	Lung function, subjective symptoms effect FVC, $FEV_{1.0}$, %$FEV_{1.0}$/ FVC Protocols: 1. Free air 2. 0.08 3. triangular mean 0.08 ppm 4. 0.06 ppm 5. triangular mean 0.06 ppm 6. triangular mean 0.04 ppm	normalised to body surface: 20 $L/m^2/min$	% change in FEV_1 and FVC for 0.08-ppm protocols were significantly greater than for all other protocols showing a mean % change from 4.5 to 5.7. With respect to hourly changes for the triangular ozone exposure averaging 0.08 ppm in % change FEV_1 was significantly decreased from pre-exposure at 4.6 h. All exposures at all-time point measured were consistently below 10 % change FEV_1 comparing to free air or pre-exposure. TSS (Total symptoms score) and PDI (Pain on deep inspiration) significantly greater at 0.08 ppm. **LOAEC: 0.08 ppm (FVC, $FEV_{1.0}$, PDI and TSS)** **NOAEC: 0.06 ppm**	Chamber 1328-M: 2.45x2.45x2.39 m Air volume: 14.3 m^3
Schelegle, E. S. et al. 2009 / 31 healthy non-smoking, non-asthmatic individuals, male and female subjects, 18 – 25 y; normal lung function	0 0.13 0.15 0.17 0.19	0 0.06 0.07 0.08 0.087	6.6 including six 50-min periods of exercise	Lung function, subjective symptoms effect FVC, $FEV_{1.0}$, %$FEV_{1.0}$/ FVC	normalised to body surface: 20 $L/m^2/min$	Significant changes were observed for FVC at 0.08 ppb upon 4.6 h, for $FEV_{1.0}$ at 0.07 ppb upon 6.6 h and for TSS at 0.07 ppb upon 5.6 h. **LOAEC: 0.07 ppm ($FEV_{1.0}$,**	Chamber 1328-M: 3.2x2.0x2.2 m Air volume: 14.1 m^3

Reference / study characteristics	ozone exposure			(Lung) function parameters	Venti-lation rate	Results	Others/ Remarks
	Conc. mg/m3	Conc. ppm	Duration hours				
						TSS) **NOAEC: 0.06 ppm**	
Kim, C. S. et al. 2011 / 59 healthy non-smoking, non-asthmatic individuals, male and female subjects, 19 – 35 y; normal lung function	0 0.13	0 0.06	6.6 including six 50-min periods of exercise, no kinetics	Lung function FVC, $FEV_{1.0}$, PMN neutrophil response (% PMN in sputum samples) GSTM1 (glutathione S-transferase mu 1) genotyping	normalised to body surface: 20 $L/m^2/min$	Significant changes were observed for FVC, $FEV_{1.0}$ (decrease) and % PMN (increase) at 0.06 ppb and 6.6 h. 10 out of 24 subjects with > 20 % PMN number increase. No significant effect of GSTM1 observed. **LOAEC: 0.06 ppm** ($FEV_{1.0}$, PMN neutrophil response) **NOAEC: not derived**	Chamber: 4x6x3.2 m Air volume: 76.8 m^3
Alexis, N. E. et al. 2010 / 15 healthy non-smoking, non-asthmatic individuals, male and female subjects, 19 – 35 y; normal lung function	0.17	0.08	6.6 including six 50-min periods of exercise, no kinetics	Airway inflammation % change in inflammatory cells (neutrophils, monocytes, and dendritic cells) after ozone exposure, detected by flow cytometry	normalised to body surface: 20 $L/m^2/min$	Before and after ozone exposure: PMNs: 349 ± 109 and 895 ± 217 Mo: 68 ± 12 and 128 ± 36 DCs: 6.0 ± 2 and 11 ± 5 MØ: 355 ± 67 and 337 ± 81 all cells/mg sputum Phenotype: Mo: CD14, CD86, HLA-DR increased, CD80 decreased MØ: CD14 increased, CD80, DR decreased DCs, PMNs not affected. Cytokines: IL-6, IL-8, IL-12p70, TNFα increased. **LOAEC: 0.08 ppm** (inflammatory cells and cytokines increased)	Total and differential cell counts of sputum samples from saliva fluid, no exercise; no information on air change, humidity. No control air exposure.
Bates, M.L. 2014 / 20 healthy smokers, 20 – 28	0.6	0.3	1 including exercise	$FEV_{1.0}$, VD: dead space, SN: slope of	n.r.	Decline FEV_1 Smokers $\Delta = -8.7 \pm 1.9$ %	Limited smoking history in all smokers:

Reference / study characteristics	ozone exposure			(Lung) function parameters	Venti-lation rate	Results	Others/ Remarks
	Conc. mg/m3	Conc. ppm	Duration hours				
y and 30 non-smokers 19 - 31 y, all non-asthmatic individuals, male and female subjects,; normal lung function				the alveolar plateau		Non-smokers Δ = −9.5 ± 1.8 % Changes in VD: Smokers Δ=−6.1± 1.2 % SN:Smokers Δ=9.1± 3.4 % **LOAEC: 0.3 ppm** (FEV_1, VD, SN) **NOAEC: not derived**	2 - 10 y, 2 - 6 packs/week The ozone -induced increase in SN suggests a loss of gas transport efficiency in their peripheral airspaces. Failure to increase VD/VT makes smokers more prone to a greater delivery of ozone to their peripheral airspaces. This smoking study was funded by a grant from the Philip Morris External Research Program.
Folinsbee, L.J. and Hazucha, M.J. 2000 / 19 healthy females, 20 – 25 y; normal lung function	0 0.8	0 0.35	1.5 including exercise	$FEV_{1.0}$, FVC, FEV25-75 %, PEF, FIVC, $FIV_{0.5}$ (inspired volume during the first 0.5 s of an FIVC manoeuvre), PIF, PC100 Raw (provocative concentration of methacholine required to double airway resistance (PC100 Raw), TGV (thoracic gas volume)	40 L/min (during exercise)	Changes in lung function Expired spirometry $FEV_{1.0}$ -19.9 % FVC -13.2 % $FEV_{25-75\,\%}$ -29.9 % PEF -22.8 % Inspired spirometry FIVC -10.6 % $FIV_{0.5}$ -20.8 % PIF -20.6 % Airway resistance PC100 Raw -23,7 IU (1 h) TGV - 0.03 L (1 h) **LOAEC: 0.35 ppm** (Spirometry parameters, PC100 RAW) **NOAEC: not derived**	Chamber: 4x6x3.2 m All effects reversible at 42 h. Participants were preselected for their responsiveness to ozone.
Hernandez M.L. et al. 2010a / 15 healthy	0, Pre-ozone	0, Pre-ozone	2 including exercise	Airway inflammation % change in sputum	30 – 40 L/min	PMN number in sputum: ozone + 21.8 %	Chamber: US EPA chamber, not

Reference / study characteristics	ozone exposure			(Lung) function parameters	Venti-lation rate	Results	Others/ Remarks
	Conc. mg/m3	Conc. ppm	Duration hours				
volunteers, 9 females, 6 males, 20-30 y, including 4 atopics, normal lung function	0.9	0.4		neutrophils, phenotype of monocytes and macrophages after ozone exposure, detected by flow cytometry		Phenotype sputum cells: Mo: CD14, CD11b, HLA-DR increased, CD86 not affected MØ: CD11b, HLA-DR increased, CD14 and CD86 not affected Cytokines: IL-1β, IL-6, IL-8, TNFα not affected. **LOAEC: 0.4 ppm** (PMNs, phenotype) **NOAEC: 0 ppm**	further specified
Hernandez M.L. et al. 2010b / 25 healthy volunteers (hv), 19 - 27y, 14 F/11 M; 14 atopic nonasthmatic volunteers (anv) / 20 - 30 y, F/7 M; 11 atopic asthmatic volunteers (aav), 19 - 32 y, 6 F/5 M normal lung function	0, Pre-ozone 0.9	0, Pre-ozone 0.4	2 including exercise	Lung function, $FEV_{1.0}$, FVC; Airway inflammation % change in sputum neutrophils, macrophages and eosinophiles, phenotype of macrophages after ozone exposure, detected by flow cytometry	30 – 40 L/min	$FEV_{1.0}$ and FVC decreased in all 3 cohorts Number/mg sputum after ozone compared to Pre- ozone: Increase n.s. for PMNs in hv Decrease s. for MØ in aav Increases n.s. for eosinophils in anv and aav, no increase in hv. Phenotype sputum cells: MØ: CD11b, CD23, FceRI, TLR4 increased in aav; CD80, TLR2, CD14, HLA-DR not affected in any cohort Cytokines: IL-1β, IL-8, IL-6 increased in aav , IL-8, IL-5 increased in anv IL-10 decreased in aav Hyaluronic acid levels increased in aav and anv **LOAEC: 0.4 ppm** (cell number, phenotype, cytokines, hyaluronic acid) **NOAEC: not derived**	Chamber: US EPA chamber, not further specified

Reference / study characteristics	ozone exposure			(Lung) function parameters	Venti-lation rate	Results	Others/ Remarks
	Conc. mg/m3	Conc. ppm	Duration hours				
Jörres R.A. et al. 2000, 23 healthy, non-smoking volunteers (15 M, 8 F), 21 - 35 y, repeated exposures	0 0.4	0 0.2	4	Lung function, $FEV_{1.0}$, FVC; Airway inflammation % change in sputum neutrophils, macrophages and eosinophiles, phenotype of macrophages after ozone exposure, detected by flow cytometry	normalised to body surface: 14.8 $L/m^2/min$)	Decrease in FEV_1: Day1 -13 % Day2 -17 % Day3 -8 % Day4 -2 % Decrease in FVC: Reported without data. Cell numbers /mm^2 in airway mucosal biopsies: MØ: 36 (FA), 41 (1d ozone), 46 (4d ozone) Lymphocytes: 23 (FA), 23 (1d ozone), 28 (4d ozone) PMN: 16 (FA), 16 (1d ozone), 30 (4d ozone) Eosinophils: 3 (FA), 2 (1d ozone), 5 (4d ozone) MC: 25 (FA), 19 (1d ozone), 23 (4d ozone) Scores for bronchitis, erythema, hypervulnerability were enhanced after 4 day ozone exposure. Soluble Components of BALF: Increased: total protein, IL-6, IL-8, reduced glutathione, and orthotyrosin, IL-10 after repeated exposure. **LOAEC: 0.2 ppm** ($FEV_{1.0}$, FVC, Airway inflammation) **NOAEC: not derived**	Plexiglas helmet (30x 30 cm), 4 exposure periods
Stenfors N. et al. 2002, 15 healthy (6 M/9 F, 19 - 31 y) and 15 mild-asthmatic (9 M/6 F, 21 - 48 y), non-smoking	0 0.4	0 (FA) 0.2	2	Lung function, FVC, $FEV_{1.0}$, PMN, IL-6, IL-8, MPO	n. r.	FVC (l): 5.1 (post air), 4.8 (post ozone), in healthy controls 5 (post air), 5 (post ozone), in mild	Exposure chamber according to Blomberg A, 1999. No further information on chamber size and conditions. Mild

Reference / study characteristics	ozone exposure			(Lung) function parameters	Venti-lation rate	Results	Others/ Remarks
	Conc. mg/m3	Conc. ppm	Duration hours				
volunteers, normal lung function						asthmatics $FEV_{1.0}$ (l/s): 4.1 (post air), 3.7 (post ozone), in healthy controls 3.7 (post air), 3.7 (post ozone), in mild asthmatics Analyses of airway lavages and bronchial biopsies: Neutrophil recruitment (PMN): Similar increase at 0.2 ppm ozone in healthy volunteers and mild asthmatics. Inflammation: no significant differences in fold-change of IL-6, IL-8 and MPO in healthy volunteers and mild asthmatics **LOAEC: 0.2 ppm** (FVC, $FEV_{1.0}$, PMN) **NOAEC: not derived**	asthmatics were subjects of this study.
Tank J. et al. 2011, 14 healthy (11 M/3 F, 22 - 47 y) volunteers, normal lung function	0 0.5	0 (CA) 0.25	3, including exercise	Lung function, FVC, $FEV_{1.0}$, ECG, finger blood pressure, brachial blood pressure, respiration, cardiac output, muscle sympathetic nerve activity (MSNA)	normalised to body surface: 20 l/min/m^2	FVC (l): 5.5 (pre ozone), 5.2 (post ozone) $FEV_{1.0}$ (l/s): 4.4 (pre ozone), 4.03 (post ozone) Airway inflammation: 16 % increase of sputum neutrophils after ozone compared to clean air. Systemic inflammation: 10.2 % increase of blood neutrophils after ozone compared to clean air.	Fraunhofer ozone exposure chamber (2.7x 2.3 x2.5 m^3), air temperature and relative humidity 20–25°C and 40–60 %, randomized, placebo controlled, crossover study

Reference / study characteristics	ozone exposure			(Lung) function parameters	Venti-lation rate	Results	Others/ Remarks
	Conc. mg/m3	Conc. ppm	Duration hours				
						Effect reversed after 24 h. Cardiovascular parameters: Resting heart rate (clean air: 59, ozone 60 bpm) blood pressure (clean air: 121/71 mmHg; ozone: 121/71mmHg) Cardiac output (clean air: 7.4 mmHg; ozone: 8 l/min) Plasma norepinephrine levels (clean air: 213 pg/ml; ozone: 202 pg/ml) MSNA (air: 23, ozone: 23 bursts/min). Acute ozone-induced airway inflammation did not increase resting sympathetic nerve traffic. **LOAEC: 0.25 ppm** (FVC, $FEV_{1.0}$, neutrophils) **NOAEC: not derived**	

10.11.1 Short summary and overall relevance of the provided information on specific target organ toxicity – single exposure

Animal studies on acute inhalation toxicity report inconsistent results on the cardiovascular system but neurotoxicological effects below 250 ppm (i.e. at least one magnitude below the guidance value of 2500 ppm) support classification for STOT SE 1; H370 (nervous system): Effects observed were morphological changes in different brain regions and behavioural changes.

NOAEC of 60 ppb was derived based on changes in lung function (FVC, FEV1.0) and symptoms score reported in the controlled human volunteer studies by Adams, W. C. 2002 and 2006. Furthermore, two 6.6 h exposure studies by Schelegle, E. S. and Kim, C. S were regarded as crucial studies and report a LOAEC of 70 ppb and 60 ppb, respectively. As supporting studies on airway inflammation reported upregulation of lung cytokines and immune cells from the level 80 ppb. AHR studies, which comprehend both hazards respiratory sensitization and STOT SE are listed in 10.6.

Conclusion on classification and labelling for Respiratory tract irritation

Laboured breathing and oedema as observed in acute toxicity studies in animals as summarized in chapter 10.3 are consistent with respiratory tract irritation. More detailed investigations on respiratory tract irritation were performed in studies of lung function parameters with human volunteers (Table 42). In two studies by Adams, the TSS (total symptoms severity) and PDI (pain on deep inspiration) both were significantly higher at 0.08 ppm. Total symptoms severity (TSS) was calculated as the sum of the severity ratings for individual symptoms throat tickle, cough, and pain on deep inspiration (PDI) indicating respiratory tract irritation. These results were confirmed by a study of Schelegle (2009) who identified significant changes of the TSS at 0.07 ppm and by a study of Kim (2011) who found polymorphonuclear neurophil (PMN) increase at 0.06 ppm.

In interpreting adverse effects in humans for respiratory tract irritation after exposure to ozone it is important to define an adverse effect. Diagnostically spirometry is performed to evaluate lung function. Spirometry is performed by deeply inhaling and forcefully exhaling into a spirometer (the device that records the various measurements of lung function). There are two measurements that are crucial in the interpretation of spirometry results. The first is called the forced vital capacity (FVC). This is a measurement of lung size (in litres) and represents the volume of air in the lungs that can be exhaled following a deep inhalation. The second is the forced expiratory volume-one second (FEV1). This is a measure of how much air can be exhaled in one second following a deep inhalation. You will also see another number on the spirometry test results, the FEV1/ FVC ratio. This ratio represents the percent of the lung size (FVC) that can be exhaled in one second. For example, if the FEV1 is 4 and the FVC is 5, then the FEV1/ FVC ratio would be 4/5 or 80 %. This means the individual can breathe out 80% of the inhaled air in the lungs in one second. The three key spirometry measurements (the FVC, FEV1 and FEV1/FVC ratio) for a given individual are compared to reference values. The reference value is based on healthy individuals with normal lung function and it tells the doctor the values that would be expected for someone of the same sex, age and height. To find the reference value on your spirometry report, look for the column marked “reference” or “predicted” value.

Interpretations of spirometry results require comparison between an individual’s measured value and the reference value. If the FVC and the FEV1 are within 80% of the reference value, the results are considered normal. The normal value for the FEV1/FVC ratio is 70% (and 65% in persons older than age 65). When compared to the reference value, a lower measured value corresponds to a more severe lung abnormality. (See table below.)

Therefore, a decrease in FEV1, FVC, FEV1/FVC of >10% would be a biologically relevant change in that parameter based on moderate abnormal findings. Ozone at 90-100 ppb is expected to result in this biologically relevant effect (decrease in FEV1 of >10%). Based on the results of Schelegle et al (2009), increases in total subjective symptoms scores were reported at 70 ppb and this is in agreement with EPA (2020) which reported no statistically significant effects in respiratory symptoms reported in any of the studies at 60 ppb ozone.

Table 43:

SPIROMETRY TEST	NORMAL	ABNORMAL	
FVC and FEV1	Equal to or greater than 80%	Mild Moderate Severe	70-79% 60-69% less than 60%
FEV1/FVC	Equal to or greater than 70%	Mild Moderate Severe	60-69% 50-59% less than 50%

10.11.2 Comparison with the CLP criteria

Toxicological results *	CLP criteria
Impact on the cardiovascular system: Inconsistent results in heart rate, arrhythmia and blood pressure in studies with high quality in the WoE-evaluation by Goodman. No classification into category STOT SE1 (cardiovascular system) (H370) is proposed by the dossier submitter. **Impact on the nervous system:** Significant toxicity to the CNS was observed after single exposure at 1 ppm. Long-term memory alteration, reduction in number of dendritic spines in hippocampus, altered motor behaviour and reduction in number of dendritic spines in striatum and prefrontal cortex are observations of severe toxic effects of relevance to human health. Based on these findings STOT SE1 (nervous system) (H370), is proposed by the dossier submitter. An SCL ≥ 0.02 % was derived but is not proposed.	Category 1 (H370): Substances that have produced significant toxicity in humans or that, on the basis of evidence from studies in experimental animals, can be presumed to have the potential to produce significant toxicity in humans following single exposure Substances are classified in Category 1 for specific target organ toxicity (single exposure) on the basis of: a. reliable and good quality evidence from human cases or epidemiological studies; or b. observations from appropriate studies in experimental animals in which significant and/or severe toxic effects of relevance to human health were produced at generally low exposure concentrations. Guidance dose/concentration values are provided below (see 3.8.2.1.9) to be used as part of weight-of-evidence evaluation.
Impact on the respiratory system: In a human study by Lin (2008) on asthma hospital admissions the risk of hospital admissions increased 22 % with a 1-ppb increase in mean ozone concentration. Acute, unspecific hyperreactivity, exacerbation or AHR (airway hyperresponsiveness) is a serious health impairment which is observed after single exposure (in a chronic study) and applies for Cat. 3 criteria. Studies on AHR are listed in 10.6, but can be used for classification STOT SE. Based on these findings STOT SE3 (H335) is proposed by dossier submitter.	Category 3 (H335): Transient target organ effects This category only includes narcotic effects and respiratory tract irritation. These are target organ effects for which a substance does not meet the criteria to be classified in Categories 1 or 2 indicated above. These are effects which adversely alter human function for a short duration after exposure and from which humans may recover in a reasonable period without leaving significant alteration of structure or function. Substances are classified specifically for these effects as laid down in 3.8.2.2
Annex 1: 3.8.2.2.1 Criteria for respiratory tract irritation The criteria for classifying substances as Category 3 for respiratory tract irritation are: (a) respiratory irritant effects (characterized by localized redness, oedema, pruritis and/or pain) that impair function with symptoms such as cough, pain, choking, and breathing difficulties are included. This evaluation will be based primarily on human data. (b) subjective human observations could be supported by objective measurements of clear respiratory tract irritation (RTI) (such as electrophysiological responses, biomarkers of inflammation in nasal or bronchoalveolar lavage fluids). (c) the symptoms observed in humans shall also be typical of those that would be produced in the exposed population rather than being an isolated idiosyncratic reaction or response triggered only in individuals with hypersensitive airways. Ambiguous reports simply of "irritation" shall be excluded as this term is commonly used to describe a wide range of sensations including those such as smell, unpleasant taste, a tickling sensation, and dryness, which are outside the scope of classification for respiratory irritation.	

Toxicological results *	CLP criteria
(d) there are currently no validated animal tests that deal specifically with RTI, however, useful information may be obtained from the single and repeated inhalation toxicity tests. For example, animal studies may provide useful information in terms of clinical signs of toxicity (dyspnoea, rhinitis etc.) and histopathology (e.g. hyperemia, edema, minimal inflammation, thickened mucous layer) which are reversible and may be reflective of the characteristic clinical symptoms described above. Such animal studies can be used as part of weight of evidence evaluation. (e) this special classification would occur only when more severe organ effects including in the respiratory system are not observed.	

10.11.3 Conclusion on classification and labelling for STOT SE

Based on the results listed above, harmonised classification and labelling for specific target organ toxicity – single exposure is proposed: STOT SE 1, H370 – "Causes damage to organs (nervous system)" and STOT SE 3, H335 "May cause respiratory irritation".

RAC evaluation of specific target organ toxicity – single exposure (STOT SE)

Summary of the Dossier Submitter's proposal

The DS proposed to classify ozone as STOT SE 1; H370 with the following target organs: cardiovascular system and respiratory system. In addition, the DS proposed classification as STOT SE 3 (H335, may cause respiratory irritation).

Cardiovascular system

The DS noted inconsistent results in heart rate (HR), arrhythmia and blood pressure in rats in the assessed studies. Therefore, the DS proposed no classification into category STOT SE 1 (cardiovascular system) (H370).

Nervous system

The DS noted significant toxicity to the central nervous system (CNS) in rats after a single exposure at 1 ppm of ozone. Long-term memory alteration, reduction in number of dendritic spines in hippocampus, altered motor behaviour and reduction in number of dendritic spines in striatum and prefrontal cortex are observations of severe toxic effects of relevance to human health. Based on these findings, STOT SE 1; H370 (nervous system), was proposed by the DS. SCLs ≥ 0.02% and ≥ 0.01 for category 1 and 2, respectively, were derived but not proposed by the DS.

Respiratory system

The DS noted that, in a human study by Lin *et al.* (2008) on asthma hospital admissions, the risk of hospital admissions increased 22% with a 0.001 ppm increase in mean ozone concentration. Acute, unspecific hyperreactivity, exacerbation or AHR (airway hyperresponsiveness) were reported. AHR is a serious health impairment which was also observed in rats after single exposure (Depuydt *et al.,* 1999). Based on these findings STOT SE 3 (H335) was proposed by the DS.

Comments received during consultation

One comment was received from a company/manufacturer concerning the lack of bioavailability of ozone, suggesting that ozone is not able to reach the CNS.

RAC acknowledges that the majority of ozone is expected to react with the tissue at the site of contact and that it is totally consumed almost immediately upon reactions with antioxidants and unsaturated fatty acids. These reactions generate the actual ozone messengers represented by either hydrogen peroxide as a fast acting compound or a variety of lipid oxidation products as late effectors.

RAC agrees, that the effects could also be caused by reaction products, which are to be expected to distribute more widely, or caused indirectly through a more complex adverse outcome pathway triggered by ozone. In any case, ozone remains the causative agent.

Assessment and comparison with the classification criteria

The DS assessment of STOT SE included effects on three target organs: the cardiovascular system, the nervous system and the respiratory system. The individual target organs will be discussed in the following sections.

Impact on the cardiovascular system

Animal studies on acute inhalation toxicity report results on the cardiovascular system. The effects seen by Farraj *et al.* (2012) were decreased HR and arrhythmia (increased number of atrial premature beats, sinoatrial block and atrioventricular block (AVB, during exposure) together with HR variability and decreased body core temperature. Arito *et al.* (1992 and 1997) confirmed significant reductions in HR at doses starting at 0.1 ppm ozone.

Table*: Summary of submitted studies for STOT SE considerations related to the cardiovascular effects*

Method, Guideline, GLP status, Reliability Ref.	**Species, Strain, Sex, No/group**	**Test Concentration**	**Results**
Farraj *et al.,* 2012 Reliability: 2 GLP: No	Rat, Charles River, spontaneously hypertensive 12 week-old Number per dose group unclear	Single doses of 0, 0.2 and 0.8 ppm 4 h exposure in whole-body chamber	0.2 ppm: increased sensitivity to aconitine-induced arrhythmia formation (compared to control) 0.8 ppm: HR and ECG: decreased HR and QTc, increased PR and RR intervals, ST depression (compared to baseline); no post-exposure effects Arrhythmia: increased number of atrial premature beats, sinoatrial block, atrioventricular block during exposure (AVB incidence 14 $\pm$7, $p<0.01$) compared to 0.2 in control); little to no post-exposure effects HR variability: increased standard deviation of NN intervals (SDNN), root mean square of successive RR interval differences (RMSSD), low-frequency (LF), high-frequency (HF), LF:HF (compared to baseline); no post-exposure effects Other: decreased core body temperature, decreased serum HDL and creatinine, increased serum sorbitol dehydrogenase, increased sensitivity to aconitine-induced arrhythmia formation (compared to control)
Arito *et al.,* 1997 Reliability:2 GLP: No	Rat, Wistar, male	Three consecutive 5 h exposures to increasing	0.1 ppm: stat. sign. decreased HR (only in young rats, ~ 80% of control), decreased tidal volume (~ 70% of control, not stat. sign, no recovery during exposure), increased breathing frequency (not stat.

		doses (0, 0.1, 0.3, 0.5 ppm) with exposure free days in-between	sign.) 0.3 and 0.5 ppm: stat. sign. decreased HR (~ 50-65% of control) less pronounced in old rats, stat. sign. decreased tidal volume (~ 50% of control), stat. sign. increased breathing frequency which recovered towards end of exposure (only in young rats)
Arito *et al.,* 1992 Reliability: 2 GLP: No	Rat, Wistar, male group size not reported	Ozone 0 ppm, 6 h 0.5 ppm, 6 h 1.0 ppm, 3 h Exposure chamber	Lower HR (% change compared to control) 0.5 ppm: 6 h: -24±3.1 ($p < 0.01$) 1.0 ppm: 3 h: -27±3.7 ($p < 0.01$) 3 h post 1 ppm: -14±3.2 ($p < 0.01$)

According to the CLP guidance Section 3.9.1. Definitions and general considerations for STOT RE:

"*Where the same target organ toxicity of similar severity is observed after single and repeated exposure to a similar dose, it may be concluded that the toxicity is essentially an acute (i.e. single exposure) effect with no accumulation or exacerbation of the toxicity with repeated exposure. In such a case classification with STOT-SE only would be appropriate*."

Studies submitted for STOT RE considerations were all describing more or less subacute toxicity or acute toxicity with a recovery within one week after exposure. Therefore, studies from the STOT RE section will be considered for STOT SE. Arito *et al.* (1990) described lower HR and increased bradyarrhythmic episodes at day 1 and 2 of exposure. Two studies provided for STOT RE, Watkinson *et al.* (2003) and Iwasaki *et al.* (1998), also observed lower HR at day 1 of exposure. The studies were only repeated over few days (2 to 5 days) and show a degree of tolerance or adaptive effects as the effects seen were recovered over time. Decreased body temperature was also observed by Watkinson *et al*. (2003), Iwasaki *et al.* (1998) and Gordon *et al.* (2014). The core body temperature was recovered few days after exposure. Observations showed a more pronounced degree of temperature changes during exercise and lower ambient temperatures.

Goodman *et al.* (2014) has carried out a critical review "*Weight of evidence evaluation of short-term ozone exposure and cardiovascular effects"* for the potential cardiovascular (CV) effects associated with short-term ozone exposure using a standardized evaluation score system (Goodman W-o-E framework). Some reference studies in the review reported partly contradicting results: Chuang *et al.* (2009) observed increased HR levels while Wang *et al.* (2013) observed no evidence of arrhythmia. Blood pressure was only measured by Chuang *et al.* (2009) and showed no changes within 8 h, but changes after 5 days. Finally, Goodman *et al.* (2014) categorize the strength of evidence for a causal relationship between short-term exposure to ambient ozone concentration and CV effects as "below equipoise."

The effects seen in cardiovascular system could be regarded as adverse: overall high and significant changes in HR together with significant increase in arrhythmia and increase in bradyarrhytmic episodes, and additionally high incidences of AVB at 0.8 ppm in study by Farraj *et al.* (2012). The guidance value (GV) for STOT SE 1 is below 2 500 ppm and therefore support classification as STOT SE 1; H370 (cardiovascular system). The effects were seen in the first days in most studies. The observed heart effects were accompanied with a transient decrease in core temperature. Therefore, RAC disagrees with the DS proposal for no classification for effects on cardiovascular system and concludes that a classification with STOT SE 1 (cardiovascular system) is warranted.

Setting of specific concentration limit (SCL) for STOT SE (cardiovascular system)

The effect dose is set to 0.1 ppm based on significant effects related to the cardiovascular effects. The GV are 2 500 ppm for SCL Cat. 1 and 20 000 ppm for SCL Cat. 2. No extrapolation in the GV for duration is needed.

***Table**: setting of SCL for STOT SE (cardiovascular effects)*

Study references	**Effective dose (ppm)**	**Species, Length of exposure**	**SCL Cat.1**	**SCL Cat.2**
Arito *et al.,* 1997	0.1 Stat. sign. decreased heart rate, heart rate variability, arrhythmia	Rat 4h	SCL Cat. 1 = (0.1 ppm / 2 500 ppm (GV)) × 100% = 0.004% → 0.002%	SCL Cat. 2 = (0.1 ppm / 20 000 ppm (GV)) × 100% = 0.0005%

RAC suggests Commission to consider the applicability of the calculated SCL values.

