Committee for Risk Assessment

RAC

Annex 1
Background document
to the Opinion proposing harmonised classification and labelling at EU level of

piperonyl butoxide (ISO); 2-(2-butoxyethoxy)ethyl 6-propylpiperonyl ether

EC Number: 200-076-7
CAS Number: 51-03-6

CLH-O-0000006819-59-01/F

The background document is a compilation of information considered relevant by the dossier submitter or by RAC for the proposed classification. It includes the proposal of the dossier submitter and the conclusion of RAC. It is based on the official CLH report submitted to 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

11 June 2020

ANNEX 1 - BACKGROUND DOCUMENT TO RAC OPINION ON PIPERONYL BUTOXIDE (ISO); 2-(2-BUTOXYETHOXY)ETHYL 6-PROPYLPIPERONYL ETHER

CLH report

Proposal for Harmonised Classification and Labelling

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

Substance Name:

2-(2-butoxyethoxy)ethyl 6-propylpiperonyl ether; piperonyl butoxide (ISO)

EC Number: 200-076-7

CAS Number: 51-03-6

Index Number: n/a

Contact details for dossier submitter:

General Chemical State Laboratory of Greece
16 Anastasiou Tsocha Str., 11521
Ampelokipi, Athens, Greece
Tel: 0030 210 6479286, 0030 210 6479408
Fax: 0030 210 6466917
Email: environment@gcsl.gr

Scientific Advisor:

Benaki Phytopathological Institute
8 Stefanou Delta Str., 145 61
Kifissia, Attica, Greece
Tel: 0030 210 8180334
Email: pcdepartment@bpi.gr

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Version number: 1.4 **Date: March 2019**

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PART A.

1 PROPOSAL FOR HARMONISED CLASSIFICATION AND LABELLING

1.1 Substance

Table 1: Substance identity

Substance name:	piperonyl butoxide (ISO); 2-(2-butoxyethoxy)ethyl 6-propylpiperonyl ether
EC number:	200-076-7
CAS number:	51-03-6
Annex VI Index number:	-
Degree of purity:	940 g/kg
Impurities:	Relevant impurities: Safrole (max. content 0.004% w/w) Dihydrosafrole (max. content ≤0.0085% w/w) Isosafrole (max. content <0.004% w/w) Dipiperonyl methane (max. content 1.95% w/w) Dipiperonyl ether (max. content 0.9%w/w) Methyl dihydrosafrole (max. content 0.5% w/w) Piperonyl Butoxide-x (Piperonyl Butoxide homologue) (max. content 0.47 % w/w) ortho-Piperonyl Butoxide (Piperonyl Butoxide homologue) (max. content 0.51 % w/w) N.N-dimethylformamide (max. content <0.04% w/w) Dichloromethane (max. content <0.05% w/w)

1.2 Harmonised classification and labelling proposal

Table 2: Harmonised classification and labelling proposal in line with CLP

Current entry in Annex VI, CLP Regulation	None
Current proposal for consideration by RAC	***STOT SE 3; H335 – May cause respiratory irritation*** ***Aquatic Acute 1; H400 – Very toxic to aquatic life*** ***Acute M-factor: 1*** ***Aquatic Chronic 1; H410 - Very toxic to aquatic life with long lasting effect*** ***Chronic M-factor: 1*** ***EUH066 – Repeated exposure may cause skin dryness or cracking***
Resulting harmonised classification (future entry in Annex VI, CLP Regulation)	***STOT SE 3; H335 – May cause respiratory irritation*** ***Aquatic Acute 1; H400 – very toxic to aquatic life***

	Acute M-factor: 1 ***Aquatic Chronic 1; H410 - very toxic to aquatic life with long lasting effect*** ***Chronic M-factor: 1*** ***EUH066 – Repeated exposure may cause skin dryness or cracking***

1.3 Proposed harmonised classification and labelling based on CLP Regulation

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	-	-	-	Conclusive but not sufficient for classification
2.2.	Flammable gases	-	-	-	Not relevant.
2.3.	Flammable aerosols	-	-	-	Not relevant.
2.4.	Oxidising gases	-	-	-	Not relevant
2.5.	Gases under pressure	-	-	-	Not relevant.
2.6.	Flammable liquids	-	-	-	Conclusive but not sufficient for classification
2.7.	Flammable solids	-	-	-	Not relevant.
2.8.	Self-reactive substances and mixtures	-	-	-	Not relevant.
2.9.	Pyrophoric liquids	-	-	-	Not relevant.
2.10.	Pyrophoric solids	-	-	-	Not relevant.
2.11.	Self-heating substances and mixtures	-	-	-	Not relevant.
2.12.	Substances and mixtures which in contact with water emit flammable gases	-	-	-	Not relevant.
2.13.	Oxidising liquids	-	-	-	Conclusive but not sufficient for classification
2.14.	Oxidising solids	-	-	-	Not relevant.
2.15.	Organic peroxides	-	-	-	Not relevant.
2.16.	Substance and mixtures corrosive to metals	-	-	-	Not relevant.
3.1.	Acute toxicity - oral	-	-	-	Conclusive but not sufficient for classification
	Acute toxicity - dermal	-	-	-	Conclusive but not

					sufficient for classification
	Acute toxicity - inhalation	-	-	-	Conclusive but not sufficient for classification
3.2.	Skin corrosion / irritation	-	-	-	Conclusive but not sufficient for classification
3.3.	Serious eye damage / eye irritation	-	-	-	Conclusive but not sufficient for classification
3.4.	Respiratory sensitisation	-	-	-	Conclusive but not sufficient for classification
3.4.	Skin sensitisation	-	-	-	Conclusive but not sufficient for classification
3.5.	Germ cell mutagenicity	-	-	-	Conclusive but not sufficient for classification
3.6.	Carcinogenicity	-	-	-	Conclusive but not sufficient for classification
3.7.	Reproductive toxicity	-	-	-	Conclusive but not sufficient for classification
3.8.	Specific target organ toxicity –single exposure	STOT SE 3; H335	-	-	-
3.9.	Specific target organ toxicity – repeated exposure	-	-	-	Conclusive but not sufficient for classification
3.10.	Aspiration hazard	-	-	-	-
4.1.	Hazardous to the aquatic environment	Aquatic Acute 1; H400 Aquatic Chronic 1; H410	Acute M-factor: 1 Chronic M-factor: 1	-	-
5.1.	Hazardous to the ozone layer	-	-	-	Not relevant

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

Labelling:

Signal word: Warning

GHS Pictograms:

GHS07 GHS09

Hazard statements:
H335: May cause respiratory irritation
H410: Very toxic to aquatic organisms with long lasting effects

Supplementary hazard wording:
EUH066: Repeated exposure may cause skin dryness or cracking

Precautionary statements:
To be considered as indicated in Regulation (EC) 1272/2008.

Proposed notes assigned to an entry: None

2 BACKGROUND TO THE CLH PROPOSAL

2.1 History of the previous classification and labelling

Piperonyl Butoxide is a synergist and a biocidal active substance in the scope of Biocidal Product Regulation (EC 528/2012), with Greece as evaluating Competent Authority (eCA). There is currently no harmonised classification for the active substance piperonyl butoxide in Annex VI of CLP.

2.2 Short summary of the scientific justification for the CLH proposal

Human Health Effects CLH proposal

Specific Target Organ Toxicity – Single Exposure

STOT SE 3: acute & 3-month inhalation toxicity studies in rats confirmed by human epidemiology data

No specific respiratory irritation (acute) study has been performed.

Epidemiological data of individuals exposed to products containing pyrethrins have revealed that respiratory symptoms such as bronchospasm, cough/choke, and dyspnea were more likely if the exposure included piperonyl butoxide (US-EPA, Memorandum, Review of Piperonyl butoxide Incident Reports, 2004). These symptoms are likely the reason for increased risk of moderate effects which typically would require medical attention. Other literature suggests that pyrethrin-based products may pose a hazard to asthmatics (Ellenhorn *et al.* 1997, Reigart and Roberts 1999, Wagner 2000).

Moreover, slight respiratory tract irritation evidenced as nasal discharge and laboured breathing accompanied by red foci in the lungs of 2/5 females was noted in the acute toxicity study by inhalation in rats (Anonymous - 5, 1991). In addition, in the 3-month inhalation study in rats red nasal discharge and histopathological alterations in the larynx including slight squamous metaplasia with minimal

hyperkeratosis and moderate inflammation were noted at 0.512 mg/L (Anonymous - 17, 1992). These findings are considered relevant as part of weight of evidence evaluation of the potential of Piperonyl butoxide to cause respiratory tract irritation and according to the criteria justify the STOT SE 3 classification.

Additional Labelling Provisions

EUH066: 21-day dermal study in New Zealand White rabbits

Irreversible skin effects (erythema, edema, desquamation, fissuring, red raised areas) were observed in the repeated dose dermal preliminary toxicity study in rabbits (Anonymous - 21, 1992) from the lowest dose tested (100 mg/kg bw/day).

Environmental Effects CLH proposal

Acute aquatic hazard: The acute (short-term) toxicity of Piperonyl Butoxide to aquatic organisms was investigated with fish, aquatic invertebrates (including daphnia, mysid shrimp and eastern oyster) and algae. The most sensitive species to Piperonyl Butoxide under acute exposure conditions was the eastern oyster *Crassostrea virginica* with a 96-hour EC_{50} of 0.23 mg Piperonyl Butoxide/L. Based on the critical acute toxicity endpoint of 0.23 mg Piperonyl Butoxide/L, Piperonyl Butoxide should be classified as "H400: Very toxic to aquatic organisms" (Aquatic Acute 1, M-factor = 1).

Chronic aquatic hazard: The chronic toxicity of Piperonyl Butoxide to aquatic organisms was investigated with fish, aquatic invertebrates (*Daphnia magna*), aquatic insects (*Chironomus riparius*) and algae. The most sensitive species to Piperonyl Butoxide under long-term exposure conditions was *Chironomus riparius* with a 28-day NOEC of 0.0148 mg Piperonyl Butoxide/L. Based on the critical chronic toxicity endpoint of 0.0148 mg Piperonyl Butoxide/L and the available information on the bioaccumulation potential (non-bioaccumulative in fish and other aquatic organisms) and readily biodegradability (not readily biodegradable), Piperonyl Butoxide should be classified as "H410: Very toxic to aquatic organisms with long lasting effects" (Aquatic Chronic 1, M-factor = 1).

2.3 Current harmonised classification and labelling

Currently there is no harmonised classification for Piperonyl Butoxide and it is not listed in Annex VI, Table 3.1 of the CLP Regulation.

Piperonyl butoxide is included in the Community Rolling Action Plan (CoRAP) list for substances that could pose risks to human health and the environment (http://echa.europa.eu/documents/10162/13628/corap_list_2016-2018_en.pdf). The initial grounds for concern were raised regarding potential endocrine disrupting properties and suspicion of being PBT.

In particular, in the Justification Document for the selection of a candidate CoRAP substance prepared for Piperonyl Butoxide (https://echa.europa.eu/documents/10162/3ea81042-f877-4caa-859d-955697c711cf) it is stated that *"In a 2-year rat carcinogenicity test amongst other effects, reduced growth and enlargement of ovaries, thyroid and small testes was noted"*.

It is noted that the aforementioned report does not include information on the study authors. Nevertheless, this CLH dossier includes 2-year rat carcinogenicity, 2-generation toxicity and developmental toxicity studies, evaluated in detail in respective sections. Considering the findings described it could be assumed that the same studies have been considered in the CoRAP document.

Overall, it is noted that effects in ovaries, testes and/or thyroid are only observed at doses where the MTD is exceeded in 2-year study in rats. Moreover, Piperonyl Butoxide is not a reproductive toxicant as presented in detail in section 4.11.

2.4 Current self-classification and labelling

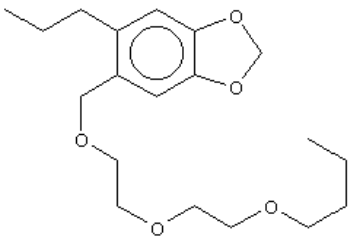
According to data presented in the C&L Inventory in the ECHA website there are currently 11 aggregated notifications.

RAC general comment

Piperonyl butoxide (PBO) is a synergist and a biocidal active substance in the scope of Biocidal Product Regulation (EC 528/2012). The insecticidal activity of pyrethroids is limited by metabolic hydrolysis and oxidation. Synergists such as piperonyl butoxide are inhibitors of the detoxifying enzymes; they may prolong the stability and enhance the potency of pyrethroids in insects.

There is currently no harmonised classification for the active substance piperonyl butoxide in Annex VI of CLP. Piperonyl butoxide is included in the Community Rolling Action Plan (CoRAP) list for substances that could pose risks to human health and the environment. The initial grounds for concern were raised regarding potential endocrine disrupting properties and suspicion of being PBT. PBO is used in a typical concentration of 94% and may contain several impurities of which four have a harmonised or self-classification as Carc. 1B, Carc. 2, or Repr. 1B. These are safrole, dihydrosafrole, N.N-dimethyl formamide, and dichloromethane. Since maximum contents of these impurities do not exceed 0.05% the dossier submitter (DS) concluded that classification of PBO is not affected.
PBO is extensively metabolised. Eight metabolites were isolated from urine and faeces and were characterised. These metabolites are formed through the oxidation and subsequent cleavage of the ether side chain and /or the methylene bridge on the 1,3-benzodioxole moiety.

The chemical structure of PBO is shown below:



3 JUSTIFICATION THAT ACTION IS NEEDED AT COMMUNITY LEVEL

Piperonyl Butoxide is an active substance approved under Biocidal Product Regulation (EC) No 528/2012 of the European Parliament and of the Council and a synergist. The Biocidal Products Committee (BPC) has issued the following opinion on the application for approval of the active substance Piperonyl Butoxide (PT18) ECHA/BPC/118/2016: https://echa.europa.eu/documents/10162/610a1218-bb3d-4ec7-935b-2ee581bf97c7

The classification and labelling proposal includes mammalian and environmental toxicity endpoints and needs to be evaluated under the CLP Regulation.

PART B.

scientific evaluation of the data

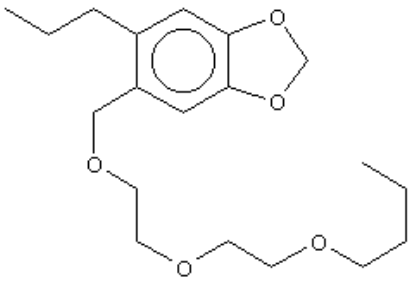
1 IDENTITY OF THE SUBSTANCE

1.1 Name and other identifiers of the substance

Table 4: Substance identity

EC number:	200-076-7
EC name:	piperonyl butoxide (ISO); 2-(2-butoxyethoxy)ethyl 6-propylpiperonyl ether
CAS number (EC inventory):	51-03-6
CAS number:	51-03-6
CAS name:	5-[[2-(2-butoxyethoxy)ethoxy]methyl]-6-propyl-1,3-Benzodioxole
IUPAC name:	5-{[2-(2-butoxyethoxy)ethoxy]methyl}-6-propyl-1,3-benzodioxole
CLP Annex VI Index number:	-
Molecular formula:	$C_{19}H_{30}O_5$
Molecular weight range:	338.43 g/mol

Structural formula:



1.2 Composition of the substance

Table 5: Constituents (non-confidential information)

Constituent	Typical concentration	Concentration range	Remarks
Piperonyl Butoxide	94%	-	-

Current Annex VI entry: -

Table 6: Impurities (non-confidential information)

Impurity	CAS number	Harmonised or Self classification	Typical concentration	Concentration range	Remarks
Safrole	94-59-7	Carc. 1B - H350; Muta. 2 - H341; Acute Tox. 4 - H302 (CLP00)	max. content 0.004% w/w	-	Does not affect classification
Dihydrosafrole	94-58-6	Carcinogen Group 2B (IARC) *	max. content ≤0.0085%	-	Does not affect classification
Isosafrole	120-58-1	- **	max. content <0.004% w/w	-	-
Dipiperonyl methane	34827-26-4	-	max. content 1.95% w/w	-	-
Dipiperonyl ether	37773-74-3	-	max. content 0.9%w/w	-	-
Methyl dihydrosafrole	4518-32-5	-	max. content 0.5% w/w	-	-
Piperonyl Butoxide-x (Piperonyl Butoxide homologue)	87437-43-2	-	max. content 0.47 % w/w	-	-
ortho-Piperonyl Butoxide (Piperonyl Butoxide homologue)	unknown	-	max. content 0.51 % w/w	-	-
N.N-dimethylformamide	68-12-2	Acute Tox. 4 - H312; Eye Irrit. 2 - H319; Acute Tox. 4 - H332; Repr. 1B - H360D (CLP00)	max. content <0.04% w/w	-	Does not affect classification
Dichloromethane	75-09-2	Carc 2 - H351 (CLP00)	max. content <0.05% w/w	-	Does not affect classification

* *"Dihydrosafrole given orally is carcinogenic in rats, in which it produces tumours of the oesophagus, and in mice, in which it produces liver tumours in males and an increased incidence of lung tumours in both males and females".* (IARC, 1998)

** *"Isosafrole is a weak rodent hepatocarcinogen; the carcinogenicity is probably mediated by a non-genotoxic mechanism. Isosafrole metabolites may give rise to only very low binding to liver DNA in mice. It cannot be excluded that high exposure to isosafrole may give rise to isomerisation of 3'hydroxy-isosafrole to 1'-hydroxysafrole, the proximate carcinogen metabolite of safrole. However, generally the exposure to isosafrole is estimated to be very low. A clear NOEL could not be demonstrated for hepatic effects in the long-term studies. Therefore, the Committee could not establish a TDI. The Committee notes that isosafrole occurs together with safrole, but at much lower concentrations. Any measure to restrict exposure to safrole in food would also cover isosafrole."* (Opinion of the Scientific Committee on Food on isosafrole (SCF/CS/FLAV/FLAVOUR/30 Final, 9 April 2003)).

