Committee for Risk Assessment

RAC

Annex 1

Background document

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

Nitrobenzene

ECHA/RAC/CLH-O-0000002350-87-01/A1
EC number: 202-716-0
CAS number: 98-95-3

Adopted

3 February 2012

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TABLES

PROPOSAL FOR HARMONISED CLASSIFICATION AND LABELLING

Substance Name:	Nitrobenzene
EC Number:	202-716-0
CAS number:	98-95-3
Registration number (s):	
Purity:	> 99.3 %
Impurities:	< 0.3 % benzene < 0.1 % dinitrobenzene < 0.1 % dinitrophenol < 0.5 % water < 0.1 % picric acid

Classification proposed by the Dossier Submitter
Proposed classification based on Directive 67/548/EEC criteria, impurities < 0.1% each

Carcinogen Category 3, R40 limited evidence for carcinogenesis
T toxic, R23/24/25 toxic by inhalation, in contact with skin and if swallowed
R48/23/24/25 toxic: danger of serious damage to health by prolonged exposure through inhalation, in contact with skin and if swallowed
Reproductive toxicant Category 3, R62 possible risk of impaired fertility
R64 may cause harm to breast-fed babies
R52-53 Harmful to aquatic organisms, may cause long-term adverse effects in the aquatic environment.

Proposed classification based on Regulation (EC) No 1272/2008 criteria: impurities < 0.1% each

H351 Suspected human carcinogen, Carc. Cat. 2
H361f Suspected human reproductive toxicant, Repr. Cat. 2
H362 May cause harm to breast-fed children, Reproductive toxicant
H301/311/331 Acute toxicity (oral, dermal, inhalation)
H372 STOT Rep. 1, causes damage to organs through prolonged or repeated oral, dermal or inhalation exposure.
H412 Harmful to aquatic life with long lasting effects, Aquatic Chronic Cat. 3

Proposed classification based on Directive 67/548/EEC criteria, containing ≥ 0.1% and < 0.3% of benzene as an impurity

Carcinogen Category 1, R45 may cause cancer
Mutagen Category 2, R46 may cause heritable genetic damage
T toxic, R23/24/25 toxic by inhalation, in contact with skin and if swallowed
R48/23/24/25 toxic: danger of serious damage to health by prolonged exposure through inhalation, in contact with skin and if swallowed
Reproductive toxicant Category 3, R62 possible risk of impaired fertility
R64 may cause harm to breast-fed babies
R52-53 Harmful to aquatic organisms, may cause long-term adverse effects in the aquatic environment.

Proposed classification based on Regulation (EC) No 1272/2008 criteria: containing ≥ 0.1% and < 0.3% of benzene as an impurity

H350 known human carcinogen, carcinogen category 1A
H340 known human mutagen, mutagen category 1B
H361f suspected human reproductive toxicant, repr. cat. 2
H362 may cause harm to breast-fed children, reproductive toxicant
H301/311/331 acute toxicity (oral, dermal, inhalation)
H372 STOT Rep. 1, causes damage to organs through prolonged or repeated oral, dermal or inhalation exposure.
H412 Harmful to aquatic life with long lasting effects, Aquatic Chronic Cat. 3

It is proposed to change the current classification to the above mentioned. The risk assessment committee is asked to review and confirm this.

Proposed labelling:

Table 1: Entry of nitrobenzene in Table 3.2 of Annex VI of Regulation (EC) No 1272/2008, extended by the proposed classifications R48/25 and R64 and classifications due to impurities as well as the reclassification of R51/53 to R52/53.

Index No	International Chemical Identification	EC No	CAS No	Classification	Labelling
609-003-00-7	nitrobenzene (containing <0.1% of each impurities except water)	202-716-0	98-95-3	Carc.Cat.3,R40 Repr.Cat.3,R62,R64 T; R23/24/25-48/23/24/25 R52-53	T R: 23/24/25-40-48/23/24/25-52/53-62-64 S: (1/2-)28-36/37-45-61
609-003-00-7	nitrobenzene (containing ≥0.1% and < 0.3% of benzene as an impurity)	202-716-0	98-95-3	Carc.Cat.1,R45 Muta.Cat.2,R46 Repr.Cat.3,R62,R64 T; R23/24/25-48/23/24/25 R52-53	T R: 23/24/25-45-46-48/23/24/25-52/53-62-64 S: (1/2-)28-36/37-53-45-61

Table 2: Entry of nitrobenzene in Table 3.1 of Annex VI of Regulation (EC) No 1272/2008 (CLP), extended by the proposed classification H362 and classifications due to impurities as well as the reclassification of H411 to H412.

Index No	International Chemical Identification	EC No	CAS No	Classification (1272/2008)		Labelling	
				Hazard Class and Category Code(s)	Hazard statement Code(s)	Pictogram, Signal Word Code(s)	Hazard statement Code(s)
609-003-00-7	nitrobenzene (containing <0.1% of impurities each)	202-716-0	98-95-3	Carc. 2 Repr. 2 +lactation Acute Tox. 3* Acute Tox. 3* Acute Tox. 3* STOT RE 1 Aquatic Chronic 3	H351 H361f† H362 H331 H311 H301 H372‡ H412	GHS06 GHS08 Dgr	H351 H361f† H362 H331 H311 H301 H372‡ H412
609-003-00-7	nitrobenzene (containing ≥0.1% and < 0.3% of benzene as an impurity)	202-716-0	98-95-3	Carc. 1A Muta. 1B Repr. 2 +lactation Acute Tox. 3* Acute Tox. 3* Acute Tox. 3* STOT RE 1 Aquatic Chronic 3	H350 H340 H361f† H362 H331 H311 H301 H372‡ H412	GHS06 GHS08 Dgr	H350 H340 H361f† H362 H331 H311 H301 H372‡ H412

Proposed specific concentration limits (if any):

Proposed notes (if any):

Benzene can be an impurity of up to 0.3%. Therefore, the classification is given twice: once for nitrobenzene with impurities of less than 0.1% (except water); and once for Nitrobenzene with impurities of benzene of up to 0.3%.

This dossier reviewed the carcinogenicity, mutagenicity and reproductive toxicity endpoints, as well as acute and repeated-dose toxicity. Corrosivity and irritation data show no effects, and respiratory sensitisation data are insufficient for a strict classification.

The classification of N R51/53 was entered in 22nd ATP of Directive 67/548/EEC. The data presented in the Risk Assessment Report (RAR 2007) do not support the current classification as N R51/53. According to this data the current classification should be changed from N R51/53 (H411) to R52/53 (H412).

* Minimum classification according to Reg. (EC) No 1272/2008, 1.2.1 (p. 338)

† Hazard statement for reproductive toxicity acc. to Reg. (EC) No 1272/2008, 1.2.3 (p. 338)

‡ Route of exposure cannot be excluded acc. to Reg. (EC) No 1272/2008, 1.2.2 (p. 338)

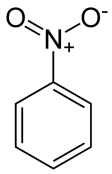
JUSTIFICATION

1 IDENTITY OF THE SUBSTANCE AND PHYSICAL AND CHEMICAL PROPERTIES

1.1 Name and other identifiers of the substance

Chemical Name:	Nitrobenzene
EC Name:	Nitrobenzene
CAS-Name:	Benzene, nitro-
CAS Number:	98-95-3
IUPAC Name:	Nitrobenzene

1.2 Composition of the substance

Chemical Name:	Nitrobenzene
EC Number:	202-716-0
CAS Number:	98-95-3
IUPAC Name:	Nitrobenzene
Molecular Formula:	$C_6H_5NO_2$
Structural Formula:	
Molecular Weight:	123 g/mol
Typical concentration (% w/w):	99.7
Concentration range (% w/w):	99 - 100

1.3 Physico-chemical properties

Table 3: Summary of physico- chemical properties

REACH ref Annex, §	Property	IUCLID section	Value	[enter comment/reference or delete column]
VII, 7.1	Physical state at 20°C and 101.3 KPa	3.1	Liquid of **pale yellow** to **yellow**-brown coloration	
VII, 7.2	Melting/freezing point	3.2	5.26 °C	BASF AG (1986)
VII, 7.3	Boiling point	3.3	210.8 °C	Lide (1991)
VII, 7.4	Relative density	3.4 density	1.2037	Lide (1991)
VII, 7.5	Vapour pressure	3.6	20 Pa at 20 °C[1)] 32.6 Pa at 25 °C[1)] 20 Pa (0.15 mmHg) at 20 °C 38 Pa (0.284 mmHg) at 25 °C 47 Pa (0.35 mmHg) at 30 °C	Auer (1988) Daubert and Danner, 1989 WHO Report, 2003, Nitrobenzene. (Environmental health criteria No. 230) by L. Davies
VII, 7.6	Surface tension	3.10	43.9 mN/m at 20 °C (pure substance)	Lide (1991)
VII, 7.7	Water solubility	3.8	1900 mg/l at 20 °C	Bayer AG (1998)
VII, 7.8	Partition coefficient n-octanol/water (log value)	3.7 partition coefficient	1.86 at 24.5 °C	BASF AG (1987)
VII, 7.9	Flash point	3.11	88 °C	BAM (1997)
VII, 7.10	Flammability	3.13	Not extremely flammable Not highly flammable Not flammable	BAM (1997)
VII, 7.11	Explosive properties	3.14	No explosive properties	BAM (1997)
VII, 7.13	Oxidising properties	3.15	From structural reasons and based on theoretical considerations as well as practical experiences has no oxidising properties according to ECC standard method A.21	BAM (2009)
	Auto flammability	3.12	480 °C (DIN 51794)	BAM (1997)

1) The vapour pressure of 0.2 hPa at 20 °C was confirmed by entries in safety data sheets of various companies. US EPA confirmed also this value (http://www.who.int/pcs/ehc/full-text/ehc230/part_I.pdf). Daubert and Danner (1989) present an experimental vapour pressure as 0.245 mm Hg equivalent to 32.6 Pa at 25 C.

2) EPA

2 MANUFACTURE AND USES

2.1 Manufacture

There is no natural source of nitrobenzene known. However, nitrobenzene may be formed by OH-initiated photooxidation of benzene which could theoretically be of natural origin. This possible source is not considered to be significant. Nitrobenzene is almost exclusively produced by nitration of benzene. Nitrobenzene is mainly used as an intermediate in the manufacture of aniline (RAR 2007).

According to available data there are 13 production and/or processing sites of nitrobenzene within the EU. The data are provided via the European Chemicals Bureau website ESIS (http://ecb.jrc.ec.europa.eu). The resultant quantity of nitrobenzene produced in the EU amounts to be 1'175'000 t/year (2000).

2.2 Identified uses

Almost all nitrobenzene is primarily used for the production of aniline and, to a much lesser extent, for the production of pharmaceuticals and various other chemicals (RAR 2007).

Type of use	Tonnage [t/a]	Appr. % in this application
Processing to aniline	1'162'900	99
Processing to pharmaceuticals	9'300	0.8
Processing to other chemicals	2'800	0.2
Total	1'175'000	100

There is a difference of about 5,000 t/a between production and processing which amounts only to around 0.42 % of the total production volume. It could not be clarified whether this amount is further used at all and if so for which application it might be used. There is no evidence that this missing tonnage in the mass balance is actually further processed and it is hence considered to be due to inaccuracies in estimates rather than due to a missing tonnage. It is not known that any quantities of nitrobenzene are imported from outside the EU.

In Germany nitrobenzene was used for perfuming soaps in the past as the so called *Oil of Mirbane*. However, the use of nitrobenzene in cosmetic products has been forbidden in Germany since the 1980s. (Cosmetic Regulation from 19th June 1985). No information is available whether nitrobenzene is or was used in soaps in EU countries other than Germany and whether this possible use may have become discontinued.

The content of nitrobenzene in different products is listed in the Danish Product Register. In 2003 nitrobenzene was present in 23 adhesive or binding products and reprographic agents in a range of 0-2 % with an approximate quantity of less than 1 tonne per year. These products might be used by professionals or consumers.

The dossier submitter has no information on any of these uses in Europe at present. It can be assumed that they are of historical relevance only and can be neglected in the future. This assumption is supported by the SPIN database where in the year 2001 nitrobenzene was only

present in 41 products in Denmark (reprographic agents) but with an amount of 0 tonnes per year. In Sweden, Norway or Finland no nitrobenzene containing products were listed (RAR 2007).

3 CLASSIFICATION AND LABELLING

3.1 Classification in Annex I of Directive 67/548/EEC

Nitrobenzene is covered by the following entries in Annex VI of Regulation (EC) No 1272/2008 (CLP).

Table 4: Entry of nitrobenzene in Table 3.2 of Annex VI of Regulation (EC) No 1272/2008 (CLP).

Index No	International Chemical Identification	EC No	CAS No	Classification	Labelling
609-003-00-7	nitrobenzene	202-716-0	98-95-3	Carc. Cat. 3; R40 Repr. Cat. 2; R60 T; R23/24/25 T; R48/23/24/25 N; R52/53	T; N R: 23/24/25-48/23/24/25-40-60-52/53 S: 2-36/37-45-46-53

Table 5: Entry of nitrobenzene in Table 3.1 of Annex VI of Regulation (EC) No 1272/2008 (CLP) as amended by the 22nd ATP of Directive (EC) No. 67/548.

Index No	International Chemical Identification	EC No	CAS No	Classification		Labelling	
				Hazard Class and Category Code(s)	Hazard statement Code(s)	Pictogram, Signal Word Code(s)	Hazard statement Code(s)
609-003-00-7	nitrobenzene	202-716-0	98-95-3	Carc. 2 Repr. 1B Acute Tox. 3 Acute Tox. 3 Acute Tox. 3 STOT RE 1 (blood) Aquatic Chronic Cat. 3	H351 H360F H331 H311 H301 H372§ H412	GHS06 GHS08 Dgr	H351 H360F H331 H311 H301 H372§ H412

§ Route of exposure cannot be excluded acc. to Reg. (EC) No 1272/2008, 1.2.2 (p. 338)

4 ENVIRONMENTAL FATE PROPERTIES

4.1 Degradation

4.1.1 Stability

No investigations are available with regard to the hydrolytic degradation behaviour of nitrobenzene. However, the substance category of the aromatic nitro compounds is generally resistant to hydrolysis (Harris JC, 1990), so that nitrobenzene is not expected to hydrolyse under environmental conditions.

4.1.2 Biodegradation

4.1.2.1 Biodegradation estimation

4.1.2.2 Screening tests

Table 6 summarised the results of screening tests of ready biodegradability for nitrobenzene.

Table 6: Tests of ready biodegradability

Test	Degradation	Conditions	Result	Reference
OECD 301C	3.3 % BOD	initial concentration: 100mg/l incubation of 14 d	not readily biodegradable	(CITI 1992)
OECD 301E	100 % DOC 88 % DOC	initial concentration: 38.5 mg/l after incubation of 21d abiotic control	Evaporation of nitrobenzene – test system is not appropriate	(BASF AG 1989a)
similar to OECD 301F	48 % BOD 0-16 % BOD	initial concentration: 60 mg/l (after incubation of 32 d and a lag phase of 25 d 100 or 120 mg/l	not readily biodegradable	(BASF AG 1989b)
Warburg respirometry test system	33 % BOD 30 % BOD 0 % BOD	initial concentration: 100 mg/l (after incubation of 14 d and a lag phase of 90 h) 300 mg/l (after incubation of 10 d) 1400mg/l	microorganisms are inhibited when c > 1000 mg/l	(Gomólka and Gomólka 1979)
Electrolytic respirometer system similar to MITI	10 % BOD	initial concentration: 100 mg/l incubation of 10 d	not readily biodegradable (but incubation <28 d)	(Urano and Kato 1986)

In a MITI I test (OECD 301C) (CITI, 1992) nitrobenzene at a concentration of 100 mg/l was tested with an inoculum (30 mg/l) containing activated sludge from a municipal sewage plant and 10 samples from 10 different sites in Japan. A degradation of 3.3 % related to Biochemical Oxygen Demand (BOD) after an incubation of 14 days has been measured.

In another standard test the biodegradability of nitrobenzene was studied according to the modified OECD screening test (OECD 301E) (BASF AG, 1989a). At a nitrobenzene concentration of 38.5 mg/l an elimination of 100 % related to Dissolved Organic Carbon (DOC) after 21 days was measured, but in the abiotic control an elimination of 88 % has also been determined. Hence, the elimination is not only based on biological processes.

In a manometric respirometry test (similar to OECD 301F) (BASF AG, 1989b) two test concentrations were tested, 60 and 120 mg/l. Concerning the test concentration of 120 mg/l there is conflicting information. The text of the test description stated that the concentration is 120 mg/l whereas the marking of the diagram says 100 mg/l. At a concentration of 60 mg/l a biodegradation rate of 48 % related to BOD after an incubation of 32 days has been determined. The lag phase was 25 days. At the higher test concentration 5 parallel assays were run and the biodegradation rate varied between 0 and 16 % related to BOD. Only few experimental details are given in the report. Due to the long lag phase it can be concluded that adaptation has taken place. In fact this study cannot be considered valid but it confirms the prediction from the other studies that nitrobenzene is not readily biodegradable.

In a study on ready biodegradability (Gomólka and Gomólka, 1979) using a Warburg respirometry test system, it was shown that at initial concentrations up to 300 mg/l, nitrobenzene was degraded slowly. 33 % related to BOD were degraded by day 14 at an initial concentration of 100 mg/l test substance with biodegradation starting after 90 hours lag time. At an initial dosage of 300 mg/l 30 % were degraded after 10 days. At this concentration nitrobenzene slowly dissolves in water so nitrobenzene concentration increases during the first 80 hours. After that the concentration declines. At an initial concentration of 1400 mg/l the nitrobenzene concentration increased at first due to slow solution in water. No decrease and no elimination of nitrobenzene were reported. The authors state that at concentrations above 1000 mg/l micro-organisms are inhibited.

Nitrobenzene at a concentration of 100 mg/l was only degraded to 10 % related to BOD after 10 days of incubation with domestic activated sludge in an electrolytic respirometer system similar to the MITI procedures (Urano and Kato, 1986). BOD, DOC and biomass were monitored, whereas no substance-specific analytic procedure was performed.

4.1.2.3 Simulation tests

Not relevant for this type of dossier.

4.1.3 Summary and discussion of persistence

It can be stated that nitrobenzene is not biodegradable with unadapted inoculum. In the screening tests of ready biodegradability nitrobenzene did not achieve the pass level (70% DOC (Dissolved Organic Carbon) or 60% ThOD (theoretical oxygen demand) , or 60% ThCO2 (theoretical carbon dioxide).

4.2 Environmental distribution

Not relevant for this type of dossier.

4.3 Bioaccumulation

4.3.1 Aquatic bioaccumulation

4.3.1.1 Bioaccumulation estimation

4.3.1.2 Measured bioaccumulation data

The following table gives an overview of different bioaccumulation studies.

Table 7: BCF of Nitobenzene based on different bioaccumulation studies

Species	Bioconcentration Factor **(BCF)**	**Reference**
Cyprinus carpio	1.7 – 7.7	(CITI 1992)
Poecilia reticulata	22.4-38.9 (related to fat weight)	(Deneer et al 1987)
Leuciscus idus melanotus	< 10 (related to wet weight)	(Freitag et al. 1982)
Chlorella fusca	24	(Freitag et al. 1982)
Chlorella fusca var. vacuolata	24	(Geyer et al. 1984)

In the MITI-list (CITI 1992) the bioaccumulation of nitrobenzene in the fresh water species *Cyprinus carpio* was ascertained. The used guideline corresponds to the guideline OECD 305 C "Bioaccumulation: Test for the degree of bioconcentration in fish". The test concentrations were 0.125 and 0.0125 mg/l, respectively, at 25 ±2 °C and the lipid content of the test organisms varied between 2 and 6 %. At a nitrobenzene concentration of 0.125 mg/l a BCF in the range of 3.1-4.8 was determined during an exposure period of 42 days. At the concentration of 0.0125 mg/l the BCF varied between 1.7 and 7.7.

Also experiments with female guppies (*Poecilia reticulata*, 5 to 8 months old) were performed (Deneer et al., 1987). The mean fat content was 8±2%. The test concentration was 1/5 of the LC_{50} (100 µmol/l=12.3 mg/l). Nitrobenzene solutions were renewed daily. After 3 days the nitrobenzene content of the individual fish was determined. The BCF_{fish} on the basis of fat weight varied from 22.4 to 38.9. The authors state that the relatively low BCF for nitrobenzene might be due to experimental difficulties in the determination of nitrobenzene in fish, due to the relatively high volatility of this compound.

The bioaccumulation of nitrobenzene in fish and algae was also examined (Freitag et al. 1982). Experimental protocols were described in detail in Korte et al., 1978. For the fish test the golden orfe *Leuciscus idus melanotus* was chosen as test organism. Five fish weighing about 1.5 g each were exposed to 50 µg/l of ^{14}C-labelled nitrobenzene for three days in a closed system. The fish were not fed during this time and no aeration took place. After three days the radioactivity in the whole fish was determined and referred to the average constant concentration of nitrobenzene in the water. A BCF of < 10 (related to wet weight) was calculated. For the algae test the green alga *Chlorella fusca* was used. Algae (20 mg d.w./200ml) were exposed to 50 µg/l ^{14}C-labelled nitrobenzene for 24 hours. After this time algal cells were separated by centrifugation and the radioactivity was measured in the algae and in the supernatant. A BCF of 24 (related to wet weight) could be determined.

In another study (Geyer et al., 1984) bioaccumulation of nitrobenzene in the alga *Chlorella fusca var. vacuolata* was examined. Algae were exposed to a nitrobenzene concentration of 50 µg/l in nutrient solution at room temperature (20–25 °C). The experimentally determined bioconcentration factor was 24.

4.3.2 Terrestrial bioaccumulation

No data available.

4.3.3 Summary and discussion of bioaccumulation

The different experiments show that nitrobenzene seems to have a low bioaccumulation potential. In all available tests the BCF values were clearly below 100.

4.4 Secondary poisoning

Not relevant for this dossier.

5 HUMAN HEALTH HAZARD ASSESSMENT

5.1 Toxicokinetics (absorption, metabolism, distribution and elimination)

Nitrobenzene is a volatile liquid that can readily gain access to the body by inhalation and skin penetration of the vapour, as well as by ingestion and dermal absorption of the liquid. Nitrobenzene activation in rats to methaemoglobin-forming metabolites appears to be mediated to a significant degree by intestinal microflora. In test animals, the major part of nitrobenzene (about 80% of the dose) is metabolized and eliminated within 3 days. The remainder is eliminated only slowly. The slow compartment is likely due to erythrocyte recycling of nitrobenzene redox forms and glutathione conjugates. Covalent binding, presumably to sulfhydryl groups of haemoglobin, was demonstrated.

In rodents and rabbits, *p*-nitrophenol and *p*-aminophenol are major urinary metabolites. In humans, part of the absorbed dose is excreted into the urine; 10–20% of the dose is excreted as *p*-nitrophenol (which thus may be used for biological monitoring). The half-times of elimination for *p*-nitrophenol are estimated to be about 5 h (initial phase) and >20 h (late phase). The urinary metabolite *p*-aminophenol is significant only at higher doses (EHC 2003).

5.2 Acute toxicity

Numerous reports on nitrobenzene poisoning in the literature are mainly dated back for many decades. An attempt is made to cover the specific criteria of nitrobenzene poisoning and exposure-related disturbances. No or only minor attempts are made to cover the aspects of treatment after nitrobenzene poisoning. In general, treatment consisted of oxygen supply, blood transfusions and intravenous injections of methylene blue.

Nitrobenzene (also called oil of mirbane) has the typical odour of bitter almonds that could be detected in the expired air or in the gastric contents.

Nitrobenzene is rapidly absorbed by the oral, inhalation and dermal route as demonstrated in the following case reports. For example, dermal absorption is in the range 1.54% after application of four micrograms of nitrobenzene to the forearm. This absorption rate is low in comparison to dinitrobenzene (53%) and to benzoic acid (43%; Feldmann and Maibach 1970) and it seems to contradict the clinical effects seen after dermal (and probably also inhalation) exposure of nitrobenzene. Case reports also demonstrate an efficient dermal absorption. However, the most prominent clinical symptoms are among others cyanosis (see Ewer 1920; Mallouh and Sarette 1993).

The most frequently reported side effect is the often life threatening methaemoglobinaemia. In addition, nitrobenzene exposure is mainly associated with the formation of Heinz bodies in erythrocytes, the toxic effects on bone marrow and lymphoid organs, neurotoxic effects and hepatotoxic effects. Large interindividual variations do exist. This is also due to the fact that often the amount of nitrobenzene absorbed is not known. Babies and children appear to be more sensitive to the effects of nitrobenzene (Beauchamp et al. 1982; David et al. 1964; Monnier 1947; Lareng et al. 1974). It should be noted that derivatives of nitrobenzene, especially m-dinitrobenzene, caused similar effects in 8 workers like nitrobenzene (Bresson et al. 1966).

In the following, a short list of case reports (consumers and workers) is documented. This list does not pretend to be complete but it covers the major aspects of nitrobenzene exposure.

A chemical company reported six cases of nitrobenzene poisoning during the years of 1970 to 1976. No data on type and duration of exposure are given. All six patients were admitted to a hospital after having shown the following symptoms: localized cyanosis, breathing problems, and conjunctivitis. No further details are given (BASF AG, unpublished report 1992).

5.2.1 Acute toxicity: oral

5.2.1.1 Human data

As residents of the maternity ward after parturition, five mothers had eaten a cake that had contained an ingredient to simulate a bitter almond taste in autumn 1944. Lacking a comprehensive chemical analysis for the causative agent, instead of natural bitter almonds and almond paste it may have contained either nitrobenzene and/ or other substances like aniline, benzaldehyde or benzonitrile. The mothers did not reveal any clinical symptoms but on the next morning (approx. 15 hours after ingestion), their breast-fed babies had developed a strong to very strong cyanosis. The children did not show any additional symptoms and the cyanosis receded largely in the next 24 hours. The children were not breast fed for 1.5 to 2 days. They received large amounts of tea, and if necessary oxygen and heart stabilizing drugs (Dollinger 1949). (see 5.9.3 et seqq.)

A middle-aged white man was brought to the hospital in a coma. He had a marked ashen-gray cyanosis. There was a very strong odour of nitrobenzene (shoe polish) about the patient, especially in his mouth. Gastric lavage revealed the presence of nitrobenzene. Respiration was decreased to about ten a minute. In spite of vigorous stimulation and oxygen supply the patient died within 45 minutes. He did not regain consciousness (Donovan 1920).

A 48-year-old habitual drinker consumed 200 ml of nitrobenzene. He vomited immediately and the contents had an intense odour of bitter almonds. He became cyanotic within a short period of time, had irregular breathing, and increased motor activity. The blood had a chocolate-brown colour. Treatment consisted among others of gastric lavage, 600 ml of bleeding, intravenous transfusion of glucose and blood transfusion. The man was in an immovable position for 4 days. Methaemoglobin and haematin was detected in urine. After about 4 weeks the man had recovered (Voll 1936).

A woman (24 years) decided to commit suicide and swallowed a mixture that contained almost 12 ml of nitrobenzene. She was deeply cyanosed after one hour. Treatment consisted among others in a blood transfusion, intravenous treatment with 10% methylene blue, saline and glucose. The urine contained methaemoglobin and an excess amount of various amino acids (e.g. alanine, serine, and glutamine). The patient complained of a severe headache, dizziness, and a bad taste in her mouth. She was afebrile and was never jaundiced. The patient made a rapid recovery within approximately four weeks (Parkes and Neill 1953). (calculated: 14g/60kg = ca. 230 mg/kg)

A woman (19 years) survived a suicidal oral dose of about 50 ml of nitrobenzene, approximately 11 g of which was absorbed from the gastro-intestinal tract. Severe symptoms, including the formation of 82% methaemoglobin, normalized entirely within 24 days due to quick and extensive treatment. Other symptoms present were unconsciousness, cyanosis (persistence for the next 10 days), irregular and shallow breathing, and sluggish reaction of the pupils to light. The venous blood had a chocolate-brown colour. There was a distinct odour of bitter almonds in the expired air (Myslak et al. 1971). (calculated: 11g/60kg = ca. 180 mg/kg)

A severe toxic methaemoglobinaemia was diagnosed at a 19 year- old male chemistry student who had accidentally ingested between 5 and 20 ml of a brown liquid while using a pipette. Analysis of the gastric aspirate revealed the presence of aniline and nitrobenzene (no further details). He became unconscious and his skin and mucous membranes were navy blue to almost black. A strong smell similar of bitter almonds was noted. Methaemoglobin level was in excess of 65% and decreased to normal levels after 3 days. The man underwent intensive treatment (blood transfusions, diuresis among others). He made an uneventful recovery in about 19 days (Harrison 1977). (calculated: max. 24g/60kg = 400 mg/kg)

A 21-year-old man was thought to have taken about 30 to 40 ml of a nitrobenzene-containing dye used in screen printing about 30 min before admission to hospital. He was reported to have peripheral and central cyanosis; pupils were normal size, heartbeat was 160 beats per minute, blood pressure was 80/54 mm Hg and respiration was 28 per minute. Blood samples were dark brown. After 1 h of positive-pressure ventilation, gastric lavage and intravenous fluids, the patient became conscious and well oriented, with a decrease in heart rate and an increase in blood pressure. Serum methaemoglobin was 4.29 g/dl. A slow intravenous infusion of ascorbic acid was started, and methylene blue was injected intravenously; after 35 min, the colour of the patient changed dramatically from brownish-blue to pink. After a second injection of methylene blue and a transfusion of packed red blood cells, methaemoglobin was 0.6 g/dl. A peripheral blood smear revealed evidence of haemolytic anaemia. There was no evidence of occult blood in the urine. The patient was discharged on the fifth day of admission (Kumar et al. 1990).

The leading clinical symptom even, after a low oral dose of nitrobenzene, is cyanosis which caused by intensive formation of methaemoglobin. Haemoglobin in blood of newborn children is much sensitive to methaemoglobin formation than haemoglobin of their mothers, however, however resulted cyanosis of acutely poisoned babies disappeared in next 24 hours and no other symptoms were reported in babies fed with milk of intoxicated mothers.

The dose of nitrobenzene taken in case of lethal intoxication of man is not known, it may be assumed that it was higher than doses inducing non-lethal acute poisoning. The amounts of nitrobenzene inducing acute poisoning in humans after oral intake are in the range of 12 – 200ml (14 – 240 g) of nitrobenzene. Assuming average human weight of 70kg, these amounts can be converted to estimated toxic doses of 14 000/70 - 240 000/70mg/kg, thus to a range of 200 – 3428,5 mg/kg. None of these intoxications were lethal, however they all received medical treatment, which has assisted in survival of intoxicated persons.

Based on a case-study of Harrison (1977) describing recovery of a man who ingested nitrobenzene in an approximate dose of 400mg/k after intensive medical treatment, it might be assumed that this dose could be lethal to man without medical treatment. On the other hand in another case a man has survived, after medical treatment, oral intake of 200 ml (240g) of nitrobenzene corresponding roughly to 240g/70kg – 3.4g/kg.

In two other recent case studies (Myslak et al. 1971; Kumar et al. 1990) patients survived oral intake of 50ml and 30-40ml of nitrobenzene, which is equal 60g and 36-48g of nitrobenzene. These

data may allow to assume that the lethal-without treatment dose of nitrobenzene could be equal to 60 000mg/70kg = 857mg/kg and 42 000/70 = 600mg/kg.

5.2.1.2 Animal data

Species	LD_{50} (mg/kg)	Observations and remarks
Rat (m)	732	In the first study a LD50 of 732 mg/kg was calculated using doses of 400, 630, 800 and 1000 mg/kg bw administered per gavage to groups of 10 male rats per group (with sesame oil as vehicle). All rats died after administration of 1000 mg/kg, 4 rats died after administration of 800 as well as 630 mg/kg and none of the animals after administration of 400 mg/kg. Mortalities occurred within 3 days, clinical signs included perturbance of equilibrium, hunched posture, closed eyes, lateral position, cyanosis and paralysis of hind legs. Necropsy revealed dark-brown discolouration of blood in the animals that died within the study, surviving animals demonstrated no macroscopically visible changes. (Hoechst AG 1977, unpublished report)
Rat (m)	588	The second study resulted in an oral LD50 of 588 mg/kg bw (0.49 ml/kg): Doses of 0.3, 0.4, 0.5, 0.6, and 0.7 ml/kg (equivalent to 360, 480, 600, 720 and 840 mg/kg) undiluted nitrobenzene were administered to 10 male rats per dose. A dose of 0.3 ml/kg did not cause mortalities, but all of the animals demonstrated clinical signs. These clinical signs included perturbance of equilibrium, piloerection, sedation, cyanosis, bloody eyes and poor reflexes. Two rats died after administration of 0.4 ml/kg, 5 rats after 0.5 ml/kg, 8 rats after 0.6 ml/kg and all 10 rats after 0.7 ml/kg. Mortalities occurred on days 2 to 4. Information on necropsy is not given. (Bayer AG 1978, unpublished report)
Rat (f)	650	In female rats oral LD50 values ranged within the same order of magnitude: In a first study an oral LD50 of 650 mg/kg bw was calculated after administration of doses of 320, 500, 630 and 800 mg/kg bw administered per gavage to groups of 10 female rats per group using sesame oil as vehicle. All rats died after administration of 800 mg/kg, 5 rats died after 630 mg/kg, 4 rats after 500 mg/kg and none of the animals after administration of 320 mg/kg. Mortalities occurred within 4 days, clinical signs included perturbance of equilibrium, hunched posture, closed eyes, lateral position, cyanosis and loss of reflexes. Necropsy revealed dark-brown discolouration of blood in the animals that died within the study, surviving animals demonstrated no macroscopically visible changes. (Hoechst AG 1977, unpublished report)

Species	LD_{50} (mg/kg)	Observations and remarks
Rat (f)	640	In a second study an oral LD50 of 640 mg/kg bw was calculated after administration of 280 to 2100 mg/kg bw to female rats as 10% gummy arabicum suspensions per gavage: Mortalities occurred within 2 days (no further information given). Clinical signs observed included restlessness and dribbling of urine; discolouration of skin and visible mucous membranes as typical signs of methaemoglobinaemia were detected. At necropsy, hyperaemia of the parenchymatous organs was detected. Histology revealed parenchymatous degeneration and fatty degeneration in liver and kidneys. Formation of methaemoglobin was assessed after oral administration of 640 mg/kg and demonstrated an 11% elevation after half an hour, 19% after 1 hour and 28% after 2 hours, intensive formation of Heinz bodies was stated. (Sziza and Magos 1959)
Rat (m) (Fischer-344)	>450	Male (80-90 day old) Fischer-344 rats weighing approximately 200 g were divided into seven groups of six rats and fasted 16 hours prior to oral administration of 50, 75, 110, 165, 200, 300 or 450 mg nitrobenzene/kg bw. Control rats received the vehicle corn oil. Histopathological changes consistently involved only liver and testes. One rat of the highest dose had cerebellar lesions (bilateral malacic areas and reactive gliosis in the cerebella pedunculus). Hepatocytic centrolobular necrosis appeared inconsistently while hepatocellular nuclear enlargement was consistently detected in rats given doses as low as 110 mg/kg. These data suggest that nuclear enlargement was independent of cell death. Testicular lesions were restricted to the seminiferous tubules, and complete destruction of the spermatocytes at days 2 and 3 after 300 and 450 mg/kg was detected. Necrotic debris and decreased numbers of spermatozoa were seen in the epididymides. No details are given on the effects of the two lowest doses of 50 and 75 mg/kg. (Bond et al. 1981)
cat	>120	In a study with cats measurement of methaemoglobin in blood after oral administration is reported: Cyanosis was detected after administration of 30 mg/kg (25 mm^3/kg). After oral administration of 3, 30, 60 and 120 mg/kg nitrobenzene to groups of 2 cats each, all animals survived. The animals of the 3 mg/kg group did not demonstrate significant elevation of methaemoglobin. After administration of 30 mg/kg slight cyanosis was observed with highest methaemoglobin level (21% and 14.5%) at the 6-hour observation time which decreased to values of 5.1% and 1.7% at the end of the fourth day. After administration of 60 mg/kg methaemoglobin levels rose to 47.3% and 34.3% after 6 hours and decreased to 5.8% and 0% after 96 hours; after administration of 120 mg/kg cyanosis, apathy and mydriasis were detected with methaemoglobin levels of 68.9% and 56.0% after 2 hours decreasing to 18.1% and 7.9% after 96 hours. (BASF AG 1970, unpublished report)

The lethal oral dose of nitrobenzene to cats was reported to be 2400 mg/kg bw and minimal lethal dose in dogs was stated to be 750 – 1000 mg/kg of body weight by von Oetttingen in 1941 (EHC 230, 2003).

Median lethal doses after oral administration of nitrobenzene to female and male rats in four animal studies listed in 5.2.1.2. above were in a range from >450 to 732 mg/kg/ bw.

The lowest, but not lethal, dose inducing cyanosis was determined in cats and was equal to 30 mg/kg. The highest concentration of methemoglobin was observed 6 hour after dosing 30 mg/kg and after that lowering within 96 hours to physiological level (ca. 1.5% MetHg).