Impact on the nervous system

The following studies from open literature were evaluated in the CLH dosser:

Method, Guideline, GLP status, Reliability **Ref.**	**Species, Strain, Sex, No/group**	**Test substance,**	**Results**
Guideline: None GLP: No Reliability: 2 Avila-Costa *et al.,* 1999	Rat, Wistar, male n = 24 animals (unclear whether per group or total)	Ozone Single doses of 0 and 1 ppm 4 h exposure, closed chamber	Long-term (24 h) memory alteration: decreased time animal remained in safety compartment before entering shock compartment (with 2.5 mA footshock) Reduction in number of dendritic spines in hippocampus
Guideline: None GLP: No Reliability: 2 Avila-Costa *et al.,* 2001	Rat, Wistar, male n = 24 animals (unclear whether per group or total)	Ozone (source not mentioned) Single doses of 0 and 1 ppm 4 h exposure, closed chamber	Altered motor behaviour: decreased exploratory and increased freezing behaviour (measured for 10 minutes, 24h post-exposure) Reduction in number of dendritic spines in striatum and prefrontal cortex
Guideline: None GLP: No Reliability: 2 Rivas-Arancibia *et al.,* 1998	Rat, Wistar, male n = 25 per dose group, divided into subgroups of 10, 10 and 5 to investigate different endpoints	Ozone generated from 98% O_2 and 5% CO_2 Single doses of 0, 0.1, 0.2, 0.5, 1 ppm 4 h exposure, closed chamber	Short-term memory: no effects Long-term (24 h) memory: 0.2 and 0.5 ppm: decreased time animal remained in safety compartment before entering shock compartment (with 2 mA footshock), compared to control All treated groups: decreased time animal remained in safety compartment before entering shock compartment (with 4 mA footshock) compared to control, but no dose-response Motor activity (measured for 10 min, 1 and 24 h post-exposure): 0.1, 0.2, 1 ppm, but not 0.5 ppm: decreased motor activity 1 h post-exposure, reversible after 24 h

			Antioxidant enzyme levels: Continuous increase in pulmonary and brain Cu/Zn SOD levels up to 0.2 ppm dose group, continuous decrease higher dose
Guideline: None GLP: No Reliability: 2 Rivas-Arancibia *et al.,* 2003	Rat, Wistar, male Experiment 1: n = 10 per dose group Experiment 2: n = 6 per dose group Experiment 3: n = 6 in ozone group, n = 5 in control Experiment 4: not reported	Ozone generated from oxygen 0 and 1 ppm 4 h exposure, closed chamber	Experiment 1: decreased exploratory behaviour and increased freezing behaviour 3 h post-exposure; reversible within 3 days Experiment 2: increased striatal lipoperoxidation levels 3 h post-exposure; reversible within 5 d Experiment 3: increased basal dopamine, glutamate and nitric oxide levels; decreased 5-HT; GABA initially decreased (3 h post exposure), then increased (3 and 5 days post exposure) Experiment 4: increased lipofucsin, neuronal cytoplasm and dendrite vacuolation, dilation of rough endoplasmic reticulum cisterns and dark cells in striatal medium spiny neurons
Guideline: None GLP: No Reliability: 2 Arito *et al.,* 1992	Rat, Wistar, male group size not reported	Ozone 0 ppm, 6 h 0.5 ppm, 6 h 1.0 ppm, 3 h Exposure chamber	Reduced amounts of wakefulness and paradoxical sleep, increased slow-wave sleep; lower EEG amplitude; lower HR
Guideline: None GLP: No Reliability: 2 Paz and Huitron-Resendiz, 1996	Rat, Wistar, male n = 10 per dose group	Ozone 0, 0.35, 0.75, 1.5 ppm 24 h exposure, closed chamber	Dose-dependent decrease in paradoxical sleep and increase in slow wave sleep; wakefulness decrease at highest dose (1.5 ppm); all during exposure Dose-dependent increase in 5-HT concentration in rat pons, however significant only at highest dose group

Summarising the assessed open literature experiments, the studies conducted by Avila-Costa *et al.* (1999 and 2001) and Rivas-Arancibia *et al.* (1998 and 2003) reported long-term memory alteration, reduction in number of dendritic spines in hippocampus, altered motor behaviour and reduction in number of dendritic spines in striatum and prefrontal cortex at dose of 1.0 ppm. The effects seen in the acute inhalation toxicity studies were also confirmed by repeated dose toxicity studies via inhalation.

Significant toxicity to the CNS was observed after single exposure at 1 ppm. The GV for STOT SE 1 is below 2 500 ppm and therefore support classification for STOT SE 1; H370 (nervous system). As the effect concentrations from the studies are very low (around 1 ppm), RAC agrees with the DS that a **classification as STOT SE 1 (nervous system) is warranted** for ozone.

Setting of specific concentration limit for STOT SE (nervous system)

The effect dose is set to 1 ppm based on long-term memory alteration, reduction in number of dendritic spines in hippocampus, altered motor behaviour and reduction in number of dendritic spines in striatum and prefrontal cortex. The GV are 25 00 ppm for SCL category 1 and 20 000 ppm for SCL Category 2. No extrapolation in the GV for duration is needed.

Table: setting of SCL for STOT SE (nervous system)

Study references	Effective dose (ppm)	Species, Length of exposure	SCL Cat.1	SCL Cat. 2
Avila-Costa *et al.,* 1999 Avila-Costa *et al.,* 2001 Rivas-Arancibia *et al.,* 1998, Environ Res. 76(1): 33-9 Rivas-Arancibia *et al.,* 2003, Pharmacol Biochem Behav. 74(4): 891-900	1.0 Long-term memory alteration, reduction in number of dendritic spines in hippocampus, altered motor behaviour and reduction in number of dendritic spines in striatum and prefrontal cortex	Rat 4 h	SCL Cat. 1 = (1 ppm / 2 500 ppm (GV)) × 100% = 0.04% → 0.02%	SCL Cat. 2 = (1 ppm / 20 000 ppm (GV)) × 100% = 0.005%

RAC suggests Commission to consider the applicability of the calculated SCL values.

Effects on the respiratory system

From the acute inhalation toxicity studies, observation of laboured breathing and oedema in animals are consistent with respiratory tract irritation.

In human volunteers, a more detailed investigations on respiratory tract irritation was performed in studies of lung function parameters. In two studies by Adams (2002 and 2006), the total symptoms severity (TSS) and pain on deep inspiration (PDI) both were significantly higher at 0.08 ppm. TSS was calculated as the sum of the severity ratings for individual symptoms, e.g. throat tickle, cough, and PDI indicating respiratory tract irritation. These results were confirmed by a study of Schelegle *et al.* (2009) who identified significant changes of the TSS at 0.07 ppm and by a study of Kim (2011) who found polymorphonuclear neutrophil increase at 0.06 ppm.

Measurements of forced expiratory volume-one second (FEV1) was done by Schelegle *et al.* (2009) in healthy humans before and after 50 minute exercise periods during 6.6 hours inhalation chamber exposures, during the last 10 minutes of each hour and at 1 and 4 hours after exposure from filtered air, 0.06-0.087 ppm ozone. The mean percent decreased in FEV1 was statistically significant ($p < 0.05$) with $11.42 \pm 2.20\%$ after the exposure protocol to 0.087 ppm ozone. A decrease in FEV1, FVC, FEV1/FVC of > 10% would be a biologically relevant change in that parameter based on moderate abnormal findings.

As discussed under Respiratory Sensitisation, a human study by Lin *et al.* (2008) on asthma hospital admissions, described in the section for respiratory sensitization, the risk of hospital admissions increased 22% with a 0.001 ppm increase in mean ozone concentration. Acute unspecific hyperreactivity, exacerbation or AHR were observed after single exposure in animals (Depuydt *et al.*, 1999).

The effects seen in the acute inhalation toxicity studies are also confirmed by studies identifying repeated dose toxicity effects via inhalation. From the submitted repeated dose toxicity studies in animals, together with the human studies, it is clear that inhaled ozone causes changes in breathing pattern, bronchial obstruction, and AHR to other bronchoconstrictive agents in animals and humans. The effects occur at ozone concentrations that are slightly higher than those necessary to cause changes in morphology, changes in mucociliary transport, and aberrant host defence. Inflammation is likely to be involved in these effects.

The significant toxicity to the respiratory system was observed after single exposure at doses in humans around 0.05 ppm. The GV for STOT SE 1 is below 2 500 ppm and therefore supports classification for STOT SE 1; H370 (respiratory system). As the effect concentrations from the

studies are very low (around 0.05 ppm), RAC disagrees with the DS proposal for STOT SE 3 (H335) classification and concludes that a classification with STOT SE 1 (respiratory system) is warranted for ozone.

Setting of specific concentration limit for STOT SE (respiratory system)

Study references	**Effective dose (ppm)**	**Species, Length of exposure**	**SCL Cat. 1**	**SCL Cat. 2**
Schelegle, *et al.,* 2009	0.087 ppm (Schelegle) 6.6 h in human. Conversion for ppm/4h 0.087 ppm × 1.65 = 0.144 ppm Changes in FEV1, FVC FEV1/FVC, TSS, PDI, AHR, lung inflammation etc.	Human 6.6 h	SCL Cat. 1 = (0.144 ppm / 2 500 ppm (GV)) × 100% = 0.00567% > 0.005%	SCL Cat. 2 = (0.144 ppm / 20 000 ppm (GV)) × 100% = 0.00072% > 0.0005%

RAC suggests Commission to consider the applicability of the calculated SCL values.

RAC concludes that **classification as STOT SE 1; H370 (cardiovascular system, respiratory system, nervous system) is warranted** with an SCL as follows:

- STOT SE 1; H370: C ≥ 0,002%;
- STOT SE 2; H371: 0,0005% ≤ C < 0,002%.
-

10.12 Specific target organ toxicity-repeated exposure

Note: Studies submitted were generally not designed in accordance with OECD Test Guidelines (TG 412, 413, 452). Typically only individual toxicological endpoints were evaluated. Purity and other technical details were not reported. Some studies (Health Effect Institute) were conducted according to FDA GLP Regulation.

Table 44: Summary table of animal studies on STOT RE

Method, guideline, deviations if any, duration, major organ target, species, strain, sex, no/group	Test substance, route of exposure, dose levels, duration of exposure	Results	Reference
Repeated dose Toxicity study; Guideline: None exposures with exposure free days in-between major organ target: age differences for effects on ventilatory rate and heart rate Rats (4-6 and 20-22 month old)	5-h exposures to increasing doses (0, 0.1, 0.3, 0.5 ppm) with exposure free days in-between duration: three 5-h	initial (1-2 min of exposure) transient rapid shallow breathing with slightly increased HR rapid shallow breathing persisted, but HR decreased (exposure hours 1 and 2) 0.1 ppm: stat. sign. decreased HR (only in young rats, ~ 80 % of control), decreased tidal volume (~ 70 % of control, not stat. sign, no recovery during exposure), increased breathing frequency (not stat. sign.) 0.3 and 0.5 ppm: stat. sign. decreased HR (~ 50-65 % of control) less pronounced in old rats), stat. sign. decreased tidal volume (~ 50 % of control),	Arito H. et al. (1997), Ind Health. 35(1):78-86

Method, guideline, deviations if any, duration, major organ target, species, strain, sex, no/group	Test substance, route of exposure, dose levels, duration of exposure	Results	Reference
		stat. sign. increased breathing frequency which recovered towards end of exposure (only in young rats)	
Repeated dose Toxicity study; Guideline: None major organ target: heart rate core body temperature Rat, Wistar, male n=9 per group	Ozone route of exposure: Inhalation dose levels: 0, 0.1, 0.3, 0.5 ppm duration: 8 h/d for 4 days	stat. sign. concentration dependent decreased heart rate during 8-h exposure and 12-h post-exposure periods on exposure days 1 and 2 (day 2 post-exposure only stat. sign. at 0.5 ppm). Recovery to or above control values on days 3 and 4. small but stat. sign. decreased core body temperature at 0.5 ppm during 8-h exposure period on days 1 and 2. No effect at 0.1 and 0.3 ppm. Recovery to control values on days 3 and 4 (above control values during post-exposure at 0.3 ppm). LOAEC: 0.1 ppm (decreased heart rate)	Iwasaki T. et al. (1998), Ind Health. 36(1):57-60
Repeated dose Toxicity study; Guideline: None major organ target: sleep and wakefulness states heart rate bradyarrhythmia Rat, Wistar, male n=8 per group	ozone generated from oxygen route of exposure: Inhalation dose levels: 0, 0.1, 0.2 ppm duration: continuous exposure for 5 d	No stat. sign. differences in amounts of wakefulness, slow-wave sleep and paradoxical sleep Stat. sign. decreased HR at 0.2 ppm on days 1 and 2. Recovery to control values on day 3. Stat. sign. concentration dependent increased number of bradyarrhythmic episodes during all states of sleep and wakefulness on days 1, 2 and 3 (no stat. sign. during paradoxical sleep period at 0.1 ppm). Recovery to control values on days 4 and 5. LOAEC: 0.1 ppm (bradyarrhythmia)	Arito H. et al. (1990), Toxicol Lett. 52(2):169-78
Repeated dose Toxicity study; Guideline: None major organ target: effect of ambient temperature (experim. 1) and exercise (experim. 2) on: ECG, HR core body temperature Rat, Fischer-344, male experim. 1: n=44-50 per group experim. 2: n=8 per group	ozone route of exposure: Inhalation dose levels: 0 and 0.5 ppm duration: experim. 1: 5 d experim. 2: 2 h	Experim. 1 (influence of different ambient temperatures: 10, 22, 34°C): decreased HR at all three ambient temperatures (with recovery on exposure day 3); effect was more pronounced at 10°C and less pronounced at 34°C) decreased core body temperature (with recovery on exposure day 3); effect was more pronounced at 10°C and less pronounced at 34°C) increased BALF biomarkers of inflammation Experim. 2 (exercising rats): decreased HR compared to control decreased core body temperature compared to control	Watkinson W.P. et al. (2003), Environ Res. 92(1):35-47
Repeated dose Toxicity	ozone	behavioural changes (increased drinking, grooming and resting; decreased rearing,	Martrette J.M. et al. (2011),

Method, guideline, deviations if any, duration, major organ target, species, strain, sex, no/group	Test substance, route of exposure, dose levels, duration of exposure	Results	Reference
study; Guideline: None major target organ: behaviour hormonal status respiratory and locomotor muscle structure Rat, Wistar, female n=12 per group	route of exposure: Inhalation dose levels: 0 and 0.12 ppm duration: 6 h/d for 15 d	jumping-play and locomotor activities) increased plasma corticosterone and free triiodothyronine (acc. to author: possibly due to stress from exposure) changes in expression of myosin heavy chains in three of five muscles studied: decreased MHC 2B and increased MHC 2A (acc. to author: possibly due to modified respiratory behaviour and hormonal changes)	Physiol Behav. 103(3-4):302-7
Repeated dose Toxicity study; Guideline: None major target organ: motor activity neurons of striatum and substantia nigra Rat, Wistar, male n=10 per group	ozone route of exposure: Inhalation dose levels: 0 and 0.25 ppm duration: 4 h /d for 15 (group 1) or 30 d (group 2)	decreased motor activity in both groups (stat. sign.), but no difference between groups increased lipid peroxidation in striatum in both groups (stat. sign.), with higher levels in 30-d group morphological alterations, loss of fibres and cell death of the dopaminergic neurons	Pereyra-Munoz N et al. (2006), J Chem Neuroanat. 31(2):114-23
Repeated dose Toxicity study; Guideline: None major target organ: olfactory bulb (memory, lipid peroxidation, estrogen receptors, dopamine β-hydroxylase) Rat, Wistar, virgin female n=240 total, divided into 6 groups	ozone route of exposure: Inhalation dose levels: 0 and 0.25 ppm duration: 4 h/d for 30 (groups 1) or 60 d (group 2)	impaired formation/retention of olfactory memory: - impaired recognition memory of a stimulus animal (at 30 days and more pronounced at 60 days) - impaired speed in locating a buried chocolate (60 days) - impairments were prevented in estradiol groups increased lipid peroxidation in olfactory bulb (30 and 60 days) reduced estrogen receptors, ER protein levels and dopamine beta-hydroxylase (effects also prevented in estradiol group)	Guevara-Guzman R. et al. (2009), Neuroscience. 159(3):940-50
Repeated dose Toxicity study; Guideline: None major organ target: memory hippocampus Rat n=22 per group	ozone route of exposure: Inhalation dose levels: 0 and 0.25 ppm duration: 4 h/d for 15, 30, 60, 90 days	- stat. sign. increased lipid peroxidation with differences between exposure-duration groups - morphological changes and swelling in neurons - decreased neurogenesis in 60- and 90-d groups - increased neurogenesis in 30-d group (possibly compensatory), but with morphological alterations in neuroblasts - decrease in Neu-N and doublecortin - increases in activated and phagocytic microglia - increased number of astrocytes - concentration dependent memory deficiency in passive avoidance test → Neurodegeneration analogous to that seen in	Rivas-Arancibia S. (2010), Toxicol Sci. 113(1):187-97

Method, guideline, deviations if any, duration, major organ target, species, strain, sex, no/group	Test substance, route of exposure, dose levels, duration of exposure	Results	Reference
		Alzheimer's disease	
Repeated dose Toxicity study; Guideline: None major organ target: heart rate core body temperature motor activity difference btw. adult and senescent rats Rat, Brown Norway, male adult (9 m) and senescent (21 m) n=not reported	ozone route of exposure: Inhalation dose levels: 1 ppm duration: 6 h/d, 2 d/wk followed by recovery period for 13 weeks	Decreased HR and core temperature, which increased during recovery period. Effect became less pronounced as exposure weeks progressed. Senescent rats less affected than adults. Motor activity was only measured during recovery period, where it was elevated in adults but not senescent rats.	Gordon C.J. et al. (2014), Inhal Toxicol. 26(7):380-90
Repeated dose Toxicity study; Guideline: None major organ target: ventilatory function HR BP motor activity markers of pulmonary inflammation and vascular disease Rat, Brown Norway adult (4 m) and senescent (20 m) n=12 per group	ozone route of exposure: Inhalation dose levels: 0 and 0.8 ppm duration: 6 h, 1d/wk for 17 weeks	Decreased ventilator function (it took longer for effect to appear in senescent rats). Residual effects were still seen 7 d post exposure. no effect on HR and BP in either age group decreased motor activity in both age groups mild neutrophilic inflammation and protein leakage in adults increased leptin, adiponectin, lipocalin and insulin in senescent rats	Gordon C.J. et al. (2013), Inhal Toxicol. 25(3):141-59
Repeated dose Toxicity study; Guideline: None major organ target: morphology of the cerebellum of rats with prenatal exposure to ozone Rat **(**Wistar), female 4 pregnant females/group Morphological analysis: 8 male born rats/group	ozone route of exposure: Inhalation dose levels: 1 ppm duration: 12 h/d for 21 days Exposure: during entire gestation (GD0 until PND0) Time point: PND90	Abnormal structures in molecular layer of cerebellum of rats born to exposed dams Decrease of total area and number of Purkinje cells 0: 10.6±0.3 mm²; 1 ppm: 4.8±0.3 mm² 0: 832±31 cells; 1 ppm: 712±34 cells →Depopulation of Purkinje cells and also degenerating Purkinje cells and cell debris Circular bodies in molecular layer Incomplete folding pattern of some lobes	Romero-Velázquez R.M. et al. (2002), Proc West Pharmacol Soc. 45:65-7
Repeated dose Toxicity study; Guideline: None	ozone route of exposure:	affected: - 0.12 ppm: decreased responsiveness to contractile stimuli in	Szarek J.L. (1994), Res Rep Health Eff

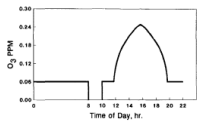
Method, guideline, deviations if any, duration, major organ target, species, strain, sex, no/group	Test substance, route of exposure, dose levels, duration of exposure	Results	Reference
major organ target: lung (contractile properties of airway smooth muscle altered airway muscle response) Rat, F344/N, male and female airway muscles examined in vitro group size: n=5-12 per group	Inhalation dose levels: 0, 0.12, 0.5, 1.0 ppm duration: 6 h/d, 5 d/wk for 20 m	small airways (but increased after abrasion of the luminal epithelium) - 0.5 ppm: increased wall area in small airways from males increased smooth muscle area in small airways in both genders decreased maximum active stress in response to stimulators in small airways in both genders decreased responsiveness to contractile stimuli in small airways (but increased after abrasion of the luminal epithelium) - 1.0 ppm: decreased maximum active stress in response to stimulators in small airways in both genders greater increase of prostaglandin release after incubation with the calcium ionophore A23187 unaffected: relation between passive tension and internal circumference in small and large airways relation between active tension and internal circumference in small and large airways wall and smooth muscle areas in large airways maximum active stress in large airways response to contractile stimuli in large airways isoproterenol-induced relaxation responses in small and large airways levels of prostaglandin E2 leukotriene C4 release	Inst. (65 Pt 2):3-63; discussion 65-74
Repeated dose Toxicity study; Guideline: None Major organ target lung GLP status no Reliability 2 Rat, Wistar, male No/ group 3-5	ozone route of exposure: Inhalation dose levels: 0 and 0.4 ppm duration: 23.5 – 24h/d for 1, 3, 7, 28, 56 days + recovery from 3 days: studied at day 7, 14, 28	time course of lung injury in rats during acute and subchronic ozone exposure and during post-exposure recovery correlated biochemical and morphological analysis of inflammatory responses (PMN and protein in BALF), structural changes, and collagen content Results: inflammatory response reached a maximum at day 1 and resolved largely within 6 days during ongoing exposure numbers of macrophages in BAL fluid increased progressively up to day 56, and slowly returned to near control levels when exposure was followed by post-exposure recovery centriacinar inflammatory responses throughout ozone exposure	Van Bree L. et al. (2001), Inhal Toxicol. 13(8):703-18

Method, guideline, deviations if any, duration, major organ target, species, strain, sex, no/group	Test substance, route of exposure, dose levels, duration of exposure	Results	Reference
	+ recovery from 7 days: studied at day 14, 28, 56 +recovery from 28 days: studied at day 35, 56 + recovery from 56 days: studied at day 136	Centriacinar thickening of septa was observed at day 7 Ductular septa, thickened progressively at days 7, 28, and 56 of exposure, showed increased collagen upon exposure at day 28, which was further enhanced at exposure at day 56 Increased collagen content in lungs was observed at exposure day 56 Collagen content was not different from control at day 56 when 7 or 28 days of exposure was followed by post-exposure recovery respiratory bronchioles were present in an increasing degree, and remained present after a recovery period continuous exposure to ozone show some acute effects, such as protein and albumin content, and neutrophil influx in BAL fluid, returned to control levels within a few days other parameters, such as the alveolar macrophage response and structural changes (presence of terminal bronchioles, thickening of ductular septa by enhanced cellularity and collagen formation) persisted or progressively increased during continued exposure <u>Conclusion:</u> Post-exposure recovery seems to partly resolve these subchronic responses (macrophages response, septal cellularity) , whereas other effects (collagen increase and respiratory bronchioles formation) do not disappear LOAEC: 0.12 ppm	
Repeated dose Toxicity study; Guideline: None Major organ target lung GLP status no Reliability 2 Rat, Wistar, male No/ group 5	ozone <u>route of exposure</u>: Inhalation <u>dose levels:</u> 0 and 0.4 ppm <u>duration:</u> 12h/day (during dark phase) at 5 consecutive days + recovery period of 5, 10, 15 or 20 days after 5-day preexposure and following a 12h ozone challenge	attenuation and recovery of pulmonary injury following short-term, repeated daily exposure to ozone inflammatory, permeability, and histopathological responses <u>Results:</u> (repeated expo and ozone -challenged rats:) (single expo: increases of alveolar–capillary permeability, inflammatory responses, cell damage in lower airways) BAL fluid values that were not different from those observed in unexposed controls attenuated responses show a gradual recovery BAL fluid levels of albumin, IL-6, and number of macrophages and neutrophils, the period for lung	Van Bree L. et al. (2002), Inhalation Toxicology, 14:883-900

Method, guideline, deviations if any, duration, major organ target, species, strain, sex, no/group	Test substance, route of exposure, dose levels, duration of exposure	Results	Reference
	(0.4 ppm) Time points: 12h after last day of exposure 6, 11, 16, 21, 26 (+ single exposure 12 h to 0.4 ppm)	tissue to regain its full susceptibility and responsiveness to ozone following a 5-day preexposure period is approximately 15–20 days the total protein and fibronectin responses in BAL fluid still exhibited an attenuated response to an ozone challenge at 20 days postexposure Morphometry: after a recovery of 5–10 days following a 5-day preexposure the response to a challenge was identical to that after a single exposure Conclusion: complete repair from lower airway inflammation caused by short-term, repeated exposure to ozone may take longer than previously assumed	
Repeated dose Toxicity study; Guideline: None Major organ target lung GLP status no Reliability 2 Monkey, rhesus monkeys (Macaca mulatta), infant male No/ group no information	ozone route of exposure: Inhalation, whole body dose levels: 0 and 0.5 ppm duration: consecutive 5 day acute and episodic exposure (I) consecutive: 5 days 8h/day (acute) (II) episodic: 5 biweekly cycles of alternating filtered air (9 consecutive days of air and 5 consecutive days of ozone (8 h/day)	characterise the distribution and magnitude of ozone-induced nasal injury in infant monkeys age-specific, 3-dimensional, epithelial maps of the nasal airways Results: principal nasal lesions: neutrophilic rhinitis, necrosis, exfoliation of epithelium lining acute: 65 % reduction (compared to filtered air controls) in the mean thickness of the nasal epithelium character, severity, and distribution of lesions in episodically exposed monkeys were similar to those in the acutely exposed infant monkeys of similar age	Carey S.A. et al. (2007), Toxicol Pathol. 35(1):27-40
Repeated dose Toxicity study; Guideline: None Major organ target: Ozone-induced persistent rhinitis und epithelial remodelling in nasal airways GLP: no Reliability 2	ozone route of exposure: Inhalation, whole body dose levels: 0 and 0.5 ppm ozone duration: acute: 8h/day for 5 consecutive days (0.5 ppm)	**Morphometric analysis and Biochemistry** 11-cycle ozone induced: persistent rhinitis, squamous metaplasia, and epithelial hyperplasia in the anterior nasal airways of infant monkeys, resulting in a 39 % increase in the numeric density of epithelial cells a 65 % increase in glutathione (GSH) concentrations at this site the persistence of epithelial hyperplasia was positively correlated with changes in GSH	Carey S.A. *et al.* (2011) Am J Physiol Lung Cell Mol Physiol. 300: L242-54

Method, guideline, deviations if any, duration, major organ target, species, strain, sex, no/group	Test substance, route of exposure, dose levels, duration of exposure	Results	Reference
Monkey, rhesus, infant male group / number: Acute: 5 Episodic cycles: 4 Control: 5	Episodic regimens: 11 biweekly cycles of alternating filtered air (9 consecutive days) and ozone (5 consecutive days, 0.5 ppm, 8h/day) Control: exposure to filtered air for 5 months	Results: indicate that early life ozone exposure causes persistent nasal epithelial alterations in infant monkeys provide a potential mechanism for the increased susceptibility to respiratory illness exhibited by children in polluted environments	
Repeated dose Toxicity study; Guideline: None Major organ target lung GLP status no Reliability 2 Monkey, bonnet (Macaca radiata), male and female No/ group 4-8	ozone route of exposure: Inhalation dose levels: 0, 0.15 and 0.3 ppm ozone duration: 8h/d Exposure groups: 1) 0.15 ppm, 6d 2) 0.15 ppm, 90d 3) 0.3 ppm, 90d	whether exposures to ambient levels of ozone induce lesions in the nasal mucosa Results: Quantitative changes were evident in the nasal transitional and respiratory epithelium (region anterior to nasal turbinates) At 6 or 90 days of exposure to 0.15 or 0.30 ppm ozone lesions consisted of ciliated cell necrosis, shortened cilia, and secretory cell hyperplasia Inflammatory cell influx and increased mucosubstances were only present at 6 days of exposure (0.15 ppm) Ultrastructural changes in goblet cells were evident at 90 days Conclusion: Ambient levels of ozone can induce significant nasal epithelial lesions, which may compromise upper respiratory defence mechanisms.	Harkema J.R. et al. (1987), Am J Pathol. 128(1):29-44
Repeated dose Toxicity study; Guideline: None Major organ target lung GLP status no Reliability 2 Monkey, bonnet (Macaca radiata), male and female No/ group 5/control group 4-7/exposure group	ozone route of exposure: Inhalation dose levels: 0, 0.15 and 0.3 ppm duration: 8h/d Exposure groups: 6 days: 0.15 ppm 90 days: 0, 0.15, 0.3 ppm	effects of ambient concentrations of ozone on the surface epithelium lining respiratory bronchioles and on the underlying bronchiolar interstitium Results: Hyperplasia of nonciliated, cuboidal epithelial cells and intraluminal accumulation of macrophages characterized ozone-induced lesions in respiratory bronchioles no significant differences in epithelial thickness or cell numbers among ozone-exposed groups Ozone-exposed epithelium was composed of 80 % cuboidal and 20 % squamous cells compared with 40 % cuboidal and 60 % squamous cells in filtered air controls Arithmetic mean thickness of the surface epithelium was significantly increased in all of the ozone-exposed groups	Harkema J.R: et al. (1993), Am J Pathol. 143(3):857-66

Method, guideline, deviations if any, duration, major organ target, species, strain, sex, no/group	Test substance, route of exposure, dose levels, duration of exposure	Results	Reference
		significant ozone-induced increase in the thickness of the bronchiolar interstitium that was due to an increase in both cellular and acellular components number of cuboidal epithelial cells per surface area of basal lamina was increased above control values by 780 % after 6 days exposure to 0.15 ppm, 777 % after 90 days to 0.15 ppm, and 996 % after 90 days exposure to 0.30 ppm Conclusion: alterations do not appear to be concentration- or time-dependent	
Repeated dose Toxicity study; Guideline: None Major organ target lung GLP status no Reliability 2 Monkey, Cynomolgus, Male No/ group single expo: 12 repeat expo: Phase 1: 4/control and 8/exposure Phase 2: following 2wk rest period, all animals exposed to ozone	ozone route of exposure: Inhalation dose levels: 0 and 1 ppm ozone duration: 6h single or repeated exposure (2wk) Time points: 1 h postexposure	cellular and molecular effects biomarker identification of ozone-evoked toxicity Results: pulmonary inflammation (BAL) + histology: evoked BAL cellular inflammation and increases in total protein, alkaline phosphatase and cytokines cellular inflammation and epithelial necrosis gene expression profiling in lung + blood: oxidative phosphorylation, immune response and cell adhesion pathways altered in response to ozone, with common and unique profiles in lung and blood Conclusion: Repeat ozone challenge evoked reproducible inflammation but attenuated cell damage	Hicks A. et al. (2010), Inflammation. 33(3):144-56
Repeated dose Toxicity study; Guideline: None Major organ target lung GLP status Reliability 2 Rat, Fisher 344, male No/ group 8/exposure group 12/control group	ozone route of exposure: Inhalation, whole body dose levels: Experiment 1: 0, 0.12, 0.25 ppm Experiment 2: 13-h background level of 0.06 ppm with exposure peak 5 days each week from 0.12 to 0.25 ppm and back	Experiment 1: relative volume of type I cells increased 13 % (0.12 ppm) and 23 % (0.25 ppm) over control magnitude of increase were clearly concentration related with fixed exposure concentration, relative volume of type I epithelium increases in proportion to exposure time Experiment 2: relative volume of type I cells increased 9 % (3 weeks) and 33 % (13 weeks) over control Conclusion: linear relationship between increase in type I cell	Chang L. et al. (1991), Toxicol Appl Pharmacol. 109(2):219-34

