Committee for Risk Assessment

RAC

Annex 1

Background document

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

styrene

EC number: 202-851-5
CAS number: 100-42-5

ECHA-RAC-CLH-O-0000002714-75-01/A1

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

Adopted

28 November 2012

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Part A.

Part A.

1 PROPOSAL FOR HARMONISED CLASSIFICATION AND LABELLING

1.1 Substance

Table 1: Substance identity

Substance name:	***Styrene***
EC number:	***202-851-5***
CAS number:	***100-42-5***
Annex VI Index number:	***601-026-00-0***
Degree of purity:	The purity varies from 99.7% to greater than 99.9% w/w. The impurities vary with the plant and production method.
Impurities:	The impurities as % w/w comprise some or all of the following: (100-41-4)Ethylbenzene <0.1 (98-82-8)Isopropylbenzene (cumene) <0.1 (98-83-9)2-Phenylpropene <0.1 (7732-18-5)Water <0.025 (122-79-2)Phenyl acetate <0.02 (106-42-3)p-Xylene <0.06 (108-38-3)m-Xylene <0.001

1.2 Harmonised classification and labelling proposal

Table 2: The current Annex VI entry and the proposed harmonised classification

	CLP Regulation		**Directive 67/548/EEC (Dangerous Substances Directive; DSD)**
Current entry in Annex VI, CLP Regulation	Flam. Liq. 3 Acute Tox. 4 * Eye Irrit. 2 Skin Irrit. 2	H226 GHS02 H332 GHS07 H319 GHS08 H315 Wng	R10 Xn; R20 Xi; R36/38
Current proposal for consideration by RAC	STOT RE. 1 (agreed by Lead Registrant). Repr. 1B	H360D H372	Xn, R48/20 T, R61
Resulting harmonised classification (future entry in Annex VI, CLP Regulation)	Flam. Liq. 3 Acute Tox. 4 * Eye Irrit. 2 Skin Irrit. 2 Repr. 1B STOT RE. 1	H226 GHS02 H332 GHS07 H319 GHS08 H315 Wng H360D H372	R10 Xn; R20 Xi; R36/38 Xn, R48/20 T, R61

1.3 Proposed harmonised classification and labelling based on CLP Regulation and/or DSD criteria

Table 3: Proposed classification according to the CLP Regulation

CLP Annex I ref	Hazard class	Proposed classification	Proposed SCLs and/or M-factors	Current classification [1)]	Reason for no classification [2)]
2.1.	Explosives				
2.2.	Flammable gases				
2.3.	Flammable aerosols				
2.4.	Oxidising gases				
2.5.	Gases under pressure				
2.6.	Flammable liquids			Flam Liq. 3	
2.7.	Flammable solids				
2.8.	Self-reactive substances and mixtures				
2.9.	Pyrophoric liquids				
2.10.	Pyrophoric solids				
2.11.	Self-heating substances and mixtures				
2.12.	Substances and mixtures which in contact with water emit flammable gases				
2.13.	Oxidising liquids				
2.14.	Oxidising solids				
2.15.	Organic peroxides				
2.16.	Substance and mixtures corrosive to metals				
3.1.	Acute toxicity - oral				
	Acute toxicity - dermal				
	Acute toxicity - inhalation			Acute Tox. 4*	
3.2.	Skin corrosion / irritation			Skin Irrit.2	
3.3.	Serious eye damage / eye irritation			Eye Irrit.2	
3.4.	Respiratory sensitisation				
3.4.	Skin sensitisation				
3.5.	Germ cell mutagenicity				
3.6.	Carcinogenicity				
3.7.	Reproductive toxicity	**Repr. 1B, H360D, "May damage the unborn child when exposed via inhalation"**			
3.8.	Specific target organ toxicity –single exposure				
3.9.	Specific target organ toxicity	**STOT RE1, H372, "causes**			

	– repeated exposure	**damage to the nervous system through prolonged or repeated exposure via inhalation”**			
3.10.	Aspiration hazard				
4.1.	Hazardous to the aquatic environment				
5.1.	Hazardous to the ozone layer				

[1)] Including specific concentration limits (SCLs) and M-factors
[2)] Data lacking, inconclusive, or conclusive but not sufficient for classification

Labelling: Signal word: Danger
Hazard statements: H226, H332, H315, H319, H360D, H372
Precautionary statements: P260, P264, P270, P314, P501

Proposed notes assigned to an entry:

Table 4: Proposed classification according to DSD

Hazardous property	**Proposed classification**	**Proposed SCLs**	**Current classification [1)]**	**Reason for no classification [2)]**
Explosiveness				
Oxidising properties				
Flammability			Flammable	
Other physico-chemical properties *[Add rows when relevant]*				
Thermal stability				
Acute toxicity			Harmful by inhalation	
Acute toxicity – irreversible damage after single exposure				
Repeated dose toxicity	R48/20 Harmful: danger of serious damage to health by prolonged exposure through inhalation			
Irritation / Corrosion			Irritating to eyes and skin	
Sensitisation				
Carcinogenicity				
Mutagenicity – Genetic toxicity				
Toxicity to reproduction – fertility				
Toxicity to reproduction – development	R61 May cause harm to the unborn child			
Toxicity to reproduction – breastfed babies. Effects on or via lactation				
Environment				

[1)] Including SCLs
[2)] Data lacking, inconclusive, or conclusive but not sufficient for classification

Labelling: Indication of danger: T
R-phrases: R10, R20, R36/38, R 48/20, R61
S-phrases: S2, S23

2 BACKGROUND TO THE CLH PROPOSAL

2.1 History of the previous classification and labelling

The substance is a transitional substance and was discussed in the TCC&L group at a number of meetings. TCC&L agreed to add R37 to the current classification and to delete specific concentration limits. It was agreed not to classify styrene for carcinogenicity and mutagenicity. For repeated dose effects it was agreed to classify with STOT RE 1. For reproductive toxicity no agreement could be reached. The minutes from the TCC&L are attached in the IUCLID file.

The discussions were based on the EU RAR prepared by UK:

EU RAR Styrene (UK, 2008)

UK has also prepared a transitional document available at the ECHA website:

Transitional Document Styrene (UK)

It also refers to the substance discussions and conclusions in the TCC&L group.

The registration dossier for styrene was carefully examined in connection with the registration of the substance November 31st 2010, in order to evaluate whether these contain relevant information not taken into account in this CLH proposal (in accordance with Annex VI, part II of the CLP regulation). However, this is not the case, as both the lead registration dossier and the CLH report are based almost solely on data from the EU RAR. There is, however a discrepancy with respect to the conclusions reached on reproductive toxicity. The CLH report proposes a harmonized classification for reproductive toxicity based primarily on one specific two-generation study. The registration dossier, however, concludes otherwise on the same study.

The Lead Registrant did also propose, according to the registration dossier, the following classification: Asp.Tox; H304 and STOT SE3; H335. (Xn; R65 and Xi; R37).

2.2 Short summary of the scientific justification for the CLH proposal

STOT RE1 (R48/20)

Styrene produces a number of serious health effects after prolonged exposure by inhalation in experimental animals and in humans. The exposure levels inducing neurotoxicity in humans are in the same order of magnitude as the exposure levels inducing neurotoxicity in animals; however, for ototoxicity and colour vision discrimination the exposure levels inducing these effects in humans seem to be a factor of 10 lower than in animals. Based on the available data, a classification as STOT RE 1 is warranted for styrene.

Although there are some indications of neurotoxic effects in the rat following repeated oral dosing of styrene, a classification is not justified for this exposure route. No repeated dermal toxicity studies are available; however, systemic toxicity following dermal contact with styrene is not expected. Therefore, a classification as STOT RE 1, with the hazard statement H372 “Causes damage to the nervous system through prolonged or repeated exposure via inhalation” is relevant.

According to the registrations dossiers, the Registrant concluded the classification STOT RE1 based on the same available data.

Repr. Cat 1B, H360D (R61)

In the rat, developmental delays postnatally including delayed neurological development and some indications of behavioural effects after weaning have been reported in a number of studies at 300 ppm styrene in the absence of maternal toxicity.

In a recent well-conducted OECD- and GLP-compliant two-generation study including developmental neurotoxicity assessment in F2 offspring, a pattern of developmental delays both before and after weaning (decreased body weights, delays in attaining some pre-weaning developmental landmarks, slight shift in the normal pattern of motor activity and delayed preputial separation), was evident mainly in the F_2 pups of the high exposure group (500 ppm). In addition, decreased swimming abilities on PND 24 and reductions in forelimb grip strength on PND 60 were found in both sexes. These effects indicate affected neuromotor functions and are evaluated as mainly a direct consequence of the styrene exposure. Significantly decreased pup body weight during the lactation period was found at 150 ppm in the absence of maternal toxicity. The results of this study shows that exposure to 500 ppm styrene causes developmental toxicity manifested as a pattern of developmental delays, including delayed neurological development, and developmental neurotoxicity effects on post-weaning behaviour, especially related to neuromotor functions. In contrast to the earlier investigations at 300 ppm, the exposure to 500 ppm induced some maternal toxicity (6-7% reduction in body weight and degeneration of the nasal olfactory epithelium). However, it is considered highly unlikely that developmental toxicity is an unspecific effect of the maternal toxicity.

Consequently, it is proposed to classify styrene as a developmental toxicant. As there is sufficient evidence to conclude that no classification is warranted for effects on fertility, the classification should be noted with a “D”.

There is evidence that a classification for developmental effects via the oral route is not warranted, and although there are no dermal investigations taken together with the highly volatile nature of styrene it is suggested to include a specific mention of the exposure via inhalation in the hazard statement H360.

The registration dossier assesses the Registrant, based on the same two-generation study, it leads to a different conclusion, and it is assessed that the observed effects are a consequence of maternal toxicity and that there is no indication of developmental toxicity. Please consider the section 4.11.

2.3 Current harmonised classification and labelling

2.3.1 Current classification and labelling in Annex VI, Table 3.1 in the CLP Regulation

Classification : Flam. Liq. 3, H226; Acute Tox. 4, H332; Eye Irrit. 2, H319; Skin Irrit. 2, H315

Labelling : GHS02, GHS07, Wng; H226, H332, H319, H315

2.3.2 Current classification and labelling in Annex VI, Table 3.2 in the CLP Regulation

Classification: R10, Xn; R20, Xi; R36/38, S2, S23. (R37 was agreed at TC C&L and will not be discussed here).

2.4 Current self-classification and labelling

2.4.1 Current self-classification and labelling based on the CLP Regulation criteria

Denmark has investigated a number of product Safety Data Sheets for products currently distributed in the EU containing styrene and none of them use the labelling in line with a STOT RE 1 classification.

2.4.2 Current self-classification and labelling based on DSD criteria

Denmark has investigated a number of product Safety Data Sheets for products currently distributed in the EU containing styrene and none of them use the labelling in line with an R48/20 classification.

3 JUSTIFICATION THAT ACTION IS NEEDED AT COMMUNITY LEVEL

Repr. 1B: A classification as a presumed human reproductive toxicant justifies that action is needed and a harmonised classification should be found at community level according to article 36 d).

STOT RE 1: Although there was agreement in the former classification and labelling group in the EU it seems that self classification for this effect is not used at a wide level in the EU. In combination with the high annual tonnages used in the EU (Styrene was a prioritized substance in the EU risk assessment process due to extensive Community-wide use) this justifies the need for a harmonised classification and labelling for this end point.

According to the registrations dossiers, the Registrant concluded the classification STOT RE1 based on the same available data.

Part B.

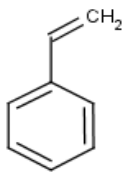
SCIENTIFIC EVALUATION OF THE DATA

1 IDENTITY OF THE SUBSTANCE

1.1 Name and other identifiers of the substance

(Taken from the EU RAR (2008)

Table 5: Substance identity

EC number:	***202-851-5***
EC name:	***Styrene / Etenylbenzene***
CAS number (EC inventory):	***100-42-5***
CAS number:	***100-42-5***
CAS name:	***Benzene, ethenyl-***
IUPAC name:	***Styrene***
CLP Annex VI Index number:	***601-026-00-0***
Molecular formula:	C_8H_8
Molecular weight (range):	***104.15 g/mol***
Structural formula:	

1.2 Composition of the substance

Table 6: Constituents (non-confidential information)

Constituent	Typical concentration	Concentration range	Remarks
Styrene		99.7% to greater than 99.9% w/w.	

Current Annex VI entry:

Table 7: Impurities (non-confidential information)

Impurity	Typical concentration	Concentration range	Remarks
(100-41-4) Ethylbenzene	<0.1		
(98-82-8) Isopropylbenzene (cumene)	<0.1		
(98-83-9) 2-Phenylpropene	<0.1		
(7732-18-5) Water	<0.025		
(122-79-2) Phenyl acetate	<0.02		
(106-42-3) p-Xylene	<0.06		
(108-38-3) m-Xylene	<0.001		

Current Annex VI entry:

Table 8: Additives (non-confidential information)

Additive	Function	Typical concentration	Concentration range	Remarks
4-tert-butylpyrocatechol (4-tert-butylbenzene-1,2-diol)			<0.006 – 0.01% w/w.	Is added as a polymerisation inhibitor

Current Annex VI entry:

1.2.1 Composition of test material

1.3 Physico-chemical properties

Table 9: Summary of physico - chemical properties

Property	Value	Reference *	Comment (e.g. measured or estimated)
State of the substance at 20°C and 101,3 kPa	Liquid		
Melting/freezing point	-30.6°C		
Boiling point	(at 1 atmosphere) 145 - 146°C		
Relative density			
Vapour pressure	5 mmHg (667 Pa)		at 20°C
Surface tension			
Water solubility	300 mg/l		at 20°C
Partition coefficient n-octanol/water	3.02 (log value)		
Density	0.906 g/cm3		
Flash point	(closed cup) 31°C		
Flammability	Autoflammability 490°C		
Explosive properties			
Self-ignition temperature			
Oxidising properties			
Granulometry			
Stability in organic solvents and identity of relevant degradation products			
Dissociation constant			
Viscosity			

*All information taken from the EU RAR (2008)

Conversion factors 1 mg/m3 = 0.23 ppm:1 ppm = 4.33 mg/m3

2 MANUFACTURE AND USES

2.1 Manufacture

Taken from the EU RAR (2008):

"Styrene is produced commercially from crude oil by a sequence of processes.
Steam cracking of naphtha, obtained from the refining of crude oil, produces ethylene, propylene and a mixture of monocyclic hydrocarbons including benzene. Ethylene and benzene, fractionated from this mixture, are then reacted together in the presence of a catalyst to produce ethylbenzene. Styrene is manufactured from ethylbenzene by one of two routes (Reinders, 1984; WHO, 1983). Firstly, it can be manufactured by dehydrogenation:

catalyst $PhC_2H_5 \longrightarrow PhCH{=}CH_2 + H_2$

Iron oxide is used as a catalyst, together with zinc and magnesium oxides. Steam is added as a dilution agent and to improve the heat transfer. The reaction is carried out at approximately 700°C and 0.8 bar. The purification of the reaction product is done by vacuum distillation. To prevent the polymerisation of the styrene, the conversion is carried out to only 60%, and there is always a reasonable dilution. The by-product gases formed in this reaction are either used as a fuel or flared.
Alternatively, styrene may be manufactured by oxidation of ethylbenzene to the hydroperoxide by bubbling air through the liquid reaction mixture. The hydroperoxide is then reacted with propylene to yield propylene oxide and a co-product, methyl phenyl carbinol, again in the liquid phase. The carbinol is dehydrated to styrene over an acid catalyst at about 225°C."

2.2 Identified uses

"Styrene is processed in closed systems as an intermediate in the chemical industry. It is the monomer for polystyrene (general purpose, GP-PS; high impact, HI-PS; and expanded, EPS) and copolymer systems (acrylonitrile-butadiene-styrene, ABS; styrene-acrylonitrile, SAN; methyl methacrylate-butadiene-styrene, MBS; and others) and in the production of styrene-butadiene rubber (SBR) and related latices (SB latex for example). It is also used as a component of unsaturated polyester (UPE) resins.

It is used in a wide range of products:

General packaging, furniture, electrical equipment (e.g. audio-visual cassettes), industrial mouldings (e.g. dental, medical), thermal insulation of refrigeration equipment and buildings, Interior and exterior automobile parts, drains, ventilation pipes, air conditioning, hobby equipment, casings etc., Tyres, radiators and heater hoses, belts and seals, wire insulation, Paper coatings, carpet backings, floor tile adhesives, Building panels, marine products, household consumer goods, trucks, Casting resins used for producing liners and seals, in putty and adhesives."

3 CLASSIFICATION FOR PHYSICO-CHEMICAL PROPERTIES

4 HUMAN HEALTH HAZARD ASSESSMENT

4.1 Toxicokinetics (absorption, metabolism, distribution and elimination)

4.1.1 Summary on toxicokinetics

Taken from the EU RAR (2008), 4.1.2.1.5

"A substantial amount of information is available on the toxicokinetics of styrene in humans, following exposure by the inhalation route; information on percutaneous absorption in humans is also available.

In humans, inhaled styrene vapour (at concentrations of 10-200 ppm) is well absorbed across the respiratory tract. Thus, a value of 100% for absorption via the inhalation route of exposure is taken forward to the risk characterisation.

Dermal absorption of the liquid has been estimated to be approximately 2% of the applied dose in an *in vitro* study using human skin samples. This value is taken forward to the risk characterisation. Dermal uptake of the vapour appears to make only a small contribution (5% or less) to the total body burden arising from combined inhalation and dermal exposure to the vapour.

No information is available on oral absorption in humans, but from the physicochemical properties of styrene and experimental animal information, one would expect extensive absorption from the gastrointestinal tract. Thus, a value of 100% for oral absorption is taken forward to the risk characterisation. Following absorption, it can be predicted from experimental animal data that styrene is widely distributed in humans, and needle biopsy investigations have shown that styrene certainly locates in adipose tissue; there was a correlation between the amount of body fat and the total body burden of styrene. Data on styrene blood levels in human volunteers following single inhalation exposures and in rats exposed via inhalation show that at identical exposure concentrations, styrene blood levels are very similar (e.g. 2.5 and 3.5 μg/ml in rats and humans respectively at 100 ppm styrene).

The rate of absorption following inhalation is much higher (2-3 fold) in mice than in rats. The absorption rate in humans is approximately the same as in rats. The rate of styrene uptake in the upper respiratory tract is partly dependent on its metabolism, and was decreased when animals were pre-treated with a P450 inhibitor.

In humans, styrene is eliminated from the body relatively rapidly, primarily in the urine. However, there is some evidence for modest biopersistence in human adipose tissue on repeated daily exposure. Styrene clearance from blood is biphasic. Half-lives for inhaled styrene were reported at 0.6 hours for the first elimination phase and 13 hours for the second elimination phase. From studies in mice, there is evidence that styrene is also rapidly eliminated from blood following either single or repeated inhalation exposure. A study in pregnant mice has shown that styrene and/or its metabolites can cross the placenta into the foetus.

The metabolism of styrene has been studied thoroughly in mice, rats and humans. A number of metabolic pathways have been identified. The evidence suggests that these pathways are active in mice, rats and humans, although there are species differences in their relative importance.

Styrene is metabolised extensively in all species. According to a PBPK model developed by Ramsey and Andersen, saturation of styrene metabolism in humans occurs at blood levels exceeding 1.7 μg/ml styrene or 200 ppm styrene in air. Below these concentrations, the rate of styrene metabolism is limited by the rate of blood perfusion in liver or other organs involved in styrene elimination. The first step in the metabolism of styrene involves oxidation of the aromatic ring or side-chain. The main route in each species is the oxidation of the side chain to give the epoxide, styrene-7,8-oxide (SO). A number of studies have demonstrated the involvement of P450 in this step and have provided information on the specific P450 isoforms involved in the production of SO (CYP2E1, CYP2B6 and CYP2C8 in the liver and CYP2F2/1 and CYP2E1 in the lung). Pre-treatment of rodents with diethyldithiocarbamate, a specific cytochrome P450 monooxygenase inhibitor, effectively inhibited the metabolism of styrene, and reduced toxicity in the mouse lung and nasal tissue was observed when the animals were pre-treated with 5-phenyl-1-pentyne, a cytochrome P4502F2 inhibitor. In isolated Clara cells and microsomes, an inhibitor of 2F2 reduced the production of SO by around 30-50%; a 2E1 inhibitor showed less inhibition indicating a lower importance of this isoform in the lung. The SO produced is enantiomeric and is produced in the R- and S-forms, probably as a result of metabolism by different P450 isoforms. Different ratios of R-SO to S-SO are found in different tissues and different species. Mouse Clara cells produce about 3 times more of the R-enantiomer than the S-enantiomer, while rat produces more of the S-enantiomer, and humans, like rats, produce more of the S-form.

SO is either metabolised further by conjugation with glutathione to give mercapturic acids, or is hydrolysed by epoxide hydrolase (EH) to phenylglycol. This is subsequently metabolised to mandelic, phenylglyoxylic and hippuric acids. P450 and EH are both microsomal enzymes in the endoplasmic reticulum. Therefore, SO produced *in situ* by P450 may potentially be rapidly detoxified if there is sufficient EH present.

Other metabolic pathways can lead to phenylacetaldehyde (PA) and phenylacetic acid (PAA) (via side-chain β-oxidation and hydroxylation), to phenylethanol and acetophenone (via side-chain α-oxidation and hydroxylation), oxidation of the aromatic ring to give 4-vinylphenol (4-VP), and products of ring opening. These metabolites are excreted in the urine. There are studies which have demonstrated that P450 enzymes are also involved in both the side-chain and ring oxidation of styrene and that 4-VP is further metabolised in lung microsomes by specific P450 isoforms to extremely reactive downstream products (e.g. an epoxide and a hydroquinone derivative). Subsequently these derivatives are conjugated with glutathione, but at present there is no information on the relative rates of 4-VP metabolites detoxification between different species.

There are some data from rodents (rats and mice) and humans to indicate the relative extent of flux through these various pathways. The approximate relative contribution of each metabolic pathway in each species, as determined from urinary metabolites, is shown in Table (a). The urinary metabolites are an indication of the overall metabolism of styrene, which is considered to occur largely in the liver.

It is clear that metabolism involving SO as an intermediate is a major route in rodents and humans. However, there are some notable species differences. In humans, almost all of styrene (95%) is metabolised to SO and further metabolised by EH; approximately 5% of styrene is metabolised via the phenylacetaldehyde pathway. No more than trace amounts of SO-GSH conjugates or ring-oxidized metabolites of styrene (4-VP) occur in humans exposed to styrene. Further metabolism of SO by EH is important but less extensive in rodents than in humans (68-72% in rats and 49-59% in mice). In rodents, conjugation of SO with GSH is an important route accounting for up to a third of the SO removal. The most significant difference between mice and rats is in relation to the production of phenylacetaldehyde (12-22% in mice against 3-5% in rats) and products of ring-oxidation (4-VP; 4-8% in mice against <1% in rats).

The data indicate significant differences in the metabolism of styrene between species and between tissues. It should be noted that, although these data arise from *in vitro* studies and PBPK modelling, they clearly mirror the toxicodynamic picture of styrene obtained *in vivo*. The tissue-specific metabolism of styrene suggests that *in situ* metabolism within each tissue may be a more important determinant of toxicity than the overall systemic metabolism and blood levels of styrene metabolites. The implication of this is that the specifics of the local metabolism in a target tissue must be considered when extrapolating findings in animals to assess the likely hazard and risks in the equivalent human tissues.

A general observation is that the human tissues investigated – apart from the liver - produce very little SO, if any, and have a greater capacity to hydrolyse SO with EH than rodents. This difference is most pronounced in human nasal and lung tissues where production of SO is minimal or undetectable, and is also associated with a greater capacity to hydrolyse SO by EH. The mouse lung and nasal tissues produce the greatest amount of SO among the species tested, and, in general, have less EH activity, suggesting that significantly high local concentrations of SO will be present in these tissues. It is also evident that other toxic metabolites, particularly 4-VP and its reactive downstream products, are produced to a far higher extent in mouse lung than in rat (14-79% of the mouse concentrations) or human lung (1.5-5% of the mouse concentrations). Although it cannot be ascertained whether or not these species differences in the formation of 4-VP metabolites in the lung may be a reflection of the different numbers of Clara cells (the metabolically active lung cells) present in the different species, since 4-VP metabolites are produced by the same cytochrome P450 enzymes involved in the production of SO, it is most likely that the species differences in the formation of 4-VP metabolites observed reflect species differences in metabolic capability."

