EUROPEAN CHEMICALS AGENCY

Committee for Risk Assessment
RAC

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

Adopted
16 March 2023

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EUROPEAN CHEMICALS AGENCY

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CLH-O-0000007279-64-01/F

OPINION OF THE COMMITTEE FOR RISK ASSESSMENT ON A DOSSIER PROPOSING HARMONISED CLASSIFICATION AND LABELLING AT EU LEVEL

In accordance with Article 37 (4) of Regulation (EC) No 1272/2008, the Classification, Labelling and Packaging (CLP) Regulation, the Committee for Risk Assessment (RAC) has adopted an opinion on the proposal for harmonised classification and labelling (CLH) of:

Chemical name: **ozone**

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

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

The proposal was submitted by **Germany** and received by RAC on **28 February 2022.**

In this opinion, all classification and labelling elements are given in accordance with the CLP Regulation.

PROCESS FOR ADOPTION OF THE OPINION

Germany has submitted a CLH dossier containing a proposal together with the justification and background information documented in a CLH report. The CLH report was made publicly available in accordance with the requirements of the CLP Regulation at ***http://echa.europa.eu/harmonised-classification-and-labelling-consultation/*** on **21 March 2022**. Concerned parties and Member State Competent Authorities (MSCA) were invited to submit comments and contributions by **20 May 2022**.

ADOPTION OF THE OPINION OF RAC

Rapporteur, appointed by RAC: **Peter Hammer Sørensen**

Co-Rapporteur, appointed by RAC: **Žilvinas Užomeckas**

The opinion takes into account the comments provided by MSCAs and concerned parties in accordance with Article 37(4) of the CLP Regulation and the comments received are compiled in Annex 2.

The RAC opinion on the proposed harmonised classification and labelling was adopted on **16 March 2023** by **consensus.**

Classification and labelling in accordance with the CLP Regulation (Regulation (EC) 1272/2008)

	Index No	Chemical name	EC No	CAS No	Classification		Labelling			Specific Conc. Limits, M-factors and ATE	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 Muta. 2 Carc. 2 Acute Tox. 1 STOT SE 1 STOT SE 3 STOT RE 1 Aquatic Acute 1 Aquatic Chronic 1	H270 H341 H351 H330 H370 (nervous system) H335 H372 (cardiovascular system, nervous system, respiratory system) H400 H410	GHS03 GHS06 GHS08 GHS09 Dgr	H270 H341 H351 H330 H370 (nervous system) H335 H372 (cardiovascular system, nervous system, respiratory system) H410		inhalation: ATE = 10 ppm (gases) M = 100 M = 1	
RAC opinion	TBD	ozone	233-069-2	10028-15-6	Ox. Gas 1 Carc. 2 Muta. 2 Acute Tox. 1 STOT SE 1 STOT RE 1 Aquatic Acute 1 Aquatic Chronic 1	H270 H351 H341 H330 H370 (nervous system, respiratory system, cardiovascular system) H372 (nervous system, respiratory system) H400 H410	GHS03 GHS08 GHS06 GHS09 Dgr	H270 H351 H341 H330 H370 (nervous system, respiratory system, cardiovascular system) H372 (nervous system, respiratory system) H410		inhalation: ATE = 10 ppmV (gases) STOT SE 1; H370: C ≥ 0,002 % STOT SE 2; H371: 0,0005 % ≤ C < 0,002 % STOT RE 1; H372: C ≥ 0,05 % STOT RE 2; H373: 0,01 % ≤ C < 0,05 % M = 100 M = 1	
Resulting Annex VI entry if agreed by COM	TBD	ozone	233-069-2	10028-15-6	Ox. Gas 1 Carc. 2 Muta. 2 Acute Tox. 1 STOT SE 1 STOT RE 1 Aquatic Acute 1 Aquatic Chronic 1	H270 H351 H341 H330 H370 (nervous system, respiratory system, cardiovascular	GHS03 GHS08 GHS06 GHS09 Dgr	H270 H351 H341 H330 H370 (nervous system, respiratory system, cardiovascular		inhalation: ATE = 10 ppmV (gases) STOT SE 1; H370: C ≥ 0,002 %	

						system) H372 (nervous system, respiratory system) H400 H410		system) H372 (nervous system, respiratory system) H410		STOT SE 2; H371: 0,0005 % ≤ C < 0,002 % STOT RE 1; H372: C ≥ 0,05 % STOT RE 2; H373: 0,01 % ≤ C < 0,05 % M = 100 M = 1	

GROUNDS FOR ADOPTION OF THE OPINION

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.

RAC evaluation of physical hazards

Summary of the Dossier Submitter's proposal

Ozone is identified as an oxidising gas in ISO 10156 which document also provides the calculation method to identify an oxidising gas (see CLP Regulation 2.4.4). 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 ISO 10156 calculation method, 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.