Method, guideline, deviations if any, duration, major organ target, species, strain, sex, no/group	Test substance, route of exposure, dose levels, duration of exposure	Results	Reference
	to 0.12 ppm over a 9-hour period duration: Experiment 1: 6 weeks, 12h/day (constant concentration) Experiment 2: 3 or 13 weeks (increasing-decreasing peak concentration)	volume and the concentration x time product (r^2=0.66) epithelial cell reactions to low-level subchronic exposure are directly related to cumulative oxidant concentration pattern of exposure did not appear to affect the resulting degree of injury low background exposure my contribute to epithelial cell injuries	
Repeated dose Toxicity study; Guideline: None Major organ target: lung; epithelial injury and interstitial fibrosis in proximal alveolar regions after prolonged ozone exposure GLP: No Reliability: 2 Rat, F344, male Number/group: 12 rats from each exposure group	ozone route of exposure: Inhalation, whole body dose levels: 0, 0.06-0.25 ppm Duration: 5-day work week: 22h/day  Weekends: 22h/day 0.06 ppm Exposure for 1, 3 ,13, 78 weeks Recovery groups: 13 weeks + 6 weeks recovery; 78 weeks + 17 weeks recovery in filtered air Controls: Rats exposed for same length of time to filtered air	Morphometric and morphological changes (proximal alveolar region) biphasic response acute: tissue reactions after 1 week of exposure (epithelial inflammation, interstitial edema, interstitial cell hypertrophy, influx of macrophages); responses subsided after 3 weeks of exposure prolonged exposure: progressive epithelial and interstitial tissue responses (epithelial hyperplasia, fibroblast proliferation, interstitial matrix accumulation) epithelial responses: type I and II epithelial cells alveolar type I cells: increase in number, thickness, smaller average surface area covered → changes persisted, no change during recovery type II epithelial cells: proliferation accumulation of interstitial matrix after chronic exposure (deposition of increased amounts of basement membrane and collagen fibres) interstitial matrix accumulation: partial recovery during follow-up periods in air (unless thickening of the basement membrane) Morphometric changes (terminal bronchioles) acute: loss of ciliated cells, differentiation of preciliated and Clara cells bronchiolar cell population stabilized on continued exposure (but: chronic exposure resulted in structural changes, suggesting injury to ciliated and Clara cells)	Chang L.-Y. *et al.* (1992) Toxicology and applied pharmacology 115: 241-252

Method, guideline, deviations if any, duration, major organ target, species, strain, sex, no/group	Test substance, route of exposure, dose levels, duration of exposure	Results	Reference
		Conclusion: chronic exposure to low levels ozone causes epithelial inflammation and interstitial fibrosis in the proximal alveolar region and bronchiolar epithelial cell injury	
Repeated dose Toxicity study; Guideline: None Major organ target: lung; chronic bronchiolitis after prolonged ozone exposure GLP: No Reliability: 2 Monkey, bonnet (*Macaca radiate*) Sex: no data Group / number: 7 days: 3 28 days: 6 90 days: 9 In total 18	ozone route of exposure: Inhalation, whole body dose levels: 0, 0.5 ppm and 0.8 ppm ozone Duration: 8h/day for 7, 28, 90 days 7 days: each 1 animal for 0.5 ppm, 0.8 ppm and filtered air 28 days: each 2 animals for 0.5 ppm, 0.8 ppm and filtered air 90 days: each 3 animals for 0.5 ppm, 0.8 ppm and filtered air	**Pulmonary function testing** not statistically different before / after exposure general trend of increased quasistatic compliance of lung in both exposed groups **Morphologic changes** low-grade chronic respiratory bronchiolitis; major features: intraluminal accumulations of macrophages hypertrophy and hyperplasia of cuboidal bronchiolar epithelial cells inflammatory response (number of intra-luminal inflammatory cells/mm of respiratory bronchiolar surface): 0.8 ppm: greatest magnitude of inflammation (at each exposure period) but: number of inflammatory cells present at 90 days less than one half that observed at 7 days tritiated thymidine labelling and counts of respiratory bronchiolar epithelium showed: up to 37-fold increase in labelling index at 7 days but: only 7-fold increase at 90 days differential cell counts showed increase in proportion of cuboidal bronchiolar cells constituting the respiratory bronchiolar epithelium 60 % of the epithelial cells were cuboidal bronchiolar cells in control monkeys more than 90 % of the respiratory bronchiolar cells were cuboidal at 90 days cuboidal bronchiolar cell does not appear secretory in control monkeys, but membrane-bound electron-dense secretory granules in exposed monkeys epithelial hyperplasia (increased number of cells/mm of airway length) persisted through 90 days of exposure at a level slightly above that present at 7 days	Eustis S. L. *et al.* (1981) Am J Pathol 105: 121-137

Method, guideline, deviations if any, duration, major organ target, species, strain, sex, no/group	Test substance, route of exposure, dose levels, duration of exposure	Results	Reference
		Conclusion: lesions observed may represent a precursor to more severe anatomic damage, such as centriacinar emphysema	
Repeated dose Toxicity study; Guideline: None Major organ target: Expression of Bcl-2 protein (regulator of apoptosis) in ozone-induced mucous cell metaplasias GLP: No Reliability: 2 Rat, F344/N, male Number/group: at least 3 per group	ozone route of exposure: Inhalation, whole body dose levels: 0 and 0.5 ppm Duration: 8h/day for 1, 3, 6 months or 3-months exposure followed by 13-week recovery period Control: filtered air	**Bcl-2 status** Adjacent metaplastic mucous cells in nasal airway epithelia that were exposed to ozone were heterogeneous in their expression of Bcl-2: some cells expressed high levels, others low levels or no Bcl-2 on Western blot analysis, Bcl-2 was detected in protein extracts from nasal epithelia of rats exposed to 0.5 ppm ozone for 1 month but not in control rats **Number of metaplastic mucous cells** increased in transitional epithelia of rat nasal airways from 0 to about 200 after 3 and 6 months of exposure to ozone; only 0 to 10 metaplastic mucous cells remained after a recovery period of 13 weeks in rats exposed to ozone for 3 months number of mucous cells of the respiratory epithelium lining the midseptum did not change after ozone exposure or recovery **Percentage of Bcl-2 positive cells** percentage of cells lining the midseptum increased from 7 to 14 % after a 3- and 6-months ozone exposure, respectively in transitional epithelia of the lateral wall and the nasoturbinates and maxilloturbinates, 35 to 55 % of cells were Bcl-2-positive after a 1-month exposure and 10 to 18 % after both a 3- and a 6-months exposure to ozone; Bcl-2 reactivity decreased to 0 to 8 % after a recovery period of 13 weeks Conclusion: the observations suggest that Bcl-2 plays a role in the development and resolution of mucous cell metaplasias	Tesfaigzy J. *et al.* (1998) Am J Physiol Respir. Cell Mol. Biol.18: 794-799
Ozone exposure during postnatal development Major organ target: Impact of ozone on pulmonary and peripheral blood responses to LPS	ozone route of exposure: Inhalation, whole body dose levels: 0 and 0.5 ppm (ozone nominal) Duration:	**Pulmonary and peripheral blood effects in the developing lung** after completion of ozone exposure regime at 6 months of age, total peripheral blood leucocyte and PMN numbers were statistically significant reduced, whereas eosinophil counts increased statistically significant in lavage, total cell numbers at 6 months were not	Maniar-Hew K. *et al.* (2011), Am J Pysiol 300: L462-L471

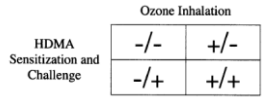
Method, guideline, deviations if any, duration, major organ target, species, strain, sex, no/group	Test substance, route of exposure, dose levels, duration of exposure	Results	Reference
GLP: No Reliability: 2 Monkey, rhesus macaque (Macaca mulatta), infant male Number/group: (1) WBC and BAL cell number/ frequency at 6 months of age: Control and treatment group: 4-9 (2) WBC and BAL cell number/ frequency at 1 year of age: Control and treatment group: 4 (3) Impact of LPS an total WBC, PMN and lymphocyte frequency at 1 year of age: Control and treatment group: 3-4 (4) Impact of LPS on total BAL cells, BAL cell frequency at 1 year of age: Control and treatment group: 3-4 (5) Impact of LPS on cytokine excretion (blood/lavage) at 1 year of age: Control and treatment group: 4	8h/day for 5 days following 9 days of filtered air for 11 cycles (controls: 11 cycles with filtered air) Animals were challenged with a single dose of inhaled LPS at 1 year of age.	affected by ozone, however there was a statistically significant reduction in in lymphocytes and statistically significant increase in eosinophils following an additional 6 months of filtered air housing, only monocytes were statistically significant increased in blood and lavage in previously exposed animals in response to LPS challenge, animals with a prior history of ozone showed an attenuated peripheral blood and lavage PMN response compared with controls *in vitro* stimulation of peripheral blood mononuclear cells with LPS resulted in reduced secretion of IL-6 and IL-8 protein in association with prior ozone exposure Conclusion: it is suggested that ozone exposure during infancy can result in a persistent effect on both pulmonary and systemic innate immune responses later in life	
Repeated dose Toxicity study; Guideline: None Major organ target: Impact of ozone on physiologic adaption, epithelial injury/repair, tracheal substance P levels GLP: No Reliability: 2 Rat, Harlan Sprague-	ozone route of exposure: Inhalation, whole body dose levels: 0 and 1 ppm Duration: Ozone exposure for up to 4 cycles (one cycle: 5-day 1 ppm ozone exposure followed by 9-day recovery)	**Impact on airway immune and structural development** each 5-day episode showed a characteristic pattern of rapid shallow breathing (days 1 and 2), epithelial injury, and interstitial and intraluminal inflammation in contrast, the neutrophil component of inflammation, tracheal substance P release, and cell proliferation became attenuated with each consecutive episode of exposure concurrent with this cyclic and attenuated response there was progressive hypercellularity and hyperplasia in all airways studied and a	Schelegle E. S. *et al.* (2003) Toxicology and Applied Pharamcology 186: 127-42

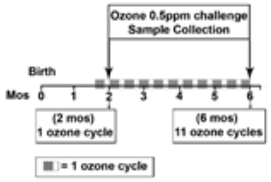
Method, guideline, deviations if any, duration, major organ target, species, strain, sex, no/group	Test substance, route of exposure, dose levels, duration of exposure	Results	Reference
Dawley, male Number/group: In total 63 animals (no details on allocation given)		progressive remodelling present in the terminal bronchioles many of the effects observed were statistically significant from air control Conclusion: the findings are consistent with the notion that the cumulative distal airway lesion is at least in part the result of a depressed cell proliferative response to injury in these airways this depressed cell proliferative response may be in part the result of diminished neutrophil inflammation and/or release of mitogenic neuropeptides in response to ozone-induced injury	
Repeated dose Toxicity study; Guideline: None Major organ target lung/nose GLP: No Reliability: 2 Rat, F344/N Hsd (Harlan Sprague-Dawley, Indianapolis, IN), male No/ group 69 animals in total, 23 animals/group Time points postexposure: 8h: 6 rats/group 4wk: 6 rats/group 13wk: 11 rats/group 13wk+2nd acute exposure (8h, 0.5 ppm) and 18h postexposure: 5 rats/group	ozone route of exposure: Inhalation, whole body dose levels: 0 (filtered air), 0.25, 0.5 ppm ozone mean chamber concentration: 0.24 ± 0.03 ppm, 0.48 ± 0.06 ppm Duration: 8h/d, 7d/wk (subchronic)	persistence of ozone-induced mucous cell metaplasia in nasal epithelium Results: mucous cell hyperplasia in nasal epithelium of rats exposed to 0.25 and 0.5 ppm 13wk postexposure: hyperplasia still evident only in 0.5 ppm group Ozone-induced mucous cell metaplasia with associated intraepithelial mucosubstances was evident only in the nasal tissues of rats exposed to 0.5 ppm ozone, though attenuated, these alterations in the nasal mucous apparatus were still detectable at 13 wk after the end of the exposure After chronic exposure+13wk recovery+8h acute exposure: induction of additional increase of mucosubstances (only for 0.5 ppm, not for control or 0.25 ppm) Conclusion: persistent nature of the ozone-induced mucous cell metaplasia in rats documented in this report suggests that ozone exposure may have the potential to induce similar long-lasting alterations in the airways of humans	Harkema J.R. et al. (1999), Am J Respir Cell Mol Biol. 20(3):517-29
Repeated dose Toxicity study; Guideline: None Major organ target: Impact on tracheobronchial epithelium and pulmonary acinus	ozone route of exposure: Inhalation, whole body dose levels: < 0.002 ppm (filtered air), 0.12 (±0.01), 1.01 (±	Effects after exposure to ozone for 3 or 20 months **Tracheobronchial airways (volume density of epithelial cells):** Ciliated cell: significant decrease at the caudal site in 1.0 ppm group: (sum of 3 and 20 months) 0: 4.95; 0.12: 5.11; 1.0: 3.44 µm³/µm² trend for increase of volume density in all regions,	Pinkerton K. E. et al.. 1998; Research Report 65: Part XIII Health Effect Institute; Library of Congress: WA754R432.

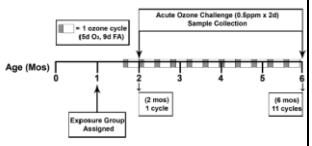
Method, guideline, deviations if any, duration, major organ target, species, strain, sex, no/group	Test substance, route of exposure, dose levels, duration of exposure	Results	Reference
GLP: unclear Reliability: 2 Rat, Fischer-344, male Number/group: In total: 42 rats Number of rats per group in general not reported (only for some endpoints n=4 per group is given in the tables of the result section)	0.05) ppm ozone (analytical) Duration: 6h/d 5d/w for 3 months (present study) or 20 months (reported in Pinkerton 1995)	except cranial bronchi Nonciliated cells: significant time effect: increase of volume density in trachea (3 months: 2.25 µm³/µm² and 20 months: 3.55 µm³/µm²) and caudal bronchi (3 months: 1.53 µm³/µm² and 20 months: 2.31 µm³/µm²) Basal cell: overall low amount of basal cells and no obvious changes in density in all exposure groups, at all-time points and all regions Total epithelial cells: No significant effect (time or concentration) and no consistent trends for both time points and concentration trachea: decrease 17.6 % (3 months, 1.0 ppm), increase 25 % (20 months, 1.0 ppm) cranial bronchus: decrease 9 % (3 months, 1.0 ppm), increase 14 % (20 months, 1.0 ppm) central bronchus: increase 8 % (3 months, 0.12 ppm), increase 21 % (20 months, 0.12 ppm) caudal bronchus: decrease 23 % (3 months, 1.0 ppm), decrease 35 % (20 months, 1.0 ppm) **Proximal and terminal airways (volume density of epithelial cells):** Ciliated cell: four-way interaction for cell volume density (site x airway x concentration x time); no significance for individual factors; conspicuous differences for 1 ppm dose group (at all sites and both time points) Nonciliated cells: significant multivariate interaction between sites and ozone concentration cranial site: significant difference between 3 months (3.42 ± 0.35 µm³/µm²) and 20 months (2.58 ± 0.12 µm³/µm²) caudal site: significant increase in 1.0 ppm group: increase 25 % (3 months, 1.0 ppm), increase 46 % (20 months, 1.0 ppm) Total epithelial cells: Proximal bronchiole was significantly greater in animals exposed for 3 months, similar trend in terminal bronchiole 3 months: - proximal, cranial: 0.12: ↑, 1.0: ↓ - proximal, caudal: 0.12: ↓, 1.0: ↑ - terminal, cranial: 0.12: ↓, 1.0: ↑ - terminal, caudal: 0.12: ↓, 1.0: ↓	

Method, guideline, deviations if any, duration, major organ target, species, strain, sex, no/group	Test substance, route of exposure, dose levels, duration of exposure	Results	Reference
		20 months: - proximal, cranial: 0.12: ↓, 1.0: ↓ - proximal, caudal: 0.12: ↓, 1.0: ↓ - terminal, cranial: 0.12: ↓, 1.0: ↓ - terminal, caudal: 0.12: ↑, 1.0: ↑ **Ventilatory units:** Volume densities for 100-µm interval to 800 µm down the alveolar duct Total epithelial volume density: Significant differences between the cranial and caudal sites Cranial site: significant multivariate effect for interaction between concentration and time; significant effects of length of exposure and concentration (volume density of epithelium, distance into the ventilatory unit); Increasing effect as a function of increasing concentration for both 3- and 20-months exposures (1.0 ppm statistically significant relative to control and changes more pronounced for 1.0 ppm/20 months exposure) Caudal site: no statistically significant multivariate differences; only for 1.0 ppm group significant changes Interstitial volume density: Significant differences between the cranial and caudal sites Cranial site: concentration-related changes for the first 500 µm and significant increase for 1.0 ppm group Caudal site: no statistically significant differences for individual distance values; increased significant elevation for 1.0 ppm group (pooled: distance values and time points) Capillary lumen volume density: No significant differences between cranial and caudal sites; significant time effect for 20 months (increase) (1.63 ± 0.09 $\mu m^3/\mu m^2$ compared to 1.20 ± 0.08 $\mu m^3/\mu m^2$ for 3 months) Macrophage volume density: No significant differences between cranial and caudal sites; after averaging the values: significant elevation in macrophages following exposure to 1.0 ppm (3 and 20 months) in the first 500 µm Antioxidant enzyme localization (after exposure for 2 months to 1.0 ppm ozone): Distribution and relative abundance of Mn-SOD	

Method, guideline, deviations if any, duration, major organ target, species, strain, sex, no/group	Test substance, route of exposure, dose levels, duration of exposure	Results	Reference
		and Cu-Zn-SOD Cu-Zn-SOD: - found in in airways down to the terminal bronchioles - all cell types labelled, but mainly Cara cells - density in tissues was higher in the airways than in the parenchym - reduced labelling of Cu-Zn-SOD in exposed animals (intensity and extent of labelling) Mn-SOD: - found in the pleura and down to the terminal bronchioles, in Clara cells - Ozone exposure: increased labelled type II cells and macrophages (contribute to overall increase of Mn-SOD in proximal alveolar region) - no significant changes in amount of Mn-SOD in Clara cells of terminal bronchioles and alveolar duct region - labelling mainly confined to mitochondria - significantly increased in type II cells immediately distal to bronchiole-alveolar duct junction (21 %) - increase in proximal alveolar region not affected - no marked induction in interstitial fibroblasts (7 %) - major site of induction: proximal portion of gas exchange region in mitochondria of type II alveolar epithelial cells → site-specific (proximal portion of alveolar duct walls and adjacent alveolar septa) and cell-specific (type II alveolar epithelial cells) increase Bronchiolarized metaplasia in alveolar duct confined to proximal alveolar region within 200 µm of a terminal bronchiole	
Repeated dose Toxicity study; Guideline: None Major organ target lung GLP: No Reliability: 2 Monkey, rhesus (*Macaca mulatta*), male No/ group 6	ozone route of exposure: Inhalation dose levels: 0 (filtered air), 0.5 ppm ozone Duration: 5 days (8h/d) followed by 9 days of filtered air for 11 episodes (5 months)	distal airway development in infants Results: ozone-exposed animals had four fewer nonalveolarized airway generations (13 or 14 in control animals, average of 10 airway generations in ozone group) terminal bronchioles of ozone exposed animals were an average of 38 % narrower and 45 % shorter → terminal and most proximal respiratory bronchiole were smaller than control hyperplastic bronchiolar epithelium and altered smooth muscle bundle orientation in terminal and respiratory bronchioles Conclusion:	Fanucchi M.V. et al. (2006), Am J Physiol Lung Cell Mol Physiol. 291(4):L644-50

Method, guideline, deviations if any, duration, major organ target, species, strain, sex, no/group	Test substance, route of exposure, dose levels, duration of exposure	Results	Reference
		results suggest that episodic exposure to environmental ozone compromises postnatal morphogenesis of tracheobronchial airways	
Ozone exposure during postnatal development Major organ target: Impact of ozone on the effects of allergen sensitization and inhalation GLP: No Reliability: 2 Monkeys, rhesus, infant, sex not reported Number/group: In total 24 animals, 6/group  Ozone: 8h/day at 0.5 ppm HDMA and HDMA + ozone group: 12 monkeys were sensitized to HDMA Sensitized group: exposed to HDMA 2h/day on day 3-5 of FA (HDMA; n=6) or ozone (HDMA + ozone, n=6) exposure Non-sensitized group: exposed to FA (FA, n=6) or ozone (ozone, n=6)	ozone route of exposure: Inhalation, whole-body dose levels: 0 (filtered air) or 0.5 ppm Duration: Ozone: 8h/day for 5 days Animals were exposed to 11 episodes of filtered air (FA), house dust mite allergen aerosol (HDMA), ozone or HDMA + ozone 5 days each followed by 9 days of FA	**Impact on airway immune and structural development** 11 repeated 5-day cycles of inhaling 0.5 ppm ozone over a 6-month period had only mild effects on the airways of nonsensitized infant rhesus monkeys similarly, the repeated inhalation of HDMA by HDMA-sensitized infant monkeys resulted in only mild airway effects, with the exception of a marked increase in proximal airway and terminal bronchiole content of eosinophils (against FA) in contrast, the combined cyclic inhalation of ozone and HDMA by HDMA sensitized infants monkeys resulted in a marked increase in serum IgE, serum histamine (statistically significant from control and ozone), and airways eosinophilia (statistically significant from control and ozone for BAL and proximal airway; statistically significant from HDMA for terminal bronchioles) furthermore, combined cyclic inhalation of ozone and HDMA resulted in even greater alterations in airway structure and content that were associated with a significant elevation in baseline airways resistance (statistically significant from FA, ozone and HDMA) and reactivity (statistically significant from FA) Conclusion: these results suggest that ozone can amplify the allergic and structural remodelling effects of HDMA sensitization and inhalation	Schelegle E. S. *et al.* (2003a) Toxicology and Applied Pharamcology 191: 74-85
Ozone exposure during postnatal development Major organ target: Impact of ozone on serotonin and serotonin receptor expression in the developing lung GLP: No Reliability: 2	ozone route of exposure: Inhalation, whole-body dose levels: 0 (filtered air) or 0.5 ppm ozone Duration: AO: 0.5 ppm 8h/d	**Impact on the developing lung** lungs were prepared for compartment-specific qRT-PCR, immunohistochemistry, and stereology - airway epithelial serotonin immunopositive staining increased in all exposure groups with the most prominent in 2-month midlevel and 6-month distal airways gene expression of 5-HTT, 5-HT_{2A}R, and 5-HT_4R increased in age-dependent manner overall expression was greater in distal compared	Murphy S. R. *et al.* (2013), Toxicological Sciences 134: 168-179

Method, guideline, deviations if any, duration, major organ target, species, strain, sex, no/group	Test substance, route of exposure, dose levels, duration of exposure	Results	Reference
Monkey, rhesus macaque (*Macaca mulatta*), infant male Number/group: In total 24 animals, 4/group at each age Animals were assigned at 1 month of age to 2 age groups (2 or 6 months) and designated to 1 of 3 exposure subgroups: (1) filtered air (FA) (2) FA + acute ozone challenge (AO) (3) episodic biweekly ozone exposure cycles + AO (EAO)  	for 2 days prior necropsy EAO: episodic exposure 0.5 ppm 8h/d for 5 days followed by 9 days of FA; repeated over a 14-day cycle and 2 consecutive acute ozone exposures on days 14 and 15 of the last cycle; then necropsy (necropsy at 2 or 6 months of age)	with midlevel airways ozone exposure disrupted both 5-HT_{2A}R and 5-HT_4R protein expression in airways and enhanced immunopositive staining for 5-HT_{2A}R (2 months) and 5-HT_4R (6 months) on smooth muscle Conclusion: ozone exposure increases serotonin in airway epithelium regardless of airway level, age, and exposure history and changes the spatial pattern of serotonin receptor protein (5-HT_{2A} and 5-HT_4) and 5-HTT gene expression depending on compartment, age, and exposure history	
Ozone exposure during postnatal development Major organ target: Impact of ozone on airway epithelial death, the neurokinin-1 receptor pathway and the postnatal developing lung GLP: No Reliability: 2 Monkey, rhesus macaque (*Macaca mulatta*), infant male Number/group: in total 24 animals, 4/group	ozone route of exposure: Inhalation, whole-body dose levels: 0 (filtered air) or 0.5 ppm ozone duration: AO: 0.5 ppm 8h/d for 2 days prior necropsy EAO: episodic exposure 0.5 ppm 8h/d for 5 days followed by 9 days of FA; repeated over a 14-day cycle and 2 consecutive acute ozone exposures	**Impact on the developing lung** - ozone increases SP/NK-1R/Nur77 pathway expression in the conducting airways (partly statistically significant) - ozone exposure cycle (5 days/cycle) delivered early at age 2 months resulted in an airway that was hypersensitive to AO exposure at the end of 2 months - continued episodic exposure (11 cycles) resulted in an airway that was hyposensitive to AO exposure at 6 months Conclusion: - observations associate with greater overall inflammation and epithelial cell death, particularly in early postnatal (2 months), distal airways	Murphy S. R. *et al.* (2014), Am J Physiol Lung Cell Mol Physiol 307: L471-L481

Method, guideline, deviations if any, duration, major organ target, species, strain, sex, no/group	Test substance, route of exposure, dose levels, duration of exposure	Results	Reference
at each age Animals were assigned at 1 month of age to 2 age groups (2 or 6 months) and designated to 1 of 3 exposure subgroups: (1) filtered air (FA) (2) FA + acute ozone challenge (AO) (3) episodic biweekly ozone exposure cycles + AO (EAO)  	on days 14 and 15 of the last cycle; then necropsy (necropsy at 2 or 6 months of age)		
Repeated dose Toxicity study; Guideline: None From 1 month to 6 months of age (5 months) Major organ target lung GLP: No Reliability: 2 Monkey, rhesus (Macaca mulatta), male No/ group 8/exposure group 9/control group	ozone <u>route of exposure</u>: Inhalation <u>dose levels:</u> 0 (filtered air), 0.5 ppm ozone <u>duration:</u> 11 successive cycles with 5 days exposure (8h/d) followed by 9 days of filtered air + recovery of 6 months in filtered air +subset of animals (filtered air and ozone exposure) were challenged with LPS, 24 prior to necroscopy Time point: 1 year of age	primary airway epithelial cell cultures derived from monkeys after ozone exposure Innate immune function was measured by expression of the proinflammatory cytokines IL-6 and IL-8 in primary cultures established following in vivo LPS challenge or, in response to in vitro LPS treatment <u>Results:</u> Postnatal ozone exposure resulted in significantly attenuated IL-6 mRNA and protein expression in primary cultures from juvenile animals IL-8 mRNA was also significantly reduced effect of ozone exposure was modulated by in vivo LPS challenge Assessment of potential IL-6-targeting microRNAs miR-149, miR-202, and miR-410 showed differential expression in primary cultures based upon animal exposure history Functional assays revealed that miR-149 is capable of binding to the IL-6 3’ UTR and decreasing IL-6 protein synthesis in airway epithelial cell lines <u>Conclusion:</u> Episodic ozone during early life contributes to the molecular programming of airway epithelium, such that memory from prior exposures is retained in the form of a dysregulated IL-6 and IL-8 response to LPS differentially expressed microRNAs such as miR-149 may play a role in the persistent modulation	Clay C.C. et al. (2014), PLoS One. 9(3):e90401

Method, guideline, deviations if any, duration, major organ target, species, strain, sex, no/group	Test substance, route of exposure, dose levels, duration of exposure	Results	Reference
		of the epithelial innate immune response towards microbes in the mature lung	
Repeated dose Toxicity study; Guideline: None From 30 days of age until 6 months of age (5 months) Major organ target lung GLP status no Reliability 2 Monkey, rhesus (Macaca mulatta), male No/ group 4	ozone route of exposure: Inhalation dose levels: 0 (filtered air), 0.5 ppm ozone Duration: 11 cycles (1 cycle = 14 days) 8h/d on days 1-5 of each exposure cycle, followed by 9 days of filtered air + 6 months recovery (filtered air) → time point + sensitised monkeys to HDMA	Airway injury in infants (persistence/recovery of the altered epithelial innervation) nerve density in intrapulmonary airways (PGP 9.5) Results (after 6 months recovery period): hyperinnervation and irregular epithelial nerve distribution was observed in both HDMA- and ozone-exposed groups, but most prominent in animals exposed to HDMA plus ozone Conclusion: adaptive mechanisms exist that re-establish epithelial innervation following cessation exposure to HDMA and/or ozone, the recovery is associated with persistent proliferative mechanisms that result in hyperinnervation of the airways	Kajekar R. et al. (2007), Respir Physiol Neurobiol. 15;155(1):55-63
Repeated dose Toxicity study; 6 months Guideline: None Major organ target lung GLP: No Reliability: 2 Monkey, rhesus macaques No/ group 6	ozone route of exposure: Inhalation dose levels: 0 (filtered air), 0.5 ppm ozone Duration: 11 episodes: 5 days (8h/d) followed by 9 days of filtered air + sensitised monkeys to house dust mite allergen (HDMA)	Atypical development of tracheal basement membrane zone (BMZ) of infants structural changes: immunoreactivity of collagen I functional changes in the BMZ: perlecan, FGF-2, FGFR-1, syndecan-4 Results: width of the BMZ was irregular in the ozone groups (=atypical development) Perlecan was also absent from the BMZ In the absence of perlecan, FGF-2 was not bound to the BMZ FGF-2 immunoreactivity was present in basal cells, the lateral intercellular space (LIS), and attenuated fibroblasts FGFR-1 immunoreactivity was downregulated, and syndecan-4 immunoreactivity was upregulated in the basal cells Conclusion: ozone effected incorporation of perlecan into the BMZ changes are associated with specific alterations in the regulation of FGF-2, FGFR-1, and syndecan-4	Evans M.J. et al. (2003), Am J Physiol Lung Cell Mol Physiol. 285(4):L931-9

Method, guideline, deviations if any, duration, major organ target, species, strain, sex, no/group	Test substance, route of exposure, dose levels, duration of exposure	Results	Reference
		in the airway epithelial-mesenchymal trophic unit	
Repeated dose Toxicity study; 18 months (chronic) Guideline: None Major organ target lung GLP: No Reliability: 2 Monkey, rhesus (Macaca fascicularis), male No/ group 6	ozone route of exposure: Inhalation dose levels: 0 (filtered air), 0.25 ppm ozone Duration: 8h/d Daily exposure or seasonal (exposure to ozone only during odd numbered months, otherwise filtered air)	comparison of daily and seasonal exposure Results: all exposed monkeys had respiratory bronchiolitis with significant increases in related morphometric parameters lung growth was not completely normal for both exposures Seasonal exposure: significantly increased total lung collagen content, chest wall compliance, inspiratory capacity larger biochemical and physiological alterations and equivalent morphometric changes as daily exposed animals Daily exposure: significantly increased volume fraction of macrophages Conclusion: long-term effects of oxidant air pollutants which have a seasonal occurrence may be more dependent upon the sequence of polluted and clean air, than on the total number of days of pollution estimation of risks of human exposure to seasonal air pollutants from effects observed in animals after daily exposure may underestimate long-term pulmonary damage equivalent changes for episodic (half the time) and continuous exposure	Tyler W.S. et al. (1988), Toxicology. 50(2):131-44
Repeated dose Toxicity study; 20 months and 90 days only for antioxidant enzyme analysis Guideline: None Major organ target lung/nose (atrophy of bone in nasal turbinates) GLP: Yes Reliability: 2 Rat, F344/N (Simonsen Laboratories, Gilroy, CA), male and female	ozone route of exposure: Inhalation, whole body dose levels: 0 (filtered air), 0.12, 0.5, 1.0 ppm ozone mean conc. (±SD): 0.12 (± 0.01), 0.51 (± 0.02), and 1.01 (± 0.05) ppm duration: 6h/d, 5d/wk 20 months	Statistics: Statistical analysis performed twice: 1. using all animals (n=32) 2. excluding animals with marked liver and spleen leukemia (n=27) → presence of leukemia no confounding factor for ozone effects, results for n=32 are reported Airways - amount of stored epithelial mucosubstances was significantly reduced in the trachea (1.0 ppm), unchanged in the central bronchus, increased six-fold in the cranial bronchus, and increased three-fold in the caudal bronchus - epithelial cell composition of the airways was	Pinkerton K.E. et al. (1995), Research Report 65: Part IX Health Effect Institute. Library of Congress:WA7 54R432