Table (a). Approximate relative contribution of metabolic pathways for styrene indicated by urinary metabolites (Cruzan *et al.*, 2002; Johanson *et al.*, 2000)

Metabolic route	Urinary metabolites (%)		
	Rat	Mouse	Human
Products of action of EH on SO	68-72	49-59	95
Conjugation of SO with GSH	23-26	20-35	Very low
Phenylacetaldehyde	3-5	12-22	5
Ring opening	ND	ND	<1
Products of 4-vinylphenol conjugation	<1	4-8	<1

ND. Not determined.

4.2 Acute toxicity

4.3 Specific target organ toxicity – single exposure (STOT SE)

4.4 Irritation

4.5 Corrosivity

4.6 Sensitisation

4.7 Repeated dose toxicity

4.7.1 Non-human information

4.7.1.1 Repeated dose toxicity: oral

Taken from the EU RAR (2008)(4.1.2.6.1., p.143)

"**Summary of Repeated Oral Exposure Studies in Animals**

Most of the available repeated oral exposure studies have been performed in rats. Few of these are of the quality, breadth and thoroughness required in standard regulatory test guidelines. Hence, most of the data do not facilitate the derivation of an overall NOAEL of the type normally sought in relation to a 90 day (or longer) exposure period. However, there is reliable information from a carcinogenicity bioassay in the rat, in which there was no evidence of treatment related toxicity in animals administered 1000 mg/kg/day for two years; a marked increase in mortality was evident at 2000 mg/kg/day. In another 2 year study, styrene did not produce any clear evidence of toxicity when administered in drinking water at a dose of 21 mg/kg/day, the highest dose level used. However it is noted that potential effects on the ear were not investigated in these studies. Ototoxicity, a clearly established effect of styrene in the rat, was seen in one study at 800 mg/kg/day (and perhaps, although less convincingly, at 400 mg/kg/day), for 2 weeks. A range of different CNS related observations have also been made with repeated oral dosing in this range, but unfortunately each one stands in isolation and lacks a surrounding, well established and validated framework within which the results can be interpreted, and in some cases contradictory results have been reported in different studies. Overall, there is no convincing evidence of clear, styrene induced neurological lesions induced by repeated oral dosing in rats. Two studies by the same authors (Srivastava et al) have reported testicular damage in rats at 200 and 400 mg/kg/day styrene. It is noted that in earlier repeated oral studies and in well conducted 2 year inhalation studies in rats at equivalent and higher doses than those used by Srivastava et al, no testicular changes or indications of any testicular effects were observed. Also, no effects on the testis and fertility parameters have been observed in a recent well-conducted OECD- and GLP-compliant rat inhalation 2-generation study with exposures up to 500 ppm (≈ 300 mg/kg/day) styrene. Therefore, despite these individual publications by the same authors reporting testicular damage, the weight of evidence indicates that styrene is not a testicular toxicant.

In mice, the most reliable information comes from a cancer bioassay, in which increased mortality and hepatic necrosis were observed at the highest dose of 300 mg/kg/day, and a NOAEL of 150 mg/kg/day was identified. The one significant observation from the remaining studies is that of

toxicity towards the lung epithelium, adding further support to the concept that the lung toxicity of styrene in mice, following oral and inhalation exposure, results from local metabolism of styrene to styrene oxide and other reactive metabolites (e.g. the downstream metabolites of 4-vinylphenol). The one report of styrene induced decrease in serum testosterone levels in young mice dosed with 12 mg/kg/day for 4 weeks is likely to be a chance finding.

A repeated oral study in dogs detected Heinz bodies in circulating erythrocytes (suggesting oxidative damage) at doses of 400 and 600 mg/kg/day, with minimal effects in females at 200 mg/kg.

Overall, in relation to repeated oral exposure, the NOAEL of 150 mg/kg/day identified from a 2 year cancer bioassay in the mouse should also be considered. But in extrapolation to humans careful consideration has to be taken of the specifics of mouse metabolism and the high sensitivity of this species for liver toxicity as compared to e.g. the rat."

4.7.1.2 Repeated dose toxicity: inhalation

Studies investigating specific organ toxicity: auditory systems

(Text is identical to the Danish proposal discussed in the TCC&L group)

"A 13-week rat inhalation study has investigated the ototoxic potential of styrene (Albee *et al,* 1992). Groups of 14 male Fischer 344 rats were exposed to 0, 50, 200 or 800 ppm (0, 217, 866 or 3464 mgm^3) styrene for 6 hours/day, 5 days/week. Evoked potential tests (12 rats per group) were included after 13 weeks. Cochleas were removed from 4 rats in the control and the 200 ppm groups and 3 rats from the 800 ppm group, and sections were specially processed for pathology. Histopathological investigations revealed lesions in the organ of Corti of animals exposed to 800 ppm styrene (two tissue samples were examined). These lesions were characterised as the loss of two outer hair cells per cross section from the upper basal turn, and the occasional absence of an outer hair cell from the lower middle turn. There were no alterations involving the inner hair cells, the Deiters' cells or the pilar cells. The organ of Corti was not affected in animals exposed to 0 or 200 ppm styrene (no tissues were examined at 50 ppm). There were no treatment-related alterations in the somatosensory evoked potential from the sensory cortex or the cerebellum. However in the 800 ppm group, auditory brainstem response (ABR) thresholds were elevated by approximately 40 dB at 16, 25 and 30 kHz. Hair cell loss at 800 ppm occurred in the cochlea in areas that relate to mid–high frequency (15-30 kHz) hearing. In this study, the NOAEC for ototoxicity was 200 ppm (866 mgm^3) with evidence of damage and impairment in the auditory system at 800 ppm (3464 mgm^3).

A further investigation of ototoxicity involved groups of 12 male Fischer 344 rats exposed to 0 or 800 ppm (0 or 3464 mgm^3) styrene for 14 hours/day, 5 days/week for 3 weeks (Yano *et al,* 1992). In styrene-exposed animals, brainstem auditory evoked potentials were "slightly" affected at 4 kHz and moderately to severely altered at 8, 16, 30 kHz. Following testing and sacrifice, cochlea's were removed from 4 controls and 4 of the styrene-exposed animals with the largest changes in auditory evoked potentials. In the styrene-treated rats, outer hair cell loss was observed in the organ of Corti, which corresponded with the hearing effects at mid frequencies. Where damage to the cochlea was severe, occasionally inner hair cells (IHC) were missing.

In another study, brainstem auditory evoked response thresholds were elevated at all frequencies tested (4, 8 and 16 kHz) in male Fischer 344 rats exposed to styrene at 800 ppm (3464 mgm^3) or

above for 14 hours/day daily for 3 weeks (Pryor *et al*, 1987). The response was greater at 8 and 16 kHz (humans can hear in this range).

These data document mid-frequency auditory dysfunction following exposure to styrene. Neither study explored the effects of styrene exposure at concentrations lower than 800 ppm (3464 mgm^3), which can be taken as a LOAEC for ototoxicity.

The ototoxicity potential of styrene in rats following inhalation exposure either alone or in combination with noise has been studied in detail by Mäkitie (1997).

In a series of experiments, groups (10-12/group) of male Wistar rats were exposed whole-body to styrene alone, noise alone, or styrene and noise combined. Exposure conditions for styrene were 100, 300 or 600 ppm (433, 1299 or 2598 mgm^3) for 12 hours/day, 5 days/week for 4 weeks. Noise exposure conditions were broadband non impulse noise in the frequency range 31.5-10 kHz with a sound pressure of 100-105 dB for 12 hours/day, 5 days/week for 4 weeks. Combined exposures were to the same noise conditions together with each of the three styrene exposure conditions. Controls were exposed to filtered air and normal noise levels. Because of the design of the study in some cases (controls, noise alone and 600 ppm styrene alone) more than one group was included overall.

Ototoxicity was assessed electrophysiologically by auditory brainstem response (ABR) and morphologically using light and electron microscopy (LM and EM). ABR was assessed using cutaneously applied electrodes with testing being carried out prior to exposure, immediately after exposure and then at specified intervals after exposure (generally about 3-6 weeks later). Auditory stimulus for ABR consisted of clicks with alternating polarity and tone bursts of 4 ms duration at 16 deliveries/second. The response to stimulus was assessed at frequencies of 1, 2, 4 and 8 kHz with testing being carried out on the right ear. ABR thresholds were defined as the lowest level giving reproducible responses in the system. Hearing loss for each rat was assessed by comparison of pre- and post-exposure thresholds as well as comparison against controls. ABR latencies (as a measure of cochlear nerve conduction) were also measured in some rats and was defined as the time between stimulus onset to the peak occurrence of the first and fifth waves of the ABR. After the electrophysiological studies had been completed, a number of morphological studies were carried out with both LM and EM. Both set of analyses were essentially designed to investigate the morphology of the organ of Corti, in particular effects of treatment on the inner and outer half cells (IHC and OHC).

No effect was seen on ABR latencies with any treatment. Styrene alone at 100 and 300 ppm had no effect on ABR thresholds compared to either pre-exposure or controls, did not increase loss of IHC or OHC, and did not induce any increases in subcellular pathology examined by EM. Exposure to styrene at 600 ppm produced mild increases in both temporary and permanent threshold shifts (TTS and PTS) of about 1-3 dB compared to pre-exposure and control values. This electrophysiological difference was accompanied by severe OHC loss in the third row of cells with less loss seen in the second and first rows respectively; no loss in IHC was seen. EM studies showed subcellular changes in OHC including increased vacuolation in the cytoplasm, formation of vesicles and alteration of mitochondria (disruption of cristae and formation of membrane-bound spherical bodies). Noise alone caused a mean threshold shift ranging from 1.8 dB (at 8 kHz) to 9.3 dB (at 2 kHz) with effects most marked at the lower frequencies (1-4 kHz). Morphological analysis by LM did not reveal any increased loss of OHC or IHC compared to controls, although EM studies revealed some stereocilia irregularity and apparent loss of stiffness in hair cells. The combination of noise with 100 or 300 ppm styrene caused a flat increase in PTS of around 5-10 dB across the whole frequency range studied but this increase was about the same as for noise alone. No increase

in IHC or PHC loss was seen under these combined conditions of exposure. The combination of noise with 600 ppm styrene, however, produced a much greater increase in PTS, which was greater than the simple summation of the response to noise and 600 ppm styrene alone, indicating a potentiation of effect. This electrophysiological change was accompanied by a loss of OHC, which was more severe than that seen with 600 ppm alone and there was also some loss if IHC. EM studies revealed structural changes such as increased vesiculation and vacuolisation of OHC cytoplasm and many cells looked to be in stages of degeneration.

Overall, this study demonstrated that ototoxicity – as assessed by electrophysiological and morphological techniques – was induced in rats exposed to 600 ppm (2596 mgm^3) styrene but not at 300 ppm (1299 mgm^3) or less, indicating a threshold in this species somewhere between 300 and 600 ppm. The combination of noise and styrene exposure was found to potentiate the effect of styrene alone at a level where both agents were found to be ototoxic.

In a further study, groups of eight male Long-Evans rats were exposed whole-body to 0, 500, 650, 850, 1000 or 1500 ppm (0, 2165, 2815, 3681, 4330 or 6495 mgm^3) styrene for 6 hours/day, five days/week for four week (Loquet *et al,* 1999). Prior to exposure rats were implanted with brain electrodes in order to record auditory stimulus evoked potentials. Audiometric testing (noise intensity thresholds for evoked potential across a range of frequencies from 2-32 kHz) was performed one month after implant at two days prior to styrene exposure and then at six weeks after the exposure period. Permanent threshold shifts (PTS) were calculated as the difference in sound intensity required to induce an evoked response between the pre- and post-exposure measured thresholds. Following audiometric testing, histopathological and scanning electron microscopic (SEM) examination was used to assess damage to the inner and outer hair cells (IHC and OHC) of the organ of Corti. Counts of surviving hair cells were used to quantitatively assess the damage.

PTS was elevated at exposure levels of 850 ppm and above. At 850 ppm there was a large PTS over the frequency range 16-20 kHz but no hearing loss at lower or higher exposure concentrations. At higher exposure levels hearing loss was measured across the frequency range with the effect increasing with airborne exposure concentration. Slight effects were seen at 650 ppm but no change in PTS compared to controls was seen at 500 ppm. Histopathology and SEM revealed no effects on IHC in treated or control animals and a background loss of OHC in controls. In contrast, animals treated with 650 ppm and above showed loss of OHC in all three rows with most loss seen at positions, which are believed to correspond to the 4 and 20 kHz detection frequencies. The effects were most marked across the exposure range in the third row of OHC with progressively less damage in the second and first rows.

Overall, this study demonstrates that styrene is ototoxic in rats following inhalation exposure at concentrations of 650 ppm (2815 mgm^3) and above, with a clear NOAEC being identified at 500 ppm (2165 mgm^3). The effects measured by audiometry were accompanied by damage to the OHC in the affected groups.

In a subsequent report, the same group using essentially the same techniques investigated the interaction between styrene and noise exposure in rats (Lataye *et al,* 2000). Groups of 16 male Long-Evans rats received implants to measure auditory stimulus evoked responses and were exposed to: noise alone (97 dB - octave noise centred at 8 kHz which was chosen where auditory sensitivity was expected to be highest) for 6 hours/day, 5 days/week for 4 weeks; styrene alone, whole-body at 750 ppm (3248 mgm^3) for 6 hours/day, 5 days/week for 4 weeks; styrene plus noise at these levels; air and normal noise levels. Audiometry was performed prior to exposure, on the

day following the end of exposure and after a six week recovery period. Histopathology and SEM studies were performed as before, immediately after the audiometric studies.

Noise alone was found to increase evoked potential thresholds (i.e. affect hearing) at both time points after exposure over the frequency range 8-20 kHz, with peak effect seen at 12 kHz: some recovery was seen after the six week recovery period although shifts in evoked potential thresholds were still observed. Styrene alone was found to affect hearing over the range 16-20 kHz but the effects were not as great as noise alone, although subsequent recovery was not observed. When styrene was combined with noise, the induced hearing loss was observed over the frequency range 8-20 kHz (i.e. the same range as noise alone) but was found to be greater than the arithmetical sum for the two agents alone over the frequency range 6-12 kHz, indicating an interaction between the two agents. Histopathological and SEM analysis showed a loss of OHC (about 17% loss) in the first row at a position associated with 17 kHz hearing frequency; OHC rows 2 and 3 showed slight losses in cells. Styrene induced large losses of OHC in row 3 at 20 kHz (86% loss) and 4 kHz (70% loss). OHC2 and OHC1 were less damaged than OHC3. Exposure to styrene and noise together induced a loss of hair cells in a pattern similar to that for styrene alone but of a more severe nature, with loss of 94% of cells in OHC3 at the 20 kHz position and 86% of cells in OHC3 at the 4 kHz position.

Overall, this study demonstrated that when rats were exposed to the two agents, noise and styrene, each at ototoxic levels, the effects on auditory function and histopathological damage were more marked than might be expected if the effects of the two were simply added, suggesting a synergistic interaction.

In a 4-week study, rats were exposed (12 hours/day, 5 days/week) to styrene at concentrations of 100, 300, or 600 ppm (433, 1299 or 2598 mgm^3) and analysed for both auditory sensitivity and structural inner ear damage after the exposure (Mäkitie *et al.* 2002). Exposure to 600 ppm styrene caused a 3 dB hearing loss at the highest test frequency (8 kHz). Quantitative morphological analysis showed that 600 ppm styrene caused a severe outer hair cell loss particularly in the third OHC row.

The authors concluded that there appears to be a threshold for styrene ototoxicity between 300 and 600 ppm (1299 and 2598 mgm^3).

The ototoxic potential of styrene in rats and guinea pigs following inhalation exposure has been studied by Lataye *et al.* (2003). Groups of 5-6 male Long-Evans rats were exposed, whole-body, to 0 or 1000 ppm (0 or 4330 mg/m^3) styrene vapour, 6 hours/day for 5 days. Cochlear function was tested before, 20 minutes after the end of the 5 days of exposure, and 2 and 4 weeks post-exposure using cubic distortion product otoacoustic emissions (DPOAE) recorded from the external ear canal in anaesthetized animals.

In styrene-exposed rats, DPOAE amplitudes were statistically significantly decreased at 2 and 4 weeks post-exposure, although the magnitude of the effect was not greater at 4 weeks than at 2 weeks. Histologically, hair cell loss was observed.

Overall, this study shows hearing loss in rats exposed to styrene at 1000 ppm (4330 mg/m^3) for 5 days.

As part of a study investigating the combined effects of styrene and ethanol, groups of 10-11 male Long-Evans rats were exposed, whole-body, to 0 or 750 ppm (0 or 3248 mg/m^3) styrene vapour 6

hours/day, 5 days/week for 4 weeks (Loquet *et al*, 2000). Audiometric thresholds for each animal were determined from 2 to 32 kHz using brainstem auditory evoked potentials before the start of the study and 6 weeks post-exposure. Histology examinations included counting hair cells in the organ of Corti from 5 animals and focussed only on auditory tissues.

The audiometry examination showed evidence of hearing loss at high and low frequencies (increased, permanent shifts in auditory threshold at 2, 16 and 20 kHz). The cytocochleogram showed outer hair cell loss of approximately 86% in regions corresponding to 8 and 22 kHz.

Overall, this study demonstrates hearing loss accompanied by histological damage in rats exposed repeatedly to 750 ppm (3248 mg/m^3) styrene for 4 weeks. Ethanol did not have any effect on auditory sensitivity, wheras styrene alone caused permanent threshold shift and outer hair cell damage. Hearing and outer hair cell loss were larger after the exposure to both ethanol and styrene than those induced by styrene alone.

In a time course experiment, male Long-Evans rats were exposed to 0 (16 animals) or to 1000 ppm (4330 mg/m^3) styrene for 6 hours/day, 5 days/week, for either 1 (16 animals), 2 (12 animals), 3 (12 animals) or 4 (12 animals) consecutive weeks (Campo *et al*., 2001). Following exposure, groups of 4-8 animals were sacrificed for the histological analysis of the cochlea. Audiometric thresholds were determined in 8 animals per group prior to exposure, the day after exposure and after a 6-week recovery period.

Hearing loss was similar despite the differences in duration of exposure, but worsened after the end of the exposure. For example, the 16 kHz threshold shift was 20 dB at the end of the exposure period (for the 1-week exposure test) but increased up to 35 dB by 6 weeks post-exposure. The histological damage in the cochlea (large losses in the third row of the outer hair cells OCH3, less severe damage in the OCH2 and no lesions in OCH1 and IHC) was also similar for the different durations of exposure. Analysis by electron microscopy showed damage and disorganisation of the plasmalemma membrane.

Overall, this study shows that ototoxicity (hearing loss accompanied by histological damage) in rats exposed repeatedly to 1000 ppm (4330 mg/m^3) styrene did not worsen with duration of exposure (from 1 to 4 weeks). Hearing loss seemed to progress after the end of the exposure period reaching its maximum at around 6 weeks post-exposure. This is likely to be the consequence of the damaged hair cells, which are still dying off after the end of the exposure. This study also shows that styrene and/or its metabolites cause a serious disturbance of the membranous organisation.

Studies on other organic solvents such as toluene (Johnson and Nylen, 1995; Pryor *et al*., 1984) show that ototoxicity appears after relatively short exposures and that continued treatment does not enhance the intensity of the ototoxic response. A clear decrease in auditory sensitivity was seen in rats exposed to 1000 ppm toluene (22 hours/day, 7 days/week) for one month, but no hearing effects were found after 19 months of exposure with a shorter daily duration (6 hours/day, 5 days/week) (Nylen *et al*., 1987).

Styrene-induced hearing loss did not appear to be counterbalanced by central compensation (GABAergic adjustment in the inferior colliculs) in Long-Evans rats exposed to 700 ppm (3031 mg/m^3) styrene 6 hours/day, 5 days/week for 4 weeks, but hearing loss caused by noise (97 dB SPL octave band noise centred at 8 kHz) did appear to be centrally compensated (Pouyatos *et al*., 2004).

Styrene-induced hearing loss was greater in 3-month old Long-Evans rats compared to 4-month old rats exposed to 700 ppm (3031 mg/m^3) styrene 6 hours/day, 5 days/week for 4 weeks, but was not body weight-sensitive. These data suggest that younger adult rats may be more susceptible to styrene ototoxicity compared to older adult animals (Lataye *et al*., 2004).

Lataye *et al*. (2005) compared styrene-induced ototoxicity in active rats and sedentary/ordinary rats and investigated the combined effects of noise and styrene on hearing. Groups of eight male Long-Evans rats were exposed whole-body under sedentary/ordinary conditions to 0, 500, 650, 850 or 1000 ppm (0, 2165, 2815, 3681 or 4330 mg/m^3) styrene for 6 hours/day, five days/week for four week. Additional groups of 4 male Long-Evans rats, forced to run (for 2 minutes every 3 minutes) in a special wheel during the exposure, were exposed whole body to 0, 300, 400, 500 or 600 ppm (0, 1299, 1732, 2165 or 2598 mg/m^3) styrene for the same exposure duration. Furthermore, groups of 6 active male Long-Evans rats were exposed to 0, 400 ppm (0 or 1732 mg/m^3) styrene alone, noise alone (an octave band noise cantered at 8 kHz) or styrene (400 ppm) and noise combined. Audiometric testing was performed prior to styrene exposure and at six weeks after the exposure period. Following audiometric testing histopathologically and scanning electron microscopic (SEM) examination was used to assess damage to the inner and outer hair cells (IHC and OHC) of the organ of Corti.

In the sedentary animals, hearing loss and OHC cell damage were observed at 650 ppm and above, but not at 500 ppm. In the active rats, functional and histological damage was observed at 400 ppm and above, but not at 300 ppm. These results show that the ototoxic potency of styrene exposure depends on the physical activity of the animals as this is related to the ventilation rate and, in turn, to the uptake of the chemical via the lungs.

Overall, based on these findings, NOAECs of 500 and 300 ppm (2165 and 1299 mg/m^3) can be identified in sedentary/ordinary rats and active rats, respectively. In the experiment investigating the combined effects of noise and styrene, noise alone or styrene alone were found without effect; however, both hearing loss and OHC cell damage were observed in the animals exposed to noise and styrene combined. These results also suggest that styrene-induced ototoxicity can be potentiated by exposure to noise.

Campo *et al*. (1999) investigated whether styrene-induced ototoxicity is caused by “tissue intoxication” or by “fluid contamination”. Long-Evans rats were exposed to styrene in air at a concentration of 1750 ppm (7578 mg/m^3) for 6 hours on the first day and a further 4 hours the following day. Immediately after exposure, blood, brain, auditory nerves, the organ of Corti, cerebrospinal fluid and inner ear fluid were sampled and the styrene concentration in each tissue determined by gas chromatography.

Styrene was found in the organ of Corti, the nerves and the brain but not in the cerebrospinal and inner ear fluids. Based on these data the authors proposed that styrene-induced ototoxicity is caused by “tissue intoxication”. Styrene, transported by blood coming from the stria vascularis or the spiral prominence, diffuses through the outer sulcus to reach the lipid-rich Hensen’s cells. These cells are in close connection with the Deiters cells that are directly located under the outer hair cells (OHC). Thus the target cells are reached by diffusion of styrene. This explains why the hair cells are lost sequentially from OHC3 to OHC1 as diffusion continues.

Conclusion on ototoxicity

Clear evidence of ototoxicity (both functional and histological) has been seen in sedentary/ordinary rats repeatedly exposed to styrene by inhalation at concentrations of 600 ppm (2598 mg/m^3) and above. In three different studies, no such effects were seen at 200 ppm (866 mg/m^3) for 13 weeks, or at 300 ppm (1299 mg/m^3) or 500 ppm (2165 mg/m^3) for four weeks. One study in active rats exposed to styrene for 4 weeks showed that styrene-induced ototoxicity tend to occur at lower exposure concentrations (400 ppm - 1732 mg/m^3) than those at which ototoxicity is observed in sedentary/ordinary rats. This is considered to be due to the increased styrene uptake, which is the consequence of the increased ventilation rate and, in turn, of the increased physical activity.

The histological damage consists of the destruction of the outer hair cells (OHC; especially of row 3) of the cochlea. These changes are accompanied by an elevation of the hearing thresholds in the mid-frequency range (10-20 kHz). The destruction of the hair cells is irreversible and occurs at slightly lower exposure concentrations than those producing the audiometric hearing threshold shifts. Mechanistic investigations indicate that styrene reaches the sensory hair cells of the cochlea via the blood stream and that styrene itself and/or its metabolites cause a serious disturbance of the membranous organisation of these target cells. However, the underlying toxicological mechanism has not been clearly elucidated. In conclusion, the ototoxic effects of styrene should be regarded as of potential relevance to human health.