Current Annex VI entry: -

Table 7: Additives (non-confidential information)

Additive	Function	Typical concentration	Concentration range	Remarks
-	-	-	-	-

Current Annex VI entry: -

1.2.1 Composition of test material

For details of the specification, which has been claimed confidential by the manufacturer, see Doc. III-A confidential of the draft Competent Authority Report.

1.3 Physico-chemical properties

Table 8: 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	Pale yellow transparent oily liquid	Mazza & Di Blasi, 2000	No comment. Visual.
Melting/freezing point	Practical experience has shown that the purified active substance Piperonyl Butoxide is a liquid both at ambient temperature and even at-10 °C.	-	Estimated.
Boiling point	Boiling temperature 203 °C at 2.78 mbar	Carloni et al., 2011	Measured. EU Method A.2 (Boiling Temperature).
Relative density	1.058 g/mL at 20 °C	Forster, 2000	Measured. EU Method A.3 (Relative Density).
Vapour pressure	2.11 x 10^{-5} Pa at 60 °C The calculated vapour pressure at 25 °C will be less than 1.33 x 10^{-5} Pa	Bowman, 1989	Measured. EPA Guideline D-63-9 gas saturation method (Vapour Pressure).
Surface tension	35.79 mN/m (active substance as supplied) temperature: 25 °C 50.39 mN/m (1% solution) temperature: 20 °C	Forster, 2000	Measured. EU Method A.5 (Surface Tension).
Water solubility	Solubility: 36.1 mg/L at 8.4 ºC and pH = 7.04	Bär, 2006	Measured. EU Method A.6 (Water Solubility)

	28.9 mg/L at 20.4 ºC and pH = 7.01 23.1 mg/L at 33.4 ºC and pH = 7.02 Solubility: 30.7 mg/L at 20.4 ºC and pH = 4.06 32.8 mg/L at 20.4 ºC and pH = 6.12 28.9 mg/L at 20.4 ºC and pH = 7.01 30.5 mg/L at 20.4 ºC and pH = 8.86		
Partition coefficient n-octanol/water	log P_{OW}= 4.8 temperature: 20 °C pH: 6.5	Bär, 2006	Measured. EU Method A.8 (Partition Coefficient)
Flash point	179.25 °C	Forster, 1999	Measured. EU Method A.8 (Flash Point)
Flammability	-	-	Not relevant.
Explosive properties	The test item has no danger of explosion.	Smeykal, 2004	Measured. EU Method A.14 (Explosive properties)
Self-ignition temperature	The self-ignition temperature of the test item is 265 °C.	Smeykal, 2004	Measured. EU Method A.15 (Auto-Ignition Temperature)
Oxidising properties	The test item has no oxidizing properties.	Tiemann, 2004	Estimated.
Granulometry	-	-	Not relevant.
Stability in organic solvents and identity of relevant degradation products	-	-	Data required.
Dissociation constant	Not required, because Piperonyl Butoxide contains no dissociative groups.	-	No comment.
Viscosity	28.7 mPa x stemperature: 20 °C 13.2 mPa x s temperature: 40 °C	Bär, 2006	Measured. OECD 114 (Viscosity of Liquids)

2 MANUFACTURE AND USES

2.1 Manufacture

Not relevant.

2.2 Identified uses

Insecticides, acaricides and products to control other arthropods (PT 18) – Biocide

3 CLASSIFICATION FOR PHYSICO-CHEMICAL PROPERTIES

Table 9: Summary table for relevant physico-chemical studies

Method	Results	Remarks	Reference
EEC A.9& EEC A.15	Flash point: 179.25 °C Self-ignition temperature: 265°C	None.	Forster, 1999 Smeykal, 2004
EEC A.14	The test item has no danger of explosion.	None.	Smeykal, 2004
Statement	The test item has no oxidizing properties.	None.	Tiemann, 2004

3.1 Insert hazard class when relevant and repeat section if needed

3.1.1 Summary and discussion of Piperonyl Butoxide

Piperonyl Butoxide does not fulfil the criteria for classification with respect to its physical and chemical properties.

3.1.2 Comparison with criteria

Not applicable.

3.1.3 Conclusions on classification and labelling

Based on the results of the physic-chemical studies no classification is proposed for Piperonyl Butoxide.

RAC evaluation of physical hazards

Summary of the Dossier Submitter's proposal

The CLH dossier contains three studies according to EC methods A.9, A.14 and A.15, plus a statement claiming the substance has no oxidising properties. Based on these data, the **DS concluded that piperonyl butoxide does not fulfil the criteria for classification with respect to its physical and chemical properties.**

Comments received during public consultation

No comments were received during the consultation

Assessment and comparison with the classification criteria

RAC re-evaluated the DS assessment of physical hazards and concludes the following.

Explosives

The substance does not contain any chemical groups that are indicative of explosive properties. A negative EC A.14 study is available as supportive evidence. **No classification is warranted**.

Flammable liquids

The substance has a flash point of 179.25 °C and an initial boiling point of 203 °C (at 2.78 mbar). As the flash point is above 60 °C, the substance does not fulfil the criteria for classification as flammable liquid. **No classification is warranted.**

Self-reactive substances and mixtures

The substance does not contain chemical groups indicative of explosives or self-reactive properties. **No classification is warranted.**

Pyrophoric liquids

No studies are available. In the absence of any information, **RAC is unable to conclude on this hazard class.**

Self-heating substances and mixtures

No suitable test data are available. According to the CLP guidance, a substance or mixture with a melting point below 160 °C should not be classified as self-heating. As the substance is a liquid at 20 °C, **no classification is warranted for this hazard class**.

Substances and mixtures which in contact with water emit flammable gases

The chemical structure of PBO does not contain metals or metalloids. Therefore, **no classification is warranted**.

2.13.

The substance chemical structure contains oxygen (no fluorine or chlorine), the oxygen atoms are chemically bound only to hydrogen and carbon atoms. Therefore, **no classification is warranted.**

Organic peroxides

The substance does not contain peroxide groups, therefore **no classification is warranted for this hazard class.**

Corrosive to metals

The substance does not contain acidic or basic functional groups, therefore **no classification is warranted for this hazard class.**

4 HUMAN HEALTH HAZARD ASSESSMENT

4.1 Toxicokinetics (absorption, metabolism, distribution and elimination)

4.1.1 Non-human information

- **Anonymous - 1 (1995)**

The absorption, distribution, metabolism and excretion (ADME) pattern of piperonyl butoxide was investigated in male and female Charles River CD rats, following administration of a single oral low (50 mg/kg bw), high (500 mg/kg bw) or a repeated oral low (50 mg/kg bw) dose of the radiolabelled compound.

Total recoveries ranged between 92.86% to 100.05% among the different dose groups. The majority of the radioactivity was excreted in the urine and faeces during the first 72 hours following administration of the ^{14}C-Piperonyl Butoxide. In all tested groups the majority of the administered radioactivity was excreted via faeces (percentages ranging from 54.76%-66.16% for both males and females). No differences were noted in the excretion pattern between the sexes. The high amount of radioactivity detected in faeces indicates that an enterohepatic circulation is involved in the excretion pattern of piperonyl butoxide. This assumption is confirmed by available literature data, which show that following intravenous administration piperonyl butoxide is largely eliminated into the bile (Fishbein, 1967 & 1969). This indicates that biliary excretion via faeces is a major route of excretion, and that the radioactivity present in faeces results from biliary metabolites (little or no parent compound was present). Therefore, biliary/faecal excretion is added to the urinary radioactivity to approximate the total bioavailability, and hence the systemic absorption. Based on the above, and taking into account the radioactivity recovered in urine and faeces the oral absorption of Piperonyl Butoxide accounts for ca 92%. In addition, rat, mice and goat data show that ca. 70-80% of a-carbon labelled Piperonyl Butoxide is eliminated via urine [Casida (1996) and Kamienski & Casida (1970) and Anonymous - 1 (1995)].

With regard to the radioactivity detected in tissues the highest portion of the administered radioactivity was found in the GI contents, carcass and liver. There was no indication of bioaccumulation of the test compound.

Piperonyl Butoxide is extensively metabolised. Eight metabolites (A, B, C, D, E, F, G & Z) were isolated from urine and faeces and characterised. These metabolites were formed through the oxidation and subsequent cleavage of the ether side chain and /or the methylene bridge on the

benzodiazole moiety. A proposed pathway of Piperonyl Butoxide metabolism is depicted in

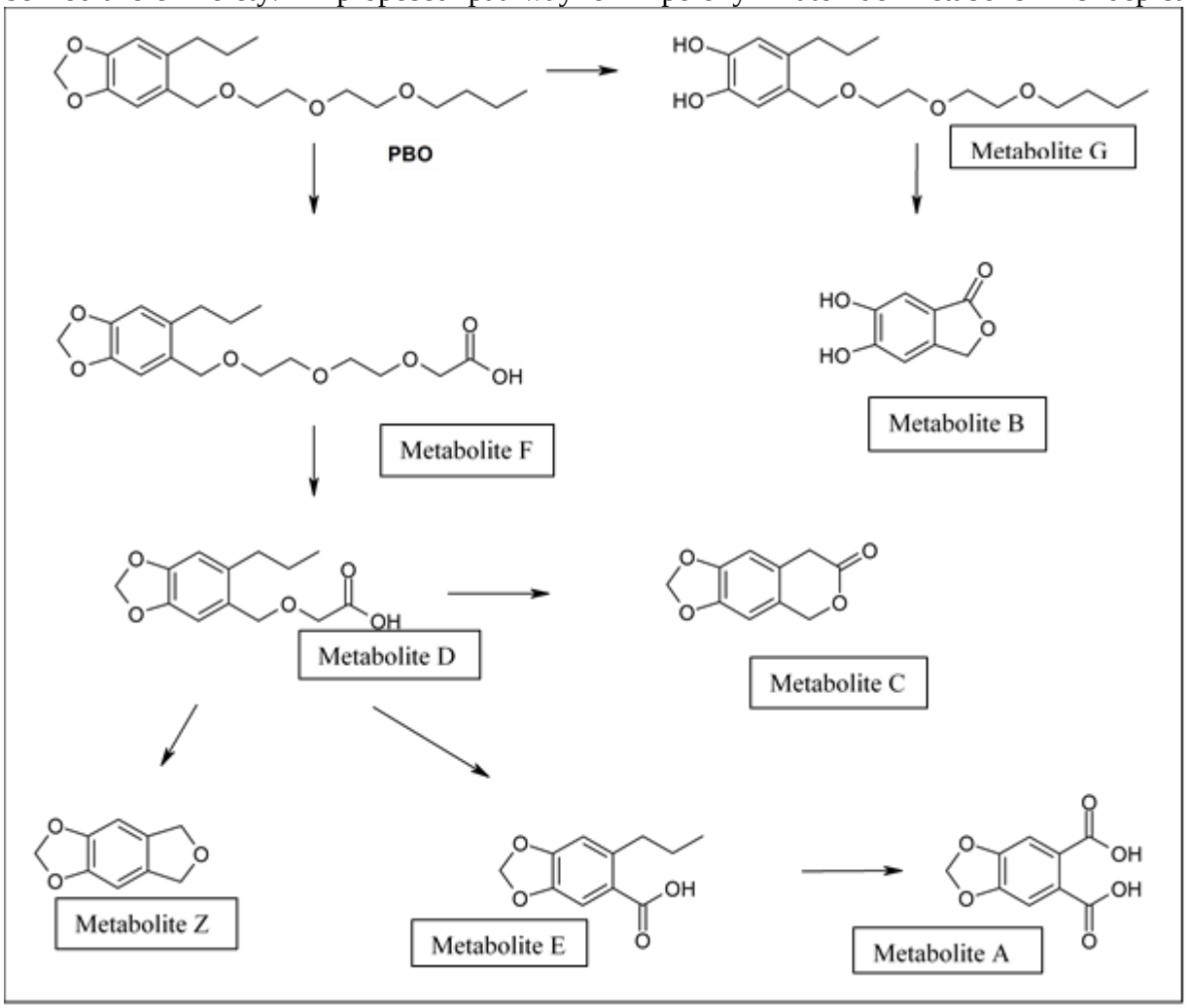


Figure 4-1 below:

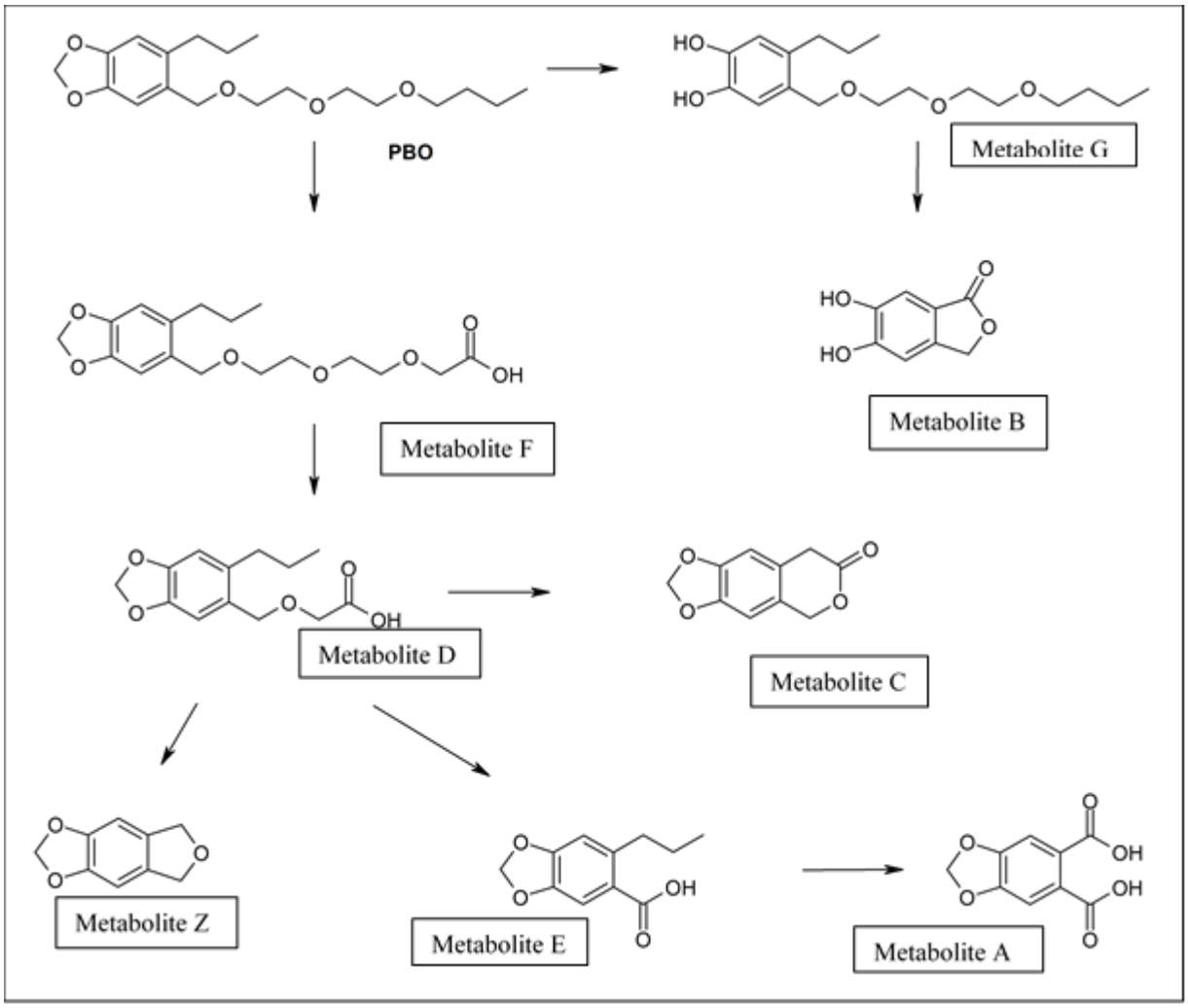


Figure 4-1: Proposed metabolic pathway for Piperonyl Butoxide in the rat

- **Anonymous - 2 (1999)**

In the dossier submitted to the eCA for the approval of Piperonyl Butoxide in the frame of Reg. (EC) No. 528/2012, there was an additional study (^{14}C-Piperonyl Butoxide: Rat-Metabolism following dosing at 50 and 500 mg/kg bw, AE F030295, **Anonymous - 2**, 1999). No Doc III was submitted for this study therefore, a detailed description cannot be given.