Method, guideline, deviations if any, duration, major organ target, species, strain, sex, no/group	Test substance, route of exposure, dose levels, duration of exposure	Results	Reference
No/ group Tracheobronchial epithelium: 4 male and 4 female animals for control and each exposure group	or 90 days (only for antioxidant enzymes analysis)	unchanged in the trachea and bronchi (all concentrations) - nonciliated bronchiolar cell volume density was significantly increased in a dose-dependent manner in terminal bronchioles in the caudal left lung arising from a long airway path relative to the trachea (stat. significant 1.0 ppm) - epithelial thickness in tracheabronchial airways decreases with increase in concentration - extension of bronchiolarized epithelium into alveolar ducts was greater in cranial regions than in caudal regions (1.0 ppm) → bronchiolarization <u>Pulmonary acinus</u> - predominant changes in the ventilatory units of the lungs: extension of bronchiolar epithelium (ciliated and nonciliated cells) into alveolar ducts and increase in interstitial volume density - depth to which bronchiolar epithelium extended beyond the brochoalveolar duct junction was concentration-dependent and site-specific - most prominent changes were noted in male rats in ventilatory units arising from a short airway path (cranial region of the left lung), rather than ventilatory units arising from a long airway path (caudal region of the left lung) - stat. significant change in the ventilatory units of animals exposed to 0.12 ppm ozone consisted of the extension of bronchiolar epithelium 200-300 µm beyond the brochoalveolar duct junction, but this alteration was significant only in male animals and was most evident in ventilatory units arising from a short airway path (cranial region of the left-lung) - interstitial volume density significantly increased at 1.0 ppm <u>Antioxidant enzymes (90 days and 20 months exposure)</u> - Glutathione S-transferase (GST), glutathione peroxide (GPx), and superoxide dismutase (SOD) significantly increased in a concentration-dependent fashion in the distal bronchiolecentral acinus - SOD increased in a concentration-dependent fashion in the distal trachea (90 days) - Antioxidant enzyme activity responded	

<table>
<tr><th>Method, guideline, deviations if any, duration, major organ target, species, strain, sex, no/group</th><th>Test substance, route of exposure, dose levels, duration of exposure</th><th>Results</th><th>Reference</th></tr>
<tr><td></td><td></td><td>differently in different lung subcompartments

SOD: minor differences (90 days); stat. significant increase (0.5 and 1.0 ppm, 20 months)

GPx: sign. concentration-dependent changes in bronchi and bronchiole-central acinus (90 days); ~50 % elevation in activity (0.5 and 1.0 ppm, 20 months, bronchiole)

GST: sign. concentration-dependent changes in bronchi and bronchiole-central acinus (1.0 ppm: 50 % increase, 90 days); no concentration-dependent changes (20 months)

- Antioxidant enzyme activities for the whole lung do not reflect changes in lung subcompartments</td><td></td></tr>
<tr><td>Repeated dose Toxicity study; 20-months

Guideline: None

Major organ target: lung study

Effects on complex carbohydrates of lung connective tissue

GLP: Yes

Reliability: 2

Rat, Fischer-344, male and female

Number/group:

<u>(1) Total glycosaminoglycan</u>
0 ppm: 7M + 7F
0.12 ppm: 3M + 3F
0.5 ppm: 6M + 7F
1 ppm: 7M + 7F
In total: 47 rats

<u>(2) Individual glycosaminoglycans</u>
0 ppm: 10
0.12 ppm: 4
0.5 ppm: 7
1 ppm: 10
In total: 31 rats</td><td>ozone

<u>route of exposure</u>: Inhalation, whole body

<u>dose levels:</u> 0 (filtered air), 0.12, 0.5, 1 ppm ozone (nominal)

Exposure: 6h/d 5d/w for 20 months</td><td>Mean body weight:
0.12 ppm: no changes
0.5 ppm: no changes
1 ppm: 8 % ↓

Total concentration of glycosaminoglycans (GAGs) in lung tissues [µg uronic acid/mg dry-defatted tissue]; tissues were either right caudal lobe or accessory lobe:

<u>Mean concentration of GAGs</u>
0.12 ppm: 16 % ↓
0.5 ppm: 18 % ↓
1 ppm: 22 % ↓ (statistically significant, but not between M and F)

→ statistically significant trend between ozone exposure and decrease in GAGs

→ also statistically significant between right caudal lobes and accessory lobes (all groups together)

<u>Concentrations of individual GAGs in lung samples:</u>
<table>
<tr><th>GAGs</th><th>0.12 ppm</th><th>0.5 ppm</th><th>1 ppm</th></tr>
<tr><td>hyaluronan (HA)</td><td>21 % ↓</td><td>36 % ↓*</td><td>44 % ↓*</td></tr>
<tr><td>heparan sulfate (HS)</td><td>4 % ↓</td><td>7.5 % ↑[1]</td><td>20 % ↑[1]</td></tr>
<tr><td>chondroitin 4-sulfate (C4-S)</td><td>19 % ↓</td><td>19 % ↓*</td><td>19 % ↓*</td></tr>
<tr><td>chondroitin 6-sulfate (C6-S)</td><td>33 % ↓</td><td>36 % ↓*</td><td>33 % ↓*</td></tr>
<tr><td>dermatan sulfate (DS)</td><td>25 % ↓</td><td>25 % ↓</td><td>23 % ↓</td></tr>
<tr><td>heparin (HEP)</td><td>72 % ↓*</td><td>Un-changed</td><td>20 % ↓</td></tr>
</table>
* statistically significant vs. control ,[1] statistically</td><td>Radhakrishnamurthy, B. 1994; Research Report 65: Part III Health Effect Institute; Library of Congress: WA754R432.</td></tr>
</table>

Method, guideline, deviations if any, duration, major organ target, species, strain, sex, no/group	Test substance, route of exposure, dose levels, duration of exposure	Results	Reference
		significant trend (for 0.12 ppm only n = 4) Gel filtration for HA, HS, C4-S+C6-S+DS (pooled fractions): 0.5 ppm: peak of HA was broader 1 ppm: molecular size for HA smaller HS and C4-S+C6-S+DS: no changes Analyses of HS fractions from lung tissues (pooled fractions): Ratio glucuronic acid:iduronic acid 0 ppm -> 80:30 0.5 ppm -> 92:8 1 ppm -> 90:10 Total sulfate (mol/mol hexosamine): 0.5 ppm: ↓ 50 % 1 ppm: ↓ 47 % Ratio of low affinity to high affinity of antithrombin III: 0 ppm -> 81.1:18.9 0.5 ppm -> 90.8:9.2 1 ppm -> 92:8	
Repeated dose Toxicity study; 20 months Guideline: None Major organ target lung (pulmonary function in anesthetised rats - plethysmography) GLP: Yes Reliability: 2 Rat, F344/N (Simonsen Laboratories, Gilroy, CA), male and female No/ group: 4-9 animals/sex per group (8-18 animals/group) (61 animals in total)	ozone route of exposure: Inhalation, whole body dose levels: 0 (filtered air), 0.12, 0.5, 1.0 ppm ozone duration: 6h/d, 5d/wk for 20 months	Time point: 1-6 days after last exposure All exposure groups for combined gender (trends): - FRC reduced - RV reduced: 0.12 ppm (29 %), 0.5 ppm (38 %), 1.0 ppm (24 %) - ERV increased: 0.12 ppm (17 %), 0.5 ppm (28 %), 1.0 ppm (22 %) - Expiratory flow at 50 % FVC (F_{50}) increased (dose-related trend) - Expiratory flow at 10 % FVC (F_{10}) decreased 0.5 ppm for combined gender: → significant differences - Significantly higher VC/TLC (6 %) - Significantly lower RV (38 %) and RV/TLC (35 %) - Significantly higher ERV/TLC (33 %) Effects were largely driven by differences of females All exposure groups for females (trends): - Quasistatic chord compliance increased - VC increased (dose-related) - VC/TLC increased: 0.12 ppm (7 %), 0.5 ppm (7 %), 1.0 ppm (5 %) - RV reduced: 0.12 ppm (40 %), 0.5 ppm (40 %), 1.0 ppm (20 %) - ERV increased: 0.12 ppm (19 %), 0.5 ppm (31	Harkema J.R. and Mauderly J.L. (1994), Res Rep Health Eff Inst. (65 Pt 5):3-17; discussion 19-26

Method, guideline, deviations if any, duration, major organ target, species, strain, sex, no/group	Test substance, route of exposure, dose levels, duration of exposure	Results	Reference
		%), 1.0 ppm (31 %) - MMEF reduced - Expiratory flow at 50 % FVC (F_{50}) increased (dose-related) - Expiratory flow at 10 % FVC (F_{10}) decreased <u>0.5 ppm for females:</u> → significant differences - Significantly higher VC/TLC (7 %) - Significantly lower RV (40 %) - Significantly reduced RV/TLC (39 %) <u>All exposure groups for males (trends):</u> - RV reduced: 0.12 ppm (18 %), 0.5 ppm (31 %), 1.0 ppm (18 %) - ERV increased - Expiratory flow at 50 % FVC (F_{50}) increased - Expiratory flow at 10 % FVC (F_{10}) decreased <u>1.0 ppm for males:</u> → significant differences - Expiratory flow at 10 % FVC (F_{10}) decreased (30 %) Very weak dose response for the concentration of ozone for 6 parameters (VC, RV/TLC, D_{CO}, FRC, MMEF/FVC, F_{10})	
Repeated dose Toxicity study; 20 months Guideline: None Major organ target lung/nose (functional and structural changes in rat nose) GLP: Yes Reliability: 2 Rat, F344/N (Simonsen Laboratories, Gilroy, CA), male and female No/ group 2-9 animals/sex per group (4-14 animals/group) (47 animals in total, 21 males and 26 females)	ozone <u>route of exposure</u>: Inhalation, whole body <u>dose levels:</u> 0 (filtered air), 0.12, 0.5, 1.0 ppm ozone <u>duration:</u> 6h/d, 5d/wk for 20 months	Time point: 7-8 days after last exposure, all effects were present in female and male rats <u>Mucous flow:</u> Concentration dependent inhibition of mucociliary function at lateral wall site of the nose (with significant changes in mucous flow, for 50-92 % of animals in 1.0 ppm group mucous flow was absent), and slight increases of mucous flow in more distal areas 1.0 ppm: extensive changes in lateral meatuses and medial maxilloturbinates (0.5 ppm only some changes and 0.12 ppm no changes observed) with areas of mucostasis and ciliastasis 1.0 and 0.5 ppm: altered mucous (milky, copious, strings of viscid mucous adhering to tissue, altered directions, vortex-like flow) in areas where mucous was flowing, except nasal septum (here: no effect) 0.12 ppm: induce increases in mucous flow in 11 tested areas <u>Histopathology:</u> 1.0 ppm: significant morphological alterations (nasal mucosa wall, nasoturbinate,	Harkema J.R. et al. (1994), Res Rep Health Eff Inst. (65 Pt 7):3-26; discussion 27-34

Method, guideline, deviations if any, duration, major organ target, species, strain, sex, no/group	Test substance, route of exposure, dose levels, duration of exposure	Results	Reference
		maxilloturbinate); nasal transitional epithelium was 4-6-times thicker (hyperplasia) with numerous mucous cells filled with mucosubstances, 0.5 ppm: some morphological alterations (see above) 1.0 and 0.5 ppm: mucous cell metaplasia (less in 0.5 ppm group) in surface epithelium throughout the nasal airway accompanied by intraepithelial gland formation; bone atrophy of maxilloturbinates and nasoturbinates; eosinophilic globules throughout the respiratory and olfactory epithelium lining the ethmoid turbinates; chronic rhinitis (moderate inflammatory cell influx of lymphocytes, plasma cells, neutrophils) <u>Morphometry of intraepithelial mucosubstances:</u> 1.0 ppm: dramatically increased mucosubstances in nasoturbinate (proximal: 317 times the amount of control; middle region: 141 x control), maxilloturbinate (proximal: 171 x control; middle region: 24 x control), lateral wall (proximal: 27 x control; middle region: 280 x control) 0.5 ppm: increased mucosubstances in nasoturbinate (proximal: 98 times the control; middle region: 62 x control), maxilloturbinate (proximal: 78 x control; middle region: 12 x control), lateral wall (proximal: 13 x control; middle region: 97 x control) 0.12 ppm: no significant differences <u>Morphology and Morphometry of nasal transitional epithelium of proximal nasal airways:</u> 1.0 and 0.5 ppm: marked increase of luminal nonciliated cells with secretory granules (mucous cells or nonciliated cuboidal cells) 1.0 ppm: total epithelial cells significantly increased (143 % →hyperplasia), due to significant increase of secretory cells (control: 0 cells; 1.0 ppm: 94±10 cells) 0.5 ppm: slight increase of total epithelial cells (12 %), significant increase of secretory cells (control: 0 cells; 0.5 ppm: 71±7 cells) and decrease of nonciliated cells (30 %) 0.12 ppm: no significant differences <u>Morphology and morphometry of respiratory epithelium in nasal septum:</u> 1.0 ppm: increase of mucous cells (74 %) and reduced amount of mucoserous (95 %) and serous	

<table>
<tr><th>Method, guideline, deviations if any, duration, major organ target, species, strain, sex, no/group</th><th>Test substance, route of exposure, dose levels, duration of exposure</th><th>Results</th><th>Reference</th></tr>
<tr><td></td><td></td><td>(67 %) cells, basal cell hyperplasia in respiratory epithelium (50 % increase) and number of total epithelial cells increased (21 %)

0.5 ppm: mild increase of basal cells (27 %)</td><td></td></tr>
<tr><td>Repeated dose Toxicity study; 20 months (NTP/HEI) and 24 months (NTP animals)

Guideline: None

Major organ target lung/nose (atrophy of bone in nasal turbinates)

GLP: Yes

Reliability: 2

Rat, F344/N (Simonsen Laboratories, Gilroy, CA), male and female

No/ group

<u>20 months:</u>
37 animals in total, 4-5 animals / sex / group

<u>24 months:</u>
127 animals in total, 4-8 males and 23-28 females per group</td><td>ozone

<u>route of exposure</u>: Inhalation, whole body

<u>dose levels:</u> 0 (filtered air), 0.12, 0.5, 1.0 ppm ozone

<u>duration:</u>
6h/d, 5d/wk for 20 months (NTP/HEI) and 24 months (NTP animals)</td><td>Time point: one week after the last exposure

<u>Morphologic changes in nasal tissues:</u>

No time related differences (similar for 20 and 24 months), no gender specific effects or specially mentioned

1.0 ppm: significant morphologic alterations (0.5 ppm less severe alterations and 0.12 ppm no detectable differences) in nasal mucosa of the lateral wall (nasoturbinate and maxilloturbinate)

1.0 and 0.5 ppm: epithelial thickening (mucous cell metaplasia and epithelial hyperplasia) and bone atrophy of nasal turbinates (results in shortening of maxilloturbinate, loss of bone matrix and bone resorption) which was more obvious in male, thinning of lamina propria, influx of inflammatory cells in nasal mucosa (more severe in highest dose group) with chronic rhinitis more prominent in males, constricted blood vessels

<u>Morphology of maxilloturbinates, 20 months exposure:</u>

Table 2. Summary of the Ozone-Related Changes in the Cross-Sectional Area of Maxilloturbinate Tissues After a 20-Month Exposure to Ozone (NTP/HEI Study)[a]

<table>
<tr><th></th><th colspan="6">Ozone Concentration (ppm)</th></tr>
<tr><th></th><th colspan="3">Male Rats</th><th colspan="3">Female Rats</th></tr>
<tr><th>Nasal Tissue</th><th>0.12</th><th>0.5</th><th>1.0</th><th>0.12</th><th>0.5</th><th>1.0</th></tr>
<tr><td>Bone</td><td>↔</td><td>↓</td><td>↓</td><td>↔</td><td>↓</td><td>↓</td></tr>
<tr><td>Lamina propria</td><td>↔</td><td>↓</td><td>↓</td><td>↔</td><td>↔</td><td>↔</td></tr>
<tr><td>Surface epithelium</td><td>↔</td><td>↔</td><td>↔</td><td>↔</td><td>↑</td><td>↑</td></tr>
<tr><td>Total turbinate</td><td>↔</td><td>↓</td><td>↓</td><td>↔</td><td>↔</td><td>↔</td></tr>
</table>
[a] ↔ = Not significantly different from, ↑ = significantly greater than, and ↓ = significantly less than control group (0 ppm).

<u>Turbinate bone:</u> reduced bony tissue in 1.0 ppm group (females: 50 %, males: 64 %) and 0.5 ppm group (both genders 52 %)

<u>lamina propria:</u> reduced in males for 1.0 ppm (40 %) and 0.5 ppm (45 %)

<u>surface epithelium</u>: dose-dependent increase in females (0.5 ppm: 55 %, 1.0 ppm: 90 %); in males also thickened epithelium, but concomitant</td><td>Harkema J.R. et al. (1997), Res Rep Health Eff Inst. (65 (Pt 12)):1-19; discussion 21-6</td></tr>
</table>

<table>
<tr><th>Method, guideline, deviations if any, duration, major organ target, species, strain, sex, no/group</th><th>Test substance, route of exposure, dose levels, duration of exposure</th><th>Results</th><th>Reference</th></tr>
<tr><td></td><td></td><td>atrophy of total maxilloturbinate (in females not counteracted as much as in males)

Total turbinate: loss in total maxilloturbinate area in males (0.5 and 1.0 ppm: 38 %)

Morphology of maxilloturbinates, 24 months exposure:

Table 3. Summary of the Ozone-Related Changes in the Cross-Sectional Area of Maxilloturbinate Tissues After a 24-Month Exposure to Ozone (NTP Study)[a]

<table>
<tr><th></th><th colspan="6">Ozone Concentration (ppm)</th></tr>
<tr><th></th><th colspan="3">Male Rats</th><th colspan="3">Female Rats</th></tr>
<tr><th>Nasal Tissue</th><th>0.12</th><th>0.5</th><th>1.0</th><th>0.12</th><th>0.5</th><th>1.0</th></tr>
<tr><td>Bone</td><td>↔</td><td>↓</td><td>↓</td><td>↔</td><td>↔</td><td>↓</td></tr>
<tr><td>Lamina propria</td><td>↔</td><td>↓</td><td>↓</td><td>↔</td><td>↓</td><td>↓</td></tr>
<tr><td>Surface epithelium</td><td>↔</td><td>↑</td><td>↑</td><td>↔</td><td>↑</td><td>↑</td></tr>
<tr><td>Total turbinate</td><td>↔</td><td>↓</td><td>↓</td><td>↔</td><td>↔</td><td>↔</td></tr>
</table>
[a] ↔ = Not significantly different from, ↑ = significantly greater than, and ↓ = significantly less than control group (0 ppm).

Turbinate bone: reduced bony tissue in 1.0 ppm group (females: 38 %, males: 52 %) and 0.5 ppm group (males: 49 %)

lamina propria: reduced areas in 1.0 ppm group (females: 22 %, males: 43 %) and 0.5 ppm group (females: 16 %, males: 28 %), changes due to reduces areas of blood vessel lumina

surface epithelium: increased areas in 1.0 ppm group (females: 60 %, males: 40 %) and 0.5 ppm group (females: 60 %, males: 25 %), changes due to hyperplasia and metaplasia

Total turbinate: turbinate atrophy in males (0.5 ppm: 23 % and 1.0 ppm: 36 %)

Nasal airway: in all dose groups increased nasal luminal area in both genders (9 % to 11 %), but only in 0.5 ppm group significantly smaller nasal luminal perimeter (females: 2.5 %, males: 11 %) resulting in no dose dependency</td><td></td></tr>
<tr><td>Repeated dose Toxicity study; 20 months

Guideline: None

Major organ target lung/nose (atrophy of bone in nasal turbinates)

GLP: Yes</td><td>ozone

route of exposure: Inhalation, whole body

dose levels: 0 (filtered air), 0.12, 0.5, 1.0 ppm ozone

duration:</td><td>Statistics:

Statistical analysis performed twice:
1. excluding 1 animal with marked lung leukemia (n=38)

2. excluding the animal with lung leukemia and all animals with liver and spleen leukemia (n=27)

→ presence of leukemia no confounding factor</td><td>Chang L.Y. et al. (1995), Res Rep Health Eff Inst. (65 Pt 8-9):3-39; discussion 99-110</td></tr>
</table>

Method, guideline, deviations if any, duration, major organ target, species, strain, sex, no/group	Test substance, route of exposure, dose levels, duration of exposure	Results	Reference
Reliability: 2 Rat, F344/N (Simonsen Laboratories, Gilroy, CA), male and female No/ group 48 animals in total in study 38 animals included in final analysis of proximal alveolar region: control: 10 0.12 ppm: 12 0.5 ppm: 8 1.0 ppm: 8 33 animals included in in final analysis for random alveolar regions control: 10 0.5 ppm: 12 1.0 ppm: 11	6h/d, 5d/wk for 20 months	for ozone effects, results for n=38 are reported No significant effect attributed to gender → effects from gender not considered <u>Effects on proximal alveolar region:</u> - Increase of total tissue volume in proximal alveolar region stat. significant - stat. significant increase in percentage of bronchiolarization at 1.0 ppm, tissue volume of interstitium at 0.5 and 1.0 ppm, total inflammatory cells at 1.0 ppm <u>Epithelium:</u> 0.12 ppm: no alterations 0.5 and 1.0 ppm: - major changes: epithelial metaplasia (change from squamous to cuboidal bronchiolar epithelium in proximal alveolar region) - no effect on mean cell surface area: total epithelial volume increased, due to metaplasia - number of type I cells increased (0.5: 64 %; 1.0: 74 %), but their size and surface area decreased (0.5: 40 %; 1.0: 50 %) - type II cells not affected <u>Interstitium:</u> (volume increased as function of concentration) 0.12 ppm: no alterations 0.5 and 1.0 ppm: - significant increase in volume of cellular and non-cellular components (0.5: 53 %; 1.0: 71 %), because matrix components (collagen, elastin, basement membrane, acellular space) increased and increase of interstitial fibroblasts - Collagen: 0.5ppm: 64 % and 1.0ppm: 78 % increase - Basement membrane thickening: Elastin: 1.0ppm: 80 % increase; Acellular space: 1.0ppm: 113 % increase <u>Endothelium and Capillaries:</u> - no sign. differences in volume of endothelium for all concentrations, only slight increase of endothelial cells at 1.0 ppm - increase of capillary surface only at 0.5 ppm <u>Evidence of Inflammation (volume of inflamm.</u>	

Method, guideline, deviations if any, duration, major organ target, species, strain, sex, no/group	Test substance, route of exposure, dose levels, duration of exposure	Results	Reference
		cells): 0.12 and 0.5 ppm: no effect 1.0 ppm: 113 % increase in alveolar macrophages in proximal alveolar region Effects on random alveolar region: (only 0.5 and 1.0 ppm animals analysed, because effects at 0.12 ppm restricted to proximal region) - no stat. significant concentration effect - no effects found for bronchiolarization, type I and II cells, interstitium (matrix volume and components), fibroblasts Effects on terminal bronchioles: - 1.0 ppm: stat. significant decrease of ciliated cell number (20 %) and increase of Clara cell number (54 %) and volume (15 %) 13 % decrease of total number of basement membrane - no effect for epithelial thickness, cell volume of ciliated cells, and surface area of ciliated and Clara cells, average diameter of bronchioles	
Repeated dose Toxicity study; 20 months Guideline: None Major organ target lung GLP: No Reliability: 2 Rat, Fisher 344, male No/ group 4 animals/group	ozone route of exposure: Inhalation, whole body dose levels: 0 (air), 1.0 ppm ozone (1.0 to 1.02 ±0.07 ppm) duration: 6h/day, 5 days/week for 20 months	distribution and degree of differentiation of ciliated and nonciliated bronchiolar epithelial (Clara) cells lining alveolar ducts of the central acinus Results: high degree of heterogeneity in the magnitude of bronchiolar epithelial cell extension into alveolar ducts was noted for each isolation and animal striking similarity was noted by scanning electron microscopy in the surface characteristics of cells lining both terminal bronchiole of exposed animals well-differentiated ciliated and nonciliated bronchiolar epithelial cells were found lining alveolar septal tips and alveoli up to a depth of 1,000 µm into the pulmonary acinus Conclusion: epithelial cell transformations in alveolar ducts is a natural consequence of lifetime exposures to oxidant gases, because there was no evidence of inflammation was present in alveolar ducts	Pinkerton K.E. et al. (1993), Am J Pathol. 142(3):947-56
Repeated dose Toxicity study; 20 months	ozone route of exposure:	Animal characteristics/lung weight: Mean final bw [g]	Last *et al.* 1994; Research Report 65: Part

Method, guideline, deviations if any, duration, major organ target, species, strain, sex, no/group	Test substance, route of exposure, dose levels, duration of exposure	Results	Reference
Guideline: None Lung study Major organ target: Content and cross-linking of lung collagen GLP: Yes Reliability: 2 Rat, Fischer-344, male and female Number/group: Biochemical analysis: 0 ppm: 6M + 6F 0.12 ppm: 3M + 3F 0.5 ppm: 6M + 6F 1 ppm: 6M + 6F In total: 42 rats Histopathology: 0 ppm: 2M + 3F 0.12 ppm: 2M + 2F 0.5 ppm: 3M + 3F 1 ppm: 3M + 3F In total: 21 rats	Inhalation, whole body dose levels: 0 (filtered air), 0.12, 0.5, 1 ppm ozone (nominal) duration: 6h/d 5d/w for 20 months	0.12 ppm M: 6 % ↓ F: 4 % ↑ 0.5 ppm M: 19 % ↓ F: 6 % ↑ 1 ppm M: 8 % ↓ 10 % ↓ Mean final lung wet weight [mg] 0.12 ppm M: 21 % ↓ F: unchanged 0.5 ppm M: 36 % ↑[1] F: 34 % ↑ 1 ppm M: 1 % ↑ F: 59 % ↑ [1] 2 rats had severe mononuclear leukemia (10 % ↓ without both animals) DNA content per lung lobe [µg]: 0.12 ppm M: 2 % ↑ F: 33 % ↑ 0.5 ppm M: 19 % ↓ F: 33 % ↑ 1 ppm M: 14 % ↓ F: 48 % ↑ Collagen content of cranial lung lobes: 4-Hydroxyproline content per lung lobe [nmol] 0.12 ppm M: 7 % ↓ F: 1 % ↑ 0.5 ppm M: 3 % ↓ F: 12 % ↑ 1 ppm M: 4 % ↑ F: 26 % ↑ -> statistically significant trend in F 4-Hydroxyproline content per lung weight [nmol/g] 0.12 ppm M: 12 % ↑ F: unchanged 0.5 ppm M: 19 % ↓ F: 7 % ↓ 1 ppm M: 6 % ↑ F: 4 % ↓ Collagen cross-links in lung: OHP per collagen [mol/mol] 0.12 ppm M: 8 % ↑ F: 27 % ↑ 0.5 ppm M: 8 % ↑ F: 18 % ↑ 1 ppm M: 8 % ↑ F: 27 % ↑ OHP per lung weight [nmol/g] 0.12 ppm M: 21 % ↑ F: 33 % ↑ 0.5 ppm M: 16 % ↑ F: 9 % ↑ 1 ppm M: 10 % ↑ F: 15 % ↑ OHP per lung lobe [nmol]	I Health Effect Institute; Library of Congress: WA754R432.

<table>
<tr><th>Method, guideline, deviations if any, duration, major organ target, species, strain, sex, no/group</th><th>Test substance, route of exposure, dose levels, duration of exposure</th><th>Results</th><th>Reference</th></tr>
<tr><td></td><td></td><td>0.12 ppm M: 2 % ↑ F: 34 % ↑
0.5 ppm M: 2 % ↑ F: 60 % ↑
1 ppm M: 8 % ↑ F: 66 % ↑
-> statistically significant trend in F

Collagen dysfunctional cross-links in lungs
<u>DHLNL:HLNL ratio</u>
0.12 ppm M: 11 % ↓ F: 4 % ↓
0.5 ppm M: 11 % ↑ F: 17 % ↑
1 ppm M: 7 % ↑ F: 17 % ↑
-> statistically significant trend in F
<u>Histopathology</u> (localization and extent of fibrosis), confirmed by second pathologist: Amount of collagen visualized in centriacinar region:
Number of Rats per Exposure Group:

0.5 ppm: epithelial hyperplasia; interstitial fibrosis; centriacinar fibrosis: average score of 2.5 for intramural and interstitial collagen in centriacinar regions
1 ppm: number of respiratory bronchioles ↑; more severe epithelial hyperplasia and interstitial fibrosis; moderate to marked centriacinar fibrosis; average score of 3.8 for interstitial collagen

Remark: number of rats in low dose group (and controls) not sufficient to draw conclusion regarding significance against controls → unclear whether NOAEL exists between 0.12 and 0.5 ppm</td><td></td></tr>
<tr><td>2-year inhalation study
Guideline: similar to TG 451
GLP: in compliance with Food and Drug Administration (FDA) Good Laboratory Practice Regulations
Reliability: 2
Rat, F344/N rats (Simonsen Laboratories (Gilroy, CA)), male and female</td><td>ozone
<u>route of exposure</u>: Inhalation, whole body
<u>dose levels:</u> 0 (Filtered air), 0.12, 0.5 and 1.0 ppm
<u>duration:</u> 6h/day 5 days/week for 2 years</td><td><u>Survival:</u>
- no differences between exposure groups and control
- males: in every group high number of moribund animals 35-40 animals;
- animals surviving to study termination:
<table><tr><td></td><td>0</td><td>0.12 ppm</td><td>0.5 ppm</td><td>1 ppm</td></tr><tr><td>Males</td><td>8</td><td>5</td><td>7</td><td>7</td></tr><tr><td>Females</td><td>28</td><td>24</td><td>30</td><td>27</td></tr></table><u>Mean body weight</u>
- no differences at 0.12 and 0.5 ppm
- slightly reduced (male: 6 %; female: 8-6 %) at 1.0 ppm during exposure</td><td>NTP, Toxicology and carcinogenesis studies of ozone and ozone/NKK in F344/N rats and B6C3F1 mice, National toxicology program, Technical report series 440 (1994)</td></tr>
</table>

<table>
<tr><th>Method, guideline, deviations if any, duration, major organ target, species, strain, sex, no/group</th><th>Test substance, route of exposure, dose levels, duration of exposure</th><th>Results</th><th>Reference</th></tr>
<tr><td>No/Group: 50 per sex and group</td><td></td><td>Clinical:
- hypoactivity, in particular at 1 ppm

Neoplastic lesions and non-neoplastic lesions: refer to Tab.22

0.5 ppm and 1.0 ppm: lung: histiocytic infiltration in alveolus, interstitial fibrosis

Conclusion:

Serious findings of interstitial fibrosis at 0.5 ppm in rat study with chronic exposure. Further details refer to Tab.22.</td><td>Boorman G. A. et al. (1994), Toxicol Pathol. 22(5):545-54

Boorman G.A. et al. (1995), Toxicol Lett. 82-83:301-6</td></tr>
<tr><td>Lifetime inhalation study

Guideline: no

GLP: in compliance with Food and Drug Administration (FDA) Good Laboratory Practice Regulations

Reliability: 1

Rat, F344/N rats (Simonsen Laboratories (Gilroy, CA)), male and female

No/Group: 50 per sex and group</td><td>ozone

route of exposure: Inhalation, whole body

dose levels: 0 (filtered air), 0.5 and 1.0 ppm

duration: 6h/day 5 days/week for 125 weeks</td><td>Survival:
- no differences between exposure groups and control
- in every group high number of moribund animals (males: 42-47 ; females: 36-40)
- animals surviving to study termination
<table><tr><td></td><td>0</td><td>0.5 ppm</td><td>1 ppm</td></tr><tr><td>Males</td><td>0</td><td>0</td><td>0</td></tr><tr><td>Females</td><td>6</td><td>6</td><td>7</td></tr></table>
Mean body weight
- mean bw and bw gains in females and males (1 ppm) were slightly lower than in controls
- final mean bw similar to controls

Clinical:
- hypoactivity, particularly at 1 ppm

Neoplastic lesions and non-neoplastic lesions: refer to Tab.22

0.5 ppm: lung: alveolar infiltration, histiocyte; interstitial fibrosis

additional 1.0 ppm: no further effects

Conclusion:

Serious findings of interstitial fibrosis at 0.5 ppm in rat study with chronic exposure. Further details refer to Tab.22.</td><td>NTP, Toxicology and carcinogenesis studies of ozone and ozone/NKK in F344/N rats and B6C3F1 mice, National toxicology program, Technical report series 440 (1994)

Boorman G. A. et al. (1995), Toxicology letters 82/83: 301-306

Herbert R. A. et al. (1996), Toxicol. Pathol. 24: 539-548</td></tr>
</table>

10.12.1 Short summary and overall relevance of the provided information on specific target organ toxicity – repeated exposure

Animal studies

A NOAEC for systemic effects was not derived due to insufficiencies in study designs and reporting as well as the potential relevance of cardiovascular and neurologic findings at the lowest tested dose level of 0.1-0.12 ppm (LOEL systemic).