Study investigating the relative ototoxicity of styrene

Studies in rats and mice have shown that some aromatic solvents have striking ototoxicity, charecterised by an irreversible hearing loss as measured by behavioural or electrophysiological methods, and associated with damage to outer hair cells in the exposed animals (Pryor *et al.*, 1983).

Gagnaire *et al.* (2005) examined the relative ototoxicity of 21 aromatic solvents compounds by comparing their average cochleograms (total cell count) obtained from rats. The rats were dosed with 8.47 mmol/kg b.w. for 5 days/week over a 2-week period. The average cochleogram was obtained from six cochleas randomly chosen from six rats. Eight of the 21 solvents were found to be ototoxic in the rat. They differed widely in their ototoxic potential. Styrene showed a greater ototoxic potential than toluene, p-xylene and n-propylbenzene. The least ototoxic solvents caused outer hair cell loss in the middle turn of the organ of Corti. The outer hair cell loss was slight in the first row, and greater in the second and third rows. The most ototoxic solvents, including styrene, caused high losses in the three rows of the outer hair cells along the entire length of the basilar membrane. There were also occasional inner hair cell losses in the most affected animals. It seemed that some structural constraint was essential to induce ototoxicity.

Studies investigating specific organ toxicity: ocular system

Groups of 10 female Sprague-Dawley rats were exposed, whole-body, to 0 or 300 ppm (0 or 1299 mg/m^3) styrene 6 hours/day, 5 days/week for 12 weeks (Vettori *et al*, 2000). Retina were removed post-mortem; the right retina was studied for tyroxine hydroxylase-immunoreactive (TH-IR) amacrine cell count, and the left retina was analysed for dopamine (DA), catecholamine (CA), tyrosine hydroxylase (TH), homovanillic acid (HVA), 3,4-dihydroxy-phenylacetic acid (DOPAC), and glutathione levels. Amacrine cells are modified neurons that produce dopamine, which is assumed to modulate the retinal cone-horizontal cell transmission that is involved in colour vision.

The number of large amacrine cells was reduced by around 30% in styrene-exposed rats when compared to controls. The density of small amacrine cells was unaffected. Dopamine and DOPAC concentrations were lower than controls (22% and 17% respectively), as were glutathione levels (28%).

Overall, this study showed effects on the number of the large amacrine cells and on the content of neuramines and glutathione of the retina of rats exposed repeatedly to 300 ppm (1299 mg/m^3) styrene for 12 weeks. This finding in rats supports the ocular toxicity reported in humans.

Studies investigating specific organ toxicity: the nervous system

Rosengren and Haglid (1989) studied brain protein in rats exposed continuously to styrene for 90 days. Groups of 8 (exposed) or 16 (control) Sprague Dawley rats inhaled 0, 90, or 320 ppm (0, 390 or 1386 mg/m^3). Animals were then kept for 4 months free of exposure, sacrificed, the brains removed, and various regions of the brain isolated by dissection. Tissue samples were homogenised and the astrological markers S-100 and glial fibrillary acidic proteins (GFAP) were measured. Relative to the controls, GFAP (a specific marker for glia cells activation) concentrations in the 320 ppm group were significantly increased in the sensory motor cortex and in the hippocampus, indicating structural damage to the neurons. These findings have been supported by two recent studies (Wang 1998 and Wang *et al,* 1998) where an increase in GFAP in cerebral cortex and hippocampus were shown.

These 3 studies show that styrene can damage neurons in different brain areas.

4.7.1.3 Repeated dose toxicity: dermal

Taken from the EU RAR (2008) (4.1.2.6.1., p. 142)

"No studies are available, although low systemic toxicity would be predicted in most conventional experimental species with the possible exception of some strains of the mouse."

4.7.1.4 Repeated dose toxicity: other routes

4.7.1.5 Human information

(Text is identical to the Danish proposal discussed in the TCC&L group)

Numerous studies investigating the neurotoxic potential of styrene in humans are available. The focus has been put on the studies investigating the ototoxicity and the effects on the colour vision as these endpoints are the most sensitive ones.

Otoneurological and audiometric studies

An otoneurological study was carried out on a small group of GRP workers (Calabrese *et al.,* 1996). Twenty workers were employed in 3 plants. It is not stated whether all the available exposed employees participated. The choice of tests was aimed at investigating any effects of styrene on the vestibular system or hearing. The workers were stated to have relatively low alcohol consumption. Exposure levels were measured in the workplace on the day before otoneurological testing. Average personal exposure levels (8-hour TWA) were 36 ± 20 ppm (156 ± 87 mg/m^3) for styrene and 65 ± 28 ppm for acetone. Audiometric tests and auditory brainstem responses (ABR) were measured and

vestibulocular (VOR) (caloric and rotation) and vestibulospinal (VSR) (static posturography) tests performed. Nine of the subjects were also examined after an exposure-free period of 3 weeks.

All workers had a normal hearing threshold. The mean ABR parameters (e.g. latencies) were not significantly different between the exposed group and an undefined control group. "Abnormal" results were obtained for 17 of the 20 workers in the VOR caloric test and in 14 of 20 in the rotation test.

Overall, there were no effects of styrene exposure on the auditory system in this study although it is noted that there was a relatively small number of workers investigated. A high incidence of undefined "abnormalities" in certain reflexes related to the vestibular system was noted among the styrene-exposed workers.

In Sweden, a small factory using styrene was closed and the workers, employed on average for 10.8 years, were no longer exposed to styrene (Möller *et al.,* 1990). The time lapse since last exposed was not reported but it appears that the same ex-workers were studied by Flodin *et al.* (1989), 7-9 months after exposure ceased. Generally, measurements of airborne styrene in the work areas had been performed annually. The measurements were obtained by personal sampling and concentrations refer to "eight hour average". "High" exposure levels had been 12-24 ppm (52-104 mg/m^3) with a very few peak exposures above 71 ppm (307 mg/m^3). An "otoneurological" study was carried out, using highly specialised tests, to examine the hypothesis that styrene exposure may affect central nervous system mechanisms relating to balance and hearing. The total workforce figure was not given but appears to have been 24 (Flodin *et al,* 1989). The 18 participating ex-workers (mean age 40) were matched for age range and alcohol consumption with 18 controls in solvent-free employment. Also, static posturography was measured in the ex-workers and in a control group of 52 men (mean age 48) from the construction industry with no exposure to solvents.

Clinical neurological assessments in the styrene-exposed workers (gait, tendon reflexes, gross motor function and cranial nerves) showed normal findings. In auditory tests, a slight high-frequency hearing loss was detected, but this was accounted for by age and/or excessive noise exposure. Abnormalities in cortical response audiometry, defined as latency above the normal reference range for the response to a frequency glide stimulus, were found in 7 ex-workers. There were no apparent effects of styrene exposure in the vestibular stimulation test by caloric irrigation of the ears. Postural sway area on a forceplate was considered by authors to be abnormally large, relative to controls, in 10 ex-workers. The actual mean sway area was small (increased to 117 mm^2 compared with 71 mm^2 in controls). There was a statistically significant increase in saccade (quick movements of the eyes) latency time and impairment of ability to visually suppress vestibulocular reflex (rotatory test) in the styrene-exposed ex-workers compared to matched controls. The actual change in mean latency was an increase from 249 to 289 ms.

Overall, the cortical response "abnormalities" detected in the absence of any effect on hearing, are not considered to have any clinical significance. However there was some evidence for an effect on vestibular function in the exposed workers.

Workers in an industry in the Netherlands were exposed to styrene; 59 male subjects of 76 employed were included in a study of hearing function at up to 16 kHz (Muijser *et al.,* 1988). Illness prevented 12 taking part and 3 others refused. A control group of 94 male photographic film workers were of similar age and were stated to be "not occupationally exposed to styrene or other chemicals". A medical history of hearing dysfunction led to exclusion of 2 exposed and 6 non-

exposed workers. The concentration of styrene in the breathing zone of some of the workers was measured on 3 consecutive days using personal absorption badges. Workers were divided into two groups according to whether they were directly (n=31) or indirectly (n=28) exposed to styrene. A task was chosen to represent "peak" exposure to styrene and measurements made while this task was conducted. The "4-hour mean" exposure levels were 32 ppm (139 mg/m^3) (directly exposed) and 14 ppm (61 mg/m^3) (indirectly-exposed); "peak exposure" was 105 ppm (455 mg/m^3). The workers had been exposed for an average of 8.6 years. Noise levels at the styrene workplaces were up to 70 DBA in the background and up to 104 DBA for short periods, at which level noise-induced high frequency hearing loss is possible. Noise exposure at the control workplace was 80-85 DBA.

Direct comparison of results in the styrene-exposed and non-exposed workers showed a higher threshold for high frequency hearing in the styrene-exposed group but on multivariate analysis to adjust for the effect of age, the difference was not statistically significant. There was also no statistically significant difference between the control and directly-exposed group. However, hearing thresholds at high frequency were statistically significantly higher in the directly-exposed versus indirectly-exposed subgroups workers. A higher threshold would be expected at higher frequencies if styrene exposure was to have an adverse effect; high frequencies are known to be more sensitive to impairment. On detailed analysis, the authors described an inconsistency in the pattern of results with respect to this expectation; a significant effect was detected at 8 kHz but not at higher frequencies. Hence the authors stated that their findings were equivocal.

In Japan, 115 male workers exposed to styrene in various workplaces were recruited for a study of the upper frequency threshold of hearing, which the authors of the study believed to be a more sensitive indicator of early hearing loss than conventional audiometry (Morioka *et al.*, 1999). In many cases they were simultaneously exposed to other solvents including toluene. On the basis of pre-existing impaired hearing, exposure to intense noise or incomplete data, 18 workers were excluded. Sound levels measured at the workplace were 53-95 DBA. Styrene was measured in the breathing zone and levels of urinary metabolite mandelic acid also measured.

Styrene levels were found to be generally less than 50 ppm (217 mg/m^3) TWA (range 0.1-100 ppm – 0.4-433 mg/m^3). Conventional audiometry at 0.5-8 kHz, performed pre-shift, demonstrated no significant difference between the exposed subjects and controls, described as "the subjects for the standard upper limit age curves for males". (It is not clear where this "standard" data was obtained, though it appears likely that it is from previous studies by the same authors.) The upper frequency limit of hearing was determined for each exposed subject and plotted on a set of "standard" percentile curves for male upper limit of hearing vs. age. Subjects whose upper frequency threshold fell below the 75th percentile curve were defined as "cases" of hearing loss, and most of the subsequent analyses are based on the percentage prevalence of such "cases". In a plot of the prevalence against duration of exposure the prevalence exceeded 25% (the expected "normal" value) at 5 years of exposure and increased linearly to 35% at 10 years of exposure, after which it remained static. For the 54 workers exposed for more than 5 years there was a significant correlation between "individual percentiles" (presumably individual results expressed in terms of position on the normal percentile curves) and styrene exposure. The prevalence of cases (defined as above) also increased with styrene exposure, becoming significantly higher than 25% when exposure exceeded 16 ppm. When analysed with respect to mandelic acid levels the prevalence of "cases" significantly greater in the group whose level exceeded 0.3 g/l compared with those below that level. There was evidence of a correlation between styrene exposure (concentration and duration) and abnormal results in a test for the upper frequency threshold of hearing.

Morata *et al*,. (2002) investigated potential auditory effects produced by occupational exposure to low levels of styrene and noise. Workers (n = 154) exposed to styrene were recruited from factories producing fibreglass products. Sixty-five were not exposed to potentially harmful levels of noise; the remaining 89 styrene-exposed workers also had noise greater than 85 dB(A) L_{eq} over an 8 hour workday. A noise-only group comprised 78 metal workers, and 81 postal workers formed the control group, without exposure to noise or styrene. Each subject answered questions concerning occupational history, medical history and lifestyle factors. These data were combined with measurements of exposure to styrene and to noise in current employment, estimates for styrene and noise exposure over each subject's lifetime, and hearing thresholds given during the study. Styrene exposure was determined by air monitoring and biological monitoring. Air from the breathing zone of each subject was sampled over the test day. Mandelic acid (MA) was measured in urine from 127 of the 154 styrene-exposed workers; samples were collected over 24 hours from the beginning of the work shift under study. Styrene concentrations in air were reported only in a scatter diagram together with MA excretion in urine. Although the correlation was statistically significant ($r = 0.27$, $p < 0.001$), there was a great variability. Noise exposures were determined as 8-hour dB(A) L_{eq} values for 185 of the workers; the remaining 128 subjects were assigned the mean exposure of other workers doing the same tasks. Each worker's previous noise exposure was estimated from questionnaire responses or database information. Using the measured or assigned exposure for current employment, and the approximate values for previous employment, lifetime noise exposure was estimated as an 8-hour L_{eq} over each working day. For the noise only group, the current exposures ranged from 75 to 116 dB(A) L_{eqbh}. An unstated number of this group did not receive "excessive" noise, above 85 dB(A) L_{eqbh}, and therefore could have been placed in the control group.

The styrene only subjects had a range of current styrene exposures from 0.05 to 23 ppm (0.2-100 mg/m^3), while for the styrene and noise group, styrene exposures ranged from 0.007 to 12 ppm (0.03-433 mg/m^3). Each subject gave an audiogram, which was evaluated for hearing loss at 1, 2, 3, 4, 6 and 8 kHz. An audiogram was considered "normal" if no threshold exceeded 25 Db Hearing Level at any frequency. A "high-frequency hearing loss" was noted if the thresholds were poorest in the frequency range 3 to 6 kHz. Over the 4 exposure groups, prevalence of high-frequency hearing loss ranged from 33% to 48%; the differences between groups were not statistically significant.

The binary hearing variable, normal versus high-frequency loss, was also employed in a further analysis. The medical, lifestyle and occupational data (including current and past exposure to noise and to styrene) were subjected to a multiple logistic regression analysis to estimate the odds of subjects developing a high-frequency hearing loss. Only age, current noise exposure and MA in the urine were significant. Neither current nor lifetime styrene exposure achieved statistical significance. The authors concluded that exposure to styrene, at concentrations below 20 ppm (87 mg/m^3), produced high-frequency hearing losses. However, considering the information on styrene exposure of the subjects and the lack of dose-response relationships, this study seems to underestimate the risk of hearing loss, due to the fact that some of the participants were exposed to very low levels of styrene.

In a cross-sectional study of 299 workers, who had each been employed for more than 6 months at either of 4 yacht-making yards and one plastics factory in Poland, visual examinations of the ear, nose and throat, and audiometric tests were conducted (Sliwinska-Kowalska *et al.*, 2003). Workers were excluded from the study if there was evidence of previous ear disease, impaired hearing, or other non-occupational risk factors for hearing loss; 9 were excluded from the original cohort. Pure-tone audiometry was performed on 290 workers (aged 20-60). Occupational noise exposures were assessed over a working day in each subjects' current employment. Those with an 8-hour average exposure greater than 85 dB(A) were defined as noise-exposed; those with lower noise exposures

were assigned to the no-noise cells of the experimental design. Hence, of the 290 workers, 194 were listed as part of a 'styrene-only' cohort (i.e. exposed to styrene and to a mean background noise level of 80 dB(A)); 26 as exposed to 'styrene and toluene' (i.e. exposed to styrene, toluene and to a mean background noise level of 80 dB(A)); 56 to 'styrene and noise' (i.e. exposed to styrene and to a mean noise level of 89 dB(A)); 14 to 'styrene, toluene, and noise' (i.e exposed to styrene, toluene and to a mean noise level of 89 dB(A)). Chemical exposures were mixed, and more than 80% of the workers in the 'styrene-only' cohort were exposed to other solvents (including toluene, dichloromethane and acetone) at levels that exceeded the OELs in Poland (100 mg/m^3, 50 mg/m^3, and 200 mg/m^3 respectively). Peak levels (unclear if these were 8-hour TWA values) were 225 mg/m^3 for toluene, 307 mg/m^3 for acetone, and 145 mg/m^3 for dichloromethane. An age-matched control group of 157 'white collars' and 66 workers from a metal factory was selected with similar exclusion criteria. Of this 'control' group, the 66 metal factory workers were exposed to mean noise levels around 89 dB(A) (noise only control group) and the 157 'white collars' to a relatively low mean noise level of 73 dB(A) (no noise, no chemicals control group). Current airborne exposure to styrene was assessed by personal monitoring during a working shift. The concentrations ranged up to a maximum of 47 ppm (204 mg/m^3). A lifetime-average styrene exposure was calculated for each of the 290 chemical-exposed groups, using environmental inspection records over the previous 15 years. These averages ranged from 4 to 309 mg/m^3 (mean 62 mg/m^3; 1-71 ppm, mean 14 ppm).

Abnormal audiograms were seen in about 63% (183/290) of the solvent-exposed workers compared to around 40% (93/223) of controls. The odds-ratio for hearing loss (based on abnormal audiogram) likely to be due to styrene exposure but also to exposure to a higher mean noise level was 5.2 (95% CI 2.9-9.8); this was calculated comparing the incidence of the abnormal audiograms in the styrene-only group (mean noise level of 80 dB(A)) with that in the cohort not exposed to any solvents but to mean levels of noise of 73 dB(A). The odds-ratio for hearing loss due to noise only was 3.4 (1.7-6.4); this was calculated comparing the incidence of the abnormal audiograms in the 'styrene and noise' group (mean noise level of 89 dB9A)) with that in the styrene-only group (mean noise level of 80 dB(A). The odds-ratio for hearing loss due to exposure to styrene and toluene but also to a higher mean noise level was 13.1 (4.5-37.7); this was calculated comparing the incidence of the abnormal audiograms in the styrene and toluene group (mean noise level of 80 dB(A)) with that in the cohort not exposed to any solvents but to mean levels of noise of 73 dB(A). The odds-ratio for hearing loss due to exposure to styrene and noise was 10.9 (4.9-24.2); this was calculated comparing the incidence of the abnormal audiograms in the styrene and noise group (mean noise level of 89 dB(A)) with that in the control cohort not exposed to any solvents but exposed to a low mean level of noise of 73 dB(A). The odds-ratio for hearing loss due to exposure to styrene, toluene and noise was 21.5 (5.1-90.1); this was calculated comparing the incidence of the abnormal audiograms in the styrene, toluene and noise group (mean noise level of 86 dB(A)) with that in the in the control cohort not exposed to any solvents but exposed to a low mean level of noise of 73 dB(A). Hearing threshold was also increased amongst styrene-exposed workers when compared to controls, and was greater when in combination with other solvents or noise.

This study shows evidence of styrene-induced hearing loss and an additive or synergistic effect with other ototoxic agents such as toluene or noise.

Studies on colour vision

The data available on colour vision and styrene exposure have recently been reviewed by Lomax *et al.* (2004).

The most common test of colour discrimination is the Lanthony Desaturated 15-hue test (D-15d). In this test the subject is presented with 15 coloured caps and asked to arrange them in a natural colour

sequence, starting from a reference blue cap and successively matching each cap to the preceding one in the sequence. The theoretically correct sequence, chosen by most subjects, follows a spectral order from blue through green, yellow and orange to red and on to purple. The colours used are desaturated ("pastel" hues in everyday English language) which increases the difficulty and consequently the sensitivity of the test. The results can be analysed both qualitatively and quantitatively.

Qualitative analysis involves plotting the results on a circular diagram of the correct colour sequence by joining the dots corresponding to caps that the subject placed consecutively. Errors appear as chords cutting across the circle. They can be classified according to whether they lie parallel to the red-green or blue-yellow axes of the circle and whether they are minor (transposition of adjacent caps) or major (spanning two or more caps). The frequency of errors is influenced by the intensity and colour of the lighting used for the test and by the visual acuity of the subject. Many subjects make minor errors in the test and a substantial number make one or more major errors, predominantly along the blue-yellow axis. The frequency of these errors increased with age. Congenital colour vision defects give very characteristic patterns of major errors of large magnitude on qualitative analysis, corresponding to the various clinically recognised forms of "colour blindness".

Quantitative analysis is based on allocating numbers to each cap corresponding to its position in a standard chromaticity chart and calculating the "colour distance" between successive pairs of caps as placed by the subject. These colour distances are summed up to give the total colour distance score (TCDS), which can be divided by the theoretically perfect score for the set of caps used, to obtain a standardised score called the colour confusion index (CCI).

Twelve cross-sectional or longitudinal studies investigating colour discrimination in reinforced plastics workers, boat builders and other workers exposed to styrene have been identified.

A recent study examined colour discrimination in styrene exposed laminators at a German boat-building plant before and after a 4 week vacation, with the assessment being repeated one year later after the introduction of improved hygiene measures (Triebig *et al.*, 2001). Thirty out of 50 male styrene-exposed laminators who had been at the factory for at least 6 months were recruited into the study; it is unclear why the other 20 laminators did not participate in the study. Twenty-seven male workers from other parts of the factory were recruited as controls. Subjects were excluded from the study for a number of reasons including congenital colour vision deficiency (identified with Ishihara colour perception tables), high alcohol consumption (above 210 g/week), poor visual acuity, diagnosis of ophthalmological disease, and, in a control subject, high mandelic acid and phenyl glyoxylic acid (MA+PGA) levels. After exclusions, 22 styrene-exposed workers and 11 control workers entered the study. The median age of the styrene-exposed workers was 38 years, and the median time working at the plant was 4.5 years (range 1-21). The control workers were similar in terms of age, alcohol consumption and duration of employment. Styrene exposure was assessed by biological monitoring of MA and PGA. These were measured in urine collected at the end of the shift on a Thursday afternoon, in order to give a measure of the peak body burden of styrene. Colour discrimination was assessed binocularly by the D-15d test, conducted under standardised lighting conditions. Testing was carried out in exposed workers and in controls on Monday morning before starting work and on Thursday afternoon of the same week. CCI values greater than the 95th percentile of the age-dependent reference value were considered abnormal.

During the first phase of the study median MA+PGA levels were 472 mg/g creatinine (range 11-2399) in the exposed group and 15 mg/g creatinine (range 5-36) in the control group. Regarding colour discrimination results, on the Monday morning, 8 of the 22 exposed workers (36%) had abnormally high CCIs, compared with 2 of the 11 controls (18%). Although the nature of the test errors was not described in detail, both tritan and red-green errors were made. At the Monday morning time point the mean CCI in the exposed workers was 1.24 (SD 0.25), higher than in the controls (mean 1.10, standard deviation (SD) 0.11), but the difference was not statistically significant. For the Thursday afternoon test the mean CCI in the styrene-exposed workers had increased to 1.29 (SD 0.09). This was significantly higher than in controls, for which the mean CCI did not change between Monday morning and Thursday afternoon. Thursday results tabulated for 19 exposed workers showed that all 7 who had abnormally high CCI values had urinary MA+PGA concentrations exceeding 450 mg/g creatinine, whereas 9 of the 12 workers with normal CCI values had MA+PGA concentrations below 450 mg/g. When the data from all the styrene-exposed workers were analysed by regression analysis, CCI was positively and significantly correlated with the urinary concentration of MA, and with the sum of MA and PGA. After a 4-week absence from the factory, the mean CCI for the exposed workers was significantly lower than the pre-vacation values, and was no longer significantly different from the mean control CCI.

A re-examination of colour discrimination was carried out in the same exposed and control subjects one year later, following the introduction of an improved ventilation system at the plant. By the time of the second phase of the study styrene exposure had decreased, shown by lower urinary styrene metabolite levels among laminators. Measurements on Thursday afternoon showed that the median end-of-shift metabolite level was 273 mg MA+PGA/g creatinine, almost half of the level found one year previously. Colour discrimination tests carried out on Thursday afternoon showed that the mean CCI among the same group of laminators was significantly lower than one year previously, at 1.16 (SD 0.14). Five laminators (23%) were still classed as having abnormally high CCI values, although for the tests conducted on the same subjects on Monday morning only 2 styrene-exposed workers gave results outside the expected range. Among the 11 control subjects, there were no changes in colour discrimination in the year between the two phases of the study. After a 4-week absence from the factory, the mean CCI value for the exposed group fell to 1.05 (SD 0.06), almost identical to that of the control group.