In brief, in this study the metabolites of BPO eliminated in urine and faeces following the administration of a single oral dose of either 50 or 500 mg/kg bw were isolated, identified and quantified.

In the results the following was reported:

"*The two major metabolites found in the faeces were Piperonyl Butoxide (M1) and the catechol M3, 4-{[2-(2-butoxyethoxy)ethoxy]mwthyl}-5-propyl-1,2-benzenediol. M1 accounted for 23.9% and 15.57% of the radioactivity present in the analysed excreta of male and female rats respectively, following a single oral dose of 500 mg Piperonyl Butoxide/kg bw M3 accounted for 19.69% and 17.36% of the radioactivity present in the analysed faecal excreta of male and female rats respectively, at the 500 mg dose level. The remaining faecal metabolites were M2, 4-{[2-(2-butoxyethoxy)ethoxy]methyl}-2-methoxy-5-propylphenol, a methylated catechol produced by a metabolic route not previously reported for Piperonyl Butoxide, M4, 2-(2-{2-[6-propyl-1,3-benzodioxol-5-yl)methoxy]ethoxy}ethoxy)ethanol and M5, 2-{2-[6-propyl-1,3-benzodioxol-5-*

yl)methoxy]ethoxy}ethanol. The structures of these metabolites were confirmed by mass spectrometry.

Metabolites M4 and M5 were produced by sequential oxidation of the 2-(2-butoxyethoxy)ethoxymethyl side chain of Piperonyl Butoxide.

The urine contained more than 20 metabolites as visualised on the HPLC radiotrace. The concentrations of each of these metabolites as a percentage of the original dose were low with none of the metabolites accounting more than 5% of the total dose. However, 12 of the metabolites (M1, M5, M6, M7, M8, M9, and M10, M11, M12, M14 (two isomers), M16) were identified by mass spectrometry. The identification of the 12 urinary and 3 faecal metabolites gave significant information to produce a metabolic profile for Piperonyl Butoxide in the rat".

4.1.2 Human information

Dermal absorption

A GLP study was conducted in human volunteers to determine the degree of dermal absorption of Piperonyl Butoxide from human skin: Radiolabelled ^{14}C-Piperonyl Butoxide was administered as a 3% (w/w) solution in isopropanol or as a 4% (w/w) solution in an aqueous formulation, to the forearm of four healthy volunteers at dose levels of 3.0 mg and 3.8 mg, respectively, corresponding to 40 µCi. After eight hours of exposure (non-occlusive) skin sites were washed and surface layers of the skin was stripped at 1h, 23 and 45h after removal of the wrapping.

Mean total recovery of administered recovery was 100.86% for the subjects administered Piperonyl Butoxide in isopropanol, and 104.22% for the subjects administered Piperonyl Butoxide in aqueous formulation.

Radioactivity recovered in urine and faeces was 1.78% and 0.46% respectively, for subjects administered Piperonyl Butoxide in isopropanol and 0.47% and 0.05% respectively, for subjects administered Piperonyl Butoxide in aqueous formulation. Less than 0.2% of administered dose was recovered from tape strips following either treatment, quickly decreasing in the day 2 and day 3 strips. Plasma samples showed evidence of minimal absorption. Dermal absorption accounted for 2.4% and 0.58% for subjects administered Piperonyl Butoxide in isopropanol and aqueous formulation, respectively taking into account the radioactivity detected in urine, faeces and tape-strips.

HPLC analysis of urinary samples indicated that Piperonyl Butoxide was quantitatively metabolised prior to excretion. At least six metabolites were detected but these were not identical with rat metabolites identified in a previous study.

It is noted that a publication on dermal absorption of Piperonyl Butoxide in human volunteers was also available [Wester et al. (1994)]. However, it was considered of limited validity due to major deficiencies i.e. it was not a GLP study, the technical material used was of unknown purity and the duration of exposure was too short compared to what is recommended in the agreed protocols for dermal absorption.

4.1.3 Summary and discussion on Toxicokinetics

^{14}C-Piperonyl Butoxide was readily absorbed and within 72 hours nearly completely excreted in the urine and faeces, mainly in form of metabolites in the rat. An oral absorption value of 100% has been set. Accumulation in tissues did not occur. Major pathways of metabolization are identified, by oxidation and hydrolysis of the glycol ether side chain, the propyl side chain or the heterocyclic methylenedioxy-ring.

Dermal absorption was investigated in human volunteers and determined to be less than 1% from aqueous and less than 3% from solvent based preparations.

4.2 Acute toxicity – Oral Route

4.2.1 Short summary and overall relevance of the provided information on acute oral toxicity

Piperonyl Butoxide exhibited low acute oral toxicity to male and female rats. The acute oral LD_{50} value was found to be equal to 4570 and 7220 mg/kg bw in male and female rats, respectively. Clinical signs of toxicity in surviving animals included yellow anogenital staining, ruffled fur, lethargy and sometimes dark nasal (and ocular) staining and ruffled skin.

Table 10: Summary table of relevant acute toxicity studies

<table>
<tr><th>Method</th><th>Results</th><th>Remarks</th><th>Reference</th></tr>
<tr>
<td>Acute Oral Rat
US EPA 81-1, OECD 401
Batch No. FEP-100 Task Force II Blend
Doses tested males (mg/kg): 2010, 3010, 3700, 5000 & 7500
Doses tested females (mg/kg): 2010, 3010, 5000, 7500 & 11250

Purity: 90.78%, GLP</td>
<td>
<table>
<tr><th colspan="6">Males (No of animals: 5/dose group)</th></tr>
<tr><th>Doses (mg/kg)</th><th>2010</th><th>3010</th><th>3700</th><th>5000</th><th>7500</th></tr>
<tr><td>Mortality</td><td>0/5</td><td>0/5</td><td>1/5</td><td>3/5</td><td>5/5</td></tr>
<tr><th colspan="6">Females (No of animals: 5/dose group)</th></tr>
<tr><th>Doses (mg/kg)</th><th>2010</th><th>3010</th><th>5000</th><th>7500</th><th>11250</th></tr>
<tr><td>Mortality</td><td>0/5</td><td>0/5</td><td>1/5</td><td>2/5</td><td>5/5</td></tr>
</table>
Male: LD_{50} 4570 mg/kg bw
Female: LD_{50} 7220 mg/kg bw
</td>
<td>Klimisch score: 1</td>
<td>Anonymous - 3 (1991a) (Piperonyl Butoxide CAR Doc IIIA6.1.1/01)</td>
</tr>
</table>

In the REACH registration dossier (individual submission), another acute oral study (2007) is included. No details regarding the batch number of the technical Piperonyl Butoxide used in this study is provided and thus its relevance to the impurity profile stated in the identity section to this report (Table 1), is unknown. The following have been concluded by the registrant:

In an acute oral toxicity study according to GLP and TG OECD 423 a group of 6 female rats received a single dose of 2000 mg/kg bw neat Piperonylbutoxide by gavage. There were no mortalities and no clinical signs. Body weights were not affected and no pathological changes were observed. LD_{50} (rat, oral) exceeded 2000 mg/kg bw.

4.2.2 Comparison with criteria

Based on the results of the available acute toxicity studies the acute oral LD_{50} value is above the lowest classification category criteria according to the CLP Regulation i.e.: Oral Category 4: 300 < ATE < 2 000 (mg/kg bw).

4.2.3 Conclusions on classification and labelling for acute oral toxicity

CLP: No classification based on available data.

4.3 Acute toxicity – Dermal Route

4.3.1 Short summary and overall relevance of the provided information on acute dermal toxicity

Dermal application of 2000 mg/ kg bw Piperonyl Butoxide did not cause death or systemic clinical signs of toxicity.

Table 11: Summary table of relevant acute toxicity studies

<table>
<tr><th>Method</th><th>Results</th><th>Remarks</th><th>Reference</th></tr>
<tr>
<td>Acute Dermal Rabbit
US EPA 81-2, OECD 402
Batch No. FEP-100 Task Force II Blend
Dose tested (males and females): 2000 mg/kg
Purity: 90.78%, GLP</td>
<td>Male & Female:
LD_{50} > 2000 mg/kg bw
<table>
<tr><th colspan="2">Males (No of animals: 5)</th></tr>
<tr><td>Dose (mg/kg)</td><td>2000</td></tr>
<tr><td>Mortality</td><td>0/5</td></tr>
<tr><th colspan="2">Females (No of animals: 5)</th></tr>
<tr><td>Dose (mg/kg)</td><td>2000</td></tr>
<tr><td>Mortality</td><td>0/5</td></tr>
</table>
</td>
<td>Klimisch score: 1</td>
<td>Anonymous - 4
(1991b)
(Piperonyl Butoxide CAR Doc IIIA6.1.2/01)</td>
</tr>
</table>

In the REACH registration dossier (individual submission), another acute dermal study (2007) is included. No details regarding the batch number of the technical Piperonyl Butoxide used in this study is provided and thus its relevance to the impurity profile stated in the identity section to this report (Table 1), is unknown. The following have been concluded by the registrant:

In an acute dermal toxicity study according to GLP and TG OECD 402 a group of 5 female and 5 male rats received a single dose of 2000 mg/kg bw of neat Piperonylbutoxide by the dermal route. There were no mortalities and no clinical signs. Bodyweights were not affected and no pathological changes were observed. LD_{50}(rat, dermal) exceeded 2000 mg/kg bw.

4.3.2 Comparison with criteria

Based on the results of the available acute toxicity study the acute dermal LD50 value is above the lowest classification category criteria according to the CLP Regulation i.e.: Dermal Category 4: 1 000 < ATE < 2 000 (mg/kg bw).

4.3.3 Conclusions on classification and labelling for acute dermal toxicity

CLP: No classification based on available data.

4.4 Acute toxicity – Inhalation Route

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

No mortalities occurred after acute inhalation exposure to Piperonyl Butoxide as an aerosol to rats. LC_{50} exceeding 5.9 mg/L Piperonyl Butoxide.

Table 12: Summary table of relevant acute toxicity studies

<table>
<tr><th>Method</th><th>Results</th><th>Remarks</th><th>Reference</th></tr>
<tr>
<td>Acute inhalation Rat
US EPA 81-3
Batch No. FEP-100 Task Force II Blend
Dose tested (males and females): 5.9 mg/L
Purity: 90.78%, GLP</td>
<td>Male & Female:
LC_{50} > 5.9 mg/L
<table>
<tr><th colspan="2">Males (No of animals: 5)</th></tr>
<tr><td>Dose (mg/L)</td><td>5.9</td></tr>
<tr><td>Mortality</td><td>0/5</td></tr>
<tr><th colspan="2">Females (No of animals: 5)</th></tr>
<tr><td>Dose (mg/L)</td><td>5.9</td></tr>
<tr><td>Mortality</td><td>0/5</td></tr>
</table>
</td>
<td>Klimisch score: 1</td>
<td>Anonymous - 5
(1991)
(Piperonyl Butoxide CAR Doc IIIA6.1.3/01)</td>
</tr>
</table>

In the REACH registration dossier (individual submission), another acute inhalation study (2007) is included. No details regarding the batch number of the technical Piperonyl Butoxide used in this study is provided and thus its relevance to the impurity profile stated in the identity section to this report (Table 1), is unknown. The following have been concluded by the registrant:

In an acute inhalation toxicity study according to GLP and TG OECD 403 a group of 5 male and 5 female rats was exposed to an aerosol of 5.2 mg Piperonylbutoxide/L air for 4h. There were no mortalities and clinical signs were confined to slightly reduced motility, slight ataxia and slight dyspnoea. Bodyweights were not affected and no pathological changes were observed. LC_{50} exceeded 5.2 ± 0.2 mg/L air.

4.4.2 Comparison with criteria

Based on the results of the available acute toxicity study the acute inhalation LC_{50} value is above the lowest classification category criteria according to the CLP Regulation i.e.: Inhalation Category 4: 1.0 < ATE < 5.0 (mg/L, dust & mist).

4.4.3 Conclusions on classification and labelling for acute inhalation toxicity

CLP: No classification based on available data.

RAC evaluation of acute toxicity

Summary of the Dossier Submitter's proposal

An acute **oral** toxicity study in rats performed according to OECD TG 401 and under GLP conditions, provided LD_{50} values of 4570 mg/kg bw and 7220 mg/kg bw in males and females, respectively. In the REACH registration dossier, another study was mentioned which the DS summarised as according to OECD TG 423 and GLP conforming. A single dose of 2000 mg/kg bw of neat PBO by gavage did not provoke mortalities in female rats.

The DS concluded that **no classification** for **Acute Oral Toxicity** is warranted.

In an acute **dermal** toxicity study in rabbits according to OECD TG 402 and GLP conditions, none of the animals died at a dose of 2000 mg/kg bw. A second study from the REACH registration dossier also performed according to OECD TG 402 and under GLP conditions, gave the same result.

The DS concluded that **no classification** for **Acute Dermal Toxicity** is warranted.

None of the five male and five female rats died in an acute **inhalation** toxicity study at an aerosol concentration of 5.9 mg/L. The study was performed according to US EPA guideline 81-3 and under GLP conditions. The DS also summarised another acute inhalation toxicity study from the REACH registration dossier. This study was GLP and OECD TG 403 conforming. No mortalities were observed at an aerosol concentration of 5.2 mg PBO/L air over 4 hours.

The DS concluded that **no classification** for **Acute Inhalation Toxicity** is warranted.

Comments received during public consultation

No comments on acute toxicity were received during public consultation.

Assessment and comparison with the classification criteria

LD_{50} values in two acute oral toxicity studies in rats were above the upper guidance value for category 4 (2000 mg/kg bw). Clinical signs included yellow anogenital staining, ruffled fur, lethargy, dark nasal and ocular staining, and ruffled skin.

No deaths or clinical signs were observed in two acute dermal toxicity studies in rabbits and rats at the upper boundary of category 4 (2000 mg/kg bw).

No deaths occurred at concentrations above 5 mg/L (upper boundary for category 4) in two acute inhalation toxicity studies in rats with an exposure time of 4 hours. In one study with 5.9 mg PBO/L air nasal discharge, excessive salivation, eye closure, and decreased activity were noted during exposure. Excessive lacrimation and salivation, nasal discharge, and laboured breathing were observed during the first week of observation. Most of the symptoms decreased during the second week. In the second study, with 5.2 mg PBO/L air, slightly reduced motility, slight ataxia, and slight dyspnoea were recorded.

Overall, RAC concurs with the DS that based on available data, **no classification for Acute Toxicity is warranted.**

4.5 Skin corrosion/Irritation

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

Table 13: Summary table of relevant skin irritation studies

Method	Results	Remarks	Reference
Skin irritation in NZW rabbits* US EPA 81-5, OECD 404 Batch No. FEP-100 Task Force II Blend Purity: 90.78%, GLP	Non-irritant	Klimisch score: 1 Slight erythema reversible within 24 hours.	**Anonymous - 6** (1991a) (Piperonyl Butoxide CAR Doc IIIA6.1.4/01)

* three males and three females

In the REACH registration dossier (individual submission), another skin irritation study (2007) is included. No details regarding the batch number of the technical Piperonyl Butoxide used in this study is provided and thus its relevance to the impurity profile stated in the identity section to this report (Table 1), is unknown. The following have been concluded by the registrant:

In a primary skin irritation study according to GLP and TG OECD 404 0.5 mL of the test substance were applied to the shaved skin of three male rabbits for 4 h with semi-occlusive dressing. No oedema and were noted. Erythema were very slight (score 1) at 1 and 24 h after patch removal for all animals and for one animal at 48 h. At 72 h there were no more skin reactions. PBO is not irritating to the skin.