Cardiac effects:

As summarized in Table **45** below, repeated exposure to ozone lead to a decrease in heart rate at doses ranging from 0.1 – 1 ppm, respectively. The effect was recovered after a few days of exposure in repeated dose studies. In one study (Watkinson, 2003; 0.5 ppm), the effect was found to be more pronounced during physical exercise and at lower ambient temperatures. Arrhythmic episodes were found to be increased after repeated exposure in a concentration dependent manner (Arito, 1990; 0.1 and 0.2 ppm). The effect was recovered on exposure day 3. Core body temperature was found to decrease under ozone exposure in both acute (0.2 – 1 ppm) and repeated dose studies (0.5 – 1 ppm). The effect recovered after a few days of exposure in the repeated dose studies and was found to be more pronounced during physical exercise and at lower ambient temperatures (Watkinson, 2003). Blood pressure was investigated in only one study (Gordon, 2013), where no effect was found. The review by Prueitt et al. (2014), "*Weight-of-evidence evaluation of long-term ozone exposure and cardiovascular effects*", confirms the study quality of Gordon et al. 2013 by assigning a total score of 4. Gordon 2013 is the only study on heart rate considered therein while Arito, 1997 and 1990, Iwasaki 1998, Watkinson 2003 and Gordon 2014 were not evaluated. However, the effects on heart rate and arrhythmia in the additional studies analysed and listed below were regarded as relevant for classification STOT RE.

Hormonal effects:

Corticosterone levels were increased after repeated exposure to 0.1 ppm ozone (Martrette, 2011) Estrogen receptors, estrogen receptor proteins and dopamine beta-hydroxylase were decreased in the olfactory bulb in a repeated dose study with 0.2 ppm (Guevara-Guzman, 2009).

Behavioural effects:

As summarized in Table 46, motor activity was decreased in repeated dose studies at doses starting at 0.25 ppm (Pereyra-Munoz, 2006, Gordon, 2013). Several behaviours, such as grooming, resting, rearing and jumping-play were affected after repeated exposure to 0.12 ppm (Martrette, 2011). In addition, olfactory memory was impaired after repeated exposure to 0.25 ppm (Guevara-Guzman, 2009). A study investigating sleep patterns reported no observed effects concerning sleep (Arito, 1990).

Table 45: Summary of cardiovascular effects observed in acute and repeated dose studies

	Cardiovascular effects							
dose/target		**0.1 ppm**	**0.2 ppm**	**0.3 - 0.35 ppm**	**0.5 ppm**	**0.75 – 0.8 ppm**	**1 ppm**	**1.5 ppm**
heart rate	acute:				**lower** heart rate (reversible) Watkinson, 2003 Iwasaki, 1998	**lower** heart rate (reversible) Gordon, 2013	**lower** heart rate (reversible) Gordon, 2014	
	repeated dose:	**lower** heart rate (with recovery) Arito, 1997 Iwasaki, 1998	**lower** heart rate (with recovery) Arito, 1990	**lower** heart rate (with recovery) Iwasaki, 1998	**lower** heart rate (with recovery; more pronounced during exercise and at lower ambient temperatures) Watkinson, 2003 Iwasaki, 1998	no effect Gordon, 2013	**lower** heart rate (reversible) Gordon, 2014	
arrhythmia	acute:		**increased** sensitivity to aconitin-induced arrhythmia formation Farraj, 2012			**increased** (reversible) increased sensitivity to aconitin-induced arrhythmia formation Farraj, 2012		
	repeated dose:	**increased** bradyarrhythmic episodes (with recovery) Arito, 1990	**increased** bradyarrhythmic episodes (with recovery) Arito, 1990					
blood pressure	acute:							
	repeated					no effect		

	Cardiovascular effects							
dose/target		**0.1 ppm**	**0.2 ppm**	**0.3 - 0.35 ppm**	**0.5 ppm**	**0.75 – 0.8 ppm**	**1 ppm**	**1.5 ppm**
	dose:					Gordon, 2013		
core body temperature	acute:					**decreased** core body temperature Farraj, 2012	**lower** core body temperature (reversible) Gordon, 2014	
	repeated dose:				**decreased** core body temperature (with recovery; more pronounced during exercise and at lower ambient temperatures) Watkinson,2003 Iwasaki, 1998		**lower** core body temperature (with recovery) Gordon, 2014	
other – repeated dose						effects on markers of vascular disease Gordon, 2013		

Table 46: Summary of behavioural effects observed in acute and repeated dose studies

	Behavioural effects							
dose/target		**0.1 - 0.12 ppm**	**0.2 – 0.25 ppm**	**0.35 ppm**	**0.5 ppm**	**0.75 – 0.8 ppm**	**1 ppm**	**1.5 ppm**
motor activity	acute	**decreased** motor activity (reversible) Rivas-Arancibia, 1998	**decreased** motor activity (reversible) Rivas-Arancibia, 1998					
	repeated dose		**decreased** motor activity Pereyra-Munoz,			**Decreased** motor activity Gordon, 2013		

	Behavioural effects							
dose/target		**0.1 - 0.12 ppm**	**0.2 – 0.25 ppm**	**0.35 ppm**	**0.5 ppm**	**0.75 – 0.8 ppm**	**1 ppm**	**1.5 ppm**
			2006					
exploratory behaviour	acute						**decreased** exploratory behaviour (reversible in one study) Rivas-Arancibia, 2003	
freezing behaviour	acute						**increased** freezing behaviour (reversible in one study) Avila-Costa, 2001 Rivas-Arancibia, 2003	
Grooming, resting, rearing, jumping-play, drinking	repeated dose	increased: resting, drinking decreased: rearing, jumping-play Martrette, 2011						
time remaining in safety compartment before entering shock compartment	acute	**decreased** time remaining in safety compartment before entering shock compartment Rivas-Arancibia, 1998	**decreased** time remaining in safety compartment before entering shock compartment Rivas-Arancibia, 1998		**decreased** time remaining in safety compartment before entering shock compartment Rivas-Arancibia, 1998		**decreased** time remaining in safety compartment before entering shock compartment Avila-Costa, 1999 Rivas-Arancibia, 1998	

	Behavioural effects							
dose/target		**0.1 - 0.12 ppm**	**0.2 – 0.25 ppm**	**0.35 ppm**	**0.5 ppm**	**0.75 – 0.8 ppm**	**1 ppm**	**1.5 ppm**
olfactory memory	repeated dose		**impaired** recognition of stimulus animal **impaired** speed locating a buried chocolate Guevara-Guzman, 2009					
wakefulness	acute				**reduced** wakefulness Arito, 1992		**reduced** wakefulness Arito, 1992	**reduced** wakefulness Paz and Huitron-Resendiz, 1996
	repeated dose	no effect Arito, 1990	no effect Arito, 1990					
paradoxical sleep	acute			**reduced** paradoxical sleep Paz and Huitron-Resendiz, 1996	**reduced** paradoxical sleep (reversible) Arito, 1992	**reduced** paradoxical sleep Paz and Huitron-Resendiz, 1996	**reduced** paradoxical sleep (reversible) Arito, 1992	**reduced** paradoxical sleep Paz and Huitron-Resendiz, 1996
	repeated dose	no effect Arito, 1990	no effect Arito, 1990					
slow-wave sleep	acute			**increased** slow-wave sleep Paz and Huitron-Resendiz, 1996	**increased** slow-wave sleep (reversible) Arito, 1992	**increased** slow-wave sleep Paz and Huitron-Resendiz, 1996	**increased** slow-wave sleep (reversible) Arito, 1992	**increased** slow-wave sleep Paz and Huitron-Resendiz, 1996
	repeated dose	no effect Arito, 1990	no effect Arito, 1990					

Table 47: Summary of effects on the central nervous system observed in acute and repeated dose studies

	CNS effects							
dose/target		**0.1 - 0.12 ppm**	**0.2 – 0.25 ppm**	**0.35 ppm**	**0.5 ppm**	**0.75 – 0.8 ppm**	**1 ppm**	**1.5 ppm**
number of dendritic spines	acute						**reduced** number of dendritic spines Avila-Costa, 1999	
neuronal changes	repeated dose		morphological alterations, cell death in dopaminergic neurons in striatum and substantia nigra Pereyra-Munoz, 2006 morphological alterations and cell swelling in hippocampus Rivas-Arancibia, 2010				Abnormal structures in molecular layer of cerebellum in offspring of dams exposed to ozone during gestation Romero-Velázquez, 2002	
neurogenesis	repeated dose		increased after 30-d (but with morphological alterations), decreased after 60 and 90 d Rivas-Arancibia, 2010					
other hippocampus changes	repeated dose		increases in activated and phagocytic microglia increased number of astrocytes decreased Neu-N and doublecortin Rivas-Arancibia, 2010					

	CNS effects							
dose/target		**0.1 - 0.12 ppm**	**0.2 – 0.25 ppm**	**0.35 ppm**	**0.5 ppm**	**0.75 – 0.8 ppm**	**1 ppm**	**1.5 ppm**
EEG amplitude	acute				**lower** EEG amplitude (reversible) Arito, 1992		**lower** EEG amplitude (reversible) Arito, 1992	
antioxidant levels	acute	**increased** antioxidant enzyme levels in brain Rivas-Arancibia, 1998	**increased** antioxidant enzyme levels in brain Rivas-Arancibia, 1998		**decreased** antioxidant enzyme levels in brain Rivas-Arancibia, 1998		**decreased** antioxidant enzyme levels in brain Rivas-Arancibia, 1998	
lipid peroxidation	acute						**increased** lipid peroxidation in brain (reversible) Rivas-Arancibia, 2003	
	repeated dose		**increased** in striatum and hippocampus Pereyra-Munoz, 2006 **increased** in olfactory bulb Guevara-Guzman, 2009					

Effects on the nervous system:

As summarized in Table 47, Pereyra-Munoz (2006) found morphological alterations, loss of fibres and cell death of the dopaminergic neurons in the striatum and substantia nigra after 4 h exposure per day for an entire period of 15 or 30 days to 0.25 ppm. This effect was accompanied by an increase in lipid peroxidation in the striatum and a decrease in motor activity. Moreover, neuronal morphological changes were also found in the hippocampus, along with swelling of neurons at 0.25 ppm (Rivas-Arancibia, 2010). In this study, the authors reported several additional effects that in their view are analogous to those seen in Alzheimer's disease (altered neurogenesis, increased lipid peroxidation, increased phagocytic microglia, increased astrocytes and memory deficiency). In addition Romero-Velázquez (2002) observed abnormal structures in the molecular layer of cerebellum in the offspring of rat dams exposed to 1 ppm ozone during entire gestation. The study did find altered morphology of pup cerebellum, confirmed with a decrease of total area and number of Purkinje cells, because of depopulation of and degenerating Purkinje cells in the cerebellum. These observations were accompanied by incomplete folding pattern of some lobes of the cerebellum, caused by ozone.

Local Respiratory effects:

Repeated ozone exposure for varying duration resulted in epithelial cell injury and pulmonary inflammation throughout ozone exposure. Cellular inflammation and the physiologic repair mechanisms were often linked to a follow up of predominant structural changes, such as epithelial hyperplasia and metaplasia, necrosis of ciliated cells and fibroblast proliferation in different parts of the respiratory system. Acute and short-term exposure was linked to inflammatory responses with the greatest magnitude seen after long-term exposure. Acute single exposure produced lung injury in animal studies showing signs of inflammation (bronchiolitis and peribronchiolar alveolitis (Hotchkiss, 1989a)), cell damage (Bassett, 1988), disruption of mucosal barrier (Bhalla, 2000), necrosis of type I epithelial cells (Pino, 1992) and progressive thickening of the walls of terminal bronchioles and proximal alveoli (Hotchkiss, 1989b). Arising acute biochemical effects (protein, albumin content and neutrophil influx in BAL) returned to control levels after cessation of exposure, but it took some time for complete repair from airway inflammation. However, structural changes, such as thickening of epithelial layer and collagen formation increased during prolonged exposure and were still present after recovery periods (Van Bree, 2001).

One dominating effect was hyperplasia of respiratory and nasal epithelium, which showed a dose-response relation and was directly related to the cumulative oxidant concentration (Chang, 1991). These effects were observed in different species.

Studies with rhesus monkeys exposed to 0.5 ppm ozone reported rhinitis, necrosis, squamous metaplasia, epithelial changes, such as exfoliation of epithelium lining and hyperplasia in nasal airways (Cary, 2011 and 2007). Moreover, ozone exposure to low concentrations, led to morphometrically detected lesions in the nose and lung (Harkema, 1987 and 1993) and even lower background exposure may contribute to epithelial cell injuries. Chang (1991) observed changes at concentrations starting from 0.12 ppm ozone. Further effects caused by subchronic exposure to low ozone levels were interstitial fibrosis in proximal alveolar region and bronchiolar epithelial injury (Chang, 1992).

Indeed, ozone exposure exert effects at critical developmental stages of infants. Because exposed infant monkeys developed 4 fewer nonalveolarized airway generations, the terminal and most proximal respiratory bronchiole were smaller and had altered smooth muscle bundle orientation in bronchioles after ozone exposure during normal distal airway development (Fanucchi, 2006). In addition Evans (2003) reported atypical development of the tracheal basement membrane of infant monkeys. Alterations in airway innervation, such as hyperinnervation due to dramatic increase in airway nerve density and irregular epithelial nerve distribution were also contributed to ozone exposure (Kajekar, 2007). Gunnison (1992) exposed rats at different ages for 2 hours and concluded age-dependent sensitivity to ozone-induced cellular damage and young neonates may be at increased risk relative to adults to some consequences of ozone exposure. These results indicate that early life ozone exposure may cause persistent alterations.

Simulated episodic exposure studies suggest that such exposures might have cumulative impacts. Functional changes during episodic exposure comprised rapid shallow breathing for the first two days accompanied with structural remodelling, such as epithelial hyperplasia and hypercellularity and also interstitial and intraluminal inflammation (Schelegle, 2003). As reported, episodically exposed animals had similar changes in physiology and biochemistry compared to continuously exposed animals for the same exposure duration, even if they were

exposed half the time (Tyler 1988). Episodic exposure in animal studies mimics seasonal, even daily changes of environmental ozone concentration and offer insight into potential effects for interim ozone exposure.

In addition, there was also evidence for persisting effects. For subchronic exposure e.g. mucous cell hyperplasia in nasal epithelium was still evident after 13 weeks recovery from 0.5 ppm ozone exposure for 13 weeks (Harkema, 1999). Tesfaigzy (1998) reported that 3-months ozone inhalation increased metaplasia of mucous cells in transitional epithelia of rat nasal airways, the increased number of mucous cells also persisted after 13 weeks recovery period.

All Health effects institute (HEI) studies listed above are considered as key studies as the GLP status (FDA regulation) was considered as fulfilled. The studies focussing on pulmonary health were performed in rats and comprised mostly an exposure duration of 20 months. In the study published by Pinkerton et al. (1998) exposure was limited to three months. In all studies animals were exposed to 0, 0.12, 0.5 or 1 ppm ozone. From the individual studies it is particular challenging to derived NOAEC and LOAEC values as it remains difficult to determine whether the effects observed are to be considered as adverse. In an integrative summary report published by the HEI in 1995 (Research report number 65) the individual HEI studies summed up above were evaluated and interpreted in the overall context of pulmonary health effects.

Pinkerton et al. (1998) found in the 3-months study modifications in the distribution of superoxide dismutase (Cu-Zn-form in terminal bronchioles and centriacinar region; Mn form in in centriacinar region). Mn superoxide dismutase increased in epithelial type 2 cells distal to brochiole-alveolar duct junction. At 1 ppm the authors reported on a statistically significant elevated volume density of nonciliated epithelial cells lining the trachea and caudal bronchi and in proximal and terminal bronchioles of the cranial region. At this dose remodelling of the centriacinar region was statistically significant. The effects observed were independent of age. The authors concluded that long-term ozone exposure is related to significant alterations of epithelial cell populations lining the airways or centriacinar region of the lung.

Pinkerton et al. (1995) detected after 20-month ozone exposure significant changes in the stored secretory product in the trachea and bronchi. Furthermore, a statistically significant increase in the volume density of non-ciliated cells in terminal bronchioles arising from caudal region (left lung) was reported. In the pulmonary acini a dose-dependent extension of bronchiolar epithelium beyond the bronchiole-alveolar duct junction into alveoli was observed. Furthermore, superoxide dismutase, glutathione S-transferase and glutathione peroxidase increased in a statistically significant manner in the distal bronchiole to central acinus at 0.5 and 1 ppm. It was further noted that the variability in antioxidant enzyme levels was more distinct in other parts of the airway. The authors concluded that 20-months exposure leads to dose-related and site-specific changes along the tracheobronchial tree and pulmonary acini of the lungs.

In another study published by Radhakrishnamurthy (1994) an ozone-related statistically significant decrease of total glycosaminoglycans was reported. After pairwise comparison with controls a statistically significant decrease of hyaluronan, chondroitin 4-sulfate, and chondroitin 6-sulfate levels was determined. However, heparan sulfate levels followed a significant trend toward increase with elevated ozone doses. Molecular size of hyaluronan decreased in ozone exposed animals. The authors further noted changes in the chemical properties and antithrombin III affinity of heparan sulfate. The authors conclude that the affected cellular metabolism of proteoglycans could contribute to functional impairments of the lung.

Harkema and Mauderly (1994) used plethysmographic techniques to assess the impact of ozone on pulmonary function. The authors reported on an ozone-related reduction of residual volume during slow lung deflation (most significant in 0.5 ppm females). The authors concluded that ozone exposure has only low relevance for integrated pulmonary function of the lung.

Harkema et al. (1994) found that mucous flow in rats after exposure to 0.5 or 1 ppm ozone was slower over the lateral wall and turbinates of the proximal third of the nasal airways. Furthermore, at these doses intranasal regions contained mucous cell metaplasia and 25-300 times more mucus in nasal transitional epithelium than the corresponding regions from controls. The authors further found at 0.5 and 1 ppm epithelial hyperplasia in nasal transitional epithelium, increases in eosinophilic globules in the surface epithelium lining the distal nasal airways and a mild-to-moderate inflammatory cell influx in the nasal mucosa in the proximal and middle nasal passages. The authors concluded that exposure to 0.5 or 1 ppm for 20 months is connected with significant changes in function and structure of the nasal mucociliary apparatus.

Harkema et al. (1997) found significant morphologic and morphometric changes in the maxilloturbinates after exposure to 0.5 or 1 ppm ozone for 20 months. Furthermore, the authors reported a significant reduction in cross-sectional area of turbinate bone and a conspicuous influx of inflammatory cells into the lamina propria surrounding the turbinate bone. Reductions in the area of lamina propria, due to blood vessel constriction, and increases in the area of the surface epithelium, due to hyperplasia and metaplasia were also related to ozone exposure. The authors concluded that ozone can cause concentration and gender-specific bony atrophy.

Drastic effects published by Chang et al. (1995) were also restricted to ozone doses of 0.5 and 1 ppm. Animals showed increases in the volume of interstitium and epithelium along the alveolar ducts. The authors concluded that the thickening of the epithelium was caused by metaplasia in which the normal squamous epithelium was modified to a cuboidal epithelium. The bronchiolar epithelial metaplasia was ozone-dependent and was in particular characterized by differentiated ciliated cells and Clara cells. At 1 ppm fibrotic responses (interstitial matrix and cellular interstitium) were observed. Components of the interstitial matrix (e.g. collagen, elastin, basement membrane) were also elevated. The authors concluded that the increase in cellular interstitium was mediated by the elevated volume of interstitial fibroblasts. At 1 ppm animals showed also inflammatory responses. The terminal bronchioles were less affected than the proximal alveolar region. The authors hypothesized that the bronchiolar epithelial metaplasia in the alveolar ducts may indicate protective mechanisms.

Last et al. (1994) investigated collagen deposition in lung tissue. Biochemical analysis indicated excess collagen in females after exposure to 0.5 or 1 ppm ozone. Furthermore, excess fibrotic lung collagen deposition was histologically determined at both doses. As the number of animals in the low dose group was too small, it is difficult to conclude whether 0.12 ppm is the true NOEL. The authors concluded that long-term exposure to ozone at 0.5 ppm or above leads to mild-to-moderate lung fibrosis.

From the studies it can be concluded that in most studies morphological and functional pulmonary changes seem to become apparent at 0.5 ppm and above. This is in agreement with the integrative summary report published by the HEI in 1995. A classification for specific target organ toxicity towards the lung is not proposed. However, respiratory tract irritation is covered with the proposed classification for STOT SE 3.

In two NTP studies with F344/N rats chronic exposure (lifetime and 2-y) to ozone at 0.5 ppm or above leads to lung fibrosis.

Table 48: Setting of specific concentration limits for STOT RE (cardiovascular system, nervous system)

Study reference	**Effective dose (ppm)**	**Species** **Length of exposure**	**SCL Cat.1**	**SCL Cat.2**
Arito H. et al. (1990), Toxicol Lett. 52(2):169-78	0.1 ppm (brady-arrhythmia) → corrected: 0.4 ppm Modification of ED for exposure duration analogous to the Guidance value (GV) unit [ppmV/6h/d]: 0.1 ppm (24h/d) *4 → 0.4 ppm (6h/d)	Rat 5d (24h/d)	Extrapolation of the GV for 90d study (inhalation (gas)) corrected for exposure duration < 9d according to CLP guidance: 50 ppm * 10 = 500 ppm SCL Cat.1= (0.4ppm/500 ppm)x100 % = 0.08 % → 0.05 %	Extrapolation of the GV for 90d study (inhalation (gas)) corrected for exposure duration < 9d according to CLP guidance:250 ppm * 10 = 2500 ppm SCL Cat.2= (0.4ppm/2500ppm)x100 % = 0.016 % → 0.01 %
Pereyra-Munoz N et al. (2006), J Chem Neuroanat. 31(2):114-23	0.25 ppm (loss of fibres and cell death of dopaminergic neurons) Modification of ED for exposure duration analogous to the Guidance value (GV) unit [ppmV/6h/d]:	Rat 15 or 30d (4h/d)	for 28d: SCL Cat.1= (0.18ppm/150 ppm)x100 % = 0.12 % → 0.1 %	for 28d: SCL Cat.2= (0.18ppm/750 ppm)x100 % = 0.024 % → 0.02 %

Study reference	**Effective dose (ppm)**	**Species** **Length of exposure**	**SCL Cat.1**	**SCL Cat.2**
	0.25 ppm (4h/d) *0.7 → 0.18 ppm (6h/d)			
Rivas-Arancibia S. (2010), Toxicol Sci. 113(1):187-97	0.25 ppm (lipid peroxidation and morphological changes in neurons) Modification of ED for exposure duration analogous to the Guidance value (GV) unit [ppmV/6h/d]: 0.25 ppm (4h/d) *0.7 → 0.18 ppm (6h/d)	Rat 15, 30, 60, 90d (4h/d)	for 28d: SCL Cat.1= (0.18ppm/150 ppm)x100 % = 0.12 % → 0.1 %	for 28d: SCL Cat.2= (0.18ppm/750 ppm)x100 % = 0.024 % → 0.02 %

GV: Guidance value

Table 49: Summary table of human data on STOT RE

Reference / study characteristics	ozone exposure			Statistical Analysis	Effect	Results	Others/ Remarks
	Conc. µg/m3	Conc. ppb	Duration hours				
Jerret, M. et al. 2009, 448,850 subjects, cohort of the American Society Cancer Prevention Study II correlated with air-pollution data from 96 US metropolitan areas. Data from April 1 to September 30 for years 1977-2000. Study included in U.S. EPA/ISA Report 2013.	71,6-114,2 -123,4 -134,2 -223,6	33.3-53.1 -57.4 -62.4 -104	Chronic exposure/ long-term, average of daily maximum values.	Standard and multilevel random-effects Cox proportional-hazard models. A total of 20 variables with 44 terms were used to control for individual characteristics that might confound or modify the association between air pollution and death.	Mortality (number of deaths by cardiovascular or respiratory cause)	The estimated relative risk of death from respiratory causes that was associated with an increment in ozone concentration of 10 ppb was 1.040 (95 % confidence interval, 1.010 to 1.067; Tab.3). Relation between exposure to ozone and death from respiratory cause: Residual Risk increases with higher ozone concentration. For every 10-ppb increase in exposure to ozone, an increase in the risk of death from respiratory causes of about 2.9 % in single-pollutant models and 4 % in two-pollutant models was observed. **LOAEC:** **33.3-53.1 ppb** (Death by respiratory cause) **NOAEC:** **No value**	The association of ozone with the risk of death from respiratory causes was insensitive to adjustment for confounders and to the type of statistical model used. Reliable study, Cox regression appropriate statistical method, large amount of confounders considered. Discrimination of ozone effects from effects by fine particular matter ≤2.5 µm in "Two-pollutant model" for rel. risk is statistically properly evaluated, study cited more than 350 times (Scopus, May 2016).
Abbey, D.E. et al. 1999, 1977–1992 mortality in a cohort of 6,338 non-smoking California Seventh-day Adventists, (27–95 y), part of Adventist Health Study (AHS). Record of California death certificate files for the years 1977–1992.	129, 172, 215, 258, 322.5	60, 80, 100, 120, 150 (Cut-offs)	8 h; monthly average of the daily 8-h average from 9:00 A.M. to 5:00 P.M.	Sex-specific adjusted mortality relative risks (RRs) by Cox proportional hazards regression	Mortality (number and relative risk of deaths by cause of lung cancer)	Ozone showed a strong association with lung cancer mortality for males with an RR of 4.19 (95 % CI: 1.81, 9.69) for the IQR difference of 551 h/yr when ozone exceeded 100 parts per billion. **LOAEC:** **100 ppb** (Mortality by lung cancer) **NOAEC:** **No value**	Only significant association for lung cancer in contrast to other associations such as cardiopulmonary causes.
Thurston, G. D., 2001, re-analysis of 19	Not applicable,	Not applicabl	Not applicable,	Different models in original studies.	Mortality, relative	Relative risk = 1.056 per 100- ppb	The higher risk in studies considering the nonlinear

Reference / study characteristics	ozone exposure			Statistical Analysis	Effect	Results	Others/ Remarks
	Conc. µg/m3	Conc. ppb	Duration hours				
different time-series epidemiological studies, single study of daily mortality in Detroit, MI, Influence of temperature specification.	daily ambient	e, daily ambient	8 - 24 h averages, association with short-term ozone exposure		risk	increase in daily 1 - h maximum ozone (95 % CI: 1.032–1.081) according to studies that specified the nonlinear nature of the temperature– mortality association **LOAEC: not applicable** (mortality) **NOAEC: No value**	nature of the temperature– mortality association indicates that past time – series studies using linear temperature–mortality specifications have under predicted the premature mortality effects of ozone. Reliable studies used, statistical method appropriate, confounders considered.
Lin, S. et al. 2008, New York State (10 regions) birth cohort with 1,204.396 eligible births; data from 1995 until 1999. Follow up each individual until first asthma hospital admission or until 31.12.2000. Hourly ambient ozone data from the New York State Depart. of Environmental Conservation (32 ozone monitoring sites), measured hourly for each day (8-hr maximum hourly value). Study included in U.S. EPA/ISA Report 2013.	80.65 to 102,73	37.51 to 47.78 Range of mean ozone concentrations over the 10 New York Regions.	Chronic exposure/ long-term.	Two-stage Bayesian hierarchical model analysis	First asthma hospital admission	Significant positive associations between chronic ozone level and asthma hospital admissions for all exposure indicators after adjusting for potential confounding variables (ORs =1.16–1.68). The risk of hospital admissions increased 22 % with a 1-ppb increase in mean ozone concentration during the ozone season. Indicators using the entire follow-up period weaker elevated risks for asthma admissions. By using the exceedance proportion, significant increase (OR = 1.68; 95 % CI, 1.64–1.73) in hospital admissions associated with an IQR (2.51 %) increase in ozone was found. **LOAEC: 37.5 ppb** (Hospital admission, asthma) **NOAEC: No value**	Impacts related to hospital admission investigated by “negative control” group of admissions due to gastroenteritis: No positive association with ozone as found for admissions due to asthma. Reliable study, statistical method appropriate, birth, maternal confounders and geographic regions considered.
Moore, K. et al. 2008, ecologic study,	64.5 - >322.5	30 - >150	Association with	Regression model, history-restricted	First asthma	A linear relation was detected for asthma hospital discharges. High	Many areas included that consistently exceeded