This well-conducted, well-reported study shows a convincing association between occupational styrene exposure (as revealed by the concentration of urinary metabolites) and poor colour discrimination, with both blue-yellow (tritan) and red-green errors being made. From the design of the study, it is not likely that this association could be accounted for by confounding factors such as age, alcohol or congenital colour vision deficiency. On the basis of the ACGIH conversion, this would suggest that elevated CCI scores occur for exposures to styrene estimated to be between 20 and 30 ppm (87-130 mg/m^3) (8-hour TWA). It is therefore unlikely from the biological monitoring data that exposures to styrene in this study would have been high enough to cause acute CNS depression. The results from both phases of the study consistently indicate that the effects of styrene on colour discrimination decrease after an exposure-free weekend, and appear to be fully reversible over a period of four weeks. The results also show an exposure-response relationship between CCI and urinary levels of MA and PGA.

Three reports described a series of linked studies investigating colour discrimination in styrene-exposed workers at a number of reinforced plastics factories in Italy.

In the first study, 75 styrene-exposed workers employed at 7 factories were recruited from a total workforce of 84 (Gobba *et al.*, 1991). The average length of time working with styrene was 7 years. Sixty workers from the rock-wool industry who were not exposed to solvents acted as controls. Two exposed workers and 3 controls were excluded from the study population on the basis of high alcohol consumption (>250 g per week), poor visual acuity or a case history of certain medical conditions including self-reported congenital colour vision deficiency. For the reinforced plastics workers, styrene exposure was monitored on a Thursday via personal sampling throughout the 8-hour work shift, and by measurement of urinary MA levels at the end of the shift. Colour discrimination was assessed by the D-15d test at the beginning of the working day, under standardised lighting conditions. For a subgroup of 39 styrene-exposed workers and matched controls, testing was also performed before, and immediately after, a one-month holiday. Analysis of the possible reversibility of the effects of styrene in these workers was described by Gobba and Cavalleri (2000), as summarised below.

The geometric mean for airborne styrene concentration was 16 ppm (69 mg/m^3) (range 0.8-131 ppm – 3.5-567 mg/m^3), and end-of-shift MA concentration was 343 mg/l (range 15-3002). With the D-15d test, some styrene-exposed workers and controls made errors along the tritan axis of colour confusion; however "a few" styrene-exposed workers made errors along the red-green axis as well. The control workers in this study were on average older than the styrene-exposed workers, and to correct for the effect of age on colour discrimination, subjects were arranged into 41 pairs matched for age to the nearest two years. In the 41 styrene-exposed workers the mean CCI was 1.26 (SD 0.22), significantly higher ($P<0.01$) than the mean CCI of 1.15 (SD 0.14) for their age-matched controls. Although not commented on by the authors, it appears from the distribution of CCI scores in the 41 styrene-exposed workers that there were two sub-populations; most exposed workers (26/41) had a CCI within one SD of the control mean, but a minority (9/41) had a CCI more than 2½ SDs greater than the control mean. In another method of analysis, all the subjects were arranged into three age groups (16-30, 30-39 and 40-64 years old). Analysis of these three groups revealed that styrene-exposed workers had higher median CCI values than controls, but the difference was only statistically significant for workers of 40 or over. In a further analysis, all exposed workers were subdivided into those with exposures above or below 50 ppm (217 mg/m^3). The results showed that the 15 workers exposed to >50 ppm had significantly greater ($P<0.05$) CCI values than the 55 workers exposed < 50 ppm, although there was no information on worker age in the two sub-groups and a high degree of overlap of individual scores between the two sub-groups of workers. In the low exposure group the median CCI was 1.08, similar to the control median, while in the high exposure group the median CCI was 1.30 with a 90th percentile of 1.64. This implies an impairment of colour discrimination. For the sub-set of 39 exposed workers who had a mean age of 35 years (SD 6), mean CCI tested before the holiday was 1.23 (SD 0.19); this is on the 95th percentile of the age-related reference value. After the holiday, mean CCI was 1.20 (SD 0.21), suggesting that a 4-week exposure-free period did not influence colour discrimination.

In the second Italian study, similar methods were used to assess colour discrimination in a group of 40 styrene exposed reinforced plastics workers from 3 factories not included in the previous study (Gobba and Cavalleri, 1993). A control group comprised of 40 subjects, matched for age, alcohol consumption and tobacco smoking, but the population from which they were recruited was not described. Subjects with alcohol consumption greater than 350 g/week or with self-reported congenital colour vision deficiency were excluded from this study population. For the reinforced plastics workers, styrene exposure was monitored on a Thursday by personal sampling, presumably throughout the 8-hour work shift, and by measurement of end-of-shift urinary styrene levels; the

frequency of monitoring was not stated. Colour discrimination was assessed by the D-15d test at the beginning of the working day, under standardised lighting conditions.

Among the exposed workers, mean styrene exposure during the 8-hour shift was again 16 ppm (69 mg/m^3). The mean CCI in styrene-exposed workers (1.21, SD 0.20) was significantly higher than the mean in the controls (1.05, SD 0.07). There was no attempt at exposure-response analysis in these workers.

In the third Italian study, the influence of changes in styrene exposure over a twelve-month period on colour discrimination was assessed in a group of 30 workers (Gobba and Cavalleri, 2000). An unexposed control group of equal numbers, matched for sex, age, alcohol consumption and smoking, was included. Mean CCI of all styrene-exposed workers at the start of the 12-month period (1.24, SD 0.21) was significantly higher than the controls (1.14, SD 0.14). Among the exposed workers there were 10 individuals for whom exposure increased (Group 1), and 20 individuals for whom exposure was unchanged or decreased slightly (Group 2). The airborne concentration of styrene was monitored by personal sampling and colour discrimination was assessed using the same methods as in the previous two studies.

In group 2 the geometric mean airborne styrene concentration was 14 ppm (61 mg/m^3) at the initial time point and 10 ppm (43 mg/m^3) one year later; over this time period mean CCI did not change significantly from the initial value of 1.27 (SD 0.18). In group 1 the geometric mean airborne styrene concentration increased over the one-year period from 11 to 16 ppm (48-69 mg/m^3); in these workers mean CCI increased from 1.18 (SD 0.16) to 1.29 (SD 0.21). None of the mean changes in styrene exposure or CCI over this 12-month period achieved statistical significance.

Overall, these three Italian studies are consistent in pointing to an association between occupational styrene exposure and impaired tritan colour discrimination. An exposure-response analysis was only carried out in the first study, and this suggested an impairment of colour discrimination in workers exposed to >50 ppm (217 mg/m^3) styrene, whereas in workers exposed to <50 ppm styrene colour discrimination was normal.

In terms of the magnitude of the effect, the median CCI in the high exposure group (>50 ppm) was 1.30. The results from a sub-group of workers suggest that the effects of styrene on colour discrimination persist even after a 4-week exposure free period.

Three studies in Japanese workers attempted to establish whether styrene has a threshold for effects on colour discrimination.

Eguchi *et al.* (1995) investigated colour discrimination in 64 styrene-exposed workers from 6 fibreglass reinforced plastics (FRP) factories and 69 controls recruited from the same and other factories. Exclusion criteria included an alcohol consumption of more than 250 g/week, the presence of congenital colour vision deficiency or poor visual acuity. The mean age of the exposed workers was 38 years (range 18-66) and duration of exposure was 7 years (range 0.2-26.8). The mean age of controls was 38 years (range 20-61). Styrene was said to be by far the most widely-used solvent in the factories; acetone was also used to clean tools. End-of-shift urinary MA concentrations were measured on the same day of colour discrimination testing, but levels were not related to creatinine concentration or ionic strength, although the subjects drank a glass of water 2

hours before urine collection to minimise the effects of differences in ionic strength. Airborne styrene concentrations were measured by area sampling. Colour discrimination was tested using the D-15d test at the beginning of the working day (usually on a Monday) to minimise the possible acute influence of styrene on colour discrimination.

The mean urinary MA concentration in styrene-exposed workers was 220 mg/l (SD 480), and the mean airborne styrene concentration was 21 ppm (91 mg/m^3) (range 6.6-36.4 ppm – 29-158 mg/m^3). In both exposed and control groups a number of subjects made errors in colour discrimination. All subjects who made errors made at least one tritan error, with no subjects making exclusively red-green errors. Paired analysis was used to compare colour discrimination in 57 age-matched pairs of styrene-exposed workers and controls. The mean CCI for the 57 styrene-exposed workers was 1.22 (SD 0.23), significantly higher than in the controls (mean 1.12, SD 0.13). The styrene-exposed workers were also divided into high and low-exposure subgroups according to whether the urinary MA concentration was above or below 420 mg/l. In the low-exposure subgroup of 40 workers the mean urinary MA level was 200 mg/l (SD 110) and the mean CCI was not significantly different from control. However, for the high-exposure subgroup of 17 workers where the mean urinary MA level was 1060 mg/l (SD 930), the mean CCI was 1.33, significantly higher than in controls and low-exposure subgroup. Stepwise regression analysis confirmed a positive correlation between CCI and MA levels, but did not show a correlation with the duration of styrene exposure. It was noted that colour discrimination was not related to alcohol consumption.

The airborne concentrations of styrene in this study related to area sampling, and so are of little use for the determination of thresholds or exposure-response relationships. The urinary MA levels were not corrected for creatinine, and given that workers were encouraged to drink water prior to urine sampling the consequent dilution effect on urinary MA levels means that any extrapolations to estimated airborne concentrations of styrene would be of uncertain reliability.

Overall, the results of this study are consistent with an exposure-response relationship for an effect of styrene on colour discrimination, and point to a threshold somewhere between the high and low exposure groups.

The same team studied the exposure-response relationship between styrene and impairment of colour discrimination in a second study using the same methods and exclusion criteria (Kishi *et al.*, 2001). Eighty-seven workers exposed to styrene in 7 FRP factories, with mean age 37.7 years (SD 13) and mean duration of exposure 6.2 years (SD 6.2), were tested. They were arranged into low-, medium- and high-exposure sub-groups on the basis of whether end-of-shift urinary MA concentrations were below 100 mg/l, between 100 and 200 mg/l, or above 200 mg/l, respectively. For each subgroup of exposed workers colour discrimination was compared with a group of age-matched controls drawn from 117 subjects from the same and other factories but not exposed to industrial solvents.

For the 21 members of the low-exposure subgroup the mean urinary MA concentration was 50 mg/l (SD 30) and the mean CCI was 1.21 (SD 0.26), not significantly different from controls. The 24 medium-exposure workers and 42 high-exposure workers had mean urinary MA concentrations of 140 mg/l (SD 30) and 650 mg/l (SD 700), respectively; for these workers mean CCI values were 1.23 (SD 0.20) and 1.27 (SD 0.27), respectively, both significantly greater than their corresponding control group values.

Overall, these results are consistent with the existence of an exposure-response relationship for an effect of styrene on colour discrimination. However, because the urinary MA levels were not

corrected for creatinine, it is not possible to reliably estimate exposure in terms of airborne styrene concentrations for the low, medium and high exposure groups.

A recent study by the same team investigated colour discrimination in a group of 57 styrene-exposed workers at a FRP boat plant (Gong *et al.*, 2002). The mean age of the exposed workers was 29 years (SD 4), and they had been working with styrene for a mean time of 6.4 years (SD 2.1). Colour discrimination testing was also carried out in a group of 69 non-exposed controls. The mean age of the controls was 38 years (SD 11). Exclusion criteria included congenital colour vision deficiency, the presence of medical conditions that may affect colour discrimination, alcohol consumption greater that 250 g/week, and occupational exposure to styrene for less than 6 months. Styrene exposure was assessed by personal sampling and by measuring urinary concentrations of MA and PGA; in this study these were corrected for the concentration of creatinine in the urine. Both contemporary and historical urinary measurements were available. A cumulative exposure index was calculated for each worker based on frequency and duration of exposure and the results of the monitoring of urinary MA concentrations. Colour discrimination was measured by the D-15d test, conducted immediately before the start of a work shift under standard lighting conditions.

The mean urinary MA concentration at the time of the study was 260 mg/g creatinine (SD 350); this was less than a third of the mean value recorded for 1993. The mean 8-hour TWA styrene concentration at the start of the working week was 50 ppm (217 mg/m^3) (SD 36). Workers were also exposed to acetone, with a mean 8-hour TWA concentration of 49 ppm (SD 25). The mean airborne concentrations of other solvents monitored were low, all less than 0.1 ppm.

CCI was compared in 43 pairs of age-matched subjects, and was significantly higher in the exposed workers than in the unexposed controls; the median values for these two groups were 1.13 and 1.04, respectively. Twenty-three control subjects made no errors (CCI=1.00), compared with 11 of the exposed workers. As in the previous studies, styrene-exposed workers were divided into subgroups. The criterion for grouping was whether the sum of contemporary urinary MA+PGA concentrations was above or below 240 mg/g creatinine. Workers were matched for age, providing 29 high-exposure workers, 29 low-exposure workers, and 29 control subjects for comparison. As found in the previous studies, CCI related to contemporary styrene exposure. For the high- and low-exposure workers, mean CCI values were 1.14 (SD 0.24) and 1.09 (SD 0.13), respectively, both statistically significantly higher than the control CCI of 1.02 (SD 0.04). However, no relationship between cumulative styrene exposure and CCI was found. The relationship between individual CCI values and individual maximum styrene exposure recorded during the previous eight years was investigated. There was a positive correlation between maximum MA concentration and CCI.

From the urinary MA+PGA cut-off value of 240 mg/g creatinine, it can be estimated that the low and high exposure groups in this study correspond to mean 8-hour TWA exposures of <12 and >12 ppm (52 mg/m^3) respectively. However, it is worth noting that this estimate of exposure is based on urinary metabolites measured at the end of a work shift, whereas visual testing was conducted pre-shift generally on a Monday. The strength of evidence for an exposure-response relationship might have been increased if visual testing had also been conducted post-shift on the same day as the urine sampling. It also needs to be noted that the results of this study imply a relationship between contemporary levels of urinary styrene metabolites and an effect on colour discrimination, but no relationship with long-term cumulative exposure was found.

Overall, all three Japanese studies consistently point to styrene having a threshold effect on colour discrimination. The Gong study, which had the most rigorous exposure assessment, indicates that

the threshold exposure level is about 12 ppm (52 mg/m^3) (8-hour TWA). In relation to severity of effect, all 3 studies conducted the colour discrimination tests pre-shift on a Monday morning, specifically to exclude an acute effect of styrene. This raises the possibility that effects would have been more severe if the tests had been conducted post-shift.

In a very recent study by Gong *et al.* (2006), the relation between colour vision loss and the exposure of styrene was investigated. Colour vision was examined by the Lanthony desaturated panel D-15 test for 76 subjects exposed to styrene in a fibreglass reinforced plastics boat plant and 102 non-exposed subjects. The exposure level was expressed by the concentration of atmospheric styrene and end-shift urinary mandelic acid (MA) and phenylglyoxylic acid (PGA) levels. The individual cumulative exposure index (CEI) was calculated based on the exposure frequency and urinary MA concentrations measure for the past eight years.

The CCI of the exposed group showed a significant difference from the age matched controls. Only a slight significant relation was found between CCI and the concentration of urinary MA plus PGA.

The exposed group was further divided into two subgroups by the median of urinary MA plus PGA of each subject. The dividing line between the subgroups was 0.24 g/g creatinine, which was equivalent to an atmospherid concentration of styrene of about 10 ppm (43 mg/m^3). The CCI values of both the subgroups were significantly higher than that of the control group.

The relation between CCI value and the maximum exposure concentration in the past eight years was examined. It was found that the CCI value of the group with the maximum exposure concentration of styrene over 50 ppm (217 mg/m^3) were significantly higher than that of the other groups.

Overall, this very recent study shows that exposure to styrene would impair colour vision even if the exposure concentration was lower than 10 ppm (43 mg/m^3). Furthermore, if the maximum concentration of styrene exposure transiently exceeded 50 ppm (217 mg/m^3) in the past, the styrene related damage might remain.

In a series of three reports, colour discrimination was investigated in styrene-exposed workers at 3 Canadian reinforced plastics plants.

The initial investigation was based on 81 workers from the 3 plants following exclusions due to self-reported congenital colour vision deficiency, other ocular disorders and injuries, poor visual acuity, or less than 6 months solvent exposure (Campagna *et al.*, 1995; Campagna *et al.*, 1992). The mean age of the workers was 29 years; mean duration of employment at the plants was 5 years and the mean alcohol consumption was 160 g/week. Styrene exposure was assessed by personal sampling over 4 hours of an 8-hour shift and by measuring end-of-shift urinary MA concentration. Visual testing was conducted on Saturday morning, 12 hours after the last exposure. Colour discrimination was assessed using the D-15d test, conducted under standardised lighting conditions. Subjects were classed as having colour vision deficiency if the coloured caps were misplaced by at least two positions, even if this occurred for only one eye.

Mean airborne styrene concentration was 48 ppm (208 mg/m^3) (SD 60) and mean urinary MA concentration was 480 mg/g creatinine (SD 690). However, about 1/3 of the workers wore respiratory protective equipment (RPE). 35 workers reported acute eye irritation associated with

styrene exposure, and some also reported tearing and blurred vision. The incidence of these symptoms was found to correlate positively with urinary MA concentration; it is unclear whether or not this adversely affected performance in the colour discrimination test. On the basis of the D-15d test, 25 workers (31%) were classified as having colour vision deficiency. Most of these workers made errors of the tritan type, although one made only errors of the red-green type and two made both types of error. Six other workers made errors with no particular pattern. When workers were dichotomised into those with or without colour vision deficiency, statistical analysis showed no significant relationship with urinary MA. The mean uncorrected CCI score for all workers (based on the mean of scores from left and right eye separately) was 1.14 (SD 0.16). Multiple regression analysis was used to investigate the relationships between CCI and age, length of service in the factory, alcohol consumption, respirator use and urinary MA. When CCI was corrected for age, alcohol consumption and length of service, it was found to have a positive correlation with urinary MA concentration but not with airborne styrene levels, presumably reflecting the use of RPE in some workers.

One statistical analysis suggested a positive relationship between CCI and urinary MA, but this was not confirmed by the analysis of those with and without colour vision deficiency. From the mean urinary MA concentration of the workers in this study and using the ACGIH conversion, it is estimated that the mean amount of styrene inhaled by workers is equivalent to an airborne concentration of 30 ppm (130 mg/m^3) (8-hour TWA). Although there was no control group, it is noted that the mean CCI score from all the exposed workers (1.14) is between the 50th and 90th percentile of normal reference value), given the mean worker age (29 years). The reports of lacrimation and blurred vision also raise doubts over the interpretation of this study.

Overall, there was no consistent evidence for a clear-cut effect of styrene exposure on colour discrimination, and it does not seem possible to draw any firm conclusions from this study.

Follow-up testing on 57 workers from the original study (T_0) was conducted two years later (T_2) (Mergler *et al.*, 1996). The mean age of these workers was 32 years (SD 9) and the mean duration of employment at the plant was 6 years (SD 6). Styrene exposure and colour discrimination were assessed as in the previous study, and this report also included the results from a neurobehavioural test battery.

Styrene exposure during the 2-year period, measured as airborne concentration or urinary MA concentration, had slightly decreased in Plant 3, but had broadly stayed the same or showed some slight increases in Plants 1 and 2. It appeared that the reductions in MA between T_0 and T_2 in Plant 3 were largely due to wearing RPE rather than to a reduction in airborne styrene levels. Exposure data for each plant were only presented graphically. The graphs revealed a lognormal distribution of exposure data and that 50% of exposures in each plant were below 50 ppm (217 mg/m^3).

Mean CCI for all 57 workers at T_2 was 1.19 (SD 0.35), similar to the mean CCI (1.18) for these workers at T_0. When the results from each plant were considered separately it was found that in Plant 3 the mean CCI had decreased by 0.20 (SD 0.78). In Plants 1 and 2 where styrene exposure had not decreased, the mean CCI had increased by 0.12 (SD 1.17) although these changes were not statistically significant. When colour discrimination test results for all 57 workers were grouped according to whether urinary MA had increased, decreased or remained unchanged, there was found to be a statistically significant correlation between urinary MA concentration and increases in CCI over the 2 years. The results of the neurobehavioural test battery showed no decline in any of the 18 test parameters between T_0 and T_2, and a statistically significant improvement in 3 parameters in workers from Plant 3, and an improvement in one parameter in workers from plants 1 and 2.

Overall, the results of the first follow-up showed no decline in colour discrimination in workers where average exposures to styrene had not changed markedly during the two-year interval. There was no information on subjective symptoms of eye irritation in this follow-up study, and the results of the neurobehavioural tests were unremarkable. However, analysis of changes in levels of urinary MA and CCI scores at the level of the individual worker suggests a correlation between increases in the body burden of styrene and deterioration in colour discrimination over the 2 years. It is not possible from the way the results are presented in this study to identify a threshold for an effect of styrene on colour discrimination or to quantify exposure-response relationships. However, qualitatively the results are suggestive of a relationship between exposure to styrene and colour discrimination.

After 9 years, colour discrimination was retested in 18 of the original workers still working at Plant 3 (Castillo *et al.*, 2001). The 18 retested workers had a mean age of 38 years and mean duration of employment of 13 years. The authors were unable to enter the plant to sample airborne styrene concentrations or access company records, but end-of-shift urinary MA measurements were made at the end of the working week as in the previous studies. In addition, personal exposure measurements were available for the period 1987 to 1998, and these were combined with individual work histories to produce a cumulative exposure index for each worker.

Urinary MA levels were lower than at year 0 and year 2, and were all below 340 mg/g creatinine. Among the retested workers age-corrected CCI had improved significantly between year 0 and year 2, during which time their styrene exposures had also fallen. However, there was no further significant change in CCI between year 2 and year 9, during which time styrene exposure had continued to fall.

Overall, the follow-up investigation at 2 years showed no changes in the mean CCI scores over this time period. In some workers within the study population there was a correlation between increases in urinary MA concentration and a decline in CCI scores. Among the small subgroup of workers re-evaluated at 9 years, no changes in mean CCI were detected, although styrene exposure had decreased. These data are suggestive of an irreversible effect of styrene on colour discrimination, but it is difficult to ascertain the magnitude of the deficiency.

Chia *et al.* (1994) investigated colour discrimination in 21 male laminator workers exposed to styrene at a concentration below 30 ppm (130 mg/m^3) and 21 carpenters from a boat-building plant in Singapore. The two groups were matched for age, smoking habits and alcohol consumption. Styrene exposure was also assessed by measurement of end-of-shift urinary MA and PGA levels. On Monday morning, after an exposure-free weekend, colour discrimination was assessed using the D-15d test under standard lighting conditions. The colour discrimination results were expressed as the geometric mean of the total colour difference score (TCDS).

Mean urinary MA and PGA concentrations for the exposed groups were 84 and 66 mg/g creatinine, respectively. Colour discrimination errors were made by the exposed workers along both the red-green and tritan axes. The exposed group ahd significantly poorer colour discrimination in the D-15 test score than the control group; the mean TCDS was 164 (SE 0.04) for the exposed group and 132 (SE 0.04) for the control group.

Overall, low exposure (30 ppm) to styrene may impair colour vision.

4.7.1.6 Other relevant information

An overwhelming number of scientific papers have addressed the neurotoxicity of styrene. The neurotoxic effect of styrene is well documented in both humans and experimental animals. Besides effect on hearing, vestibular function and colour discrimination, other studies in both animal and humans have shown effects on the peripheral nerve conducting velocities (EU-RAR 2006, Yamamoto *et al.* 1997).

The acute toxicity of styrene is mainly dominated by a CNS depressive effect or narcotic effect affecting – among others – reaction time as shown by Gamberale *et al*. (1976). The EEG might also be affected and some more permanent effects have been reported. Also effects on permanent changes of neurotransmitter concentrations have been shown.

Styrene is both vistibulotoxic and may impare the balance (Toppila *et al.,* 2006).

ACGIH as well as several other Occupational TLV-authorities have reduced the TLV of styrene to 20 ppm because loss of colour discrimination is considered as a serious effect.

Toluene is classified Xn;R48/20 based on functional impairment of hearing in experimental animals, progressing to permanent hearing loss, accompanied by loss of hair cells in the outer cochlea, observed at dose levels relevant for classification.

4.7.1.7 Summary and discussion of repeated dose toxicity

4.7.1.8 Summary and discussion of repeated dose toxicity findings relevant for classification according to DSD

Dose response estimation including weight of evidence consideration

Ototoxicity

A substantial number of scientific papers have addressed the ototoxicity of styrene observed in animal studies and epidemiological studies.