5.2.2 Acute toxicity: inhalation

5.2.2.1 Human data

It is stated that if a worker was exposed all day at a threshold level value of 1 ppm, approximately 25 mg of nitrobenzene would be absorbed, of which about one-third would be by skin absorption, the remainder by inhalation (Piotrowski 1967).

It is reported that 200 ppm (ca. 1 mg/l) is the maximum concentration that can be inhaled for one hour without serious disturbance, and 1 to 5 ppm (ca. 0.005 to 0.025 mg/l) is considered a safe level for daily exposure (Henderson and Haggard 1943).

Seven volunteers were exposed for six hours with nitrobenzene vapours in the range of 0.005 to 0.03 mg/l. The exposure was a nose-only-exposure. Retention of nitrobenzene diminished from 87% to 73% during the 6-hour exposure, indicating a low rate of conversion of nitrobenzene in the body that leads to blood saturation. In urine, the metabolite p-nitrophenol was present at about 13% of the inhaled concentration of nitrobenzene. The metabolite p-aminophenol could not be detected in urine. The conversion of nitrobenzene to p-nitrophenol was in the range of 16% (Salmowa et al. 1963).

The case provided by Henderson and Haggard (1943) indicate that one hour exposure of humans to nitrobenzene in the concentration ca. 1mg/l, which is slightly below the saturated vapour concentration (SVC) of nitrobenzene equal to 1.014 mg/l at 20^{o}C did not resulted in a serious alteration of health, which most probably means that observed symptoms did not require medical treatment and were not interpreted as a serious threat to health.

The other studies (Piotrowski, 1967; Salmowa et al. 1963) have a limited value for assessment of severity of acute inhalation toxicity as the concentration applied were very low, but they have a value for knowledge on toxicokinetics of nitrobenzene in humans.

5.2.2.2 Animal data

Species	LC_{50} (mg/l)	Exposure time (h/day)	Observations and remarks

Species	**LC_{50} (mg/l)**	**Exposure time (h/day)**	**Observations and remarks**
Rat (m)	2.847	4h	In a LC50 study according to OECD TG 403, groups of 8 week old male rats were exposed, head-only, to atmospheres of nitrobenzene for single 4-hour periods. The LC50 was determined to be 556 ppm (2847 mg/m^3, 2.847 mg/l). Findings for dose groups, ppm / $^{mg}/_{L}$ (deaths/exposed) were as follows: 439 / 2.24 (0/10), 514 / 2.63 (0/10), 542 / 2.78 (1/10), 555 / 2.84 (7/10), 578 / 2.96 (8/10), 714 / 3.70 (10/10). Clinical signs observed during exposure included cyanosis, prostration, slight to severe corneal clouding, lacrimation, pallor, tremors, tachypnea, rales, laboured breathing, hyperactive / aggressive behaviour, white foamy mouth and nasal discharge. An 8 - 21% loss of weight was observed 1 to 4 days post-exposure, but normal weight gain was achieved thereafter. The extent to which those clinical signs appeared was generally concentration related. Deaths usually occurred within 1 to 2 days following exposure; time span was shortened with increased concentration. (DuPont 1981, unpublished report) The saturated vapour concentration (SVC) of nitrobenzene as calculated below is equal 1.014 mg/l, thus the concentration in this study was 2.8 times higher than SVC. It should be assumed that in this study the animals were exposed to a mixture of vapour and mist of nitrobenzene.
Rat		3h; 7h	In an inhalation risk test with rats 3/12 animals died after 7 hours of exposure to nitrobenzene vapours saturated at 20°C. The saturated nitrobenzene vapours were generated by conducting 200 l/h of air through undiluted nitrobenzene at 20°C. *None of 12 animals exposed for 3 hours died within a 14-days observation period*. After exposure for 7 hours, 3/12 animals died demonstrating severe irritation of mucous membranes. At necropsy, dilatation of the heart, brown discolouration of muscles and organs, swelling of the lungs and infarct-like blood status were detected. (BASF AG 1977, unpublished report) The saturated vapour concentration (SVC) of nitrobenzene as calculated below is equal to 1.014 mg/l. Having in mind possibility of mist formation the actual exposure level could be even slighlty higher that 1.014 mg/l. Having in mind that none of the 12 animals exposed for 3 hours died within a 14-day observation period the LC_{50} of nitrobenzene after 4 hour inhalation exposure must be above this concentration. This hypothesis is strengthen by a fact that more than doubling the time of exposure (to 7 hours) thus most probably doubling the dose absorbed in lungs resulted in 25% mortality.

Species	**LC_{50} (mg/l)**	**Exposure time (h/day)**	**Observations and remarks**
Rat (m)		8h	In a second study 6 male rats survived **an 8-hours inhalation of vapours saturated at 23.1°C**. In this study the saturated nitrobenzene vapours were generated by conducting 400 l/h of air through undiluted nitrobenzene at 23.1°C. The animals demonstrated restlessness, hunched posture, lateral position, closed eyes, uncontrolled movements of the head and enhanced respiration during the first hour of exposure. Between 6 and 7 hours after the start of the exposure white discolouration of eyelids, ears and noses and dark discolouration of iris was detected. At the end of the exposure period animals demonstrated lateral position and tumbling movements. All animals survived and recovered within 4 days after exposure. Necropsy at the end of the 14-days observation period revealed no macroscopically visible changes. (Hoechst AG 1977, unpublished report). Vapour pressure of nitrobenzene increases rapidly with an increase of temperature (see Table 1) thus at temp. 23.1°C VP of nitrobenzene would be much higher than in temperature of 20°C, although it is not known. VP at 25°C is 38 Pa. Saturated vapour concentration at 25°C calculated using the following formula: SVC [mg/l] = 0.0412 x MW x VP equals to 1.93 mg/l, while SVC at 20°C equals to 1.014 mg/l. Assuming proportionality a SVC of nitrobenzene at 23.1°C could be in a range 1.55-1.60 mg/l. Thus based on results of this experiment LC_{50} of nitrobenzene is above 1.6 mg/l. Using as an example the conversion factors provide in section 3.1.2.2.of Guidance on the Application of Regulation 1272/2008 in table 3.1.1. note (b) on relation between length of time of exposure and level cut-off level of exposure criteria - the exposure for 8 hour at concentration of 1.6 mg/l, would correspond to exposure for 4 hours at concentration of 3.2mg/l for mist of nitrobenzene, thus LC_{50} of nitrobenzene mist might be even above this value.

Species	LC_{50} (mg/l)	Exposure time (h/day)	Observations and remarks
Rat (m/f)		7h	In a third study 6 female and 6 male rats survived a 7-hours nose-only exposure to vapours saturated at 20°C. Saturated nitrobenzene vapours were generated by conducting 600 l/h of air through undiluted nitrobenzene at 20°C. The animals demonstrated enhanced respiration, paleness of the skin and passivity during the exposure. All rats survived and one hour after exposure all had recovered. Necropsy at the end of the 14-days observation period revealed no macroscopically visible changes.(Hoechst AG 1981, unpublished report) The saturated vapour concentration (SVC) of nitrobenzene as calculated below is equal to 1.014 mg/l. Having in mind possibility of mist formation the actual exposure level could be even slighlty higher that 1.014 mg/l. Having in mind that none of the 12 animals exposed for 7 hours died within a 14-day observation period the LC_{50} of nitrobenzene after 4 hour inhalation exposure must be above this concentration. This hypothesis is strengthen by a fact that the time of exposure (7 hours) was almost two times longer than 4 hours which is required in standard acute inhalative toxicity tests
dog; rabbit; guinea pig; rat; cat; hen; pigeon; certain parasites			In 1919 "fumigation" experiments were conducted with dogs, rabbits, guinea pigs, rats, cats, hens, pigeons and certain parasites. The following conclusions were stated: "Apart from a possible disturbance of the digestive functions and a possible asphyxia due to direct action on the blood, most of the symptoms of poisoning by nitrobenzene may be explained on the basis of disturbances of the cerebellum or cerebellar path. Inhalation of nitrobenzene vapours in toxic doses produces chromatolytic degeneration of the Purkinje cells of the cerebellum. Microscopic examinations have shown only the degeneration and morphological changes in the erythrocytes. The size of the lethal dose depends on certain conditions such as the amount and kinds of fat in the blood. These conditions govern the concentration of nitrobenzene in the vicinity of the nerve cells. A latent period clapses between administration of nitrobenzene and the onset of the symptoms of poisoning". (Chandler 1919)

The saturated vapour concentration (SVC) for a volatile substance (nitrobenzene), have been calculated according the equation provided in to Guidance on the Application of Regulation (EC) No 1272/2008 (see Section 3.1.2.3.2): SVC [mg/l] = 0.0412 x MW x VP. Taking into account:

MW – molecular weight in g/mol = 123.06
VP - vapour pressure in hPa at 20°C = 0.2

$SVC_{nitrobenzene}$ = 0.0412 x 123.06 x 0.2 = 1.014 mg/l

The saturated vapour concentration at 20°C for nitrobenzene is 1.014 mg/l, however at 25°C it may be as high as 1.93 mg/l . The data presented in section 5.2.2.2 of Background document indicate that LC_{50} for rats exposed for 3 and 7 hours hour to saturated vapour concentration (SVC) of nitrobenzene would be higher than 1.5 mg/l, and most probably higher than 3mg/l (BASF AG, 1977). Therefore based on these data it would be rather difficult to state that acute inhalative toxicity of vapour of nitrobenzene meets the classification criteria for T, R23 within DSD being for gases and vapours $0.5 < LD_{50} \leq 2$mg/l/4hr, or CLP criteria for Acute Tox. 2 being $0.5 < LD_{50} \leq 2$mg/l/4hr because of lack mortality of rats in conditions corresponding to upper limit of a LD_{50} range defined in this criterion.
Even after large extension of time of inhalation exposure to 8 hour at saturated vapour concentration (SVC) at 23.1°C none of 6 rats died (Hoechst AG, 1977). None out 12 rats died after 7-hour exposure to SVC of nitrobenzene at 20°C , B Hoechst AG, 1981).

According to Guidance on the Application of Regulation (EC) No 1272/2008 (Annex I: 3.1.2.3. "Specific considerations for classification of substances as acutely toxic by the inhalation route", page 197) an LC_{50} well below the SVC will be considered for classification according to the criteria for vapours; whereas an LC_{50} close to or above the SVC will be considered for classification according to the criteria for mists

5.2.3 Acute toxicity: dermal

5.2.3.1 Human data

Five babies aged between 16 days and 11 weeks were exposed to a cloth that was marked with a hospital stamp that contained nitrobenzene. The babies exhibited cyanosis, irregular pulse, breathing problems and convulsions. Two of the five babies with skin problems (no further details) showed more severe signs than the other three babies without skin problems. All babies recovered within a few days (Ewer 1920).

A 2-year old boy developed a dirty, greyish blue colour of the skin, lips, and nails after he had worn shoes for a few hours that had been dyed with nitrobenzene. While asleep he had wet his shoes and socking. His breathing was shallow and irregular, with short periods of apnoea. The boy was treated by rest in bed and by oxygen inhalation. The next day his colour was normal (Levin 1927).

As recently as 1993, in Saudi Arabia a two-month-old baby developed a chocolate-coloured cyanosis but was otherwise healthy-looking with no evidence of pulmonary, cardiac or central nervous symptoms. Methaemoglobin level was 31.5%. The mother admitted that she had rubbed the child with "Oleum Dulcis", a locally available hair oil which is imported from India. This mixture had a strong almond odour and contained 1% of nitrobenzene. As the patient was asymptomatic apart from being cyanosed, he was observed without treatment. The methaemoglobin level dropped during the three day period (Mallouh and Sarette 1993).

A girl received a lice treatment with a nitrobenzene containing oil. After the third treatment the girl had developed a cyanosis and her room had the odour of bitter almonds. The expired air also had the odour of bitter almonds. Urine contained urobilin and urobilinogen. The girl recovered within about 2 days (Bohland 1919).

The case reports described here indicate an ability of nitrobenzene to penetrate through undamaged human skin , even from diluted solutions, leading to formation of methemoglobin and visible

cyanosis which somehow act as a warning signal leading to cessation of exposure. In all cases the induced symptoms disappeared in few days without medical treatment, except in one case, after cessation of exposure and no permanent damage was reported in the acutely intoxicated children after recovery.

5.2.3.2 Animal data

Species	LD_{50} (mg/kg)	Observations and remarks
rabbit	760	Dermal LD50 values were calculated for rabbits resulting in 760 mg/kg bw. Doses of 560, 760 and 1000 mg/kg bw in ethanol were dermally applied to the clipped skin of 5 rabbits per dose in a well-ventilated area (chemical hoods) to minimize inhalation hazard to both experimenters and animals. Ventilation was maintained throughout the animal exposure period in an effort to keep conflicting inhalation effects at a minimum. The dosage sleeves were secured with extra layers to retard evaporation due to the increased air movement. The animals were immobilized during the exposure period of 24 hours. No mortality occurred after application of 560 mg/kg and 4/5 rabbits died each after application of 760 mg/kg and of 1000 mg/kg. Clinical signs included manifestations of methaemoglobinaemia with symptoms evident within less than 20 minutes. Animals that died (deaths within 4 days) exhibited lethargy and collapse as well as loss of motor coordination. Surviving animals demonstrated lethargy and persisting discolouration of skin and eyes. Within a pre-screening test, blue discolouration of skin and eyes were observed after dermal application of 330 mg/kg to one rabbit. Data on necropsy are not mentioned. (Harton and Rawl 1976)
rabbit	301	In a Draize test with 6 rabbits a quantity of 0.5 ml of undiluted nitrobenzene was occlusively applied to the skin of each rabbit for an exposure period of 24 hours. Three of the animals died within 2 days exhibiting signs of cyanosis. The results of this Hoechst AG 1977 unpublished study reported by the Dossier Submitter in section 5.3.1. Skin Irritation indicate that LD_{50} of undiluted nitrobenzene applied for 24 hours by dermal route equals 301mg/kg bw.
rat	2100	A dermal LD^{50} of 2100 mg/kg bw was detected in a percutaneous application study with female rats using undiluted nitrobenzene (no further technical information). Mortalities occurred between 12-72 h and loss of weight and cyanosis were observed as clinical signs. No relationship was observed between dose applied and time of death. At necropsy, hyperaemia of the parenchymatous organs was detected. Histology revealed parenchymatous degeneration and fatty degeneration in liver and kidneys. Formation of methaemoglobin was assessed after dermal application of 2100 mg/kg and demonstrated a 16% elevation after half an hour, 25% after 1 hour and 35% after 2 hours. Intensive formation of Heinz bodies was observed after 24 h. (Sziza and Magos 1959)

The case reports described in this background document indicate an ability of nitrobenzene to penetrate through undamaged human skin, even from diluted solutions, leading to formation of methemoglobin and visible cyanosis which somehow act as a warning signal leading to cessation of exposure. In all cases the induced symptoms disappeared in few days without medical treatment, except in one case, after cessation of exposure and no permanent damage was reported in the acutely intoxicated children after recovery.

The reported dermal LD_{50} for rats equal to 2100mg/kg and to rabbits amount to 301 mg/kg and to 760 mg/kg.

5.2.4 Acute toxicity: other routes

5.2.4.1 Comparison With criteria

Acute Oral toxicity :

Based on a case-study of Harrison (1977) describing recovery of a man who ingested nitrobenzene in an approximate dose of 400mg/k after intensive medical treatment, it might be assumed that this dose could be lethal to man without medical treatment. In another case a man has survived, after medical treatment, oral intake of 200 ml (240g) of nitrobenzene corresponding roughly to 240g/70kg = 3400mg/kg. In two other case studies (Myslak et al. 1971; Kumar et al. 1990) patients survived oral intake of 50ml and 30-40ml of nitrobenzene, which is equal 60g and 36-48g of nitrobenzene. These data may allow to assume that the lethal-without treatment dose of nitrobenzene could be equal to 60 000mg/70kg = 857mg/kg and 42 000mg/70kg = 600mg/kg. The lowest dose of nitrobenzene reported to induce serious intoxication of a women was equal approximately to 230mg/kg (Parkes and Neil,1953).

Having in mind the known interspecies variation in sensitivity to toxicity of chemicals believed to be well estimated by a factor of 10, it may be reasonable assumed that nitrobenzene even at doses lower than 230mg/k (Parkes and Neil, 1953) may be seriously toxic to humans. Thus the doses inducing serious toxicity in humans may be much lower than a range of median lethal doses for rats used as criteria for classification of acute toxicity..

In addition it is known that humans are more sensitive than rats to MetHb- formation under influence of chemical substances. The lowest oral dose of aniline significantly increasing level of methemoglobin (from 1.2% to 2.5%) in human volunteers receiving this substance once a day for three consecutive days was 25 mg/man, which may be converted to 25mg/70kg= 0.36mg/kg (Jenkins et al., 1972 quoted from Aniline EU Risk Assessment Report, ECB, 2004) . The lowest dose of aniline causing a slight increase in Met-Hb in rats (3.3% versus 2.4% in controls) amounted 20mg/kg (Jenkins at al., 1972 from ECB, 2004). Thus humans are 56 times (20mg/kg/0.36mg/kg) more sensitive to Met-Hb formation than rats, which are used to determine medial lethal doses as a basis for classification of acute toxicity. This ratio of sensitivities to Met-Hb formation between humans and rats is much higher than 10 usually taken as a default value for interspecies differences in susceptibility to toxic action of chemicals (guidance). Higher susceptibility of humans is likely to be consequence of the interspecies differences in the activity of methaemoglobin reductase, which reduces methaemoglobin to haemoglobin. The activity of this enzyme is five and ten times higher in rat erythrocytes and mouse erythrocytes, respectively than in human erythrocytes (Smith, 1986 quoted from ECB, 2004)).

Taking into account that formation of Met-Hb, in response to a single exposure to nitrobenzene, may cause secondary toxic effects in internal organs such as spleen, liver, kidney, brain and lungs, the high difference between humans and rats in susceptibility to induction of Met-Hb should be taken in hazard classificaon.

Therefore, in the opinion of RAC the classification derived solely on animal data - Xn with a risk phrase R 22 Harmful if swallowed (DSD) and Acute Tox. 4 with hazard statement H302 Harmful if swallowed – should be made relevant to humans and reflect higher sensitivity of humans, thus acute oral toxicity of nitrobenzene should by classified T: R26 Toxic if swallowed (DSD) and Acute Tox. 3, H301 Toxic if swallowed.

Acute inhalation toxicity:

The case-study provided by Henderson and Haggard (1943) indicate that one hour exposure of humans to nitrobenzene in the concentration ca. 1mg/l, which is slightly below the saturated vapour concentration (SVC) of nitrobenzene equal to 1.014 mg/l at 20^{o}C did not resulted in a serious alteration of health, which most probably means that observed symptoms did not require medical treatment and were not interpreted as a serious threat to health.

The saturated vapour concentration at 20°C for nitrobenzene is 1.014 mg/l, however at temperature of 25°C it may be as high as 1.93 mg/l . The data presented in section 5.2.2.2 of Background document indicate that LC50 for rats exposed for 3 and 7 hours hour to saturated vapour concentration (SVC) of nitrobenzene would be higher than 1.5 mg/l, and most probably higher than 3mg/l (BASF AG, 1977). Therefore based on these data it would be rather difficult to state that acute inhalation toxicity of vapour of nitrobenzene meets the classification criteria for T, R23 within DSD being for gases and vapours 0.5 □ LD50 ≤ 2mg/l/4hr, or CLP criteria for Acute Tox. 2 being 0.5 □ LD50 ≤ 2mg/l/4hr because of lack mortality of rats in conditions corresponding to upper limit of a LD50 range defined in this criterion.

Even after large extension of time of inhalation exposure to 8 hour at saturated vapour concentration (SVC) at 23.1°C none of 6 rats died (Hoechst AG, 1977). None out 12 rats died after 7-hour exposure to SVC of nitrobenzene at 20°C , B Hoechst AG, 1981).

According to Guidance on the Application of Regulation (EC) No 1272/2008 (Annex I: 3.1.2.3. “Specific considerations for classification of substances as acutely toxic by the inhalation route”, page 197) an LC_{50} well below the SVC will be considered for classification according to the criteria for vapours; whereas an LC_{50} close to or above the SVC will be considered for classification according to the criteria for mists

The data of DuPont report (1981) indicate that LC_{50} for rats of a mixture of mist and vapour of nitrobenzene equals 2.847 mg/l which is within a range of 1.0 < LD_{50} ≤ 5 mg/l/4hr which is a criterion for category Harmful “Xn” and risk phrase R20 within DSD system as well as criterion for Acute Tox. 4 and with hazard statement H332 within CLP system. The results of other animal studies on acute inhalation toxicity also support such a classification.

Taking into account that formation of Met-Hb, in response to a single exposure to nitrobenzene, may cause secondary toxic effects in internal organs such as spleen, liver, kidney, brain and lungs, the high difference between humans and rats in susceptibility to induction of Met-Hb should be taken in hazard classification.

Therefore, in the opinion of RAC the classification derived solely on animal data - Xn with a risk phrase R 20 (DSD) and Acute Tox. 4 with hazard statement H332 Harmful if inhaled – should be made relevant to humans and reflect higher sensitivity of humans, thus acute oral toxicity of nitrobenzene should by classified T: R23 Toxic by inhalation (DSD) and Acute Tox. 3, H301 Toxic if inhaled.

This classification is in agreement with the existing classification, a proposal of Dossier Submitter, opinion of TC C&L and opinions some MSCAs expressed during public consultation.

Acute dermal toxicity:

The case reports described in background document indicate an ability of nitrobenzene to penetrate through undamaged human skin, even from diluted solutions, leading to formation of methemoglobin and visible cyanosis which somehow act as a warning signal leading to cessation of exposure. In all cases the induced symptoms disappeared in few days without medical treatment, except in one case, after cessation of exposure and no permanent damage was reported in the acutely intoxicated children after recovery.

The reported dermal LD_{50} for rats equal to 2100mg/kg and to rabbits amount to 301 mg/kg and to 760 mg/kg. The lowest reported LD_{50} equal 301mg/kg falls into a range of 50 – 400mg/kg, which is according to Directive 67/548/EEC a criterion of category Toxic "T" with risk phrase R 24 - toxic in contact with skin

However, the criteria for acute dermal toxicity according to CLP regulation are different. Taking only into account study on rats nitrobenzene could not be classified to acute dermal toxicity since dermal LD_{50} for rats (2100mg/kg) is outside classification range for category Acute Tox. 4 equal 1000 -2000 mg/kg in CLP Regulation, what demonstrate the rats are not appropriate model for assessment acute dermal toxicity for humans. Based on study on rabbits nitrobenzene would be classified into category Acute Tox. 3, H311 since both LD50 for rabbits (301 mg/kg and 760 mg/kg) are within a range of 200 – 1000 mg/kg. Taking as basis results obtained in more sensitive species the classification for dermal toxicity Acute Tox 3 with a hazard statement H311 is proposed which is in agreement with a proposal of the dossier submitter.

This classification is in agreement with the existing classification, a proposal of Dossier Submitter, opinion of TC C&L and opinions some MSCAs expressed during public consultation.

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Conclusions on classification and labeling

Proposed classification for acute toxicity based on Directive 67/548/EEC criteria

T, Toxic, R23/24/25 – toxic by inhalation, in contact with skin and if swallowed

Proposed classification based on Regulation (EC) No 1272/2008 criteria

Acute Tox. 3 - H301 Toxic if swallowed
Acute Tox. 3 - H311 Toxic in contact with skin
Acute Tox. 3 - H331 Toxic if inhaled

5.3 Irritation

5.3.1 Skin

Species	No. of animals	Exposure time (h/day)	Conc. (w/w)	Dressing: (occlusive, semi-occlusive, open)	Observations and remarks (specify degree and nature of irritation and reversibility)
rabbit	6			occlusive	Nitrobenzene demonstrated only slight local irritant properties in Draize tests with rabbits. Very slight irritation was detected after 24 hours occlusive exposure of rabbit skin to 0.05 ml (20 mg) "chemically pure" nitrobenzene (6 rabbits). At the 24-hours observation time mild irritation grade 1 was detected which had reversed at the 48 hours observation time. (Sziza and Magos 1959)
Rabbit	6	24		occlusive	In a second Draize test with 6 rabbits a quantity of 0.5 ml of undiluted nitrobenzene was occlusively applied to the skin of each rabbit for an exposure period of 24 hours. Three of the animals died within 2 days exhibiting signs of cyanosis. Slight skin irritation was detected. In a similar test with a 10% dilution of nitrobenzene in sesame oil no mortality occurred, the animals demonstrated mild skin irritation (irritation index 1.2 according to FDA regulations) (Hoechst AG 1977, unpublished report) The results of this study using undiluted nitrobenzene can be used to estimate LD_{50} of nitrobenzene by dermal route, taking into account that dermal exposure lasted 24 hours. 50% mortality was observed at the dose of 0.5ml, of undiluted nitrobenzene with relative density 1.2037, per rabbit of approximately 2kg bw. Thus LD_{50} of nitrobenzene would be 0.5ml x 1.2037 ml/g/2kg = 301mg/kg bw.
Conclusion: R-phrase none (see Summary and discussion).					

5.3.2 Eye

Species	No. of animals	Exposure time (hours)	Conc. (w/w)	Observations and remarks (specify degree and nature if irritation, any serious lesions, reversibility)

Species	No. of animals	Exposure time (hours)	Conc. (w/w)	Observations and remarks (specify degree and nature if irritation, any serious lesions, reversibility)
rabbit	6			In a Draize eye test with 6 rabbits 0.1 ml nitrobenzene was instilled into the conjunctival sac of each animal. Conjunctival irritation was highest 1 hour after instillation (irritation index of 2 according to FDA regulations). The substance is assessed as "causes no conjunctival irritation" according to FDA regulations (no further information). (Hoechst AG 1977)
rabbit	2			Two rabbits were tested in a second Draize eye test using 0.05 ml of "chemically pure" nitrobenzene each. Slight conjunctival irritation disappeared within 48 hours, no corneal lesions were observed. (Sziza and Magos 1959)
Rabbit	1			One rabbit was tested in a third Draize eye test with 0.1 ml of undiluted nitrobenzene. A moderate area of slight corneal opacity was observed at the 1-hour observation time, mild conjunctival redness and slight conjunctival swelling was detected. The eye returned to normal within one day. In a parallel test with one rabbit the eye was washed 20 seconds after instillation of the substance demonstrating less irritation than the unwashed eye. (DuPont de Nemours Co. Inc. 1977, unpublished report)
				In vitro: In a study investigating *in vitro* alternatives to the Draize test for eye irritation was concluded that nitrobenzene could be considered as a non-irritant according to the HET-CAM test, a test performed on the chorioallantoic membrane of hen eggs (Spielmann et al. 1991).
Conclusion: R-phrase none (see Summary and discussion).				

5.3.3 Respiratory tract

No data available

5.3.4 Summary and discussion of irritation

Nitrobenzene is not a corrosive substance. Very slight to slight skin irritation was observed in rabbits; three out of six rabbits died after a 24-hour occlusive exposure with 0.5 ml undiluted nitrobenzene after exhibiting signs of cyanosis. Slight eye irritation was observed in rabbits which disappeared within 24 hours. None of the tests were conducted according to OECD TG 404/405. Nevertheless, from the data presented here it can be concluded that a classification and labelling for irritation/ corrosion is not warranted.

Data on effects on the skin and eyes of humans are not available, but data obtained from the case reports do not warrant a classification and labelling for these effects either.

5.3.5 Comparison with classification criteria

According to Directive 67/548/EEC to be classified as "Irritating to skin" a substance after exposure period for up to 4 hour should induce significant inflammation on rabbit skin with a mean values of scores for either erythema and eschar formation or oedema of 2 or more, which was not observed in tests in which dermal exposure lasted even longer. Therefore, no classification is warranted.

The symptoms observed on rabbit skin did not also reach any of the skin irritation criteria determined in Regulation 1272/2008 such as:

(1) Mean value of ≥ 2,3 - ≤ 4,0 for erythema/eschar or for oedema in at least 2 of 3 tested animals from gradings at 24, 48 and 72 hours after patch removal or, if reactions are delayed, from grades on 3 consecutive days after the onset of skin reactions; or

(2) Inflammation that persists to the end of the observation period normally 14 days in at least 2 animals, particularly taking into account alopecia (limited area), hyperkeratosis, hyperplasia, and scaling; or

(3) In some cases where there is pronounced variability of response among animals, with very definite positive effects related to chemical exposure in a single animal but less than the criteria above.

Nitrobenzene after instillation into eye of rabbit did not produce effects meeting criteria set in the Directive 67/548/EEC for a substance considered irritating to eyes with a risk phrase R 36. The mean scores of the eye irritation tests was below any of the following values:

—cornea opacity equal to or greater than 2 but less than 3,
— iris lesion equal to or greater than 1 but not greater than 1,5,
— redness of the conjunctivae equal to or greater than 2,5,
— oedema of the conjunctivae (chemosis) equal to or greater than 2,
Therefore, no classification is warranted.

The symptoms observed after instillation of nitrobenzene into rabbit eye did not also reach any of the criteria determined in Regulation 1272/2008 for category for reversible eye effects such as:

- at least in 2 of 3 tested animals, a positive response identified as corneal opacity ≥ 1 and/or iritis ≥ 1, and/or conjunctival redness ≥ 2 and/or conjunctival oedema (chemosis) ≥ 2 calculated as the mean scores following grading at 24, 48 and 72 hours after installation of the test material, and which fully reverses within an observation period of 21 days

5.3.6 Conclusions on classification and labelling

The properties of nitrobenzene do not warrant classification for skin or eye irritancy neither in DSD nor in CLP classification system

5.4 Corrosivity

See 5.3.4

5.5 Sensitisation

5.5.1 Skin

In a review paper on allergies caused by aromatic amino- and nitro-chemicals it is mentioned that the potential of nitrobenzene to cause cross-reactivity in patients that were sensitised by p-phenylenediamine or azo-dyes was low. Three weakly positive cases out of 15 patients were reported (Schulz 1962, test concentration: 1%; vehicle not mentioned).

Species	Type of test	No. of animals	Incidence of reactions observed
guinea pig	ear-flank test	6	An ear-flank test with guinea pigs resulted also in no skin sensitisation: A 10% dilution of nitrobenzene in dimethyl formamide was applied over three days to the ears of 6 guinea pigs; the flanks were challenged one week later. The erythematous reaction produced 24 hours after challenge was rated and compared with that in unsensitized controls. In this comparative study, the method is reported to demonstrate good reproducible results with many classes of chemical compounds. However, the number of tested animals is too low according to international criteria (ECETOC 1999). (Stevens 1967)
			<u>Additional data: QSAR</u> The existing data are not sufficient to assess the potential of nitrobenzene to cause sensitisation. Hence, a search on structurally related compounds, which are known to cause sensitisation, was performed. In the paper from Schlede et al. (2003), six substances are listed, which consist of a benzene ring and, among other substituents, contain a nitro group. These structures were categorised as "significant contact allergen" (six structures) or "solid-based indication for a contact allergenic potential" (one structure). Most closely related to nitrobenzene are 2,4-dinitrochlorobenzene and 2,4-dinitrofluorobenzene (both categorised as "significant contact allergen"). Basketter et al. (1996) reported that dichloronitrobenzene, which has one nitro group, shows a reduced potential to cause skin sensitisation compared to 2,4-dinitrochlorobenzene. However, these structural data indicate that also nitrobenzene may bear some sensitising potential. Furthermore, p-aminophenol, an important metabolite of nitrobenzene, is categorised as "significant contact allergen". (Schlede et al. 2003)
Conclusion: insufficient data; R-phrase none (see Summary and discussion).			

5.5.2 Respiratory system

No data available

5.5.3 Summary and discussion of sensitisation

Skin

The animal data are insufficient to assess the intrinsic property of nitrobenzene to cause sensitisation, since the available studies (ear-flank test) were performed with methods that do not meet international guideline requirements and are considered to be too insensitive. In humans, three weakly positive cases out of 15 patients were reported from a study on cross-reactivity. These data are insufficient to conclude on classification.

This lack of knowledge is paired with a concern from several structurally related compounds which are known to cause skin sensitisation. In case that workers or consumers may be exposed to nitrobenzene, the conduction of a Local Lymph Node Assay (LLNA) or a Magnusson Kligman Test should be considered within a substance evaluation procedure to appropriately assess the skin sensitisation potential of nitrobenzene.

5.5.4 Comparison with classification criteria :

The studies described above on skin sensitization properties of nitrobenzene were not performed with methods described in Council Regulation (EC) No 440/2008 or equivalent, internationally recognized methods. The effects observed in these available studies for nitrobenzene are not sufficient to assess sensitization properties of nitrobenzene.

5.5.5 Conclusions on classification and labelling

The available data are not sufficient to conclude on classification

5.6 Repeated dose toxicity

5.6.1 Repeated dose toxicity: oral

Species/ strain, group size	Dose (mg/kg bw, mg/kg diet)	Duration of treatment	Observations and remarks (effects of major toxicological significance)

rats F344 (6m+6f)	0, 5, 25, 125 (gavage , mg/kg bw/day)	28 d (plus 2 weeks of recovery for control and high dose groups)	Blood: anaemia (RBC↓ haemoglobin↓, haematocrit ↓, MCV↑, reticulocytes↑, leucocytosis ≥25 mg/kg (no methb data) Liver: extramedullary haematopoiesis↑, Kupffer cell pigmentation at 125 mg/kg, liver weight ↑ ≥5 mg/kg Spleen: pigmentation (haemosiderosis) extramedullary haematopoiesis congestion ≥5 mg/kg, spleen weight↑ ≥25 mg/kg Testis: Tubular degeneration& atrophy, hypospermia at 125 mg/kg Kidneys: brown pigmentation in tubules (haemosiderosis) at 125 mg/kg CNS: cerebellar spongiosis and perivascular pigmentation at 125 mg/kg Other results: premature death(1/6 f), decreased movement, pale skin, gait abnormalities, reduced body weight & bw gain & thymus atrophy at 125 mg/kg LOAEL 5 mg/kg (Shimo et al. 1994)
Mouse B6C3F1 (7-8 f)	0,30,100, 300 (gavage , mg/kg bw/day)	14 d	Blood: RBC↓ at 300 mg/kg, MCH↑ MCV↑, reticulocytes↑ ≥100 mg/kg (no methb data) Liver: hydropic degeneration, haemosiderin pigmentation at 300 mg/kg Spleen: haemosiderin pigmentation, extramedullary haematopoiesis& congestion red pulp ≥100 mg/kg Testis: ND Kidneys: ∅ CNS: ND Other results: morbidity at 300 mg/kg, bone marrow: cell counts↑, proliferation rate↑ & number of monocytic/granulocytic stem cells↑ ≥30 mg/kg altered immune responses ≥100 mg/kg LOAEL 30 mg/kg (Burns et al. 1994)
Mouse B6C3F1; Rat Fischer-344 (m+f)	38, 300, 600 mg/kg bw/day (gavage)	14 d	Range finding study: Other results: mortalities or sacrificed in a moribund status at 600 mg/kg (rats & mice) and at 300 mg/kg (rats). Treated animals were inactive, ataxic, prostate, cyanotic and dyspnoeic. Reduced weight gain in mice at ≥37.5 mg/kg. Liver, spleen, lung, kidney, brain: significant histological changes in both species (no further details). (NTP, 1983a, cited from EHC Report 2003)
Mouse B6C3F1 (10m+10f)	0, 19, 38, 75, 150, 300 mg/kg bw/day (gavage)	13 weeks	Liver: weight increase significant in all female dose groups and in two highest groups of male mice. Brain: acute necrosis in vestibular nucleus in 1 male at 300 mg/kd Other results: mortalities in week 4 and 5. Clinical signs included ataxia, lethargy, dyspnoea, convulsions, irritability and rapid head-bobbing movements. (NTP, 1983a, cited from EHC Report 2003)

Rat F-344 (10m+10f)	0, 9.4, 19, 38, 75, 150 mg/kg bw/day (gavage)	13 weeks	Brain: lesions in brain stem areas (facial, olivary & vestibular nuclei), cerebellar nuclei consisting of demyelination, loss of neurons, varying degrees of gliosis, haemorrhage, occasional neutrophil infiltration and occasionally haemosiderin-laden macrophages. Other results: mortalities (7 males, 1 female) and sacrification due to moribundity in week 6-9 (2 females) and in week 10-13 (2 males) at 150 mg/kg. Clinical signs: ataxia, left head tilt, lethargy, trembling, circling, dyspnoea, cyanosis at ≥75 mg/kg. (NTP, 1983a, cited from EHC Report 2003)
Rat Sprague-Dawley (10m+10f)	0, 20, 60, 100 mg/kg bw/day (gavage)	54 d (females: throughout premating (14d), mating(14d), gestation (22d) and lactation (4d), males sacrificed on Day 41 or 42)	OECD Combined Repeat Dose and Reproductive/ Developmental Toxicity Screening test protocol, TG 422: Effects in surviving males sacrificed on day 41 or 42: Blood: RBC↓, haemoglobin↓, haematocrit ↓ at 100 mg/kg, MCH↑ MCV↑, reticulocytes↑, erythroblasts↑, leucocyte no.↑ ≥60 mg/kg Liver: serum cholesterol ↑ at 100 mg/kd, liver weight ↑ (all dose groups),centrilobular swelling of hepatocytes, haemosiderin deposition in Kupffer cells, extramedullary haematopoiesis Spleen: weight ↑ (all dose groups), extramedullary haematopoiesis and haemosiderin deposition (also in the renal tubular epithelium and bone marrow) Brain: neuronal necrosis and gliosis in nuclei areas of cerebellar medulla and pons at 60 mg/kg (3/10 males) and at 100 mg/kd (10/10 males) Testes: atrophy of seminiferous in 10/10 males at ≥60 mg/kg and in 1 male at 20 mg/kg Other results: mortalities (2 males) at 100 mg/kg on Day 21 and 35 LOAEL: 20 mg/kg (Mitsumori et al. 1994, cited from EHC Report 2003)

A 28-day repeated dose toxicity study of nitrobenzene in F344 rats (Shimo et al. 1994).