4.5.1.1 Non-human information

Dermal application of undiluted Piperonyl Butoxide to the skin of albino rabbits induced very slight erythema in four out of six animals (Anonymous - 6, 1991a). No oedema was formed. No irritation effects were observed 24h post exposure.

Table 14: Mean skin irritation scores (Anonymous - 6, 1991a)

		Rabbit No					
	Time	**1**	**2**	**3**	**4**	**5**	**6**
Erythema	After 24 hours	0	0	0	0	0	0
	After 48 hours	0	0	0	0	0	0
	After 72 hours	0	0	0	0	0	0
	Mean score 24-72 hr	0	0	0	0	0	0
Oedema	After 24 hours	0	0	0	0	0	0
	After 48 hours	0	0	0	0	0	0
	After 72 hours	0	0	0	0	0	0
	Mean score 24-72 hr	0	0	0	0	0	0

4.5.1.2 Human information

No data.

4.5.1.3 Summary and discussion of skin irritation/corrosion

Slight erythema was observed on the skin of four out of six albino rabbits. This was fully reversible within 24 hours.

4.5.2 Comparison with criteria

Only slight sighs of irritation were observed in rabbits, which were reversed within 24 hours. Estimated skin irritation scores (0.00) are below the criteria triggering classification and labelling (according to CLP).

4.5.3 Conclusions on classification and labelling for skin corrosion/irritation

CLP: No classification based on the available data.

RAC evaluation of skin corrosion/irritation

Summary of the Dossier Submitter's proposal

In one skin irritation study in rabbits according to OECD TG 404 and performed under GLP conditions with undiluted PBO, all mean scores for erythema and oedema were 0 for three males and three females. In another skin irritation study, the substance was applied to the shaved skin of three male rabbits for 4 hours under semi-occlusive dressing. No oedemas were observed, very slight erythema which were present at the 1- and 24-hours observations in all animals and in one animal at the 48 hours observation, were resolved after 72 hours.

The DS concluded that **no classification** for skin irritation is warranted.

Comments received during public consultation

No comments were received on this endpoint.

Assessment and comparison with the classification criteria

In one guideline conforming study no irritation was observed in three male and three female rabbits. In another guideline conforming study with three male rabbits very slight erythema, reversible after 72 hours post-exposure, was observed. All scores were below classification criteria (mean scores at least 2.3 in 2 out of 3 tested animals).

Thus, RAC concurs with the DS that **no classification for Skin Irritation/ Corrosion is warranted.**

4.6 Serious eye damage/eye irritation

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

Table 15: Summary table of relevant eye irritation studies

Method	Results	Remarks	Reference
Eye irritation in NZW rabbits* US EPA 81-4, OECD 405 Batch No. FEP-100 Task Force II Blend Purity: 90.78%, GLP	Non-irritant	Klimisch score: 1 Slight reversible conjunctival redness and chemosis.	**Anonymous - 7** (1991) (Piperonyl Butoxide CAR Doc IIIA6.1.4/02)

* Sex of animals not stated in the study report

In the REACH registration dossier (individual submission), another eye irritation study (2007) is included. No details regarding the batch number of the technical Piperonyl Butoxide used in this study is provided and thus its relevance to the impurity profile stated in the identity section to this report (Table 1), is unknown. The following have been concluded by the registrant:

In a primary eye irritation study according to TG OECD 405 and GLP 0.1 mL PBO were instilled into one eye of each of three Himalayan rabbits. Treated eyes were washed after 24 h. There were no systemic reactions. Corneal opacity was observed in two animals at 24 and 48 h (grade 2) and 72 h (grade 1) after instillation. Irritation of the iris (grade 1) was observed in two animals at 24 and 48 h after instillation. Conjunctival redness (grade 1) was observed in all animals at 1 and 24 h, in two animals until 48 h after instillation. Chemosis (grade 1) was observed in two animals at 24 h and in one anima at 48 h after instillation. No classification and labelling with regard to eye irritation is required.

4.6.1.1 Non-human information

After ocular instillation of undiluted Piperonyl Butoxide to albino rabbits slight conjunctival redness (grade 1) was observed one hour after instillation (Anonymous - 7, 1991). Within the first 24 hours the symptoms became milder (redness grade 1, at 2/6 animals). Slight conjunctival chemosis (grade 1) was also observed one hour after instillation in 4 out of 6 albino rabbits. The signs disappeared within 24 hours after treatment. Within 48 hours the majority of animals did not show effects any longer. Within 72 hours all animals were free from irritation. The cornea and iris were not affected at all. Piperonyl Butoxide is considered as slightly irritant to the rabbit eye.

Table 16: Primary eye irritation scores (Anonymous - 7, 1991)

		Rabbit No					
	Time	**1**	**2**	**3**	**4**	**5**	**6**
Cornea opacity	After 24 hours	0	0	0	0	0	0
	After 48 hours	0	0	0	0	0	0
	After 72 hours	0	0	0	0	0	0
	After 4 days	0	0	0	0	0	0
	After 7 days	0	0	0	0	0	0
	Mean score 24-72 hr	0	0	0	0	0	0
Iris	After 24 hours	0	0	0	0	0	0
	After 48 hours	0	0	0	0	0	0
	After 72 hours	0	0	0	0	0	0
	After 4 days	0	0	0	0	0	0
	After 7 days	0	0	0	0	0	0
	Mean score 24-72 hr	0	0	0	0	0	0
Conjunctivae Redness	After 24 hours	0	0	1	1	0	0
	After 48 hours	0	0	1	0	0	0
	After 72 hours	0	0	0	0	0	0
	After 4 days	0	0	0	0	0	0
	After 7 days	0	0	0	0	0	0
	Mean score 24-72 hr	0	0	0.67	0.33	0	0
Conjunctivae Chemosis	After 24 hours	0	0	0	0	0	0
	After 48 hours	0	0	0	0	0	0
	After 72 hours	0	0	0	0	0	0
	After 4 days	0	0	0	0	0	0
	After 7 days	0	0	0	0	0	0
	Mean score 24-72 hr	0	0	0	0	0	0
Conjunctivae Discharge	After 24 hours	0	0	0	0	0	0
	After 48 hours	0	0	0	0	0	0
	After 72 hours	0	0	0	0	0	0
	After 4 days	0	0	0	0	0	0
	After 7 days	0	0	0	0	0	0
	Mean score 24-72 hr	0	0	0	0	0	0

4.6.1.2 Human information

No data.

4.6.1.3 Summary and discussion of serious eye damage/eye irritation

Slight reversible conjunctival redness and chemosis were observed one hour after instillation. Within 48 hours the majority of animals did not show effects any longer. Within 72 hours all animals were free from irritation. The cornea and iris were not affected at all.

4.6.2 Comparison with criteria

The mean eye irritation scores are below the criteria for classification as irritating to the eyes according to CLP, (conjunctival redness ≥ 2 only effect observed).

4.6.3 Conclusions on classification and labelling for serious eye damage/eye irritation

CLP: No classification based on available data.

RAC evaluation of serious eye damage/irritation

Summary of the Dossier Submitter's proposal

The DS summarised one OECD TG 405 study in New Zealand White (NZW) rabbits and cited the summary of another in Himalayan rabbits from the REACH registration dossier. Both studies were performed under GLP conditions.

One hour after instillation of undiluted PBO slight conjunctival redness was observed in all animals and slight chemosis in four out of six animals in the first study. Symptoms resolved within 72 hours in all affected rabbits.

In the second study, symptoms were slightly more severe: corneal opacity was observed in two animals up to 72 hours after instillation, slight iritis, slight conjunctival redness, and slight chemosis were observed in at least two animals up to 48 hours after instillation.

Because mean scores for observed effects were below guidance values for eye irritation in the first study, the DS concluded that **no classification for Serious Eye Damage/Irritation is warranted.**

Comments received during public consultation

No comments on this endpoint were received.

Assessment and comparison with the classification criteria

In one eye irritation study in NZW rabbits, the only effect observed after instillation of undiluted PBO was conjunctival redness in all six animals after one hour, in two after 24 hours, and in one after 48 hours. Means scores for the two affected animals at 24 hours were 0.67 and 0.33, respectively.

In the eye irritation study from the REACH registration dossier, the following grades were observed (table below). RAC notes that scores for conjunctival redness at the 72-h observation were not stated in the CLH report.

Table: *Scores for effects observed in the registration dossier study as provided in the registered substances factsheet on the ECHA website.*

Effect	Time point/ hours after instillation	Number of animals out of three (grade)
Corneal opacity	24	2 (grade 2)
	48	2 (grade 2)
	72	2 (grade 1)
	mean scores	**1.67; 1.67; 0**
Iritis	24	2 (grade 1)
	48	2 (grade 1)
	72	0
	mean scores	**0.67; 0.67; 0**
Conjunctival redness	24	3 (grade 1)
	48	2 (grade 1)
	72	2 (grade 1)
	mean scores	**1.0; 1.0; 0.33**
Chemosis	24	2 (grade 1)
	48	1 (grade 1)
	72	0
	mean scores	**0.67; 0.33; 0**

RAC notes that the registrant (and DS) concluded that no classification was required based on these study results. However, the study was conducted before the introduction of the CLP regulation in 2008.

According to CLP guidance substances should be classified as eye irritants, when mean scores for corneal opacity are ≥ 1, and/or ≥ 1 for iritis, and/or ≥ 2 for conjunctival redness, and/or ≥ 2 for chemosis in at least two out of three tested animals. A mean score of 1.67 for corneal opacity were observed in two out of three animals. **Classification as Eye Irrit. 2, H319 is warranted** according to classification criteria.

4.7 Respiratory sensitisation

No data on respiratory sensitisation is available.

RAC evaluation of respiratory sensitisation

Summary of the Dossier Submitter's proposal

Data on respiratory sensitisation are not available. RAC notes that the DS provided "conclusive but not sufficient for classification" as reason for no classification in table 3 of the CLH report.

Comments received during public consultation

No comments on this endpoint were received.

Assessment and comparison with the classification criteria

RAC was unable to assess this hazard class due to lack of data.

4.8 Skin sensitisation

4.8.1 Short summary and overall relevance of the provided information on skin sensitisation

Table 17: Summary table of relevant skin sensitisation studies

<table>
<tr><th>Method</th><th>Results</th><th>Remarks</th><th>Reference</th></tr>
<tr>
<td>Guinea pig
US EPA 81-6 (1984), OECD 406 (1981), Modified Buehler method
Batch No. FEP-100 Task Force II Blend
Dose for induction: used as delivered (100%)
Dose for challenge: used as delivered (100%)
Purity: 90.78%, GLP</td>
<td>Not sensitising
<table>
<tr><th></th><th colspan="3">Number of animals with signs of allergic reactions / number of animals in group</th></tr>
<tr><th></th><th>Negative control</th><th>Test group</th><th>Positive control</th></tr>
<tr><td>scored after 24h</td><td>0 / 10</td><td>0 / 10</td><td>9 / 10</td></tr>
<tr><td>scored after 48h</td><td>0 / 10</td><td>0 / 10</td><td>8 / 10</td></tr>
</table>
</td>
<td>Klimisch score: 1</td>
<td>Anonymous - 8
(1991b)
(Piperonyl Butoxide CAR Doc IIIA6.1.5/01)</td>
</tr>
</table>

In the REACH registration dossier (individual submission), another skin sesnitization study (2007) is included. No details regarding the batch number of the technical Piperonyl Butoxide used in this study is provided and thus its relevance to the impurity profile stated in the identity section to this report (Table 1), is unknown. The following have been concluded by the registrant:

In a dermal sensitization study PBO in sesame oil was tested in a group of young Dunkin-Hartley guinea pigs using the method of Magnusson and Kligman. Benzocaine in a parallel served as positive control material. No signs of systemic toxicity were noted. No skin sensitization was induced by the test material. In this study, Piperonyl butoxide was not a dermal sensitiser.

4.8.1.1 Non-human information

In a modified Buehler sensitisation assay, a group of 10 adult male Hartley guinea pigs were tested with a 6-hour application of undiluted Piperonyl Butoxide, which had been determined in screening studies to be non-irritating. Nine applications of 0.4 mL per site, every other day, were performed over a 3-week period under occluded conditions. After the 9th induction, the animals were rested for a two-week period. A challenge application was applied for 6 hours. A naive group (previously not exposed to Piperonyl Butoxide) was tested at the time of the challenge similar to the challenged guinea pigs. An additional group of guinea pigs were studied using 1-chloro-2,4-dinitrobenzene (DNCB), as a positive control.

The naive positive control animals were negative at challenge. For the DNCB positive control sensitisation was demonstrated.

Piperonyl Butoxide exhibited no sensitizing potential under the conditions of the Modified Buehler Test.

4.8.1.2 Human information

No data.

4.8.1.3 Summary and discussion of skin sensitisation

Dermal sensitisation was investigated a modified Buehler test in guinea pigs. There was no response consistent with dermal sensitisation.

4.8.2 Comparison with criteria

In a modified Buehler sensitisation assay none of the animals tested showed a sensitisation to Piperonyl Butoxide. A response in at least 15% of the animals in responding to >20% topical induction dose is required for classification.

4.8.3 Conclusions on classification and labelling for skin sensitisation

No sensitisation effects were detected in the modified Buehler test and therefore no classification is necessary.

CLP: No classification based on available data.

RAC evaluation of skin sensitisation

Summary of the Dossier Submitter's proposal

The DS summarised one modified Buehler assay with induction and challenge concentrations of 100% PBO and one Magnusson-Kligman test from the registration dossier with 50% PBO in sesame oil (both OECD TG 406 and GLP conforming). None of the 10 tested animals showed any skin reactions. In both studies, positive controls gave clearly positive results.

The DS concluded that no classification for Skin Sensitisation is warranted.

Comments received during public consultation

No comments were received.

Assessment and comparison with the classification criteria

Since PBO at a concentration of 100% did not induce any skin reactions in 10 out of 10 animals in a guideline conforming Buehler assay, RAC concurs with the DS that **no classification for Skin Sensitisation is warranted.**

4.9 Germ cell mutagenicity (Mutagenicity)

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

A summary of relevant *in vitro* and *in vivo* mutagenicity studies is included in Table 18.

Table 18: Summary table of relevant *in vitro* and *in vivo* mutagenicity studies*

Method	Results*	Remarks	Reference
In vitro			
Reverse Mutation Test Using Bacteria EU B.13/14 *S. typhimurium:* TA98, TA100, TA1535, TA1537 & TA 1538 100 – 5000 µg/plate ± S9	Negative	Klimisch Score: 2 No cytotoxicity	Lawlor, 1991 (Piperonyl Butoxide CAR Doc IIIA 6.6.1/01)
Reverse Mutation Test Using Bacteria EU B.13/14 • *S. typhimurium:* TA98, TA100, TA1535 & TA1537 31.6, 100, 316, 1000, 2500 µg/plate ± S9 • *E. coli:* 5000 µg/plate ± S9	Negative	Klimisch Score: 1 Cytotoxicity: in TA 100 in experiment I and in TA 100, TA 1537 and TA 1535 in experiment II	Wallner, 2010
Mammalian cells clastogenicity chromosomal aberrations EPA F, 84-2 Chinese hamster ovary cells (CHO) 25 - 251 µg/mL ± S9	Negative	Klimisch Score: 2 Cytotoxicity at the high dose in both the non-activated and the S-9 activated studies	Murli, 1991 (Piperonyl Butoxide CAR Doc IIIA6.6.1/02)
Mammalian cells gene mutation OECD 476 Chinese hamster ovary cells (CHO) 10-100 µg/mL +S9 25-500 µg/mL –S9	Negative	Klimisch Score: 2 Cytotoxicity at 500 µg/mL in S-9 activated & at 75 µg/mL in the non-activated system	Tu, 1986 (Piperonyl Butoxide CAR Doc IIIA 6.6.3/01)
In vivo			
MICRONUCLEUS MOUSE No guideline Swiss mice: Male and female 5animals/sex/dose 0 (vehicle), 300, 1000, 3000 mg/kg bw	Negative	Klimisch Score: 3 Short study report; limited presentation of raw data Accepted only as indicative	**Anonymous - 9** 1989 (Piperonyl Butoxide CAR Doc IIIA6.6.4)

* More details on the studies results are presented in Section 4.9.1.1.