Clear evidence of ototoxicity (both functional and histological) has been seen in sedentary/ordinary rats repeatedly exposed to styrene by inhalation at concentrations from 600 ppm (2598 mg/m^3). In three different studies, no such effects were seen at 200 ppm (866 mg/m^3) for 13 weeks, or at 300 ppm (1299 mg/m^3) or 500 ppm (2165 mg/m^3) for four weeks. One study in active rats exposed to styrene for 4 weeks showed that styrene-induced ototoxicity tend to occur at lower exposure concentrations (400 ppm - 1732 mg/m^3) than those at which ototoxicity is observed in sedentary/ordinary rats. This is considered to be due to the increased styrene uptake, which is the consequence of the increased ventilation rate and, in turn, of the increased physical activity.

The histological damage consists of the destruction of the outer hair cells (OHC; especially of row 3) of the cochlea. These changes are accompanied by an elevation of the hearing thresholds in the mid-frequency range (10-20 kHz). The destruction of the hair cells is irreversible and occurs at slightly lower exposure concentrations than those producing the audiometric hearing threshold shifts. Mechanistic investigations indicate that styrene reaches the sensory hair cells of the cochlea via the blood stream and that styrene itself and/or its metabolites cause a serious disturbance of the

membranous organisation of these target cells. However, the underlying toxicological mechanism has not been clearly elucidated.

From the studies in animals it can be concluded that a NOAEC for hearing loss in the rat is between 300 and 600 ppm (1300-2600 mg/m^3). A marked loss in the number of OHC has been shown at 600 ppm (2600 mg/m^3). An interaction between styrene exposure and noise, or ethanol seems to be at least additive.

The available human data indicate a relationship between styrene exposure and hearing loss as well as effects on vestibular reflexes in some workers.

One study has concluded that exposure to styrene, at concentrations below 20 ppm (87 mg/m^3), produced high-frequency hearing losses. However, considering the information on styrene exposure of the subjects in this study and the lack of dose-response relationships, this study seems to underestimate the risk of hearing loss, due to the fact that some of the participant were exposed to very low level of styrene.

Another study has shown evidence of styrene-induced hearing loss and an additive or synergistic effect with other ototoxic agents such as toluene or noise.

Therefore, these human data indicate that the observations of ototoxicity in animals are relevant to humans. In addition, the human studies indicate that the sensitivity for developing hearing loss might be substantial greater in man than in the rat. A similar relationship is also seen for toluene, already classified Xn;R48/20.

Effects on colour vision

Twelve cross-sectional or longitudinal studies investigating colour discrimination in reinforced plastics workers, boat builders and other workers exposed to styrene have been identified. These studies provide evidence that styrene causes changes in colour discrimination relative to age-matched controls. Generally, the effect was on the tritan (blue-yellow) type, although some workers also had evidence of red-green colour vision deficiency. The most recent study (Gong *et al.*, 2006) shows that exposure to styrene would impair colour vision even if the exposure concentration was lower than 10 ppm (43 mg/m^3). Furthermore, if the maximum concentration of styrene exposure transiently exceeded 50 ppm (217 mg/m^3) in the past, the styrene related damage might remain. Also the data from the Canadian studies are suggestive of an irreversible effect of styrene on colour discrimination. Similarly, the results from the Italian studies also suggest that the effects of styrene on colour discrimination persist even after an exposure free period.

The ocular effects of styrene in experimental animals have not been studied in depth, but there is one study, which has shown effects on the number of the large amacrine cells as well as on the content of neuramines and glutathione of the retina of rats exposed repeatedly to 300 ppm (1299 mg/m^3) styrene for 12 weeks. The number of large amacrine cells was reduced by around 30% in styrene-exposed rats when compared to controls. The density of small amacrine cells was unaffected. Dopamine and DOPAC content were lower than controls (22% and 17% respectively), as were glutathione levels (28%). This finding in rats supports the ocular effects reported in humans.

Neurotoxicity

The neurotoxicity of styrene is well documented in both humans and experimental animals. Besides effect on hearing, vestibular function and colour discrimination, other studies in both animals and humans have shown a number of different effects in the nervous system.

4.7.1.9 Comparison with criteria of repeated dose toxicity findings relevant for classification according to DSD

Xn;R48/20 Harmful: danger of serious damage to health by prolonged exposure through inhalation.

Effects

Styrene causes a number of different neurotoxic effects, which are relevant for assigning the R-phrase R48 to styrene as these effects are not covered by other R-phrases. R48 is justified because styrene causes several types of serious damage to health by prolonged exposure by inhalation and is even more potent than toluene, which is similar to styrene in chemical structure, physico-chemical properties as well as toxicological properties.

Ototoxicity:

Styrene-induced chronic impairment of auditory function has been demonstrated in a number of animal studies and several human studies. This has been substantiated by morphological evidence of hair cell loss in the rat cochlea as well as by functional investigations in humans. The available data suggest that humans are sensitive to this effect and that styrene is more potent than toluene, which already has been classified Xn;R48/20.

Effects on colour vision:

Several human studies show that low-level exposure to styrene (< 50 ppm / 217 mg/l) may impair colour vision. Some of the human studies may have underestimated the risk because some individuals were exposed to very low levels of styrene (< 8 ppm / 35 mg/l). Some studies argue that the effect is reversible, but scientifically this has not been documented. ACGIH as well as several other Occupational TLV-authorities have reduced the TLV of styrene to 20 ppm (87 mg/l) because loss of colour discrimination is considered as a serious effect.

Neurotoxicity:

Several different kinds of investigations of e.g. EEG, peripheral nerve conduction velocity, and ototoxicity have been performed in both experimental animals and in humans. Being a neurotoxin might imply that styrene induces vestibulotoxicity and several studies in humans and experimental animals have confirmed this. In addition, styrene causes irreversible changes in the central nervous system of animals as documented in a substantial number of papers reviewed in the EU-RAR.

Criteria for classification

According to the criteria for classification and labelling section 3.2.4. 'Comments regarding the use of R48' *"…serious damage to health is to be considered to include death, clear functional disturbance or morphological changes which are toxicologically significant. It is particularly important when these changes are irreversible."* Hearing loss and colour vision discrimination are to be considered as serious damage to health.

Furthermore, *"It is also important to consider not only specific severe changes in a single organ or biological system but also generalised changes of a less severe nature involving several organs, or severe changes in general health status."* Thus, for a substance such as styrene, which affects a number of endpoints, the criteria emphasise the importance to consider the whole scale of effects, not only individual studies.

According to section 3.2.4.1 (b) (i) *"major functional changes in the central or peripheral nervous systems, including sight, hearing and the sense of smell, assessed by clinical observations or other appropriate methods (e.g. electrophysiology)"*. The four effect types observed following exposure to styrene – as addressed above – are all considered as being "serious" according to the classification criteria and thus, R48 is warranted.

According to the classification criteria, exposure cut-off guide values are stated when the basis for R48 is a 90-day or 28-day rat study. Substances are classified at least as harmful by inhalation when these effects are observed at levels of the order of: rat ≤ 0.25 mg/l, 6 hours/day. This guide value can apply directly when severe lesions have been observed in a subchronic (90 days) toxicity test. When interpreting the results of a subacute (28 days) toxicity test, this figure should be increased approximately threefold. However, when a two-year study is available *"…it should be evaluated on a case-by-case basis"*. Furthermore, *"If results of studies of more than one duration are available, then those from the study of the longest duration should normally be used."* The classification criteria do not give any exact guidance when studies have been performed with other durations or other species than the rat. For styrene, for which a large number of studies with varying exposure durations are available, and for which the studies have focussed on different endpoints, the classification criteria indicate a duty to apply an individual approach.

In section 3.2.4.1 it is stated *"When considering data from practical experience special attention should be given to exposure levels."* No further guidance is given.

In the general introduction to Annex VI, section 1.1 it is stressed that *"… all the toxicological … properties of substances … which may constitute a risk during normal handling and use …"* should be identified.

In section 3.2.3.1, the possibility to classify very volatile substances as "harmful" on a case-by-case basis *"… when there is appropriate evidence that such substances may present a risk in normal handling and use… "*, even when the specified criteria for health effects are not fulfilled, is described.

Exposure levels

Ototoxicity:

Data from epidemiological studies suggest that humans are sensitive to this effect; however, these studies do not allow a determination of a LOAEC/NOAEC. In the rat, exposure levels above 500 ppm (2165 mg/m^3) have caused impaired hearing function. In humans, several epidemiological studies support the findings in the animal studies. However the studies suggest that humans are much more sensitive than the rat.

Effects on colour vision:

The most recent study (Gong *et al.*, 2006) shows that exposure to styrene would impair colour vision even if the exposure concentration was lower than 10 ppm (43 mg/m^3). Furthermore, if the maximum concentration of styrene exposure transiently exceeded 50 ppm (217 mg/m^3) in the past, the styrene related damage might remain.

Neurotoxicity:

The data on exposure are sufficient to decide whether effects could occur at exposure levels, which may be encountered in the working environment as several symptoms or signs of neurotoxicity may occur at exposure levels similar to the present TLV. See the EU-RAR.

Thus, the critical effects observed following repeated exposure cannot be excluded to occur under normal handling and use and should be taken into account when considering R48 for styrene.

4.7.1.10 Conclusions on classification and labelling of repeated dose toxicity findings relevant for classification according to DSD

Styrene produces a number of serious health effects after prolonged exposure by inhalation in experimental animals and in humans. The exposure levels inducing neurotoxicity in humans are in the same order of magnitude as the exposure levels inducing neurotoxicity in animals; however, for ototoxicity and colour vision discrimination the exposure levels inducing these effects in humans seem to be a factor of 10 lower than in animals. Based on the available data, a classification as Xn; R48/20 is warranted for styrene."

4.8 Specific target organ toxicity (CLP Regulation) – repeated exposure (STOT RE)

4.8.1 Summary and discussion of repeated dose toxicity findings relevant for classification as STOT RE according to CLP Regulation

See the summary and discussion of relevant findings above in 4.7.1.8

4.8.2 Comparison with criteria of repeated dose toxicity findings relevant for classification as STOT RE

STOT RE 1 H372 Causes damage to the nervous system through prolonged or repeated exposure by inhalation.

Effects

Styrene causes a number of different neurotoxic effects, which are relevant for assigning STOT RE to styrene. STOT RE is justified because styrene causes significant health effects that can impair function by prolonged exposure by inhalation and these effects are not covered by other hazard classes. Styrene is even more potent than toluene, which is similar to styrene in chemical structure, physico-chemical properties as well as toxicological properties and already has been classified STOT RE.

Ototoxicity:

Styrene-induced chronic impairment of auditory function has been demonstrated in a number of animal studies and several human studies. This has been substantiated by morphological evidence of hair cell loss in the rat cochlea as well as by functional investigations in humans. The available data suggest that humans are sensitive to this effect and that styrene is more potent than toluene.

Effects on colour vision:

Several human studies show that low-level exposure to styrene (< 50 ppm) may impair colour vision. Some of the human studies may have underestimated the risk because some individuals were exposed to very low levels of styrene (< 8 ppm). Some studies argue that the effect is reversible, but scientifically this has not been documented. ACGIH as well as several other Occupational TLV-authorities have reduced the TLV of styrene to 20 ppm because loss of colour discrimination is considered as a serious effect.

Neurotoxicity:

Several different kinds of investigations of e.g., EEG, peripheral nerve conduction velocity, and ototoxicity have been performed in both experimental animals and in humans. Being a neurotoxin might imply that styrene induces vestibulotoxicity and several studies in humans and experimental animals have confirmed this. In addition, styrene causes irreversible changes in the central nervous system of animals as documented in a substantial number of papers reviewed in the EU-RAR.

Criteria for classification

According to the classification criteria for substances in Annex 1: 3.9.2.1, Category 1 is assigned to *"Substances that have produced significant toxicity in humans or that, on the basis of evidence from studies in animals, can be presumed to have the potential to produce significant toxicity in humans following repeated exposure"* and *"Substances are classified in Category 1 … 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"*. 'Significant' is defined as *"… changes which clearly indicate functional disturbance or morphological changes which are toxicologically relevant*" and 'severe' is defined as effects that *"… are generally more profound or serious than 'significant' effects and are of a considerably adverse nature which significantly impact on health"*.
Category 1 is justified for styrene as hearing loss, demonstrated in a number of animal studies and several human studies, and colour vision discrimination, demonstrated in several human studies, are to be considered as severe toxic effects being of a considerable adverse nature, which significantly impact on health.

According to Annex 1: 3.9.2.10.2, *"When well-substantiated human data are available showing a specific target organ toxic effect that can be reliably attributed to repeated or prolonged exposure to a substance, the substance shall normally be classified. Positive human data, regardless of probable dose, predominates over animal data. Thus, if a substance is unclassified because no*

specific target organ toxicity was seen at or below the dose/concentration guidance value for animal testing, if subsequent human incident data become available showing a specific target organ toxic effect, the substance shall be classified."
STOT RE 1 is justified for styrene as hearing loss and colour vision discrimination have been demonstrated in relatively new human studies, which predominate over the animal data.

According to Annex 1: 3.9.2.7.3, *"… 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:"* and in (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)."*

Ototoxicity: Styrene-induced chronic impairment of auditory function has been demonstrated in a number of animal studies and several human studies.
Effects on colour vision: Effects on colour vision discrimination has been demonstrated in several human studies.
Neutotoxicity: Several different kinds of investigations have revealed neurotoxic effects of styrene in both experimental animals and in humans. Styrene causes irreversible changes in the central nervous system of animals as documented in a substantial number of papers reviewed in the EU-RAR.

The types of effect observed following exposure to styrene are all considered as being "significant" according to the classification criteria and thus, STOT RE 1 is warranted.

According to Annex 1: 3.9.1.4, *"Assessment shall take into consideration not only significant changes in a single organ or biological system but also generalised changes of a less severe nature involving several organs."*
Thus, the criteria emphasise the importance to consider the whole scale of effects for styrene, not only individual studies.

4.8.3 Conclusions on classification and labelling of repeated dose toxicity findings relevant for classification as STOT RE

Styrene produces a number of serious health effects after prolonged exposure by inhalation in experimental animals and in humans. The exposure levels inducing neurotoxicity in humans are in the same order of magnitude as the exposure levels inducing neurotoxicity in animals; however, for ototoxicity and colour vision discrimination the exposure levels inducing these effects in humans seem to be a factor of 10 lower than in animals. Based on the available human data and support from animal data, a classification as STOT RE 1 is warranted for styrene.

Although there are some indications of neurotoxic effects in the rat following repeated oral dosing of styrene, a classification is not justified for this exposure route. No repeated dermal toxicity studies are available; however, systemic toxicity following dermal contact with styrene is not expected. Therefore, a classification as STOT RE 1, with the hazard statement H372 "Causes damage to the nervous system through prolonged or repeated exposure via inhalation" is relevant.

RAC evaluation of specific target organ toxicity (CLP) – repeated exposure (STOT RE) and repeated dose toxicity (DSD)

Summary of the Dossier submitter's proposal

The present proposal concerns one of the endpoints previously agreed at TC C&L i.e., repeated dose toxicity (CLP STOT RE 1; H372 "Causes damage to the nervous system through prolonged or repeated exposure by inhalation" and DSD Xn; R48/20 "Harmful: danger of serious damage to health by prolonged exposure through inhalation").

Styrene is a transitional substance and was discussed in the TC C&L group prior to the transfer of responsibility for classification and labeling to ECHA. At a TC C&L meeting in September 2007, it was agreed to classify with R48/20 (equivalent to STOT RE 1 under CLP) for repeated dose effects.

The key supporting studies are summarised below (copied from the Danish proposal).

Ototoxicity: Styrene-induced chronic impairment of auditory function has been demonstrated in a number of animal studies and several human studies. This has been substantiated by morphological evidence of hair cell loss in the rat cochlea as well as by functional investigations in humans. The available data suggest that humans are sensitive to this effect and that styrene is more potent than toluene.

Effects on colour vision: Several human studies show that low-level exposure to styrene (< 50 ppm) may impair colour vision. Some of the human studies may have underestimated the risk because some individuals were exposed to very low levels of styrene (< 8 ppm). Some studies argue that the effect is reversible, but scientifically this has not been documented. ACGIH[i] as well as several other Occupational TLV[ii]-authorities have reduced the TLV of styrene to 20 ppm because loss of colour discrimination was considered to be a serious effect.

Neurotoxicity: Several different neurotoxicological investigations (including e.g., EEG, peripheral nerve conduction velocity, and ototoxicity) have been performed in both experimental animals and in humans. Styrene causes irreversible changes in the central nervous system of animals as documented in a substantial number of papers reviewed in the EU-RAR.

The proposed classification is STOT RE 1, with the hazard statement H372 "Causes damage to the nervous system through prolonged or repeated exposure via inhalation".

Comments received during public consultation

Industry stakeholders submitted several published studies that had not been included in the CLH dossier. These studies added useful information, but were not considered to affect the interpretation of findings described in the overall database. A general comment expressed by industry was that although the proposal draws on the studies already evaluated in the EU RAR, the dossier submitter has omitted relevant qualifying comments and negative criticism of studies important for their proposal. RAC has

[i] American Conference of Governmental Industrial Hygienists

[ii] Threshold Limit Value

therefore also considered the detailed industry comments and consulted the EU RAR.

All comments received on repeated dose toxicity were in support of classification with STOT RE 1 based on the evidence of ototoxicity. However, industry did not agree that effects on colour vision supported this classification, because (for example) two recent studies have not found any effects on colour vision in exposed workers (Seeber *et al*. 2009, Vyskocil *et al*. 2012). Industry is of the view that even if changes in colour discrimination were caused by styrene, these changes were reversible and so slight that they cannot be considered adverse health outcomes of styrene exposure.

Assessment and comparison with the classification criteria

Key data and arguments that are relevant to the proposal
Clear evidence of ototoxicity has been seen in 10 repeated dose toxicity studies at concentrations of 600 ppm (2598 mg/m^3) and above, where rats were exposed to styrene by inhalation. The evidence includes findings of hearing loss in the mid-frequency range (10-20 kHz) and histopathological evidence of destruction of the outer hair cells of the cochlea.

One study compared the effects in active and resting rats, and found that styrene-induced ototoxicity tends to occur at lower exposure concentrations in active than in resting rats (presumably because of higher systemic exposure at the higher ventilation rate). Styrene-induced ototoxicity has also been studied in the absence or presence of noise, and the findings indicate that simultaneous noise increases the ototoxicity of styrene.

The effect of styrene on hearing has also been observed in studies conducted in a number of occupational settings. The occupational co-exposure to other agents affecting hearing (noise and solvents) decreases the power of the epidemiological studies. However, the three largest studies on occupational exposure to styrene indicated effects on hearing at concentrations occurring in these occupational settings, i.e., below 50 ppm. Thus, there is some evidence to suggest that humans are more sensitive to the ototoxicity of styrene than rats, and that noise and other ototoxic solvents may potentiate the ototoxicity of styrene. In conclusion, the RAC finds ototoxicity to be well documented in rats, and that humans also are likely to be sensitive to this adverse effect of styrene.

The dossier also refers to effects on colour vision in humans as a basis for the classification proposal. There are a number of studies in occupational settings on the effects of styrene on colour vision. It is quite clear that styrene has an effect on colour vision, decreasing the ability to discriminate colour in humans. Studies submitted during the public consultation included a meta-analysis study (Paramei *et al*. 2004) which, based on the studies available at that time, concluded that there were effects on colour vision, as well as two more recent studies which did not show such effects (Seeber *et al*. 2009, Vyskocil *et al*. 2012; the latter published and submitted after the public consultation). Some studies indicate the effect to be reversible, whereas others indicate the irreversibility of this effect. Although the effect is clear, it is difficult to judge the extent to which it can be considered adverse.

The proposal also refers to neurotoxicity as a basis for the STOT RE 1 classification, but as no specific neurotoxicity studies are mentioned in the documents, RAC did not comment on neurotoxicity in relation to this proposal. However, RAC notes that the European Commission has acknowledged that styrene exposure above 50 ppm over a period of 5-10 years may induce chronic encephalopathy in occupationally exposed humans (Information notices on occupational diseases: a guide to diagnosis; European Commission 2009), indicating that classification based on neurotoxicity may be warranted. Due to lack of such data in the CLH dossier, the RAC could not address

neurotoxicity in the current opinion.

Comparison with criteria and RAC conclusions
The CLP criteria state that a substance should be placed in STOT RE if it causes significant toxicity in humans or if, on the basis of evidence from studies in experimental animals, it can be presumed to have the potential to produce significant toxicity in humans following repeated exposure. Examples of significant toxicity relevant to this opinion mentioned in the CLP include "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)".

The RAC is of the view that appropriate studies in experimental animals in combination with reliable epidemiological studies provide evidence for adverse effects on hearing (ototoxicity), and that this is a sufficient basis for classification with STOT RE. Regarding the category, RAC finds that the evidence for ototoxicity occurring in humans at concentrations below 50 ppm warrant classification in category 1, even though the concentrations needed to cause ototoxicity in rats would suggest a lower category. In this case the human data are considered to be more relevant when deciding on the category, since reliable human toxicity data normally lead to classification in category 1 (there are no guidance values for human data).

The effects of styrene on colour vision in humans can be viewed as supportive of the STOT classification, but the degree to which this effect can be considered to be adverse is difficult to establish based on the proposal. Therefore, RAC does not find that this effect, as described in the CLH proposal, as such is a sufficient basis for classification.

The proposed classification is STOT RE 1, which is supported by RAC. However, RAC considers that the hazard statement should be rephrased as follows, since the affected organs have been clearly identified, and could be affected via different routes of exposure (at least after oral and inhalation exposure): H372 "*Causes damage to the hearing organs through prolonged or repeated exposure*" (corresponding to Xn; R48/20 according to the DSD).

4.9 Germ cell mutagenicity (Mutagenicity)

Taken from the EU RAR (2008) 4.1.2.7.4.

"Summary of genotoxicity
A large number of studies have been published which have aimed to investigate the genotoxic potential of styrene in humans by examination of various endpoints in styrene exposed workers. Very low levels of DNA adducts were found in some styrene exposed workers but it has been stated that such low levels should be viewed with caution. There is also some evidence of DNA damage (SSBs) induced in styrene exposed workers. Both these endpoints are indicative of exposure but are not necessarily associated with heritable effects. The results of several studies on another indicator endpoint of unclear health significance, SCEs, did not provide evidence of a positive response, despite these being induced in animals exposed to styrene. There are also many studies investigating endpoints (gene mutations, chromosome aberrations and micronuclei) known to lead to heritable effects. The number of studies assessing gene mutation is very limited and no conclusions can be drawn from them. Although 5 studies appear to present evidence that styrene may be weakly clastogenic in humans, there are 11 robust negative studies also. Together with a lack of evidence of a dose-response relationship and the negative response for induction of

micronuclei when studied concurrently in two of the positive chromosome aberration studies, no clear conclusion on *in vivo* clastogenicity of styrene in humans can be made.

Overall, given the lack of evidence of consistent relationships between exposure levels and study outcome, the lack of any consistent profile of endpoints and the absence of information on the relevance of the types of adducts seen and their mutagenic potential *in vivo*, there is no convincing evidence that styrene has shown mutagenic activity in humans. Hence, information from studies in experimental animals and other systems needs to be considered.

The overall picture presented by the *in vitro* assay results available is that at least in some text systems (including Ames tests *in vitro* chromosome aberration studies in mammalian cells), styrene does posses some genotoxic potential *in vitro*. Metabolic activation (presumably to styrene oxide) is required for this activity. Styrene has been exhaustively studied in clastogenicity studies in animals up to dose levels producing severe toxicity in some cases. There is no convincing evidence of styrene clastogenicity when the quality of the studies and the plausibility of the test results are considered. Equivocal results were obtained after exposure to high doses causing lethality. However, overall, negative results were obtained from *in vivo* chromosome aberration and micronucleus studies in the rat, hamster and the mouse following single or repeated exposures to styrene up to concentrations and/or doses causing systemic toxicity, via the inhalation, oral and intraperitoneal route in the tissues examined (bone marrow, peripheral lymphocytes, splenocytes and whole blood). Furthermore, a recently published micronucleus test in bone marrow cells of mice conforming to the current OECD guideline was clearly negative.

The general pattern of SCE results in the wide range of tissues examined (lymphocytes, spleenocytes, bone marrow, alveolar macrophages, regenerating liver cells) from both the rat and the mouse following inhalation or i.p exposure to styrene has been positive. However, it is important to note that, in most cases, concomitant chromosome aberration and/or micronucleus assays involving the same animals and in some cases the same tissues were carried out and that negative results were obtained for these indicators of chromosome damage. Therefore, this clearly reduces the significance of the SCE findings in relation to mutagenicity.