Nitrobenzene at dosages of 0, 5, 25 and 125 mg/kg/day was administered in a 28-day repeat dose toxicity study on male and female F344 rats. All rats in each group consisting of 6 males and 6 females received a daily intragastric administration of this chemical for 28 days. Additional two groups of animals exposed to 0 and 125 mg/kg/day were used for examinations of subsequent recovery for 2 weeks.

Decreased movement, pale skin, gait abnormalities and decreased body weight gain of their gains were seen in the 125 mg/kg group.

Hematology revealed decreases of RBC, Hb and Ht in the 25 and/or 125 mg/kg groups.

Hematologic and clinical chemistry parameters in rats treated with nitrobenzene for 28 days, with or without a recovery period of 14 days

	28-Day dosing study[a]				14-Day recovery group[a]	
Parameter	**Control**	**5 mg/kg**	**25 mg/kg**	**125 mg/kg**	**Control**	**125 mg/kg**
Males						
RBC (× 10^4/mm^3)	761 ± 117	670 ± 54	524 ± 36[b]	412 ± 54[b]	727 ± 93	724 ± 100
Hb (g/dL)	16.9 ± 0.6	16.6 ± 0.6	14.5 ± 0.5[b]	14.2 ± 0.5[b]	16.7 ± 0.7	17.7 ± 0.6[c]
Hct (%)	41.6 ± 6.3	35.6 ± 3.3	32.3 ± 2.4[c]	34.9 ± 3.4[c]	38.2 ± 4.9	45.7 ± 6.6[c]

MCV (fL)	54.7 ± 0.8	53.0 ± 0.9	61.3 ± 2.7[b]	84.8 ± 5.5[b]	52.7 ± 1.4	63.0 ± 1.4
WBC (× 10^2/mm^3)	44 ± 14	45 ± 8	122 ± 44[b]	1426 ± 521[b]	46 ± 5	40 ± 16
Females						
RBC (× 10^4/mm^3)	708 ± 63	718 ± 129	635 ± 126	458 ± 43[b]	694 ± 79	674 ± 86
Hb (g/dL)	17.5 ± 0.9	16.3 ± 1.0	15.5 ± 0.6[b]	14.5 ± 0.8[b]	16.8 ± 0.4	18.0 ± 1.2
Hct (%)	38.1 ± 3.2	37.8 ± 6.5	37.7 ± 7.4	35.4 ± 3.4	36.7 ± 4.6	39.5 ± 5.1
MCV (fL)	53.8 ± 1.2	52.7 ± 0.5	59.5 ± 1.6[b]	77.2 ± 1.6[b]	52.8 ± 0.8	58.3 ± 5.2[c]
WBC (× 10^2/mm^3)	40 ± 12	43 ± 8	73 ± 44	1990 ± 298[b]	42 ± 4	47 ± 6

[a] Values are means ± standard deviations for six animals/group, except for the 125 mg/kg-day female group with five animals. The limited information available did not clarify the disposition of the additional female that apparently started on study in the 125 mg/kg-day group.

[b] $p < 0.01$ versus controls, as calculated by the authors.

[c] $p < 0.05$ versus controls, as calculated by the authors. Source of data: Shimo et al. 1994

In the organ weight, increases of the liver, spleen, kidney weight and decreases of the testis and thymus were seen in the 125 mg/kg group.
Histopathology revealed spongiotic changes and brown pigmentation in perivascular region of the cerebellum, increased extramedullary hematopoiesis of the liver, brown pigmentation of renal tubular epithelium and degeneration of seminiferous tubular epithelium and atrophy of seminiferous tubule in the 125 mg/kg group.
Livers of both sexes in the high-dose group had increased incidences of extramedullary hematopoiesis in 5/6 males an 2/5 females and brown pigmentation in Kupffer's cell 5/6 males and 4/5 frmales.
The only histopathology finding in the kidney of the high-dose group animals was moderate brown pigmentation in the tubular epithelium in both sexes (5/6 males and 5/5 females).
In the 125 mg/kg group all 6 males had severe congestion, severe increased brown pigmentation and severe extramedullary haematopoiesis in spleen, while in females these alterations occur respectively in 5 (severe congestion), 2 (severe increased brown pigmentation) and 5 females.

In the 25 mg/kg group histopatological alterations were seen mainly in spleen: 4 out of 6 males and 4 out of 6 females had moderate congestion, 5 out of 6 males and all 6 females had moderately increased brown pigmentation in red pulp of spleen and all animals had moderately increased extramedullary haematopoiesis in spleen. 3 out of 6 female rats in the 25 mg/kg group had moderately increased haematopoiesis in bone marrow.
In the 5 mg/kg group no histopatological changes were found in internal organs except moderately increased extramedullary hematopoiesis in spleen of 3 out of 6 males and 3 out of 6 female rats, and moderately increased hematopoiesis in bone marrow in 1 out of 6 female rats.

However, the most sensitive changes were dose-dependent hematologic changes (see table above). There were approximately 30 % reductions in RBC count in 25 and 125 mg/kg group, hematocrit (Hct), and 13 % - 16% reduction in Hb concentration in 25 and 125 mg/kg group, in addition to increases in MCV and the WBC count; metHb concentrations were not reported.

The NOAEL and LOAEL for these reported changes according to US EPA (US EPA, 2009) were 5 and 25 mg/kg-day, respectively.

Comparison with classification criteria:
Taking into account that the duration of exposure was only 28 days the guidance values for specific target organ toxicity categories should be increased threefold in comparison with guidance values for 90-day exposure. Thus, in order to warrant classification the significant, severe effects should

be observed in a range of 30 - 300 mg/kg/day (3 x 10 – 100mg/kg) for STOT RE 2 and for Xn, R 48/22 at a dose of ≤ 150 mg/kg/day(3 x ≤50mg/kg) respectively.
The guidance dose level for classification in STOT RE 1 based on 28-day study would be below 30mg/kg/day and for Xn, R 48/25 to be below 15mg/kg.

In conditions of repeated oral exposure for 28 days at the dose 125mg/kg nitrobenzene induced significant toxicological effects such as pallor and gait abnormalities, large reduction in RBC count (-30%), level of haemoglobin (-16%) and hematocrit accompanied with severe congestion in spleen, severe or moderate increased brown pigmentation in spleen red pulp, increased extramedullary haematopoiesis in spleen and liver, hemosiderosis in kidney and cerebellum spongiotic changes in cerebellum, atrophy of seminiferous tubules and degeneration of seminiferous tubules,. All exposed animals had increased haematopoiesis in bone marrow. MetHb level was not measured.
At the repeated dose 25 mg/kg the haematological effects in males were comparable with that induced at 125 mg/kg, but in females the reduction of haematological indices were less pronounced than in females exposed at 125 mg/kg. Histopatological effects at the dose of 25 mg/kg were limited to spleen where moderate congestion, increased brown pigmentation and moderately increased extramedullary haematopoiesis were observed .
At the dose of 5 mg/kg reduction of haematological indices was not statistically or toxicologically significant. The histopatological changes were limited to moderately increased extramedullary haematopoiesis in spleen in 50% of animals and one females exhibited moderately increased haematopoiesis in bone marrow.
Thus severity of effects observed at the dose of 125 mg/kg meet criteria defined in section 3.9.2.7.2 of Annex i of CLP Regulation: any consistent and significant adverse changes in clinical haematology or significant organ damage noted at necropsy. The toxic effects observed at the dose of 25mg/kg should be considered as reversible, small changes in haematology and adaptive changes in internal organs that are not considered toxicologically relevant.

The observed effects warrant classification of nitrobenzene in category STOT RE 2 or in category Xn,R48 taking into account that severe effects at the dose of 125 mg/kg were observed after 28 days of exposure , thus below the guidance values for levels of oral exposure required for classification based on 28-day study results for STOT RE 2 (30 to 300 mg/kg/day) and for Xn, R 48/22 (≤ 150 mg/kg/day).

Study of Immunotoxicity of nitrobenzene in female B6C3F1 mice (Burns et al. 1994 quoted according to US EPA, 2009)

Burns et al. (1994) carried out a 14-day gavage study of nitrobenzene in corn oil in which female B6C3F1 mice were administered 0, 30, 100, and 300 mg/kg of the compound. The primary focus of the study was the immunotoxicity of the compound.
Examination of the mice at autopsy 24 hours after the final exposure showed hepatomegaly and splenomegaly in the 100 and 300 mg/kg groups, although the overall liver changes were slight.
The affected spleens were dark red in color, with mild congestion in the red pulp areas and the appearance of occasional nucleated erythrocytes.
Hemosiderin pigment was noted in the red pulp areas, a response thought to be indicative of erythrocyte dysfunction.

A number of apparently compound-related effects in hematologic responses to nitrobenzene were observed, consistent with the concept of the erythrocyte as a primary target organ of nitrobenzene toxicity.
The changes included decreases in erythrocyte number (7.64 ± 0.15 × 106 cells/μL in controls versus 6.94 ± 0.14 × 106 cells/μL in mice exposed to 300 mg/kg-day nitrobenzene) but increases in mean corpuscular volume (MCV) (56 ± 1 fL in controls versus 63.7 ± 1.4 fL in mice receiving 300 mg/kg-day) and mean cell hemoglobin concentration (MCHb) (18.1 ± 0.3 pg in controls versus 20.6 ± 0.6 pg in animals receiving 300 mg/kg).

However, there were no treatment-related changes in Hb concentration or hematocrit (Hct). Although no treatment-related differences in leukocyte differentials were observed after 14 days, there were striking changes in the percentage of circulating reticulocytes as a result of treatment (4.57 ± 0.48% in mice receiving 300 mg/kg versus 1.03 ± 0.9% in controls). MetHb was not evaluated.

***Comparison with classification criteria**:* The reduction in levels of HB did not meet ≥ 20% required by CLP and DSD classification criteria for repeated toxicity, and hemosiderin deposits in spleen of rats exposed at the level of 100 mg/kg can not be taken as meeting classification criteria defined in the Guidance on the Application of Regulation (EC) No 1272/2008 because they were not associated with reduction of Hb ≥ 10% or with microscopic changes like necrosis, fibrosis or cirrhosis

Nitrobenzene (14-day and 90-day gavage studies) in Fischer 344 rats and B6C3F1 mice (NTP 1983a cited from U.S. EPA (2009)

The National Toxicology Program (NTP, 1983) conducted a 90-day oral gavage study of nitrobenzene in F344 rats (10/sex/group) exposed to 0, 9.38, 18.75, 37.5, 75, and 150 mg/kg-day and B6C3F1 mice (10/sex/group) exposed to 0, 18.75, 37.5, 75, 150, and 300 mg/kg-day.

Fischer 344 rats
Clinical signs of toxicity in rats, such as ataxia, head tilt, lethargy, and trembling, were evident, mostly in animals receiving 150 mg/kg-day and, to a lesser extent, 75 mg/kg-day.

Nine male and three female rats at the 150 mg/kg-day dose level died prior to study completion. Overall, there was little change in body weight gain between control and treated groups, and the final body weights were not significantly different from controls at any dose level.

Organ weights appeared to have been dose dependently affected by nitrobenzene exposure, most notably in the case of liver, kidney, and testis (males). The liver weights and their ratios to body weight were dose dependently increased over control levels and achieved statistical significance compared with controls at all dose levels. Right kidney weight was increased over controls at all dose levels, and the ratio of kidney weight to final body weight was significantly increased over controls at the 9.38, 18.75, and 75 mg/kg-day dose levels. Right testis weight and its ratio to body weight were decreased in the 18.75–75 mg/kg dose range.

There were statistically significant changes in some hematologic parameters in rats exposed to nitrobenzene via gavage. As shown in Tables 5.6.1.1. and 5.6.1.2 the principal effects were dose-dependent decreases in hematocrit (Hct), Hb, and RBC count and dose-dependent increases in reticulocyte counts and metHb. In males, these changes achieved statistical significance compared

with controls at a dose of 9.38 mg/kg-day for metHb and Hb and 18.75 mg/kg-day for the other parameters. In females, the changes achieved statistical significance compared with controls at 37.5 mg/kg-day and above for the RBC count and at 9.38 mg/kg-day for the other parameters. Toxicologically these effects were mild or moderate and they were not exceeding 10% of Hb values in control rats. The functional reduction of Hb level including increase in MetHb levels did not exceed 20% which is a criterion for significant toxicity for substance inducing haemolytic anemia according to Guidance on the Application of Regulation (EC) No 1272/2008. However, the reduction in functional Hb male rats exposed for 90 days due to a combination of Hb reduction and MetHb increase amounted approximately to 5%, 15% and 16% in the 9,38mg/kg, 37.5 mg/kg and 75 mg/kg groups, respectively. In female rats the reductions in functional Hb were even lower than in males.

Table 5.6.1.1. Hematologic parameters, reticulocytes, and metHb levels in male F344 rats exposed to nitrobenzene via gavage for 90 days

Dose (mg/kg-day)	Hb (g/dL)[a]	Hct (%)[a]	RBCs (× 10_6)[a]	Reticulocytes (%)[a]	MetHb (%)[a]
0	16.24 ± 0.42	47.82 ± 3.2	9.06 ± 0.41	2.23 ± 0.44	1.13 ± 0.58
9.38	15.73 ± 0.29[b]	44.19 ± 4.98	9.01 ± 0.23	2.62 ± 0.45	2.75 ± 0.58[b]
18.75	15.54 ± 0.37[b]	41.84 ± 1.88[b]	8.70 ± 0.37[b]	3.72 ± 0.65[b]	4.22 ± 1.15[b]
37.5	14.72 ± 0.30[b]	37.66 ± 0.93[b]	7.97 ± 0.34[b]	4.75 ± 0.62[b]	5.62 ± 0.85[b]
75	14.87 ± 0.41[b]	38.08 ± 1.96[b]	7.61 ± 0.41[b]	6.84 ± 0.72[b]	7.31 ± 1.44[b]
150	16.2	38	6.31	15	12.22

[a] Values are means ± standard deviations, where n = 10 in each group except for the 150 mg/kg-day group with one male. [b] Significantly different from controls, as calculated by the authors. Source: NTP (1983a).

Table 5.6.1.2. Hematologic parameters, reticulocytes, and metHb levels in female F344 rats exposed to nitrobenzene via gavage for 90 days

Dose (mg/kg-day)	Hb (g/dL)[a]	Hct (%)[a]	RBCs (× 10_6)[a]	Reticulocytes (%)[a]	MetHb (%)[a]
0	15.82 ± 0.22	42.27 ± 3.41	8.39 ± 0.49	2.60 ± 0.37	0.94 ± 0.03
9.38	15.53 ± 0.29[b]	39.37 ± 1.26[b]	8.05 ± 0.28	3.69 ± 0.32[b]	2.06 ± 0.45[b]
18.75	15.49 ± 0.39[b]	39.59 ± 1.79[b]	8.01 ± 0.35	4.75 ± 0.68[b]	3.62 ± 1.09[b]
37.5	15.43 ± 0.38[b]	38.95 ± 0.62[b,c]	7.83 ± 0.35[b]	6.28 ± 0.90[b]	5.27 ± 0.76[b]
75	14.86 ± 0.52[b]	37.52 ± 1.11[b]	7.33 ± 0.30[b]	8.72 ± 1.49[b]	6.85 ± 2.25[b]
150	15.62 ± 0.60	35.88 ± 1.30[b]	5.86 ± 0.35[b]	32.07 ± 3.56[b]	12.77 ± 1.83[b]

For the reticulocyte and metHb effects, statistical significance compared to controls was achieved at all dose levels.

At necropsy, rats receiving 150 mg/kg-day nitrobenzene had enlarged spleens. Males at this dose level had enlarged livers, and those receiving 75 mg/kg-day and 150 mg/kg-day showed signs of testicular atrophy.

Histopathologic examination of the major organs and tissues revealed compound-related effects in the spleen, which appeared to be congested.

Splenic congestion is an abnormality that leads to elevated splenic vein pressure, which in turn results in higher sinusoidal pressure, and is commonly observed in laboratory animals in response to a variety of circumstances, including agonal death, method of euthanasia, or exposure to chemicals (US EPA, 2009). Administration to rodents of aromatic amine-type chemicals (e.g., aniline) may cause splenic congestion and hemorrhage, which are accompanied by hemosiderin deposition

(brown intracellular pigmentation due to insoluble iron), fatty change, and extramedullary hematopoiesis and fibrosis. These changes have been suggested to result from methemoglobinemia or accumulation in erythrocytes of toxic metabolites that are released in the spleen when RBCs are broken down in the red pulp. Sustained congestion causes the spleen to become more firm, enlarged, and fibrotic and renders the organ susceptible to trauma (US EPA).

Splenic corpuscles, i.e., small nodular masses of lymphoid tissue attached to the sides of the smaller arterial branches (splenic lymph follicles, malpighian corpuscles) in spleen of exposed rats were small, and the red pulp contained hemosiderin. The red pulp (also called splenic pulp), which may act as a reservoir for storing blood, is a soft mass of dark reddish-brown color resembling coagulated blood, and it is made of a fine reticulum of fibers divided into splenic sinuses and splenic cords. The splenic red pulp may undergo changes due to a variety of factors, including immune stimulation, changes in circulation, accumulation of macrophages, and connective tissue or pigment, and in response to increased demand for filtration of abnormal RBCs (cited from US EPA, 2009).

The severity of splenic congestion was graded by the study authors (NTP, 1983a). Control rats had no splenic congestion or minimal splenic congestion (grade 1). Congestion increased in severity up to moderate in the highest dose group. The incidence of these and other histopatological lesions in relation to dose is shown in Tables 5.6.1.3. and 5.6.1.4. These tables also report the incidence of splenic congestion of grade 2 or higher.

The toxicologically significant changes were observed in male rats of the 75 and 150 mg/kg group under a form of moderate spleen congestion and depletion of lymphoid tissue, although no fibrotic lesions were reported. The female rats seemed to be more prone for induction of such changes in spleen as they started to occur at the dose of 18.75mg/kg and their incidence increased with increase of the dose.

Significant increase of incidence of histopatological changes in testis occurred at the dose of 75 and 150 mg/kg.

The treatment related effects were seen in the brain stem of male and female rats at the dose of 150 mg/kg/day under a form of malacia and degeneration. Hemorrhage and vacualization frequency in the brain stem were not dose-dependent, so most probably they were not treatment related.

Liver congestion was observed in male rats at the dose 150mg/kg and kidney pigmentation suggesting haemosiderin deposits in kidneys of female rats at the dose of 75 mg/kg and 150 mg/kg.

Table 5.6.1.3. Selected histopathology findings in male F344 rats exposed to nitrobenzene for 90 days via gavage

	Nitrobenzene dose (mg/kg-day)					
Tissue examined	**0**	**9.38**	**18.75**	**37.5**	**75**	**150[a]**
Spleen						
Congestion	1/10	4/10	7/10	6/10	10/10	10/10
Congestion ≥ grade 2	0/10	0/10	0/10	0/10	5/10	10/10
Lymphoid depletion	0/10	0/10	0/10	1/10	9/10	10/10
Liver						
Congestion	0/10	0/10	0/10	0/10	0/10	6/10
Testis	0/10	0/10	0/10	1/10	9/10	9/9
Atrophy	0/10	0/10	0/10	0/10	10/10	9/9

Hypospermatogenesis Multinucleate giant cells	0/10	0/10	0/10	0/10	10/10	8/9
Brain stem						
Hemorrhage	0/10	1/10	4/10	4/10	5/10	2/10
Vacuolization	7/10	0/10	4/10	0/10	3/10	0/10
Degeneration	0/10	0/10	0/10	0/10	0/10	4/10
Malacia	0/10	0/10	0/10	0/10	0/10	4/10

[a] Includes tissue findings in nine rats that died between days 67 and 88. Source: NTP (1983a).

Table 5.6.1.4. Selected histopathology findings in female F344 rats exposed to nitrobenzene for 90 days via gavage

	Nitrobenzene dose (mg/kg-day)					
Tissue examined	**0**	**9.38**	**18.75**	**37.5**	**75**	**150[a]**
Spleen						
Congestion	2/10	5/10	10/10	10/10	10/10	10/10
Congestion ≥ grade 2	0/10	1/10	3/10	5/10	8/10	9/10
Lymphoid depletion	0/10	0/10	2/10	4/10	8/10	10/10
Kidney						
Pigmentation	0/10	0/10	0/10	0/10	5/10	9/10
Brain stem						
Hemorrhage	4/10	2/10	3/10	1/10	1/10	7/10
Vacuolization	6/10	3/10	1/10	1/10	1/10	5/10
Degeneration	0/10	0/10	0/10	0/10	0/10	4/10
Malacia	0/10	0/10	0/10	0/10	0/10	3/10

[a] Includes tissue findings in three rats that died between days 38 and 60. Source: NTP (1983a).

It should be noted that the recorded histopathology lesions in the high-dose male and female rats included the findings from animals that died prior to the full 90-day study duration (days 67–88 in males and days 38–60 in females). The extent to which some observed histopathologic effects in the liver were compound related is unclear, because hematopoietic foci and hepatocellular necrosis were evident in both treated and control rats. Hyaline droplets were noted in the cortical tubule cells of the kidney, and some pigmented granules were evident in the cells of a few treated rats. There were obvious compound-related histopathologic effects on the seminiferous tubules of the testis of male rats. In some cases, the tubules contained spermatogonia and spermatocytes, while in others there were very few or no spermatids, spermatozoa, and Sertoli cells. Some tubules appeared to contain only a lacy fibrinous material, and others contained multinucleate giant cells.
Histopathologic changes in the brains of treated rats included hemorrhage, vacuolization, and a wide range of inconsistent degenerative changes.

Based on the changes in absolute and relative organ weights and the dose-dependent increases in reticulocyte count and metHb concentration US EPA had identified a lowest-observed-adverse-effect level (LOAEL) of 9.38 mg/kg-day for the subchronic oral effects of nitrobenzene in F344 rats in this study (US EPA, 2009).

It should be noted that according to the CLP regulation the criterion for classification into STOT category is not any toxic effects observed in animals, but significant toxicological effects which have affected the function or morphology of a tissue or organ. Small, although statistically significant increase of a percentage of reticulocytes, changes in organ weight or low increase in level of MetHb should not be considered automatically as basis for classification in STOT category, although they should be taken into account in risk assessment and management. Further guidance on severity of toxic effects to be regarded as warranting classification in STOT category is

provided by the Guidance on the Application of Regulation (EC) No 1272/2008 and, in case of classification of hematoxic substance within DSD classification system, by the Hazard classification of chemicals inducing haemolytic anaemia: An EU regulatory perspective. Regulatory Toxicology and Pharmacology, 2006, 54, 3, pp 229-241(Muller et al. 2006).

The severity of effects observed at the dose levels of 37.5 - 150mg/kg such as lethality (only high dose), pigmentation (hemosiderosis) in kidney of female rats (75-150 mg/kg) combined with 10% or more reduction in level of functional hemoglobin, reduction in hematocrite, number of red blood celss, increase in percentage of reticulocytes linked with higher incidence of moderate spleen congestion (in females 37.5- 150 mg/kg, and in males 75-150mg/kg) and liver congestion (males 150 mg/kg) , although without fibrotic lesions, increased incidence of degeneration and malacia in brain stem (150 mg/kg) are judged as meeting criteria of significant hematoxicity and organ toxicity defined in the Guidance on the Application of Regulation (EC) No 1272/2008, section 3.9.2.5.2 point.

The intensity of the hematological effects leading to less than 10% reduction in level of functional hemoglobin (reduction of Hb plus increase in MetHb) at the dose of 9.38 – 18.75 mg/kg without significant microscopic changes in liver, kidney and brain and only with low increase in incidence of mild histopatological changes in spleen (congestion) without fibrotic changes in liver, kidney or spleen do not meet criteria of significant hematoxicity as defined CLP guidance or guidance developed for classification of substances inducing haemolytic anaemia within DSD framework (Muller et al. 2006).
Conclusion: The effects observed in this study meet classification criteria of STOT RE 2 because they demonstrated significant hematotoxicity below the guidance value of $\leq$ 100 mg/kg/day (CLP criteria) and meet classification criteria of category Xn, R48 because there were induced below a guidance value of $\leq$ 50 mg/kg/day for this category in DSD. However, please see section 5.6.5 for further discussion.

Mice.

Three male B6C3F1 mice receiving 300 mg/kg-day died prior to study completion, most likely as a result of nitrobenzene exposure. Some surviving animals at this dose level showed clinical signs of toxicity, including ataxia, hyperactivity, and irritability.

Mice exhibited signs of toxicity reflective of neurological impairment, increased liver and kidney weights, and decreased testis weight in male mice or decreased thymus in female mice. However, there were no compound-related changes in body weight gain at any dose level. Absolute and relative organ weight changes were confined to liver, kidney, and testis in male mice and to the liver, kidney, and thymus in females. For example, liver weight and its ratio to body weight were dose dependently increased in male mice, the increases achieving statistical significance at the 150 and 300 mg/kg-day dose levels. Relative kidney weight was significantly increased at 75 and 300 mg/kg-day in males. Absolute and relative testis weights were decreased at dose levels of 300 mg/kg-day. Treatment-related increases in absolute liver weights in female mice were evident at 18.75 mg/kg-day and above, with relative liver weights achieving statistical significance at a dose level of 37.5 mg/kg-day and above.

Hematologic responses observed in mice were similar to those in rats, with dose-dependent increases in reticulocytes and metHb (starting at a dose of 18.5mg/kg) and progressively lower levels of Hb, Hct, and RBCs (starting at the dose of 75 mg/kg) . These changes are documented in Tables 5.6.1.5 and 5.6.1.6.

Table 5.6.1.5. Hematologic parameters, reticulocytes, and metHb levels in male B6C3F1 mice exposed to nitrobenzene via gavage for 90 days

Dose (mg/kg-day)	n	Hb (g/dL)[a]	Hct (%)[a]	RBCs (× 10^6)[a]	Reticulocytes (%)[a]	MetHb (%)[a]
0	10	15.20 ± 0.66	41.77 ± 2.29	9.27 ± 0.75	5.02 ± 1.0	1.07 ± 0.32
18.75	10	14.59 ± 0.66	39.76 ± 2.89	8.87 ± 0.50	5.81 ± 0.88[c]	2.16 ± 0.32[b,c]
37.5	10	15.02 ± 0.92	41.13 ± 3.48	9.17 ± 0.76	6.95 ± 0.82[b,c]	3.42 ± 0.61[b,c]
75	9	14.63 ± 0.35[b]	39.56 ± 2.66	8.68 ± 0.52	7.85 ± 0.74[b]	4.75 ± 1.03[b]
150	10	14.44 ± 0.47[b]	37.62 ± 1.94[b]	8.25 ± 0.37[b]	9.30 ± 1.12[b]	5.98 ± 0.97[b]
300	7	15.45 ± 0.52[d]	36.26 ± 3.30[b,d]	7.79 ± 0.29[b,e]	10.45 ± 1.58[b]	6.72 ± 1.28[b]

[a] Values are means ± standard deviations. [b] Significantly different from controls, as calculated by the authors. [c] Summary statistics represent nine samples. [d] Summary statistics represent six samples. [e] Summary statistics represent five samples.
Source: NTP (1983a).

Table 5.6.1.6. Hematologic parameters, reticulocytes, and metHb levels in female B6C3F1 mice exposed to nitrobenzene via gavage for 90 days

Dose (mg/kg-day)	n	Hb (g/dL)[a]	Hct (%)[a]	RBCs (× 10^6)[a]	Reticulocytes (%)[a]	MetHb (%)[a]
0	9	15.66 ± 0.61	44.33 ± 3.41	9.54 ± 0.67	4.17 ± 0.35	0.87 ± 0.23
18.75	9	15.70 ± 0.60	44.24 ± 2.32	9.52 ± 0.35	5.54 ± 0.51[b]	1.20 ± 0.22[b]
37.5	10	15.24 ± 0.83	43.86 ± 2.30	9.21 ± 0.60	6.29 ± 0.61[b]	1.45 ± 0.34[b]
75	10	14.98 ± 0.50[b]	41.66 ± 1.71[b]	9.06 ± 0.44	6.72 ± 0.60[b]	1.82 ± 0.30[b]
150	10	14.96 ± 0.33[b]	40.98 ± 2.24[b]	8.81 ± 0.35[b]	7.31 ± 0.48[b]	2.25 ± 0.40[b]
300	10	15.99 ± 0.59	38.66 ± 2.69[b]	8.11 ± 0.61[b]	11.08 ± 1.96[b]	3.54 ± 1.39[b]

[a] Values are means ± standard deviations. [b] Significantly different from controls, as calculated by the authors.
Source: NTP (1983a).

Table 5.6.1.7. Selected histopathology findings in male B6C3F1 mice exposed to nitrobenzene for 90 days via gavage

Tissue examined	Nitrobenzene dose (mg/kg-day)					
	0	18.75	37.5	75	150	300
Spleen						
Lymphoid depletion	0/10	0/10	0/10	0/10	0/10	1/10
Liver						
Cytomegaly	0/10	0/10	0/10	1/10	2/10	10/10
Testis						
Atrophy	0/10	3/10	2/10	0/10	5/10	5/10
Hypospermatogenesis	0/10	0/10	0/10	0/10	0/10	4/10
Multinucleate giant cells	0/10	0/10	0/10	0/10	0/10	2/10
Brain stem						
Hemorrhage	3/10	1/10	3/10	0/10	0/10	2/10
Degeneration	0/10	0/10	0/10	0/10	0/10	1/10

Source: NTP (1983a).

Table 5.6.1.8. Selected histopathology findings in female B6C3F1 mice exposed to nitrobenzene for 90 days via gavage

Tissue examined	Nitrobenzene dose (mg/kg-day)					
	0	**18.75**	**37.5**	**75**	**150**	**300**
Spleen Lymphoid depletion	0/10	0/10	0/10	0/10	2/10	5/10
Liver Cytomegaly	0/10	0/10	0/10	0/10	0/10	8/10
Adrenal Fatty change	0/10	0/10	0/10	0/10	0/10	8/10
Brain stem Hemorrhage	2/10	2/10	1/10	2/10	0/10	3/10

Source: NTP (1983a).

Conclusion: The effects observed in mice do not meet classification criteria of STOT RE 2 because hematotoxicity linked with microscopic changes in internal organs were demonstrated only at doses of 150-300 mg/kg above a guidance value of $\leq$ 100 mg/kg/day (CLP criteria) and a guidance value of $\leq$ 50 mg/kg/day for Xn, R48 in DSD classification criteria. However, please see section 5.6.5 for further discussion.

5.6.2 Repeated dose toxicity: inhalation

Species, group size	conc. (mg/l)	Exposure time	Duration of treatment	Observations and remarks (effects of major toxicological significance)
rats F-344 (m+f)	0,10,35,125 ppm (0, 0.05, 0.18, 0.64 mg/L)	6h/d, 5d/w	14 d	**Subacute toxicity**: Blood: RBC↓, methb↑ ≥10 ppm Liver: ∅ Spleen: congestion, haematopoiesis↑, haemosiderosis↑ ≥10 ppm, capsular fibroblastic hyperplasia in m ≥35 ppm Testis: Germ cell degeneration, phagocytosis & maturation arrest, hypospermia, Sertoli cell hyperplasia at 125 ppm Kidneys: hyaline nephrosis at 125 ppm (in 10/10 males and 2/10 females) CNS: ∅ Other results: $LOAEC_{sys}$ 10 ppm, $NOAEC_{local}$ 125 ppm (Medinsky and Irons 1985)

rats CD (m+f)	0,10,35,125 ppm (0, 0.05, 0.18, 0.64 mg/L)	6h/d, 5d/w	14 d	Blood: anaemia at 125 ppm; RBC↓ & other red cell parameters ∅ at 10+35 ppm, methb↑ in f ≥10 ppm, in m ≥35 ppm, WBC↑ in m ≥35 ppm Liver: centrilobular or periportal degeneration at 125 ppm, single cell necrosis >35 ppm Spleen: congestion, haematopoiesis↑, haemosiderosis↑ ≥10 ppm Testis: Germ cell degeneration, phagocytosis & maturation arrest, hypospermia, at 125 ppm Kidneys: degeneration of cortical tubular cells at 125 ppm CNS: cerebellar haemorrhage, edema, malacia at 125 ppm Other results: morbidity, pulmonary vascular edema & congestion at 125 ppm $LOAEC_{sys}$ 10 ppm, $NOAEC_{local}$ 125 ppm (Medinsky and Irons 1985)
rats CD (m)	0,12,39,112 ppm (0, 0.06, 0.20, 0.57 mg/L)	6h/d, 5d/w	14 d	Blood: anaemia, methb↑, ≥12 ppm, immature RBCs and neutrophilia at 112 ppm Liver: ∅ Spleen: haemosiderosis↑≥39 ppm, lymphoid cell atrophy at 112 ppm Testis: Germ cell atrophy, oligospermia at 112 ppm Kidneys: creatinine↑ at 112 ppm CNS: cerebellar haemorrhage/edema in cerebellum/mid-brain/cervical spinal cord at 112 ppm Other results: morbidity thymus atrophy, pulmonary edema, ocular keratitis at 112 ppm $LOAEC_{sys}$ 12 ppm, no N(L)OAEC $_{local}$ (DuPont 1981)
mice B6C3F1 (m+f)	0,10,35,125 ppm (0, 0.06, 0.20, 0.57 mg/L)	6h/d, 5d/w	14 d	Blood: MCV↑,methb↑at 125 ppm Liver: centrilobular necrosis in m at 125 ppm hydropic degeneration ≥35 ppm Spleen: congestion, haematopoiesis↑, (occasionally) haemosiderosis↑ ≥35 ppm Testis: Tubular degeneration, aspermia, germ cell maturation arrest at 125 ppm Kidneys: tubular degeneration at 35 ppm CNS: cerebellar haemorrhage at 125 ppm Other results: morbidity at 125 ppm, bronchial hyperplasia >35 ppm ppm NOAECsys10 ppm, NOAEClocal10 ppm (Medinsky and Irons 1985)

Rats F-344 (m+f)	0,5,16, 50 ppm (0, 0.025, 0.08, 0.26 mg/L)	6h/d,5d/w	90 d	**Subchronic toxicity**: Blood: haemolytic anaemia ≥5 ppm, methb↑ in m≥5 ppm, f≥16 ppm Howell-Jolly bodies in m at 125 ppm Liver: focal centrilobular degeneration, liver cell cord disorganization ≥5 ppm Spleen: congestion, haematopoiesis↑, haemosiderosis↑, capsular fibroblastic hyperplasia ≥ 5 ppm stromal hyperplasia at 50 ppm Testis: Germ cell maturation arrest, tubular degeneration Leydig cell hyperplasia at 50 ppm Kidneys: nephrosis: cytoplasmatic eosinophilic droplets in proximal tubules in m ≥5 ppm, f at 50 ppm CNS: ND Other results: adrenals: medullary basophilia bronchial hyperplasia, bone marrow erythroid hyperplasia at 50 ppm $LOAEC_{sys}$ 5 ppm, $NOAEC_{local}$ 16 ppm (Hamm 1984)
Rats CD (m+f)	0,5,16, 50 ppm (0, 0.025, 0.08, 0.26 mg/L)	6h/d,5d/w	90 d	Blood: haemolytic anaemia ≥16 ppm, methb↑ in m≥16 ppm, in f at 50 ppm, leucocytosis, immature RBCs at 50 ppm Liver: hepatocytic basophilia/vacuolation & centrilobular hypertrophy, Kupffer cell pigmentation ≥16 ppm Spleen: congestion, haematopoiesis↑, haemosiderosis↑, capsule thickness↑ ≥5 ppm Testis: Tubular atrophy, Leydig cell hyperplasia, aspermia at 50 ppm, occasionally ≥5 ppm Kidneys: nephrosis at 50 ppm CNS: ND Other results: bone marrow erythroid hyperplasia ≥ 16 ppm, rhinitis, epithelial & goblet cell hyperplasia of nasal turbinates ≥16 ppm $LOAEC_{sys}$ 5 ppm, $NOAEC_{local}$ 5 ppm (Hamm 1984)

Mice B6C3F1 (m+f)	0,5,16, 50 ppm (0, 0.025, 0.08, 0.26 mg/L)	6h/d,5d/w	90 d	Blood: methb↑ at 50 ppm Liver: centrilobular hyperplasia/ hypertrophia, m ≥16 ppm, f ≥5 ppm Spleen: congestion, haematopoiesis↑, haemosiderosis↑ ≥5 ppm Testis: ND Kidneys: ND CNS: ND Other results: adrenals: cortical vacuolization ≥5 ppm bone marrow hyperplasia, bronchial hyperplasia at 50 ppm $LOAEC_{sys}$5 ppm, $NOAEC_{local}$16 ppm (Hamm 1984)
Rat F-344 (10m+10f)[§]	0,1,5,25 ppm (0, 0.005, 0.025, 0.13 mg/L)	6h/d,5d/w	15 mo	**Chronic toxicity**: Interim sacrifice groups of the CIIT cancer study: Blood: anaemia, polychromatic cells, Howell-Jolly bodies, methb↑ at 25 ppm, nucleated RBCs, leucocytosis in f at 25 ppm Liver: bilirubin↑ in m at 25 ppm cystic degeneration, eosinophilic cell foci, centrilobular hypertrophy in m≥5 ppm Spleen: haematopoiesis↑, congestion, haemosiderosis↑ ≥1 ppm Testis: ND Kidneys: increased severity or incidence of chronic nephropathy m≥5 ppm, slight increase in incidence of chronic nephropathy in f at 25 ppm CNS: ND Other results: endometrial polyps f≥1 ppm, pigmentation of olfactory ≥25 ppm, $LOAEL_{sys}$ 1 ppm $LOAEL_{local}$ 25 ppm (CIIT 1993)
rat Sprague - Dawley (CD) [§] (10m)	0,1,5,25 ppm (0, 0.005, 0.025, 0.13 mg/L)	6h/d,5d/w	15 mo	Interim sacrifice groups of the CIIT cancer study: Blood: anaemia, macrocytes, Howell-Jolly bodies, polychromasia in m at 25 ppm, methb↑ in m≥1 ppm Liver: centrilobular hypertrophy Kupffer cell pigmentation (haemosiderosis) m≥5 ppm Spleen: congestion ≥1 ppm, haematopoiesis↑, haemosiderosis↑ at 25 ppm Testis: ND Kidneys: ∅ CNS: ND Other results: nasal (resp.) epithelium hyperplasia, pigmentation of olfactory epithelium ≥25 ppm, $LOAEL_{sys}$ 1 ppm, $LOAEL_{local}$ 25 ppm. (CIIT 1993)

Abbreviations:
ALAT Alanin-Aminotransferase, d day/s, m males, f females, ND no data, ∅ no histopathological abnormalities, RBC red blood cell, methb methaemoglobin, MCH mean corpuscular haemoglobin, MCHC mean corpuscular haemoglobin concentration, MCV mean corpuscular volume, $N/LOAEC_{sys}$ No/Lowest observed adverse effect concentration for systemic effects, $N/LOAEC_{local}$ No/Lowest observed adverse effect concentration for local effects on the respiratory tract; § nonneoplastic lesions observed in the final sacrifice groups (2 y) were reported in Section 5.8 Carcinogenicity

CIIT study, 1985. In a Chemical Industry Institute of Toxicology (CIIT) study (Medinsky & Irons ,1985) 8- to 9-week-old Fischer-344 rats, Sprague-Dawley (CD) rats and B6C3F1 mice (10 per sex per dose) were exposed to nitrobenzene at concentrations of approximately 0, 51, 180 or 640 mg/m3 (0, 10, 35 or 125 ppm) via inhalation for 6 h per day, 5 days per week, for 2 weeks.