It is noted that in the REACH registration dossier two further assays (unscheduled DNA synthesis in mammalian cells *in vitro*, 1991 and 1996) are included. No details regarding the batch numbers of the technical Piperonyl Butoxide use in these studies is provided and thus their relevance to the impurity profile stated in the identity section to this report (Table 1), is unknown. Moreover, both of

these assays are considered by the registrants as supporting data. The following are concluded by the registrants:

1. Unscheduled DNA synthesis in mammalian cells *in vitro*, 1996 (Exp Supporting Genetic toxicity *in vitro*. 001)

"The results of the dose range-finding study indicate that no appreciable toxicity was observed up to 5000 µg per plate. No positive responses were observed with any of the strains used, in the presence as well as in the absence of microsomal enzymes. These results were confirmed in an independent assay Piperonyl Butoxide was not mutagenic when tested on S. typhimurium strains TA98, TA100, TA1535, TA1537 and TA1538, with or without S9-mix activation."

2. Unscheduled DNA synthesis in mammalian cells *in vitro*, 1991 (Exp Supporting Genetic toxicity *in vitro*. 006)

"The test material didn't induce significant changes in the nuclear labeling of rat primary hepatocytes in two independent trials for an applied concentration range of 50ug/mL to 2.50ug/mL. Piperonyl Butoxide was therefore evaluated as inactive in the Assay for UDS in Rat Primary Liver Cell Cultures with a Confirmatory Assay."

Overall, it is not considered necessary to request the original study reports in order to include them in the CLH dossier.

4.9.1.1 *Non-human information*

In vitro data

Two bacterial reverse mutation assays (Table 19 & Table 20), a mammalian cell clastogenicity chromosomal aberration assay (Table 21) and a mammalian gene mutation assay (Table 22) both conducted using Chinese hamster ovary cells produced negative results in the presence and absence of S9. Based on the results of these studies, no genotoxic potential of Piperonyl Butoxide was detected *in vitro*. More details on the studies results are presented, below:

Table 19: Mean number of revertant colonies/plate (Lawlor, 1991)

Piperonyl Butoxide µg/plate		Average revertants per plate									
	Strain:	TA98		TA100		TA1535		TA1537		TA1538	
	S9-mix:	-	+	-	+	-	+	-	+	-	+
Vehicle*		19	36	111	158	13	16	8	12	12	21
100		23	36	99	132	11	15	8	11	11	25
333		18	35	98	154	11	12	8	12	11	21
667		15	42	96	149	10	15	3	13	10	22
1000		22	36	68	154	12	17	8	9	12	21
3330		21	28	107	181	10	11	5	11	10	22
5000		20	33	94	143	10	9	4	9	8	23
Positive control**		141	863	376	856	307	108	475	127	252	903

* DMSO

** 2-nitrofluorene 1.0 µg/plate at TA98 (-), TA1538 (-)
2-aminoanthracene 2.5 µg/plate at TA 98(+), TA100 (+), TA1535 (+), TA1537 (+), TA1538 (+)
Sodium azide 2.0 µg/plate at TA100 (-), TA 1535 (-)
ICR-191 2.0 µg/plate at TA1537 (-)

Table 20: Mean number of revertant colonies/plate in the Plate Incorporation test (Wallner, 2010)

Piperonyl Butoxide µg/plate		Average revertants per plate									
	Strain:	TA98		TA100		TA1535		TA1537		E. coli WP2	
	S9-mix:	-	+	-	+	-	+	-	+	-	+
H2O dest.		24	31	120	127	8	12	9	6	73	65
Vehicle*		30	30	122	138	12	9	8	6	47	51
31.6		25	36	121	144	10	7	10	7	41	50
100		28	42	104	137	12	5	6	7	53	58
316		26	29	84	125	11	11	8	9	51	60
1000		27	31	80	103	10	7	8	8	52	53
2500		23	32	75	100	8	9	9	7	53	49
5000		21	33	81	94	10	8	6	8	45	46
Pos. control**		741	2062	571	1738	992	73	98	243	467	199

* DMSO

** 4-NOPD 40 µg/plate at TA98 (-) at 10 µg/plate, TA1537 (-), E. coli WP2 (-)
2-AA 2.5 µg/plate at TA 98(+), TA100 (+), TA1535 (+), TA1537 (+), E. coli WP2 (+)
NAN3 10 µg/plate at TA100 (-), TA 1535 (-)

Table 21: Chromosome aberrations by Piperonyl Butoxide in Chinese Hamster Ovary (CHO) cells after different harvest times (Murli, 1991)

Treatment	S-9 Activation	Harvest time (hrs)	Cells scored	Aberrations per cell (mean ± SD)	Cells with aberrations (%)
Untreated,	-	10	100	0.03	3.0
DMSO	-	10	100	0.01	1.0
Piperonyl Butoxide:					
25 µg/mL	-	10	200	0.01	0.5
49.9 µg/mL	-	10	200	0.00	0.0
MMC, 1.0 µg/mL	-	10	25	0.48	36**
Untreated	+	10	100	0.020	2.0
DMSO	+	10	100	0.00	0
Piperonyl Butoxide:					
62.6 µg/mL	+	10	200	0.01	1.0
125 µg/mL	+	10	200	0.00	0
188 µg/mL	+	10	200	0.02	1.0
251 µg/mL	+	10	106	0.01	1.0
CP, 25 µg/mL	+	10	25	0.72	24**
Untreated	-	20	100	0.00	0
DMSO	-	20	100	0.00	0
Piperonyl Butoxide:					
49.9 µg/mL	-	20	200	0.01	0.0
74.9 µg/mL	-	20	200	0.00	2.0
99.9 µg/mL	-	20	200	0.06	1.5
MMC, 0.08 µg/mL	-	20	25	0.48	28**
Untreated	+	20	200	0.01	1.0
DMSO	+	20	200	0.02	2.0

Treatment	S-9 Activation	Harvest time (hrs)	Cells scored	Aberrations per cell (mean ± SD)	Cells with aberrations (%)
Piperonyl Butoxide:					
62.6 µg/mL	+	20	200	0.030	2.5
125 µg/mL	+	20	200	0.08	3.5
188 µg/mL	+	20	200	0.01	0.5
251 µg/mL	+	20	106	0.01	1.0
CP, 12.5 µg/mL	+	20	25	2.6	88.0**

* $p \leq 0.05$ ** $p \leq 0.01$; Fisher's exact test.

Table 22: Effects of Piperonyl Butoxide on gene mutations at the HGPRT-locus of Chinese hamster ovary (CHO) cells (Tu, 1986)

Treatment	S9 activation	Dose (µg/mL)	Cloning efficacy (CE)	Relative[1] cloning efficacy (%) (RCE)	Cloning efficiency at selection (%) (MCE)	Mutant frequency[2] (MF)	Statistical significance ($p < 0.01$)
None	-	0	44.5		66.0	3.03	-
DMSO	-	0	42.5	100	71.0	2.11	-
Piperonyl butoxide	-	10	43.5	102	72.0	2.78	No
	-	25	39	92	72.5	7.53	No
	-	50	24	56	77.5	6.41	No
	-	75	12	28	78.5	14.56	Yes
	-	100	5	11	85.0	4.12	No
EMS	-	248	12.5	29	41.0	763	Yes
None	+	0	40.5	-	58.5	2.54	-
DMSO	+	0	37.5	100	61.5	3.23	-
Piperonyl butoxide	+	25	43.5	116	53.5	11.11	No
	+	50	44.0	117	73.5	6.76	No
	+	100	48.0	128	-#	-#	-#
	+	250	43.0	115	67	1.49	No
	+	500	16.5	44	68	8.09	No
DMNA	+	500	3.0	8	23.5	589	Yes

1) values are given relative to that of the vehicle
2) mutant frequency per 10^6 clonable cells
one plate contaminated, one plate not subcultured by error

In vivo data

An *in vivo* micronucleus test in mice was submitted with negative result. The test was considered as indicative due to major deficiencies (not GLP, purity not reported, limited study report). The study results are confined to the data included in Table 23.

Table 23: Summary of micronucleus results in male and female mice (Anonymous - 9, 1989)

Dose (mg/kg b.w.)	Micronuclei per 2000 polychromatic erythrocytes per animal	
	Male	Female
0	1.8	1.4
300	1.6	1.6
1000	1.6	2.0
3000	1.2	1.8
Triethanolamine 0.25	24.4	21.0

4.9.1.2 *Human information*

No data.

4.9.1.3 *Other relevant information*

No other relevant information

4.9.1.4 *Summary and discussion of mutagenicity*

Piperonyl Butoxide was negative in all systems tested *in vitro* and *in vivo*.

4.9.2 Comparison with criteria

Under CLP to be classified as a Cat. 2 germ cell mutagen, the substance needs to show positive results in mammals and /or in some cases *in vitro* experiments.

In the case of Piperonyl Butoxide, all 4 *in vitro* studies were negative as well as the supportive *in vivo* study in mice. Overall, no classification for mutagenicity is proposed for Piperonyl Butoxide.

4.9.3 Conclusions on classification and labelling for germ cell mutagenicity

No classification.

RAC evaluation of germ cell mutagenicity

Summary of the Dossier Submitter's proposal

The DS presented results from four *in vitro* mutagenicity studies (two bacterial reverse mutation tests, one chromosomal aberration test, and one gene mutation assay, both in mammalian cells), and one non-guideline micronucleus test in mice with limited reliability due to lacking details on raw data. Additionally, the DS referred to summaries of results included in one REACH registration dossier from two unscheduled DNA synthesis tests in mammalian cells. RAC notes that for the first UDS test the summary copied to the CLH report was from a bacterial test. All tests were deemed negative.

DS concluded that **no classification** for Germ Cell Mutagenicity based on the available data is warranted.

Comments received during public consultation

One MSCA supported the DS's assessment.

Assessment and comparison with the classification criteria

In vitro

Two bacterial reverse mutation assays (EU B.13/14) with PBO up to a concentration of 5000 µg/plate gave negative results in several *S. typhimurium* strains and *E. coli* WP2 with and without S9 enzymatic activation.

PBO up to a concentration of 251 µg/mL did not produce higher numbers of CHO cells with chromosomal aberrations neither with nor without S9 activation in a mammalian clastogenicity test (US EPA F84-2).

In a gene mutation assay (OECD TG 476) in CHO cells, PBO at a concentration of 75 µg/mL without S9 activation induced a slight but statistically significantly higher mutant frequency of the HGPRT locus as compared to DMSO controls. None of the other concentrations (up to 100 µg/mL without S9, up to 500 µg/mL with S9) with or without S9 activation induced significant changes in mutation frequency.

In the first non-guideline but GLP compliant UDS test, cultured human liver slices were exposed to 0.05, 0.2, 0.5, 1.0, 1.5, or 2.5 mM PBO. None of the concentrations tested induced UDS.

In the second GLP compliant UDS test according to CFR guidance 21CFR 58, 40CFR 792, and 40CFR 160, rat primary liver cell cultures were exposed to PBO up to 100 µg/mL in trial 1 and up to 74.9 µg/mL in trial 2. Cytotoxicity was observed from concentrations above 49 µg/mL. The results were deemed negative in a concentration range from 2.5 µg/mL to 50 µg/mL.

In vivo

One micronucleus test in mice with mayor deficiencies is available. Study details are not reported. In this test, PBO up to a dose of 3000 mg/kg bw did not induce an increase in micronuclei in male and female erythrocytes. RAC concurs with the DS that this study is of low reliability.

All presented *in vitro* assays were negative. Both UDS tests are considered as supportive evidence. Since there is no reliable *in vivo* assay available to assess the mutagenicity of PBO in germ cells, **RAC proposes not to classify PBO for Germ Cell Mutagenicity due to insufficient data.**

4.10 Carcinogenicity

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

A summary of the relevant carcinogenicity studies is included in Table 24.

Table 24: Summary table of relevant carcinogenicity studies

Method	Results	Remarks	Reference
Rat (Sprague-Dawley): 2-year, feeding US EPA 83-5 (OPPTS	500 mg/kg bw/d hepatotoxicity*, nephrotoxicity*	Klimisch score: 2	**Anonymous - 10** , 1987

Method	Results	Remarks	Reference
870.4200), OECD 453 Doses: 0, 30, 100, 500 mg /kg/d Purity: 87.67-89.71%	100 mg/kg bw/d hepatotoxicity * testes weight ↓ kidney weights (females) glomerulonephritis (females) NOAEL: 30 mg/kg bw/d	Deviations from study protocol: 1. There were no treated satellite groups (20 rats/sex/group) for the evaluation of pathology other than tumours. 2. In addition to the time points of blood sampling, an additional blood sampling should have been performed at approximately 12 weeks. 3. Regarding Haematology, there was no measurement of Packed Cell Volume (PCV). *4.* The highest dose level depressed body weight gain by more than 10%.	(Piperonyl Butoxide CAR Doc IIIA6.7/01)
Rat (Fisher 344/DuCrj): 2-year feeding Not a guideline study. Doses: 0, 6000, 12000, 24000 ppm 0, 547, 1052 and 1877 mg/kg/day for males 0, 537, 1061 and 2002 mg/kg/day for females (all values calculated based on food consumption in a preliminary trial)	**Excessive toxicity at all doses (> MTD):** gastric and caecal haemorrhage, renal lesions, anaemia, platelet alteration, hyperplastic and neoplastic nodular lesions of the liver **Additional evidence of toxicity at different doses:** 24000 ppm body weight ↓, haematological changes hepatotoxicity adenoma, carcinoma, nephrotoxicity 12000 ppm hepatotoxicity, adenoma, carcinoma (males) body weight ↓, nephrotoxicity, haematological changes 6000 ppm haematological changes	STUDY NOT ACCEPTABLE (The MTD was exceeded at all dose levels due to excessive toxicity evidenced primarily as gastric and caecal haemorrhage – findings not reliable).	Takahashi, 1994a (Piperonyl Butoxide CAR Doc IIIA6.7/03)
Mouse (Charles River CD-1): 18-months, feeding OECD 451 Doses: 0, 30, 100, 300 mg /kg/d	300 mg /kg bw/d body weight↓, hepatotoxicity, adenoma (males and females) 100 mg /kg bw/d hepatotoxicity, adenoma (males) NOAEL: 30 mg/kg bw/d	Klimisch score: 1 *The study was discussed in the WG-II-2016: "The WG considered that based on the information available, not having the phase III studies, Piperonyl Butoxide should be considered as a potential carcinogen with a threshold mode of action."* Mechanistic data (including Phase III studies) submitted since WG-II-2016, indicated that hepatic neoplasms are of no human relevance (see section 4.10.1.1).	**Anonymous - 11**, 1993 (Piperonyl Butoxide CAR Doc IIIA6.7/02)

Method	Results	Remarks	Reference
Mouse (Crj:CD-1): 1-year feeding Not a guideline study. Doses: 0, 6000, 12000 ppm 0, 900 and 1800 mg/kg/day (Note: there are no details on food consumption in the published report; conversion of ppm in the diet to mg/kg bw per day is based on default values as included in Appendix F of the WHO guideline for the preparation of toxicological working papers on food additives, 2000[1])	12000 ppm Increased mortality, hepatotoxicity, reduced body weight, hepatic adenomas and carcinomas 6000 ppm hepatocellular adenomas & carcinomas in male mice	Klimisch score: 3 Accepted only as indicative. The high dose (12000 ppm) exceeded the MTD. The high dose was considered to be over the MTD based on the survival effects (81% compared to 94% in controls) and 29% decrease in terminal body weights as compared to controls and considering the study was conducted only for 12 months instead of 18 months.	Takahashi, 1994b (Piperonyl Butoxide CAR Doc IIIA6.7/04)

* Details on tumour and lesion incidences are included in

Table 28.

4.10.1.1 Non-human information

A summary of the relevant carcinogenicity studies is included in Table 24. Further details on these studies are included, below:

Carcinogenicity: oral

Chronic toxicity and oncogenicity of Piperonyl Butoxide has been assessed in rats and mice.

In rats, Piperonyl Butoxide was not found to be carcinogenic in a two-year dietary study (Anonymous - 10, 1987) at doses up to 500 mg/kg bw/day. The NOAEL was set at 30 mg/kg bw/day on the basis of effects on the liver and kidneys (see tables, below). There was no evidence of a carcinogenic potential.