The binding of styrene metabolites to DNA was very low and did not indicate any specificity for the target tissue (mouse lung). Induction of alkali-labile single-strand breaks has also been produced *in vivo* in rats and mice exposed to styrene. Again the significance of these findings is unclear, given the repeated failure of styrene to demonstrate mutagenic activity in standard clastogenicity assays.

In summary, the available data suggest that styrene is weakly positive in indicator tests detecting SCEs, DNA stand breaks and DNA adducts. In contrast, an in vivo UDS test performed in accordance with international guidelines did not reveal a genotoxic effect of styrene in mouse liver.

Overall, based on standard regulatory tests, there is no convincing evidence that styrene possesses significant mutagenic/clastogenic potential *in vivo* from the available data in experimental animals."

4.10 Carcinogenicity

Taken from the EU RAR (2008), 4.1.2.8.3.

"**Summary of carcinogenicity**

In relation to human studies, several cohort and case-control studies covering workers exposed to styrene are available. In large, well-conducted studies, cancer mortality was investigated in the GRP industry with relatively high exposure to styrene and no significant exposures to other chemicals. In these studies, and in studies in styrene production workers, there was no clear and consistent evidence for a causal link between specific cancer mortality and exposure to styrene. The increased

risks for lymphatic and haematopoietic neoplasms observed in some of these studies are generally small, statistically unstable and often based on subgroup analyses. These findings are not very robust and the possibility that the observations are the results of chance, bias or confounding by other occupational exposures cannot be ruled out. In the styrene-butadiene rubber industry, several studies have pointed to an increased risk of cancer of the lymphatic and haematopoietic systems. However, detailed analysis of these data, together with the general toxicological picture for styrene and butadiene (see butadiene EU RAR), suggests that where increases are due to occupational exposure, it is butadiene, not styrene, that is the more likely causative agent. In conclusion, based on human studies, there is no clear and consistent evidence for a causal link between specific cancer mortality and exposure to styrene.

The carcinogenic potential of styrene has been explored in rats and mice, using the inhalation and oral routes of exposure. A carcinogenic effect of styrene towards the lung is evident in the mouse. This has been shown in a well-conducted lifetime inhalation study in CD1 mice at exposure concentrations of ≥ 20 ppm styrene and, somewhat less convincingly, in an oral study in mice of the $B6C3F_1$ strain. The inhalation study, which included extensive histopathological examination, showed that the tumours (prevalently adenomas) were preceded by cytotoxicity characterised by early Clara cell toxicity followed by progressive bronchiolar epithelial hyperplasia and bronchiolar-alveolar hyperplasia.

In the rat, styrene has not exhibited any clear evidence of carcinogenic potential by the inhalation or oral route. In individual studies there have been isolated findings of statistically significantly higher incidences of various particular tumour types in particular groups of styrene-treated animals, compared with the in-study controls. However, the findings have been within historical background ranges, not reproducible between studies, in some cases have not shown an upward trend with increasing dose, and have not been associated with evidence of underlying styrene-induced changes at the site in question.

On the question of the relevance of the mouse lung tumours for human health, consideration of the available toxicokinetic information and data from single and repeated inhalation exposure studies in experimental rodents suggests the following as the most plausible toxicological mechanism for the mouse lung tumours. Styrene is metabolised by cytochrome P450 enzymes in the metabolically active Clara cells (non-ciliated bronchiolar epithelial cells involved in the metabolism of xenobiotics, but also in the secretion of surfactants and in the renewal process of the bronchiolar epithelium) of the bronchiolar epithelium of the mouse, producing cytotoxic metabolites of styrene including styrene 7,8 oxide (SO) and oxidative metabolites of 4-vinylphenol (4-VP). These metabolites cause early Clara cell toxicity/death and sustained regenerative bronchiolar cell proliferation which, in turn, leads to compensatory bronchiolar epithelial hyperplasia and ultimately tumour formation. Clara cell toxicity could also be a consequence of the long term depletion of glutathione, because of conjugation with SO. Genotoxicity of SO (an EU-category 2 and IARC group 2A carcinogen) or other reactive styrene metabolites is unlikely to be involved in tumour development as minimal binding of styrene metabolites to DNA has been detected in mouse lung with no species- or tissue-specificity.

All of the key events of this postulated mode of action are less operative in the non-responsive rat (which does not develop lung tumours at exposure concentrations up to 1000 ppm) and even less operative in humans.

The number of Clara cells (being responsible for both the formation of toxic metabolites and the target for their toxic action) is very low in humans, even less than in rats. While Clara cells comprise about 85% of bronchiolar epithelium in mice and 25% in rats, in humans such cells are rare.

Although the enzymes CYP2E1 and CYP2F2 required for the formation of the Clara cell toxicants such as SO (including the highly pneumotoxic R-enantiomer) and the downstream metabolites of 4-VP have been detected in human lung, their activities are low (at least 400 times lower than in the mouse) and metabolic activation of styrene to SO is minimal or undetectable.

In human lung, detoxification of SO (if formed at all in human pulmonary tissue) takes place predominantly *via* epoxide hydrolase (located on the endoplasmatic reticulum in close proximity to the toxifying cytochrome P450s). The close proximity of the "detoxifying" enzymes to any "toxifying" enzymes ensures the efficient removal of any toxic metabolites. Rodents use both epoxide hydrolase and glutathione-S-transferase as detoxification pathways with the mouse relying on glutathione conjugation more so than the rat. As glutathione S-transferase is located in the cytosol, this makes this detoxification pathway less efficient than the epoxide hydrolase pathway. In comparison to the rodent species, in humans, SO detoxification proceeds nearly exclusively via epoxide hydrolase and glutathione S-transferase accounts for only 0.1% of SO detoxification. Taking account both of the toxification to SO and its detoxification, PBPK-modelling has shown that the SO content of human lungs is very small, if there is any.

Formation of 4-VP and its downstream metabolites occurs at a far higher extent in mouse lung than in rat (14-79% of the mouse concentrations) or human lung (1.5-5% of the mouse concentrations). Although it cannot be ascertained whether or not these species differences in the formation of 4-VP metabolites in the lung may be a reflection of the different numbers of Clara cells (the metabolically active lung cells) present in the different species, since 4-VP metabolites are produced by the same cytochrome P450 enzymes involved in the production of SO, it is most likely that the species differences in the formation of 4-VP metabolites observed reflect species differences in metabolic capability.

As indicated by PBPK-modelling, glutathione depletion caused by SO does not occur in humans. Also, as reactive downstream metabolites of 4-VP are formed in human lung only to a very small extent, the 4-VP metabolic pathway is not expected to cause any glutathione depletion in human pulmonary tissue.

There is no evidence from extensive epidemiological investigations that long term exposure to styrene has produced lung damage or lung cancer in humans.

Hence, overall, the weight of evidence appears to indicate that the consequences of long term exposure to styrene in mouse lung cannot be replicated in the human situation at relevant levels of exposure. Although there are still some uncertainties in this postulated mode of action and in its relevance to humans, namely the lack of data on the relative rates of 4-VP metabolites detoxification in different species, no alternative modes of action that logically present themselves can be supported by as significant a body of evidence as the one presented in this assessment. Consequently, it is felt that the level of confidence in the postulated mode of action can be reasonably high and that, in view of the extensive negative lung epidemiology, it is reasonable to conclude that the lung tumours seen in mice are unlikely to be of any relevance for human health. A more detailed analysis (according to the IPCS framework for evaluating a mode of action in chemical carcinogenesis) of the evidence in support of the proposed mode of action and of its relevance for human health is presented in Annex A to this document.

The carcinogenicity of styrene was evaluated by IARC in 2002. Styrene was considered *possibly carcinogenic to humans* (Group 2B). The Working Group concluded that based on metabolic considerations, it is likely that the proposed mechanism involving metabolism of styrene to styrene 7,8-oxide in mouse Clara cells is not operative in human lungs to a biologically significant extent. However, based on the observations in human workers regarding blood styrene 7,8-oxide, DNA

adducts and chromosomal damage, it cannot be excluded that this and other mechanisms are important for other organs.

In the Rapporteur's[iii] view, pointing to a possible carcinogenic potential of styrene in other organs is highly speculative as: a) Several large cohort and case-control studies of workers exposed to styrene have shown no evidence for a causative association between styrene exposure and cancer in humans at any site; b) No consistent evidence for styrene-induced toxicity in any organ has emerged from studies of exposed workers; c) The level of DNA damage found in workers exposed to styrene is very low (10-fold lower than that produced by endogenously-generated genotoxic substances such as ethylene oxide) and thus cannot be considered to be of any relevance for subsequent tumour formation. Mechanistic studies have shown that styrene-oxide (SO) and its genotoxicity are not the driving force for lung tumour formation in mice, the only experimental tumour site observed so far. Furthermore, DNA adducts in animals after styrene exposure do not show any specific species or target organ relationship. For example, there is no excess of SO-adduct formation in tissues where SO is formed (e.g. in the liver) at high levels; d) Chromosomal damage caused by styrene exposure in humans is far away from being conclusive. Although 5 studies appear to present evidence that styrene may be weakly clastogenic in humans, there are 11 robust negative studies also. Together with a lack of evidence of a dose-response relationship and the negative response for induction of micronuclei when studied concurrently in two of the positive chromosome aberration studies, no clear conclusion on *in vivo* clastogenicity of styrene in humans can be made. Furthermore, at much higher exposures such effects were not observed in experimental animals."

4.11 Toxicity for reproduction

4.11.1 Effects on fertility

Taken from the EU RAR (2008), 4.1.2.9.2

"In an OECD- and GLP-compliant two-generation reproduction toxicity study, the effects of styrene on reproductive performance and fertility were evaluated (Unpublished, Stomp et al., 2003; Cruzan et al., 2005a). Included in the study was an assessment of the potential developmental neurotoxicity of styrene in the F_2 generation (Cruzan et al., 2005b). 25/sex/group Sprague-Dawley rats were exposed for 6 hours daily to either clean air or styrene vapour in a stainless steel and glass whole-body inhalation chamber. Styrene concentrations were 50, 150 and 500 ppm (216.5, 649.5, and 2165mg/m^3). Reproductive performance (i.e. mating behaviour and fertility), gestation length, litter data (number of pups, sex ratio), postnatal survival, sperm evaluations and primordial follicle counts were not adversely affected by styrene exposure across the generations. The mean length of the estrous cycle in the high-exposure females of the F_0 generation was shorter (4.2 days) and differed statistically from that of controls (5.8 days). However, the value was similar to the laboratory's historical control mean value (4.3 days) and within the historical control range (4.1-5.1 days) and not affected in subsequent generations. Hence, it is not considered to be exposure-related.

A 3-generation study in conjunction with a 2-year continuous-exposure study has been conducted in the Sprague-Dawley derived rat (Beliles et al, 1985). Styrene was administered in drinking water at 0, 125 or 250 ppm. Consumption of styrene was estimated to be 14 (males) or 21 (females) mg/kg/day at the higher dose level. To produce F_1 pups, 10 males and 20 females were mated from

[iii] ESR Rapporteur.

each group approximately 90 days after initiation of the study. At weaning, F_1 pups were randomly selected to be mated to produce the F_2 generation and were treated as before up to about 110 days, and then mated as before to produce an F_3 generation.

Only minor general toxic effects (slight but significant reduction in bodyweight in the F_0 females after 2 years) were observed. There were no treatment-related effects on reproduction apart from a slight and not statistically significant reduction in the proportion of F_2 females producing litters at 250 ppm (75% compared with 86% in controls and 95% at 125 ppm); there was no evidence of such an effect in the F_0 and F_1 generations. Overall, this was a poorly designed study because higher dose levels could have been used. Hence, although negative results were obtained, they do not provide adequate reassurance of an absence of potential to impair fertility for styrene.

Three studies by the same authors (Srivastava et al., 1982, 1989 and 1992b) have reported testicular damage in rats at 200 and 400 mg/kg/day styrene (see section 4.1.2.6 for further details of these studies). However, a number of methodological weaknesses in the conduct of these studies put into question the reliability of these findings. It is also noted that in earlier repeated oral studies and in well conducted 2 year inhalation studies in rats at equivalent and higher doses than those used by Srivastava et al, no testicular changes or indications of any testicular effects were observed. Also, no effects on the testis and fertility parameters have been observed in a recent well-conducted OECD- and GLP-compliant rat inhalation 2-generation study with exposures up to 500 ppm (≈ 300 mg/kg/day) styrene. Therefore, despite these individual publications by the same authors reporting testicular damage, the weight of evidence indicates that styrene is not a testicular toxicant.

No evidence of any adverse effects on the female gonads has been reported in several well conducted carcinogenic and chronic toxicity studies in both rats and mice exposed via both the inhalation and oral routes to dose levels giving rise to clear evidence of toxicity and death. No signs of effects of the gonads have been reported in chronic inhalation studies in rabbits, guinea pigs or dogs at exposure concentrations giving rise to toxicity (see section 4.1.2.6).

Summary of animal studies investigating potential effects on fertility

A well-conducted two-generation inhalation study found no effects on fertility and reproductive performance in rats exposed to up to 500 ppm (2165 mg/m^3 ≈ 300 mg/kg/day) styrene, a concentration causing parental toxicity (degeneration of the olfactory epithelium and reductions in body weights).

From the other relevant studies available, there is no convincing evidence that styrene can impair reproductive performance, produce testicular toxicity, sperm abnormalities or adversely affect the reproductive organs. Thus, taken together, the data available indicate that styrene does not have the potential to impair fertility and reproductive performance in animals."

4.11.2 Developmental toxicity

The most recent EU Draft Risk Assessment Report on styrene Draft January 2006[iv] (EU-RAR 2006) has formed the basis for the description of the developmental toxicity studies. However, the evaluation of the selected key studies has been based on the original references.

[iv] Section 4.11.2 has been prepared based on the EU RAR 2006 draft version and discussed at the TC C&L, September 2007. No changes in the text later.

An overview of the relevant studies performed for developmental toxicity on styrene is shown in the table below:

Species	**Route**	*** Dose mg/kg/day ppm ** Conc. (mg/l)**	**Exposure time (h/day)**	**Exposure period:**	**Observations and Remarks**	**Ref. No.**
Rats (14, 3, 7/dose) (Mol: WIST)	Whole-body inhalation	0 ppm, 50 ppm, 300 ppm (0, 0.21, 1.26 mg/l).	6 hr/day	GD 7-21	No signs of maternal toxicity No treatment-related deaths of pups Pup body weights statistically significantly reduced on day 1 (8-11% at 50 and 300 ppm), on 21post-partum (19 and 15% at 50 and 300 ppm respectively) and in females only on day 77 post-partum (8 and 7% at 50 and 300 ppm respectively), but not on day 125 post-partum. No significant difference in brain weight. Statistically significant decreases in certain brain neurotransmitter levels (e.g. serotonin) at 300 ppm. Pre-weaning development: Statistically significant effects on the mean litter day of eye-opening, righting reflex attained, auditory startle reflex and incisor eruption at 300ppm (delays < 2days compared to control). Statistically significantly delayed neurodevelopment (pivoting, bar holding, surface righting) at 300 ppm. No effects on cliff drop avoidance or negative geotaxis. Post-weaning: Rotarod performance significantly affected at 300 ppm on day 30 and 60 indicating neuromotor effects; no effect on day 120. Spontaneous activity significantly increased on days 30-31 and 60-61 at 300 ppm; no effect on days 127-128. No effects on barbiturate sensitivity and no histopathological findings	Kishi et al. 1992, Kishi et al. 1995

Species	Route	* Dose mg/kg/day ppm ** Conc. (mg/l)	Exposure time (h/day)	Exposure period:	Observations and Remarks	Ref. No.
					(in dams or pups). In summary, decreased pup body weight, delayed pup neurodevelopment and behavioural effects at the age of 1 and 2 months is reported. However, the number of litters was small, especially in the neurobehavioural studies, and no firm conclusions can be drawn based on this study alone.	
Rats (9-14 pregnant females per group) (Mol: WIST)	Whole-body inhalation	0 ppm, 50 ppm, 300 ppm (0, 0.21, 1.26 mg/l). Two control groups: a pair-fed control group matched with the 300 ppm exposed animals and a group fed ad libitum	6 hr/day	GD 6-20	No effect on number of live offspring per litter. Statistically significant reduction (by up to 21% of the ad-lib control values) in food consumption in dams exposed to 300 ppm. Slight reduction (by 8 and 4% of the ad-lib control and pair-fed control value respectively) in body weight gain in dams exposed to 300 ppm; not statistically significant. Statistically significant increase in neonatal death in the 300 ppm group (7.3%) compared to both control groups (1.2% and 1.3% in ad lib and pair-fed respectively). Analysis based on litters was not statistically significant, indicating that the increase may be due to a high rate of death in one or a few litters Offspring body weight at birth unaffected, however by day 21 post-partum a statistically significant reduction in body weight in males pups at 300 ppm (by 8% of the pair-fed controls), suggesting that the effect is related to styrene exposure and not to food intake. Offspring cerebellum brain weights similar across groups on day 0 and day 21 post-partum. Cerebrum weights statistically significantly lower (by 13%) on day 0 and 21 at 300 ppm compared with ad libitum fed controls but similar to pair-fed controls suggesting that the effect was related to reduced food intake. Neurotransmitter analyses on days 0 and 21 post-partum showed statistically significantly decreased	Katakura et al. 1999, Katakura et al. 2001

Species	Route	* Dose mg/kg/day ppm ** Conc. (mg/l)	Exposure time (h/day)	Exposure period:	Observations and Remarks	Ref. No.
					levels of some neurotransmitters (homovanillic acid, 5-hydroxytryptamine and 5-hydroxyindoleacetic acid) at 300 ppm. 5-hydroxytryptamine levels similar to pair-fed controls, suggesting that the effect was related to reduced food intake. Levels of homovanillic acid and 5-hydroxyindoleacetic acid decreased compared to both control groups suggesting that these effects were related to styrene exposure. No effects on the levels of dopamine, 3,4-dihydroxyphenyl - acetic acid, 3-methoxytyramine, and norepinephrine Delayed eye-opening, incisor eruption and air righting-reflex at 300 ppm compared to both control groups, suggesting that the effect was related to styrene exposure. Surface righting-reflex and ear-opening unaffected.	
Mice (18-19 mated females/dose) (Mol: ICR)	Whole-body inhalation	0, 2, 20, 100 ppm (0, 0.0087, 0.087, 0.433 mg/l)	Continuously	GD 0-15	No adverse effects in non-pregnant females exposed to styrene under the same conditions. Dams at 100 ppm showed signs of hyper-activity and reduced body weight gain (45% lower than controls). There were no mortalities. No effects on the number of implantations, resorptions, or live foetuses. Reduced placental weight (18%) and foetal weight (25%) at 100 ppm	(Ninomiya *et al*, 2000)
Rats (6/3 litters per group)	Oral	0, 200 mg/kg	see ->	GD1-Parturition After parturition 4 groups : control dams and their natural pups;	No overt signs of maternal toxicity No effect on number of pups per litter or pup body weights No evidence effect on protein content of the striatal region of the	Zaidi *et al*, 1985

Species	Route	* Dose mg/kg/day ppm ** Conc. (mg/l)	Exposure time (h/day)	Exposure period:	Observations and Remarks	Ref. No.
				styrene-exposed dams and their natural pups (gestation and lactation exposure); control dams and fostered in utero styrene-exposed pups (gestational exposure only); styrene-exposed dams and fostered unexposed pups (lactational exposure only). Treatment of pups and dams continued accordingly until week 3.	brain Pups exposed during gestation only: no effect on ^{3}H-spiroperidol binding Pups exposed during gestation and lactation or during lactation only: binding of ^{3}H-spiroperidol statistically significantly increased compared with controls (both by 20-26%). Further analysis revealed the increased ^{3}H-spiroperidol binding was due to an increased number of dopamine receptors; there was no change in binding affinity. Pups exposed during gestation and lactation, or during lactation only: significant increase in amphetamine-induced locomotor activity and apomorphine-induced stereotypy.	
Rats (25/sex/group) (Sprague-Dawley)	Whole-body inhalation	0, 50, 150 and 500 ppm (0, 0.2165, 0.6495, and 2.165 mg/l)	6hr/day	F_0 : 10 weeks prior to mating and throughout two weeks of mating. The females during gestation and lactation, except from gestation day 21 to lactation day 4, when styrene was administered in olive oil by gavage at dose levels of 66, 120 and 300 mg/kg/day (divided into 3 equal doses approx. 2 hours apart). F_1: From PND 22 and followed same protocol as F_0 generation.	Parental animals, F_0 and F_1, 500 ppm: degeneration of the olfactory epithelium lining of the nasal cavity at 500 ppm; incidence and degree less pronounced in F_1 than F_0 . Body weight of males statistically significantly reduced by 7-8% (F_0) and 8-13% (F_1) and in females by 7-8%. Body weights during gestation reduced by 5% (not statistically significant) in F_0 and by 6-7% (statistically significant) in F_1 Parental animals, 150 ppm: body weight of males statistically significantly reduced by 6-7%. No statistically significant effects on body weights in females, although reductions of up to 5-6% in F_1 females during the study including gestation. Water consumption during gestation and lactation: statistically significantly increased during	Unpublished, Stump et al., 2003; Cruzan et al., 2005a, Cruzan et al., 2005b

Species	Route	* Dose mg/kg/day ppm ** Conc. (mg/l)	Exposure time (h/day)	Exposure period:	Observations and Remarks	Ref. No.
				F_2: not directly exposed	gestation in 150 and 500 ppm F_1 dams (by 11-14% and 20-24% respectively) and in 500 ppm F_0 dams (by 13-24%). No effects on pre-weaning body weights of F_1 pups (PND 1-21). Body weights of 500 ppm F_1 pups decreased (by 7-7.6%, not statistically significant) during the post-weaning period (PND22-28) and body weight gain statistically significantly lower (by 11%). Delayed (approx. 2 days) preputial separation in 500 ppm F_1 males. Statistically significant, exposure-related decreases in body weight of 10-13% and 7-10% in 500 ppm and 150 ppm in F_2 pups respectively in pre-weaning period (PND 0-21). Reductions in body weight of 500 ppm F_2 pups continued throughout post-weaning period even though exposure had stopped. No macroscopic findings attributable to exposure evident at necropsy. Statistically significant reductions in mean absolute pituitary gland weight in 500 ppm F_2 male pups (by 34%) and in 150 and 500 ppm F_2 female pups (by 19% and 24% respectively). Mean relative (to final body weight) pituitary gland weight statistically significantly reduced in 500 ppm F_2 male pups (by 22%). Attainment of pre-weaning developmental landmarks (pinna detachment, surface righting response, incisor eruption and hair growth) and acquisition of preputial separation delayed in 500 ppm F_2 pups. Statistically significant reduction in forelimb grip strength (by 24-28% of the control values) in both sexes	

Species	Route	* Dose mg/kg/day ppm ** Conc. (mg/l)	Exposure time (h/day)	Exposure period:	Observations and Remarks	Ref. No.
					on PND 60 in 500 ppm F_2 offspring. Hindlimb grip strength reduced by 18% in males on PND 45. No effects on PND 22. Grip strength has been related with body weight. Thus, the reduction in forelimb grip strength observed on PND 60 might be a consequence of the reduced body weight seen in these pups. An estimation of the reduction in grip strength per 100 g body weight using mean group values show that styrene induced a 17-24% reduction of grip stregth after correction for body weight. Consequently, the reduced grip strength is (mainly) a consequence of the styrene exposure. The “normal” age-related pattern of motor activity (increases PND 13-17 and decreases PND 17-21) slightly shifted in 500 ppm pups: activities of both sexes lower, but not statistically significantly different from control group at PND 13, rose at PND 17, did not return to control levels at PND 21. Activity in both sexes similar to control group by PND 61. No exposure-related effects on startle Response At PND 24, mean time to escape in straight channel swimming trial (day 1) statistically significantly increased in 500 ppm male offspring (by 38%) and an increase of similar magnitude but not statistically significant in females of the same group. No difference in straight channel swimming trial at PND 62. However, the decreased swimming ability at PND 24 cannot be interpreted as a ”temporary” effect based on this, because the sensitivity at PND 62 may likely have been too low. This view is strongly supported by the positive control data (appendix G), as both	

Species	Route	* Dose mg/kg/day ppm ** Conc. (mg/l)	Exposure time (h/day)	Exposure period:	Observations and Remarks	Ref. No.
					PTU and methimazole did not induce any effects on swimming ability at PND 62, but only on PND 21/22. No exposure-related differences suggestive of an impairment of learning or memory ability observed in either sex at the two ages tested (PND 24 and PND 62). No notable differences between exposed offspring and controls in neuropathologic evaluations.	