At an exposure level of 640 mg nitrobenzene/m3, there were severe clinical signs and a 40% rate of lethality in Sprague-Dawley rats after the fourth day and morbidity of all B6C3Fl mice, necessitating their early sacrifice; surviving Sprague- Dawley rats, exhibiting rapid shallow breathing, wheezing and orange urogenital staining, were sacrificed at the end of the first week.

In contrast, Fischer rats tolerated this level for 2 weeks without any adverse clinical signs. Significant concentration-dependent increases in relative liver, spleen and kidney weights were reported, primarily in Fischer rats; relative spleen weights were increased as much as 3 times those of control in Fischer rats and were still greater than controls in recovery animals (n = 5) at 14 days after exposure. Kidney and liver weights had recovered by day 14, but not by day 3, after exposure (quoted from EHC 230).

Comparison with criteria: Adverse significant clinical effects were seen at 0.64mg/l when exposed for 14 days, thus a level much higher then set in the criteria for STOT category 1 for effects seen in 90 day studies (≤ 0.2 mg/l/6h/day), but within the levels for category STOT 2 (a range between 0.2 and ≤1.0 mg/l/6h/day) and within the category Xn, R48 inhalation, rat 3 x ≤ 0,25 mg/l, 6 h/day = 0.75 mg/l, 6 h/day. However, please see section 5.6.5 for further discussion.

CIIT subchronic study, 1984/ Hamm, 1984. Central Institute of Industrial Toxicology reported in 1984 (CIIT, 1984) a subchronic study (reported also in this draft report as a study of Hamm, 1984), in which F344 rats, CD rats, and B6C3F1 mice, 10/sex/group, were exposed via inhalation to 0, 5, 16, or 50 ppm (respectively 0, 0.026, 0.082 and 0.260 mg/l) of nitrobenzene), 6 hours/day, 5 days/week **for 90 days** (quoted from Toxicology Review of Nitrobenzene,USA EPA, 2009)

There were no compound-related effects on body weight, mortality, or the occurrence of behavioral signs in the subchronic 90-day study. However, increases in spleen weights were evident in all strains and sexes of rats and mice exposed to nitrobenzene at the high concentration (0.26 mg/l) and at 16 ppm (0.082 mg/l) in male F344 and CD rats.

Gross Necropsy Results

In F-344 rats the only dose related gross findings were a uniformly distributed pattern of alternating pale brown and red areas measuring approximately $1mm^2$ on the surface of livers, pale circumscribed foci consistent with necrosis on the cut surface of liver, and enlarged spleens in the 50 ppm (0.26 mg/L) groups of both sexes. Small testicless were found in the 50 ppm (0.26 mg/L) male group. In CD rats the only dose related gross findings were prominent lobular markings in the livers of 50 ppm males, enlarged spleens in 50 ppm males and females, and small testes in 50 ppm

(0.26 mg/L) males. In B6C3F1 mice the only dose related gross finding was enlarged spleens in 50 ppm (0.26 mg/L) males and females.

Examination of the internal organs of exposed animals at necropsy confirmed that the liver, spleen, and testis were the primary target organs of nitrobenzene. For example, in high-concentration rats of either strain, males presented with testicular atrophy, enlarged spleens, and the presence of irregular blotches on the surface of the liver. Similarly, both sexes of B6C3F1 mice had enlarged spleens in response to nitrobenzene at 50 ppm. However, increases in spleen weights were evident in all strains and sexes of rats and mice exposed to nitrobenzene at the high concentration and at 16 ppm (0.082mg/l) in male F344 and CD rats. Most marked among the potential compound-related changes in hematologic or clinical chemistry parameters were the increased concentrations of serum metHb (Table 5.6.2.1) and a 50% increase in the concentration of bilirubin in male F344 rats exposed at concentration of 0.082 and 0.26 mg/l (16 and 50 ppm) to nitrobenzene.

Table 5.6.2.1. Concentrations of metHb in plasma of F344 and CD rats and B6C3F1 mice in response to nitrobenzene inhalation

		Concentration of nitrobenzene (ppm/mg/l)			
Strain/ species	**Sex**	**0/0**	**5ppm/ 0.026mg/l**	**16/0.082 mg/l**	**50/0.26 mg/l**
		Concentration of metHb in plasma (%)[a]			
F344 rat	Males	1.2 ± 0.4	3.0 ± 1.0[b]	4.4 ± 1.3b	10.1 ± 1.2b
	Females	1.6 ± 0.8	3.2 ± 0.9	3.9 ± 1.3b	10.5 ± 1.5b
CD rat	Males	0.6 ± 0.2	0.9 ± 0.6	3.2 ± 0.7b	10.1 ± 2.0b
	Females	2.1 ± 1.2	2.3 ± 0.6	3.7 ± 0.2	9.6 ± 2.5b
B6C3F1 mouse	Males	0.7 ± 0.6	1.6 ± 0.4	2.1 ± 1.3	5.8 ± 1.7b
	Females	1.3 ± 0.9	0.8 ± 0.5	2.0 ± 0.6	5.1 ± 0.8b

[a] Values are means ± standard deviations, where n = 5 except for the 16 ppm F344 rat female group with 4 animals.

[b] $p < 0.05$, as calculated by the authors. Source: CIIT (1984), Hamm, 1984.

Histologic sections of organs and tissues of nitrobenzene-receiving rats and mice demonstrated treatment-related lesions in the spleen, testis, liver, epididymides, kidney, and bone marrow, plus other possible target organs of nitrobenzene, such as the adrenals, lymph nodes, and lungs.

F344 rats

Proliferative capsular lesions were noted in the spleen. Three main morphological variants were encountered: focal or multifocal fibroblastic hyperplasia of the splenic capsule often containing encysted aggregates of lymphoid cells. This lesion was observed in one female out of 10 examined in the 0.026mg/L group, in one male out of 10 examined males in the 0.082mg/L group and in all animals exposed to 0.26mg/l. The second alteration was a proliferation of mesenchymal cells on the serosl surface, often encapsulated by a single layer of differentiated mesothelial cells, which occurred in 8/10 males and 10/10 females in the 0.26 mg/L group. A third histopatological lesion was detected in 10/10 males and in 6/10 females in the 0.26mg/L group and consisted of a focal accumulation of lymphocytes and macrophages immediately beneath the capsule, often accompanied by stromal hyperplasia extending into the spenic parenchyma. The results strongly suggest multifocal or diffuse fibrosis in the spleen of all animals exposed at 0.26/mg/l, while changes in the animals exposed at lower concentration were obsereved in single animals, were not dose-dependent and most probably not related to the treatment.

A hyaline droplet degeneration (Hamm, 1984) was observed in kidneys of nitrobenzene exposed F-344 rats. The lesion consisted of an accumulation of hyaline or eosinophilic droplets in the cytoplasm of proximal

tubular epithelial cells (droplet hyaline denegeration). The severity of the lesion, as well as its frequency, tended to vary with both concentration and sex. The lesion was of minimal severity in 6 out of 10 males in the 0.026 mg/L (5 ppm) dose group, mild to moderate severity in males exposed to 0.082 mg/l (16 ppm), and moderate severity in both males and females at the 0.26 mg/L (50 ppm) exposure concentration (Hamm et al. 1984). Such changes are due to disturbances in readsorption of protein by tubular epithelium cells form primary urine and they are reversible after cessation of the cause.

Moderate to severe degeneration of tubular epithelial cells was noted in the testes of all F344 rmales exposed to 0.26 mg/L (50 ppm) . This consisted of a maturation arrest at the level of primary and secondary spermatocytes and was usually accompanied by interstitial edema and Leydig cell hyperplasia. An absence of mature sperm was noted in the epididymus of these animals, together with the presence of proteinacious material within the lumen of the ductus.

In the liver, disorganization of hepatic cord architecture was noted in one female and one male rat exposed to 5 ppm and was accompanied by a slight degree of vascular dilatation and focal centrilobular hepatocyte degeneration. This lesion was not observed in any male rats and in only one female rat exposed to 16 ppm but was found in 7 of l0 males and one female exposed to 50 ppm (0.26 mg/L).

Proliferative changes were occasionally noted in lymph nodes of animals exposed to nitrobenzene. These consisted of a proliferation of plasma cells arranged in clusters or sheets extending from the subcapsular sinusoids to the medulla, containing numerous mitotic figures. This was sometimes accompanied by increased numbers of mast cells and an accumulation of macrophages at the margins of the lesion and the variable presence of multinucleated giant cells in the subcapsular cortical spaces.

Erythroid hyperplasia was evident in the bone marrow of a majority of male and female rats exposed to 50 ppm (0.26 mg/L) nitrobenzene.

Other histopathologic effects evident in F344 rats included basophilia of the medullary cells of the adrenal in 5/10 high-concentration males and in 3/10 high-concentration (0.26 mg/l) females, plus an increased incidence of bronchial hyperplasia in both sexes receiving the highest dose.

CD rats

The splenic lesions in CD rats consisted of sinusoidal congestion, increased extramedullary haematopoiesis, and numbers of hemosiderin-laden macrophages infiltrating the red pulp. An increase in the thickness of the splenic capsule was noted in 4/10 male and 3/10 female CD rats exposed to 0.26 mg/l (50 ppm) nitrobenzene; however no focal hyperplastic lesions were encountered.

In a manner similar to F344 rats, CD rats displayed dose-dependent toxic nephrosis, with 10/10 male and 5/10 female rats exposed to 0.26 mg/l (50 ppm) nitrobenzene displaying this condition. Additional minor renal lesions included an accumulation of lipid in proximal convoluted tubules, encountered only in 4 of 10 female rats exposed at 16 ppm, and a slight increase in glomerular cellularity, observed in one male exposed to 16 ppm, 2 males exposed to 50 ppm, and in one female exposed to 50 ppm.

Liver lesions in CD rats included an increase in basophilic cytoplasm (and/or vacuolization in hepatocytes) predominantly in periportal areas, centrilobular hepatocyte hypertrophy, some cells containing enlarged nucleoli, and increase in pigment-laden Kupffer cells.

CD rats also displayed a marked bilateral testicular atrophy in response to nitrobenzene, as indicated by a loss of seminiferous epithelium with only a few scattered spermatogonial cells

present, interstitial cell hyperplasia, oedema, and the absence of mature sperm in the epididymal lumen. These testicular lesions were found in 9 of 10 males exposed to nitrobenzene at the highest concentration – 0.26 mg/L , in one male exposed to 0.026mg/L (5ppm), and a slight reduction in mature sperm was found in 2 animals exposed to 0.082 mg/l (16 ppm).

There was erythroid hyperplasia in the bone marrow of approximately one-half the male and female rats exposed to 16 ppm (0.082 mg/l) and in almost all animals exposed to 50 ppm.

Lesions observed in the nasal passages consisted of rhinitis in association with epithelial hyperplasia on the free margins of the turbinates in the anterior passages and goblet cell hyperplasia on the ventral septum. These lesions were confined primarily to males exposed to 16 and 50 ppm (0.082 mg/l and 0.26 mg/L) and to females exposed to 50 ppm (0.26 mg/L).

A reactive lymphoid hyperplasia, together with a variety of other inflammatory changes, was noted in a number of rats exposed primarily to 16 or 50 ppm. These changes are complicated by the presence of inflammatory lesions in the lungs of some of these animals including interstitial and granulomatous pneumonitis and aggregates of macrophages and lymphocytes occurring primarily in perivascular areas.

These lung lesions are consistent with a low grade infectious agent and raise the possibility that lymph node changes were secondary to infection rather than treatment. These animals were negative when tested for serum antibodies to mycoplasma and common rat viruses.

S6C3F1 Mice

Extramedullary hematopoiesis was found in the spleen in all dose groups of both sexes of mice. This consisted of an increase in the total number and the proportion of precursor cells of erythroid and myeloid/megakaryocyte cell ines. This was apparent even though extramedullary hematopoiesis is a frequent finding in young untreated mice. These changes were accompanied by dose dependent increases in congestion and in the number of hemosiderin-laden macrophages present in the red pulp. A minimal to moderate decrease in cellularity in lymphoid dependent areas was noted in approximately half the animals of both sexes exposed to 50 ppm (0.26 mg/L) but was encountered in only 2 males exposed to 16 ppm (0.082mg/l). No splenic capsular lesions were noted in any of the mice.

An adrenal lesion, which was evident at the 5 ppm concentration and increased in severity with dose, was confined to female mice and consisted of a prominant cellular vacuolization that was restricted to the zona reticularis contiguous with the medulla.

There was a sex difference in the severity of treatment-related liver lesions. Male mice exhibited hepatocyte hyperplasia which was primarily centrilobular in distribution, the incidence and severity of which was dependent an exposure (4/9 at 16 ppm; 9/9 at 50 ppm). Affected cells had basophllic cytoplasm and contained enlarged and hyperchromatomatjc nuclei. Numerous multinucleated hepatocytes were observed, same with as many as 5 nuclei. A majority of female mice exposed to 50 ppm (7/9) exhibited a relatively less severe centrilobular hyperpiasla and hypertrophy resulting in same disorganization of normal cord architecture.

Additional lesions were noted in mice of both sexes exposed to 50 ppm, including a mild hyperplasia of the bronchial epithelium and a generalized bone marrow hyperplasia.

Comparison with criteria: Repeated exposure (90 days) to 0.26 mg/l nitrobenzene induced marked increase in the concentration of methoglobine up to 10% in F344 and CD rats. In addition at this concentration nitrobenzene caused increased spleen weight in rats and mice, congestion, proliferative fibrotic capsular lesions and increases in extramedullary hematopoiesis in spleen of all

F344 rats of either sex, sinusoidal congestion, increased extramedullary hematopoiesis, and numbers of hemosiderin-laden macrophages infiltrating the red pulp in CD rats with an increase in the thickness (fibrosis) of the splenic capsule was noted in 4/10 male and 3/10 female. In both strain of rats (F344 and CD) exposure to nitrobenzene at the concentration of 0.26 mg/l induced toxic nephrosis and in male F344 rats centrilobular degeneration of liver. These effects meet the criteria of significant hematotoxicity in the Guidance on the Application of Regulation (EC) No 1272/2008 such as e.g. significant increase in hemosiderosis in the spleen, liver or kidney in combination with microscopic effects like necrosis, fibrosis or cirrhosis with reduction in Hb level and increase in MetHb level.

At the concentration of 0.026 and 0.082 mg /l, lower than the upper limit value for STOT Category 1 of 0.2 mg/l/6h/day nitrobenzne induced statistically significant increases in concentration of methemoglobine in blood, but they were 2-3 times lower than in animals exposed at 0.26 mg/l/6h/day, and there is no clear evidence in study report on significant heamosiderosis in combination with microscopic changes such as necrosis, fibrosis or cirrhosis associated with increase in methemoglobin concentration or with other effects meeting classification criteria for substances causing haemolytic anamia.

Taking into account that these effects were observed at 0.26mg/l thus in a range of 0.2 and $\leq$ 1.0 mg/l/6h/day nitrobenzene meets criteria for STOT RE 2. However, please see section 5.6.5 for further discussion. Since the concentration of 0.26mg/l inducing significant organ toxicity is very close to DSD guidance value of $\leq$ 0.25 mg/l/6h/day for category Xn, R48 it is proposed to classify nitrobenzene to this category.

CIIT chronic study, 1993. A chronic inhalation study of nitrobenzene was conducted in F344 rats, Sprague-Dawley (CD) rats, and B6C3F1 mice (Cattley et al., 1994; CIIT, 1993) cited from US EPA 2009. .

A total of 70 male and female F344 rats and 70 male Sprague-Dawley (CD) rats were exposed to **0, 0.005, 0.026, 0.13 mg/l (**0, 1, 5, or 25 ppm) nitrobenzene, and a total of 70 male and female B6C3F1 mice were exposed to nitrobenzene at **0, 0.026, 0.13 and 0.26mg/l** (0, 5, 25, or 50 ppm), 6 hours/day, 5 days/week, excluding holidays, **for 2 years**, resulting in a total of 505 exposures (quoted after US EPA, 2009). Ten rats/sex/strain/group were terminated 15 months into the study to provide samples for an interim evaluation of hematologic parameters. Mice were evaluated at study termination but not at 15 months.

Effects of nitrobenzene on clinical signs, body weight changes, and survival appeared to be sporadic and unrelated to dose.

Both male and female F344 rats in the 0.13 mg/l (25 ppm) group displayed treatment-related statistically significant reductions in RBCs, Hct, and Hb concentration, with mean levels that were lower in animals sacrificed at term compared with animals sacrificed at 15 months. In F 344 male and female rats in the 0.13 mg/l (25 ppm) group the functional reductions of haemoglobin being a sum of reduction of Hb level in blood (ca. 10%) and an increase of MetHb (ca. 2%) was approximately 12% at the end of 2 years exposure. They were not larger in comparison to contral rats as after 15 months of exposure. The functional reduction of Hb level in blood of CD rats was lower than in F344 rats.

Concentrations of metHb increased with increasing nitrobenzene exposure, though time-related trends in this parameter were less clear-cut. Most notable among the hematologic responses in CD rats were the increases in metHb in the 15-month interim blood samples, as shown in Table 5.6.2.2. These achieved statistical significance ($p < 0.01$) versus controls at all dose concentrations employed in the study. No histopathology was performed on the spleens of CD rats at interim or

final sacrifice to determine if effects in the spleen accompanied the statistically significant increase in metHb levels. It should be noted, however, that, at final sacrifice, metHb levels were only increased in the 25 ppm exposure group, which may indicate a compensatory response to metHb formation. It should also be pointed out that background MetHb levels in both strains of control rats were consistently higher at 24 months than at 15 months, resulting in apparently less pronounced relative changes at 24 months than at 15 months among exposed animals (Table **5.6.2.2**).

In mice, RBCs and Hct were statistically significantly lower in 0.26mg/l (50 ppm) males than in controls (8.70 ± 0.12 versus 9.61 ± 0.29 × 106 cells/μL and 41.64 ± 0.52 versus 45.06 ± 1.15%, respectively). In common with the rats, there were statistically significant increases in metHb concentrations in high-dose mice of both sexes compared with controls. In mice exposed at 0.26mg/l the functional reduction of haemoglobin being a sum of reduction of Hb level in blood (ca. 4 %) and an increase of MetHb (ca. 2%) was approximately 6 % at the end of 2 years exposure.

Table 5.6.2.2. Percentage metHb formation in response to inhaled nitrobenzene

Treatment group ppm/mg/l	MetHb (%)			
	Interim sacrifice (15 months)		Terminal sacrifice (24 months)	
	Males	Females	Males	Females
B6C3F1 mice				
0	NA[a]	NA	1.97 ± 0.24	1.39 ± 0.20
5/0.026	NA	NA	1.94 ± 0.34	1.37 ± 0.18
25/0.13	NA	NA	3.02 ± 0.41	2.22 ± 0.26[b]
50 /0.26	NA	NA	3.97 ± 0.48[c]	2.79 ± 0.24[c]
F344 rats				
0	2.90 ± 0.31	2.35 ± 0.36	3.88 ± 0.33	2.68 ± 0.37
1/0.005	3.21 ± 0.18	3.33 ± 0.40	3.31 ± 0.32	2.13 ± 0.16
5/0.026	3.18 ± 0.43	3.17 ± 0.39	4.19 ± 0.53	2.54 ± 0.30
25/0.13	4.73 ± 0.52[c]	5.90 ± 0.96[c]	5.27 ± 0.33[c]	5.00 ± 0.45[c]
CD rats				
0	1.18 ± 0.34	NA	2.75 ± 0.52	NA
1/0.005	4.08 ± 0.80[c]	NA	2.87 ± 0.34	NA
5/0.026	6.22 ± 1.60[c]	NA	2.35 ± 0.32	NA
25/0.13	5.85 ± 0.83[c]	NA	4.60 ± 0.53[c]	NA

[a] NA = not applicable. [b] $p < 0.05$. [c] $p < 0.01$. Source: US EPA, 2009

Numerous noncancerous histopathologic lesions resulted from nitrobenzene inhalation, though some of these responses were not clear-cut because of a high incidence of the same effect in controls, which left the possibility that the response might be a nonspecific lesion due to age. For example, chronic nephropathy and extramedullary hematopoiesis of the spleen occurred in controls and at all concentration levels in both sexes of F344 rats and in male Sprague-Dawley rats. However, a number of histopathologic effects of nitrobenzene appeared to be compound related, including those in the nose, spleen, liver, kidney, and testis (Table 5.6.2.3).

Pigmentation of the olfactory epithelium was dose-dependently increased in male and female rats, with incidences of 99% in male F344 rats versus 60% of controls, 95% in male CD rats versus 67% of controls, and 100% in female F344 rats versus 55% of controls in the high-exposure groups. An increased incidence of focal inflammation and hypertrophy of the submucosal glands in areas lined by respiratory epithelium was observed in the nasal region of high-exposure male and female F344

rats. In CD rats, exposure-related lesions in nasal sections consisted of a slight increase in the incidence and severity of inflammatory changes in the anterior section of the nose.

Splenic pigmentation was assessed in male and female F344 rats. In male F344 rats, an exposure-related increase was observed (100% of 25 ppm exposed animals versus 80% of controls).

In contrast, 99% of female rats were found with this endpoint in the highest exposure group compared to 90% of controls.

Liver effects exhibited a mixed response with respect to exposure-dependent changes. Hepatic eosinophilic foci were observed in a dose-dependent manner in 81 and 23% of male and female F344 rats at the highest dose (25 ppm) compared with 38 and 8.6% of controls, respectively.

Male F344 rats exhibited an exposure-dependent increase in spongiosis hepatis (83% of animals at 25 ppm versus 36% of controls), whereas this endpoint was observed with only the high-exposure groups in 57% of male CD rats compared to 40% of controls and 9% of female F344 rats versus 0% of controls.

Table 5.6.2.3. Selected noncancer histopathologic changes in rats as a result of exposure to nitrobenzene via inhalation for 2 years
Sources: Cattley et al. (1994); CIIT (1993).

Target tissue	Exposure concentration (ppm/mg/l)							
	Males				Females			
	0/0	**1/0.005**	**5/0.026**	**25/0.13**	**0/0**	**1/0.005**	**5/0.026**	**25/0.13**
F344 rats								
Liver								
Eosinophilic foci	26/69	25/69	44/70a	57/70a	6/70	9/66	13/66	16/70a
Centrilobular hepatocytomegaly	0/69	0/69	8/70a	57/70a	0/70	0/66	0/66	0/70
Spongiosis hepatic	25/69	24/69	33/70	58/70a	0/70	0/66	0/66	6/70a
Kidney								
Tubular hyperplasia	2/69	2/68	2/70	13/70a	0/70	0/66	2/66	2/70
Nose								
Pigmented olfactory epithelium	40/67	53/67	67/70	68/69a	37/67	54/65	60/65	66/66a
Spleen								
Pigmentation	55/69	63/69	64/70	70/70a	62/69	61/66	60/66	68/69a
CD rats								
Liver								
Centrilobular hepatocytomegaly	3/63	1/67	14/70a	39/65a				
Spongiosis hepatic	25/63	25/67	25/70	37/65a				
Nose								
Pigmented olfactory epithelium	42/63	49/64	60/66	58/61a				
Testis								
Bilateral atrophy	11/62	17/66	22/70	35/61a				
Epididymis								
Bilateral hypospermia	8/60	13/65	15/67	32/59a				

[a] Statistically significantly different from control values, as calculated by the authors.

The number of male rats presenting with centrilobular hepatocytomegaly at necropsy was increased at 5 and 25 ppm nitrobenzene, with 81% of F344 rats and 60% of CD rats afflicted at the highest exposure level compared with 0 and 5% of controls, respectively; however, this endpoint was not detected in female F344 rats, regardless of exposure level. Changes in the kidney were restricted to the high-exposure group in male F344 rats, with less clear exposure-related changes in female F344 rats. Tubular hyperplasia was detected in 19% of male F344 rats versus 3% of controls, only 3% of female F344 rats at 5 and 25 ppm nitrobenzene, and none of the controls. Testicular changes were assessed in male CD rats. Clear exposure-dependent changes were observed for bilateral atrophy of the testis (57% at the highest dose; 18% of controls) and bilateral hypospermia of the epididymis (54% at the highest dose; 13% of controls).

Table 5.6.2.4. Selected noncancer histopathologic changes in B6C3F1 mice as a result of exposure to nitrobenzene via inhalation for 2 years

	Exposure concentration (ppm/mg/l)							
	Males				Females			
Target tissue	**0/0**	**5/0.026**	**25/0.13**	**50/0.26**	**0/0**	**5/0.026**	**25/0.13**	**50/0.26**
Liver								
Centrilobular hepatocytomegaly	1/68	15/65	44/65a	57/64a	0/51	0/61	0/64	7/62a
Multinucleated hepatocytes	2/68	14/65	45/65a	56/64a	0/51	0/61	0/64	2/62a
Lung								
Hyperplasia	1/68	2/67	8/65a	13/66a	0/53	2/60	5/64a	1/62
Bronchiolization	0/68	58/67a	58/65a	62/66a	0/53	55/60a	63/64a	62/62a
Thyroid								
Follicular cell hyperplasia	1/65	4/65	7/65a	12/64a	2/49	1/59	1/61	8/61
Nose								
Pigmented olfactory epithelium	0/67	7/66	46/65a	49/66a	0/52	6/60a	37/63a	29/61a
Degenerated olfactory epithelium	1/67	1/66	32/65a	41/66a	0/52	19/60a	47/63a	42/61a

[a] Statistically significantly different from control values, as calculated by the authors. Sources: Cattley et al. (1994); CIIT (1993) quoted .

In mice, tissue sites displaying increased incidence of nonneoplastic lesions included lung, olfactory epithelium, and, in the males, thyroid follicular cells and hepatocytes (Table 5.5.2.4). Histopathologic endpoints for the lung included hyperplasia and alveolar bronchiolization. In male mice, a clear exposure-dependent increase in hyperplasia was found, up to 20% in high-exposure animals versus 1.5% of controls. In contrast, female mice displayed a mixed response, with findings of hyperplasia in 3% of animals at 5 ppm, 8% at 25 ppm, and 2% at 50 ppm versus controls. Bronchiolization of the alveoli was increased at all exposure levels (male mice: 5 ppm, 87%; 25 ppm, 89%; and 50 ppm, 94%; female mice: 5 ppm, 92%; 25 ppm, 98%; and 50 ppm, 100%). This endpoint was not detected in any controls.

Additional effects of nitrobenzene on the respiratory tract were noted with statistically significant increases in the number of animals presenting with pigmentation and degeneration of the olfactory epithelium in the nasal region. Pigmented olfactory epithelium was detected in 74 and 48% of high-dose male and female mice, respectively. Similarly, an exposure-dependent increase in degenerated olfactory epithelium occurred in mice of both sexes (male mice: control, 1%; 5 ppm, 2%; 25 ppm, 49%; and 50 ppm, 62%; female mice: control, 0%; 5 ppm, 32%; 25 ppm, 75%; and 50 ppm, 69%). Lesions noted in nasal sections increased in severity with increasing dose (CIIT, 1993); however, severity scores were not reported.

A differential response was observed between male and female mice with histopathologic endpoints in the thyroid and liver.

In the thyroid, an exposure-dependent increase in follicular cell hyperplasia, up to 19% at 50 ppm, was found in male mice versus 2% of controls, whereas this effect was only observed in females up to 13% compared to 4% of controls at the highest exposure (50 ppm).

In the liver, male mice presented with exposure-dependent changes in centrilobular hepatocytomegaly and multinucleated hepatocytes, up to 89 and 88%, respectively. In contrast, centrilobular hepatocytomegaly was undetectable in female mice, except for the highest dose (11% above controls), as were multinucleated hepatocytes (3% above controls).

Comparison with classification criteria:

Formation of methemoglobin and haemolytic anaemia caused by nitrobenzene

In the chronic inhalation studies the hematotoxic effects in rats exposed at 0.13mg/l and mice exposed at 0.26mg/l were limited to mild increase in level of methemoglobin not exceeding 6% (with 1.39 – 2.75% in control animal), reduction in functional Hb level in blood of approximately 12 % in comparison with controls, reduction in RBC counts not exceeding 10% in comparison with control values, thus in the light of legal requirements and guideline on the application of the CLP regulation or DSD regulation such toxic changes were alone not sufficient to classify them as significant hematotoxicity. The observed haematological changes were not accompanied in by the histopatological changes in spleen, liver and kidney typical for long-lasting haemolytic anaemia. Although pigmentation in spleen was slightly more frequent (hemosiderosis) in highest exposed group than in controls, it was not leading to fibrosis of spleen, which was observed in the 90-day inhalation study at higher nitrobenzene concentration of 0.26mg/l. Thus results of this chronic experiment do not warrant classification of nitrobenzene to STOT RE category due to its hematotoxic effect.

Local effects in alveolar and olfactory epithelium

Bronchiolization. In all mice exposed to nitrobenzene at concentration of 0.026, 0.13 and 0.26 mg/l there was a dose dependent increase in frequency of animals with bronchialization of alveolar epithelium, i.e. metaplasia of a plane epithelium naturally existing in alveoli. The alveoli were re-lined by a proliferation of bronchiolar epithelium. Such changes may be followed by interstitial fibrosis of intraalveolar septa. None of control animals were showing such change. The lungs of rats were not examined histopatologically in this study (Cattley at al. 1994). In this study no signs of interstitial fibrosis were reported, thus only change in the type of alveoli epithelium cells should be considered. It is known that alveoli are lined with two main types of epithelial cells: type I pneumocytes (squamous pneumocytes) which are lining most of alveoli walls, and pneumocytes type II secreting surfactant which are cuboidal in shape and are disposed throughout pneumocytes type I. In case of inhalation exposure to mild irritant substances cuboidal pneumocytes type 2 may proliferate and re-line the alveoli (Glaister, 1986) so it may resemble that in bronchioli. The type II pneumocytes may subsequentially differentiate into type I of epithelium, thus such change of epithelium may be fully reversible after cessation of exposure. Since in this study no interstitial fibrosis was observed, the changes of reversible type in the composition of alveolai epithelium should not be interpreted as severe biological changes. The presence of such changes without other changes in interstitial tissue are not sufficient to warrant classification for STOT category.

The other biological change which occurred in male and female mice exposed for 2 years at concentration 0.13 and 0.26 mg/l, and in female mice exposed at conc. of 0.026 mg/l, was increased pigmentation in olfactory epithelium and increased frequency of degeneration of olfactory epithelium. The increased pigmentation in olfactory epithelium was observed also in female and male F344 rats and male CD rats. The increased pigmentation was most probably due to deposition of nitrobenzene in epithelial cells of nose, particularly in olfactory epithelium which is located in upper part of nose (fornix) of nasal cavity, which is also without ciliated and goblet cells assisting in cleaning of other parts of nasal epithelium of mice and rats. Lower self-cleaning ability of olfactory epithelium was most probably linked to increased frequency of degenerative and inflammatory lesions of that part of nose in

mice and rats. Such changes occurs frequently in rodents exposed to mild irritants since they breath only through nose. The significance of such changes for humans who are breathing through nose and mouth might be overestimated. The changes in olfactory epithelium are not sufficiently characterized in an article of Cattley et al. (1994) to warrant classification for STOT category.

5.6.3 Repeated dose toxicity: dermal

Species group size	Dose mg/kg	Exposure time	Duration of treatment	Observations and remarks (effects of major toxicological significance)
Mouse B6C3F1; Rat F-344 (m+f)	200 1600 3200		14 d	Range finding study: Nitrobenzene was administered to $B6C3F_1$ mice and Fischer-344 rats (both sexes) by skin painting at doses in the range 200–3200 mg/kg of body weight per day for 14 days. All rats and mice at the 1600 and 3200 mg/kg bw/d doses died or were sacrificed moribund prior to the end of treatment. Treated animals were inactive, ataxic, prostrate and dyspnoeic. Significant depression of weight gain (>10%) was seen in mice from all dose groups. Histologically, mice and rats showed changes in the brain, liver, spleen and testes, with mice less affected than rats. Reticulocyte counts and methaemoglobin levels were increased in mice and rats (all dosage groups except mice receiving lowest dose, 200 mg/kg bw/d); haemoglobin and RBC were decreased in rats (no details were given in the report). (NTP, 1983b, cited from EHC Report 2003)
Mouse B6C3F1 (10m+10f)	0, 50, 100, 200, 400 or 800, daily, on shaved skin in the interscapular area		13 weeks	Nitrobenzene was administered to $B6C3F_1$ mice (10 per sex per group) by skin painting (in acetone vehicle) at 0, 50, 100, 200, 400 or 800 mg/kg of body weight per day for 13 weeks (NTP, 1983b); the chemical was applied to a shaved area of the skin in the intercapsular region.