[1] https://www.who.int/foodsafety/chem/jecfa/en/tox_guidelines.pdf

Table 25: Organ weights of rats sacrificed at termination – Males (Anonymous - 10, 1987)

Treatment mg/kg bw/d	Body weight (g)	Liver weight (g)	Relative liver weight (%)	Brain weight (g)	Kidney weight (g)
Control 1	651	19.9	3.11	2.33	5.55
Control 2	640	20.6	3.25	2.29	6.40
30	643	22.0	3.49	**2.39**[1]	6.70
100	582	22.1	**3.99**[1,2]	2.29	7.03
500	**503**[1,2]	**24.4**[1,2]	**4.87**[1,2]	**2.23**[2]	5.72

[1]significantly ($p<0.05$) different from control 1,
[2]significantly ($p<0.05$) different from control 2

Table 26: Organ weights of rats sacrificed at termination – Females (Anonymous - 10, 1987)

Treatment mg/kg bw/d	Body weight (g)	Liver weight (g)	Relative liver weight (%)	Brain weight (g)	Kidney weight (g)
Control 1	405	11.8	2.96	2.03	3.06
Control 2	465	12.4	2.74	2.01	3.08
30	471	14.5[1,2]	3.16	2.05	3.52[1,2]
100	422	15.1[1,2]	3.63[1,2]	2.06	3.44[1,2]
500	320[1,2]	15.4[1,2]	4.87[1,2]	1.99	3.44[1]

[1]significantly ($p<0.05$) different from control 1,
[2]significantly ($p<0.05$) different from control 2

Table 27: Kidney chronic interstitial glomerulonephritis – Females (Anonymous - 10, 1987)

Treatment mg/kg bw/day	N	Incidence	Chi-square	
			Control 1	Control 2
Control 1	60	32	-	-
Control 2	60	38	-	-
30	60	44	4.3*	1.0
100	60	51	12.7**	6.3**
500	60	54	18.1**	10.5**

** significantly ($p<0.05$) different from control*
*** significantly ($p<0.01$) different from control*

Table 28: Total tumour or lesion incidences (Anonymous - 10, 1987)

Cases	Sex	N	Control 1	Control 2	30	100	500
Liver – hypertrophy of hepatocytes	m	60	4	2	1	4	29*#
	f	60	4	2	0	2	47*#

Liver – focal mixed cells	m	60	1	1	4	1	5
	f	60	3	3	3	13*#	20*#
Hepatocellular Carcinoma	m	60	1	1	0	0	1
	f	60	0	0	0	1	1
Hepatocellular Adenoma	m	60	0	0	0	0	2
	f	60	0	0	0	0	0
Thyroid – hyperplasia of follicles	m	60	4	11	13*	8	21*#
	f	60	0	4	2	9*	11*#
Thyroid – pigment in follicles	m	60	24	27	22	22	48*#
	f	60	8	10	6	9	44*#
Testes – bilateral atrophy	m	60	11	9	20*#	28*#	26*#
Ovaries – hyperplasia of Sertoli-like cells	f	60	29	22	11	16	28

* different from control 1, # different from control 2

It is noted that the hyperplasia of Sertoli-like cells observed in the ovaries, is a geriatric background lesion which is usually accompanied by atrophy of the germinal epithelium, as also mentioned by the study author. The statistical analysis did not show differences in incidence or severity between control and the high dose group.

There was a significant difference between controls and all dose levels in the incidence of bilateral atrophy. The severity of bilateral testicular atrophy was further evaluated as shown in Table 29.

Table 29: Distribution of severity of lesions for bilateral atrophy in testis (Anonymous - 10, 1987)

Treatment mg/kg bw/day	**N**	**Severity Grade**			
		0	**1 Slight**	**2+3 Mild + Moderate**	**4 Severe**
Control 1 + 2	120	100 (83%)	3 (2.5%)	7 (5.8%)	10 (8.3%)
30	60	40 (67%)	10 (16.6%)	6 (10%)	4 (6.7%)
100	60	32 (53%)	9 (15%)	14 (23%)	5 (8.3%)
500	60	34 (57%)	7 (11.7%)	5 (8.3%)	14 (23%)

Statistical analysis of the severity of bilateral testicular atrophy indicates differences in patterns of response in treated groups. Examination of the data shows a tendency towards increased severity of lesions as the dose increases. At the low dose level, the increase of severity appears to be only at the lowest level of severity and therefore, of equivocal biological significance.

In an open literature study in rats (Takahashi, 1994a) Piperonyl Butoxide induced hepatocellular carcinomas in males and females in a dose–dependent manner when administered orally in the diet at daily doses exceeding the MTD (greater than 6000 ppm) for 2 years. Findings from the Takahashi (1994a) study were not considered reliable, due to the excessive toxicity observed in animals of all dose groups evidenced primarily as gastric and caecal haemorrhage. Thus, overall it may be concluded that Piperonyl Butoxide is not carcinogenic in rats.

In mice, hepatotoxicity was evidenced after oral administration of Piperonyl Butoxide at doses greater than 100 mg/kg bw/day (Anonymous - 11, 1993). The NOAEL in this study was set at 30 mg/kg bw/day on the basis of liver effects summarised in Table 30 - Table 34.

Two peer reviews on the liver histopathology were performed at different time points since there was disagreement between the first diagnosis of the pathologists of the testing facility (BRRC first diagnosis) and the independent pathologist report which was reviewed at BRRC (BRRC 1st peer review). A 2nd peer review was organised by the independent pathologist and included the opinion of two (2) other independent pathologists (Table 33). The DS accepts the opinion of the majority on the incidence of hepatocellular carcinomas and adenomas in mice. The statistical evaluation of the results (Table 34) is based on this incidence, as well. A positive dose-related trend in the incidence of adenomas and the incidence of combined adenomas and carcinomas with statistical increases in the middle and high doses was observed in male mice.

The relevant contemporaneous historical control data (HCD) from BRRC submitted by the Industry (Table 35), showed that in male mice the incidence of hepatocellular adenomas is in the range of 6/60 – 13/60, whereas the incidence of hepatocellular carcinomas is in the range of 1-60 – 3/60. The DS concludes that the incidences of hepatocellular adenomas in male mice treated with 100 and 300 mg/kg bw/day and of hepatocellular carcinomas in male mice treated with 300 mg/kg bw/day are outside the respective HCD of BRRC contemporaneous studies.

Table 30: Absolute and relative liver weights of mice treated with Piperonyl Butoxide (Anonymous - 11, 1993)

Dose level (mg/kg bw/day)	**0**	**0**	**30**	**100**	**300**
Males					
Mean final body weight (g)	42.8	44.8	43.6	43.2	41.9
Mean absolute liver weight (g)	2.560	2.639	2.817	3.037 [a]	3.037 [bd]
Mean relative liver weight (%)	6.060	5.870	6.492	7.038 [c]	10.130 [bd]
Females					
Mean final body weight (g)	38.1	37.5	37.6	38.9	38.1
Mean absolute liver weight (g)	2.208	2.153	2.182	2.446 [ad]	2.657 [bd]
Mean relative liver weight (%)	5.822	5.772	5.779	6.308 [c]	6.955 [bd]

a Significantly different from the first control group ($p < 0.05$)
b Significantly different from the first control group ($p < 0.01$)
c Significantly different from the second control group ($p < 0.05$)
d Significantly different from the second control group ($p < 0.01$)

Table 31: Group incidence of liver adenomas in mice treated with Piperonyl Butoxide (Anonymous - 11, 1993)

Dose level (mg/kg bw/day)	**0**	**0**	**30**	**100**	**300**
Males					

Dose level (mg/kg bw/day)	**0**	**0**	**30**	**100**	**300**
Basophilic adenomas	7	6	10	15	10
Eosinophilic adenomas	4	1	3	8	19
Mixed cell adenomas	0	1	2	2	5
Females					
Basophilic adenomas	1	0	0	0	2
Eosinophilic adenomas	1	2	1	1	9
Mixed cell adenomas	0	0	0	0	1

Table 32: Distribution of hepatocellular pathology in mice treated with Piperonyl Butoxide (Anonymous - 11, 1993)

Dose level (mg/kg bw/day)	**0**	**0**	**30**	**100**	**300**
Males					
Mice examined	60	60	60	60	60
Mice with adenoma	8	7	13	21	25
Mice with carcinoma	3	3	2	2	5
Mice with both adenoma and carcinoma	0	0	1	0	5
Mice with hyperplastic foci	0	2	1	2	5
Mice with liver hypertrophy	6	11	11	16	43
Mice with hepatocellular necrosis	21	20	11	14	29
Females					
Mice examined	60	60	60	60	60
Mice with adenoma	2	2	1	1	10
Mice with carcinoma	0	0	0	0	0
Mice with hyperplastic foci	0	0	0	1	4
Mice with liver hypertrophy	0	4	0	1	9
Mice with hepatocellular necrosis	6	13	13	17	7

Table 33: Differences in the assessment of carcinomas in male mice (Anonymous - 11, 1993)

Dose level (mg/kg bw/day)	**A Control**	**E Control**	**B 30 mg/kg**	**C 100 mg/kg**	**D 300 mg/kg**
Male mice examined	60	60	60	60	60
Male mice with carcinoma					
BRRC first diagnosis	1	0	3	2	5

Dose level (mg/kg bw/day)	**A Control**	**E Control**	**B 30 mg/kg**	**C 100 mg/kg**	**D 300 mg/kg**
BRRC 1st peer review	2	2	3	2	7
Majority opinion in 2nd peer review	3	3	2	2	5

* BRRC: Testing facility

- BRRC first diagnosis: The results of the initial microscopic diagnosis at BRRC
- BRRC peer review: The results of the 1st peer review conducted at BRRC.
- Majority opinion: A further review was conducted and the majority opinion was reported.

Table 34: Distribution of hepatocellular pathology in mice treated with Piperonyl Butoxide (new statistical analyses for male mice)

		Statistical analysis of tumours Fisher Exact Test					
Group		**1**	**2**	**3**	**4**	**5**	
Dosage level (mg/kg/day)		**Control A**	**30mg/kg**	**100mg/kg**	**300mg/kg**	**Control E**	**Trend**
Liver: carcinoma: Males	n	3	2	2	5	3	
	%	5.00	3.33	3.33	8.33	5.00	
	P	C	0.9318	0.9318	0.5683	C	0.2917
Liver: adenoma: Males	n	8	13	21	25	7	
	%	13.33	21.67	35.00	41.67	11.67	
	P	C	0.1706	0.0010 ++	0.0000 +++	C	0.0000 +++
Liver: adenoma or carcinoma: Males	n	11	15	23	30	10	
	%	18.33	25.00	38.33	50.00	16.67	
	P	C	0.3228	0.0046 ++	0.0000 +++	C	0.0000 +++

Table 35: Historical Control Data on the incidence of hepatocellular neoplasms in CD-1 mice

Summary of the Bushy Run Research Center (BRRC) Neoplasm Historical Control Database in CD-1® Mice

CODE	START DATE	SPECIES	STRAIN	SUPPLIER
1	28-SEP-83	MOUSE	CD-1	CHARLES RIVER MI
2	5-DEC-85	MOUSE	CD-1	CHARLES RIVER NY
3	27-APR-88	MOUSE	CD-1	CHARLES RIVER MI
4	27-APR-88	MOUSE	CD-1	CHARLES RIVER MI
5	5-JUL-88	MOUSE	CD-1	CHARLES RIVER MI
6	5-JUL-88	MOUSE	CD-1	CHARLES RIVER MI
7	21-MAR-90	MOUSE	CD-1	CHARLES RIVER MI
8	21-MAR-90	MOUSE	CD-1	CHARLES RIVER MI

MALES

FINDING TEXT	CODES	1	2	3	4	5	6	7	8	TOTAL	(%)
LI- LIVER Number of tissues examined		70	69	59	60	55	60	60	60	493	
HEMANGIOMA	#B	0	0	1	1	0	0	0	0	2	0.41
HEMANGIOSARCOMA	#M	0	0	1	0	0	0	1	1	3	0.61
HEPATOBLASTOMA	#M	0	0	0	0	0	0	0	1	1	0.20
HEPATOCELLULAR ADENOMA	#B	12	6	8	13	7	6	8	7	67	13.59
HEPATOCELLULAR CARCINOMA	#M	2	0	2	3	0	1	1	3	12	2.43
LYMPHOSARCOMA	#M	2	1	2	2	1	2	0	0	10	2.03

Liver toxicity was also observed in an open literature study in mice (Takahashi 1994b) were Piperonyl Butoxide was administered orally in the diet at daily doses of 6000 and 12000 ppm. The high dose was considered to be over the MTD based on the survival effects (81% compared to 94% in controls) and 29% decrease in terminal body weights as compared to controls and considering the study was conducted only for 12 months instead of 18 months. A dose dependent increase in the hepatocellular adenomas and carcinomas in male mice with the incidence of combined tumours at 1.9%, 24.5% and 75% in the control, low and high dose groups were observed respectively. Hemangioendothelial sarcoma of liver was present in the high dose group (42% versus none in controls).

The US-EPA has classified Piperonyl Butoxide as Group C Carcinogen (Possible human carcinogen – i.e. limited evidence of carcinogenicity in animals in the absence of human data) based primarily on statistically significant increases in hepatocellular tumors in both sexes of the CD-1 mouse (adenomas, carcinomas and combined adenomas/carcinomas in males and adenomas in females).

IARC (International Agency on Research for Cancer) has classified Piperonyl Butoxide in Group 3 "Not classifiable as to its carcinogenicity to humans", based on an evaluation dated from 1987, preceding the conduction of the study in CD-1 mice (Anonymous - 11, 1993, See Doc IIIA_7_2).

JMPR evaluation in 1995, reviewed the study in CD-1 mice and concluded that Piperonyl Butoxide was carcinogenic at doses which were toxic to the liver and caused general toxicity. The incidence of hepatocellular adenomas composed of large, polyhedral, densely-packed cells with abundant granular, notably eosinophilic cytoplasm was significantly increased in Piperonyl Butoxide-treated mice. This appearance is different from that of spontaneous adenomas found in CD-1 mice, where small to medium, well-differentiated, basophilic cells, distributed in solid to normal sinusoidal patterns, are found.

Industry submitted the following reports and studies in the frame of the investigation of a mode of action (MoA) for Piperonyl Butoxide-induced liver tumour formation in male mice:

1. **Lake B.G, Boobis A.R.: Piperonyl Butoxide Module 1: Review of Rodent Liver Tumour Formation by Piperonyl Butoxide and Evidence for a Putative Mode of Action for Mouse Liver Tumour Formation, 2011a:**

The report reviewed all the available chronic toxicity and carcinogenicity studies of Piperonyl Butoxide in rats and mice including open literature publications, NTP studies and studies on the mechanism of action of Piperonyl Butoxide. The reviewers concluded the following:

- chronic administration of Piperonyl Butoxide results in altered hepatic foci and liver tumours in mice.
- a putative MoA for Piperonyl Butoxide-induced liver tumour formation can be identified, since Piperonyl Butoxide appears to be a constitutive androstane receptor (CAR) activator in mouse liver. The scientific weight of evidence suggests that CAR mediated effects that lead to the formation of liver tumours in mice (and rats) are not relevant to humans.

The stipulated MoA includes stimulation of microsomal CYP2b forms after treatment of mice with Piperonyl Butoxide, increased liver weight with morphological evidence of hepatocyte hypertrophy and a transient stimulation of replicative DNA synthesis. The postulated MoA is similar to that established for rodent tumour formation by phenobarbital and related compounds. There are mechanistic data available that support this hypothesis (See Doc IIIA Section A6.10). However, the authors identified major data gaps in the establishment of a MoA for Piperonyl Butoxide-induced mouse liver tumour formation. The testing strategy for addressing these data gaps is described in the following points.

2. **Lake B.G, Boobis A.R.: Piperonyl Butoxide Module 2: Proposed studies to confirm the putative Mode of Action for Mouse Liver Tumour Formation by Piperonyl Butoxide, 2011b:**

The proposed studies are briefly outlined below. The authors indicate that the studies should be conducted in 3 phases of which Phase I should be performed before Phase II:

Phase I: Comparison of the hepatic effects of Piperonyl Butoxide and phenobarbital in CD-1 mice. The aim of these studies is to obtain additional data on the comparability of the hepatic changes produced by Piperonyl Butoxide and phenobarbital in the mouse. In addition, this study should examine whether Piperonyl Butoxide can produce peroxisome proliferation in mouse liver and information on the reversibility of the hepatic effects of Piperonyl Butoxide will also be obtained.

Phase II: Comparison of the hepatic effects of Piperonyl Butoxide in wild type mice, CAR/PXR knockout mice and CAR/PXR humanised mice. The aim of these studies would be to confirm unequivocally that the hepatic effects of Piperonyl Butoxide in mouse liver are mediated through CAR and to obtain data on species differences between effects on mouse and human CAR/PXR receptors.