Reference / study characteristics	ozone exposure			Statistical Analysis	Effect	Results	Others/ Remarks
	Conc. µg/m3	Conc. ppb	Duration hours				
California's South Coast Air Basin (195 spatial grids), children who ranged in age from birth to 19 years, from 1983 to 2000, measurements for 3-month periods along with demographic variables (U.S. Census Bureau's decadal surveys for years 1980, 1990 and 2000). Average concentrations of the 1-hr daily maximum ozone. Study included in U.S. EPA/ISA Report 2013.		(quarterly 1-hr maximum ozone)	short-medium term ozone exposure	marginal structural models (HRMSMs)	hospital admission (parameter: discharge)	correlation between median 1-hr and 8-hr maximum average ozone levels (r =0.99). During 1980–2000, ozone concentrations showed moderate correlation with particulate matter with aerodynamic diameter ≤ 10 µm (PM_{10}) and little correlation with the pollutants NO_2, CO, SO_2. A 10-ppb increase above the median ozone concentration of 87.7 ppb is estimated to lead to a 4.6 % increase in the proportion of discharges (3.26 × 10–4). **LOAEC: 87.7 ppb** (Hospital admission, asthma) **NOAEC: No value**	National Ambient Air Quality Standards for ozone during the 1980–2000 study period (U.S. EPA 2000). Reliable study, statistical method appropriate, confounders considered.
Mortimer, K.M. et al. 2002, cohort of 846 asthmatic children (4-9 y) in 8 urban areas of the USA, data from the National Cooperative Inner-City Asthma Study (NCICAS), daily air pollution concentrations from the Aerometric Information Retrieval System database from US EPA.	103.2	48, daily ambient, across all urban areas	8-h average ozone (10:00–18:00 h), association with short-term ozone exposure	Linear mixed effect models (SAS Proc Mixed)	Peak expiratory flow rate (PEFR) and symptoms (cough, chest tightness, wheeze)	A 15 ppb increase in 5-day moving average ozone was associated with a 0.59 % decline in morning PEFR (95 % CI 0.13–1.05) and with a sign. increased incidence of a ≥10 % decline in morning PEFR (OR=1.14, 95 % CI 1.02–1.27). **LOAEC: 63 ppb** (PEFR, asthma symptoms) **NOAEC: No value**	This longitudinal analysis supports previous time-series findings that at levels below current USA air-quality standards, summer-air pollution is significantly related to symptoms and decreased pulmonary function among children with asthma. Reliable study, statistical method appropriate, confounders considered.
Silverman, R.A. and Ito, K (2010). Daily time-series analysis of 6008 asthma ICU	< 172	< 80; daily ambient, NAAQS	Risks for interquartile range	Adjusted regression model	asthma hospitalisation, ICU: life	Susceptibility to ozone is age-dependent, with children at highest risk for non-ICU hospitalizations and ICU	There appear to be severe adverse health effects to exposures even below the currently

Reference / study characteristics	ozone exposure			Statistical Analysis	Effect	Results	Others/ Remarks
	Conc. µg/m3	Conc. ppb	Duration hours				
admissions and 69,375 general (non-ICU) asthma admissions in 4 age groups (<6, 6-18, 19-49, 50+ y) in 74 New York City hospitals for the months April to August from 1999 to 2006. Ozone data from UC EPA`s Air Quality System.		(the 3-year average of the fourth-highest daily concentrations should not exceed this value); exceeded on 46 days.	increases in the a priori exposure time window of the average of 0-day and 1-day lagged pollutants, association with short-term ozone exposure.		threatening episodes requiring intensive care unit admission	admission. For each 22-ppb increase in ozone, there was a 19 % (95 % CI, 1 % to 40 %) increased risk for ICU admissions and a 20 % (95 % CI, 11 % to 29 %) increased risk for general hospitalizations. **LOAEC: < 80 ppb** (ICU, asthma hospitalisation) **NOAEC: No value**	accepted standard of 80 ppb. Reliable study, statistical method appropriate, confounders considered.
Atkinson, R.W. et al. (2016). Meta-Analysis of evidence from a total of 14 publications from 8 cohorts on correlation of mortality with long-term ozone exposure. Studies from EMBASE, MEDLINE until 9/2015 and PubMed until 10/2015. For mortality associated with respiratory effects, studies from Jerret (2009), and Bentayeb (2015) for the warm season, studies from Carey (2013), Jerret (2013), and Lipsett (2011) for all year were considered.	Not reported	Not reported	Long-term	Meta-analysis; statistics included in original cohort studies. Adjustment for key confounders age, sex, body mass, index, smoking, socioeconomic status. Analysis of hazard ratio (HR) and relative risk (RR)	Respiratory associated mortality	Hazard ratio respiratory causes of death derived from 3 cohorts was 1.03 (95 % CI 1.01 to 1.05) for the warm season and 0.94 (95 % CI .81 to 1.10) all year per 10 ppb ozone. No evidence on association between long-term annual ozone concentrations and respiratory mortality.	The Jerret study (Jerret, M. et al. 2013, Am J Respir Crit Care Med.188:593-9, not part of this dossier) included in the meta-analysis by Atkinson, provides only data for 73,711 subjects from the American Cancer Society Cancer Prevention II Study cohort. This is only one part (California) of the complete analysis of the same Cancer Prevention II Study also published by Jerret, M. et al. in 2009 correlating data from 96 metropolitan areas in US and analysing data from

Reference / study characteristics	ozone exposure			Statistical Analysis	Effect	Results	Others/ Remarks
	Conc. µg/m3	Conc. ppb	Duration hours				
							448,850 subjects. In Jerret 2009, however, an association between ozone exposure and relative risk of death from respiratory causes was reported (for details see above, Jerret 2009).
Turner, M.C., Jerret, M. et al. (2016). Large-scale prospective study, data from 669,046 participants, among whom 237,201 deaths occurred through 2004. Associations between long-term exposure and all-cause and cause-specific mortality in an extended analysis of the American Cancer Society Cancer Prevention II study investigated using new national-level estimates of ambient ozone.	60 - 131.8	23.7-61.3	Long-term	Cox proportional hazards regression models for associations between mean ozone (2002–2004), PM2.5 (1999–2004), and NO_2 (2006) concentrations and all-cause and cause specific mortality.	Respiratory associated mortality	In single-pollutant models, significant positive associations between ozone, PM2.5, and NO_2 concentrations and all-cause and cause-specific mortality. In two-pollutant models adjusted for PM2.5, significant positive associations remained between ozone and all-cause (hazard ratio [HR] per 10 ppb, 1.02; 95 % confidence interval [CI], 1.01–1.04) respiratory mortality (HR, 1.12; 95 % CI, 1.08–1.16) that were unchanged with further adjustment for NO_2. Findings suggest that long-term ambient ozone contributes to risk of respiratory mortality.	Follow-up analysis of the American Cancer Society Cancer Prevention II study. In accordance with Jerret, M. et al. (2009), an association between ozone exposure and relative risk of death from respiratory causes was reported. Reliable study, statistical method appropriate, confounders considered.

Human studies

The classification and SCL calculation is based on the study by Jerrett et al. in 2009, published in New England Journal of Medicine, because of substantial population size of 448,850 subjects, the duration and the high quality standards of the statistics. With respect to the statistical analyses in the study by Jerrett et al. 2009, the applied Cox proportional-hazard models are clearly and precisely described, many confounders were considered. The dossier submitter assumes that the assessment of proportionality of hazards (covariate effect is constant throughout duration of the study) was verified, even though not described in detail. Considering supplementary information given by Jerrett et al. 2009, the formal analysis to evaluate a possible threshold for the association between exposure to ozone and the risk of death are also coherent.
The results are convincing as the study is based on a substantial population size and was well conducted, so the association of ozone with the risk of death from respiratory causes can be assumed.
Regarding the safe dose, the interpretation is not obvious. The authors evaluated two kinds of dose-response relationship, the linear model through the origin as well as a threshold model. Statistical testing for superiority of the threshold model exhibited p=6 % for the most likely threshold of 56 ppb. The authors seem to favour the threshold model although this test was not significant on the 5 % level.

In summary, when no other information can be used, the linear model should be assumed; only in the case that prior knowledge is in favour of the threshold model, a threshold should be assumed, with the most likely threshold based on the present data being 56 ppb.

Setting of specific concentration limit according to Jerrett 2009 for STOT RE Cat.1, resp. system the LOAEC of 33.3 ppb is used for calculation according to CLP Guidance Tab. 3.9.2-a:

Table 50: Setting of specific concentration limits for STOT RE (respiratory system)

Study reference	**Effective dose (ppm)**	**Species, Length of exposure**	**SCL Cat.1**	**SCL Cat.2**
Jerrett et al. 2009	0.033 ppm (LOAEC) (Death by respiratory cause)	Human Chronic	Extrapolation of the GV for 90d study (inhalation (gas)) corrected for chronic exposure: 50 ppm / 2 = 25 ppm SCL Cat.1= (0.033ppm/25 ppm)x100 % = 0.13 % → 0.1 %	Extrapolation of the GV for 90d study (inhalation (gas)) corrected for chronic exposure: 250 ppm / 2 = 125 ppm SCL Cat.2= (0.033ppm/125 ppm)x100 % = 0.026 % → 0.02 %

GV: Guidance value

10.12.2 Short summary and overall relevance of the provided information on specific target organ toxicity – repeated exposure

STOT RE 1 for cardiovascular system (animal studies):

The significant functional disturbance at low exposure concentrations after long-term exposure indicate, that ozone exerts specific target organ toxicity towards the cardiovascular system. The decisive effects, including the increased number of bradyarrhythmic episodes and decreased heart rates occurred at concentrations starting at the lowest tested doses of 0.1 ppm. Therefore a classification for STOT RE 1 for the cardiovascular system is proposed.

STOT RE 1 for nervous system (animal studies):

Alterations with significant organ damage in different brain regions with cell death and altered neurogenesis were reported after repeated ozone exposure. These could be directly linked to adverse behavioural changes as decreased motor activity. This indicates significant organ damage, therefore a classification for STOT RE 1 is proposed.

STOT RE 1 for respiratory system (human studies): Epidemiological studies indicated a correlation of ozone exposure with an increased risk of deaths by respiratory cause and that ozone exerts specific target organ toxicity towards the respiratory system after repeated exposure. For every 10-ppb increase in exposure to ambient ozone concentration, an increase in the risk of death from respiratory causes of about 2.9 % in single-pollutant models and 4 % in two-pollutant models was observed. Therefore a classification for STOT RE 1 is proposed.

10.12.3 Comparison with the CLP criteria

Toxicological results *	CLP criteria
Impact on the cardiovascular system: The detrimental impact of ozone on the cardivascular system was reported in repeated dose studies accompanied by changes in decreased heart rate and increased arrhythmia at very low doses ≤ 0.1 ppm ozone. The effects were seen after short-term exposure (5d) as well as after long-term exposure (90d) and were in the range of the equivalent guidance values for Cat.1. A classification into category STOT RE 1(cardivascular system) (H372) is proposed by the dossier submitter. An SCL ≥ 0.05 % was derived but is not proposed (refer to Table 48) **Impact on the nervous system:** Significant toxicity to the CNS was observed at 0.25 ppm, including morphological changes, cell death and altered neurogenesis in different brain regions accompanied with oxidative stress. Based on these findings STOT RE 1 (nervous system) (H372) is proposed by the dossier submitter. An SCL ≥ 0.03 % was derived but is not proposed (refer to Table 48) **Impact on the respiratory system**: Reliable and good quality evidence from epidemiological studies for significant toxicity to the respiratory system in humans including a high number of deaths by respiratory cause. Based on these findings STOT RE 1(respiratory system) (H372) is proposed by the dossier submitter. An SCL ≤ 0.1 % was derived but is not proposed (refer to Table 50)	Category 1 (H372): Substances that have produced significant toxicity in humans or that, on the basis of evidence from studies in experimental animals, can be presumed to have the potential to produce significant toxicity in humans following repeated exposure. Substances are classified in Category 1 for target organ toxicity (repeat exposure) on the basis of: reliable and good quality evidence from human cases or epidemiological studies; or observations from appropriate studies in experimental animals in which significant and/or severe toxic effects, of relevance to human health, were produced at generally low exposure concentrations. Equivalent guidance values for 90-day studies: Inhalation (rat), gas: C ≤ 50 ppmV/6h/day Equivalent guidance values for 28-day studies: Inhalation (rat), gas: C ≤ 150 ppmV/6h/day

10.12.4 Conclusion on classification and labelling for STOT RE

Based on the significant pathological changes on (1) the cardiovascular system, (2) the nervous system and (3) a high number of deaths by respiratory cause after repeated inhalative exposure, harmonised classification and labelling for specific target organ toxicity – repeated exposure is proposed as:

(1) STOT RE 1, H372 - Causes damage to the cardiovascular system through prolonged or repeated exposure, (2) STOT RE 1, H372 - Causes damage to the nervous system through prolonged or repeated exposure and
(3) STOT RE 1, H372 - Causes damage to the respiratory system through prolonged or repeated exposure.

RAC evaluation of specific target organ toxicity – repeated exposure (STOT RE)

Summary of the Dossier Submitter's proposal

The DS proposed to classify ozone as STOT RE 1; H372 with the following target organs: cardiovascular system, nervous system and respiratory system.

Cardiovascular system

The significant functional disturbance at low exposure concentrations after long-term exposure indicate that ozone exerts specific target organ toxicity towards the cardiovascular system in animal studies. The adverse effects, including the increased number of bradyarrhythmic episodes and decreased HRs, occurred at concentrations starting from the lowest tested doses of 0.1 ppm. Therefore, a classification as STOT RE 1 for the cardiovascular system was proposed by the DS. SCLs ≥ 0.05% and ≥ 0.01% were derived for Category 1 and 2, respectively, but not proposed by the DS.

Nervous system

Significant brain damage in different regions with cell death and altered neurogenesis were reported after repeated ozone exposure in animal studies. These alterations could be directly linked to adverse behavioural changes as decreased motor activity. Therefore a classification for STOT RE 1 was proposed by the DS. SCLs ≥ 0.1% and ≥ 0.02% were derived for category 1 and 2, respectively, but not proposed by the DS.

Respiratory system

Epidemiological studies indicated a correlation of ozone exposure with an increased risk of deaths by respiratory cause and that ozone exerts specific target organ toxicity towards the respiratory system after repeated exposure. For every 0.010 ppm increase in exposure to ambient ozone concentration, an increase in the risk of death from respiratory causes of about 2.9% in single-pollutant models and 4% in two-pollutant models was observed. Therefore, a classification for STOT RE 1 was proposed by the DS. SCLs ≥ 0.1% and ≥ 0.02% were derived for category 1 and 2, respectively, but not proposed by the DS.

Comments received during consultation

One comment was received from Company-Manufacture regarding the bioavailability of ozone when inhaled.

Assessment and comparison with the classification criteria

The DS proposal for a classification for STOT RE included three target organs for discussion: the cardiovascular system, the nervous system and the respiratory system. The individual target organs will be discussed in the following sections.

Impact on the cardiovascular system

As summarized in the table below, repeated exposure to ozone lead to a decrease in heart rate at doses ranging from 0.1–1 ppm. The effect was recovered after a few days of exposure in repeated dose studies. In the study by Watkinson *et al.* (2003), the effect was found to be more pronounced during physical exercise and at lower ambient temperatures.

***Table**: Summary of effects observed regarding heart rate*

Dose/ppm	Heart rate	
	Acute dose studies	Repeated dose studies (days)
0.1	-	Lower heart rate (with recovery) Arito *et al*., 1997 (3 d) Iwasaki *et al*., 1998 (4 d)
0.2	-	Lower heart rate (with recovery) Arito *et al*., 1990 (5 d)
0.3-0.35	-	Lower heart rate (with recovery) Iwasaki *et al*., 1998 (4 d)
0.5	Lower heart rate (reversible) Watkinson *et al*., 2003 Iwasaki *et al*., 1998	Lower heart rate (with recovery; more pronounced during exercise and at lower ambient temperatures) Watkinson *et al*., 2003 (2 or 5 d) Iwasaki *et al*., 1998 (4 d)
0.75-0.8	Lower heart rate (reversible) Gordon *et al*., 2013	No effect Gordon *et al*., 2013 (6h, 1 d/week, 17 week)
1	Lower heart rate (reversible) Gordon *et al*., 2014	Lower heart rate (reversible) Gordon *et al*., 2014 (6 h, 2 d/week, 13 week)

Arito *et al.* (1997) tested ozone with 5 h exposure for three days with exposure free days in-between using doses of 0, 0.1, 0.3 and 0.5 ppm. At 0.1 ppm: statistically significantly decreased HR (only in young rats, ~ 80% of control), decreased tidal volume (~ 70% of control, not statistically significant, no recovery during exposure), and increased breathing frequency (not statistically significant) were observed. At 0.3 and 0.5 ppm: statistically significantly decreased HR (~ 50-65% of control, less pronounced in old rats), decreased tidal volume (~ 50% of control, statistically significant), and increased breathing frequency which recovered towards end of exposure (statistically significant, only in young rats) were observed.

Iwasaki *et al.* (1998) tested ozone exposure with the dose levels of 0, 0.1, 0.3 and 0.5 ppm 8 h/d for 4 days. Statistically significant concentration dependent decreased HR during 8 h

exposure and 12 h post exposure periods on exposure days 1 and 2 (day 2 post-exposure only statistically significant at 0.5 ppm). HR recovery to control values or above on days 3 and 4.

Watkinson *et al.* (2003) tested ozone in ozone/temperature experiments (5 d exposures), and in ozone/exercise experiments (2 h exposures) at 0 and 0.5 ppm doses. Decreased HR was reported at all three ambient temperatures tested in the ozone/temperature experiments (10, 22, 34°C) with recovery on exposure day 3. The effect was more pronounced at 10°C and less pronounced at 34°C. In the ozone/exercise experiments with exercising rats, decreased HR was observed.

Gordon *et al.* (2014) tested 1 ppm ozone with exposure for 6 h/d, 2 d/week for 13 weeks. They reported decreased HR and core temperature (bradycardic and hypothermic effects), which increased (tachycardic and hyperthermic effects) during recovery period after 2 d exposure. The effect became less pronounced as exposure weeks progressed. Senescent rats were less affected than adults. In a study from 2013, Gordon *et al.* tested ozone exposure with dose levels of 0 and 0.8 ppm (6 h/d, 1 d/week for 17 weeks) and reported no effects on HR.

Arrhythmic episodes were found to be increased after repeated exposure in a concentration dependent manner (Arito, 1990; 0.1 and 0.2 ppm). The effect was recovered on exposure day 3.

***Table**: Summary of effects regarding arrhytmia*

Dose/ppm	Arrhythmia	
	Acute dose studies	Repeated dose studies
0.1	-	-
0.2	Increased sensitivity to aconitin-induced arrhythmia formation Farraj *et al*., 2012	Increased bradyarrhythimc episodes (with recovery) Arito *et al*., 1990 (5d)
0.75-0.8	Increased (reversible) Increased sensitivity to aconitin-induced arrhythmia formation Farraj *et al*., 2012	Increased bradyarrhythimc episodes (with recovery) Arito *et al*., 1990 (5d)

Arito *et al*. (1990) reported statistically significantly decreased HR at a dose 0.2 ppm on days 1 and 2 of exposure which recovered to control values on day 3 of exposure. Further, statistically significantly concentration dependent increase in number of bradyarrhythmic episodes during all states of sleep and wakefulness on days 1, 2 and 3 was reported (not statistically significant, during paradoxical sleep period at 0.1 ppm, recovery to control values on days 4 and 5).

Blood pressure was investigated in only one study (Gordon *et al.*, 2013), where no effect of ozone exposure was reported. The review by Prueitt *et al.* (2014), weight-of-evidence evaluation of long-term ozone exposure and cardiovascular effects, confirms the study quality of Gordon *et al.* (2013) with regard to HR effects while Arito *et al.*, (1997 and 1990), Iwasaki *et al*. (1998), Watkinson *et al*. (2003) and Gordon *et al.* (2014) were not evaluated. However, the effects on HR and arrhythmia in the additional studies analysed and listed below were regarded as relevant for classification STOT RE.

Core body temperature was found to decrease under ozone exposure in both acute (0.2–1 ppm) and repeated dose studies (0.5–1 ppm). The effect recovered after a few days of exposure in

the repeated dose studies and was found to be more pronounced during physical exercise, and at lower ambient temperatures (Watkinson *et al.*, 2003).

***Table**: Summary of effects regarding core body temperature*

Dose/ppm	Core body temp	
	Acute dose studies	Repeated dose studies
0.5	-	Decreased core body temperature (with recovery; more pronounced during exercise and at lower ambient temperatures) Watkinson *et al*., 2003 (2 or 5 d), Iwasaki *et al*., 1998 (4 d)
0.75-0.8	Decreased core body temperature, Farraj *et al*., 2012	-
1	Lower core body temperature (reversible) Gordon *et al*., 2014	Lower core body temperature (with recovery) Gordon *et al*., 2014 (6 h, 2 d/week, 13 week)

Iwasaki *et al.* (1998) reported small but statistically significantly decreased core body temperature at 0.5 ppm during 8 h exposure period on days 1 and 2. No effect at 0.1 and 0.3 ppm. Recovery to control values on days 3 and 4 of exposure (above control core body temperature values during post-exposure period in 0.3 ppm group were reported).

Watkinson *et al.* (2003) also reported decreased core body temperature (with recovery on exposure day 3); effect was more pronounced at 10°C and less pronounced at 34°C).

RAC does not support the proposal by the DS for classification for STOT RE based on available data. RAC considers the described cardiovascular effects as related to acute toxicity and notes the recovery after few days even during dosing. RAC considers the effects more relevant for consideration for STOT SE as described in the CLP guidance, section 3.9.1. Definitions and general considerations for STOT-RE:

"*Where the same target organ toxicity of similar severity is observed after single and repeated exposure to a similar dose, it may be concluded that the toxicity is essentially an acute (i.e. single exposure) effect with no accumulation or exacerbation of the toxicity with repeated exposure. In such a case classification with STOT-SE only would be appropriate*."

Therefore, RAC disagrees with the DS proposal for STOT RE 1 (cardiovascular system) and concludes that no classification for STOT RE for cardiovascular effects is warranted.

Impact on the nervous system

Effects on CNS are summarised in the tables below. The effects are divided according to acute or repeated dose studies.

***Table**: Summary of neural changes and numbers of dendritic spins*

Dose/ppm	Neuronal changes (no. dendritic spines)	
	Acute dose studies	Repeated dose studies
0.2-0.25	-	Morphological alterations, cell death in dopaminergic neurons in striatum and substantia nigra Pereyra-Munoz *et al*., 2006 (15 or 30 d) Morphological alterations and cell swelling

		in hippocampus. Rivas-Arancibia *et al*., 2010 (15-90 d)
1	Reduced number of dendritic spines, Avila-Costa *et al.*, 1999	-

In the acute and STOT SE sections, Avila-Costa (1999 and 2001) reported reduction in number of dendritic spines in striatum and prefrontal cortex after one single ozone dose of 1 ppm/4 h in rats.

Pereyra-Munoz *et al.* (2006) found morphological alterations, loss of fibres, and cell death of the dopaminergic neurons in the striatum and substantia nigra after 4 h/d repeated exposure to 0.25 ppm ozone for a period of 15 or 30 days in rats. This effect was accompanied by an increase in lipid peroxidation in the striatum and a decrease in motor activity.

Moreover, neuronal morphological changes were also found in the hippocampus, along with swelling of neurons at exposure to 0.25 ppm ozone for 4 h/d for 15-90 days in rats (Rivas-Arancibia *et al.,* 2010). In this study, the authors reported several additional effects including altered neurogenesis, increased lipid peroxidation, increased phagocytic microglia, increased astrocytes and memory deficiency.

The effects observed from the other studies are summarized in the tables below.

***Table**: Summary of effects - neurogenesis*

Dose/ppm	Neurogenesis	
	Acute dose studies	Repeated dose studies
0.2-0.25	-	Increased after 30 d (but with morphological alterations), decreased after 60 and 90 d Rivas-Arancibia *et al*., 2010 (15-90 d)

***Table**: Summary of effects – other hippocampus changes*

Dose/ppm	Other hippocampus changes	
	Acute dose studies	Repeated dose studies
0.2-0.25	-	Increases in activated and phagocytic microglia increased number of astrocytes decreased Neu-N and doublecortin. Rivas-Arancibia *et al*., 2010 (15-90 d)

Table: *Summary of effects – Lipid peroxidation*

Dose/ppm	Lipid peroxidation	
	Acute dose studies	Repeated dose studies
0.2-0.25	Increased in striatum and hippocampus Pereyra-Munoz *et al*., 2006 Increased in olfactory bulb Guevara-Guzman *et al*., 2009	Increased in striatum and hippocampus Pereyra-Munoz *et al*., 2006 (15-30 d) Increased in olfactory bulb Guevara-Guzman *et al*., 2009 (30-60 d)
1	Increased lipid peroxidation in brain (reversible) Rivas-Arancibia *et al*., 2003	-

Together with the mentioned effects related to the CNS, also motor activity was decreased in repeated dose studies at doses starting at 0.25 ppm (Pereyra-Munoz *et al*., 2006 and Gordon *et al*., 2014). Several behaviours, such as grooming, resting, rearing and jumping-play were affected after repeated exposure to 0.12 ppm (Martrette *et al*., 2011). In addition, olfactory memory was impaired after repeated exposure to 0.25 ppm (Guevara-Guzman *et al*., 2009). A study investigating sleep patterns reported no observed effects concerning sleep (Arito *et al*., 1990). The effects are illustrated with dose ranges below.

***Table**: Summary of effects - motor activity*

Dose/ppm	Motor activity	
	Acute dose studies	Repeated dose studies
0.1-0.12	Decreased motor activity (reversible) Rivas-Arancibia *et al*., 1998	-
0.2-0.25	Decreased motor activity (reversible) Rivas-Arancibia *et al*., 1998	Decreased motor activity Pereyra-Munoz *et al*., 2006 (15-30 d)
0.75-0.8	-	Decreased motor activity Gordon *et al*., 2013 (6h, 1d/week,17 weeks)

***Table:** Summary of exploratory behaviour*

Dose/ppm	Exploratory behavior	
	Acute dose studies	Repeated dose studies
1	Decreased exploratory behaviour (reversible in one study) Rivas-Arancibia *et al*., 2003	-

***Table:** Summary of freezing behaviour*

Dose/ppm	Freezing behavior	
	Acute dose studies	Repeated dose studies
1	Increased freezing behaviour (reversible in one study) Avila-Costa *et al.*, 2001 Rivas-Arancibia *et al*., 2003	-

***Table:** Summary of behavioural effects*

Dose/ppm	Grooming, resting, rearing, jumpin-play, drinking	
	Acute dose studies	Repeated dose studies
0.1-0.12	-	Increased: resting, drinking decreased: rearing, jumping-play Martrette *et al*., 2011 (15 d)

***Table**: Summary of other behavioural effects*

Dose/ppm	Time remaining in safety compartment before entering shock compartment	
	Acute dose studies	Repeated dose studies
0.1-0.12	Decreased time remaining in safety compartment before entering shock compartment Rivas-Arancibia *et al*., 1998	-
0.2-0.25	Decreased time remaining in safety compartment before entering shock compartment Rivas-Arancibia *et al*., 1998	-
0.5	Decreased time remaining in safety compartment before entering shock compartment Rivas-Arancibia *et al*., 1998	-
1	Decreased time remaining in safety compartment before entering shock compartment Avila-Costa, 1999 Rivas-Arancibia *et al*., 1998	-

***Table**: Summary of effects – olfactory memory*

Dose/ppm	Olfactory memory	
	Acute dose studies	Repeated dose studies
0.2-0.25	-	Impaired recognition of stimulus animal Impaired speed locating a buried chocolate Guevara-Guzman *et al*., 2009 (30-60 d)

***Table**: Summary of effects – wakefulness*

Dose/ppm	Wakefulness	
	Acute dose studies	Repeated dose studies
0.1-0.12	-	No effect Arito *et al*., 1990 (5 d)
0.2-0.25	-	No effect Arito *et al*., 1990 (5 d)
0.5	Reduced wakefulness Arito *et al*., 1992	-
1	Reduced wakefulness Arito *et al*., 1992	-
1.5	Reduced wakefulness Paz and Huitron-Resendiz, 1996	-

***Table**: Summary of effects – paradoxical sleep*

Dose/ppm	Paradoxical sleep

	Acute dose studies	Repeated dose studies
0.35	Reduced paradoxical sleep Paz and Huitron-Resendiz, 1996	-
0.5	Reduced paradoxical sleep (reversible) Arito *et al*., 1992	-
0.75-0.8	Reduced paradoxical sleep Paz and Huitron-Resendiz, 1996	-
1	Reduced paradoxical sleep (reversible) Arito *et al*., 1992	-
1.5	Reduced paradoxical sleep Paz and Huitron-Resendiz, 1996	-

***Table**: Summary of effects – slow-wave sleep*

Dose/ppm	Slow-wave sleep	
	Acute dose studies	Repeated dose studies
0.1-0.12	-	No effect Arito *et al*., 1990 (5 d)
0.2-0.25	-	No effect Arito *et al*., 1990 (5 d)
0.35	Increased slow-wave sleep Paz and Huitron-Resendiz, 1996	-
0.5	Increased slow-wave sleep (reversible) Arito *et al*., 1992	-
0.75-0.8	Increased slow-wave sleep Paz and Huitron-Resendiz, 1996	-
1	Increased slow-wave sleep (reversible) Arito *et al*., 1992	-
1.5	Increased slow-wave sleep Paz and Huitron-Resendiz, 1996	-

In addition, the DS included in the CLH report STOT RE section a study by Romero-Velázquez *et al.* (2002) reporting abnormal structures in the molecular layer of cerebellum in the offspring of female rat exposed to 1 ppm ozone during entire gestation. The study reported altered morphology of pup cerebellum with a decrease of total area and number of Purkinje cells (depopulation of and degenerating Purkinje cells in the cerebellum). These observations were accompanied by incomplete folding pattern of some lobes of the cerebellum. However, this study investigated only offspring and no information is available to support STOT RE assessment. The study will therefore be considered only under reproductive toxicity section for its developmental effects.

Conclusion for STOT RE (CNS)

In the repeated dose toxicity studies and in some acute inhalation studies, significant toxicity to the CNS was reported, including alterations with significant organ damage in different brain regions with cell death and altered neurogenesis combined with oxidative stress. These effects could also be directly linked to adverse behavioural changes as described above. This indicates significant organ damage and therefore RAC agrees with the DS that a classification for STOT RE 1 (nervous system) is warranted.

Setting of specific concentration limits for STOT RE (nervous system)

Study reference	**Effective dose (ppm)**	**Species, Length of exposure**	**SCL Cat. 1**	**SCL Cat. 2**
Pereyra-Münoz *et al.,* 2000 (15-30 d) Rivas-Arancriba, 2010 (15-90 d)	0.25 ppm, 4 h, converted to 6 h > 0.18 ppm. Morphological alterations, cell death in dopaminergic neurons in stratum and substantia nigra.	Rat 15-30 days. Effects were evident at day 15 of exposure	Extrapolation of the GV for a 28 d study (inhalation (gas)) corrected for 15 days exposure. 150 ppm (GV 28d) × 2 = 300 ppm SCL Cat 1 = 0.18 ppm / 300 ppm) × 100% = 0.06% > 0.05%	Extrapolation of the GV for a 28 d study (inhalation (gas)) corrected for 15 days exposure. 750 ppm (GV 28 d) × 2 = 1 500 ppm SCL Cat. 1 = 0.18 ppm / 1 500 ppm) × 100% = 0.012% > 0.01%

RAC suggests Commission to consider the applicability of the calculated SCL values.

Impact of respiratory system

Ozone induced toxicity in the respiratory system in experimental animals has been described in an enormous number of repeated dose toxicity studies submitted from the public literature. Changes in the lung morphology constitute an early sign of the effects of ozone.

Repeated ozone exposure for varying durations resulted in epithelial cell injury and pulmonary inflammation throughout the exposure. Cellular inflammation and the physiological repair mechanisms were often linked to a follow up of predominant structural changes, such as epithelial hyperplasia and metaplasia, necrosis of ciliated cells, and fibroblast proliferation in different parts of the respiratory system. Acute and short-term exposure were linked to inflammatory responses, with the greatest magnitude seen after long-term exposure.