Species group size	Dose mg/kg	Exposure time	Duration of treatment	Observations and remarks (effects of major toxicological significance)
				(contd.) No effect on mean final body weights. Six high-dose males were sacrificed moribund, and three died between weeks 3 and 10; seven high-dose females were sacrificed moribund, and one high-dose female and one female of the 100 mg/kg bw/d group died between weeks 2 and 9. Clinical signs in some animals at the high dose included inactivity, leaning to one side, circling, dyspnoea, prostration and, in one, head tilt, whereas a number of dosed females had extremities cold to the touch. One high-dose female exhibited tremors, and two were insensitive to painful stimuli. Inflammation of the skin (diffuse or focal and of minimal to mild severity) was seen at the site of nitrobenzene application at the two highest doses; inflammatory cells were present in the dermis, with varying degrees of involvement of the subcutaneous tissue. There was acanthosis and hyperkeratosis of the epidermis, with occasional thick crusts of necrotic cells or focal areas of necrosis extending deep into the epidermis. Liver weights in treated male mice from the 400 mg/kg bw/d group and females from the 400 and 800 mg/kg bw/d groups were significantly increased compared with controls. At the high dose, a number of periportal hepatocytes were smaller than those in control livers and in treated mice, and there was a noticeable variation in the size of hepatocyte nuclei, especially in the centrilobular zone. The cytoplasm of hepatocytes in many treated mice had a homogeneous eosinophilic appearance, whereas that in controls had a vacuolated appearance characteristic of glycogen-containing cells. While degeneration of the "X" zone of the adrenal glands (the zone of cells adjacent to the medulla) in female mice was noted, the degree of vacuolation in treated animals was reported to be greater than normally seen in controls. Brain lesions were found in 2 of 10 males and 3 of 10 females at 800 mg/kg bw/d; the lesions appeared to be localized in the brain stem in the area of the vestibular nucleus and/or cerebellar nuclei; one high-dose female had a mild bilateral lesion in a nucleus of the ventrolateral thalamus. Such lesions were probably responsible for the clinical behavioural findings of head tilt, leaning to one side and circling. Brain vascular lesions (as described in the rat dermal study; see below) were not observed in this mouse dermal study.

Species group size	Dose mg/kg	Exposure time	Duration of treatment	Observations and remarks (effects of major toxicological significance)
				(cont.) No clear NOAEL was established in this study, with the following findings (among others) noted at the lowest dose of 50 mg/kg bw/d: lung congestion, adrenal cortical fatty change and variation in the size of hepatic nuclei, especially the centrilobular zone. (NTP, 1983b, cited from EHC Report 2003)
Rat F-344 (10m+10f)	0, 50, 100, 200, 400 or 800, daily		13 weeks	Nitrobenzene was administered to Fischer-344 rats (10 per sex per group) by skin painting (in acetone vehicle) at 0, 50, 100, 200, 400 or 800 mg/kg of body weight per day for 13 weeks (NTP, 1983b); the chemical was applied to a shaved area of the skin in the intercapsular region. Mean final body weights were not significantly affected; the body weights in the high-dose groups were not analysed due to a high incidence of early deaths. Seven high-dose male rats died and 3 of 10 were sacrificed moribund between weeks 4 and 10; five high-dose females died and five were sacrificed between weeks 2 and 12. Clinical signs in high-dose males included ataxia, head tilt, lethargy, trembling, circling, dyspnoea, forelimb paresis, splayed hind limbs, diminished pain response and reduced righting response. Except for dyspnoea in a few females, the other clinical signs were not noted in females. The extremities of a number of rats (both sexes) were cold to the touch and/or cyanotic. Brain lesions were found in both sexes at 800 mg/kg bw/d; the lesions appeared to be localized in the brain stem to areas of the facial, olivary and vestibular nuclei and to cerebellar nuclei and probably correlate with the clinical behavioural findings. These lesions were characterised by demyelination, loss of neurons, varying degrees of gliosis, haemorrhage, fibrin in and around small vessels and occasional capillary proliferation. The brain vascular lesions were characterised by fibrin in and around vessel walls; red blood cells within macrophages at the site of haemorrhage indicated that the effect was real, not an agonal change or secondary to tissue mishandling at sacrifice. Perivascular haemosiderin-containing macrophages were occasionally observed. Brain vascular lesions as described in this dermal study were not observed in the Fischer-344 rat gavage study or in the B6C3F1 mouse dermal study (see above). No clear NOAEL was established in this study, with lung congestion and fatty change in the adrenal cortex in addition to the haematological findings noted at the lowest dose of 50 mg/kg bw/d. (NTP, 1983b, cited from EHC Report 2003)

Species group size	Dose mg/kg	Exposure time	Duration of treatment	Observations and remarks (effects of major toxicological significance)
Conclusion: R-phrase 48/24 is confirmed (see Summary and discussion).				

NTP sponsored a 90-day skin painting toxicological study (NTP, 1983b cited from US EPA, 2009) with nitrobenzene in F344 rats and B6C3F1 mice. The authors treated F344 rats and B6C3F1 mice (10 animals/sex/group) with 50, 100, 200, 400, and 800 mg/kg-day nitrobenzene in acetone, the responses being compared with those in animals painted with acetone alone. \

At 800 mg/kg-day, all rats and 9/10 male and 8/10 female mice died before the end of the experiment. Furthermore, surviving animals in the other exposure groups (dose levels not stated) displayed profound clinical signs of acute toxicity, including ataxia, dyspnea, circling, lethargy, and insensitivity to pain.

Only female mice showed a dose-related increase in metHb concentration.

Among the histopathologic findings, there was a marked degeneration of the testes in the males of both species and all nitrobenzene-receiving rats displayed congestion of the spleen. The incidence of congestion of the lungs was dose-dependently increased in males and females of both species. Vacuolization of the brain or brain stem was another characteristic histopathologic finding, the effects becoming apparent in rats exposed to nitrobenzene at 100 mg/kg or higher, in male mice exposed to 800 mg/kg, and in female mice exposed to 400 and 800 mg/kg nitrobenzene. Tables 5.6.3.1., 5.6.3.2., 5.6.3.3., and 5.6.3.4. document these histopathologic changes.

Table 5.6.3.1. Incidence of histopathologic lesions in male F344 rats exposed to nitrobenzene for 90 days via dermal exposure

Target tissue	Dose (mg/kg-day)					
	0	**50**	**100**	**200**	**400**	**800**
Lung Congestion	1/10	1/10	7/10	4/10	4/10	10/10
Spleen						
Congestion	0/10	10/10	10/10	10/10	10/10	10/10
Hematopoiesis	10/10	10/10	10/10	10/10	10/10	10/10
Lymphoid atrophy	0/10	0/10	7/10	7/10	10/10	10/10
Liver						
Congestion	0/10	1/10	0/10	0/10	0/10	6/10
Kidney						
Congestion	0/10	0/10	0/10	0/10	0/10	7/10
Testis						
Atrophy	0/10	0/10	0/10	0/10	10/10	10/10
Hypospermatogenesis	0/10	0/10	0/10	0/10	10/10	10/10
Multinucleate giant cells	0/10	0/10	0/10	0/10	9/10	10/10
Brain						
Hemorrhage	1/10	4/10	0/10	0/10	2/10	2/10

Table 5.6.3.2.. Incidence of histopathologic lesions in female F344 rats exposed to nitrobenzene for 90 days via dermal exposure

Target tissue	Dose (mg/kg-day)					
	0	**50**	**100**	**200**	**400**	**800**
Lung Congestion	1/10	1/10	3/10	1/10	6/10	9/10
Spleen						
Congestion	8/10	10/10	10/10	9/10	10/10	10/10
Hematopoiesis	0/10	10/10	10/10	10/10	10/10	10/10
Lymphoid atrophy	0/10	0/10	0/10	1/10	9/10	10/10
Liver						
Congestion	0/10	0/10	0/10	0/10	0/10	4/10
Kidney						
Congestion	0/10	0/10	0/10	0/10	4/10	4/10
Uterus						
Atrophy	0/10	0/10	0/10	0/10	0/10	6/10
Brain						
Hemorrhage	0/10	1/10	5/10	2/10	1/10	2/10
Cerebrum						
White matter vacuolization	0/10	0/10	10/10	10/10	4/10	3/10
Cerebellum						
White matter vacuolization	0/10	0/10	8/10	4/10	7/10	6/10
Brain stem						
Hemorrhage	0/10	1/10	1/10	4/10	7/10	6/10
Vacuolization	0/10	0/10	10/10	8/10	4/10	3/10

Source: NTP (1983b).

Table 5.6.3.3. Incidence of histopathologic lesions in male B6C3F1 mice exposed to nitrobenzene for 90 days via dermal exposure

Target tissue	Dose (mg/kg-day)					
	0	**50**	**100**	**200**	**400**	**800**
Lung Congestion	2/10	6/10	4/10	4/10	10/10	9/10
Spleen						
Congestion	0/10	0/10	0/10	0/10	0/10	10/10
Hematopoiesis	1/10	3/10	3/10	9/10	9/10	10/10
Lymphoid atrophy	0/10	0/10	0/10	0/10	0/10	3/10
Liver						
Congestion	0/10	0/10	0/10	1/10	10/10	10/10
Pigmentation	0/10	0/10	0/10	0/10	0/10	6/10
Thymus						
Atrophy	0/10	0/10	0/10	0/10	0/10	7/7
Testis						
Atrophy	0/10	0/10	0/10	0/10	5/10	10/10
Hypospermatogenesis	0/10	0/10	0/10	0/10	2/10	10/10
Multinucleate giant cells	0/10	0/10	0/10	0/10	0/10	4/10
Brain						
Hemorrhage	1/10	1/10	3/10	1/10	0/10	2/10
Brain stem						
Hemorrhage	1/10	1/10	2/10	1/10	1/10	6/10
Degeneration	0/10	0/10	0/10	0/10	0/10	3/10
Skin						
Inflammation	0/10	0/10	0/10	0/10	8/10	3/10

Table 5.6.3.4. Incidence of histopathologic lesions in female B6C3F1 mice exposed to nitrobenzene for 90 days via dermal exposure

Target tissue	Dose (mg/kg-day)					
	0	**50**	**100**	**200**	**400**	**800**
Lung						
Congestion	4/10	3/10	2/10	4/10	8/10	10/10
Spleen						
Congestion	0/10	0/10	1/10	0/10	2/10	9/10
Hematopoiesis	7/10	4/10	3/10	7/10	10/10	9/10
Lymphoid atrophy	0/10	0/10	1/10	0/10	0/10	3/10
Liver						
Cytomegaly	0/10	0/10	0/10	0/10	0/10	8/10
Thymus						
Atrophy	0/10	0/10	0/10	0/10	0/10	9/9
Ovary						
Atrophy	0/10	0/10	0/10	0/10	0/10	3/10
Uterus						
Atrophy	0/10	0/10	0/10	1/10	1/10	5/10
Adrenal cortex						
Fatty change	0/10	6/10	9/10	10/10	8/10	2/10
Brain						
Hemorrhage	0/10	1/10	0/10	1/10	3/10	2/10
Brain stem						
Hemorrhage	1/10	0/10	0/10	0/10	2/10	4/10
Degeneration	0/10	0/10	0/10	0/10	1/10	3/10
Skin						
Inflammation	0/10	0/10	0/10	0/10	9/10	7/10

Comparison with classification criteria:

The following significantly toxic effects, meeting classification criteria in CLP and DSD systems, were seen in F344 rats and B6C3F1 mice expose dermally at 50 and 100 mg/kg bw/day for 13 weeks:

in male rats - spleen congestion starting from 50 mg/kg, lung congestion and lymphoid atrophy starting at 100 mg/kg;

in female ratss lung congestion at 100mg/kg,

in male mice: lung congestion starting at 50 mg/kg, in female mice fatty change in the adrenal cortex in addition to the haematological findings noted at the lowest dose of 50 mg/kg bw/d. These effects meet criteria of advers health effects at the dose levels in a range between 20 and 200 mg/bw /day for STOT REP, Category 2 (CLP) or dose levels ≤100 mg/kg (bodyweight)/day for category Xn, R48/24. However, please see section 5.6.5 for further discussion.

5.6.4 Other relevant information

Species group size	Dose mg/kg	Exposure time	Duration of treatment	Observations and remarks (effects of major toxicological significance)

Species group size	Dose mg/kg	Exposure time	Duration of treatment	Observations and remarks (effects of major toxicological significance)
Rat F-344 (≈ 50m total)	200,400,600 (gavage)		Single application following a 28 d feeding, groups with different diets (with/ without 5-8% pectin)	Methaemoglobinaemia: Elevated Methb levels up to 60%, concentration raised at 1 h, maximum at 4 h (Goldstein et al. 1984)
Rat SD (5m/group)	50 study start at an age of 6, 8, 10 or 40 weeks		2 or 4 weeks	Dysspermatogenesis: Reduced sperm numbers and testes weight (aggravation with duration), depressed sperm activity (no effect of duration), (Koida and coworkers, 1985, abstract only)
Rat Wistar			5 or 10 weeks	Neurotoxicity: Microscopic examination of the olfactory bulb revealed a degeneration of the mitral cell layer, representing the principal relay neurones, with the most densely degeneration in the ventral region. (Pinching and Doving 1974)
Rat F-344 (12 m/group)	550 (oral)		Single treatment and sacrifice after 6, 24, or 48 h	Petechial haemorrhages in the brain stem and cerebellum, and bilaterally symmetric degeneration (malacia) in the cerebellum and cerebellar peduncles developed within 48 hours after treatment. (Morgan et al. 1985)
				In vitro studies: Dysspermatogenesis: Nitrobenzene was directly toxic on testicular cells in vitro. In Sertoli cell cultures and Sertoli-germ cell cocultures vacuolation of Sertoli cells (at 10-3 M), exfoliation of germ cells (5x10-4 M), secretion of lactate and pyrovate by Sertoli cells (>5x10-4 M). Inhibin secretion by Sertoli cells as a potential marker for stimulation of FSH hormone release was altered in a biphasic manner, with low (10^{-8} to 10^{-6} M) and high (10^{-4} to 10^{-3} M) doses enhancing inhibin secretion while intermediate (10^{-5} M) doses had no effect. (Allenby et al. 1990)

5.6.5 Summary and comparison with classification criteria

Nitrobenzene is already classified and labelled as Toxic, T, R 48/23/24. According to the criteria of the Directive 67/543/EEC, the extension of the labelling to R 48/23/24/25 is proposed. According to the Directive 1272/2008 labelling to H372 STOT-RE 1 (inhalative; haematopoietic system, liver, testis, CNS, kidneys, adrenals, bronchial/nasal passages) is proposed. "causes damage to organs through prolonged or repeated exposure”

5.6.5.1 Comparison with classification criteria

Classification criteria to be met According to Regulation No. 1272 (section 3.9.2.) substances are classified as specific target organ toxicants following repeated exposure by the use of expert judgement on the basis of the weight of all available evidence. To category 1 for STOT-RE are classified 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.

Substances are classified in category 2 for target organ toxicity (repeat exposure) on the basis of observations from appropriate studies in experimental animals in which significant toxic effects, of relevance to human health, were produced at generally moderate exposure concentrations.

As defined in 3.9.2.7.3. of CLP regulation the evidence from appropriate studies in experimental animals can furnish much more detail, in the form of clinical observations, haematology, clinical chemistry, and macroscopic and microscopic pathological examination, and this can often reveal hazards that may not be life-threatening but could indicate functional impairment. Consequently all available evidence, and relevance to human health, shall be taken into consideration in the classification process, including but not limited to the following toxic effects in humans and/or animals:

(a) morbidity or death resulting from repeated or long-term exposure. Morbidity or death may result from repeated exposure, even to relatively low doses/concentrations, due to bioaccumulation of the substance or its metabolites, and/or due to the overwhelming of the de-toxification process by repeated exposure to the substance or its metabolites;

(b) significant functional changes in the central or peripheral nervous systems or other organ systems, including signs of central nervous system depression and effects on special senses (e.g. sight, hearing and sense of smell);

(c) any consistent and significant adverse change in clinical biochemistry, haematology, or urinalysisparameters;

(d) significant organ damage noted at necropsy and/or subsequently seen or confirmed at microscopic examination;

(e) multi-focal or diffuse necrosis, fibrosis or granuloma formation in vital organs with regenerative capacity;

(f) morphological changes that are potentially reversible but provide clear evidence of marked organ dysfunction (e.g., severe fatty change in the liver);

(g) evidence of appreciable cell death (including cell degeneration and reduced cell number) in vital organs incapable of regeneration.

Guidance on dose/concentration values from 90 day studies to assist classification for Category 1 are provided in table 3.9.2 and for Category 2 in table 3.9.3 of CLP regulation.

- For oral exposure (rat) they are either ≤ 10 mg/kg/day for Category 1 or in a range between 10 and 100mg/bw /day for Category 2,

- For vapour inhalation (rat) they are ≤ 0.2 mg/l/6h/day for Category 1 or in a range between 0.2 and ≤1.0 mg/l/6h/day for Category 2

- For dermal exposure (rat or rabbit) they are ≤ 20 mg/kg/day for Category 1 or in a range between 20 and 200 mg/bw /day for Category 2

It should be noted that as defined in point 3.9.2.8.1. of Annex I of the Regulation (EC) No 1272/2008 there are some effects in humans and/or animals that do not justify classification. Such effects include, but are not limited to:

(a) clinical observations or small changes in bodyweight gain, food consumption or water intake that have toxicological importance but that do not, by themselves, indicate 'significant' toxicity;

(b) small changes in clinical biochemistry, haematology or urinalysis parameters and/or transient effects, when such changes or effects are of doubtful or minimal toxicological importance;

(c) changes in organ weights with no evidence of organ dysfunction;

(d) adaptive responses that are not considered toxicologically relevant;

(e) substance-induced species-specific mechanisms of toxicity, i.e. demonstrated with reasonable certainty to be not relevant for human health, shall not justify classification.

In the Guidance on the Application of Regulation (EC) No 1272/2008, section 3.9.2.5.2. **Hematotoxicity** the following examples of effects not warranting classification are listed

- Significant decrease in Hb without any other significant indicators of haemolytic anaemia.
- Minimal to slight increase in MetHb formation without any other indications of significant haemolytic anaemia.

As summarized in section 5.6. Repeated dose toxicity nitrobenzene is inducing methemogobinemia and other hematological effect, therefore a detailed comparison also of these effects with classification criteria is needed before classification can be made. There are two guidance documents which could be helpful in this comparison:

- Guidance on the Application of Regulation (EC) No 1272/2008, section 3.9.2.5.2. and
- Hazard classification of chemicals inducing haemolytic anemia: An EU regulatory perspective by EU Working Group on Haemolytic Anaemia (2006).

In the assessment of all individual hematological effects as well as totality of findings it should be to judged whether they constitute an adaptive response or an adverse toxicologically significant effect.

There are several studies on animals exposed by:

- oral route (NTP 1983a cited from US EPA 2009; Shimo et al. 1994; Burns st al;1994; Mitsumori et al. 1994),
- inhalation (Medinsky and Iron 1985; DuPont 1981; Hamm 1984, CIIT 1993) or
- through skin (NTP 1983b cited form US EPA 2009)

which have been reviewed in the background document and can be used for assessment specific target organ toxicity.

These studies demonstrate that the most sensitive cells to toxicity of nitrobenzene are erythrocytes where nitrobenzene induces formation of methemoglobin, which leads to premature destruction of erythrocytes, reduction of haemoglobin level in blood, increased medullary and extramedullary

haematopoiesis, increased in percentage of reticulocytes in blood, deposition of hemosiderin in spleen, liver and kidney with eventual fibrosis of these organs.

According to Regulation No. 1272 (section 3.9.2.) substances are classified as specific target organ toxicants following repeated exposure by the use of expert judgement on the basis of the weight of all available evidence. To STOT-RE categories are classified 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. According to Directive 67/548/EEC only effects causing serious damage to health such as death, clear functional disturbance or morphological changes should be considered for classification. It should be noted that as defined Annex I of the Regulation (EC) No 1272/2008 (3.9.2.8.1.) there are some effects in humans and/or animals that do not justify classification. Such effects include, but are not limited to:

(a) clinical observations or small changes in bodyweight gain, food consumption or water intake that have toxicological importance but that do not, by themselves, indicate 'significant' toxicity;

(b) small changes in clinical biochemistry, haematology or urinalysis parameters and/or transient effects, when such changes or effects are of doubtful or minimal toxicological importance;

(c) changes in organ weights with no evidence of organ dysfunction;

(d) adaptive responses that are not considered toxicologically relevant;

(e) substance-induced species-specific mechanisms of toxicity, i.e. demonstrated with reasonable certainty to be not relevant for human health, shall not justify classification.

Oral route

The NTP 1983a study was chosen as key study to demonstrate the specific target organ toxicity due to oral exposure of nitrobenzene. The results of other studies as reviewed and compared with classification criteria in background document are in support of the conclusion drawn on the results of this key study.

Effects in rats justifying classification

In the 90-day gavage study on rats (NTP, 1983a cited from US EPA, 2009) the severity of effects observed in F344 rats at the dose levels of 37.5 - 150mg/kg such as lethality (only high dose), pigmentation (hemosiderosis) in kidney of female rats (75-150 mg/kg) combined with 10% or more reduction in level of functional haemoglobin, reduction in hematocrit, number of red blood cells, increase in percentage of reticulocytes linked with higher incidence of moderate spleen congestion (in females 37.5- 150 mg/kg, and in males 75-150mg/kg) and liver congestion (males 150 mg/kg), although without fibrotic lesions are judged as meeting criteria of significant hematoxicity and organ toxicity defined in the Guidance on the Application of Regulation (EC) No 1272/2008, section 3.9.2.5.2 point.

Effects in rats not justifying classification

The intensity of the haematological effect in rats leading to less than 10% reduction in level of functional haemoglobin (being a sum of % reduction of Hb and % increase in MetHb) at the dose of 9.38 – 18.75 mg/kg not accompanied with significant microscopic changes in liver, kidney and brain and only with low increase in incidence of mild histopathological changes in spleen without fibrotic changes in liver, kidney or spleen do not meet criteria of significant hematoxicity as defined CLP guidance or guidance developed for classification of substances inducing haemolytic anaemia within DSD framework (Muller et al. 2006).

Effects in mice not justifying classification

In the 90-day gavage study on mice (NTP, 1983a cited from US EPA, 2009) the effects observed in B6C3F1 mice exposed by gavage to nitrobenzene for 90 days do not meet classification criteria of STOT RE 2 because hematotoxicity linked with microscopic changes in internal organs were demonstrated only at doses of 150-300 mg/kg above a guidance value of $\leq$ 100 mg/kg/day (CLP criteria) and above a guidance value of $\leq$ 50 mg/kg/day for Xn, R48 in DSD classification criteria.

Conclusion: The effects observed in this study in F344 rats meet classification criteria of STOT RE 2 (erythrocytes) with a hazard statement H373 because they demonstrated significant hematotoxicity below the guidance value of $\leq$ 100 mg/kg/day (CLP criteria) and meet classification criteria of category Xn, R48/22 because there were induced below a guidance value of $\leq$ 50 mg/kg/day for this category in DSD. The effects observed in this study in mice do not meet classification criteria at the level of exposure above those defined by the DSD and CLP guidance values.

However, as it was already considered, humans are much more sensitive then rats to formation of Met-Hb, which should be taken into account in the weight of evidence approach underpinning decision on classification. Formation of Met-Hb, in response to repeated exposure to nitrobenzene, may cause secondary toxic effects in internal organs such as spleen, liver, kidney, brain and lungs, thus the high difference between humans and rats in susceptibility to induction of Met-Hb should be taken into account in hazard classification.

Therefore, in the opinion of RAC the classification derived solely on animal data - Xn, R48/22 (DSD) and STOT RE 2 (erythrocytes) with a hazard statement H373 – should be made relevant to humans and reflect higher sensitivity of humans, thus the repeated specific target organ toxicity of nitrobenzene should by classified T: R48/25 (DSD) and STOT RE 1 with a hazard statement H372 Causes damage to organs (blood)

This classification is in agreement with the existing classification, a proposal of Dossier Submitter, opinion of TC C&L and opinions of some MSCAs expressed during public consultation.

Inhalation exposure

The 90-day inhalation study on rats and mice (Hamm 1984) was chosen as key studies to demonstrate the specific target organ toxicity due to inhalation exposure of nitrobenzene. The results of other studies as reviewed and compared with classification criteria in background document are in support of the conclusion drawn on the results of this key study.

Effects in rats justifying classification

In a 90-day inhalation study on rats and mice (CIIT study,1984; Hamm,1985) a repeated exposure (90 days) to nitrobenzene at conc. of 0.26 mg/l has induced marked increase in the concentration of methemoglobin up to 10% in F344 and CD rats. In addition at this concentration nitrobenzene caused in all F344 rats of either sex an increase in spleen weight, congestion of spleen, proliferative fibrotic capsular lesions and increases in extramedullary haematopoiesis in spleen. In CD rats the sinusoidal congestion, increased extramedullary haematopoiesis, and numbers of hemosiderin-laden macrophages infiltrating the red pulp in spleen, and an increase in the thickness (fibrosis) of the splenic capsule were noted. In both strain of rats (F344 and CD) exposure to nitrobenzene at the concentration of 0.26 mg/l induced toxic nephrosis. These effects meet a criteria of significant hematotoxicity in the Guidance on the Application of Regulation (EC) No 1272/2008 such as e.g. significant increase in hemosiderosis in the spleen, liver or kidney in combination with microscopic effects like necrosis, fibrosis or cirrhosis with reduction in Hb level and increase in MetHb level.

Effects in rats not justifying classification

At the concentration of 0.026 and 0.082 mg /l, lower than the limit value for STOT Category 1 of 0.2 mg/l/6h/day nitrobenzene induced statistically significant increases in concentration of methemoglobine in blood, but they were 2-3 times lower than in animals exposed at 0.26 mg/l/6h/day, and there is no clear evidence in study report on significant heamosiderosis in combination with microscopic changes such as necrosis, fibrosis or cirrhosis associated with increase in methemoglobin concentration or with other effects meeting classification criteria for substances causing haemolytic anaemia (CIIT study,1984; Hamm,1985). Therefore classification for category STOT RE 1 in the basis of this study is not warranted.

In the chronic inhalation study (Cattley et al. 1994; CIIT, 1993) the hematotoxic effects in rats exposed for 2 years at 0.13mg/l and mice exposed at 0.26mg/l were limited to mild increase in level of methemoglobin not exceeding 6% (with 1.39 – 2.75% in control animal), reduction in functional Hb level in blood of approximately 12 % in comparison with controls, reduction in RBC counts not exceeding 10% in comparison with control values, thus in the light of legal requirements and guideline on the application of the CLP regulation or DSD regulation such toxic changes were alone not sufficient to classify them as significant hematotoxicity. The observed haematological changes were not accompanied in by the histopatological changes in spleen, liver and kidney typical for long-lasting haemolytic anaemia. Although pigmentation in spleen was slightly more frequent (hemosiderosis) in highest exposed group than in controls, it was not leading to fibrosis of spleen, which was observed in the 90-day inhalation study at higher nitrobenzene concentration of 0.26mg/l. Thus results of this chronic experiment do not warrant classification of nitrobenzene to STOT RE 1 due to its hematotoxic effect.

Conclusions:

Taking into account that the significant and severe haemtotoxic effects in animals were observed in blood and internal organs at 0.26mg/l, thus in a range of 0.2 and ≤ 1.0 mg/l/6h/day nitrobenzene meets criteria for STOT RE 2 (blood) with a hazard statement H 373. Since the concentration of 0.26mg/l inducing significant organ toxicity is very close to DSD guidance value of ≤ 0.25 mg/l/6h/day for category Xn, R48/20 it is considered appropriate to classify nitrobenzene to this category based on data derived from studies on rodents.

However, as it was already considered in this opinion, humans are much more sensitive then rats to formation of Met-Hb, which should be taken into account in the weight of evidence approach underpinning decision on classification. Formation of Met-Hb, in response to repeated exposure to nitrobenzene, may cause secondary toxic effects in internal organs such as spleen, liver, kidney, brain and lungs, thus the high difference between humans and rats in susceptibility to induction of Met-Hb should be taken in hazard classification.

Therefore, in the opinion of RAC the classification derived solely on animal data - Xn, R48/20 (DSD) and STOT RE 2 (blood) with a hazard statement H373 – should be made relevant to humans and reflect higher sensitivity of humans, thus the repeated specific target organ toxicity of nitrobenzene by inhalation should by classified T: R48/23 (DSD) and STOT RE 1 with a hazard statement H372 Causes damage to organs (blood)

This classification is in agreement with the existing classification, a proposal of Dossier Submitter, opinion of TC C&L and opinions of some MSCAs expressed during public consultation.

Dermal route

NTP sponsored a 90-day skin painting toxicological study (NTP, 1983b cited from US EPA, 2009) with nitrobenzene in F344 rats and B6C3F1 mice. The authors treated F344 rats and B6C3F1 mice (10 animals/sex/group) with 50, 100, 200, 400, and 800 mg/kg-day nitrobenzene in acetone, the responses being compared with those in animals painted with acetone alone.

Effects in rats and mice justifying classification

The following significantly toxic effects, meeting classification criteria in CLP and DSD systems, were seen in F344 rats and B6C3F1 mice expose dermal at 50, 100 and 200 mg/kg bw/day for 13 weeks:

- in male F344 rats - spleen congestion starting from 50 mg/kg, lung congestion and lymphoid atrophy starting at 100 mg/kg;
- in female rats - spleen haematopoiesis and congestion, lung congestion at 100mg/kg, haemorrhage in brain starting with a dose 100mg/kg

- in male mice: lung congestion and spleen haematopoiesis starting at 50 mg/kg,
- in female mice - fatty change in the adrenal cortex in addition to the haematological findings noted at the lowest dose of 50 mg/kg bw/d.

Conclusions: These effects meet criteria of severe adverse health effects at the dose levels in a range between 20 and 200 mg/bw /day for STOT RE, Category 2 (CLP) with a hazard statement H 373 or in dose levels >10 mg/kg to ≤100 mg/kg (bodyweight)/day for category Xn, R48/21 (DSD).

However, as it was already considered in this opinion, humans are much more sensitive then rats to formation of Met-Hb, which should be taken into account in the weight of evidence approach underpinning decision on classification. Formation of Met-Hb, in response to repeated exposure to nitrobenzene, may cause secondary toxic effects in internal organs such as spleen, liver, kidney, brain and lungs, thus the high difference between humans and rats in susceptibility to induction of Met-Hb should be taken in hazard classification.

Therefore, in the opinion of RAC the classification derived solely on animal data - Xn, R48/21 (DSD) and STOT RE 2 (blood) with a hazard statement H373 – should be made relevant to humans and reflect higher sensitivity of humans, thus the repeated specific target organ toxicity of nitrobenzene by inhalation should by classified T: R48/24 (DSD) and STOT RE 1 with a hazard statement H372 Causes damage to organs (blood)

This classification is in agreement with the existing classification, a proposal of Dossier Submitter, opinion of TC C&L and opinions of some MSCAs expressed during public consultation.

5.6.6 Conclusions on classification and labelling

Having in mind the above arguments the RAC is of the opinion that specitic target organ toxicity of nitrobenzene should be classify according to CLP regulation for category STOT RE 1 (blood) with a hazard statement H372 Causes damage to organs (blood) and according to DSD regulation for category T, R48/23/24/25.

This classification is in agreement with the existing classification, a proposal of Dossier Submitter, opinion of TC C&L and opinions of some MSCAs expressed during public consultation.

5.7 Mutagenicity

5.7.1 In vitro data

Test	Cell type	Conc. range	Observations and remarks
Bacterial gene mutation test	S. typhimurium, TA92, TA1535, TA100, TA94, TA98	30 - 3000 µg/plate with S-9 mix; 10 - 3000 µg/plate without S-9 mix	Result: negative Toxicity: at 1000 and 3000 µg/plate study does not completely meet requirements of OECD TG 471 (use of only four strains and three to four analysable concentrations, results only presented as summarised tables) but is considered as sufficiently reliable for risk assessment due to clear negative results (Miyata et al. 1981)
Bacterial gene mutation test	S. typhimurium TA1535, TA1537, TA100 and TA98	10 - 1000 µg/plate with and without S-9 mix	Result: negative Toxicity: at highest dose study does not completely meet requirements of OECD TG 471 (use of only four strains and three to four analysable concentrations, results only presented as summarised tables) but is considered as sufficiently reliable for risk assessment due to clear negative results (Haworth et al. 1983)
Bacterial gene mutation test	S. typhimurium TA 98 and TA100	36.93 - 3693,3 µg/plate with S-9 mix; not tested without S-9 mix	Result: negative Toxicity: at highest dose Remarks: Flavine mononucleotide supplementation (Dellarco and Prival 1989)
Bacterial gene mutation test	S. typhimurium TA97, TA98 and TA100	33 - 3333 33, 100, 333, 1000 and 3333 µg/plate with and without S-9 mix	Result: negative Toxicity: no data Remarks: non standard method only raw data and abstract available (Hughes et al. 1984)
Bacterial gene mutation test	S. typhimurium TA98 and TA100	200, 1000 µg/plate with S-9 mix; not tested without S-9 mix	Result: positive Toxicity: no data Remarks: positive only in the presence of the comutagen norharman (Suzuki et al. 1983)
Mammalian cell gene mutation test	Chinese hamster lung fibroblasts (V79)	0.1 - 1 µg/ml with and without S-9 mix	Result: inconclusive Toxicity: no data Remarks: methodical insufficiencies (no data on cytotoxicity and plating efficiency; low statistical power of test since only 2 x 10^5 cells / culture were inoculated for the selection of resistant cells; effects are not dose related and not reproduced) (Kuroda 1986)
mammalian cell chromosomal aberration test	Chinese hamster lung fibroblasts (V79)	0.125 - 500 µg/ml with S-9 mix; not done without S-9	Result: negative Toxicity: no data

Test	Cell type	Conc. range	Observations and remarks
		mix	(Ishidate, 1988)
Mammalian cell chromosomal aberration test	human lymphocytes	6.1 mg/ml with S-9 mix; not done without S-9 mix	Result: inconclusive Toxicity: no data Remarks: insufficient study description (no detailed information on nitrobenzene concentrations, toxicity, cell preparation and solvent controls; results only as summarised table) (Huang et al. 1996)
mammalian cell micronucleus test	Chinese hamster lung fibroblasts (V79)	0.123 to 12.31 µg/ml with S-9 mix; not done without S-9 mix	Result: weakly positive Toxicity: no toxicity Remarks: insufficient data presentation (data for micronuclei frequencies were only presented as a figure.) (Bonacker et al. 2004)
mammalian cell UDS test	human and rat hepatocytes	1.23 to 123 µg/ml with S-9 mix; not done without S-9 mix	Result: negative Toxicity: no data (Butterworth et al. 1989)
mammalian cell micronucleus test and comet assay	rat and human kidney cells	7.63 to 61.5 µg/ml with S-9 mix; not done without S-9 mix	Result: inconclusive for both genotoxic endpoints Toxicity: toxic at least at the highest tested dose Remarks: methodical insufficiencies (non-routine method; confusion about the incubation time of 20 h or 48 h; data on genotoxic effects were only presented as ratios treated/control cultures in a figure) (Robbiano et al. 2004)

5.7.2 In vivo data

Test	Species (tissue)	Dose groups	Harvest time	Observations and remarks (include route of administration)
Micro-nucleus test	mice bone marrow	62.5-250 mg/kg bw 1 x i.p.	24, 48 h	Result: negative Toxicity: at highest dose tested Remark: OECD TG 474 (BASF AG 1996)
Chromo-somal aberrations & SCE	rat spleen and blood lympho-cytes	5, 16, or 50 ppm (0.025, 0.082, 0.25 mg/L) inhalation 6h/day, 5 days/week for 21 days	beginning of primary cell cultures less than 1 h after termination of exposure, cytogenetic analysis after 54 h (blood lympho-cytes) or 72 h (spleen lympho-cytes)	Result: negative for both endpoints Toxicity: mitotic index decreased in blood lymphocytes, cell cycle delay (Kligerman et al. 1983)
UDS test	rat hepatoc	200, 500 mg/kg bw	12 h	Result: negative Toxicity: no data

Test	Species (tissue)	Dose groups	Harvest time	Observations and remarks (include route of administration)
	ytes	1 x p.o.		(Mirsalis et al. 1982)
DNA-binding	rat liver & kidney, mouse liver & lung	4 mg/kg bw 1 x sc	24 h	Result: weak positive Toxicity: no data Remark: non-routine method (Novartis 1997)
DNA-binding	rat liver	0.1 µg - 10 mg/kg bw 1 x i.p. 4.1 µg/kg bw 1 x i.p.	2 h 4, 12, 24 h, 3, 7, 14, 21 d	Result: positive Toxicity: no data Remark: non-routine method (Li et al. 2003)
DNA damage & Micronuclei	rat kidney	300 mg/kg bw 1 x p.o.	unclear	Result: inconclusive for both endpoints Toxicity: no data Remark: methodically inadequate (Robbiano et al. 2004)
Conclusion: no classification.				

5.7.3 Human data

5.7.4 Other relevant information

5.7.5 Summary and discussion of mutagenicity

Nitrobenzene was negative in several bacterial tests with a number of Salmonella typhimurium strains. For genotoxicity of nitrobenzene in mammalian cells in vitro no test according to current guidelines was available. The two most reliable tests - a chromosomal aberration test in Chinese hamster lung cells and a test on unscheduled DNA synthesis in human hepatocytes - revealed negative results. Inconclusive results were obtained in a mammalian cell gene mutation test, a chromosomal aberration test in primary human lymphocytes and further non-routine tests and a weak positive result were reported from micronucleus test in Chinese hamster lung cells. These studies were either methodically inadequate or insufficiently described and were not considered as relevant for risk assessment.