Phase III: Studies in cultured mouse and human hepatocytes. The aim of these studies would be to provide further evidence that the effects of Piperonyl Butoxide resemble those of phenobarbital by studying the induction of CYP forms and to obtain data on species differences (mouse versus human)

in the effect of Piperonyl Butoxide on replicative DNA synthesis. The studies would be performed with male CD-1 mouse hepatocytes and hepatocytes from human donors.

The author presented in tabulated form (Table 36) a comparison of the hepatic effects of Piperonyl Butoxide in mice and humans on the key events for the proposed MoA for Piperonyl Butoxide-induced mouse liver tumour formation. The Table contains data that were already available at the time of the Lake and Boobis (2011b) report, together with data to be obtained for the proposed phase I, II and III studies:

Table 36: Comparison of the hepatic effects of Piperonyl Butoxide in mice and humans

Key event	Evidence in mice	Evidence in humans
Activation of CAR	To be demonstrated by studies with CAR/PXR knockout mice	Probable at high doses
Induction of CYP forms	Direct experimental evidence *in vivo*. To be confirmed *in vitro* in cultured hepatocytes	Probable at high doses. To be confirmed in cultured hepatocytes
Liver hypertrophy	Direct experimental evidence *in vivo*	Possible at very high doses
Increased cell proliferation	Direct experimental evidence *in vivo*. To be confirmed *in vitro* in cultured hepatocytes	Not likely. To be studied in cultured hepatocytes and *in vivo* with CAR/PXR humanised mice
Inhibition of apoptosis	Data to be obtained.	Not likely
Altered hepatic foci	Direct experimental evidence *in vivo*. Dose-response to be better characterised.	Not likely
Hepatocellular tumours	Direct experimental evidence *in vivo*	Not likely

Industry has followed the proposed testing strategy. The three Phase studies (I, II, III) have now been finalised (See below), and the study summaries were submitted by the Industry. The e-CA for Piperonyl Butoxide in the frame of Reg. (EC) No. 528/2012 has requested the submission of the full study reports as part of preparation of the draft CAR (See Piperonyl Butoxide CAR Doc I). The evaluation of these studies is presented below:

- **Anonymous - 12** (2012). Phase I study: A 14-day dietary study comparing the hepatic effects of Piperonyl Butoxide and sodium phenobarbital in male CD-1 mice. Ref. No: 5491/1, Performing Laboratory: Leatherhead Food Research, Surrey, UK.

Study design: Male CD-1 mice were administered diets containing Piperonyl Butoxide to provide intakes of 0 (control), 30, 100 and 300 mg/kg bw/day for 14 days. To serve as a positive control, mice were also administered 0.05% sodium phenobarbital (NaPB) in the diet for 14 days. In addition, a recovery study was performed where mice were administered 0 (control) and 300 mg/kg bw/day Piperonyl Butoxide and 0.05% NaPB for 14 days followed by 28 days recovery period. To assess the effect of Piperonyl Butoxide on hepatic cytochrome P450 (CYP) forms, microsomal protein and total CYP content, the activities of the CYP1A marker 7-ethoxyresorufin O-deethylase, the CYP2B marker 7-pentoxyresofurin O-depentylase, the CYP3A marker testosterone 6β-hydroxylase and the CYP4A marker lauric acid 12-hydroxylase were determined along with microsomal CYP1A, CYP2B,

CYP3A and CYP4A protein levels (determined by enzyme-linked immunosorbent assay) and hepatic CYP1A2, CYP2B10, CYP3A11 and CYP4A10 mRNA levels. In addition, to assess whether Piperonyl Butoxide could induce hepatic peroxisome proliferation in mouse liver (which is associated with CYP4A form induction), whole homogenate cyanide-insensitive palmitoyl-CoA oxidation activity was determined.

Results: The treatment of male CD-1 mice with 30, 100 and 300 mg Piperonyl Butoxide/kg bw/day for 14 days resulted in a statistically significant dose-dependent induction of hepatic microsomal total CYP content, CYP2B-dependent 7-pentoxyresorufin O-depentylase activity and CYP2B10 mRNA levels (Table 37). Apart from the effect in markers of CYP2B form induction, treatment with Piperonyl Butoxide also resulted in statistically significant increases in markers of CYP1A and CYP3A form induction (Table 37 & Table 38).

Table 37: Hepatic CYP mRNA levels (14-day sacrifice, 28-day recovery, fold induction)[a,b]

	0 mg/kg/day PBO	30 mg/kg/day PBO	100 mg/kg/day PBO	300 mg/kg/day PBO	100 mg/kg/day NaPB
CYP1A2	1.00 ± 0.192	1.40 ± 0.269**	2.29 ± 0.449***	3.50 ± 1.242**	3.82 ± 0.828***
28-d recovery	1.00 ± 0.116	–	–	0.84 ± 0.298	0.84 ± 0.174*
CYP2B10	1.00 ± 0.120	1.63 ± 0.714*	4.27 ± 1.041***	16.97 ±8.358***	30.25 ± 11.090***
28-d recovery	1.00 ± 0.056	–	–	0.52 ± 0.229**	0.62 ± 0.421*
CYP3A11	1.00 ± 0.118	1.39 ± 1.032	1.67 ± 0.398**	5.07 ± 3.237*	2.83 ± 1.367**
28-d recovery	1.00 ± 0.437	–	–	0.69 ± 0.285	0.88 ± 0.190
CYP4A10	1.00 ± 0.270	1.12 ± 0.966	1.80 ± 0.709*	0.37 ± 0.318**	0.57 ± 0.248**
28-d recovery	1.00 ± 0.246	–	–	0.88 ± 0.329	3.09 ± 3.090

[a] Results are mean ± SD for groups of 7 or 8 animals.
[b] Values statistically significantly different from control are: *p<0.05; **p<0.01; ***p<0.001.

Table 38: Hepatic microsomal CYP protein levels (14-day sacrifice, 28-day recovery, fold induction)[a,b]

	0 mg/kg/day PBO	30 mg/kg/day PBO	100 mg/kg/day PBO	300 mg/kg/day PBO	100 mg/kg/day NaPB
CYP1A	1.00 ± 0.240	1.32 ± 0.207**	1.84 ± 0.109***	3.11 ±0.584***	3.32 ± 0.423***
28-d recovery	1.00 ± 0.205	–	–	0.98 ± 0.260	1.20 ± 0.606
CYP2B	1.00 ± 0.434	1.33 ± 0.291	2.51 ± 1.307***	5.39 ± 2.649***	4.77 ± 1.221***
28-d recovery	1.00 ± 0.347	–	–	1.21 ± 0.366	1.44 ± 0.697
CYP3A	1.00 ± 0.420	0.95 ± 0.165	1.40 ± 0.401	1.75 ± 0.665**	1.52 ± 0.463*
28-d recovery	1.00 ± 0.189	–	–	1.25 ± 0.211*	1.51 ± 0.697
CYP4A	1.00 ± 0.437	0.84 ± 0.460	1.39 ± 0.315*	0.41 ± 0.117**	0.44 ± 0.156**
28-d recovery	1.00 ± 0.565	–	–	0.96 ± 0.222	1.88 ± 1.438

[a] Results are mean ± SD for groups of 7 or 8 animals.
[b] Values statistically significantly different from control are: *p<0.05; **p<0.01; ***p<0.001.

Conclusion: Overall, the hepatic effects of Piperonyl Butoxide were similar to those of NaPB, which suggests that Piperonyl Butoxide is a constitutive androstane receptor (CAR) activator in this species. The statistically significant induction of CYP2B-dependent enzyme activity, CYP2B protein levels and CYP2B10 mRNA levels at carcinogenic Piperonyl Butoxide dose levels (100 and 300 mg/kg bw/day Piperonyl Butoxide) lends support to the activation of CAR being a key event in the MoA for Piperonyl Butoxide-induced mouse liver tumor formation. Treatment with 30-300 mg Piperonyl Butoxide/kg/day did not produce any marked effects on the markers of hepatic peroxisome

proliferation examined in this study. Finally, the hepatic effects of Piperonyl Butoxide and NaPB were essentially reversible within 28 days of recovery following 14 days of treatment.

- **Anonymous - 13** (2013). Phase II study: Comparison of the hepatic effect of Piperonyl Butoxide in male constitutive androstane receptor (CAR)/pregnane X receptor (PXR) double knockout and wild type C57BL/6J mice; Ref, No: 5507/1, Performing Laboratory: Leatherhead Food Research, Surrey, UK.

Summary: This study was part of a series of investigations aimed at elucidating the MoA for Piperonyl Butoxide-induced mouse liver tumour formation. The hypothesis under investigation was that the MoA for Piperonyl Butoxide-induced mouse liver tumour formation is similar to that of sodium phenobarbital (NaPB), which is an activator of the constitutive androstane receptor (CAR) (Elcombe *et al*., 2014[2]).

The purpose of this study was to demonstrate that the hepatic effects of Piperonyl Butoxide in mouse liver are primarily mediated through CAR. Thus, if the hepatic effects of Piperonyl Butoxide are CAR-dependent, no CAR-mediated effects (liver hypertrophy, increased replicative DNA synthesis, induction of Cyp2b enzymes) of Piperonyl Butoxide would be observed in mice lacking CAR. The CAR/PXR double receptor knockout mouse was used in this study to ensure that no mitogenic or other effects of Piperonyl Butoxide, which may be attributable to receptor crosstalk between CAR and PXR, would be observed.

Study design: In this study, the effect of Piperonyl Butoxide treatment on hepatic cytochrome P450 (CYP) enzymes and markers of peroxisome proliferation was studied in male C57BL/6J wild type mice and in male mice lacking both the constitutive androstane receptor (CAR) and the pregnane X receptor (PXR) (CAR/PXR double knockout mice).
Male C57BL/6J wild-type and CAR/PXR double knockout mice were administered diets containing 0 (control) and 1243-1354 ppm Piperonyl Butoxide for 14 days. The calculated mean intakes for the C57BL/6J wild-type and CAR/PXR double knockout mice were 291 and 236 mg/kg bw/day Piperonyl Butoxide, respectively. Liver whole homogenates from control and Piperonyl Butoxide treated wild-type and CAR/PXR double knockout mice were assayed for cyanide-insensitive palmitoyl-CoA oxidation activity to assess the induction of hepatic peroxisome proliferation in mouse liver by Piperonyl Butoxide. Liver microsomes from all mice were assayed for protein and total CYP content and the activities of the CYP1A marker 7-ethoxyresorufin O-deethylase, the CYP2B marker 7-pentoxyresofurin O-dependentylase, the CYP3A marker testosterone 6β-hydroxylase and the CYP4A marker lauric acid 12-hydroxylase. Levels of microsomal CYP1A, CYP2B, CYP3A and CYP4A proteins were determined by enzyme-linked immunosorbent assay and hepatic acyl-CoA oxidase, CYP1A2, CYP2B10, CYP3A11 and CYP4A10 mRNA levels were determined by real-time quantitative reverse transcription-polymerase chain reaction methodology.

Results: The treatment of male C57BL/6J wild type mice with Piperonyl Butoxide for 14 days resulted in a marked statistically significant induction of microsomal CYP2B marker 7 pentoxyresorefin O-dependentylase activity and hepatic CYP2B10 mRNA levels (Table 39). Treatment with Piperonyl Butoxide also resulted in statistically significant increase in hepatic microsomal

[2] Elcombe CR, Peffer RC, Wolf DC, Bailey J, Bars R, Bell D, Cattley RC, Ferguson SS, Geter D, Goetz A, Goodman JI, Hester S, Jacobs A, Omiecinski CJ, Schoeny R, Xie W, Lake BG. Mode of action and human relevance analysis for nuclear receptor-mediated liver toxicity: A case study with phenobarbital as a model constitutive androstane receptor (CAR) activator. Crit Rev Toxicol. 2014 Jan;44(1):64-82. doi: 10.3109/10408444.2013.835786. Epub 2013 Nov 4. Review. PubMed PMID: 24180433; PubMed Central PMCID: PMC4019974.

protein and total CYP content and in CYP2B protein levels (Table 40). Apart from the effects of the markers of CYP2B enzyme induction, treatment with Piperonyl Butoxide also resulted in statistically significant increase in marker of CYP1A and CYP3A enzyme induction (Table 40). In contrast to male C57BL/6J wild type mice, the treatment of male CAR/PXR double knockout mice with Piperonyl Butoxide had no statistically significant effect on microsomal protein content and statistically significantly reduced microsomal total CYP content. Treatment with Piperonyl Butoxide resulted in a statistically significant reduction in hepatic CYP2B10 mRNA levels (Table 39) and produced only small statistically significant increases in microsomal 7 pentoxyresorufin O-dependentylase activity and CYP2B protein levels (Table 40). The treatment of CAR/PXR double knockout mice with Piperonyl Butoxide also resulted in statistically significant increases in the markers of CYP3A enzyme induction examined (Table 40).

Table 39: Hepatic CYP and Acyl-CoA oxidase mRNA levels (14-day sacrifice)[a,b]

	Control - C57BL/6J	PBO - C57BL/6J	Control – CAR/PXR k.o.	PBO – CAR/PXR k.o.
Acyl-CoA oxidase	1.00 ± 0.167	1.07 ± 0.323	1.00 ± 0.150	1.73 ± 0.370***
Cyp1a2	1.00 ± 0.133	3.95 ± 0.878***	1.00 ± 0.148	5.16 ± 1.258***
Cyp2b10	1.00 ± 0.088	1297 ± 444.0***	1.00 ± 0.091	0.39 ± 0.420***
Cyp3a11	1.00 ± 0.292	8.90 ± 5.465***	1.00 ± 0.374	1.56 ± 0.644
Cyp4a10	1.00 ± 0.164	1.32 ± 0.823	1.00 ± 0.243	4.50 ± 1.552***

[a] Results are mean ± SD for groups of 8 animals.
[b] Values statistically significantly different from control are: **p<0.01; ***p<0.001.

Table 40: Hepatic microsomal CYP protein levels (14-day sacrifice, 28-day recovery, fold induction)[a,b]

	Control - C57BL/6J	PBO - C57BL/6J	Control – CAR/PXR k.o.	PBO – CAR/PXR k.o.
Cyp1a	1.00 ± 0.456	4.03 ± 0.692***	1.00 ± 0.281	4.63 ± 1.183***
Cyp2b	1.00 ± 0.333	5.76 ± 1.115***	1.00 ± 0.353	1.73 ± 0.325***
Cyp3a	1.00 ± 0.318	3.05 ± 0.731***	1.00 ± 0.367	0.97 ± 0.199
Cyp4a	1.00 ± 0.189	1.37 ± 0.203**	1.00 ± 0.295	2.12 ± 0.127***

[a] Results are mean ± SD for groups of 8 animals.
[b] Values statistically significantly different from control are: **p<0.01; ***p<0.001.

Conclusion: Overall, the hepatic effects of Piperonyl Butoxide in male C57BL/6J mice are consistent with previous studies conducted in CD-1 mice. The studies with CAR/PXR double knockout mice demonstrate that the induction of CYP2B and CYP3A enzymes by Piperonyl Butoxide is largely abolished in mice lacking CAR and PXR, but that non CAR- and PXR- dependent pathways are involved in the induction of CYP1A enzymes. While Piperonyl Butoxide was shown to produce some increase in the markers of hepatic peroxisome proliferation studies, the effects were more marked in CAR/PXR double knockout mice than in wild type mice. In conclusion, the data from this study supports the hypothesis under investigation that Piperonyl Butoxide produces liver tumors in mice by a MoA involving CAR activation.

As a remark, it is noted that CAR/PXR humanised mice were not tested as proposed for Phase II studies. Therefore, no data can be obtained on species differences between effects on mouse and human CAR/PXR receptors.

- **Phase III studies: Studies comparing the effects of Piperonyl Butoxide on hepatocellular proliferation and Cytochrome P450 enzyme expression in primary mouse and human hepatocytes.**

The two studies (Mouse Study - Elcombe, 2017a and Human Study - Elcombe, 2017b) are part of the third phase of a series of investigations aimed at elucidating the MoA for Piperonyl Butoxide-induced mouse liver tumour formation. The hypothesis under investigation was that the MoA for Piperonyl Butoxide-induced mouse liver tumour formation was similar to that of sodium phenobarbital (NaPB), which is an activator of the constitutive androstane receptor (CAR) (Elcombe *et al*., 2014[3]).

To investigate the MOA of Piperonyl Butoxide in human liver it was necessary to utilise *in vitro* studies using isolated human liver hepatocyte cultures. To enable a direct comparison, the first study conducted used isolates mouse hepatocytes which also enabled the *in vitro* findings to be "bridged" to the Phase I and II *in vivo* mouse studies as a consistency check for the liver response to Piperonyl Butoxide and NaPB, and thus further 'externally' validate the utility of the *in vitro* model.

a) *In vitro* mouse study: Piperonyl Butoxide: MoA Phase III - Cytochrome P450 Enzyme and Replicative DNA-Synthesis Induction in Cultured Male CD-1 Mouse Hepatocytes; Elcombe, B. and Vardy, A., (2017a), Report Number: CXR1621, Performing Laboratory: CXR Biosciences Ltd, Dundee, UK.