As summarised in the CLH report STOT SE section, Hotchkiss *et al.* (1989a) reported that acute single exposure of ozone produced lung injury in animal studies showing signs of inflammation (bronchiolitis and peribronchiolar alveolitis starting from 0.66 ppm/6 h). Other local respiratory effects (noted in CLH report STOT RE section) include cell damage from 1.8 ppm/4 h (Bassett *et al.,* 1988), disruption of mucosal barrier from 0.8 ppm/3 h (Bhalla, 2000), necrosis of type I epithelial cells at ~1 ppm/8 h (Pino *et al.,* 1992), and progressive thickening of the walls of terminal bronchioles and proximal alveoli from 0.8–1.5 ppm (Hotchkiss *et al.,* 1989b). Arising acute biochemical effects (protein, albumin content and neutrophil influx in BAL) returned to control levels after cessation of exposure, but recovery from airway inflammation required longer time. However, structural changes, such as thickening of epithelial layer and collagen formation, increased during prolonged exposure, were still present after recovery periods as observed at 0.4 ppm/24 h by Van Bree *et al.* (2001).

Chang *et al.* (1991) reported a dose-response relationship in hyperplasia of respiratory and nasal epithelium which was directly related to the cumulative oxidant concentration. These effects were observed in different species with doses from 0.06–0.25 ppm 12–13 h/d over 3 to 13 weeks.

Studies in the monkeys (Carey *et al.* 2007 and 2011) investigated ozone exposure with 0.5 ppm 8 h/d for 5 days and reported rhinitis, necrosis, squamous metaplasia, epithelial changes, such as exfoliation of epithelium lining and hyperplasia in nasal airways.

Moreover, Harkema *et al*. (1987 and 1993) reported that exposure to low concentrations of ozone (up to 0.3 ppm 8 h/d for 90 days) in monkeys led to morphometrically detected lesions in the nose and lung and that even lower background exposure may contribute to epithelial cell injuries. Chang *et al.* (1991) reported changes at concentrations starting from 0.12 ppm ozone in rats. Further reported effects caused by sub-chronic exposure to low ozone levels were interstitial fibrosis in proximal alveolar region and bronchiolar epithelial injury (Chang *et al.,* 1992).

At developmental stage on infants, effects in monkeys were reported by Fanucchi *et al.* (2006). Exposed infant monkeys developed 4 fewer non-alveolarized airway generations, and the terminal and most proximal respiratory bronchiole were smaller and had altered smooth muscle bundle orientation in bronchioles after ozone exposure during normal distal airway development. In addition, Evans *et al.* (2003) reported atypical development of the tracheal basement membrane of infant monkeys. Alterations in airway innervation, such as hyperinnervation due to dramatic increase in airway nerve density and irregular epithelial nerve distribution were also contributed to ozone exposure (Kajekar *et al.,* 2007).

A Research Report No. 65 by the Health Effect Institute (HEI), investigated the consequences of prolonged inhalation of ozone on F344/N rats including the commentary of the institute's Health Review Committee. This NTP/HEI collaborative ozone project was designed to measure a variety of endpoints in order to form some generalized, comprehensive conclusions about ozone exposure in rats. As such, the data from this functional study by Harkema *et al.* (1994) are essential for subsequent correlations with data from studies conducted by other investigators from the NTP/HEI collaboration.

Harkema *et al.* (1994) used plethysmography techniques to assess the impact of ozone on pulmonary function. The authors reported on an ozone-related reduction of residual volume during slow lung deflation (most significant in 0.5 ppm females). The authors concluded that ozone exposure had only low relevance for integrated pulmonary function of the lung.

Harkema *et al.* (1994) reported that mucous flow in rats after exposure to 0.5 or 1 ppm ozone was slower over the lateral wall and turbinates of the proximal third of the nasal airways. Furthermore, at these doses intranasal regions contained mucous cell metaplasia and 25-300 times more mucus in nasal transitional epithelium than the corresponding regions from controls. The authors further reported at 0.5 and 1 ppm epithelial hyperplasia in nasal transitional epithelium, increases in eosinophilic globules in the surface epithelium lining the distal nasal airways, and a mild-to-moderate inflammatory cell influx in the nasal mucosa in the proximal and middle nasal passages. The authors concluded that exposure to 0.5 or 1 ppm for 20 months was connected to significant changes in function and structure of the nasal mucociliary apparatus.

In most studies morphological and functional pulmonary changes seem to become apparent at 0.5 ppm and above. This is in agreement with the integrative summary report published by the HEI.

Human data on respiratory tract effects

Many studies have been performed on rodents and the modelling of data obtained from studies on rodents suggest that existing anatomical differences within primates would cause the rodents to be more sensitive to damaging effects of the gas in the distal airways and alveoli compared to humans.

The amount of human data is extensive and consist of controlled human studies and epidemiological studies on ozone.

The DS based the classification and SCL calculation on the study by Jerrett *et al.* (2009). The reason for using this study was the substantial population size of 448850 subjects, the duration and the high quality standards of the statistics allowing the association of ozone with the risk of death from respiratory causes be assumed. The DS assumed that the assessment of proportionality of hazards (covariate effect is constant throughout duration of the study) was verified, even though not described in detail. Considering supplementary information given by Jerrett *et al.* (2009), the formal analysis to evaluate a possible threshold for the association between exposure to ozone and the risk of death are also coherent.

Combined with exercise, available studies indicated a correlation of ozone exposure with an increased risk of deaths by respiratory cause and that ozone exerts specific target organ toxicity towards the respiratory system after repeated exposure. For every 0.01 ppm increase in exposure to ambient ozone concentration, an increase in the risk of death from respiratory causes of about 2.9% in single-pollutant models, and 4% in two-pollutant models were reported.

Setting of specific concentration limits for STOT RE (respiratory system)

The DS based the classification and SCL calculation on the study by Jerrett *et al.* (2009). The LOAEC from this study was 0.0333–0.0531 ppm (death by respiratory cause), which was consistent, or at least in the same order of magnitude, with other epidemiological studies on humans related to effects of the respiratory system.

***Table**: Summary of the study by Jerret et al. (2009) and the derivation of specific concentration limits*

Study reference	**Effective dose (ppm)**	**Species, Length of exposure**	**SCL Cat.1**	**SCL Cat.2**
Jerrett *et al.,* 2009	0.033 ppm (LOAEC) Death by respiratory cause	Human Chronic	Extrapolation of the GV for 90 d study (inhalation (gas)) corrected for chronic exposure: 50 ppm (GV 90 d) / 2 = 25 ppm SCL Cat. 1 = (0.033 ppm / 25 ppm) × 100% = 0.13% → 0.1%	Extrapolation of the GV for 90 d study (inhalation (gas)) corrected for chronic exposure: 250 ppm (GV 90 d) / 2 = 125 ppm SCL Cat. 2 = (0.033 ppm / 125 ppm) × 100% = 0.026% → 0.02%

RAC suggests Commission to consider the applicability of the calculated SCL values.

Conclusion on STOT RE on respiratory system:

According to the CLP criteria for STOT RE category 1; H372:

Substances that have produced significant toxicity in humans or that, on the basis of evidence from studies in experimental animals, can be presumed to have the potential to produce

significant toxicity in humans following repeated exposure. Substances are classified in Category 1 for target organ toxicity (repeat exposure) on the basis of:

- *reliable and good quality evidence from human cases or epidemiological studies; or*
- *observations from appropriate studies in experimental animals in which significant and/or severe toxic effects, of relevance to human health, were produced at generally low exposure concentrations.*

Equivalent GVs for 90-day studies: Inhalation (rat), gas: C ≤ 50 ppmV/6 h/day Equivalent GVs for 28-day studies: Inhalation (rat), gas: C ≤ 150 ppmV/6 h/day

Epidemiological studies indicated a correlation between ozone exposure and an increased risk of deaths by respiratory cause, and that ozone exerts specific target organ toxicity towards the respiratory system after repeated exposure. For every 0.01 ppm increase in exposure to ambient ozone concentration, an increase in the risk of death from respiratory causes of about 2.9% in single-pollutant models, and 4% in two-pollutant models were observed.

Based on reliable and good quality evidence from epidemiological studies for significant toxicity to the respiratory system in humans, including a high number of deaths by respiratory cause, RAC agrees with the DS to **classify ozone as STOT RE 1 (respiratory system); H372**.

Supplemental information - In depth analyses by RAC

Ozone treatment for hypertension is described. Ozone regulates blood pressure and is effective in preventing the progression of hypertensive disease. The mechanisms are associated with anti-vasoconstrictor effects through reducing the levels of serum endothelin-1 and ET-receptor, and mRNA expression in the heart and vascular tissue.

10.13 Aspiration hazard

No data submitted by the applicant.

11 EVALUATION OF ENVIRONMENTAL HAZARDS

11.1 Rapid degradability of organic substances

Ozone is not an organic, but an inorganic substance. It is not considered to degrade, but to decompose or self-decompose to oxygen and hydroxyl radicals (please also see chapter 11.3)

11.1.1 Ready biodegradability

Biodegradation is not considered as relevant for ozone since it is an inorganic compound which quickly reacts and decomposes when coming into contact with organic and inorganic matter.

11.1.2 BOD_5/COD

No information available.

11.1.3 Hydrolysis

Ozone does not have any hydrolysable groups within its structure and is therefore considered not susceptible to hydrolysis.

11.1.4 Other convincing scientific evidence

11.1.4.1 Field investigations and monitoring data (if relevant for C&L)

No relevant field investigations or monitoring data available.

11.1.4.2 Inherent and enhanced ready biodegradability tests

Biodegradation is not considered as relevant for ozone since it is an inorganic compound which quickly reacts and decomposes when coming into contact with organic and inorganic matter.

11.1.4.3 Water, water-sediment and soil degradation data (including simulation studies)

Biodegradation is not considered as relevant for ozone since it is an inorganic compound which quickly reacts and decomposes when coming into contact with organic and inorganic matter.

11.1.4.4 Photochemical degradation

Phototransformation in water is considered negligible for the environmental fate and behaviour since self-decomposition and decomposition in contact with organic matter are more relevant.

Ozone is much more stable in air than in water, especially under dry conditions.

There are many factors influencing the fate of ozone in the atmosphere, therefore it is hard to define a general half-life value for ground-level ozone in air. The half-life of ozone in ambient air has been examined by the US EPA to be in the order of 12 hours (Rice and Browning, 1980). This value is often cited in the ozone literature and seems reliable and conservative enough to be selected as key value.

In the chamber study by McClurkin et al (2013) the stability of ozone in air within the context of disinfection of storage containers was tested. The half-life time of ozone as a function of air movement, temperature and humidity was determined. Half-life in still air at 24 °C and zero humidity was as high as 1524 min (25.4 h). As airflow, temperature and humidity increased, half-life time decreased to as low as 39 min. The self-decomposition of ozone in indoor air (in the absence of pollutants and light) is therefore strongly influenced by relative humidity, temperature and air flow (McClurkin et al, 2013).

The phototransformation of ozone in ambient air is well studied, and humidity has been found to play an important role. A degradation rate constant with OH radicals is not relevant since in photolytic ozone decomposition the emphasis is on a range of radical chain reactions. Molecular ozone reacts very easily with $O•^{2-}$ ($kA = 1.6 \cdot 10^9\ M^{-1}s^{-1}$) and $HO•^{2-}$ ($kA = 3 \cdot 106\ M^{-1}s^{-1}$). When O_3 reacts with O_2(-I)/ $O•^{2-}$, OH• is produced.

Jans and Hoigné (2000) performed laboratory experiments to study transformation of ozone into OH radicals (OH•) in waters of chemical compositions that reflect the characteristics of atmospheric waters (droplets of clouds and fog). This transformation is mainly accomplished by sensitised photoreactions promoted by radical-type chain reactions and to a much lesser extent by direct photolysis of ozone. Aqueous-phase direct photolysis of ozone (in atmospheric water) is too slow to be of environmental interest (time scale of hours). The main reason is that the wavelength region of sunlight that is absorbed by aqueous tropospheric ozone (and needed for direct photolysis) is already highly screened by stratospheric ozone. In comparison, thermal chemical transformations (sensitised photoreactions) are much quicker.

According to existing models, a radical-type chain reaction is initiated by any process that generates O_2(I). Photolysis of iron-oxalate complexes which are present in cloud droplets is a potential source of O_2(-I) in atmospheric waters. Other organic iron complexes which also occur in cloud waters and also act as photolytic sources of O_2(-I) show similar behaviour. When O_2(-I) reacts with ozone, OH• is generated. Reactions of OH• with other compounds like formaldehyde, formate, methanol, carbohydrates convert the very reactive and unselective OH• fast and at a high yield into highly selective O_2(-I). At the pH of typical cloud waters it is then $O•^{2-}$ (pKa of $HO_2•$ = 4.8) that further transforms O_3 into OH• or that reduces Cu(II) to Cu(I) that also reacts with O_3 to reproduce OH•. This chain of reactions can however be inhibited in the presence of compounds that scavenge OH• without converting a significant fraction of it into O_2(-I). Atmospheric waters also contain some hydrogen peroxide (H_2O_2). When dissociated ($HO_2•$) it also reacts highly selectively with ozone. However, due to the high pKa of H_2O_2 (pKa = 11.3), the reactions of $HO_2•$ are not relevant at the low pH values (pH < 5) encountered in typical cloud and fog waters. O_3 and $HO_2•$ are both steadily supplied from the gas-phase reservoir to the droplets in clouds.

Ozone in atmospheric water (fog and cloud droplets) is continuously involved in complex radical-type chain reactions responsible for the photolytic transformation of ozone. In contrast, direct photolysis of ozone is too slow to be of environmental interest – (time scale of hours, Jans and Hoigné, 2000).

Apart from chemical reactions in the air, the main removal process for ozone in the earth's boundary layer is deposition to the surface, known as dry deposition, where the ozone is 'absorbed' by soil and vegetation. The surfaces of soil and vegetation react with ozone, also in the dark, and hence form an important sink for ozone (Stella et al, 2011; Ainsworth et al, 2012).

Ozone decomposes to oxygen and short-lived radicals.

11.2 Environmental transformation of metals or inorganic metals compounds

Ozone is neither a metal nor an inorganic metal compound. Information on its environmental fate and behaviour can be found in Section 11.1.

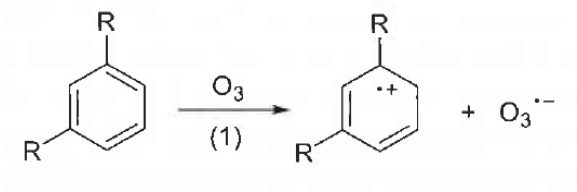
11.3 Environmental fate and other relevant information

Ozone is unstable in water. Reactions of ozone in water can be generally distinguished by direct reactions with other compounds (molecules, radicals etc.) and indirect reactions which involve hydroxyl radicals that are produced by ozone decay, and other compounds. No general rules have been described which can explain the influence of different parameters on the decay of ozone in both natural and wastewater.

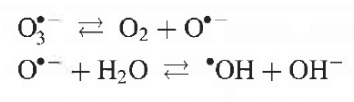
Several factors (pH, alkalinity, concentration of organic carbon, temperature, concentration of anions/cations, and hydrodynamic conditions) could have relevant effects on the decay constant (Gardoni et al, 2012). Depending on the water quality, the half-life of ozone is in the range of seconds to hours (Sotelo et al 1987).

According to von Sonntag and von Gunten (2012) "in natural waters, the dissolved organic matter (DOM) contributes significantly to ozone decay, and waters that have a low DOM and high bicarbonate content show relatively high ozone stability."

"The stability of ozone in drinking water and in wastewater is largely determined by its reaction with the DOM. The nature of DOM varies among waters of different origin as does its concentration. For example, in drinking waters, DOM, measured as DOC, is typically below 4 mg/L, while in wastewaters it ranges between 5 and 20 mg/L. The nature of DOM has an influence on the rate of its reaction with ozone and thus on the ozone lifetime in these natural waters, drinking waters and wastewaters. Carbonate alkalinity influences ozone stability by scavenging •OH. When ozone reacts with DOM, •OH radicals are produced. The •OH radical is an important intermediate in the decomposition of ozone in water. In a study on wastewater, it has been suggested that ozone reacts with the electron-rich aromatic components of DOM by electron transfer" (von Sonntag and von Gunten, 2012) – as follows:



The O3●- radical gives rise to ●OH, through the following reactions:



A study performed in five Swiss natural waters with various compositions (DOC and alkalinity), at pH 8 and 15 °C, indicated that ozone's DT_{50} values in groundwater (DOC 0.7 mg/L, carbonate alkalinity 6.7 mM), spring water (DOC 0.9 mg/L; carbonate alkalinity 5.4 mM), lake water 1 (DOC 1.3 mg/L; carbonate alkalinity 2.5 mM), lake water 2 (DOC 1.6 mg/L; carbonate alkalinity 3.6 mM) and lake water 3 (DOC 3.2 mg/L; carbonate alkalinity 3.4 mM) were approximately 50, 19, 3, 3 and 5 minutes, respectively which results in an average DT_{50} value of 16 minutes. And when considering only the half-lives of the lake waters, which have higher DOC concentration, an average DT_{50} of 4 minutes is calculated. Based on the results, ozone stability decreased in the sequence groundwater > spring water > lake water 1, 2 > lake water 3. This corresponds to an increasing trend in DOC concentration and a decreasing trend in alkalinity (von Sonntag and von Gunten, 2012).

Table 51: Summary of relevant information on environmental fate and other relevant information

Method, Guideline, GLP status	**pH**	**Temp. [°C]**	**Initial TS concen-tration, C_0[mol/l]**	**Half-life, DT_{50} [minutes]**	**Coefficient of correlation, r^2**	**Remarks**	**Reference**
GLP not stated	8	15 ºC	-	16	-	DT_{50} in surface waters. Average half-life value estimated from a study performed in five Swiss natural waters (with DOC ranging from 0.7 and 3.2 mg/L)	von Sonntag and von Gunten, 2012
GLP not stated	8	15 ºC	-	4	-	Assumed DT_{50} in the sewer. Average half-life value estimated from three Swiss natural waters with DOC ranging between 1.3 and 3.2 mg/L. Since wastewaters have DOC ranging between 5 and 20 mg/L, the DT_{50} of 4 min is considered to be a worst-case for the STP.	von Sonntag and von Gunten, 2012

11.4 Bioaccumulation

11.4.1 Estimated bioaccumulation

No studies are available, that examined the bioaccumulation potential of ozone to aquatic organisms.

Bioaccumulation of ozone is not expected based on the log Kow of – 0.87 and based on the fact that ozone is an atmospheric and highly reactive gas. In the case that ozone is released to ecosystems, it will react very rapidly with organic matter. Thus, it is evident that ozone has no potential for bioconcentration or bioaccumulation in aquatic organisms.

11.4.2 Measured partition coefficient and bioaccumulation test data

No studies are available, that examined the bioaccumulation potential of ozone to aquatic organisms.

Bioaccumulation of ozone is not expected based on the log Kow of – 0.87 and based on the fact that ozone is an atmospheric and highly reactive gas. In the case that ozone is released to ecosystems, it will react very rapidly with organic matter. Thus, it is evident that ozone has no potential for bioconcentration or bioaccumulation in aquatic organisms

11.5 Acute aquatic hazard

For the effects assessment of ozone to aquatic organisms no studies according to internationally accepted guidelines are available. Instead the applicant for authorization as biocidal active substance provided numerous studies from peer-reviewed literature on the effects of ozone on fish, invertebrates and algae, covering both freshwater and marine species.

In all the studies cited in Table 52 and Table 53, the test material was introduced into the test systems as 100 % ozone gas.

Table 52: Summary of relevant information on acute aquatic toxicity

Method	Species	Test material	Results[1]	Remarks	Reference
Fish					
EPA 1975 No GLP Reliability[2] 2	*Oncorhynchus mykiss* *(Salmo gairdneri)*	ozone	96h-LC_{50}= 0.0093 mg/L (measured)	continuous ozone flow (water flows set at 800 mL/min, resulting in a 95% replacement time of 0.5 h) O_3 concentrations measured spectrophotometrically with DPD method as Cl_2 and converted to ozone; sublethal effects (gill tissue morphology, elevated hemoglobin & hematocrit levels) at 7-29 µg/L	Wedemeyer at al. 1979 (published study)
Not specified No GLP Reliability 2	*Atherinops affinis (marine)* (larvae)	ozone	2h-LC_{50} = 0.31 mg/L TRO (measured as Br_2) 0.093 mg/L (expressed as equivalent conc. of O_3 using the factor 0.3[3] calculated by eCA)	continuous ozone flow TRO (total residual oxidant) concentration was measured spectrophoto-metrically with DPD method as CL_2; In the paper, TRO concentrations (mg/L) were calculated and expressed as equivalent concentrations of bromine (Br_2);using the factor 0.44 (molecular weight Cl_2/molecular weight Br_2)	Jones et al., 2006 (published study)
Not specified No GLP Reliability 2	*Cyprinodon variegatus* *(marine)* (juveniles)	ozone	4h-LC_{50} = 0.35 mg/L TRO (measured as Br_2) 0.105 mg/L (expressed as equivalent conc. of O_3 using the factor 0.3[2] 2calculated by eCA)		
Not specified No GLP Reliability 2	*Atherinops affinis* *(marine)* (juveniles)	ozone	48h-LC_{50} = 0.26 -0.34 mg/L TRO (as Br_2) 0.078 – 0.102 mg/L (expressed as equivalent conc. of O_3 using the factor	static different water sources (artificial and natural seawater) analytical monitoring at test start to confirm nominal conc.	

[1] Indicate if the results are based on the measured or on the nominal concentration

[2] Reliability ratings given by dossier submitter

[3] the factor 0.3 was calculated from the molecular weight ratio between O_3 (48 g/mol) and Br_2 (160 g/mol)

Method	Species	Test material	Results[1]	Remarks	Reference
			0.3[2] calculated by eCA)	TRO (total residual oxidant) concentration was measured spectrophoto-metrically with DPD method as Cl_2; In the paper, TRO concentrations (mg/L) were calculated and expressed as equivalent concentrations of bromine (Br_2);using the factor 0.44 (molecular weight Cl_2/molecular weight Br_2)	
Not specified No GLP Reliability 2	*Cyprinodon variegatus* *(marine)* (juveniles)	ozone	48/96h-LC_{50}= 0.17 mg/L (TRO, as Br_2) 0.05 mg/L (expressed as equivalent conc. of O_3 using the factor 0.3[2] calculated by eCA); no difference in water source		
Not specified No GLP Reliability 2	*Cyprinus carpio* (larvae)	ozone	48h_LC_{50} = 0.03 mg/L	continuous ozone flow; O_3 concentration measured spectophoto-metrically with the indigo method	Leynen et al., 1998 (published study)
Not specified No GLP Reliability 2	*Leuciscus idus* (larvae)	ozone	48h-LC_{50} = 0.036 mg/L		
Not specified No GLP Reliability 2	*Clarias gariepinus* (larvae)	ozone	48h-LC_{50} = 0.035 mg/L		
Not specified No GLP Reliability 2	*Ictalurus punctatus* (eggs, lavae)	ozone	3h-LC_{50} = 4 mg/L (eggs) 3h-LC_{50} = 0.47 mg/L (larvae)	static, O_3 concentration measured spectophoto-metrically by neutral buffered iodometric method; aim of study was the determination of effective methods for treatment of river water (destroy of entained eggs and larvae of fish)	Coler & Asbury, 1980 (published study)
Not specified No GLP Reliability 2	*Perca flavescens* (eggs, larvae)	ozone	3h-LC_{50} > 2.06 mg/L (eggs) 3h-LC_{50} = 0.21 mg/L (larvae)		
Not specified No GLP Reliability 2	*Alosa sapidissima* (eggs)	ozone	3h-LC_{50} = 0.39 mg/L		
Not specified No GLP	*Oncorhynchus mykiss* *(Salmo gairdneri)*	ozone	3h-LC_{50} = 0.19 mg/L		

Method	Species	Test material	Results[1]	Remarks	Reference
Reliability 2	(larvae)				
Not specified No GLP Reliability 2	*Lepomis macrochirus* (larvae)	ozone	3h-LC_{50} = 0.33 mg/L		
Not specified No GLP Reliability 2	*Notropis hudsonius* (postlarvae)	ozone	3h-LC_{50} = 1.22 mg/L		
American Public Health Association 1971 No GLP Reliability 2	*Lepomis macrochirus*	ozone	24h-LC_{50} = 0.06 mg/L	continuous, O_3 concentration measured spectophoto-metrically via oxidation of a buffered iodine solution and measurement of the triiodide ion liberated by ozone delayed mortality of surviving fish after 2-7 days caused by severe fungus infection	Paller & Heidinger, 1979 and 1980 (published study)
Invertebrates					
Not specified No GLP Reliability 2	*Daphnia magna*	ozone	48h-NOEC = 0.011 mg/L 24h-EC_{100} = 0.021 mg/L	continuous ozone flow, O_3 concentration measured spectophoto-metrically with the indigo method	Leynen et al, 1998 (published study)
Not specified No GLP Reliability 2	*Americamysis bahia* (marine)	ozone	3h-LC50 = 0.62 mg/L TRO (as Br_2) after 3h, 0.051 mg/L (expressed as equivalent conc. of O_3 using the factor 0.3[4]calculated by eCA)	continuous ozone flow, TRO (total residual oxidant) concentration was measured spectrophoto-metrically with DPD method as Cl_2; In the paper, TRO concentrations (mg/L) were calculated and expressed as equivalent concentrations of bromine (Br_2);using the factor 0.44 (molecular weight	Jones et al, 2006 (published study)
Not specified No GLP Reliability 2	*Leptocheirus plumulosus* (marine)	ozone	5h-LC50 > 5.63 mg/L TRO (as Br_2) after 3h, > 1.69 mg/L (expressed as equivalent conc. of O_3 using the factor		

[4] the factor 0.3 was calculated from the molecular weight ratio between O_3 (48 g/mol) and Br_2 (160 g/mol)

Method	Species	Test material	Results[1]	Remarks	Reference
			0.3[3]calculated by eCA)	Cl_2/molecular weight Br_2)	
Not specified No GLP Reliability 2	*Rhepoxinius abronius* (marine)	ozone	4h-LC50 = 0.94 mg/L, 0.28 mg/L (expressed as equivalent conc. of O_3 using the factor 0.3[3]calculated by eCA)		
Not specified No GLP Reliability 2	*Americamysis bahia* (marine)	ozone	48h-LC50 = 0.34-0.46 mg/L TRO (as Br_2), 0.1 – 0.138 mg/L (expressed as equivalent conc. of O_3 using the factor 0.3[3] calculated by eCA)		
Not specified No GLP Reliability 2	*Litopenaenus vannamei (marine)*	ozone	96h-LC_{50} = 0.5 mg/L OPO (Ozone-produced oxidants measured as chlorine equivalent) 0.33 mg/L (expressed as equivalent conc. of O_3 using the factor 0.67 (molecular weight O_3/molecular weight Cl_2) calculated by eCA)	O_3 concentrations measured spectrophoto-metrically with DPD method as Cl2,	Schroeder et al. 2010 (published study)
Algae					
No data available					

11.5.1 Acute (short-term) toxicity to fish

For fish, the lowest effect value was reported in a published study (Wedemeyer et al. 1979) for the rainbow trout *Oncorhynchus mykiss*. In a 96 h-study under continuous ozone exposure, a 96h-LC_{50} of 9.3 µg/L was derived. The EC_0 was determined as 8 µg/L, thus indicating a very steep dose-response-curve of ozone. Mortality of the fish apparently results from massive destruction of the gill lamellare epithelium together with a severe hydromineral imbalance. Water samples were analyzed twice daily during the test by spectrophometrically measuring the residual ozone. This procedure has at best ± 15 % precision and is not specific for ozone but gives total oxidants present.

The other available studies with fish reported effect values in the range of 0.031 mg/L to 1.43 mg/L. Both freshwater and marine fish species were tested. It has to be considered, that both the exposure regimes as well as the life stages of the exposed fish differ from each other as well as from standard fish tests, therefore, a comparison of species sensitivity is not possible.

11.5.2 Acute (short-term) toxicity to aquatic invertebrates

For invertebrates, the lowest effect values were found for *Daphnia magna* under continuous ozone exposure for 48 h. The 48h-NOEC is reported as 11 µg/L and the 24 h EC_{100} as 21 µg/L. Although no EC_{50} could be derived from this study, the study is selected as key study, as the difference between the NOEC and the EC_{100} is just a factor of 2 and therefore, the NOEC of 11 µg/L can be considered as surrogate for the EC_{50}. Again, a very steep dose-response-curve was found for ozone.

The other available studies with invertebrates reported effect values between 0.051 mg/L to > 1.69 mg/L.

Both freshwater and marine invertebrate species were tested.

11.5.3 Acute (short-term) toxicity to algae or other aquatic plants

For green algae no studies determining EC50-values are available.