In vivo no mutagenic effect was detected in a bone marrow micronucleus test in mice (OECD TG 474) (BASF AG 1996) and in a test on chromosomal aberrations and SCE in lymphocytes from peripheral blood and spleen from subacute exposed rats via inhalation (Kligerman et al. 1983). In rats, no UDS was induced in rat liver after single high oral doses (Mirsalis et al. 1982). However, a DNA-binding capacity was detected in vivo in two studies after subcutaneous or i.p. application in liver and lung of mice and in liver and kidney of rats (Novartis 1997 and Li et al. 2003).

Due to the DNA-binding capacity a tissue specific genotoxic potential of nitrobenzene responsible for a genotoxic mechanism of carcinogenesis cannot be excluded, but due to the low binding capacity alone a genotoxic mode of carcinogenicity is rather unlikely. From the available negative data for micronuclei formation, chromosomal aberrations, SCE and UDS in rodents in vivo it can

concluded that nitrobenzene is not suspected to exert mutagenic effects on germ cells. Nitrobenzene should not be classified as a mutagen.

5.7.6 Comparison with classification criteria

Criteria to be met:
A substance can be classified according to Directive 67/548/EEC to Muta, category 2 if it there is a sufficient evidence to provide a strong presumption that human exposure to the substance may result in the development of heritable genetic damage, generally on the basis of:

- — appropriate animal studies,
- — other relevant information.

To be classified to Muta, cat. 3 (DSD classification system) there should be evidence from appropriate mutagenicity studies, but this is insufficient to place the substance in category 2.

Within the CLP classification system the classification in Category 1B is based on:

— positive result(s) from in vivo heritable germ cell mutagenicity tests in mammals; or

— positive result(s) from in vivo somatic cell mutagenicity tests in mammals, in combination with some evidence that the substance has potential to cause mutations to germ cells. It is possible to derive this supporting evidence from mutagenicity/genotoxicity tests in germ cells in vivo, or by demonstrating the ability of the substance or its metabolite(s) to interact with the genetic material of germ cells; or

— positive results from tests showing mutagenic effects in the germ cells of humans, without demonstration of transmission to progeny; for example, an increase in the frequency of aneuploidy in sperm cells of exposed people.

Existing evidence: The results of the *in vivo* mutagenicity tests (BASF AG, 1996, Kligerman et al. 1983, Miralis et al. 1982) summarized in the table above provide evidence that nitrobenzene do not induce mutations in somatic cells. No mutagenicity was also observed in *in vitro* mutagenicity tests summarized in section 5.7.1. Sporadic inconclusive results in some tests were due to methodological insufficiencies.

5.7.7 Conclusions on classification and labelling

The properties of nitrobenzene do not warrant classification for mutagenicity neither in DSD nor in CLP classification system

5.8 Carcinogenicity

5.8.1 Carcinogenicity: oral

5.8.2 Carcinogenicity: inhalation

Species/strain group size	conc. mg/l	Exposure time	Duration of treatment	Observations and remarks (effects of major toxicological significance)
Rat F-344 (m/f) **(60 animals/ sex/ group, plus 10 animals/ sex/group for interim sacrifice at 6 months)**	**0, 1, 5, 25 ppm** (0, 0.005, 0.025, 0.13 mg/L)	**6h/d** **5d/week**	**107 weeks**	**F-344 rats (Table 6, Table 9)** In male F-344 rats, the incidences of hepatocellular adenoma, hepatocellular adenoma or carcinoma, and renal tubular adenoma were increased. Male F344 rats had a marginally increased incidence of thyroid follicular neoplasia (adenoma or adenocarcinoma). In female F344 rats, the incidence of endometrial stromal polyps was increased. The tumor rates at multiple target organs increased with dose-dependency (see Table 6). The increases in tumor incidences reached significance at 25 ppm in rats. Although there may be some increase in tumor rates, 5 ppm appear as the dose without significant tumor response. Nitrobenzene exposure was associated to increased relative and absolute organ weights of the liver and kidneys in the 25 ppm exposed rats of both sexes. Higher incidences of rough granular cortical surfaces noted in the rats of the final sacrifice groups (males ≥5 ppm and 25 ppm females) were considered indicative of chronic progressive nephrosis. Exposure-related anaemic effects were seen in 25 ppm males and females. Red blood cell counts, haematocrit and haemoglobin levels were depressed, methaemoglobin concentrations were elevated at 25 ppm (0.13 mg/L)) nitrobenzene. An increase of MCV, nucleated RBCs, polychromatic cells, macrocytes, the presence of Howell-Jolly bodies and leucocytosis were noted in one or both sexes of this dose group. An elevation of GGT (males) and elevated bilirubin (both sexes) were noted in 25 ppm exposed groups. In general, the incidence and severity of microscopic lesions at the final sacrifice were greater in males than in females. Several lesions represent a progression from what was observed at the interim sacrifice. In addition to the above reported non-neoplastic lesions at tumor sites, there was an increased incidence of extramedullary haematopoiesis of the spleen (1 and 5 ppm males), pigment-laden macrophages (25 ppm exposed females and males of all dose groups), an increased incidence and severity of sinusoidal congestion of the red pulp (all dose groups). Stromal hyperplasia of the spleen was found in two 5 ppm and two 25 ppm males and one 5 ppm and one 25 ppm females versus none in the control groups. An increased severity of chronic nephropathy and an increase in the number of convoluted tubules containing intracytoplasmatic and intraluminal

				eosinophilic droplets, an increase in the amount of yellowish-brown pigment, and an increase in
				suppurative tubular inflammation was noted in exposed males and females (significance at exposure ≥5 ppm). Hyperplasia and inflammation of the submucosal glands in the anterior portion of the nose (level 1 + 2) lined by respiratory epithelium were present in 25 ppm males and females. Because of high incidences of spontaneous lesions of the testis no treatment-related effect could be identified in F-344 males. (CIIT 1993; Cattley et al. 1994)
Rat Sprague-Dawley (CD) **(m) (60 animals/sex/ group, plus 10 animals/sex/ group for interim sacrifice at 6 month)**	**0, 1, 5, 25 ppm** (0, 0.005, 0.025, 0.13 mg/L)	**6h/d** **5d/week**	**107 weeks**	**CD rats (Table 8, Table 9:)** In male CD rats, the incidences of hepatocellular adenoma and the combined incidences of hepatocellular adenoma and carcinoma were increased; the incidence of hepatocellular carcinoma alone was not affected by nitrobenzene exposure. Except renal changes associated with chronic progressive nephropathy, no other macroscopic finding was found in CD males at the end of the study. An increased incidence of sinusoidal congestion was evident at all nitrobenzene concentrations. A minor exposure-related increase in the severity of splenic extramedullary haematopoiesis and degree of pigmentation was also noted. Testes atrophy was present both in control and nitrobenzene exposed males. There was a positive trend in exposed rats, in that increased incidences of this lesion were present in 25 ppm exposed and 5 ppm exposed rats. An increased incidence of bilateral hypospermia in the epididymides was observed in the 25 ppm exposed males. Chronic nephropathy was noted in both the control and nitrobenzene-exposed males, with only a slight increase in severity of the change noted in the 25 ppm exposure group. In addition, the secondary lesions associated with severe chronic nephropathy (parathyroid hyperplasia, fibrous osteodystrophy, soft tissue mineralization) were increased in these animals. Nasal changes consisted of inflammation in the anterior nasal passages. Increased incidences and severity of suppurative exudate, subacute inflammation, and mucosal epithelial hyperplasia were seen in males of all dose groups. Brown pigment in the submucosa of the olfactory epithelium, which was commonly found in control and exposed animals, was evident in a slightly increased amount in males of the 5 and 25 ppm groups. (CIIT 1993; Cattley et al. 1994)
Mouse B6C3F1 **(70m/70f)**	**0, 5, 25, 50 ppm** (0, 0.025, 0.13, 0.26	**6h/d** **5d/week**	**107 weeks**	**B6C3F1 mouse (Table 10, Table 11)** In male B6C3F1 mouse, the incidences of alveolar/bronchiolar adenoma, alveolar/ bronchiolar carcinoma, and thyroid adenoma were increased. In female B6C3F1 mouse, the incidence of mammary

	mg/L)			gland adenocarcinoma was increased.
				(contd.) In addition, female B6C3F1 mice exposed to nitrobenzene had a marginally increased incidence of hepatocellular adenomas. In mouse, increases in tumor incidences were also evident in multiple target organs and significant increase was seen at the lowest dose tested (5 ppm) and above. At 2 year sacrifice, RBC counts and haematocrit were lower for 50 ppm males and 5 and 25 ppm females, females of these groups also had decreases in haemoglobin. The MCH and MCHC were higher for males and females of the 50 ppm groups. Males of the 50 ppm exposure group and female 25 and 50 ppm exposure groups (0.13 and 0.26 mg/L) had increases in methaemoglobin. Clinical chemistry revealed higher activities of ALAT in males of the 25 and 50 ppm groups. In 50 ppm exposed female mice, absolute and relative organ weights of the liver and kidney were increased, 25 ppm exposed male mice also showed increased relative liver weights. In addition to the non-neoplastic lesions of organs with treatment-related tumors already reported above, non-neoplastic lesions were also observed in other organs. Nasal inflammatory lesions consisting of increased incidence of secretion of respiratory epithelial cells (in female mice of all dose groups, and 50 ppm exposed males), and glandularization of respiratory epithelium (50 ppm exposed males and females) were evident. In addition, increased incidences of degeneration and loss of olfactory epithelium (females of all dose groups, 25 ppm and 50 ppm exposed males) along with dilation of submucosal glands and accumulation of submucosal brown pigment-containing macrophages (all nitrobenzene exposure groups) was observed. Nitrobenzene inhalation also resulted in increased incidence of lymphoid hyperplasia of the spleen (50 ppm exposed females), bone marrow hypercellularity (5 ppm and 50 ppm exposed males), increased incidence of adrenal cortical vacuolization (25 ppm and 50 ppm females), increased incidence of thymic involution (50 ppm females), increased incidence of testicular atrophy (50 ppm males), bilateral hypospermia of the epididymis (50 ppm males), increased incidence of renal cysts (50 ppm males), and mononuclear cell infiltration in pancreas (50 ppm females). (CIIT 1993; Cattley et al. 1994)
Conclusion: Classification as Carcinogen Category 3 and R-phrase R 40 is confirmed (see Summary and discussion).				

Table 8: Incidence of selected neoplastic lesions in F-344 and Sprague-Dawley CD rats following nitrobenzene exposure

		Female F-344 incidence (%)				Male F-344 incidence (%)				Male CD incidence (%)			
Tissue	Diagnosis	0 ppm 0 mg/L	1 ppm 0.005 mg/L	5 ppm 0.026 mg/L	25 ppm 0.128 mg/L	0 ppm 0 mg/L	1 ppm 0.005 mg/L	5 ppm 0.026 mg/L	25 ppm 0.128 mg/L	0 ppm 0 mg/L	1 ppm 0.005 mg/L	5 ppm 0.026 mg/L	25 ppm 0.128 mg/L
Liver	Hepatocellular adenoma	0/70 (0)	2/66 (3)	0/66 (0)	3/70 (4)	1/69 (1) T	3/69 (4)	3/70 (4)	15/70 (21) P	1/63 (2) T	1/67 (1)	2/70 (3)	7/65 (11) P
	Hepatocellular carcinoma	0/70 (0) T	0/66 (0)	0/66 (0)	2/70 (3)	0/69 (0) T	1/69 (1)	2/70 (3)	4/70 (6)	2/63 (3)	0/67 (0)	2/70 (3)	2/65 (3)
	Hepatocellular adenoma or carcinoma	0/70 (0) T	2/66 (3)	0/66 (0)	4/70 (6)	1/69 (1) T	4/69 (6)	5/70 (7)	16/70 (23) P	2/63 (3) T	1/67 (1)	4/70 (6)	9/65 (14) P
Kidney	Tubular adenoma	0/70 (0)	0/66 (0)	0/66 (0)	0/70 (0)	0/69 (0) T	0/68 (0)	0/70 (0)	5/70 (7) P	2/63 (3)	0/67 (0)	2/70 (3)	0/65 (0)
	Tubular carcinoma	0/70 (0)	0/66 (0)	0/66 (0)	0/70 (0)	0/69 (0)	0/68 (0)	0/70 (0)	1/70 (1)	0/63 (0)	1/67 (1)	0/70 (0)	0/65 (0)
	Tubular ademona or carcinoma	0/70 (0)	0/66 (0)	0/66 (0)	0/70 (0)	0/69 (0) T	0/68 (0)	0/70 (0)	6/70 (9) P	2/63 (3)	1/67 (1)	2/70 (3)	0/65 (0)
Thyroid	Follicular cell adenoma	0/69 (0)	—	—	2/68 (3)	0/69 (0)	0/69 (0)	2/70 (3)	2/70 (3)	2/63 (3)	4/64 (6)	2/68 (3)	3/64 (5)
	Follicular cell adeno-carcinoma	0/69 (0)	—	—	1/68 (1)	2/69 (3) T	1/69 (1)	3/70 (4)	6/70 (9)	4/63 (6)	1/64 (2)	1/68 (1)	2/64 (3)
	Follicular cell adenoma or adeno-carcinoma	0/69 (0)	—	—	3/68 (4)	2/69 (3) T	1/69 (1)	5/70 (7)	8/70 (11)	5/63 (8)	5/64 (8)	3/68 (4)	5/64 (8)
Uterus	Endometrial stromal polyp	11/69 (16) T	17/65 (26)	15/65 (23)	25/69 (36) P	—	—	—	—	—	—	—	—
Testes	Interstitial cell tumor	—	—	—	—	61/69 (88)	52/56 (93)	58/61 (95)	65/70 (93)	3/62 (5)	6/66 (9)	7/70 (10)	4/61 (7)
Multiple	Mononuclear cell leukaemia	11/70 (16)	5/66 (8)	4/66 (6)	0/70 (0)	12/69 (17)	5/69 (7)	4/70 (6)	0/70 (0)	1/63 (2)	4/67 (6)	0/70 (0)	0/65 (0)

Note: T, significantly positive nitrobenzene exposure-related trend in incidence determined by Cochran-Armitage trend test, $p < 0.05$.
P, significantly different from incidence in 0-ppm control group determined by Fisher Exact Test, $p < 0.05$.

Table 9: Incidence of nonneoplastic lesions in rats

Tissue	Diagnosis	Female F344 incidence (%)				Male F344 incidence (%)				Male CD incidence (%)			
		0 ppm 0 mg/L	1 ppm 0.005 mg/L	5 ppm 0.026 mg/L	25 ppm 0.128 mg/L	0 ppm 0 mg/L	1 ppm 0.005 mg/L	5 ppm 0.026 mg/L	25 ppm 0.128 mg/L	0 ppm 0 mg/L	1 ppm 0.005 mg/L	5 ppm 0.026 mg/L	25 ppm 0.128 mg/L
Liver	Eosinophilic foci	6/70 (9) T	9/66 (14)	13/66 (20)	16/70 (23) P	26/69 (42) T	25/69 (36)	44/70 (63) P	57/70 (81) P	11/63 (17) T	3/67 (4)	8/70 (11)	19/65 (29)
	Centrilobular hepato-cytomegaly	0/70 (0)	0/66 (0)	0/66 (0)	0/70 (0)	0/69 (0) T	0/69 (0)	8/70 (11) P	57/70 (81) P	3/63 (5) T	1/67 (1)	14/70 (20) P	39/65 (60) P
	Spongiosis hepatis	0/70 (0) T	0/66 (0)	0/66 (0)	6/70 (9) P	25/69 (36) T	24/69 (35)	33/70 (47)	58/70 (83) P	25/63 (40) T	25/67 (37)	25/70 (36)	37/65 (57) P
Kidney	Chronic nephropathy	58/70 (83)	51/66 (77)	60/66 (91)	67/70 (96)	69/69 (100)	64/68 (94)	70/70 (100)	70/70 (100)	54/63 (86)	60/67 (90)	63/70 (90)	59/65 (91)
	Tubular hyperplasia	0/70 (0)	0/66 (0)	2/66 (3)	2/70 (3)	2/69 (3) T	2/68 (3)	2/70 (3)	13/70 (19) P	3/63 (5)	1/67 (1)	5/70 (7)	6/65 (9)
Thyroid	Follicular cell hyper-plasia	1/69 (1)	—	—	0/68 (0)	0/69 (0) T	1/69 (1)	2/70 (3)	4/70 (6)	2/63 (3)	2/64 (3)	1/68 (1)	4/64 (6)
Nose[a]	Pigment deposition olfactory epith.	37/67 (55) T	54/65 (83) P	60/65 (92) P	66/66 (100) P	40/67 (60) T	53/67 (79) P	67/70 (96) P	68/69 (99) P	42/63 (67) T	49/64 (77)	60/66 (91) P	58/61 (95) P
Testes	Atrophy, bilateral	—	—	—	—	61/69 (88)	50/56 (89)	59/61 (97)	61/70 (87)	11/62 (18) T	17/66 (26)	22/70 (31)	35/61 (57) P
Epidi-dymis	Hyposper-mia, bilateral	—	—	—	—	15/69 (22)	21/54 (39)	12/59 (20)	12/70 (17)	8/60 (13) T	13/65 (20)	15/67 (22)	32/59 (54) P
Spleen	Extramedul-lary haemato-poiesis	60/69 (87)	62/66 (94)	60/66 (91)	65/69 (94)	53/69 (77)	62/69 (90) P	65/70 (93) P	61/70 (87)	58/63 (92)	56/67 (84)	61/69 (8)	60/65 (92)
	Pigmentation	62/69 (90) T	61/66 (92)	60/66 (91)	68/69 (99) P	55/69 (91) P	63/69 (91) P	64/70 (91) P	70/70 (100) P	59/63 (94) T	58/67 (87)	67/69 (97)	65/65 (100)

Note: T, significantly positive nitrobenzene exposure-related trend in incidence determined by Cochran-Armitage trend test, $p < 0.05$.
P, significantly different from incidence in 0-ppm control group determined by Fisher Exact Test, $p < 0.05$.

Table 10: Incidence of selected neoplastic lesions in B6C3F1 mice following nitrobenzene exposure

Tissue	Diagnosis	Male incidence (%)				Female incidence (%)			
		0 ppm 0 mg/L	5 ppm 0.026 mg/L	25 ppm 0.128 mg/L	50 ppm 0. 256 mg/L	0 ppm 0 mg/L	5 ppm 0.026 mg/L	25 ppm 0.128 mg/L	50 ppm 0. 256 mg/L
Lung	A/B adenoma	7/68 (10) T	12/67 (18)	15/65 (23) P	18/66 (27) P	4/53 (8)	11/60 (18)	3/64 (5)	2/62 (3)
	A/B carcinoma	4/68 (6)	10/67 (15)	8/65 (12)	8/66 (12)	2/53 (4)	0/60 (0)	4/64 (6)	4/62 (6)
	A/B adenoma or carcinoma	9/68 (13)T	21/67 (31) P	21/65 (32) P	23/66 (35) P	6/53 (11)	11/60 (18)	6/64 (9)	6/62 (10)
Thyroid	Follicular cell adenoma	0/65 (0) T	4/65 (6)	1/65 (2)	7/64 (11) P	2/49 (4)	0/59 (0)	3/61 (5)	2/61 (3)
Mammary gland	Adeno-carcinoma	—	—	—	—	0/48 (0)	—	—	5/60 (8) P
Liver	Hepatocellular adenoma	14/68 (21)	18/65 (28)	15/65 (23)	14/64 (22)	6/51 (12) T	5/61 (8)	5/64 (8)	13/62 (21)
	Hepatocellular carcinoma	12/68 (18)	13/65 (20)	12/65 (18)	8/64 (13)	1/51 (2)	2/61 (3)	3/64 (5)	1/62 (2)
	Hepatocellular adenoma or carcinoma	25/68 (37)	30/65 (46)	22/65 (34)	21/64 (33)	7/51 (14)	7/61 (11)	7/64 (11)	14/62 (23)

Note: T, significantly positive nitrobenzene exposure-related trend in incidence determined by Cochran-Armitage trend test, $p < 0.05$.
P, significantly different from incidence in 0-ppm control group determined by Fisher Exact Test, $p < 0.05$.

Table 11: Incidence of selected nonneoplastic lesions in B6C3F1 mice following nitrobenzene exposure

		Male incidence (%)				Female incidence (%)			
Tissue	Diagnosis	0 ppm 0 mg/L	5 ppm 0.026 mg/L	25 ppm 0.128 mg/L	50 ppm 0. 256 mg/L	0 ppm 0 mg/L	5 ppm 0.026 mg/L	25 ppm 0.128 mg/L	50 ppm 0. 256 mg/L
Lung	A/B hyperplasia	1/68 (1) T	2/67 (3)	8/65 (12) P	13/66 (20) P	0/53 (0)	2/60 (3)	5/64 (8) P	1/62 (2)
	Bronchializatio	0/68 (0) T	58/67 (87) P	58/65 (89) P	62/66 (94) P	0/53 (0) T	55/60 (92) P	63/64 (98) P	62/62
Thyroid	Follicular cell hyperplasia	1/65 (2) T	4/65 (6)	7/65 (11) P	12/64 (19) P	2/49 (4) T	1/59 (2)	1/61 (2)	8/61 (13)
Liver	Centrilobular hepatocytomegaly	1/68 (1) T	15/65 (23) P	44/65 (68) P	57/64 (89) P	0/51 (0) T	0/61 (0)	0/64 (0)	7/62 (11) P
	Multinucleated hepatocytes	2/68 (3) T	14/65 (22) P	45/65 (69) P	56/64 (88) P	0/51 (0)	0/61 (0)	0/64 (0)	2/62 (3)
Nose[a]	Glandularization of respiratory	10/67 (15) T	0/66 (0)	0/65 (0)	27/66 (41) P	0/52 (0) T	0/60 (0)	0/63 (0)	7/61 (11) P
	Increased secretory product. respiratory epith.	0/67 (0) T	0/66 (0)	3/65 (5)	6/66 (9) P	2/52 (4) T	7/60 (12)	19/63 (30) P	32/61 (52) P
	Degeneration/loss. olfactory epith.	1/67 (1) T	1/66 (2)	32/65 (49) P	41/66 (62) P	0/52 (0) T	19/60 (32) P	47/63 (75) P	42/61 (69) P
	Pigment deposition. olfactory epith.	0/67 (0) T	7/66 (11) P	46/65 (71) P	49/66 (74) P	0/52 (0) T	6/60 (10) P	37/63 (59) P	29/61 (48) P
Testes	Diffuse atrophy	1/68 (1)	—	—	6/66 (9)	—	—	—	—
Epididymis	Hypospermia	3/68 (4)	—	—	11/66 (17) P	—	—	—	—
Bone marrow. Femur	Hypercellularity	3/68 (4) T	10/67 (15) P	4/64 (6)	13/66 (20) P	4/52 (8)	—	—	9/62 (15)
Thymus	Involution	10/48 (21)	—	—	10/44 (23)	7/41 (17)	—	—	22/57 (39) P
Kidney	Cyst	2/68 (3)	—	—	12/65 (18) P	0/51 (0)	—	—	0/62 (0)
Pancreas	Mononuclear cell infiltrate	3/65 (5)	—	—	3/64 (5)	1/46 (2)	—	—	8/62 (13) P

Note: T, significantly positive nitrobenzene exposure-related trend in incidence determined by Cochran-Armitage trend test, $p < 0.05$.
P, significantly different from incidence in 0-ppm control group determined by Fisher Exact Test, $p < 0.05$.
[a] Level 3

5.8.3 Carcinogenicity: dermal

5.8.4 Carcinogenicity: human data

5.8.5 Other relevant information

5.8.6 Summary and discussion of carcinogenicity

Following long-term inhalation of nitrobenzene tumor incidences at six organ sites were significantly increased. (see Table 12)

Liver tumors

Hepatocellular neoplasms (adenoma and adenoma or carcinoma) were induced by nitrobenzene in male F344 rats and in male CD rats but not in F344 females. Female CD rats had not been tested. Increased incidences of eosinophilic foci were seen in mid and high dose male F344 rats and female F344 rats of the high dose group. Spongiosis hepatis (used as a synonym to focal cystic degeneration) was present in all high dose groups of both strains and sexes. In mid and high dose males of both rat strains, a dose-related increase of centrilobular hepatomegaly (syn. centrilobular hypertrophy) was observed. Spongiosis hepatis and eosinophilic foci did not show a coincidental occurrence with the liver tumor rates. The only lesion which may be considered as a possible critical event preceding the tumor development in nitrobenzene exposed mammals was the occurrence of centrilobular hypertrophy. This hypothesis is supported by solely males of both rat strains showing hypertrophy and liver cell tumors. Assuming that hypertrophy represents the precursor lesion in liver cell tumor development, the absence of the hypertrophy may be indicative for the prevention of tumor growth in the low dose group. Whereas liver tumors were only seen at the high concentration, hypertrophy was evident in the mid and high dose groups. - Hypertrophy of centrilobular hepatocytes in rodents is commonly associated with a metabolic activation of microsomal enzymes. Up to now, there is no evidence that nitrobenzene activates liver cell enzymes. Due to the differences in the occurrence, hypertrophy was obviously not associated with other hepatotoxic effects. Degenerative lesions consisting of spongiotic hepatis were also described in female F344 rats, but none of them showed hypertrophy - In B6C3F1 mice, the incidences of centrilobular hypertrophy were increased significantly in all male dose groups and in high dose females, but no significant increase of liver cell tumors was seen. Female mice exclusively showed a non-significant higher rate of liver adenomas at the high dose group. Another non-neoplastic lesion described in male B6C3F1 mice of all treatment groups was the occurrence of multinucleated hepatocytes which did not show any association to tumor development in the liver.

In summary, liver cell tumors in males of two rat strains appeared to be linked to nitrobenzene. Looking for nonneoplastic liver effects as possible underlying toxic events in the tumor development, none of the toxic effects observed supported enough evidence to explain the tumor development. There was no coincidence of toxic lesions and neoplasia and no concurrent dose-response relationships except an apparent coherence of centrilobular hypertrophy and liver cell tumors.

Kidney tumors

Nitrobenzene exposure resulted in higher rates of tubular adenomas and of combined incidence of tubular adenomas or carcinomas in high dose males of F344 rats. In this dose group, the incidence of tubular hyperplasia, considered to represent a preneoplastic lesion, was also increased significantly. Chronic nephropathy observed at rates of 77-96% in female (control and dose) groups

and at 94-100% in male (control and dose) groups of F344 rats was not correlated to the tumor response in high dose male F344 rats only. Intratubular eosinophilic (hyaline) droplet inclusions were observed in F344 rats (10/10 males, 2/10 females at 125 ppm) following 14 day-inhalation and in males at 5 ppm and above and in females at 25 ppm following a 90 day period of exposure. This finding may indicate a degenerative effect in renal tubular cells of rats of this strain with males more sensitive than females, which could be considered as a toxic effect preceding tumor growth.

Thyroid tumors

Chronic nitrobenzene exposure was associated with significantly increased incidence of thyroid follicular cell adenomas in male B6C3F1 mice. The observed tumor rates (none in control group, 6%, 2% and 11% in low, mid and high dose groups) were not dose-related. Tumor rates in high dose female mice were lower (3%) than in the control group (4%). The incidence of thyroid follicular cell adenocarcinoma was increased in nitrobenzene exposed male F344 rats (3% in controls, 1%, 4%, or 9% in low, mid and high dose groups), but this effect was considered marginal since only the Trend test was positive, and the higher incidences in the mid and high dose groups were not significantly different from control values. No treatment-related effect was observed in female F344 rats or male CD rats. In both male mice and male F344 rats, the increased incidence of follicular cell neoplasms was associated with an increased incidence of follicular cell hyperplasia (significant only for male mice). In mice, it has been suggested that hyperplasia of the thyroid follicular epithelium represents a preneoplastic change (McConnell 1992).

No other toxic effect on the thyroid was observed in studies with repeated administration of nitrobenzene.

Thyroid carcinogenesis in rodents may occur as a secondary response to microsomal enzyme induction in hepatocytes, which elevates glucuronidation and excretion of thyroid hormones. This causes a continuously stimulated TSH production and chronic activation of thyroid. The observed hepatocytic hypertrophy in rats and mice could be interpreted as indicative for enzyme induction and could hint on a rodent-specific mechanism. However, hypertrophy of hepatocytes was also significantly increased in male CD rats which did not develop thyroid tumors. Therefore at least in the rat strains tested the occurrence of hepatocyte hypertrophy is not consistent to the development of thyroid tumors as a secondary mechanism. Also, no data on enzyme induction, no proof of altered serum levels of thyroid hormones and TSH and no data on biliary excretion are available to support this mode of action. Life-long metabolic activation is also known to induce liver tumors. In opposite to other hepatic enzyme inducers where the treated rat is much more sensitive towards thyroid effects than the mouse, the nitrobenzene-treated mice developed increased rates of follicular cell hyperplasia and thyroid tumors but no liver tumors and males of both rat strains had liver tumors, while only marginal increases in follicular cell hyperplasia were found in both strains and marginal increase in thyroid adenocarcinomas were only observed in the F344 male rats.

While for the rat the induction of UDP-glucuronosyltransferase is often supposed to contribute to this mechanism, an induction of UDP-glucuronosyltransferase is unknown for the mouse. The knowledge of species differences among the rats' and the humans' regulation of thyroid homeostasis (such as a higher turnover of thyroid hormones, higher TSH serum levels, lack of thyroxine binding globulin (TBG) in rats than in humans) could not simply be applied on the mouse thyroid status (e.g. the mouse TBG is similar to humans).

In principle, UDP-glucuronosyltransferases are inducible in humans through a number of substances (Griem et al., 2002). However, such an inductive mechanism is not known for nitroaromatic compounds.

Finally, a rodent-specific mode of thyroid carcinogenesis could not completely be ruled out, but at present no sufficient evidence is available to postulate a likely mode of action.

Uterus tumors

High dose F344 females exposed to nitrobenzene had an increased incidence of endometrial stromal polyps, a relatively common spontaneous lesion of the uterus in this strain. The overall incidences of endometrial stromal polyp in all exposure groups (23-36% vs. 16% in control females) were within the range of historical data (up to 37%, Leininger and Jokinen 1990). Toxic nitrobenzene-related effects on the uterus were not observed in this study or any other repeated dose study. Because of the high spontaneous rate in the F344 females and that the tumor rate of high dose females was within historical control values, the association of these benign uterus tumors to nitrobenzene exposure was considered as equivocal.

Lung tumors

The incidence of alveolar/bronchiolar adenomas in male mice increased related to the dosage, but it gained significance only at 50 ppm nitrobenzene. A higher rate of adenocarcinomas was seen in all dose groups, but their incidences did not reach significance and were not dose-related. The spontaneous incidences in control groups were 6 and 10% for lung alveolar/bronchiolar adenomas, respectively adenocarcinomas for males and 4 and 8% for females. The combined incidence of lung adenomas and carcinomas in male B6C3F1 mice did not exceed the 2-year historical control ranges (up to 42%, Rittinghausen et al., 1996). Consistently, the incidences of alveolar/bronchiolar hyperplasia considered as a preneoplastic lesion were increased in males of the 25 and 50 ppm groups. Another nonneoplastic lesion, the alveolar bronchialization was evident with dose-related higher incidences in all male and female groups of B6C3F1 mice. Other repeated dose inhalation studies revealed hyperplasia of the bronchial epithelium in male and female B6C3F1 mice (Medinsky and Irons 1985). Females did not show lung tumors after nitrobenzene treatment, but alveolar bronchialization, respectively bronchial hyperplasia, was evident.

The moderate spontaneous lung tumor rates, the lack of dose-relationship (for combined adenomas and adenocarcinomas and for adenocarcinomas alone) and the fact that increased tumor rates are still in the historical control range are uncertainties to consider lung tumors as nitrobenzene-related.

Mammary tumors

Increased incidence of mammary gland adenocarcinomas was seen in female mice exposed to 50 ppm nitrobenzene. No other adverse effect was seen in this or other repeated dose study.

Conclusion and rationale for classification

Nitrobenzene was classified with Carc. Cat. 3, R40 in 1994 and introduced in Annex I of 67/548/EEC with the 22. ATP, corresponding to Carc. Cat. 2, H351 under Regulation (EC) No 1272/2008 (CLP). With respect to carcinogenicity, there are no new relevant data available.

Chronic inhalation of nitrobenzene induced increased incidence of tumors of the lung and thyroid in male B6C3F1 mice, and higher tumor rates of the mammary gland in the female mice. No clear causal relationship of nitrobenzene to the murine lung tumors could be recognized. Although a clear dose-response-relationship was not present for the low and mid dose groups, the thyroid tumors (only adenomas) at the high dose must be considered as nitrobenzene-induced since no rodent-specific mechanism could be applied. Due to the absence of tumors in untreated controls the mammary tumors were also contributed to the nitrobenzene treatment.

The tumor sites observed in nitrobenzene exposed mice did not clearly show coincidence with the tumor sites in the rat strains. In male F344 rats exposed to nitrobenzene higher rates of liver and

kidney tumors were seen and female F344 rats had higher incidences of uterine neoplasms. Although not gaining significance, it could not be excluded that increased rates of thyroid adenocarcinomas in F344 rats were associated to nitrobenzene. A single tumor site was related to nitrobenzene treatment in CD rats; males of this strain had liver cell adenomas and adenocarcinomas similar to F344. The treatment relationship of the uterine tumors appears unequivocal due to the high spontaneous rates while the liver tumors in two rat strains and the kidney tumors in one rat strain have to be considered as caused by nitrobenzene treatment.

Table 12: Incidences of the significantly increased tumors after inhalation of nitrobenzene

	F344 rats f	F344 rats m	CD rats m	B6C3F1 mice f	B6C3F1 mice m
Lung: Adenoma or carcinoma*					13% (0 ppm) [(T)], 31% (5 ppm)[(1)], 32% (25 ppm), 35% (50 ppm)
Liver: Hepatocellular adenoma or carcinoma*		1% (0 ppm) [(T)], 4% (1 ppm), 4% (5 ppm), 21% (25 ppm)	3% (0 ppm), 1% (1 ppm), 6% (5 ppm), 14% (25 ppm)		
Kidney: Tubular Adenoma or carcinoma*		0% (0 ppm) [(T)], 0% (1 ppm), 0% (5 ppm), 9% (25 ppm)			
Thyroid: Follicular cell adenoma or adeno-carcinoma*		3% (0 ppm) [(T)] 1% (1 ppm) 7% (7 ppm) 11% (25 ppm)			0% (0 ppm) [(T)], 6% (5 ppm), 2% (25 ppm), 11% (50 ppm)
Uterus: Endometrial stromal polyp	16% (0 ppm) [(T)], 26% (1 ppm), 23% (5 ppm), 36% (25 ppm)				
Mammary gland: Adeno-carcinoma				0% (0 ppm), 8% (50 ppm)	

[(1)]underlined values: significantly different from incidence in 0-ppm control group determined by Fisher Exact Test, $p<0.05$.
[(T)] only significantly positive exposure-related trend in incidence determined in Cochran-Armita Trend test
*combined incidences

In summary, nitrobenzene is carcinogenic in two species, mice and rats, and in two rat strains. For the kidney tumors a cytotoxic mode of action might be acceptable as the likely mode, but for the other tumors observed, a toxic effect possibly preceding the tumor development was not clearly identified. Other target sites with marked toxicity such as hematopoietic system (erythrocytes and spleen), nose or testes did not show a tumor response.

From a conservative view, the findings support the classification as a carcinogen of category 2 (CLP: 1B) although no mutagenic potential has been identified. Supportive arguments for category 2 (CLP: 1B) may be given by a general concern for carcinogenicity of nitroaromatic compounds. A number of substances with structural similarities to nitrobenzene were already classified as

carcinogens, category 2 (CLP: 1B) such as 2-nitrotoluene (CAS 88-72-2), 2,4-dinitrotoluene (CAS 121-14-2), 2,6-dinitrotoluene (CAS606-20-2), 2,3-dinitrotoluene (602-01-7), 3,4-dinitrotoluene (CAS 618-85-9), 3,5-dinitrotoluene (CAS 618-85-9), 2,5-dinitrotoluene (CAS 619-15-8), 4-nitrobiphenyl (CAS 92-93-3), 2-nitroanisole (CAS 91-23-6), 5-nitroacenapthalene (CAS 602-87-9), and 2-nitronapthalene (CAS 581-89-2).

Weighing the evidence for the distinction between category 2 (CLP: 1B) and 3 (CLP: 2), there are also arguments to propose a classification as category 3 (CLP: 2) carcinogen:

- The genotoxicity data available did not give a substantial concern that nitrobenzene is mutagenic. Testing in vitro (bacterial tests, chromosomal aberrations test, UDS in human hepatocytes) and in vivo (MN test in the mouse, tests on chromosomal aberrations and SCE on rat lymphocytes, UDS in rat hepatocytes) were negative. Although DNA binding for rat liver and kidney and for mouse liver and lung could in principle indicate a mutagenic effect, relatively low DNA binding activities were estimated in the study of Novartis (1997). Also, the DNA binding activities did not reflect the distribution of tumors among sexes since no sex-specific distribution of activities was found in the rat liver, the rat kidneys and the mouse liver. Covalent binding indices were equally low in both sexes for the rat liver and the mouse liver, however liver tumors were only observed in the male rat liver. The weak positive DNA binding alone was interpreted as an insufficient argument for a genotoxic mode of action. At present, nitrobenzenes' carcinogenicity is thought to be mediated by a non-identified, non-genotoxic mechanism.