HUMAN HEALTH HAZARD EVALUATION

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. 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.

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

Method, Guideline, GLP status, Reliability Ref.	Test substance, Species	Results
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.**

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.

Type of data/ report, Reliability, Ref.	Test substance	Relevant information about the study	Observations
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.

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, Guideline, GLP status, Reliability, Reference	Species, Strain, Sex, No/group Dosis	Results
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**1: 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.

Reference / study characteristics	ozone exposure			Results
	Conc. µg/m3	Conc. ppb	Duration hours	
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.**

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±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 doses (0, 0.1, 0.3, 0.5 ppm) with exposure free days in-between	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), stat. sign. increased breathing frequency which recovered towards end of exposure (only in young rats)

Method, Guideline, GLP status, Reliability Ref.	Species, Strain, Sex, No/group	Test Concentration	Results
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.

<u>Setting of specific concentration limit (SCL) for STOT SE (cardiovascular system)</u>

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 4 h	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	Ozone generated from oxygen 0 and 1 ppm	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

Method, Guideline, GLP status, Reliability Ref.	Species, Strain, Sex, No/group	Test substance,	Results
	Experiment 2: n = 6 per dose group Experiment 3: n = 6 in ozone group, n = 5 in control Experiment 4: not reported	4 h exposure, closed chamber	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 2 500 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 ± 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/ h 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%.

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 (6 h, 1 d/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.

<u>Conclusion for STOT RE (CNS)</u>

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.

<u>Setting of specific concentration limits for STOT RE (nervous system)</u>

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**.

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 mentioned 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.

<u>Summary of the submitted human studies</u>

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.

<u>Other reviews of genotoxicity data</u>

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.

<u>Final conclusion for mutagenicity</u>

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**.

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 treatment.	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 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, Guideline, GLP status, Reliability	**Species, Strain, Sex, No/ group NOAEC/ LOAEC**	**Ozone, Dose levels, Duration of exposure**	**Results**
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 <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
Hasset *et al.*, 1985 Method: 6-month inhalation study for ozone Guideline: no GLP: no Rel. 2	Mice, A/J, female No/Group: 40 Neoplastic LOAEC: 0.5 ppm (derived from Exp.2) Non-neoplastic NOAEC/LOAEC not available under the conditions of the study.	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	Neoplastic findings <u>Exp. 1 (0.31 ppm, age of animals at sacrifice: ~ 1 year):</u> - <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><th>Method, Guideline, GLP status, Reliability</th><th>Species, Strain, Sex, No/ group NOAEC/ LOAEC</th><th>Ozone, Dose levels, Duration of exposure</th><th>Results</th></tr>
<tr><td></td><td></td><td>exposure
Exp. 2:
102 h/first week of each month for 6 months; sacrifice 3 months after final ozone dose (whole body)</td><td>
<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, $p < 0.005$)

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

- Control:
No. of tumour-bearing animals: 8/45
% Mice with tumours: 18
Total no. of lung tumours: 9
Average no. of tumours/mouse: 0.20
- Ozone:
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)
- Focal bronchiolar alveolar hyperplasia (10% males treated with ozone)
- Bronchiolar epithelium hyperplasia (10% males and 10%</td></tr>
</table>

Method, Guideline, GLP status, Reliability	Species, Strain, Sex, No/ group NOAEC/ LOAEC	Ozone, Dose levels, Duration of exposure	Results
			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: 0.12 ppm: - Nose: inflammation (only males), lateral wall hyaline degeneration (only females) Additional 0.5 ppm and 1.0 ppm: - 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 Additional 1.0 ppm: - 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: NOAEC: < 0.5 ppm LOAEC: 0.5 ppm	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,

Method, Guideline, GLP status, Reliability	Species, Strain, Sex, No/ group NOAEC/ LOAEC	Ozone, Dose levels, Duration of exposure	Results
inhalation study Guideline: no GLP: in compliance with Food and Drug Administration (FDA) Good Laboratory Practice Regulations Reliability: 1			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)
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: 0.12 ppm: - 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) 0.5 ppm and 1.0 ppm: - 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**.

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 After 42 days of exposure males stayed 8 days with unexposed females for mating	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 (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 ossifications)	Long-Evans female pregnant rats Group size: Maternal: n = 14-37 Offspring: Animals: n = 38-102 Litters: n = 8-18	Ozone gestation days 6-9: continuous exposure to 0 or 1.04 ppm 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	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 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 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 Resorption: 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). Visceral anomalies: enlarged renal pelvis in 5.8% of foetuses in treated early-term group (none in control) and

Method, Guideline, GLP status, Reliability	Species, Strain, Sex, No/ group	Ds	Results
		exposure to 0 or 0.44 ppm	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 Skeletal ossification and malformations: 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: 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)
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	Treatment groups: -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	Ozone 0 or 1.0 ppm during 12 h darkness phase for first 20 days of gestation	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