11.6 Long-term aquatic hazard

Table 53: Summary of relevant information on chronic aquatic toxicity

Method	Species	Test material	Results[1]	Remarks	Reference
Fish					
EPA 1975 No GLP Reliability 2	*Oncorhynchus mykiss*	ozone	3-months-NOEC = 2.3 µg/L	Limit test (0.002 mg/L) Continuous ozone flow Limited feeding of the fish O_3 concentrations measured spectrophoto-metrically with DPD method as Cl_2 and converted to ozone; No mortality, mild thrombo-cytosis Average ozone conc: 0.0023 mg/L	Wedemeyer et a. 1979 (published study)
				Limit test (0.005 mg/L) Continuous ozone flow Limited feeding of the fish O_3 concentrations measured spectrophoto-metrically with DPD method as Cl_2 and converted to ozone; No mortality,	

Method	Species	Test material	Results[1]	Remarks	Reference
Fish					
				Significant effects on growth, mild hypoglycaemia, polycythemia and lymphocytopenia Average ozone conc: 0.005 mg/L	
Invertebrates					
No GLP Reliability 2	*Litopenaeus vannamei* (marine)	ozone	21-NOEC = 0.06 mg OPO/L 21d-LOEC = 0.1 mg OPO/L OPO: Ozone-produced oxidants measured as chlorine equivalent[1]) 21d-NOEC = 0.04 mg/L (expressed as equivalent conc. of O_3 using the factor 0.67 calculated by eCA)	Continuous ozone flow, O_3 concentrations measured spectrophoto-metrically with DPD method as Cl2, At 0.1 and 0.15 mg/L OPO increased cannibalistic behaviour; After the 21 day exposure 69% and 35% of the survivors showed clear indications of soft shell syndrome at OPO concentrations of 0.10 and 0.15 mg/l	Schroeder et al. 2010 (published study)
Algae					
Not specified No GLP Reliability 2	*Nannochloropsis oculate* (marine)	ozone	3d-NOEC = 0.05 mg/L TRO (0.006 mg/L measured as TRO in algae solution 5d-NOEC = 0.24 mg/L TRO (0.014 mg/L measured as TRO in algae solution)	static, 5 concentrations 0.05 - 0.92mg/L (measured as TRO in seawater without algae) produced from different ozone exposure durations (0.5,	Kureshy et al, 1999 (published study)

Method	Species	Test material	Results[1]	Remarks	Reference
Fish					
				0.75, 1, 1.5, 2 min) of test water analytical monitoring of TRO conc.. in treatments with algae: 0.006 – 0.48 TRO = total residual oxidants including ozone, chloramines and bromamines measured spectrophoto-metrically with the indigo method cell cultures were exposed to ozone for up to 2 min and then were cultured for further 5 days	
Not specified No GLP Reliability 2	*Isochyris galbana* (marine)	ozone	3d-NOEC = 0.23 mg/L TRO (0.03 mg/L measured as TRO in algae solution) 4d-NOEC = 0.34 mg/L TRO (0.08 mg/L measured as TRO in algae solution)	static, 5 concentrations 0.05 – 0.9 mg/L (measured as TRO in seawater without algae) produced from different ozone exposure durations (0.5, 1, 1.5, 2, 3 min) of test water; analytical monitoring of TRO conc. in treatments with algae: 0.03 – 0.48 mg/L) TRO = total residual oxidants including	

Method	Species	Test material	Results[1]	Remarks	Reference
Fish					
				ozone, chloramines and bromamines measured spectrophoto-metrically with the indigo method cell cultures were exposed to ozone for up to 3 min and then were cultured for further 4 days	
Not specified No GLP Reliability 2	*Chaetoceros gracilis* (marine)	ozone	3d-NOEC = 0.06 mg/L TRO (0.01 mg/L measured as TRO in algae solution) 5d-NOEC = 0.31 mg/L TRO (0.05 mg/L measured as TRO in algae solution)	static, 5 concentrations 0.06 -0.92 mg/L (measured as TRO in seawater without algae) produced from different ozone exposure durations (0.5, 1, 1.5, 2, 3 min) of test water, analytical monitoring of TRO conc. in treatments with algae: 0.01 – 0.25 mg/L TRO = total residual oxidants including ozone, chloramines and bromamines measured spectrophoto-metrically with the indigo method cell cultures were exposed to ozone for up to 3 min	

Method	Species	Test material	Results[1]	Remarks	Reference
Fish					
				and then were cultured for further 5 days	

11.6.1 Chronic toxicity to fish

Only one relevant long-term study with fish is available for ozone. Wedemeyer et al. (1979) examined the long-term toxicity of ozone to Oncorhynchus mykiss for an exposure period of 3 month. Juvenile rainbow trouts (10 – 13 cm) were exposed to ozone in a flow-through system. Two tests were performed, one with a ozone concentration of 2 µg/L (average measured conc. during the 3-months exposure period: 2.3 µg/L), and the other with an ozone concentration of 5 µg/L (nominal and average measured conc.). One replicate was used in both tests. At both tested concentrations, no mortality occurred. In the 2.3 µg/L exposure, no significant effects on haematology, blood chemistry or growth was found, except for a mild thrombocytosis in the test fish. In the test with 5 µg/L, significant effects on growth, together with a mild hypoglycaemia, a mild polycythaemia and lymphocytopenia were observed. Thus, a NOEC of 2.3 µg/L can be derived from this study. Growth of juvenile fish is a sensitive indicator of toxicity and is also a recommended endpoint in OECD 215 (Fish juvenile growth test). This test is recommended to cover the long-term toxicity for fish for substances with a log K_{ow} < 5. Although the study by Wedemeyer et al. was not performed according to OECD 215, and the juvenile fish used were greater than recommended in OECD 215, it can be considered as a long-term toxicity tests for fish, as the exposure time was 3 times longer than the 28 days foreseen in OECD 215 and thus can be regarded to partly compensate for the higher size of the test organisms. Therefore, the NOEC of 2.3 µg/L is used for the effects assessment of ozone,. In addition, the study was performed with the test species that was most sensitive in the acute studies.

The applicant provided further studies with fish in which sublethal endpoints were examined. These studies had an exposure duration between 10 hours and 21 days and focused on sublethal endpoints like changes in blood osmolarity and gill morphology (effects at 0.17 mg/L in Lepomis macrochirus) (Paller & Heidinger, 1980), gill histopathology and stress response (gst, Hsp70 & Hsp90 expression) (effects at 0.1 – 0.15 mg/L OPO / 0.14 – 0.22 mg/L O3 in Psetta maxima) (Reiser et al, 2011), genotoxicity and hematological alterations (effects at 0.15 mg/L TRO as Br2 / 0.45 mg/L O3 in Scophthalmus maximus) (Silva et al, 2011). Further studies were assessed as not valid by the eCA because no effect concentrations were derived and/or the concentration of ozone during the exposure period fluctuated considerably. However, as these studies were either assessed as not valid by the eCA or investigated sublethal endpoints not relevant for the environmental effect assessment, the endpoints from these studies have not been reported in this document. All effect values from these studies are higher than 2.3 µg/L from the study by Wedemeyer et al. and therefore the additional studies are considered as not relevant for the environmental effects assessment of ozone.

11.6.2 Chronic toxicity to aquatic invertebrates

For aquatic invertebrates, also only one long-term study is available (Schroeder et al. 2010). In a 21d-study, juvenile marine white pacific shrimp Litopenaeus vannamei were exposed to three OPO (ozone-produced oxidants) concentrations (0.06, 0.1 and 0.15 mg/L) to a continuous ozone flow. In the lowest concentration of 0.06 mg/L OPO (0.04 mg/L O3) no mortality or behaviour effects occurred during the exposure period. Even at higher OPO concentrations no behavioural impairment such as loss of equilibrium, lethargy or reduced feeding activity could be observed. However, an obvious increase of cannibalistic behaviour in shrimp exposed to the 0.10 and 0.15mg/L OPO treatments was evident and mortality levels reached 47 % and 43 % after 21 days of exposure, respectively. However, mortality did not appear until day 12 and 9 in 0.10 and 0.15 mg/L OPO treatments, respectively. After the 21 day exposure 69 % and 35 % of the survivors showed clear indications of soft shell syndrome at OPO concentrations of 0.10 and 0.15 mg/L (0.067 – 0.1 mg/L O_3),

respectively. The affected treated shrimp had a soft, paper-like carapace with a gap between muscle tissue and exoskeleton.

11.6.3 Chronic toxicity to algae or other aquatic plants

Kurshey et al. (1999) examined the toxicity of ozone to three marine algae species in a static exposure system. The algae were exposed to ozone for 0.5 to 3 minutes, resulting in initial ozone concentrations between 0.006 and 0.48 mg/L (measured as total residual oxidants). Thereafter, the algae were cultured for further 4 or 5 days. It was shown that with increasing culture time the effects on cell counts decreased. NOEC values were between 0.006 mg/L TRO for *Nannochloropsis oculate* after 3 days and 0.08 mg/L TRO for *Isochyris galbana* after 4 days. No EC_{50} could be determined from the study and the algae were exposed only in a static system with fast decreasing ozone concentrations, thus underestimation of ozone toxicity to algae is probable. However, based on the unlikely direct exposure of ozone to the environment, it was decided not to request an algae study with continuous exposure, from which a relevant EC_{50} could be derived. In addition, the mode of action of ozone does not indicate that algae would react by orders much more sensitive to ozone than fish and invertebrates. Thus, the NOEC value of 0.006 mg/L for *Nannochloropsis oculate* was considered as key value for algae toxicity of ozone.

11.6.4 Chronic toxicity to other aquatic organisms

No studies are available.

11.7 Comparison with the CLP criteria

11.7.1 Acute aquatic hazard

Acute studies are available for fish, invertebrates and algae. The lowest acute effect value (96h-LC_{50} = 0.0093 mg/L) was found for *Oncorhynchus mykiss* in a test system with continuous ozone flow and analytical monitoring.

The criterion for classification as **H400 "Very toxic to aquatic life**" is a $LC_{50} \leq 1$ mg/L. Hence, Ozone fulfils this criterion and has to be classified as H400. Due to an acute toxicity in the range of $0.001 < EC_{50} \leq 0.01$ mg/L an **M-factor = 100** has to be applied.

11.7.2 Long-term aquatic hazard (including bioaccumulation potential and degradation)

Biodegradation is not considered as relevant for ozone since it is an inorganic compound which quickly reacts and decomposes when coming into contact with organic and inorganic matter. Regarding the abiotic degradation processes, ozone decomposes to oxygen and short-lived radicals and does not have any hydrolysable groups within its structure and is therefore considered not susceptible to hydrolysis.

In conclusion, ozone has to be considered as highly reacting substance, for which no classification criteria are defined in the framework of classification and labelling. Hence, for the purpose of classification and labelling ozone will be considered as rapidly degradable (c.f. section 11.3).

Based on a log Kow of -0.87, ozone is not expected to have a bioaccumulation potential.

For effects assessment of ozone adequate chronic toxicity data is available for fish and invertebrates. For algae, NOEC values are available from a static test system with fastly decreasing ozone concentration.

For ozone a 3-month test with *Oncorhynchus mykiss* under continuous ozone flow and analytical monitoring is available, with a **NOEC** for growth of **0.0023 mg/L**. For the marine shrimp *Litopenaeus vannamei* a 21d-NOEC for mortality and behaviour of 0.004 mg/L was derived in a continuous flow-through system. For the marine algae *Nannochloropsis oculate* a 3d-NOEC of 0.006 mg/L(cell count) was derived in a static test system. These effect values are all in the same order, supporting the assumption that ozone acts unspecifically on aquatic organisms.

For rapidly degradable substances the criterion for classification as H410 "**Very toxic to aquatic life with long lasting effects"** is EC_{10}/NOEC ≤ 0.01 mg/L. Ozone fulfils this criterion and has to be classified accordingly. Due to a chronic toxicity in the range 0.001 < NOEC ≤ 0.01 mg/L an **M-factor = 1** has to be applied.

11.8 CONCLUSION ON CLASSIFICATION AND LABELLING FOR ENVIRONMENTAL HAZARDS

Classification of ozone:

Aquatic Acute 1; H400, M = 100

Aquatic Chronic 1; H410, M = 1

Labelling:

Signal word: Warning

Pictogram: GHS 09

Hazard statement: H410: "Very toxic to aquatic life with long lasting effects"

RAC evaluation of aquatic hazards (acute and chronic)

Summary of the Dossier Submitter's proposal

Ozone is generated *in situ* as a biocidal active substance from oxygen and used to disinfect water and ambient air. Ozone is not currently listed in Annex VI of the CLP Regulation (EC) 1272/2008, however as ozone is an active substance in the meaning of Regulation (EU) No. 528/2012 (BPR), the DS proposed harmonised classification and labelling based on the draft risk assessment report (draft CAR for BPR).

Overall, the DS concluded that for the purpose of classification and labelling, ozone should be considered as rapidly degradable, is not expected to have a bioaccumulation potential and proposed classification as:

Aquatic Acute 1 with an M-factor of 100, based on the 96-hour LC_{50} value of 0.0093 mg/L for *Oncorhynchus mykiss*, and

Aquatic Chronic 1 with an M-factor of 1, based on the three-months NOEC value of 0.0023 mg/L for *Oncorhynchus mykiss* and 3-days NOEC value of 0.006 mg/L for *Nannochloropsis oculate*.

Degradation

Biodegradation is not considered as relevant for ozone since it is an inorganic compound which quickly reacts and decomposes when coming into contact with organic and inorganic matter.

Ozone decomposes to oxygen and short-lived radicals and does not have any hydrolysable groups within its structure and is, therefore, considered not susceptible to hydrolysis.

Photo transformation in water is considered negligible for environmental fate and behaviour since self-decomposition and decomposition in contact with organic matter are more relevant.

Ozone is unstable in water. Reactions of ozone in water can generally be distinguished by direct reactions with other compounds (molecules, radicals, etc.) and indirect reactions, which involve hydroxyl radicals that are produced by ozone decay, and other compounds. No general rules have been described which can explain the influence of different parameters on the decay of ozone in both natural and wastewater. Several factors (pH, alkalinity, concentration of organic carbon, temperature, concentration of anions/cations, and hydrodynamic conditions) could have relevant effects on the decay constant. Depending on the water quality, the half-life of ozone is in the range of seconds to hours. A study performed in five Swiss natural waters with different DOC and alkalinity at pH 8 and 15°C indicate that ozone half-live in groundwater, spring water, and 3 lake waters were approximately 50, 19, 3, 3 and 5 minutes, respectively which results in an average DT_{50} value of 16 minutes. Based on the results, ozone stability decreased by increasing DOC concentration and decreasing alkalinity.

Ozone is much more stable in air than in water, especially under dry conditions. There are many factors influencing the fate of ozone in the atmosphere, therefore it is hard to define a general half-life value for ground-level ozone in air. Still, the half-life of ozone in ambient air has been examined by the US EPA to be in the order of 12 hours (Rice and Browning, 1980). This value is often cited in the ozone literature and seems reliable and conservative enough to be selected as the key value. Ozone in atmospheric water (fog and cloud droplets) is continuously involved in complex radical-type chain reactions responsible for the photolytic transformation of ozone. Apart from chemical reactions in the air, the main removal process for ozone in the earth's boundary layer is deposition to the surface, known as dry deposition, where the ozone is 'absorbed' by soil and vegetation.

Overall, the DS considered that ozone is highly reactive substance, for which no classification criteria are defined in the framework of classification and labelling. Nevertheless, due to the results summarised above, for the purpose of classification and labelling, the DS considered ozone as rapidly degradable.

Aquatic Bioaccumulation

As there are no experimental results on BCF values, the bioaccumulation potential for classification purposes was based on its n-octanol/water partition coefficient (K_{ow}) and on the fact that ozone is an atmospheric and highly reactive gas (reacts very rapidly with organic matter). The log K_{ow}, estimated by the atom/fragment contribution method for estimating octanol–water partition coefficients, was -0.87.

Overall, based on the information summarised above, the DS considered that ozone has a low potential for bioaccumulation.

Aquatic Toxicity

The aquatic toxicity test results from available acute and chronic studies of ozone are summarised in the following table and sections. Although no studies according to internationally accepted guidance were available, the DS provided numerous studies from peer-reviewed literature on the effects of ozone on fish and aquatic invertebrates, covering both freshwater and marine species. Reliability ratings for the studies were given by the DS. In all studies, the test material was introduced into the test systems as 100% ozone

gas. The most sensitive trophic group for aquatic (acute and chronic) toxicity was fish (*Oncorhynchus mykiss*).

Aquatic Acute toxicity

Test method / Reliability	Test organism	Short-term result (endpoint)	Reference
Fish			
EPA 1975 / No GLP / reliability 2	*Oncorhynchus mykiss*	**96 h LC_{50} = 0.0093 mg/L (measured)**	Wedemeyer *et al*., 1979
Not specified / No GLP / reliability 2	*Atherinops affinis* (marine, larvae)	2 h LC_{50} = 0.093 mg/L(*)	Jones *et al*., 2006
	Cyprinodon variegatus (marine, juveniles)	4 h LC_{50} = 0.105 mg/L(*)	
	Atherinops affinis (marine, juveniles)	48 h LC_{50} = 0.078–0.102 mg/L(*)	
	Cyprinodon variegatus (marine, juveniles)	48/96 h LC_{50} = 0.05 mg/L(*)	
Not specified / No GLP / reliability 2	*Cyprinus carpio* (larvae)	48 h LC_{50} = 0.03 mg/L (measured)	Leynen *et al*., 1998
	Leuciscus idus (larvae)	48 h LC_{50} = 0.036 mg/L (measured)	
	Clarias gariepinus (larvae)	48 h LC_{50} = 0.035 mg/L (measured)	
Not specified / No GLP / reliability 2	*Ictalurus punctatus* (eggs, larvae)	3 h LC_{50} = 4 mg/L (eggs, measured) 3 h-LC_{50} = 0.47 mg/L (larvae, measured)	Coler & Asbury, 1980
	Perca flavescens (eggs, larvae)	3 h LC_{50} > 2.06 mg/L (eggs, measured) 3 h LC_{50} = 0.21 mg/L (larvae, measured)	
	Alosa sapidissima (eggs)	3 h LC_{50} = 0.39 mg/L (measured)	
	Oncorhynchus mykiss (larvae)	3 h LC_{50} = 0.19 mg/L (measured)	
	Lepomis macrochirus (larvae)	3 h LC_{50} = 0.33 mg/L (measured)	
	Notropis hudsonius (post-larvae)	3 h LC_{50} = 1.22 mg/L (measured)	
American Public Health Association 1971 / No GLP / reliability 2	*Lepomis macrochirus*	24 h LC_{50} = 0.06 mg/L (measured)	Paller & Heidinger, 1979 and 1980
Aquatic invertebrates			
Not specified / No GLP / reliability 2	*Daphnia magna*	48 h EC_{50} = 0.011 mg/L (**)	Leynen *et al*., 1998
	Americamysis bahia (marine)	3 h LC_{50} = 0.051 mg/L(*)	Jones *et al*., 2006

Not specified / No GLP / reliability 2	*Leptocheirus plumulosus* (marine)	3 h LC_{50} >1.69 mg/L(*) 4 h LC_{50} = 0.28 mg/L(*)	
	Americamysis bahia (marine)	48 h LC_{50} = 0.1–0.138 mg/L (*)	
Not specified / No GLP / reliability 2	*Litopenaenus vannamei* (marine)	96 h LC_{50} = 0.33 mg/L (*)	Schroeder *et al*., 2010
Algae / other aquatic plants			
No data available			

(*) expressed as equivalent conc. of O_3 using the factor 0.3 calculated by eCA. The factor 0.3 was calculated from the molecular weight ratio between O_3 (48 g/mol) and Br_2 (160 g/mol).
(**) 48h-NOEC of 0.011 mg/L and the 24h-EC_{100} of 0.021 mg/L were reported. Although no EC_{50} could be derived from this study, the study was selected as key study, as the difference between the NOEC and the EC_{100} was by a factor of 2 and therefore, the NOEC of 0.011 mg/L was considered as surrogate for the EC_{50}.

A number of studies have been submitted on the acute toxicity of ozone to freshwater and marine fish. However, it should be noted that both exposure regimes, as well as the life stages of the exposed fish, differ from each other as well as from standard fish tests. Therefore, a comparison of species sensitivity was not possible. The lowest 96h LC_{50} value of 0.0093 mg/L for *Oncorhynchus mykiss* was determined under continuous ozone exposure following EPA Standard bioassay procedures (EPA 1975). The EC_0 was determined as 0.008 mg/L, thus indicating a very steep dose-response-curve for ozone. Fish mortality was apparently due to massive destruction of the gill lamellae epithelium together with a severe hydromineral imbalance. Water samples were analysed twice daily during the test by spectrophotometrically measuring the residual ozone. This procedure has at best a precision of ± 15% which is not specific for ozone but gives the total oxidants present. The study was considered as valid and reliable for classification of ozone by the DS. The other reported LC_{50} values from the available fish studies ranged from 0.03 to 1.22 mg/L.

A number of studies have been submitted on the acute toxicity of ozone in freshwater and marine invertebrates. A 48h NOEC value of 0.011 mg/L and a EC_{100} value of 0.021 mg/L for *Daphnia magna* was determined under continuous ozone exposure. Although no EC_{50} could be derived from this study, the study was selected as key study by the DS, as the difference between the NOEC and the EC_{100} was a factor of just 2. Therefore, the NOEC of 0.011 mg/L was considered by the DS as a surrogate for the EC_{50}. Again, a very steep dose-response-curve was found for ozone. This study was considered as valid and reliable for the classification of ozone by the DS. The other available studies with invertebrates reported effect values between 0.051 mg/L to > 1.69 mg/L.

No data on the aquatic acute toxicity to algae were available.

Overall, the DS proposed classification of ozone as Aquatic Acute 1 based on the 96h LC_{50} for *Oncorhynchus mykiss* of 0.0093 mg/L, based on measured concentrations. As this acute toxicity value falls within the $0.001 < L(E)C_{50} \leq 0.01$ mg/L range, the acute M-factor proposed by the DS was 100.

<u>Aquatic Chronic toxicity</u>

Test method / reliability	Test organism	Long-term result (endpoint)	Reference / Test item
Fish			
Not specified / No GLP / reliability 2	*Oncorhynchus mykiss*	**3 months-NOEC = 0.0023 mg/L (average measured con.)**	Wedemeyer *et al*., 1979
Aquatic invertebrates			
Not specified / No GLP / reliability 2	*Litopenaeus vannamei* (marine)	21d NOEC = 0.06 OPO/L (*) 21d NOEC = 0.04 mg/L (**)	Schroeder *et al*., 2010
Algae / other aquatic plants			
Not specified / No GLP / reliability 2	*Nannochloropsis oculate* (marine)	3d NOEC = 0.006 mg/L (***) 5d NOEC = 0.014 mg/L (***)	Kureshy *et al*., 1999
	Isochyris galbana (marine)	3d NOEC = 0.03 mg/L (***) 5d NOEC = 0.08 mg/L (***)	
	Chaetoceros gracilis (marine)	3d NOEC = 0.01 mg/L (***) 5d NOEC = 0.05 mg/L (***)	

(*) ozone-produced oxidants (OPO) measured as chlorine (Cl_2) equivalent
(**) expressed as equivalent conc. of O_3 using the factor 0.67 calculated by eCA. The factor 0.67 was calculated from the molecular weight ratio between O_3 (48 g/mol) and Cl_2 (70,906 g/mol).
(***) measured as TRO (Total Residual Oxidants including ozone, chloramines and bromamines measured spectrophotometrically with the indigo method) in the treatment with algae.

One study has been submitted on the chronic toxicity of ozone in fish, performed with the same test species that was also the most sensitive in the acute studies (*Oncorhynchus mykiss*). Juvenile rainbow trout (10–13 cm) were exposed to ozone in a flow-through system for an exposure period of 3 months. Two tests were performed, one with an ozone concentration of 0.002 mg/L (average measured concentration during the 3-months exposure period was 0.0023 mg/L), and the other with an ozone concentration of 0.005 mg/L (nominal and average measured concentration). One replicate was used in both tests. At both tested concentrations, no mortality occurred. In the 0.0023 mg/L exposure, no significant effects on haematology, blood chemistry or growth were found, except for a mild thrombocytosis in the test fish. In the test with 0.005 mg/L, significant effects on growth, together with hypoglycaemia, polycythaemia, and lymphocytopenia were observed. Thus, a NOEC of 0.0023 mg/L was derived from this study.

Growth of juvenile fish is a sensitive indicator of toxicity and is also a recommended endpoint in OECD TG 215 (Fish juvenile growth test). This test is recommended to cover the long-term toxicity for fish for substances with a log K_{ow} < 5. Although the study was not performed according to OECD TG 215 and the juvenile fish used were larger than recommended in OECD TG 215, the DS considered the test acceptable and reliable for long-term toxicity of fish as the exposure time was 3 times longer than the 28 days and it could therefore be regarded as partly compensating for the larger the test organisms.

One study has been submitted on the chronic toxicity of ozone in aquatic invertebrates. In a 21-day study, whiteleg shrimp (*Litopenaeus vannamei*) juveniles were exposed to three OPO (ozone-produced oxidants) concentrations (0.06, 0.1 and 0.15 mg/L) in a continuous

ozone flow. In the lowest concentration of 0.06 mg/L OPO (or 0.04 mg/L expressed as equivalent concentration of O_3), no mortality or behaviour effects occurred during the exposure period. However, an increase of cannibalistic behaviour in shrimp exposed to the 0.10 and 0.15 mg/L OPO (or 0.067 and 0.1 mg/L O_3) treatments was evident and mortality levels reached 47% and 43% after 21 days of exposure, respectively. After the 21-day exposure, 69% and 35% of the survivors showed clear indications of soft-shell syndrome at OPO concentrations of 0.10 and 0.15 mg/l (or 0.067 and 0.1 mg/L O_3). Therefore, a 21-day NOEC value of 0.04 mg/L expressed as O_3 was considered by the DS as the lowest reliable chronic toxicity value for aquatic invertebrates.

One study with three marine algae species performed in a static exposure system was available. The NOEC values were between 0.006 mg/L TRO (Total Residual Oxidants) for *Nannochloropsis oculate* after 3 days and 0.08 mg/L TRO for *Isochyris galbana* after 4 days. No EC_{50} could be determined from the study and the algae were exposed in only a static system with fast decreasing ozone concentrations. Thus, underestimation of ozone toxicity to algae is probable. However, based on the unlikely direct exposure of ozone to the environment, no further algae studies with continuous exposure, from which a relevant EC_{50} could be derived, have been provided. In addition, the DS considered that the mode of action of ozone does not indicate that algae would be a more sensitive trophic level than fish and/or invertebrates. Therefore, the NOEC value of 0.006 mg/L for *Nannochloropsis oculate* was considered by the DS as the key value for algae toxicity of ozone.

Overall, the DS proposed to classify ozone as Aquatic Chronic 1 based on the three-month NOEC for *Oncorhynchus mykiss* of 0.0023 mg/L, based on average measured concentrations. For rapidly degradable substances this chronic toxicity value falls within the $0.001 < \text{NOEC} \leq 0.01$ mg/L range, the chronic M-factor proposed by the DS was 1.

Comments received during consultation

One MSCA and one National Authority (NA) commented on the environmental part of DS's proposal. The MSCA agreed with the proposed classification and had only minor comments which did not have an impact on the proposed classification. Nevertheless, the MSCA agreed that chronic endpoints are available and support that any available acute endpoints on algae would not change the classification. Still, the MSCA noted that according to the CLP guidance, the classification may be altered in the future, if additional information becomes available.

The NA did not explicitly express support for the proposed classification and commented regarding to the applicability and relevance of M-factors as ozone is highly unstable and will not, in their opinion, be placed on the market as part of a mixture. In answer to the comment on the applicability of the aquatic M-factors, RAC would like to stress that in the legal text of CLP regulation, Article 10, it is clearly stated that "*M-factors for substances classified as hazardous to the aquatic environment, acute category 1 or chronic category 1, shall be established by manufacturers, importers and downstream users*". In addition, CLP regulation, Annex I, Table 4.1.0, indicates that "*When classifying substances as Acute Category 1 and/or Chronic Category 1 it is necessary at the same time to indicate then appropriate M-factor(s)"*.

Although M-factors are used to derive by the summation method the classification of a mixture in which the substance is present, there are not explicitly stated in the CLP

regulation or guidance that M-factors should or could not be established if the substance is not intended to use in the mixture.

Therefore, RAC is of opinion that setting of M-factors is a part of legal requirements for substances which are classified as aquatic acute/chronic in category 1 according to CLP regulation and do not depend on the intended use of substance.

Assessment and comparison with the classification criteria

Degradation

Ozone is an inorganic substance, therefore the concept of degradability as applied to organic compounds has limited or no meaning.

Biodegradability studies are not required for inorganic substances as they cannot be tested for biodegradability. Ozone quickly reacts and decomposes to oxygen and short-lived hydroxyl radicals and does not have any hydrolysable groups within its structure. Phototransformation in water is negligible for the environmental fate and behaviour since self-decomposition and decomposition in contact with organic matter are more relevant.

Ozone is unstable in water. Several factors such as pH, alkalinity, concentration of organic carbon, temperature, concentration of anions/cations, and hydrodynamic conditions could have relevant effects on the decay constant of ozone. Depending on the water quality, the half-life of ozone is in the range of seconds to hours. A study performed in five Swiss natural waters with different DOC and alkalinity at pH 8 and 15 ^{0}C indicate that ozone half-live in groundwater, spring water, and 3 lake waters were approximately 50, 19, 3, 3 and 5 minutes, respectively which results in an average DT_{50} value of 16 minutes.

Ozone is much more stable in air than in water, especially dry conditions, nevertheless there are many factors influencing the fate of ozone in the atmosphere, therefore it is hard to define a general half-life value for ground-level ozone in air. Nevertheless, the half-life of ozone in ambient air has been examined by the US EPA to be in the order of 12 hours.

Overall, RAC considers that ozone is highly reacting substance in contact with organic/inorganic matter and decomposes or self-decompose to oxygen and short-lived radicals. There are no hydrolysable groups within ozone structure and photo transformation is negligible. The study performed in five Swiss natural waters indicates that half-live results of ozone is an average DT_{50} value of 16 minutes. Half-life of ozone in ambient air is in the order of 12 hours. Consequently, RAC agrees with DS that ozone in scope of classification according to the CLP criteria should be considered as **rapidly degradable**.

Aquatic Bioaccumulation

The estimated log K_{ow} -0.87 is well below the CLP trigger value of ≥ 4. Although for inorganic substances log K_{ow} cannot be considered as a measure of the potential to accumulate (CLP guidance, Annex IV), it is an atmospheric and highly reactive gas which will react very rapidly with organic matter. Thus, RAC considers that the potential for bioaccumulation is unlikely. Consequently, RAC agrees with the DS that ozone is not bioaccumulative according to the CLP criteria.

Aquatic Toxicity

RAC notes that there are no studies available according to internationally accepted guidelines, with the DS providing numerous studies from peer-reviewed literature. RAC agrees with the reliability ratings of the studies given by the DS.

Regarding aquatic acute toxicity to invertebrates (*Daphnia magna*), RAC agrees that, in this specific case, the NOEC value can be considered as a surrogate EC_{50} as the difference between the NOEC and the EC_{100} was just a factor of 2. However, RAC still notes that the actual numerical acute toxicity value for invertebrates (*Daphnia magna*) could not be derived from this study.

Additionally, RAC notes that although there are studies with the algae, no relevant EC_{50} values for algae could be derived. Overall, although most sensitive trophic group for aquatic acute toxicity seems to be fish (*Oncorhynchus mykiss*), RAC is of opinion that the classification might need to be revised in the future in light of any future additional relevant data becoming available due to the current absence of aquatic acute data for algae and, partially, for invertebrates.

Regarding aquatic chronic toxicity, RAC acknowledges that valid and reliable data is available for all trophic levels. RAC notes that the aquatic chronic toxicity study with fish (*Oncorhynchus mykiss*) was not performed according to OECD TG 215 and the size of the juvenile fish used was greater than recommended in OECD TG 215. However, RAC agrees with the DS that the test is acceptable and reliable for long-term toxicity test for fish, because the exposure time was 3 times longer than the 28 days and is therefore regarded to partly compensate for the larger test organisms. Therefore, RAC is of opinion that the most sensitive trophic group for aquatic chronic toxicity is fish (*Oncorhynchus mykiss*). In addition, in the same order of magnitude were results from aquatic chronic toxicity study with algae (*Nannochloropsis oculate*).

Consequently, RAC agrees that the lowest acute endpoint for aquatic acute classification is the 96h LC_{50} for *Oncorhynchus mykiss* of **0.0093 mg/L**, based on measured concentrations. The lowest chronic endpoint for aquatic chronic classification is the three-months NOEC value for *Oncorhynchus mykiss* of **0.0023 mg/L**, based on average measured concentrations.

Conclusion on classification

Ozone is considered as rapidly degradable and does not fulfil the CLP criteria for bioaccumulation. Based on the available and reliable information, RAC agrees with the DS that ozone warrants classification as:

Aquatic Acute 1 based on LC_{50} = 0.0093 mg/L for *Oncorhynchus mykiss.* As this acute toxicity value falls within the $0.001 < L(E)C_{50} \leq 0.01$ mg/L range, the **acute M-factor is 100**.

Aquatic Chronic 1 based on NOEC = 0.0023 mg/L for *Oncorhynchus mykiss*. As this chronic toxicity value falls within the $0.001 < NOEC \leq 0.01$ mg/L range for rapidly degradable substances, the **chronic M-factor is 1.**

12 EVALUATION OF ADDITIONAL HAZARDS

12.1 Hazardous to the ozone layer

12.1.1 Short summary and overall relevance of the provided information on ozone layer hazard

No studies or information have been provided on ozone layer hazard of the active substance. Waiving this data has been considered acceptable as the active substance is ozone and thus poses no hazard to the ozone layer.

12.1.2 Comparison with the CLP criteria

As the active substance in question is ozone, no hazard to the ozone layer as defined by the CLP criteria is to be expected.

12.1.3 Conclusion on classification and labelling for hazardous to the ozone layer

Not applicable.

13 ADDITIONAL LABELLING

14 REFERENCES

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15 ANNEXES

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