4.11.2.1 Non-human information

Rats

In an OECD- and GLP-compliant two-generation reproduction toxicity study including developmental neurotoxicity assessment of the F_2 generation, the effects of styrene on fertility and postnatal development were evaluated (Stump et al., 2003; Cruzan et al., 2005a, Cruzan et al., 2005b). 25/sex/group Sprague-Dawley rats were exposed for 6 hours daily to either clean air or styrene vapour in a stainless steel and glass whole-body inhalation chamber. Styrene concentrations were 50, 150 and 500 ppm (216.5, 649.5, and 2165mg/m^3).

The F_0 generation was exposed for 10 weeks prior to mating and throughout the subsequent two weeks of mating, during which males and females from each group were randomly paired and co-habited. The females continued inhalation exposure during gestation and lactation, except from gestation day 21 through to lactation day 4, when styrene was administered in olive oil by gavage at dose levels of 66, 120 and 300 mg/kg/day (divided into 3 equal doses approx. 2 hours apart). This was done because this period is critical to pup neurological and neuroendocrine development and hence, there were concerns that stress on the pups arising from the removal of the dams for the 6h exposure session might have affected pup development. These oral dose levels were chosen (based on the Sarangapani physiologically based pharmacokinetic (PBPK) modelling) to generate peak blood levels of styrene after each gavage dose that closely matched the predicted blood level of styrene from each of the 3 inhalation exposure levels.

At weaning on PND 21, offspring (25/sex/group) were randomly selected to constitute the F_1 generation. Inhalation exposure of the F_1 animals was initiated on post-natal day (PND) 22 and, followed exactly the same protocol as for the F_0 generation. The F_2 generation was not directly exposed to the test article but was potentially exposed *in utero* and through nursing during PND 0-21. At weaning on PND 21, 40 F_2 pups/sex/group were selected for post-weaning developmental landmarks and neurobehavioural evaluation (i.e. functional observatory battery evaluations, locomotor activity, acoustic startle response and learning and memory evaluations). No exposure to

styrene occurred during this period. In addition, 10 F_2 pups/sex/group were selected for neuropathological assessment (performed on PND 21), which included brain weight and brain dimension measurements, brain morphometric analysis and central and peripheral nerve evaluation.

Developmental landmarks (pinnal detachment, surface righting response, hair growth, incisor eruption, eye opening, preputial separation and vaginal perforation) were assessed in all the selected F_1 and F_2 pups.

In the F_0 and F_1 parental animals degeneration of the olfactory epithelium lining of the nasal cavity was observed at 500 ppm only. The incidence and degree of degeneration were less pronounced in the F_1 generation compared to the F_0 generation.

In the 500 ppm groups of both the F_0 and F_1 generations, the mean body weight of the males was statistically significantly reduced by 7-8% (F_0) and 8-13% (F_1) and in females by 7-8%. Mean body weights of the 500 ppm females during gestation were reduced by 5% (not statistically significant) in the F_0 dams and by 6-7% (statistically significant) in the F_1 dams. In the 150 ppm groups of the the F_0 and F_1 generations, the mean body weight of the males was statistically significantly reduced by 6-7%. There were no statistically significant effects on body weights in the 150 ppm females of the F_0 and F_1 generations, although reductions of up to 5-6% were observed in the F_1 females during the study including gestation. Overall, there were no statistically significant maternal effects on body weight at 150 ppm.

Water consumption measured in females during the gestation and lactation periods only was statistically significantly increased during gestation in the 150 and 500 ppm groups of the F_1 generation (by 11-14% and 20-24% respectively) and in the 500 ppm group of the F_0 generation (by 13-24%).

No exposure-related effects were observed on the pre-weaning body weights of the F_1 pups (PND 1-21). However, the body weights of the 500 ppm F_1 pups were decreased compared to controls (by 7-7.6%, not statistically significant) during the post-weaning period (PND22-28) and the bodyweight gain in this group was statistically significantly lower than that of the controls (by 11%). A delay (approx. 2 days) in preputial separation was observed in the 500 ppm F_1 males. The correlation between body weight and preputial separation in rats is established; therefore, the delay in preputial separation in the 500 ppm F_1 male offspring may have been a consequence of the lower body weight observed in this group following direct exposure to styrene from weaning to preputial separation.

Statistically significant, exposure-related decreases in body weight of 10-13% and 7-10% were observed in the 500 and 150 ppm pups of the F_2 generation respectively throughout the pre-weaning period (PND 0-21). The reductions in body weight of the F_2 pups in the 500 ppm group continued throughout the post-weaning period even though exposure had stopped. No macroscopic findings attributable to exposure were evident at necropsy. Statistically significant reductions in mean absolute pituitary gland weight compared to controls were noted in the 500 ppm F_2 male pups (by 34%) and in the 150 and 500 ppm F_2 female pups (by 19% and 24% respectively). Also, the mean relative (to final body weight) pituitary gland weight was statistically significantly reduced in the 500 ppm F_2 male pups by 22%. The magnitude of the decreases in the pituitary gland weight at 150 and 500 ppm is relatively large and cannot be accounted for by the reduced body weights in the high-exposure F2 male pups. Information on the normal growth rate of the pituitary gland in fast-developing organisms and especially its relationship to body weight development would be useful for evaluating this effect. However, in the absence of such information it is assumed that the reduced pituitary weight may represent adverse developmental effects of styrene exposure.

The attainment of the pre-weaning developmental landmarks (pinnal detachment, surface righting response, incisor eruption and hair growth) and the acquisition of the preputial separation were also delayed in the 500 ppm F_2 pups. These effects may be due to the delay in growth (10-13% reduced body weights) observed in these pups.

Functional observations incl. grip strength

Detailed functional observatory evaluations were assessed in 20/sex/group on PND 4, 11, 22, 45 and 60. Statistically significant reductions in the forelimb grip strength (by 24-28% of the control values) were found in both sexes of the 500 ppm group on PND 60. Hindlimb grip strength was also reduced by 18% of the control value in males only on PND 45. No effects were seen on PND 22, which was the earliest age of grip strengths measurements.

Grip strength has been related with body weight (Maurissen et al., 2003). Thus, the reduction in forelimb grip strength observed on PND 60 might be related to the reduced body weight seen in these offspring. However, in the Maurissen et al 2003 study, the body weight reduction was around 18%, while for styrene, the reduction in body weight on PND 63 was 8.6% in the males and 6.3% in the females (not statistically significant), i.e. less than half of the body weight reduction in the Maurissen study. In addition, the grip strength was reduced 17-18% in the Maurissen study compared to 24-28% reduction in the styrene study, i.e. styrene induces a more marked reduction in grip strength.

We have estimated the reduction in grip strength per 100 g body weight using the mean group values (see tables below). The results show that styrene actually induced a 17-24% reduction in grip strength after correction for body weight. Consequently, the marked reduction in grip strength observed in both sexes on PND 60 is mainly a direct consequence of the styrene exposure.

Males, forelimb grip strength (FGS) at PND 60 and body weight (bw) on PND 63

	bw	FSG	FSG per 100 g bw
Control	361	591	163,7
Styrene	330	451	136,7
% reduction	8,6	23,7	**16,5**

Females, forelimb grip strength (FGS) at PND 60 and body weight (bw) on PND 63

	bw	FSG	FSG per 100 g bw
Control	227,1	522	229,9
Styrene	212,7	374	175,8
% reduction	6,3	28,4	**23,5**

Motor activity

Motor activity in selected F_2 pups (20/sex/group) was assessed on PND 13, 17, 21 and 61 using a SDI Photobeam Activity System. The “normal” age-related pattern of motor activity (increases between PND 13-17 and decreases between PND 17-21) appeared to be slightly shifted in the 500 ppm pups: the activities of both sexes were lower, but not statistically significantly different from those in the control group at PND 13, rose at PND 17 but did not return to expected control levels at

PND 21. This shift in the age-related pattern of motor activity may be related to the growth delay seen in this group of animals particularly in the pre-weaning stage.

The activity in both sexes was similar to that of the control group by PND 61.

Startle Response

The same animals used in the motor activity assessment (20 rats/sex/group) were used for the acoustic startle response test on PND 20 and 60 using the SR-Lab Startle Response System. No exposure-related trends were apparent in either sex of any of the exposed groups compared with controls.

Learning and memory

Twenty rats/sex/group were analysed for learning and memory in the Biel Maze swimming trials. Using a water-filled eight unit T-maze, animals were required to cross from one end to the other of the maze and escape by locating a platform hidden under the water surface. The time taken to swim across the maze and the number of errors for all trials were recorded. The evaluation was performed at two different ages (PND 24 and PND 62) using a different set of animals for each age and consisted of three phases conducted over seven consecutive days. The first day of testing (phase one) involved a straight channel swimming trial designed to evaluate the animals' swimming ability and motivation to escape. Each animal was placed in a straight channel opposite the escape platform and the time taken to escape recorded. Each animal was allowed four trials. Phase two (days 2-6) trials were designed to measure sequential learning (learning and short-term memory). Each animal was allowed three minutes in two trials/day for two days to solve the maze in path A and two trials/day for three consecutive days to solve the maze in the reverse path (path B). Animals failing to escape within the allotted time (3 mins) were removed and placed on the escape platform for 20 secs; then removed from the maze. The long-term memory of the animals was probed on day 7 (phase three) by challenging them to solve the maze in path A again. Biel maze data were evaluated as the mean time to escape over all trials for each of the three phases.

At PND 24, the mean time to escape in the straight channel swimming trial (day 1) was statistically significantly increased in the 500 ppm male offspring (10.58 secs compared to 7.53 secs in controls). An increase of similar magnitude (11.43 secs compared to 7.8 secs in control) was observed in the females of the same group, but the difference was not statistically significant. An increase in swimming time around 3 secs may seem small, but this has to be evaluated in relation to the total swimming time of only 8 sec in the controls, i. e. the increase is actually quite marked (38%). The body weight of the pups was reduced around 12-14% on PND 28. The absence of clear knowledge on the relationship between body weight and swimming ability makes it is difficult to evaluate whether a 12-14% reduction in body weight solely can explain an increase of 38% in swimming time. Consequently, it is evaluated that the effect on the swimming ability may be a direct effect of the styrene exposure.

No difference was seen in the straight channel swimming trial at PND 62. However, the decreased swimming ability at PND 24 cannot be interpreted as a "temporary" effect based on this, because the sensitivity of the testing of swimmming ability at PND 62 may likely have been too low. This view is strongly supported by the positive control data (appendix G), as the two positive controls used (PTU and methimazole) did not induce any effects on swimming ability at PND 62, but only on PND 21/22. Actually, this means that the endpoint swimming ability at PND 62 have not been validated using positive controls.

For the subsequent assessments, the mean time to escape was initially relatively high and decreased throughout repeated testing in the forward path in all groups. Overall, no exposure-related differences suggestive of an impairment of learning or memory ability were observed in either sex at the two different ages tested (PND 24 and PND 62).

Neuropathological evaluations

Ten F_2 pups/sex/group were randomly selected and subject on PND 21 to brain weight measurement, morphometry and neuropathological evaluation of the brain and spinal cord. An additional 10 F_2 offspring/sex/group from the 20 used in the evaluation of motor activity were selected at random and subject on PND 72 to the same neuropathological assessments with the inclusion of the peripheral tissues. Histological and morphometric evaluations were conducted only in the control and 500 ppm groups.

No notable differences were found between the exposed animals and the controls in the neuropathologic evaluations.

Summary

Degeneration of the olfactory epithelium of the nasal cavity was observed in the 500 ppm group of the F_0 and F_1 parental animals. Also, body weights were statistically significantly reduced during the pre-mating interval in the 150 ppm males of the F_1 generation (by 6-7%) and in the 500 ppm males and females of both the F_0 and F_1 generations (by 6-13%). Body weights of females during gestation were statistically significantly reduced (by 6-7%) only at 500 ppm in the F_1 generation.

Styrene exposure caused a statistically significant decrease in body weight gain of the 500 ppm F_1 pups (by 11%) and in body weight of the 150 ppm (by 7-10%) and 500 ppm F_2 pups (by 10-13%). Generally, a pattern of developmental delay was evident mainly in the F_2 at 500 ppm. The delay in attaining some pre-weaning developmental landmarks (pinna detachment, surface righting response, incisor eruption and hair growth), the slight shift in the normal pattern of motor activity, and the delayed preputial separation around PND 40 may be related to the reduced body weight of the animals and may as such represent an indirect expression of styrene developmental toxicity extending several weeks after exposure was stopped. The reduced weight of the pituitary gland, the decreased swimming ability on PND 24 and especially the reduction in forelimb grip strength on PND 60 in both sexes cannot be explained by the reduced body weights and are therefore considered as mainly a direct consequence of the styrene exposure.

Overall, the results of this OECD- and GLP-compliant two-generation reproduction toxicity study including developmental neurotoxicity assessment of F_2 offspring show that styrene causes developmental toxicity manifested as a pattern of developmental delay, including delayed neurological development, and developmental neurotoxicity effects on post-weaning behaviour, especially neuromotor function.

In another study, 24 pregnant Wistar rats were assigned to groups and exposed to 0 (n=14), 50 (n=3) or 300 ppm (n=7) styrene vapour whole-body, for 6 hours/day on days 7-21 of gestation (Kishi *et al.*, 1992 and Kishi *et al.*, 1995). The pups of 5 dams at 0 ppm, 2 dams at 50 ppm and 5 dams at 300 ppm were evaluated in neurobehavioural studies over the course of the study. Pups were examined daily for development (startle reflex, eye opening, incisor eruption and vaginal

patency). During the pre-weaning phase of the study (day 1 to 22) pups were also examined with respect to surface righting, pivoting locomotion, bar holding ability, negative geotaxis and cliff drop avoidance. Post-weaning (days 23 to 120) offspring were examined with respect to open-field behaviour, motor-coordination, activity, operant conditioning and sensitivity to barbiturates. Dams and pups were examined histopathologically (brain, lungs, liver and kidneys): dams at day 0 (parturition) and pups on days 21 and 160 post-partum. Statistical analysis was conducted initially on a litter basis and subsequently on an individual basis.

No overt signs of maternal toxicity were observed. Maternal body weight gains, gestational lengths and the number of offspring delivered were comparable with controls. No treatment-related deaths occurred in the pups of any group. Pup body weights were significantly reduced around 8-11% on day 1 at both 50 and 300 ppm styrene. For the pups used for neurobehavioural studies, pup body weights were statistically significantly reduced in both sexes on day 21 post-partum (by 19 and 15% of the control value at 50 and 300 ppm respectively) and in females only on day 77 post-partum (by 8 and 7% of the control value at 50 and 300 ppm respectively) but not on day 1 and 125 post-partum. Brain samples were taken from 10 pups per group on day 1 post-partum. There was no significant difference in brain weight but statistically significant decreases in certain brain neurotransmitter levels (e.g. serotonin) occurred at 300 ppm.

Statistically significant differences in terms of the mean litter date of eye opening, righting reflex attained, auditory startle reflex apparent and incisor eruption were reported between controls and the 300 ppm group (delays in each case were < 2 days compared to the control values). With regard to the neurobehavioural delopment of the preweaning pups, a statistically significantly delayed development (surface righting, pivoting, bar holding) was reported for the 300 ppm group, while no differences compared with controls were observed in cliff drop avoidance or negative geotaxis.

Rotarod performance was significantly affected at 300 ppm on days 30 and 60 indicating effect on neuromotor coordination. No exposure related differences were seen at the age of 120 days.

Spontaneous activity of pups on days 30-31 and 60-61 was significantly increased at 300 ppm, but at the age of 127-128 days there were no differnces between controls and styrene-exposed offspring. No differences in barbiturate sensitivity and no histopathological findings (in dams or pups) were observed in any test group.

Overall, decreases in pup body weight and delays in pup development and in the acquisition of pre-weaning behavioural characteristics were reported in this study following prenatal exposure to 300 ppm styrene. In addition, behavioural effects including indication of neuromotor effects were reported postweaning at the age of 1 and 2 months. However, the number of litters was small, especially in the neurobehavioral studies, and consequently no firm conclusions can be drawn based on this study alone. No overt maternal toxicity was reported in this study up to 300 ppm styrene.

In another study by the same workers, groups of 9-14 pregnant Wistar rats were exposed whole-body to 0, 50 or 300 ppm styrene for 6 hours/day on days 6-20 of gestation (Katakura *et al*, 1999 and Katakura *et al*, 2001). Two control groups were included: a pair-fed control group (food consumption matched with 300 ppm exposed animals) and a group fed ad libitum. Implantation sites were investigated for the number of resorptions. At parturition all pups were weighed, counted and examined for external malformations. Eight pups per group (4 males and 4 females, where possible) were selected at random and were left to be reared by their natural mothers. Two pups per sex per dam were sacrificed at day 0 of parturition and the prefrontal cortex, striatum, hippocampus, hypothalamus, cerebrum, cerebellum, and midbrain were assessed with respect to levels of neurotransmitters (dopamine, 3,4-dihydroxyphenylacetic acid, 3-methoxytyramine, homovanillic

acid, 5-hydroxytryptamine, 5-hydroxyindoleacetic acid and norepinephrine). At day 21 post-partum 4 pups per group (2 males and 2 females) were sacrificed and neurotransmitter levels determined. The remaining dams and pups were sacrificed at day 21 post-partum and microscopic pathology of the brain, liver, lung and kidneys conducted. Post-parturition, developmental parameters such as ear-unfolding, eye-opening, incisor eruption and righting reflex were recorded daily.

The number of live offspring litter were similar across all 4 groups. A statistically significant reduction (by up to 21% of the ad-lib control values) in food consumption was observed in dams exposed to 300 ppm. A slight reduction (by 8 and 4% of the ad-lib control and pair-fed control value respectively) in body weight gain was also observed in dams exposed to 300 ppm, but this did not attain statistical significance.

There was a statistically significant increase in neonatal death in the 300 ppm group (7.3%) compared to both control groups (1.2% and 1.3% in ad lib and pair-fed respectively). However, analysis based on litters was not statistically significant, indicating that the increase may have been due to a high rate of death in one or a few litters. Offspring bodyweight at birth was unaffected, however by day 21 post-partum a statistically significant reduction in body weight was observed amongst males born to dams that had been exposed to 300 ppm styrene (by 8% of the pair-fed controls), suggesting that the effect is related to styrene exposure and not to food intake. Offspring cerebellum brain weights were similar across all groups at day 0 and day 21 post-partum. Cerebrum weights were statistically significantly lower (by 13%) in animals of the 300 ppm group at day 1 and 21 compared with ad libitum fed controls but were similar to pair-fed controls suggesting that the effect was related to reduced food intake.

Neurotransmitter analyses on days 0 and 21 post-partum showed statistically significantly decreased levels of some neurotransmitters (homovanillic acid, 5-hydroxytryptamine and 5-hydroxyindoleacetic acid) in animals exposed at 300 ppm, compared with controls. 5-hydroxytryptamine levels were only reduced when compared to ad-lib fed controls, suggesting that the effect was related to reduced food intake. However, the levels of homovanillic acid and 5-hydroxyindoleacetic acid were decreased in the 300 ppm animals compared to both control groups, suggesting that these effect were related to styrene exposure. Levels of other neurochemicals analysed were similar across all groups.

Delayed eye-opening, incisor eruption and air righting-reflex were observed amongst pups at 300 ppm compared to each of the control groups suggesting that the effect is related to styrene exposure and not to food intake. Impaired air righting-reflex was also seen at 50 ppm. However, this only attained statistical significance when compared to ‘ad-lib’ controls. There were no statistically significant differences between the two control groups, thus the paired-feeding was concluded by the authors to have no effect on righting reflexes. Surface righting-reflex and ear-opening were unaffected.

Overall, decreases in male pup body weight on day 21 post-partum and delays in the acquisition of some developmental landmarks (eye-opening, incisor eruption, air-righting reflex) were reported in this study following prenatal exposure to 300 ppm styrene. In addition, reduced levels of some brain neurotransmitters were found on day 0 and 21 postpartum. Comparisons to a pair-fed control group sugggest that these effects were related to styrene exposure and not to food intake. No overt maternal toxicity was reported in this study up to 300 ppm styrene in comparison with the ad-lib and pair-fed controls.

The draft RAR includes another publication on the possible neurobehavioural effects of styrene in rats exposed prenatally from the same group (Bingqing *et al*, 1989, as described in the styrene RAR). As both the methods and the results in this publication seems quite similar to those reported

in Kishi *et al.*, 1992 and Kishi *et al.*, 1995, it is assumed to be an early publication (in Japanese) on the same study. Consequently, the paper from Bingqing *et al*, 1989 is not included here.

Mice

Groups of 18-19 mated ICR female mice were exposed, whole body, to 0, 2, 20, and 100 ppm (0, 8.7, 87, 433 mg/m^3) styrene vapour continuously between days 0 and 15 of gestation (Ninomiya *et al*, 2000). No adverse effects were seen in the non-pregnant females exposed to styrene under the same conditions. At the highest exposure level the dams showed signs of hyper-activity and reduced body weight gain (44% lower than controls). There were no mortalities. There were no substance-related effects on the number of implantations, resorptions, or live foetuses. Reduced placental weight was noted amongst animals exposed to 100 ppm (mean 0.09g compared to 0.11g in controls, 18% reduction). Reduced foetal weight was also noted at 100 ppm (mean 0.36g compared to 0.48g in controls, 25% reduction). Overall, evidence of markedly reduced placental and foetal weight were found at a level of exposure (100 ppm for 24h/day) associated with a marked impairment of maternal growth during pregnancy.

A study is available which was designed principally to investigate the effect of styrene on the dopamine receptors in pre- and postnatally exposed rats (Zaidi *et al*, 1985). Pregnant dams (probably 12 per group) were administered 0 or 200 mg/kg styrene in groundnut oil by oral gavage from day one of gestation to parturition. At parturition pups were randomised and litter sizes adjusted to 8 pups per dam. Animals were then divided into 4 exposure groups each containing 3 litters: group A comprised control dams and their natural pups (controls); group B comprised styrene-exposed dams and their natural pups (gestation and lactation exposure); group C comprised control dams and fostered in utero styrene-exposed pups (gestational exposure only); and group D comprised styrene-exposed dams and fostered unexposed pups (lactational exposure only). Treatment of the pups and dams was then continued accordingly until week 3.

Average litter weights and number of pups were recorded both at parturition and at week 3 post-parturition. Behavioural studies (not specified) were carried out on 8 pups/group at week 3. Measurements of amphetamine-induced locomotor activity and apomorphine-induced stereotypy were also taken as parameters of dopamine receptor sensitivity. Six pups/sex were then sacrificed and the brains removed and the corpus striata dissected for assessment of dopamine receptor binding by measuring the binding of ^{3}H-spiroperidol, as a specific ligand.

No significant difference in the number of pups per litter or in average body weights between control and exposed pups were observed at either time point. No overt signs of maternal toxicity were observed during the study. No evidence of any significant treatment-related effect on the protein content of the striatal region of the brain was observed at dissection. In pups exposed during gestation only there was no effect on ^{3}H-spiroperidol binding compared with controls. However, in pups exposed during gestation and lactation or during lactation only the binding of ^{3}H-spiroperidol was statistically significantly increased compared with controls (both by 20-26%). Further analysis revealed the increased ^{3}H-spiroperidol binding was due to an increased number of dopamine receptors; there was no change in binding affinity. Pups exposed during gestation and lactation, or during lactation only, also showed a significant increase in amphetamine-induced locomotor activity and apomorphine-induced stereotypy. These data suggest that exposure to styrene may alter brain dopamine receptor levels during maturation and affect amphetamine-induced locomotor activity in the rat.

4.11.2.2 Human information

Birth weights were analysed in a US study of mothers who worked when pregnant in the reinforced plastics industry (Lemasters *et al*, 1989). There were 819, 154, and 75 pregnancies in the no, low and high exposure groups. These groups were comparable in age, gravidity and length of education. A lower proportion of women in the high exposure group were from families with income >$15000. Regression analysis was used to take into account many factors influencing pregnancy outcome such as age. There was no statistically significant effect of exposure to styrene on birth weight. For the 50 births in a subgroup working in jobs expected to have the highest styrene exposures (about 50 ppm), mean birth weight was 4% lower than that for unexposed births after adjustment for other factors but this finding was not statistically significant.