- Nitrobenzene is readily metabolized in humans and animals via all exposure routes to a number of nitroaromatic compounds. A nitro-reductive enzyme activity in organs or intestinal nitro-reduction produces aniline probably via nitrosobenzene, and phenylhydroxylamine. Aniline is classified as a carcinogen, category 3 (CLP: 2) and a mutagen, category 3 (CLP: 2). The sparse data available for nitrosobenzene and phenylhydroxylamine do not allow a conclusion about their genotoxic potential (Bomhard and Herbold, 2005), no data are available to conclude on their carcinogenic potential. Aniline might be applied for comparative evaluation sharing with nitrobenzene the same metabolites (nitrosobenzene and phenylhydroxylamine), and its classification as carcinogen, category 3 (CLP: 2) would support the same category for nitrobenzene. But this comparison is limited by differences in the observed tumor spectrum: associated to the haemolytic toxicity - the spleen was the only tumors site for aniline.

- Although there is supportive evidence from category 2 (CLP: 1B)-classified nitroaromates with structural similarities, it must be considered that the spectrum of metabolites from which one or multiple metabolites should be suspected to be active as the ultimate carcinogen is quite different to those of nitrobenzene. Multiple tumor sites appeared to be common for representatives of the compound group; however the spectrum of target tumor sites could differ considerably. Liver tumors were also observed from 2-4-dinitrotoluene and 2-nitroanisole, but tumor types or tumor sites were not consistent to those of nitrobenzene.

- A consistency of tumor findings does only exist for liver tumors found in one sex of two rat strains. Other target organs did not show consistency across rodent species, sex and across strains. The observed diversity of tumor sites among rats and mice may be explained by differences in the metabolic pattern. For example, F344 rats are known to form p-hydroxyanilide at a higher rate than CD rats or B6C3F1 mice (ratio 19:9:3.6, see RAR 2007, Table 4.1 in 4.1.2.1.1). In rats no excretion of p-aminophenol was found and a higher percentage of p-nitrophenol and n-hydroxyacetanilide was seen (see RAR 2007, Table 4.1 in Section 4.1.2.1.1). Differences in intestinal nitro-reduction and its contribution to the generation and absorption of metabolites could also exist between species, strains and sexes. Although the exact mechanisms of tumor production remain unknown for

nitrobenzene, the absence of consistency for tumor responses among species and sexes weakens the evidence for category 2 (CLP: 3).

Based on the evidence from the present database it is proposed to confirm the classification and labelling is confirmed:

Carcinogen, Category 3, Harmful, Xn, R 40, Limited evidence of a carcinogenic effect.

(CLP: Carcinogen category 2, H351 Suspected human carcinogen)

5.8.7 Comparison with classification criteria

Criteria to be met:
A substance can be classified according to Directive 67/548/EEC as category 2 carcinogenic substance if there is sufficient evidence to provide a strong presumption that human exposure to a substance may result in the development of cancer, generally on the basis of: —

- appropriate long-term animal studies,

— - other relevant information.

or to Category 3 of carcinogenic substances which cause concern for man owing to possible carcinogenic effects but in respect of which the available information is not adequate for making a satisfactory assessment. There is some evidence from appropriate animal studies, but this is insufficient to place the substance in category 2.

Within the CLP classification system the classification in Category 1B presumed to have carcinogenic potential for humans, classification is largely based on animal evidence.
Such evidence may be derived from:

— animal experiments for which there is sufficient (1) evidence to demonstrate animal carcinogenicity (presumed human carcinogen). In addition, on a case-by-case basis, scientific judgement may warrant a decision of presumed human carcinogenicity derived from studies showing limited evidence of carcinogenicity in humans together with limited evidence of carcinogenicity in experimental animals. The placing of a substance in Category 2 is done on the basis of evidence obtained from human and/or animal studies, but which is not sufficiently convincing to place the substance in Category 1A or 1B, based on strength of evidence together with additional considerations (see section 3.6.2.2). Such evidence may be derived either from limited (1) evidence of carcinogenicity in human studies or from limited evidence of carcinogenicity in animal studies.

Existing evidence: There are no data on carcinogenicity of nitrobenzene in humans thus classification has to be based on results of animal studies. The long-term carcinogenicity study of nitrobenzene has been performed by one scientific centre (CITT,1993; Cattley et al. 1994) on male and femaleF-344 rats, male Cd rats exposed to nitrobenzene vapour at concentrations 0, 0.005, 0.025, 0.13 mg/L (0, 1, 5, 25 ppm) for 6h/d, 5d/w for 107 weeks and male and female B6C3F1 mice exposed to nitrobenzene vapour at concentrations 0, 0.005, 0.025, 0.13 and 0.26 mg/L (0, 1, 5, 25 and 50 ppm) for 6h/d, 5d/w for 107 weeks . The value of the study is reduced because some internal organs considered by the authors as nontarget tissue such as lungs, kidney, adrenals, prostate, testes, ovaries, uterus were not microscopically examined in all exposed animals, but were examined only in control and highest exposed group animals.
Mice. In male mice there was statistically significant increase in frequency of animals with benign lung adenoma and thyroid adenoma in the 0.13mg/L group , but not in frequency of lung or thyroid carcinoma. In female the frequencies of lung adenoma and carcinoma were not different form control. The frequency of lung adenoma and combined adenoma and carcinoma in control male and female mice were 13% and 11 %, while in the highest dose group they were more frequent

35% and 10%. There was increase in frequency of mammary gland carcinoma (8%) in female mice exposed at 0.26 mg/L, but female mice exposed at lower concentrations were not microscopically examined. Historical control values were not given. There was a relatively high frequency of hepatocellular adenoma and carcinoma in liver of control and exposed mice, apparently not treatment related, reaching 37 % and 33% in control and highest dosed males, respectively, and 14% in control female and 23% in highest dose animals
Rats. There was as statistically significant increase in frequency of animals with hepatocellular adenoma in liver in male F344 and CD rats, but not in female F344 rats, exposed at the highest concentration, however there no increase in frequency of these adenomas in rats exposed at lower concentrations. There was also an increased frequency of animals with tubular adenoma in kidney in male F344 rats exposed at highest concentration of 0.13 mg/l, but not in other exposed F 344 females and male CD rats. There was not treatment related increase in hepatocellular carcinoma nor in tubular carcinoma in male or female rats.

This evidence of carcinogenicity should be interpreted as limited because there was significant increase in frequency of benign neoplastic changes such as adenoma in lung and thyroid only in male mice, but not in female mice. Increase in mammary gland carcinoma in highest group of female mice could not by supported by the results in low exposed group because they were not microscopically examined. The location of benign tumours in rats were different than in mice. They were located only in liver of maleF344 and CD rats, and in kidney of F344 rats , but not in female F344 rats. So there is inconsistency in neoplastic responses between mice and rats and between females and males, which is lowering a strength of evidence.
Based on this limited evidence it is proposed to classify nitrobenzene in CLP system for category 2 carcinogens, H 351 and in the DSD system to Category 3, R 40 limited evidence for carcinogenicity

5.8.8 Conclusions on classification and labelling

Based on this limited evidence it is proposed to classify nitrobenzene in CLP system for category 2 carcinogens, H 351 and in the DSD system to Category 3, R 40 limited evidence for carcinogenicity

5.9 Toxicity for reproduction

5.9.1 Effects on fertility

Species	Route Dose	Exposure time	Number of gen. exposed	Observations and remarks
Rat Sprague-Dawley (m/f) (30 animals/ sex/ group)	Inhalation 0 (air), 1, 10, and 40 ppm (0.005, 0.051 and 0.204 mg/L)	Premating (10 wks): 6 hr/day, 5 days/week mating and gestation: 6 hr/day, 7 days/week dams		2-generation reproductive toxicity study Mating procedure and exposure regimen for the F1 animals identical with those for the F0 rats. F2 pups were never exposed to nitrobenzene by inhalation. F1 males not sacrificed after mating were used for recovery studies: high-dose and control group males were allowed a 9-week (one spermatogenesis cycle) nonexposure period after the 2-week mating period. At the end of the recovery period, they were mated to nonexposed virgin females on a one-to-

		exposed until g.d. 19 after delivery from p.n. d. 5 on dams only (without litters) returned to exposure until p.n. day 21 F_1 animals were allowed a 2-week growth period without nitro-benzene exposure.		one basis. No mortalities and no treatment-related clinical signs of abnormality in the F0 and F1 rats during the entire exposure period and during recovery. No biologically significant alterations in absolute body weights or body weight gains due to nitrobenzene exposure, during gestation. The F_0 and F_1 female rats exposed to 40 ppm had lower body weight gains when compared to controls, however, this finding was attributed to the decreased number of pregnant rats. Differences in female body weight during gestation in the recovery phase of the study were also attributed to the lack of pregnancies in the females mated with the F_1 males formerly exposed to 40 ppm nitrobenzene. The fertility index (number of pregnancies/number of females mated) clearly decreased in the 40-ppm group in F_0, F_1 and the recovery groups (16/30, 3/30, and 14/30, respectively). No statistically significant alterations in fertility were observed in the 1- or 10-ppm groups, Also, in F_1 females of the 40-ppm group the gestation index (numbers of pregnancies with live litters/number of pregnancies) and the number of implantations were decreased: of the three pregnant F_1 females only one delivered and in two of the three uteri examined a decreased number of implantations were observed. No biologically significant differences in gestation index, number of implantations, number of resorptions, resorption index (number resorptions/number of implantations) or duration of gestation were observed in the 1- or 10-ppm groups, no biologically significant differences in litter size at birth, number of viable pups, sex ratio, and survival indices on p.n. day 1, 4, or 21 of any generation.
				(cont.) F_1 offspring body weights of the male and female pups of the 40 ppm group were approximately 12% lower than respective control values on p.n. day 21; there were no differences in body weight between control and 40-ppm pups of the recovery generation. Comment on Lactation effects: There were no treatment-related clinical signs of abnormality in the F_0 and F_1 rats during the nitrobenzene exposure regimen which included premating, mating, gestational, and postnatal periods. The live birth index, the survival index on day 1 and on day 4 and lactation index of F_1 and F_2 generations were not affected by inhalation exposure to nitrobenzene at concentration of 0.005 , 0.051 and 0.204 mg/L. The mean survival indices for all groups, all generations, ranged between 93 and 100%. Instances of ecchymosis, hypoactivity,

				hypothermia, and partial cannibalism of pups were evenly distributed among control and nitrobenzene-treated groups for all generations. For the F_1 generation, body weight means of the male and female pups of the 40-ppm group (0.204mg/L group) were approximately 12% lower than respective control values on Postnatal Day 21. The increase in the 1-ppm (0.005mg/l) F_1 male body weights on Postnatal Days 14 and 2 1 was considered spurious. For the F_2 generation, male body weights on Postnatal Day 0 of both the 1 - and 10-ppm groups were lower than the male control value only (numerical data not provided). Although male body weights of both the 1 and 10 ppm groups were lower than control values on postnatal Day 0, these decreases were only 5% below mean and were not observed on Postnatal Days 4, 7, 14, and 2 1. Thus, the body weight decreases in the F2 male pups were not considered to be biologically significant. The survival index for Postnatal Days 1, 4, and 2 1 (lactation index) was unaltered for all nitrobenzene exposure groups of each generation (F_1, F2,). Beginning with Exposure Day 15 (Week 3) of the F1 exposure regimen, clinical signs of sialodacryoadenitis (SDA) viral infection were observed in several males and females. These signs included swollen glands of the neck region, lacrimation, and conjunctivitis and were common to all exposure groups (including control). Signs of SDA viral infection were observed through Exposure Day 26 (Week 6). During the recovery phase, no nitrobenzene exposure-related clinical signs were observed in the F1 male rats.
				Reductions in the size of the testes occurred, and stat. sign. reductions in both abs. and rel. weights of testes and epididymides in F_0 males of the 40-ppm group after 12 weeks of exposure. There were similar observations in the F_1 males of the 40-ppm group after 9 weeks of recovery; mean weights of testes and epididymides for the 1- and 10-ppm groups of F_0 and F_1 generations were similar to control values. There were no microscopic changes in the reproductive organs of the female rats that could be attributed to nitrobenzene exposure. Biologically significant histopathological findings were limited to the testes and epididymides of F_0 and F_1 rats exposed to 40 ppm nitrobenzene; specifically, the testes of the F_0 generation males of the 40-ppm group had seminiferous tubule atrophy and spermatocyte degeneration. Degree and distribution of the atrophy were marked to severe and multifocal or diffuse, respectively, in 14/30 animals. In addition, there were giant syncytial spermatocytes observed in the seminiferous tubules of

				22/30 animals. The epididymides of these males had degenerated spermatocytes in the tubular lumina and decreased numbers of spermatids. The microscopic findings for the testes of the F_1 generation males exposed to 40 ppm for 12 weeks followed by a 9-week recovery period were similar to those for lesions of 40 ppm F_0 males. Marked or severe atrophy of seminiferous tubules persisted in 21/30 animals. However, giant syncytial spermatocytes were nearly absent and the active stages of spermatocyte degeneration in the seminiferous tubules were much less frequent. As with the F_0 males, the epididymides of these F_1 males contained degenerated spermatocytes and reduced numbers of spermatids. With regard to male reproductive organ toxicity and spermatogenesis a NOAEC of 10 ppm (equivalent to 51 mg/m^3) is derived from this study. No signs of systemic toxicity were observed in this study up to and including the highest tested concentration of 40 ppm (equivalent to 205 mg/m^3). (Dodd et al. 1985, 1987)
Rat (CD, 16/ group); Sprague Dawley and F344; Mouse B6C3F1 (10/ sex/ group)	Inhalation 0.573 mg/L 0.640 mg/L	14 days		see also 5.6.2: Persistent testicular and epididymal lesions as well as severe spermatotoxic effects were reported from histopathological examination of the gonads from two 14-day inhalation studies with Sprague Dawley and F344 rats as well as with B6C3F1 mice at high concentration levels of 112 ppm (573 mg/m^3) and 125 ppm (640 mg/m^3). Considering testicular toxicity, respectively dysspermatogenesis, a NOAEC in the range of 35 to 39 ppm (179 to 200 mg/m^3) can be derived from these subacute toxicity studies. (DuPont 1981; Medinsky and Irons 1985)
Rat F344 and CD; Mouse B6C3F1	Inhalation 0.256 mg/L	90 days		see also 5.6.2: Likewise, in a 90-day inhalation study with F344 and CD rats as well as with B6C3F1 mice moderate to severe degeneration of tubular epithelial cells of the testes, Leydig cell hyperplasia and aspermia in the epididymis were found at concentration levels of 50 ppm (256 mg/m^3) for the male rats but not for male mice. The NOAEC for testicular and spermatotoxic effects in this study for rats was 16 ppm (82 mg/m^3). (Hamm 1984)
Rat Sprague-Dawley (10m/10f)	Oral 0, 20, 60, and 100 mg/kg	Comment on exposure duration: Male and female rats were dosed	Study according to OECD TG 422	Some of the high dose animals exhibited neurological signs, and 2 males and 9 females (7 during pregnancy, 2 during lactation) died during the study. Food consumption and body weight gain was also reduced in this group. Haemolytic anaemia due to methaemoglobin formation was evident in treated males. There were significant increases in

		once a day for 14 days prior mating and during mating (up to 14 days) and gestation (22 days) periods to day 3 of lactation		absolute and relative organ weights of the liver and spleen in treated males along with significant decreases in testis and epididymidis weights (60 and 100 mg/kg bw dose groups). Toxic changes were observed in the liver, kidney, spleen, bone marrow and brain. Histopathologically, all males in the high and middle dose groups and one male in the low dose group (20 mg/kg bw) showed atrophy of seminiferous tubules, the severity being dose-dependent. In addition, Leydig cell hyperplasia and decreased numbers of cells with round nuclei per seminiferous tubule in the testes and loss of intraluminal sperm in the epididymidis were observed. With respect to reproduction, there were no evident effects on copulation, fertility, and implantation indices in treated dam, although the survival index of the dams was dramatically decreased in the high dose group. There were no abnormalities in the gestation period and in delivery conditions in remaining treated females and controls. One dam died in the 20 and 60 mg/kg bw groups as well as the remaining two dams of the 100 mg/kg bw group during day 1 and 3 of lactation. The number of pups alive on day 0 of lactation and the live birth index were significantly decreased in the high dose group and no pubs were alive on day 4 of lactation. The viability index was significantly decreased at that day also in the 60 mg/kg bw dose group. The pup body weights were decreased in the middle and high dose group on day 0 and on day 4 in male pups of all treatment groups and in the females of the middle dose group. No pups showed any external or visceral malformations. A LOAEL systemic toxicity of 20 mg/kg bw/d was derived from this study based on changes in haematological parameters in males from each treated group. No dosage without adverse effect on male reproductive system (LOAEL 20 mg/kg bw/d, atrophy of seminiferous tubules) was investigated in this study. (Mitsumori et al. 1994)
Rat Sprague-Dawley (70 males/group)	Oral, gavage 60 mg/kg bw/d controls received 1 ml/kg sesame oil	Up to 70 days	“ReproTox-Protocol” (OECD-proposed OECD 1990)	A further experiment was conducted to determine which spermatogenic endpoints were affected by nitrobenzene, how changes were related to male fertility and how long a treatment period is needed before damage can be detected. An experimental group (n=70) of male Sprague-Dawley rats was given nitrobenzene via gavage (10% in sesame oil) each morning for up to 70 consecutive days at a dosage of 60 mg/kg bw/d. 70 control male rats received 1 ml/kg sesame oil. Groups of treated and control males were mated to normal proestrus females on day 7, 14, 21, 28, 42, 56, or 70 of treatment. Male rats were sacrificed on the day after

				mating, and testes and epididymides weights, sperm count and sperm morphology, sperm motility, progressive motility of sperm, as well as copulation and fertility indices were examined. No change in testicular and epididymal weight was observed in the 7-day treatment group. Significant and pronounced organ weight decreases however were observed in all groups sacrificed thereafter. Histopathological observations of the testes revealed a decrease in elongated spermatids and the appearance of multi-nuclear giant cells in the day 14 group. No change in sperm count was observed in the 7-day group. The sperm count of the 14-day group was significantly reduced to 34% of the control value. Sperm counts of the 21-day group and all groups thereafter were dramatically decreased mostly to less than 10% of the control values. Sperm motility was decreased beginning on day 14 of treatment as was progressive motility, and no progressive motility was observed beginning on day 21. Sperm viabilities of the 7-day and 14-day treatment groups were comparable to control values, whereas it was significantly decreased to 20% at 21 days. Thereafter sperm viability was less than 10%. Abnormal sperm rate increased from treatment day 21 on to about 40 to 50% in the later treatment groups. Copulation indices were comparable in the control and all treatment groups. In the control group all females were fertilized. Fertility indices of the 7- and 14-day treatment group were unaffected. A significant decrease in fertility index was observed in the 21-day treatment group. No more pregnant animals were obtained from groups in which rats were treated for 28 days or longer. Data from this study thus demonstrated, that the fertility index due to oral nitrobenzene exposure was not affected until sperm count was depressed at or below 10%. (Kawashima et al. 1995)
Rat Mouse	Oral 18.75, 75, and 300 (mouse) or 9.4, 37.5, and 75.0 (rats) mg/kg bw/d	13 weeks		For mice as well as for rats reduced organ weights for testes and epididymides as well as a decrease in sperm density and sperm motility and an increase in percentage of abnormal sperm were indicated for dosages of 18.75, 75, and 300 (mice) or 9.4, 37.5, and 75.0 (rats) mg/kg bw/d (testing of lower dosages was not indicated). For the female sex no data were given, since monitoring of vaginal cytology was revealed not to be a suitable screening parameter. For the oral route of administration the dosage of 9.4 mg/kg bw/d (LOAEL) investigated in this study in rats was the lowest dose tested. (Morrissey et al. 1988)
Rat F344	Oral 50, 75, 110, 165, 200,	One single treatment		In an oral study with F344 rats groups of six rats each received single dosages of 50, 75, 110, 165, 200, 300, or 450 mg nitrobenzene/kg bw in corn oil for dose response

	300, or 450 mg/kg bw in corn oil			evaluations. Three rats at each dosage were sacrificed 2 and 5 days after administration. For time-response evaluations groups of three rats each were orally dosed with 300 mg nitrobenzene/kg bw in corn oil and sacrificed at 1, 2, 3, 4, 7, and 10 days after administration. Liver, testes, and epididymides were histologically examined. Testicular lesions were restricted to the seminiferous tubules in this study. The early lesion consisted of enlarged, pale staining cytoplasm of the primary and secondary spermatocytes. Progressive necrosis of these layers was seen with complete destruction of the spermatocytes at days two and three after 300 and 450 mg nitrobenzene/kg bw. Within three days after administration, multinucleated giant cells within the seminiferous tubules were detected. In addition, necrotic debris and decreased number of spermatozoa were noted in the epididymis as early as three days and as late as ten days after nitrobenzene administration. No apparent effect on the epididymal epithelium was reported from this study. (Bond et al. 1981)
Rat F-344	Oral 300 mg/kg bw in corn oil	Single dose with observation for 100 days		To investigate the possible regeneration of the seminiferous epithelium after single dose administration, in a further study sperm production had been continuously monitored in F344 rats, the vas deferentia of which had been anastomosed with the urinary bladder to allow chronic monitoring of sperm output by microscopically counting the number of sperm in collected urine. Six weeks after surgery rats were dosed p.o. with a single dose of 300 mg nitrobenzene/kg bw in corn oil and followed up to for up to 100 days. Degenerative changes in the seminiferous tubules were observed histologically as early as 3 days after dosing. Pachytene spermatocytes and step 1-2 spermatids were shown the most susceptible stages and were observed forming giant cell stages as early as three days after treatment. A 17-day period of aspermia resulted: sperm were not detected in the urine of treated rats between 32 and 48 days after treatment. By days 76 - 100, the rate of sperm output recovered and reached 78% of the control group. By day 100 after treatment, an approximately 90% regeneration of the seminiferous epithelium could be observed. (Levin et al. 1988)
Rat Sprague-Dawley (6m)	Oral 300 mg nitrobenzene/kg bw	Single dose with observation for 14 days		Nitrobenzene was further investigated within in a short duration test design evaluated for screening of reproductive responses. Groups of six male Sprague-Dawley rats each were orally treated once with a single dose of 300 mg nitrobenzene/kg bw, sacrificed after 2, respectively 14 days, and investigated for organ weight and histopathology of testes and epididymides, sperm count and sperm morphology. No quantitative data are available from this

				study. It is reported that fourteen days after treatment testes and epididymides weights had decreased as well as had epididymal sperm count and an increase in abnormal sperm morphology was observed. Histopathology revealed degeneration of spermatocytes as soon as two days after treatment. (Linder et al. 1992)
Rat Sprague-Dawley (6m)	Oral 300 mg/kg bw in corn oil			Nitrobenzene was further tested within a comparative *in vivo*/*in vitro* test design using modulation of the Sertoli cell immunoreactive inhibin secretion as an indicator for early detection of adverse effects of chemicals on spermatogenesis. Groups of six male Sprague-Dawley rats were gavaged with doses of 300 mg nitrobenzene/kg bw in corn oil and sacrificed 1 and 3 days after treatment for collection of testicular interstitial fluid. Testicular weight was significantly reduced at 3 days post-treatment, and there was a significant increase in the levels of immunoreactive inhibin in testicular fluid at both 1 and 3 days after treatment. Also in cultures of isolated seminiferous tubules or Sertoli cells of untreated adult males the incubation with 0.01 or 1 mM nitrobenzene for 1-3 days induced a dose-related increase in both basal and stimulated secretion of immunoreactive inhibin. (Allenby et al. 1991)
Rat Wistar (m)	Oral 300mg/kg bw			Nitrobenzene was further evaluated within an *in vitro*/*ex vivo* test design where male Wistar rats received a single oral dose of 300 mg nitrobenzene/kg bw or the vehicle. Seminiferous tubules were isolated 1 or 3 days after treatment at different stages of the spermatocytic cycle and cultured in the presence of radiolabelled methionine for 24 hr. The culture medium was then analyzed for secreted proteins containing radiolabelled methionine. Testicular weight was significantly reduced after 1 and 3 days post-treatment. Incorporation of methionine into the secreted proteins was significantly decreased in treated groups and dependent on the stage of the spermatogenic cycle at which the tissues had been isolated. A similar effect was noted, when tissues from control rats were incubated in vitro with 0.1 mM nitrobenzene for 24 or 72 hr. The relative abundance of several potential marker proteins secreted by seminiferous tubules was changed dramatically upon treatment. (McLaren et al. 1993)
Rat/ Mouse	Dermal male rats: 0.05, 0.2 and 0.4 mg/kg bw/d male mouse: 0.05, 0.2 and 0.4	13 weeks		For male rats reduced organ weights for testes and epididymides as well as a decrease in sperm density and sperm motility and an increase in percentage of abnormal sperm were indicated for dosages of 0.05, 0.2 and 0.4 mg/kg bw/d. For male mice decreased testicular weight and sperm motility as well as increased percentage of abnormal sperm were indicated for dosages of 0.05, 0.2 and 0.4 mg/kg

	mg/kg bw/d			bw/d. (Morrissey et al. 1988)

5.9.2 Developmental toxicity

Inhalation exposure

Species/Strain	Dose (mg/kg)	Exposure	Observations and remarks
Rat CD (26 f)	0.005, 0.051 and 0.205 mg/L	g.d. 6 to 15 for 6 hr/day (whole chamber administra-tion)	Female rats were exposed to nitrobenzene vapours at 0, 10, and 40 ppm (5.1, 51.2 and 204.8 mg/m^3) The animals were observed daily for clinical signs throughout the study (g. d. 0 to 21), and maternal body weights were taken on g.d. 0. 6, 9, 12, 15, 18, and 21. After sacrifice on g.d. 21 maternal liver, spleen, kidney, and uteri weights were taken and the ovarian corpora lutea of pregnancy were counted. All live and dead foetuses as well as late and early resorption sites were noted and recorded. All live foetuses were weighed and sexed and examined for external malformations including cleft palate. One-half of the foetuses in each litter were examined for thoracic and abdominal visceral abnormalities including craniofacial structures, the other half was examined for skeletal alterations. There were no maternal deaths, early deliveries, or abortions. The pregnancy rate was high and equivalent for the control and all treatment groups. There were no exposure-related or concentration-related clinical signs of toxicity reported. In the 40 ppm group maternal weight gain was transiently reduced during the treatment period, however, at sacrifice maternal body weight was equivalent across all groups. Spleen weights (absolute and relative) were statistically significantly increased at 10 and 40 ppm with a clear-cut exposure-related response. Absolute and relative liver weights were also increased at 40 ppm but the differences were not statistically significant. Histological examination of maternal organs and measurement of methaemoglobin levels were not performed. Gestational parameters were unaffected by treatment. The control and treatment groups did not differ in number of corpora lutea per dam, in number of resorptions, dead and live foetuses per litter, in percentage pre- or postimplantation loss, in sex ratio or in foetal body weight per litter. Foetal evaluations revealed that there was no significant increase in the number of litters with one or more affected foetuses at any exposure concentration relative to controls for individual and total external, visceral, or skeletal malformations. There was a significant increase in the incidence of total malformations at 1 ppm but not at 10 or 40 ppm relative to that of controls. In the absence of an increased incidence of any specific malformation and in the absence of any concentration response, this finding was not considered treatment related. The incidences of variations did not indicate foetal toxicity, likewise there were no indications of reduced

			foetal body weights or any other signs of foetal toxicity. In terms of developmental toxicity, a NOAEC of 40 ppm (205 mg/m^3) can be derived from this study. In terms of maternal toxicity a NOAEC of 10 ppm (51 mg/m^3) can be derived. (Tyl 1984; Tyl et al. 1987)
New Zealand White rabbits	10, 40, and 100 ppm (0.051, 0.205, 0.513 mg/L)	g.d. 7 to 19 for 6 h/d (whole chamber administra-tion)	Groups of 22 pregnant females were exposed to nitrobenzene at target concentration levels of 10, 40, and 100 ppm (equivalent to 67, 302 and 660 mg/m^3) on g.d. 7 to 19 for 6 h/d (whole chamber administration). Animals were weighed and given detailed physical evaluations at regular intervals during gestation. At sacrifice on g.d. 30 each female was given a gross post-mortem evaluation and the livers as well as a blood sample were taken for analysis of haemoglobin and methaemoglobin levels. Corpora lutea and uterine implantation data were also recorded. Foetuses were measured for body weight and crown-rump length. After gross external examination all foetuses were evaluated for visceral and skeletal malformations or variations in ossification. No adverse effect of treatment was evident from maternal mortality data. Mean body weight data during gestation were comparable between the control and treated groups. No adverse effect of treatment was evident from physical in-life evaluations or from gross post-mortem evaluations. Mean liver weight (absolute and relative) were increased in the mid-dose (relative liver weight 2.81 +/- 0.56 at 40 ppm compared to 2.52 +/- 0.6 for controls) and high-dose group animals (relative liver weight 2.82+/- 0.53 at 100 ppm). While haemoglobin values at sacrifice were comparable between control and treated groups mean methaemoglobin values were significantly higher than controls (40 and 60% increase) at the mid-dose and high-dose group. No adverse effect of treatment was evident from pregnancy rate data, premature delivery or abortion data. Corpora lutea and uterine implantation data were comparable between the control, the 10 and the 40 ppm group. In the high-dose group, the mean number of resorption sites, the mean percentage of resorptions to implants and the incidence of females with resorptions were slightly higher than control; however, these differences from control data were not statistically significant. No adverse effect of treatment was evident from foetal weight or crown-rump distance data or foetal sex distribution data. External, visceral and skeletal evaluation of foetuses from treated females did not reveal an increase in malformation rate nor an increase in the incidence of external, visceral or ossification variations. In terms of developmental toxicity, a NOAEC of 40 ppm (205 mg/m^3) can be derived from this study. For maternal toxicity a NOAEC of 10 ppm (51 mg/m^3) based on increased methaemoglobin levels and increased liver weights is derived. (Bio/dynamics Inc. 1984)
Conclusion: no classification			

5.9.3 Human data

As residents of the maternity ward after parturition, five mothers had eaten a cake that had contained an ingredient to simulate a bitter almond taste in autumn 1944. Lacking a comprehensive chemical analysis for the causative agent, instead of natural bitter almonds and almond paste it may have contained either nitrobenzene and/ or other substances like aniline, benzaldehyde or benzonitrile. The mothers did not reveal any clinical symptoms but on the next morning (approx. 15 hours after ingestion), their breast-fed babies had developed a strong to very strong cyanosis. The children did not show any additional symptoms and the cyanosis receded largely in the next 24 hours. The children were not breast fed for 1,5 to 2 days. They received large amounts of tea, and if necessary oxygen and heart stabilizing drugs (Dollinger 1949).

5.9.4 Other relevant information

5.9.5 Summary and discussion of reproductive toxicity

Fertility

Acording to section 3.7.1.3 of CLP regulation adverse effects on sexual function and fertility encompass any effect of substances that has the potential to interfere with sexual function and fertility. This includes, but is not limited to, alterations to the female and male reproductive system, adverse effects on onset of puberty, **gamete production and transport**, reproductive cycle normality, sexual behaviour, fertility, parturition, pregnancy outcomes, premature reproductive senescence, or modifications in other functions that are dependent on the integrity of the reproductive systems.
According to Directive 67/548/67 effects on male or female fertility, includes adverse effects on libido, sexual behaviour, any aspect of spermatogenesis or oogenesis, or on hormonal activity or physiological response which would interfere with the capacity to fertilise, fertilisation itself or the development of the fertilised ovum up to and including implantation.

Effects of nitrobenzene meeting classification criteria

Practically all studies of repeated toxicity demonstrated that nitrobenzene causes serious adverse effects on spermatogenesis in different species of animls:

1. degeneration of seminiferous tubular epithelium and atrophy of seminiferous tubule of rats exposed by gavage for 28 days at the dose of 125 mg/kg (Shimo et al. 1994)

2. atrophy of seminiferous epithelium, hypospermatogenesis and presence of multinucleate giant cells in testes of male F344 rats exposed at the dose of 75 and 150 mg/kg to nitrobenzene for 90 days via gavage, and in testes of male B6C3F1 mice exposed at the dose of 150 and 300 mg/kg to nitrobenzene for 90 days via gavage (NTP 1983a cited from U.S. EPA (2009)

3. moderate to severe degeneration of tubular epithelial cells was noted in the testes of all F344 males exposed by inhalation to 0.26 mg/L (50 ppm) for 90 days . This consisted of a maturation arrest at the level of primary and secondary spermatocytes and was usually accompanied by interstitial edema and Leydig cell hyperplasia. An absence of mature sperm was noted in the epididymis of these animals, together with the presence of proteinacious material within the lumen of the ductus. (CIIT subchronic study, 1984/ Hamm, 1984.)

4. CD rats exposed by inhalation for 90 days at the highest concentration – 0.26 mg/L displayed a marked bilateral testicular atrophy in response to nitrobenzene, as indicated by a loss of seminiferous epithelium with only a few scattered spermatogonial cells present, interstitial cell hyperplasia, oedema, and the absence of mature sperm in the epididymal lumen. (CIIT subchronic study, 1984/ Hamm, 1984.)

5. bilateral atrophy of the testis (57% at the highest dose vs 18% of controls) and bilateral hypospermia of the epididymis (54% at the highest dose vs13% of controls) was observed in CD male rats exposed for 2 years by inhalation to nitrobenzene at the highest concentration of 0.13mg/l as well as increased incidence of hypospermia in epididymis in male mice exposed for 2 years by inhalation at concentration of 0.26 mg/l (Cattley et al. (1994); CIIT (1993)

6. atrophy of seminiferous epithelium, hypospermatogenesis and presence of multinucleate giant cells in testes of male F344 rats exposed to nitrobenzene for 90 days via dermal exposure at doses of 400 and 800mg/kg and in testes of male B6C3F1 mice exposed by dermal route for 90 days at doses of 400 and 800 mg/kg to nitrobenzene (NTP 1983a cited from U.S. EPA (2009) (NTP,1983b study cited from US EPA, 2009).

7. atrophy of the seminiferous tubules, hyperplasia of Leydig cells, and loss of intraluminal sperm in the epididymides in male SD rats exposed for 40 - 54 days by gavage at doses of 60 or 100 mg/kg-day nitrobenzene in sesame oil (Mitsumori at al. 1994)

The alteration of fertility due to disturbances in spermatogenesis were confirmed in 2-generation reproductive toxicity study and other studies

1. reduced fertility index (number of pregnancies/number of females mated) SD rats in F0 and F1 generations and reduced gestation index (number of pregnancies with live litters/number of pregnancies) in a 2-generation study, in which rats were exposed by inhalation at concentration of 0.204mg/l 6hr/day, 5 days /week for two generations. These reductions were associated with atrophy of seminiferous epithelium in testes of F0 and F1 generations exposed at the highest concentration of 0.204mg/l (Dodd at al. 1987).

2. reduced fertility index of rats receiving 60mg/kg of nitrobenzene by gavage after 3 weeks of exposure. The value of fertility index was further dropping down with duration of exposure leading to infertility of male rats after 28 days of exposure. The fertility index due to oral nitrobenzene exposure was not affected until sperm count was depressed at or below 10%. (Kawashima et al. 1995)

The studies reviewed in the background document provide sufficient evidence that nitrobenzene has a property of causing serious alterations of spermacytogenesis and spermiogenesis in two animal species: rats and mice; leading to reduced fertility and to infertility of male animals. Thus there is sufficient evidence providing a strong presumption that human exposure to the nitrobenzene may result in the same adverse effects on the basis of clear evidence in animal studies of impaired fertility. These toxic effects were occurring at around the same or lower dose levels as other toxic effects and they were not a secondary non-specific consequence of the other toxic effects.

Developmental toxicity

Investigations in rats and rabbits with the inhalation route of application did not reveal any developmental toxicity (including teratogenicity) associated with the exposure to nitrobenzene during organogenesis at concentration levels that produced no observable maternal toxicity or produced some slight maternal toxicity.

Effects on or via lactation

Very young children are more susceptible to methaemoglobinaemia, the main cause of toxicity of nitrobenzene in man and animals (Beauchamp et al. 1982). This is due to newborns still having foetal Hb[**], which is more susceptible to metHb formation than adult Hb (Goldstein et al. 1969). Also, the activity of NADH-cytochrome b5 reductase, the enzyme required for the conversion of ferric iron to ferrous iron in Hb, is not fully developed in infants and very young children (Wentworth et al. 1999) and neither is G6PD activity, an enzyme required to replenish NADPH (Goldstein et al. 1969). Lastly, the neonates' clearance capacity is estimated to be about half of an adults' in the first four weeks post parture (Begg 2000).