Method, Guideline, GLP status, Reliability	Species, Strain, Sex, No/ group	Ds	Results
	and non-exposed mothers 8 per group	Exposure chamber	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 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
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	I. GD 9-12 (mid gestation exposure) Continuously 1.0, 1.5 ppm II. GD 17-20	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%;

Method, Guideline, GLP status, Reliability	**Species, Strain, Sex, No/ group**	**Ds**	**Results**
	II. 1.0 ppm: 6 II. 1.5 ppm: 6 Litters reduced to 8 pups	(late gestation) Continuously 1.0, 1.5 ppm	male weight reduction: 1.0: 8%, 1.5:12% <u>PND60</u> 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. <u>Behavioural testing:</u> 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)) <u>Open field tests:</u> 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 <u>grooming (II.):</u> 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 <u>rearing (II.):</u> 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 <u>activity</u> in open field unaffected. Maternal: Not analysed Offspring: <u>NOAEL:</u> not determinable because LOAEL set at lowest dose <u>LOAEL:</u> 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 <u>male and female</u> No/group each 10/ group Litters reduced to 7 and culled to 8 pups (4 male and 4 female)	0.3, 0.6, 0.9 ppm Exposure: 6 days before formation of breeding pairs until PND26 (males and females exposed)	Retardation of postnatal body weight gain PND2-40, PND100 significant reduction of body weight gain for 0.9 ppm group (specific values not shown) <u>Paw preference test PND70:</u> 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 <u>Hot plate response test PND100 (injection of morphine or a saline):</u> Reduced drug sensitivity (morphine) after 0.9 ppm ozone exposure: Shorter latency + higher frequency in hind limp withdrawal

Method, Guideline, GLP status, Reliability	Species, Strain, Sex, No/ group	Ds	Results
	On PND21 reduction to 3 pups/litter	Continuous	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

Method, Guideline, GLP status, Reliability	**Species, Strain, Sex, No/ group**	**Ds**	**Results**
			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 GLP: No Reliability: 2 Sharkhuu *et al.,* 2011	Species mice Strain Balb/c No/group 20 pregnants/group exposed (Experiment was performed 3 times) Analysis of offspring: BALF: 3-6/sex LDH/prot.: 3-7/sex	Ozone 0.4, 0.8, 1.2 ppm ozone for 4h/d at GD 9-18	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: 25% less productive dams compared to control) No effect on litter size and sex ratio 1.2 ppm: reduced weight gain in offspring PND1: 13%; PND3: 22%; PND7: 15% and in male still at PND42: 9% lower weight Inflammation: 1.2 ppm: increased LDH activity in BALF at PND42 in female offspring and same trend for protein →Lung injury, normal lung development altered

Method, Guideline, GLP status, Reliability	**Species, Strain, Sex, No/ group**	**Ds**	**Results**
	DTH: 6-8/sex Sensitised offspring: BALF: 3-6/sex		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 OVA-sensitised female offspring 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: NOAEL: 0.8 ppm (pregnancies) LOAEL: 1.2 ppm (pregnancies) Offspring: NOAEL: 0.8 ppm (bw) LOAEL: 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 pregnancies 0 and 0.3: 9/10; 0.6: 6/10 (not significant) No effect on body weight of pups (no data given) Somatic and neurobehavioural development PND2-20: →No concentration dependent effects, only effects in 0.3 ppm group Maternal: NOAEL: 0.3 ppm (pregnancies) LOAEL: 0.6 ppm (pregnancies) Offspring: NOAEL: 0.6 ppm LOAEL: ≥ 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

Method, Guideline, GLP status, Reliability	Species, Strain, Sex, No/ group	Ds	Results
			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

Effects on dams

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.

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

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.

<u>Other effects on offspring</u>

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**.

ENVIRONMENTAL HAZARD EVALUATION

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
Not specified / No GLP / reliability 2	*Americamysis bahia* (marine)	3 h LC_{50} = 0.051 mg/L(*)	Jones *et al.*, 2006
	Leptocheirus plumulosus (marine)	3 h LC_{50} >1.69 mg/L(*) 4 h LC_{50} = 0.28 mg/L(*)	
	Americamysis bahia	48 h LC_{50} = 0.1–0.138 mg/L (*)	

Test method / Reliability	Test organism	Short-term result (endpoint)	Reference
Fish			
	(marine)		
Not specified / No GLP / reliability 2	*Litopenaeus 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.

Aquatic Chronic toxicity

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 $0.001 < NOEC \leq 0.01$ 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.**

ANNEXES:

Annex 1 The Background Document (BD) gives the detailed scientific grounds for the opinion. The BD is based on the CLH report prepared by the Dossier Submitter; the evaluation performed by RAC is contained in 'RAC boxes'.

Annex 2 Comments received on the CLH report, response to comments provided by the Dossier Submitter and RAC (excluding confidential information).