A range of other epidemiological studies focussing on developmental effects have been conducted but most of these lacked adequate exposure information and were too small to be conclusive. Nevertheless, the studies have been generally negative and the available human data provides no reliable evidence for styrene exposure-related adverse effects in relation to spontaneous abortions, congenital abnormalities or birth weight, within the exposure ranges investigated.

No epidemiological studies focussing on delayed postnatal development or developmental neurotoxicity effects have been found.

Overall, there is no clear evidence of an effect of styrene on human reproduction, but data are too limited to exclude the possibility for effects.

4.11.3 Other relevant information

4.11.4 Summary and discussion of reproductive toxicity relevant for classification according to DSD

Developmental

In the rat, inhalation exposure produced no evidence of significant effects on conventional parameters (i.e. malformations, death) assessed in the foetus at non-maternally toxic exposure concentrations of up to 600 ppm styrene. However, developmental delays postnatally including delayed neurological development and some indications of behavioural effects after weaning have been reported in a number of studies at 300 ppm styrene in the absence of maternal toxicity.

In a recent well-conducted OECD- and GLP-compliant two-generation study including developmental neurotoxicity assessment in F2 offspring, a pattern of developmental delays both before and after weaning (decreased body weights, delays in attaining some pre-weaning developmental landmarks, slight shift in the normal pattern of motor activity and delayed preputial separation), was evident mainly in the F_2 pups of the high exposure group (500 ppm). In addition, decreased swimming abilities on PND 24 and reductions in forelimb grip strength on PND 60 were found in both sexes. These effects indicate affected neuromotor functions and are evaluated as mainly a direct consequence of the styrene exposure. Significantly decreased pup body weight during the lactation period was found at 150 ppm in the absence of maternal toxicity. The results of this study shows that exposure to 500 ppm styrene causes developmental toxicity manifested as a pattern of developmental delays, including delayed neurological development, and developmental neurotoxicity effects on post-weaning behaviour, especially related to neuromotor functions. In

contrast to the earlier investigations at 300 ppm, the exposure to 500 ppm induced some maternal toxicity (reductions in body weights of 6-7% and degeneration of the nasal olfactory epithelium). However, it is considered highly unlikely that the developmental toxicity is unspecific effects of the maternal toxicity.

4.11.5 Comparison with criteria relevant for classification according to DSD

Repr. Cat. 2; R61 May cause harm to the unborn child

According to the classification criteria, section 4.2.3.3 'Comments regarding the categorisation of substances toxic to reproduction', a substance can be placed in Category 2 for developmental toxicity when *"… clear evidence of adverse effects in well conducted studies in one or more species."* It is also stated *"Since adverse effects in pregnancy or postnatally may result as a secondary consequence of maternal toxicity, reduced food or water intake, maternal stress, lack of maternal care, specific dietary deficiencies, poor animal husbandry, intercurrent infections, and so on, it is important that the effects observed should occur in well conducted studies and at dose levels which are not associated with marked maternal toxicity."*

Classification in category 3 is based on similar criteria as for category 2 but may be used where the experimental design has deficiencies which make the conclusions less convincing, or where the possibility that the effects may have been due to non-specific influences such as generalised toxicity cannot be excluded.

Exposure levels

The classification criteria for developmental toxicity are evidence based – and do not have to fulfil specific criteria in relation to effect concentration levels.

Classification

As clear evidence of adverse effects has been obtained in a well-conducted OECD- and GLP-compliant two-generation study including developmental neurotoxicity assessment in F2 offspring, and as it is considered highly unlikely that the developmental toxicity is unspecific effects of the maternal toxicity, classification of styrene as a developmental toxicant in Category 2 with R61 (May cause harm to the unborn child) is warranted.

4.11.6 Conclusions on classification and labelling relevant for classification according to DSD

In the rat, developmental delays postnatally including delayed neurological development and some indications of behavioural effects after weaning have been reported in a number of studies at 300 ppm styrene in the absence of maternal toxicity.

In a recent well-conducted OECD- and GLP-compliant two-generation study including developmental neurotoxicity assessment in F2 offspring, a pattern of developmental delays both before and after weaning (decreased body weights, delays in attaining some pre-weaning developmental landmarks, slight shift in the normal pattern of motor activity and delayed preputial separation), was evident mainly in the F_2 pups of the high exposure group (500 ppm). In addition, decreased swimming abilities on PND 24 and reductions in forelimb grip strength on PND 60 were found in both sexes. These effects indicate affected neuromotor functions and are evaluated as

mainly a direct consequence of the styrene exposure. Significantly decreased pup body weight during the lactation period was found at 150 ppm in the absence of maternal toxicity. The results of this study shows that exposure to 500 ppm styrene causes developmental toxicity manifested as a pattern of developmental delays, including delayed neurological development, and developmental neurotoxicity effects on post-weaning behaviour, especially related to neuromotor functions. In contrast to the earlier investigations at 300 ppm, the exposure to 500 ppm induced some maternal toxicity (6-7% reduction in body weight and degeneration of the nasal olfactory epithelium). However, it is considered highly unlikely that the developmental toxicity are unspecific effects of the maternal toxicity.

Consequently, it is proposed to classify styrene as a developmental toxicant in Category 2 with R61 (May cause harm to the unborn child).

4.12 Summary and discussion of reproductive toxicity relevant for classification according to CLP

Please consult the section 4.11.4

4.12.1 Comparison with criteria relevant for classification according to CLP

The classification criteria for hazard categories for reproductive toxicants within CLP are quite similar to the classification criteria within DSD. This applies both to the evaluation of developmental toxicity effects as well as the evaluation of the relevance of maternal toxicity. The main difference is the Hazard statements and the titles for the categories where CLP Category 1B is similar to DSD Category 2.

According to the CLP criteria a substance should be placed in Category 1B Presumed human reproductive toxicant when "… *clear evidence of an adverse effect on sexual function and fertility or on development in the absence of other toxic effects, or if occurring together with other toxic effects the adverse effect on reproduction is considered not to be a secondary non-specific consequence of other toxic effects*."

4.12.2 Conclusions on classification and labelling relevant for classification according to CLP

Please see section 4.11.6. It is proposed to classify styrene in Category 1B Presumed human reproductive toxicant.

As there is sufficient evidence to conclude that no classification is warranted for effects on fertility, the hazard statement should be noted with a "D".

There is evidence that a classification for developmental effects via the oral route is not warranted, and although there are no dermal investigations taken together with the highly volatile nature of styrene it is suggested to include a specific mention of the exposure via inhalation in the hazard statement H360D: "May damage the unborn child when exposed via inhalation".

The generic concentration limit of styrene if classified as a presumed human reproductive toxicant in category 1B in a mixture would be $\geq$ 0.3%. As styrene is a volatile chemical the need for a specific concentration limit has been considered. The consideration is based on Guidance for setting specific concentration limits for reproductive toxicants within the "*CLP regulation (EC/1272/2008), Draft 1, July 2010" from EChA working group on human health guidance for CLP*. Here it is stated that "*For substances with a saturated vapour pressure concentration above 130 mg/m^3 the derived*

ED10 should be decreased by a factor covering the higher inhalative exposure. This factor should be the ratio of 130 mg/m³ and the saturated vapour pressure concentration of the substance under consideration when the saturated vapour pressure is below 2000 mg/m^3 and the ratio of 130 mg/m³ and a maximum exposure of 2000 mg/m³ which is used as reasonable worst case estimate (Schneider et al) when the saturated vapour concentration is above 2000 mg/m^3. This results in a maximum correction factor of 0.065 for rat studies."

As styrene has a very high saturated vapour concentration (ca. 28.000 mg/m3) the maximum concentration factor of 0.065 for rat studies is used. The dose level of 300 ppm (1290 mg/m3) has been used as a surrogate for ED10 and this exposure leads to around 130 mg/kg bw/day. The corrected ED10 becomes 130 mg/kg bw/day * 0.065 = 8.45 mg/kg bw/day. The guidance suggest that the border for moving a chemical from the medium potency group with the generic concentration limit to the high potency group with a specific concentration limit is below 3-5 mg/kg bw/day. Consequently, the generic concentration limit appears relevant for styrene.

RAC evaluation of reproductive toxicity

Summary of the Dossier submitter's proposal

The present proposal includes classification for developmental toxicity (Repr. 1B; H360D "May damage the unborn child when exposed via inhalation" according to CLP, and Repr. Cat. 2; R61 "May cause harm to the unborn child" according to DSD).

Previous discussions
Styrene is a transitional substance and was discussed in the TC C&L group at a number of meetings. For reproductive toxicity no agreement could be reached at that time, and the case was handed-over to ECHA[v].

Key supporting studies
In a well-conducted OECD and GLP-compliant two-generation study in rats, which included developmental neurotoxicity assessment in F2 offspring, a pattern of developmental delays both before and after weaning (decreased body weights, delays in attaining some pre-weaning developmental landmarks, slight shift in the normal pattern of motor activity and delayed preputial separation), was evident mainly in the F2 pups of the high exposure group (500 ppm). In addition, decreased swimming abilities on post-natal day (PND) 24 and reductions in forelimb grip strength on PND 60 were found in both sexes. These data indicate that neuromotor functions were affected and are assessed as being mainly a direct consequence of the styrene exposure. Significantly decreased pup body weight during the lactation period was found at 150 ppm in the absence of maternal toxicity. The results of this study show that exposure to 500 ppm styrene causes developmental toxicity manifested as a pattern of developmental delays, including delayed neurological development, and developmental neurotoxicity effects on post-weaning behaviour, especially related to neuromotor functions. In contrast to the earlier investigations at 300 ppm, the exposure to 500 ppm induced some maternal toxicity (reductions in body weights of 7-8% and degeneration of the nasal olfactory epithelium). However, it is considered unlikely that the developmental toxicity is a non-specific secondary effect of the maternal toxicity.

Comments received during public consultation

[v] Follow up III of the meeting of the Technical Committee on Classification and Labelling in Arona, 26-28 September 2007 (Ispra, 29 May 2008)

No new experimental studies specifically addressing the toxicity of styrene were submitted during the Public Consultation, but industry stakeholders provided extensive comments, mainly addressing the interpretation of the studies, accompanied by additional references to published literature to support their arguments. A general comment expressed by industry stakeholders was that although the proposal draws on the studies already evaluated in the EU RAR, the dossier submitter has omitted relevant qualifying comments and negative criticism of studies important for assessing reproductive toxicity. RAC has therefore also considered the detailed industry stakeholders comments and consulted the EU RAR. Overall, RAC agrees with industry that the data are not sufficient for classification with Repr. 1B. Some responses to the industry stakeholders comments follow here.

Response to comments on effects on pup body weights (pages 25-30 in main document)

The industry stakeholders noted the following points:

- in addition to the effects on pup body weights, maternal body weights were also lower relative to controls, although generally not statistically significant,
- due to the variability in pup body weights, the statistically significant effects may still be chance findings,
- the apparently decreased body weights of the exposed pups are not actually caused by decreases in the exposed animals but rather are explained by the unusually high body weights of the control pups,
- with reference to a publication by Piersma *et al*. (2011), effects seen only in F2 pups and not in F1 pups must be chance findings,
- decreased pup body weights
    - were due to non-specific general toxicity and are not evidence of specific developmental toxicity,
    - may be caused by impaired maternal care, which may be a consequence of maternal olfactory degeneration and transient narcotic effects,
    - are of minor toxicological relevance and not a reason for classification.

The RAC has studied the detailed comments from industry, and agrees that the effects on body weights in F2 pups are rather small, but also notes that it is consistent in F2 pups exposed to 500 ppm styrene over time, and larger than the potential effects on the dams. There are therefore no reasons to not trust the statistical evaluation of the data, or to speculate on chance findings. One cannot totally rule out that the decreased pup body weight is caused by impaired maternal care, but on the other hand, there are little firm data to substantiate that olfactory degeneration or, if occurring, slight transient narcotic effects could lead to decreased growth of the offspring. The RAC is of the opinion that the decreased pup growth provides some evidence of developmental effects of styrene.

Response to comments on effects on relative pituitary gland weight (pages 47-51 in the main document)

The comments from industry stakeholders note:

- the great variability in pituitary gland weights in PND 21 pups, making comparisons of

weights very uncertain,

- that effects were only noted in F2 pups and not in F1 pups or adult F1 animals,
- that with reference to a publication by Piersma *et al.* (2011), effects seen only in F2 pups and not in F1 pups must be considered as chance findings,
- that if there is an effect on pituitary gland weight, the effect cannot be regarded as severe,
- that the lack of histopathological alterations in the pituitary gland raises questions regarding the relevance of the effect on pituitary weight,
- that if there is a decreased pituitary weight, it is a consequence of non-specific delay of development and not evidence of specific developmental toxicity.

The RAC has studied the detailed comments, and agrees that the variability of the pituitary gland weight at PND 21, combined with the fact that the effect only occurs in F2 males, makes the original finding less robust. Because of the magnitude of the effect (22%) and its possible relationship to a delay of development (the lack of histopathological findings would support a delayed development rather than pituitary toxicity), the finding cannot be disregarded. The RAC is of the opinion that the decreased pituitary gland weight gives some evidence of developmental effects of styrene.

Response to comments on effects on grip strength (pages 42-46 in the main document)

The comments from industry stakeholders note:

- the large variation in grip strength between animals, making comparisons between groups very uncertain, exemplified by increased grip strength in the 150 ppm group at day 45,
- that grip strength is not linearly correlated with body weight over time, making the body weight correlated conclusions uncertain,
- that the decreased body weight-correlated grip strength might be explained by larger decreases in body weight at earlier ages than when the grip testing was performed,
- the lack of consistency between results at different time points, as well as in hind-limb versus fore-limb grip strength data,
- that the data from this study are within the historical control data from that laboratory
- that the decreased grip strength does not correlate with any histopathological effects, this leads to a conclusion of doubtful significance of this minor effect, which may possibly be explained by a non-specific delay in development.

The RAC has noted the lack of consistency between time points, which is a weakness in the argument for this being a toxicologically significant effect. However, although the grip strength varies considerably, this is a well-established parameter and it is difficult to ignore the findings of a statistically significant effect on grip strength. The body weight correction might not be perfect, but seems sufficiently reliable to draw the conclusion that the effects on grip strength are larger than the effects on body weight. Histopathological findings could have strengthened the findings on grip strength, but the lack of pathology is not a sufficient reason to disregard the grip strength findings. A comparison with historical control data may be relevant when the effects lie at the

borderline of biological and/or statistical significance, but the RAC notes that in view of the magnitude of the effect in this case (24-28% for forelimb grip strength), the concurrent controls should be given greater weight than historical control data. Overall, the RAC is of the opinion that the decreased grip strength gives some evidence of developmental effects of styrene, although not warranting classification Repr. 1B as proposed by the DS.

Response to comments on swimming trials (pages 36-41 in the main document)

The comments from industry stakeholders note:

- that the increased swimming time in the short swim trial (straight channel; 10 sec) is of doubtful biological significance considering that there were no effects on swimming time in the longer swimming trial (time to escape; 50-150 sec),
- that the effect was confined only to the first of four trials,
- that historical control data would indicate an unusually short swimming time for the control group rather than long times for the groups exposed to styrene,
- that long swimming times can be related to lower body weights.

This led industry to the conclusion that if there is an effect, it has minor toxicological relevance, and could be caused by a non-specific delay of development associated with maternal toxicity combined with "chance" variation in data.

The RAC has noted that swimming time was affected only in the short swim and not in the long swim, but as no mechanism has been identified this is not considered a reason to disregard the effects. Furthermore, although the effect mainly arises in the first trial, the data for the first trial is rather convincing as a dose-related effect (approximately 13-16-17-21 and 15-21-18-24 seconds in males and females, respectively, of the control-50-150-500 ppm groups. It is likely that swim time correlates with body weight, but the effects at 500 ppm seems larger than expected based on the observed changes in body weights. Regarding historical control data, the RAC notes that if the effect would be caused by unusual control data, statistically significant effects would have been expected in all the exposed groups and not only in the 500 ppm group. Overall, while accepting that the data are not entirely consistent, the RAC is of the opinion that the increased swim time gives some evidence of developmental effects of styrene.

Other stakeholder comments

Very diverging views on this proposal were received, with one Member State and one Labour Union supporting the proposal, two Member States instead supporting classification as Repr. 2, one Member State expressing this as being a borderline case between Repr. 2 and no classification, and two Member States saying that there should be no classification for developmental toxicity. Those disagreeing with the proposal felt that the observed effects were not convincing or consistent between endpoints or generations, being rather mild, and were probably caused by maternal toxicity.

Assessment and comparison with the classification criteria

Key data and arguments that are relevant to the proposal
The proposal high-lights the effects in the two-generation study in rats as the main reasons for classification. The following findings in offspring are stressed in the dossier;

- a decreased pup growth in F2 offspring at 150 and 500 ppm (7-10% and 10-13%, respectively),

- a decreased relative pituitary gland weight (22% in F2 males),
- a decreased forelimb grip strength (24-28%), and
- an increased time to escape in straight channel swimming trials (38% in males).

RAC notes the dose-dependent decrease in pup weights in the second generation offspring (F2), and although there were some effects on the F1 maternal body weight at the top dose (reduction by 7-8%), the reduced growth of the pups at the mid dose supports that this could be a direct effect on the offspring. There were no effects on the weights of the first generation offspring (F1), even though F0 maternal weights were clearly affected (7-8%).

The relative pituitary weight was clearly decreased in males at the top dose, and of such a magnitude to indicate this to be an adverse effect even in the absence of any pathological findings. It has been argued in the PC comments that the large variability in the weight of the pituitary between animals at PND 21 (but not in adults) makes it difficult to draw firm conclusions from the mean values observed on PND 21. On the other hand, if it is the developmental rate of the pituitary that is affected, the lack of pathological findings may be consistent with the decreased weight. Although this finding may constitute some evidence of developmental effects, the robustness of this finding is decreased by the lack of pituitary effects in F2 females (PND 21) or the F1 generation (PND 21 offspring or adults).

Forelimb grip strength was reduced (24-28%) in both sexes at 500 ppm at day 60 (but not on days 22 and 45). The magnitude of the effect on day 60 was larger than the effect on body weight, potentially indicating a neuromuscular effect. However, it cannot be ruled out that the effect is caused by the decreased growth rate. Hind limb grip strength was decreased (18%) at day 45, but only in 500 ppm exposed males, and without any effects on days 22 and 60. Overall, the grip strength tests give some evidence of neuromuscular effects, but the findings are clearly weakened by only being observed on one of the three occasions when it was studied, and by the observed effects on forelimb and hind limb grip strength not occurring on the same occasions. Discrepancies between effects on fore and hindlimb strength have, however, been reported in other studies (Maurissen *et al*., 2003).

Time to escape in straight channel swimming trials is assumed to reflect swimming ability and motivation to escape. The time to escape was increased by 38% in males of the 500 ppm group at day 24. Effects of a similar magnitude were observed in the females, but were not statistically significant (information from the EU RAR). Findings of similar effects on day 62 when it was studied again would have strengthened this observation. However, the dossier notes that the positive controls PTU and methimazole also only affected this parameter on day 24 but not on day 62. It is not explained in the dossier why PTU and methimazole can be considered as "positive controls".

Observations of delayed development at 300 ppm in the two rat inhalation developmental studies are referred to as supporting information. RAC notes that in both studies there are observations of effects on time of eye opening, righting reflex, and incisor eruption, and that these effects fit the pattern of effects observed in the two-generation study. The use of pair-fed control dams in one of these studies indicates that the effects are not caused by a decreased pup growth rate. The two developmental studies have some methodological deficiencies and can only be used as supportive studies.

Comparison with criteria and RAC conclusions
The CLP criteria state that a substance should be placed in Category 1B 'Presumed human reproductive toxicant', when the data; "*provide clear evidence of an adverse effect on….. development in the absence of other toxic effects, or if occurring together with other toxic effects the adverse effect on reproduction is considered not to be a secondary non-specific consequence of other toxic effects. However, when there is*

mechanistic information that raises doubt about the relevance of the effect for humans, classification in Category 2 may be more appropriate".

For styrene, there are indications of effects on development but the rather inconsistent effects (e.g., decreased pup growth in F2 but not in F1, decreased grip strength only at some time points, and effects on swimming trials at day 24 but not on day 62) cannot qualify asthe 'clear evidence' required by the CLP. Furthermore, some relationship between decreased pup growth and the other effects cannot be completely ruled out. Thus, in the opinion of RAC, classification with Repr. 1B, H360 (CLP) is not appropriate. As the criteria for DSD are very similar to the CLP criteria, classification with Repr. Cat 2; R61 according to the DSD is likewise not warranted.

The CLP criteria state that a substance should be placed in Category 2 'Suspected human reproductive toxicant' when the data provide; "*some evidence from humans or experimental animals, possibly supplemented with other information…..on development, and where the evidence is not sufficiently convincing to place the substance in Category 1. If deficiencies in the study make the quality of evidence less convincing, Category 2 could be the more appropriate classification*. *Such effects shall have been observed in the absence of other toxic effects, or if occurring together with other toxic effects the adverse effect on reproduction is considered not to be a secondary non-specific consequence of the other toxic effects*".

The generally well performed two-generation study provided some evidence of long-lasting, delayed pup development, as exemplified by dose-dependently decreased F2 pup body weights at 150 and 500 ppm (10-13% at 500 ppm), a decreased pituitary weight in male 500 ppm F2 pups (22%), and decreased grip strength (24-28% forelimb grip strength) and swimming abilities at 500 ppm. In the weight of evidence assessment made by the RAC, it has been taken into consideration that styrene did not affect other parameters studied in the two-generation study. Two developmental studies in rats, in the absence of maternal toxicity, also indicated a delayed development of newborn pups (delayed eye opening, righting reflex, and incisor eruption), and decreased pup weights (8-11% at day 1 and 15% at day 21 at 300 ppm in one study and 8% at day 21 at 300 ppm in the other study) although there are some deficiencies in these studies. There might be a relationship between decreased pup growth and the other findings, but it is noted that the effect in the two-generation study on the pituitary weight, and decreased grip strength cannot be fully explained by the decreased growth rate. The possibility of the effects being caused by general pup toxicity rather than by specific developmental toxicity is discussed in the comments, but RAC finds it difficult to distinguish between the two based on the available data.

Maternal toxicity was also discussed in the comments received during the public consultation as potentially explaining the observed effects. Maternal effects were only noted at the top dose (500 ppm) in the two-generation study. They consisted of nasal toxicity and a reduced body weight gain, such that the final body weights of the females were 7-8 % lower than control weights in both F0 and F1. It is not likely that the maternal nasal toxicity can explain the effects noted on the pups. Likewise, the reduced maternal weight gain does not seem to be of a sufficient magnitude to constitute marked maternal toxicity or to explain the pup effects.

Whether the pup effects were caused by the pre- or postnatal exposure has also been raised, and it is acknowledged that it is always difficult to determine when such effects have been initiated. However, in the two-generation study, F2 pup body weights were reduced already on day 0 ("*decreases in body weight…were observed…throughout the pre-weaning period (PND 0-21*"). In the two developmental toxicity studies (where treatment of the dams stopped prior to birth), pup body weights were reduced and developmental landmarks were delayed, occurring later during the pre-weaning phase, indicating that the effects were attributable to the gestational exposure. Placental

transfer of styrene has also been shown in mice.

In adult rats, styrene causes ototoxicity (loss of hearing) and toxicity to the nasal epithelium. In humans, styrene causes hearing loss, affects colour vision and long-term exposure may also lead to brain damage (chronic encephalopathy). In rat pups, styrene consistently affects the growth of the pups, resulting in delayed development of the offspring. There are also indications of neurological/neuromuscular deficits in the offspring, and although there are inconsistencies in these data, these effects should be interpreted in the context of the neurotoxic effects of styrene on adult animals. There is evidence of developmental toxicity noted in three different studies.

Overall, RAC is of the opinion that there is sufficient evidence of developmental effects to warrant classification as Repr. 2, H361d (CLP).

The criteria also state that if the effects are considered to be of low or minimal toxicological significance (e.g., small effects on foetal weights, or small differences in postnatal developmental assessments), classification may not necessarily be the outcome. The types of effects observed in the styrene studies might initially suggest that this is a borderline case for classification, but RAC considers that the overall pattern of long-lasting developmental delays and neurological/neuromuscular deficits fulfill the requirements for classification with Repr. 2, H361d (CLP). As the criteria for reproductive toxicity classification under DSD are very similar to the CLP criteria, classification with Repr. Cat 3; R63, is warranted according to the DSD.

5 ENVIRONMENTAL HAZARD ASSESSMENT

6 OTHER INFORMATION

7 REFERENCES

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