Based on its low molecular weight of 123 $^{g}/_{mol}$ and its lipophilicity with a partition coefficient logP of 1.85 at 30°C, a good permeability into the lactiferous glands after systemic distribution is reasonable. Nitrobenzenes water solubility is sufficient for toxic doses, notwithstanding the lipophilic components of milk. There is a high likelihood of accumulation and milk concentrations in excess of the maternal plasma concentration. Even substances with a comparable molecular weight and lower lipophilicity such as p-acetylaminophenol (Paracetamol, MW 151.17 $^{g}/_{mol}$, logP 0.49) and α-methylphenethylamine (Amphetamine, MW 135.21 $^{g}/_{mol}$, logP 1,80) exhibit milk/plasma-ratios in ranges of 1.0 to 3.0 and half-lives in mammary tissue extended by 50% (Berlin et al. 1980, Findlay et al. 1981, Steiner et al. 1984).

The case of breast-fed cyanosed babies whose mothers had eaten an aromatized cake (Dollinger 1949) is an indication of a nitrobenzene-associated effect, since nitrobenzene was frequently found in solutions of false bitter-almond-oil (Zeitoun 1959) and other substances used to substitute natural ingredients even in Europe during and right after the war (Högl 1952). Other substances with bitter almond smell such as benzaldehyde (LDoral 1.3g/kg, rat; 1.0g/kg, guinea pig) or benzonitrile (LD50oral 971mg/kg; mouse) are less toxic, or do not induce clinical symptoms of cyanosis such as hydrogen cyanide (HCN), and therefore less likely to have caused the effect. The report of Dollinger, 1949, does not provide a proof that mothers were exposed to nitrobenzene. In addition the hypothesis which could be based on this report that nitrobenzene may induce toxic effects on or via lactation is not supported by results of 2-generation study (Dodd et al. 1987) and Mitsumori et al. 1994 study.

In a two-generation study in Sprague-Dawley rats (Dodd et al. 1987) the only significant finding in the litters derived from rats exposed at the highest concentration of 0.204mg/L (40 ppm) was an approximate 12% decrease in the mean body weight of F1 pups on Postnatal Day 21. There was no reduction of body weight of F2 weanlings on postnatal day 21 in any exposed groups, which suggest that this body weight decrease in F1 generation was not treatment related. No weight differences had been noted between all dose groups and control animals at parturition. The live birth index, the survival index on day 1 and on day 4 as well as lactation index of F1 and F2 generations were not affected by inhalation exposure to nitrobenzene at concentration of 0.005 , 0.051 and 0.204 mg/L. The mean survival indices for all groups, all generations, ranged between 93 and 100%. The maternal toxicity of F0 lactating dams exposed at 0.0204 mg/L was not demonstrated in a 2-generation study of Dodd at al. (1987), but it was only based on lack of visible clinical signs of abnormalities. The levels of haemoglobin and methemoglobin were not measured in a 2-generation study of Dodd at al. (1987), however it is highly probable that their levels in lactating mothers exposed at 0.204mg/l were severely affected. In rats exposed for 90 days to nitrobenzene by inhalation at concentration of 0.26 mg/l (Hamm,1984) comparable to concentration used in the study of Dodd et al. (1987), the level of methemoglobin were significantly increased up to ca. 10%,

[**] More fetal Hb than adult Hb until about 10 months of age. Fetal Hb is present until about 2 years of age.

followed by increase in bilirubin, fibrosis of spleen and hyaline degeneration in kidney. Thus slight reduction of weight of weanlings only in first generation could be due to marked maternal toxicity leading to haemolytic anaemia of lactating mothers not investigated in a study of Dodd at al. (1987) A screening study (Mitsumori et al. 1994, largely along OECD TG 422, which is insufficient for postnatal effects) reports methaemoglobinaemia in the F0 generation, and reduced body weights (F1) in males of the low dose group on day 4 as compared to day 0 after parturition, additional to reduced body weights of the middle and high dose group at parturition and reduced viability among those groups (67%, 0%). In the study of Mitsumori et al. 1994 female rats were receiving nitrobenzene 14 days before mating, during mating , gestation and up to 3rd day after parturition at doses of 20, 60 and 100mg/kg. None of pups born by female rats exposed at 100mg/kg survived till 4 day after parturition, viability of pups during first 4 days of life of females exposed at 60mg/kg was reduced to 66.9%, while viability of those exposed at 20 mg/kg (99%) was equal viability of control pups (99.1%). Mean body weight gain of pups in the control group for male and female pups were : 3.1 – 3.1g, in the 20mg/kg group: 2.6 – 2.7g and in the 60mg/kg were: 1.7 – 1.7 g, which demonstrated that nitrobenzene significantly influence body weight gain during first 4 days of lactation. No data were provided on further development. Taking into account that in studies of developmental toxicity on female rats and rabbits exposed by inhalation at 0.051; 0.205 and 0.503mg/L during organogenesis did not reveal developmental toxicity, showing nevertheless toxic effect on mother, the results of reduced viability and reduced body weight gain of surviving pups observed in the study of Mitsumori at al. (1994) could be interpreted as showing effect on or via lactation, if the lactating mothers were not severly intoxicated. However, the doses of 60 and 100 mg/kg given by gavage in the study of Mistsumori et al. 1994 were causing severe intoxication of mothers. The animals exposed at 100mg/kg/day from 13 day of exposure exhibited piloerection, salivation, emaciation and anaemia. In some of these animals; torticolitis, circling movement and abnormal gait was observed. Both males and females showed decrease in food consumption and decrease in body weight gain. Seven females out of 10 in the 100mg/kg group died during gestation and 2 during lactation period, which demonstrate severity of intoxication with nitrobenzene at this dose. In the 60mg/kg group the anaemia was seen in 6 of rats from day 19 of pregnancy and neurological signs such as abnormal gait and torticolitis were observed in one rat from day 1 of lactation. The females in the 60mg/kg group also showed decreased food consumption and inhibition of body weight gain during lactation. One female each from the 60 and 20 mg/kg died during lactation (Mitsumori et al. 1994). The levels of haemoglobin and RBC in males rates exposed at doses of 20, 60 and 100mg/kg were significantly reduced (females were not examined) and level of methemoglobin was significantly increased in comparison with control rats. Having that in mind the reduction viability of pups during 4 first days of lactation and reduced body weight gain of pups on day 4 of lactation was due to maternal toxicity leading to anaemia, methemoglobinemia, reduced body weight and death of lactating female rats.

5.9.5.1 Comparison with criteria

Fertility

Reduced male fertility and/or atrophy of spermatogenic epithelium, or degeneration of tubular epithelium in testes were observed at exposure levels inducing low increases of methemoglobin

concentration in blood (well below 10%), but not high enough to result in significant hypoxia in peripheral tissue.

The following reprotoxic effects were observed:

1. in rats exposed for 10 weeks by inhalation at relatively low exposure levels of 40 ppm (0.204mg/l) (Dodd et al. 1985, 1987), no methemoglobin measurements

2. in male rats exposed by inhalation for 2 years at concentrations of 5 and 25 ppm (0.026—0.13mg/l) with very small increase in percentage of methemoglobin amounting in control rats and rats exposed at concentration of 1, 5 and 25 ppm for 24 months to 2.7%, 2.9% 2.4% and 4.6%

3. in male rats exposed for 90 days by inhalation at concentration of 50 pp (0.26mg/l) with methemoglobin level of 10%, (Hamm1984, CIIT, 1984)

4. in male rats exposed for 90 days by gavage at doses of 75 and 150mg/kg with methemoglobin level of 7.3 and 12.2 % respectively (NTP, 1983a)

5. in male mice exposed for 90 days via gavage at doses 18.75; 37.5; 150 and 300mg/kg with methemoglobin level of 2.2%, 3.4%, 5.98% and 6.72%, Concentration of metHb in control male mice – 1.07 % (NTP, 1983a)

6. in male rats and mice exposed for 90 days to nitrobenzene via dermal route at doses of 400 and 800mg/kg, Only female mice showed a dose-related increase in MetHb concentration (NTP, 1983b).

7. As a consequence of damage to spermatogenic epithelium and reduced spermatozoa, the reduced fertility in terms of reduced number of pregnancies and offspring was demonstrated in a rat two-generation inhalation study (Dodd et al. 1985, 1987).

The results of these studies provide sufficient evidence that nitrobenzene has a property of causing serious alterations of spermacytogenesis and spermiogenesis in two animal species: rats and mice; leading to reduced fertility and to infertility of male animals. Thus there is sufficient evidence providing a strong presumption that human exposure to the nitrobenzene may result in the same adverse effects on the basis of clear evidence in animal studies of impaired fertility. These toxic effects were occurring at dose levels which did not induce severe methemoglobinemia and they were not a secondary non-specific consequence of the other toxic effects.

Therefore RAC is of the opinion that there is sufficient evidence to classify nitrobenzene according to the CLP regulation for the reproductive toxicity category 1B Presumed human reproductive toxicant with hazard statement H360F (Repr. 1B, H360F) and according to Directive 548/67 to category 2 with a risk phrase R60 – may impair fertility

The rationale for the RAC opinion is different from the rationale of the dossier submitter and TC C&L (2007) proposing to classify nitrobenzene to Repr.Cat.3; R62 and Repr. 2; H 361f.

TC C&L has acknowledged that :"*Numerous studies with rats and mice revealed nitrobenzene to persistently adversely affect male reproductive organs (atrophy of the germ epithelium) and spermatogenesis independently from the route of administration (inhalation, oral, dermal)"* however it has decided to weaken this evidence based on the following recognitions: .

- *"haematotoxicity is the predominating toxic effect after treatment with nitrobenzene and that these latter effects were also observed in the available reproduction toxicity studies with nitrobenzene.*
- *humans in comparison to the rat species are much more sensitive to the induction of methaemoglobinaemia and that* ***the rat as an experimental model rather may underestimate the significance of methaemoglobin-induced haematotoxicity of nitrobenzene****.*
- *as far as both haematological as well as reproduction parameters had been evaluated in the studies available with nitrobenzene, haematotoxicity was consistently induced at dose levels clearly below those inducing testes toxicity. Therefore, nitrobenzene is not considered to represent a specific reproductive toxicant".*

It its opinion RAC noted that reproductive toxicity of nitrobenzene is not related to its hematotoxicity as was assumed earlier. The increase in levels of methemoglobin up to 10% by exposure to aniline is not leading to a damage of spermatogenic epithelium, therefore the latter is not a secondary effects of increased level of methmoglobin. Such a conclusion may be derived from results of studies with repeated exposure of animals to aniline, which is also, like nitrobenzene, a strong methemoglobin-forming substance. No alterations of spermatogenic epithelium were reported in rodents repeatedly exposed to aniline despite increased level of MetHb (SCTEE, 2003; ECB, 2004) Thus, the damage to spermatogenic epithelium and reduced fertility is a specific effect of nitrobenzene independent from its ability to induce MetHb and related hematotoxic effects.

The other incorrect argument for downgrading classification of reprotoxicity of nitrobenzene from Repr Cat. 2 to Repr. Cat. 3 is an observation that Met-Hb formation appears often, although not always, in animals at slightly lower exposure level than the degeneration or atrophy of spermatogenic epithelium. Thus, in line with this argument, lowering the exposure to protect against formation of Met-Hb will also protect against spermatotoxicity. This argument is valid and appropriate in the process of risk assessment and management which is different from hazard identification. The supporters of this view do not take into account that the aim of both DSD and CLP classification systems is to identify inherent hazardous properties of a substance and that hazard identification is separate from risk assessment or risk management. Consequent use of this argument would lead to a limitation of the classification of substances to the most sensitive endpoints and to distortion of a process of hazard identification, which is a starting point for risk

assessment and management. It should be noted that nitrobenzene is the reproductive toxicant for animals in a range of doses that would allow to classify that substance to "STOT RE 1 (male gonads)" because the histopatological damage of spermatogenic epithelium was detected in animals exposed for 2 years by inhalation at dose level of 0.026—0.13mg/l, which is well below guidance value for STOT RE category 1 equal to 0.2 mg/L/6hour/day (CLP regulation) and below 0.25 mg/L/6hour/day which is a guidance value for category Xn, R48/20(male gonads) for studies of 90 days duration (DSD regulation).

The methemoglobin formation and spermatotoxicity should be thus regarded as inherent toxic properties of nitrobenzene with different mode of action and classified in accordance with criteria given in DSD and CLP regulations.

Taking the above consideration into account, RAC is of the opinion that there is sufficient evidence to classify nitrobenzene according to the CLP regulation for the reproductive toxicity category 1B Presumed human reproductive toxicant with hazard statement H360F (Repr. 1B, H360F) and according to Directive 548/67 to category 2 with a risk phrase R60 – may impair fertility

Effects on or via lactation

The rationale for the RAC opinion which is different from the proposal of the dossier submitter

In a two-generation study in Sprague-Dawley rats (Dodd et al. 1987) the only significant finding in the litters derived from rats exposed at the highest concentration of 0.204mg/L (40 ppm) was an approximate 12% decrease in the mean body weight of F1 pups on Postnatal Day 21. There was no reduction of body weight of F_2 weanlings on postnatal day 21 in any exposed groups, which suggest that this body weight decrease in F_1 generation was not treatment related. No weight differences had been noted between all dose groups and control animals at parturition. The live birth index, the survival index on day 1 and on day 4 as well as lactation index of F1 and F2 generations were not affected by inhalation exposure to nitrobenzene at concentration of 0.005 , 0.051 and 0.204 mg/L. The mean survival indices for all groups, all generations, ranged between 93 and 100%. The maternal toxicity of F0 lactating dams exposed at 0.0204 mg/L was not demonstrated in a 2-generation study of Dodd at al. (1987), but it was only based on lack of visible clinical signs of abnormalities. The levels of haemoglobin and methemoglobin were not measured in a 2- generation study of Dodd at al. (1987), however it is highly probable that their levels in lactating mothers exposed at 0.204mg/l were severely affected. In rats exposed for 90 days to nitrobenzene by inhalation at concentration of 0.26 mg/l (Hamm,1984) comparable to concentration used in the study of Dodd et al. (1987), the level of methemoglobin were significantly increased up to ca. 10%, followed by increase in bilirubin, fibrosis of spleen and hyaline degeneration in kidney. Thus slight reduction of weight of weanlings only in first generation occurred as indirect effect of marked maternal toxicity leading to haemolytic anaemia of lactating mothers not investigated in a study of Dodd at al. (1987) .

In a study of Mitsumori et al. 1994 female rats were receiving by gavage nitrobenzene at doses of 20, 60 and 100 mg/kg for 14 days before mating, during mating , gestation and up to 3rd day after parturition at doses of 20, 60 and 100mg/kg. None of pups born by female rats exposed at 100mg/kg survived till 4 day after parturition, viability of pups during first 4 days of life of females exposed at 60mg/kg was reduced to 66.9%, while viability of those exposed at 20 mg/kg (99%) was equal viability of control pups (99.1%). The results of reduced viability and reduced body weight gain of pups observed in this study only for 4 first days of life (Mitsumori at al. 1994) could be interpreted as showing effect on or via lactation, if the lactating mothers were not severely intoxicated. In fact, the doses of 60 and 100 mg/kg given by gavage in the study of Mistsumori et al. 1994 were causing severe intoxication of mothers. The animals exposed at 100mg/kg/day starting from 13 day of exposure exhibited piloerection, salivation, emaciation and anaemia. In some of these animals; torticolitis, circling movement and abnormal gait was observed. Both males and females showed decrease in food consumption and decrease in body weight gain. Seven females out of 10 in the 100mg/kg group died during gestation and 2 during lactation period, which demonstrate severity of intoxication with nitrobenzene at this dose. In the 60mg/kg group the anaemia was seen in 6 of rats from day 19 of pregnancy and neurological signs such as abnormal gait and torticolitis were observed in one rat from day 1 of lactation. The females in the 60mg/kg group also showed decreased food consumption and inhibition of body weight gain during lactation. One female each from the 60 and 20 mg/kg died during lactation (Mitsumori et al. 1994). The levels of haemoglobin and RBC in males rates exposed at doses of 20, 60 and 100mg/kg were significantly reduced (females were not examined) and level of methemoglobin was significantly increased in comparison with control rats. Having that in mind the reduction viability of pups during 4 first days of lactation and reduced body weight gain of pups on day 4 of lactation was due to maternal toxicity leading to anaemia, methemoglobinemia, reduced body weight and death of lactating female rats.

The case of breast-fed cyanosed babies whose mothers had eaten an aromatized cake (Dollinger 1949) is an indication of a nitrobenzene-associated effect, since nitrobenzene was frequently found in solutions of false bitter-almond-oil (Zeitoun 1959) and other substances with bitter almond smell such as benzaldehyde or benzonitrile are less toxic, or do not induce clinical symptoms of cyanosis such as hydrogen cyanide (HCN), and therefore less likely to have caused the effect. However, a report of Dollinger, 1949, does not provide any proof that mothers were exposed to nitrobenzene. In addition the hypothesis which could be based on this report that nitrobenzene may induce toxic effects on or via lactation is not supported by results of 2-generation study (Dodd et al. 1987) and Mitsumori et al. 1994 study.

Taking into account the data in the background document summarized above showing that alterations in viability and body weight gain of pups during lactation observed in studies on rats were due to severe maternal toxicity RAC is of the opinion, that nitrobenzene should not be classified within CLP system for hazard category for lactation effects with hazard statement H362 - may cause harm to breast-fed children (Lact. H362), or in DSD classification system the risk phrase R64 - may cause harm to breast-fed babies (R64). Thus the opinion proposed by the Dossier Submitter is not supported.

During public consultation some MSCAs were not in favour to classify nitrobenzene for lactational effects and some were in favour to do so.

5.9.5.2 Conclusions on Reproductive Toxicity

Conclusions on fertility

There is sufficient evidence to classify nitrobenzene according to the CLP regulation for the reproductive toxicity category 1B Presumed human reproductive toxicant with hazard statement H360F (Repr. 1B, H360F) and according to Directive 548/67 to category 2 with a risk phrase R60 – may impair fertility (T, R60)

Conclusions on developmental toxicity

Taking into account that studies in rats and rabbits with the inhalation route of application did not reveal any developmental toxicity (including teratogenicity) associated with the exposure to nitrobenzene during organogenesis at concentration levels that produced some slight maternal toxicity RAC shares an opinion of Dossier Submitter that nitrobenzene should not be classified as developmental toxicant.

Conclusions on lactation

Taking into account the data in the background document showing that alterations in viability and body weight gain of pups during lactation observed in studies on rats were due to severe maternal toxicity RAC is of the opinion, that nitrobenzene should not be classificed within CLP system for hazard category for lactation effects with hazard statement H362 - may cause harm to breast-fed children (Lact. H362), or in DSD classification system the risk phrase R64 - may cause harm to breast-fed babies (R64). Thus the opinion proposed by the Dossier Submitter is not supported.

5.10 Other effects

5.11 Aspiration Hazard

The dossier submitter originally proposed classification for aspiration toxicity (Asp. Tox 1 – H304 (CLP), Xn; R65 (DSD), based on an estimated kinematic viscosity of less than 20.5 mm^2/s at 40°C. However, during public consultation, a comment was received from an industrial association arguing that the classification criteria in Annex I of CLP (section 3.10.2) should only be applied for pure hydrocarbons. The dossier submitter argues that pure hydrocarbons should be considered an example of classes likely to exhibit aspiration hazardous properties. Nevertheless, upon further reflection and based on a high surface tension (42 mN/m) and existing classification for Acute Toxicity, the dossier submitter no longer considers the classification of Nitrobenzene for Apsiration Toxicity warranted. The withdrawal of the proposal was reflected in the revised CLH report submitted after public consultation. RAC agrees with the dossier submitter that classification for Aspiration Toxicity is not warranted.

5.12 Derivation of DNEL(s) or other quantitative or qualitative measure for dose response

Not relevant for this type of dossier.

6 HUMAN HEALTH HAZARD ASSESSMENT OF PHYSICO-CHEMICAL PROPERTIES

6.1 Explosivity

Including C&L

6.2 Flammability

Including C&L

6.3 Oxidising potential

Including C&L

7 ENVIRONMENTAL HAZARD ASSESSMENT

7.1 Aquatic compartment (including sediment)

7.1.1 Toxicity test results

7.1.1.1 Fish

Short-term toxicity to fish

The following table gives an overview of the sensitivity of different fish species to nitrobenzene in short-term tests. It covers the full range of species tested. For each species the lowest available valid test was selected, respectively.

Table 13: Acute toxicity data to fish

Species	Endpoint	Effect concentrations [mg/l]	Test system	Reference
Brachydanio rerio	96 hours LC_{50}	92 (mc)	Flow-through (OECD Guideline)	(Roederer 1990)
Lepomis macrochirus	96 hours LC_{50}	43 (nc)	Static (method: US-EPA)	(Buccafasso et al. 1981)
Pimephales promelas	96 hours LC_{50}	119 (mc)	Flow-through (no standard method)	(Geiger et al. 1985)
Pimephales promelas	96 hours LC_{50}	44 (nc)	Flow-through larval test (method: US-EPA)	(Marchini et al. 1992)
Cyprinodon variegatus (saltwater)	96 hours LC_{50}	59 (nc)	Static (method: US-EPA)	(Heitmuller et al. 1981)
*Oryzias latipes**	48 hours LC_{50}	1.8 (no information)	No information	(Yoshioka et al. 1986)

(mc) - measured concentration
(nc) - nominal concentration
*Only very few information are given on the test design and test conditions. The results are presented only in tabular form and can therefore not be valided.

In acute toxicity tests to fresh (and one salt) water species values in the range from 43 mg/l to 119 mg/l were obtained.

Heitmuller et al (1981) tested the acute toxicity of *Cyprinodon variegatus*. The method was described in "Methods for acute toxicity tests with fish, macroinvertebrates, and amphibians" (U.S. EPA 1975). The tests were performed in natural seawater (saltwater) and without aeration. After 96 hours a LC_{50} value of 59 mg/l was achieved.

The same test method was used by Buccafasso et al. (1981) without seawater but deionized water. To control volatilization, the jars with high volatile substances were capped. For substances which appeared to be soluble in water (like nitrobenzene) a concentrated stock solution was prepared or the appropriate amount of the compound was added directly in the deionized water in the jars. For *Lepomis macrochirus* an LC_{50} (96 hours) of 43 mg/l was observed.

At the fathead minnow (*Pimephales promelas*) larval survival and growth test the fish larvae were exposed to nitrobenzene (Marchini et al. 1992). This test was chosen because the larval stage is the most sensitive stage and consequently the heaviest toxic effects are frequently exhibited in early

larval development. Marchini et al. compared the results of the larvae stage with values from the literature at the juvenile stage (28-33 days old). For larvae a 96 hours LC_{50} of 44 mg/l was detected. Compared to literature data for juveniles 96 hours LC_{50} of 119 mg/l was reported (Geiger et al. 1985).

The experimental values are in reasonable agreement with the QSAR estimation according to the TGD (1996) which results in a fish (96 hours) LC_{50} of 37 mg/l for polar narcotic acting substances.

The several results from Table 13 fulfilled the criteria of Aquatic Chronic 3 and R52/53 (10 mg/l < LC_{50} 96 hours ≤ 100mg/l).

Long-term toxicity to fish

Table 14: Long-term toxicity data to fish

Species	Endpoint	Effect concentrations [mg/l]	Test system	Reference
Oryzias latipes	NOEC	1.9 *)	Semistatic	(Canton et al.1985)
Oncorhynchus mykiss	27d NOEC	< 1µg/l *)	Flow-trough	(Black et al. 1982)

*) no data whether mc or nc

The effect values found by Black et al. (1982) for several substances other than nitrobenzene (e.g. benzene, toluene) are usually very low compared to effect values found by other authors. No explanation for these large discrepancies could be found. A careful examination of the entire information provided by Black et al. gave no plausible reason for the inconsistency of the data. It was not possible to confirm the low effect values for the other substances. Hence it can be assumed that the values for nitrobenzene (e.g. 27 days NOEC < 1 µg/l) are not representative as well. Because of the doubt about valididity of the results, the values were also not used in the risk assessment (RAR 2007, WHO 2003).

A semichronic test by Canton et al. (1985) showed a NOEC of 1.9 mg/l. No information about test conditions was given in this article, but for the performance of the standard tests the authors referred to some of their former publications (e.g. Slooff and Canton, 1983a; Slooff and Canton, 1983b). It is not conclusive which test method and which test conditions (for example test duration) were used.

7.1.1.2 Aquatic invertebrates

Short-term toxicity to aquatic invertebrates

Table 15shows the available test results for nitrobenzene obtained in short-term tests with aquatic invertebrates.

Table 15: Acute toxicity data to aquatic invertebrates

Species	Endpoint	Effect concentrations [mg/l]	Test system	Reference
Daphnia magna	48 hours EC_{50}	35 (nc)	Semistatic (OECD proposal 1979) Endpoint: behaviour	(Canton et al. 1985)
Daphnia magna	24 hours EC_{50}	50 (nc)	Static (German DIN method) Endpoint: immobilisation	(Bringmann and Kühn 1982)

Daphnia magna	48 hours LC_{50}	27 (nc)	Static (method: U.S. EPA) Endpoint: mortality	(LeBlanc 1980)
Ceriodaphnia dubia	24 hours LC_{50}	54 (mc)	Static (method: U.S. EPA) Endpoint: mortality	(Marchini et al. 1993)

(mc) - measured concentration
(nc) - nominal concentration

Only the EC_{50} 48 hours is a criterion for C&L. But the other result supports an EC_{50} between 10mg/l and 100mg/l in 48 hours.

The test of short-term toxicity (OECD proposal 1979) was a semisatic test (Canton et al. 1985). The test solution was daily refreshed. A stability test in water showed no loss within 1 day. For *Daphnia magna* an EC_{50} (48 hours) of 35 mg/l was measured.

The experimental EC_{50} values (48 hours) for *Daphnia* are in reasonable agreement with QSAR estimations according to the TGD (1996) which result in a *Daphnia* (48 hours) EC_{50} of 18 mg/l for polar narcotic acting substances.

The several results from Table 15 fulfilled the criteria of Aquatic Chronic 3 and R52/53 (10 mg/l < EC_{50} 48hours ≤ 100mg/l).

Long-term toxicity to aquatic invertebrates

Table 16: Long-term toxicity data to aquatic invertebrates

Species	Endpoint	Effect concentrations [mg/l]	Test system	Reference
Daphnia magna	21d NOEC	1.9	in analogy to the rules of the Dutch Standardisation Organisation	(Canton et al. 1985)
Daphnia magna	21d NOEC	2.6 (mc)	Semistatic (Proposed Preliminary Testing Method: published as "Recommendation of the Federal Environmental Agency for the Performance of Testing according to §5, para.1 ; No.3 of the Regulatios on Documents to be Submitted and Evidence of Testing under the Chemical Act"	(Kühn et al. 1988)

(mc) - measured concentration

The lowest long-term effect value for *Daphnia magna* was a 21 days NOEC of 1.9 mg/l (nominal) with the endpoint reproduction rate (Canton et al., 1985). No information about test conditions was given in this article, but for the performance of the standard tests the authors referred to their former publications (Canton and Slooff, 1982). According to this all daphnids (one day old) had been obtained from standardised laboratory cultures, whereas the tests were carried out in analogy to the rules of the Dutch Standardisation Organisation (NEN 6501, 6502, 6504 and 6506 DSO 1980). 25 organisms per group were used and the test volume per group was 1 litre. Daphnids were fed with Chlorella and the test solution was renewed three times a week. In addition to the test description of the Dutch Standardisation Organisation, where only nominal concentrations were reported, the actual concentrations of the test substance were measured in the present test.

In a semi-static chronic test to daphnia magna a 21-day NOEC of 12.5 mg/l based on nominal concentrations was found. Based on the measured concentration at day 3 after renewal of the test solution the NOEC is 2.6 mg/l (Kühn et al., 1988) There are some doubts concerning NOEC established in this study, since it is not known what was a concentration throughout the whole study, a measurement was done on day 3 and it is not know whether solution was replace every day.

The test was performed in accordance with "Proposed Preliminary Testing Method: Prolonged Toxicity Test on *Daphnia magna* (Determination of NOEC for reproduction rate, mortality and time of the first appearance of offspring; 21d)" (Effective: 01.01.1984).

7.1.1.3 Algae and aquatic plants

In the following table the toxicity data to algae are listed.

Table 17: Toxicity data to algae

Species	Endpoint	Effect concentrations [mg/l]	Test system	Reference
Chlorella pyrenoidosa	96 hours ErC_{50}	18*)	Static (OCED-Guideline 201) Endpoint: growth inhibition	(Maas-Diepeveen and van Leeuwen 1986)
Selenastrum capricornutum	96 hours ErC_{50}	23.8*)	Static (US-standard test) Endpoint: growth inhibition	(Bollmann et al. 1989)
Chlorella pyrenoidosa	72 hours ErC_{50} NOEC	28 (mc) 9.2	Static (OECD-Guideline 201) Endpoint: growth inhibition	(Ramos et al. 1999)
Scenedesmus obliquus	48 hours ErC_{50}	67.7 (nc)	Static (OCED-Guideline 201) Endpoint: growth inhibition	(Liu and Lang 1995)
Scenedesmus pannonicus	NOEC	16	Derived from minium inhibitory concentrations by a factor 2	(Canton et al. 1985)

(mc) - measured concentration
(nc) - nominal concentration
*) no data whether mc or nc

EC_{50}-values for different algal species are in the range from 18 mg/l to 28 mg/l (96 or 72 hours). The lowest effect value from a test with a standardized exposure time of 96 hours was found by Maas-Diepeveen and van Leeuwen (1986) with *Chlorella pyrenoidosa* with a 96 hours EC50 of 18 mg/l.

The effects of nitrobenzene on the same algal species have been studied in 72 hours growth inhibition tests (Ramos et al. 1999). The EC_{10} and EC_{50} were established by fitting the relative growth rate as a function of the test concentration using the Weibull function. An EC_{10} of 8.5 mg/l and an EC_{50} of 28 mg/l was estimated. The NOEC of 9.2 mg/l was calculated using the EPA methods implanted in the TOXCALC toxicity data analysis software (v 5.0.9).

The NOEC of 16 mg/l for *Scenedesmus pannonicus* was derived by dividing the minimum inhibitory concentration by a factor 2 (Canton et al. 1985). No information about test conditions was given in this article, but the authors referred to Bringmann and Kühn (1978). It is not clear which species is used, because Bringmann and Kühn tested *Scenedesmus quadricauda* and, *Microcystis aeruginosa* The toxicity threshold for *Scenedesmus quadricauda* was 33 mg/l and for *Microcystis aeruginosa* 1.9 mg/l .

The several results from Table 17 fulfilled the criterion of Aquatic Chronic 3 and R52/53 (10 mg/l < ErC_{50} 96 hours/72 hours ≤ 100mg/l).

7.1.1.4 Sediment organisms

Not relevant for this type of dossier.

7.1.1.5 Other aquatic organisms

Not relevant for this type of dossier.

7.1.2 Calculation of Predicted No Effect Concentration (PNEC)

Not relevant for this type of dossier.

7.2 Terrestrial compartment

Not relevant for this type of dossier

7.3 Atmospheric compartment

Not relevant for this type of dossier

7.4 Microbiological activity in sewage treatment systems

Not relevant for this type of dossier

7.5 Calculation of Predicted No Effect Concentration for secondary poisoning (PNEC_oral)

Not relevant for this type of dossier.

7.6 Summary and discussion on the environmental classification and labelling

7.6.1 Discussion with rationale for existing classification

The existing classification of N R51/53 has been added to Annex I of Directive 67/548/EEC by the 22nd ATP in 1996. The summary record of the TC CnL meeting held on 13/14 Dec 1994, provides very limited information about the acute toxicity data basis for this classification. As it is described in the summary report of Working Group on the Classification and Labeling of Dangerous Substances: Environmental Effects; Meeting at ECB Ispra, 13-14 December 1994 (European Commission, Directorate General XII – JRC; ECBI/24/94 - Rev. 1) for Nitrobenzene (C160, 609-003-00-7): "DE reported that the initial proposal was R52-53. However, based on acute toxicity data, Industry proposed R51 instead and stated that the compound was biodegradable and recommended no classification. However, it was proposed that the compound be classified as R51-53. Conclusion: It was agreed that the compound be classified as N; R51-53."

However almost 14 years later, in the Risk Assessment Report prepared under the Existing Substances Regulation (RAR 2007,), all available ecotoxicological test data had been scrutinised and validated. Very few acute test results would meet the criterion for R51 classification, but all of these are neither sufficiently documented nor reliable (cf. pp. 33-39 of RAR 2007). In contrast, all valid information presented in this background document provides consistent justification for the proposed classification R52/53 (H412).

7.6.2 Comparison with classification criteria:

Classification criteria for substances

According to the 2nd ATP to CLP environmental classification consists of one acute hazard classification category and three long-term hazard classification categories. The acute and the long-term hazard classification categories are applied independently.

The criteria for classification of a substance in category Acute 1 are defined on the basis of acute aquatic toxicity data only (EC_{50} or LC_{50}). The criteria for classification of a substance into the Chronic categories 1 to 3 follow a tiered approach where the first step is to see if available information on chronic toxicity merits long-term hazard classification. In absence of adequate chronic toxicity data, the subsequent step is to combine two types of information, i.e. acute aquatic toxicity data and environmental fate data (degradability and bioaccumulation data).

According to the 2nd ATP to CLP, if there are adequate chronic toxicity data available for all three trophic levels, than the substance should be classify according to the criteria given in Table 4.1.0(b) (i) or 4.1.0(b) (ii) depending on information on rapid degradation. If we do not have adequate chronic toxicity data available for all three trophic level, than during the classication procedure we have to assess both:

- Classification according to the criteria given in Table 4.1.0(b) (i) or 4.1.0(b) (ii) depending on information on rapid degradation, and
- Classification according to the criteria given in Table 4.1.0(b) (iii)

and classify according to the most stringent outcome.

According to Directive 67/548/ECC (28th ATP – Directive 2001/59/EC) environmental classification consists of one acute hazard classification category and three long-term hazard classification categories. The criteria for classification of a substance in acute category are based on acute aquatic toxicity data only (EC_{50} or LC_{50}). The criteria for classification of a substance into the Chronic categories combine two types of information, i.e. acute aquatic toxicity data and environmental fate data (degradability and bioaccumulation data).

Effects of nitrobenzene meeting classification criteria

According to CLP and Directive 67/548/EWG nitrobenzene does not meet the criteria for acute environmental classification. The acute aquatic toxicity indices such as LC_{50} for fish or EC_{50} for invertebrates and ErC_{50} for alga are higher than 1 mg/l.

Nitrobenzene is not rapidly degradable (in 28-day ready-biodegradation studies nitrobenzene did not achieve the pass level. See section 4.1.2 of BD).

There are no adequate chronic toxicity data for nitrobenzene for all three tropic levels. During classification nitrobenzene for chronic toxicity, according to CLP, both criteria given in Table 4.1.0(b) (i) or 4.1.0(b) (ii) and in Table 4.1.0(b) (iii) should be assess.

The classification of nitrobenzene, according to CLP, was based on Table 4.1.0(b) (iii) – the most stringent outcome. The following information were used for classification:

- aquatic acute toxicity indices for fish, invertebrates and alga obtained during tests were between 10 mg/l and 100 mg/l

- the substance is not rapidly degradable.

The same information should be used during classification nitrobenzene according to Directive 67/548/EWG.

7.6.3 Conclusions on environmental classification

Nitrobenzene did not fulfill the pass level of ready biodegradability. There are no adequate chronic toxicity data available for all three trophic levels (studies of long-term toxicity to fish are not valid or not reliable). Because of this acute data were used for classification. EC_{50}/LC_{50} for fish, invertebrates and alga were observed between 10mg/l and 100mg/l. These results were conform to the criteria of classification for R52/53 (based on Directive 67/548/EEC) respectively aquatic chronic Cat. 3 H412 (based on Regulation (EC) No 1272/2008).

JUSTIFICATION THAT ACTION IS REQUIRED ON A COMMUNITY-WIDE BASIS

All endpoints have been addressed within this C&L proposal, since nitrobenzene was a priority substance in the existing chemicals program (EEC) 793/93. The proposal to add R48/25 was already submitted to TC C&L and was agreed on in September 2007. In addition to the previously agreed classification, R64 is now suggested. Furthermore, the Risk Assessment Report (RAR 2007) did support the reclassification from N R51/53(H411) to R 52/53(H412).

Additionally, nitrobenzene is a substance with very high production volumes and widespread use in EU countries, with production volumes exceeding one million metric tonnes per year. Although its main use is in the production of aniline, several thousand tonnes per year are unaccounted for in their use. It is therefore proposed to extend and amend the current harmonized classification listed in Table 3.2 of Annex VI of Regulation (EC) No 67/548 and Table 3.1 of Annex VI of Regulation (EC) No 1272/2008 (CLP) by those reasoned for in this dossier.

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