Summary: This study investigated the potential for Piperonyl Butoxide to stimulate hepatocellular proliferation (measured as the change in replicative DNA synthesis [S-phase of the cell cycle]) and cytochrome P450 (Cyp) 2b10 and 3a11 mRNA levels in isolated male CD-1 mouse hepatocyte cultures. Cytotoxicity, as evaluated by ATP depletion, was assessed in parallel. NaPB and epidermal growth factor (EGF) served as Positive Control items, where appropriate.

Piperonyl Butoxide caused strong concentration-dependent increases in replicative DNA synthesis (S-phase) using BrdU uptake as observed in Phase I studies *in vitro*, as did the positive controls NaPB and EGF, indicating the sensitivity of the system. However, Piperonyl Butoxide did not increase Cyp2b10 mRNA at any concentration assessed and Cyp3a11 mRNA was only slightly increased at the lowest concentration, whereas NaPB did however give rise to the expected increases. The reason for the lack of response for Piperonyl Butoxide with respect to the mRNA levels is unclear, and is discussed further in the Phase II report. With Piperonyl Butoxide at high concentrations, substantial cytotoxicity was observed both microscopically and from ATP depletion results.

The study confirmed that Piperonyl Butoxide induces replicative DNA synthesis in cultured mouse hepatocytes, which is consistent with the *in vivo* observations (Phase I studies).

Study design: Treatment with ≥200 μM Piperonyl Butoxide caused severe cytotoxicity (as determined by depletion in ATP levels) in male CD-1 mouse primary hepatocytes in the preliminary study

[3] Elcombe CR, Peffer RC, Wolf DC, Bailey J, Bars R, Bell D, Cattley RC, Ferguson SS, Geter D, Goetz A, Goodman JI, Hester S, Jacobs A, Omiecinski CJ, Schoeny R, Xie W, Lake BG. Mode of action and human relevance analysis for nuclear receptor-mediated liver toxicity: A case study with phenobarbital as a model constitutive androstane receptor (CAR) activator. Crit Rev Toxicol. 2014 Jan;44(1):64-82. doi: 10.3109/10408444.2013.835786. Epub 2013 Nov 4. Review. PubMed PMID: 24180433; PubMed Central PMCID: PMC4019974.

(Elcombe, 2017; CXR1619) resulting in 500 μM Piperonyl Butoxide being the top concentration assessed in the current study. Therefore, primary monolayer cultures of hepatocytes were prepared and exposed to Piperonyl Butoxide at 6 concentrations (5, 10, 20, 50, 200 and 500 μM) or to a vehicle control (0.1% DMSO). Separate cultures were exposed to NaPB at 3 concentrations (10, 100 and 1000 μM) as a positive control. There were 3 replicates for each concentration for Cyp2b10 and Cyp3a11 Taqman mRNA measurements, 5 replicates for each concentration for replicative DNA synthesis (incorporation of 5-bromo-2´-deoxyuridine [BrdU]) and 6 replicates for each concentration for cytotoxicity measurements (measured as the change in cellular ATP). EGF was tested at a single concentration (25 ng/mL) as a positive control agent for replicative DNA synthesis.

Results: Treatment with ≥200 μM Piperonyl Butoxide caused severe cytotoxicity in male CD-1 mouse primary hepatocytes both in the preliminary study (Elcombe, 2017; CXR1619) and in the current study (Table 41). NaPB had no biologically-significant effect on ATP levels at concentrations up to 1000 μM.

Treatment with ≥5 μM Piperonyl Butoxide caused strong concentration-dependent, statistically-significant increases in replicative DNA synthesis as determined by the S-phase labelling index, peaking at 5.9-fold (Table 42). Treatment with NaPB or EGF resulted in statistically-significant increases in replicative DNA-synthesis, as expected within the range of historical data.

Treatment with 5 μM Piperonyl Butoxide produced a small statistically significant 1.4-fold increase in Cyp3a11 mRNA levels and a 1.6-fold increase in Cyp2b10 mRNA levels, which was not statistically significant. However, treatment with ≥20 μM Piperonyl Butoxide for 96 hours resulted in a marked decrease in both Cyp2b10 and Cyp3a11 mRNA levels (Table 43). The reasons for the reduction in Cyp2b10 and Cyp3a11 mRNA levels at the higher Piperonyl Butoxide concentrations are unknown, but may either be a consequence of CYP enzyme inhibition by Piperonyl Butoxide (as has been observed with other CYP inhibitors) or may be reflective of the time point that the mRNA measurements were made (i.e. following 96 hours in culture), when an increase in CYP mRNA levels may have already occurred and hence subsequently decreased prior to harvest. At Piperonyl Butoxide concentrations ≥200 μM, substantial cytotoxicity was noted both microscopically and from the ATP depletion results (Table 41).

Treatment with NaPB resulted in statistically-significant increases in both Cyp2b10 and Cyp3a11 mRNAs, as expected (Table 43).

The results of this study provided data to help assess the human relevance of the proposed MoA for Piperonyl Butoxide-induced liver tumour formation.

Conclusion: The treatment of isolated male CD-1 mouse hepatocyte cultures with Piperonyl Butoxide caused strong concentration-dependent increases in replicative DNA-synthesis as determined by the S-phase labelling index. Cyp2b10 and Cyp3a11 mRNA levels were somewhat increased by treatment with 5 μM Piperonyl Butoxide, whereas treatment with ≥20 μM Piperonyl Butoxide resulted in a marked reduction in Cyp2b10 and Cyp3a11 mRNA levels.

Treatment with the positive control items NaPB and EGF gave the expected set of responses, indicating the suitability of the test system.

The study confirmed that Piperonyl Butoxide induces replicative DNA synthesis in cultured mouse hepatocytes, which is consistent with the *in vivo* observations (Phase I studies).

b) *In vitro* human study: Piperonyl Butoxide: MoA Phase III - Cytochrome P450 Enzyme and Replicative DNA-Synthesis Induction in Cultured Male and Female Human Hepatocytes; Elcombe, B. and Vardy, A., (2017b), Report Number: CXR16212, Performing Laboratory: CXR Biosciences Ltd, Dundee, UK.

Summary: This study investigated the potential for Piperonyl Butoxide to stimulate hepatocellular proliferation (measured as the change in replicative DNA synthesis [S-phase of the cell cycle]) and cytochrome P450 (CYP) 2B6 and 3A4 mRNA levels in isolated male and female human hepatocyte cultures (both from caucasian donors, aged 51 and 52 years, respectively). Cytotoxicity, as evaluated by ATP depletion, was assessed in parallel. NaPB and EGF served as Positive Control items, where appropriate.

While Piperonyl Butoxide like NaPB showed evidence of CAR/PXR activation, neither Piperonyl Butoxide nor NaPB caused any increase in replicative DNA synthesis in either male or female human hepatocytes in culture. This was in strong contrast to the situation in the mouse study described above and is considered to reflect a key difference in response to CAR activation between the species.

The assay was validated as EGF produced a robust statistically increase in replicative DNA synthesis, demonstrating that the human liver cells could respond to a proliferative stimulus.

Piperonyl Butoxide showed some evidence of CAR/PXR activation, although this was more marked in male hepatocytes as indicated by induction of CYP2B6 and CYP3A4 mRNA levels. NaPB treatment of male and female hepatocytes resulted in statistically significant increases in both mRNA levels.

Study design: Treatment with ≥50 μM Piperonyl Butoxide caused cytotoxicity in male and female human primary hepatocytes in the preliminary study (Elcombe, B. 2017; CXR1620). However, 500 μM Piperonyl Butoxide was selected as the top concentration to be assessed in the current study to allow comparison with the study conducted using isolated hepatocytes from male CD-1 mice (Elcombe, B. 2017; CXR1621). Therefore, primary monolayer cultures of hepatocytes were prepared and exposed to Piperonyl Butoxide at 6 concentrations (5, 10, 20, 50, 200 and 500 μM) or to a vehicle control (0.1% DMSO). Separate cultures were exposed to NaPB at 3 concentrations (10, 100 and 1000 μM) as a positive control. There were 3 replicates for each concentration for CYP2B6 and CYP3A4 Taqman mRNA measurements, 5 replicates for each concentration for replicative DNA synthesis (incorporation of BrdU) and 6 replicates for each concentration for cytotoxicity measurements (measured as the change in cellular ATP). EGF was tested at a single concentration (25 ng/mL) as a positive control agent for replicative DNA synthesis.

Results: Treatment with ≥50 μM Piperonyl Butoxide caused cytotoxicity (as determined by depletion in ATP levels) in both male and female human hepatocyte cultures. However, 500 μM Piperonyl Butoxide was the top concentration assessed in the current study to allow a direct comparison to be made with the CD-1 mouse hepatocyte study (Elcombe, B, 2017b; CXR1621). NaPB had no biologically-significant effect on ATP levels.

Neither Piperonyl Butoxide nor NaPB caused any increase in replicative DNA synthesis, as determined from the S-phase labelling index (Table 42). However, treatment with EGF resulted in statistically-significant increases in replicative DNA-synthesis in both sets of human hepatocytes, demonstrating their ability to respond to a proliferative stimulus (Table 42).

There was some evidence of Piperonyl Butoxide-mediated induction of CYP2B6 and CYP3A4 mRNA levels in both the male and female human hepatocytes. Although there was no strong evidence of a concentration-dependency, CYP2B6 and CYP3A4 mRNA levels peaked at increases of 3.8-fold and 2.6-fold, respectively in male human hepatocytes. Some statistically significant increases in

CYP2B6 and CYP3A4 mRNA levels were also observed in female human hepatocytes (Table 43). These increases in CYP mRNA levels suggest that Piperonyl Butoxide may activate both the human constitutive androstane receptor (CAR) and the pregnane X receptor (PXR).

Treatment with NaPB resulted in statistically-significant increases in both CYP2B6 and CYP3A4 mRNAs in both sets of human hepatocytes, as expected (Table 43).

The results of this study provided data to help assess the human relevance of the proposed MoA for Piperonyl Butoxide-induced liver tumour formation.

Conclusion: Both Piperonyl Butoxide and NaPB caused induction of CYP2B6 and CYP3A4 mRNAs, suggesting that Piperonyl Butoxide may activate both human CAR and PXR nuclear hormone receptors.

However, while Piperonyl Butoxide like NaPB showed evidence of CAR/PXR activation, neither Piperonyl Butoxide nor NaPB caused any increase in replicative DNA synthesis in either male or female human hepatocytes in culture. This was in strong contrast to the situation in the mouse study and is considered to reflect a key difference in response to CAR activation between the species.

EGF produced a robust increase in replicative DNA synthesis, demonstrating that the test system could respond to a proliferative stimulus.

Comparison of data between mouse and human hepatocytes

Significant cellular toxicity (as determined by depletion in ATP levels) was measured at ≥ 200 µM PBO both in mouse and human hepatocyte cultures, while treatment with NaPB had no biologically-significant effect on ATP levels in either culture (Table 41).

Table 41: ATP Assay Following NaPB or Piperonyl Butoxide Administration in Mouse and Human Hepatocytes.

Test/Control Item & concentration	ATP content (luminescence units)		
	Mouse	Human (male)	Human (female)
Vehicle control (0.1% [v/v] DMSO)	636,307 ± 15,523 (100.0 ± 2.4)	322,975 ± 20,879 (100.0 ± 6.5)	221,455 ± 29,746 (100.0 ± 13.4)
10 µM NaPB	577,847 ± 26,202*** (90.8 ± 4.1)	345,970 ± 21,063 (107.1 ± 6.5)	253,858 ± 13,162* (114.6 ± 5.9)
100 µM NaPB	553,202 ± 13,573*** (86.9 ± 2.1)	334,248 ± 19,846 (103.5 ± 6.1)	247,029 ± 7265 (111.5 ± 3.3)
1000 µM NaPB	551,556 ± 6499*** (86.7 ± 1.0)	298,116 ± 22,608 (92.3 ± 7.0)	209,706 ± 7256 (94.7 ± 3.3)
5 µM PBO	550,593 ± 16,143*** (86.5 ± 2.5)	336,931 ± 25,681 (104.3 ± 8.0)	210,210 ± 10,116 (94.9 ± 4.6)
10 µM PBO	451,026 ± 9897*** (70.9 ± 1.6)	302,913 ± 23,259 (93.8 ± 7.2)	213,697 ± 28,759 (96.5 ± 13.0)
20 µM PBO	388,820 ± 18,112*** (61.1 ± 2.8)	315,910 ± 14,037 (97.8 ± 4.3)	207,197 ± 19,586 (93.6 ± 8.8)
50 µM PBO	381,907 ± 12,522*** (60.0 ± 2.0)	200,257 ± 6661*** (62.0 ± 2.1)	168,962 ± 26,203** (76.3 ± 11.8)
200 µM PBO	10,185 ± 3065*** (1.6 ± 0.5)	16,832 ± 6562*** (5.2 ± 2.0)	25,216 ± 23,724*** (11.4 ± 10.7)
500 µM PBO	947 ± 124*** (0.1 ± 0.0)	2972 ± 173*** (0.9 ± 0.1)	2348 ± 1104*** (1.1 ± 0.5)
Values are Mean ± SD. Values in parentheses are mean % control ± SD; n=6 per group. A Student's t-test (two-tailed) was performed on the results; statistically different from control *p<0.05; ** p<0.01; *** p<0.001			

In cultured mouse hepatocytes, treatment with PBO caused a strong concentration-dependent increase in DNA replicative synthesis, as determined by the S-phase labelling index. Treatment with NaPB or EGF resulted also in statistically-significant increases in replicative DNA synthesis, as expected (Table 42). However, no increase in replicative DNA synthesis was observed in cultured human hepatocytes, although treatment with the positive control EGF produced a marked mitogenic response, indicating the functional viability of the *in vitro* test system (Table 42). Treatment with NaPB also had no impact on replicative DNA synthesis in either the male or the female human hepatocytes.

Table 42: Replicative DNA Synthesis (S-Phase) Following NaPB, EGF or Piperonyl Butoxide Administration in Mouse and Human Hepatocytes

Test/Control Item & concentration	S-phase labelling index		
	Mouse	Human (male)	Human (female)
Vehicle control (0.1% [v/v] DMSO)	1.34 ± 0.33 (100.0 ± 24.3)	0.49 ± 0.09 (100.0 ± 18.1)	0.54 ± 0.08 (100.0 ± 15.5)
10 µM NaPB	2.01 ± 0.11** (150.2 ± 8.0)	0.50 ± 0.18 (100.2 ± 36.0)	0.55 ± 0.09 (101.1 ± 16.6)
100 µM NaPB	2.63 ± 0.37*** (196.8 ± 27.9)	0.44 ± 0.10 (88.4 ± 20.8)	0.53 ± 0.22 (98.7 ± 40.5)
1000 µM NaPB	1.88 ± 0.29* (140.1 ± 21.8)	0.40 ± 0.08 (80.8 ± 16.5)	0.49 ± 0.07 (90.0 ± 13.4)
5 µM PBO	3.44 ± 0.28*** (256.9 ± 21.2)	0.45 ± 0.11 (91.7 ± 22.8)	0.63 ± 0.04 (117.0 ± 8.3)
10 µM PBO	4.61 ± 0.48*** (344.6 ± 36.2)	0.49 ± 0.07 (98.6 ± 14.8)	0.56 ± 0.12 (103.9 ± 22.4)
20 µM PBO	7.91 ± 0.65*** (590.6 ± 48.6)	0.52 ± 0.12 (105.0 ± 24.9)	0.56 ± 0.06 (103.6 ± 1 0.9)
50 µM PBO	6.72 ± 0.48*** (501.9 ± 35.8)	0.46 ± 0.07[#] (93.7 ± 14.9)	0.52 ± 0.04[#] (96.4 ± 6.5)
200 µM PBO[#]	0.64 ± 0.28** (47.7 ± 20.7)	0.21 ± 0.10**[##] (42.1 ± 21.0)	ND
500 µM PBO	ND	ND	ND
25 ng/mL EGF	8.43 ± 1.47*** (629.3 ± 109.6)	7.20 ± 0.70*** (1457.0 ± 141.5)	18.95 ± 1.48*** (3509.8 ± 273.6)

Values are Mean ± SD. Values in parentheses are mean % control ± SD; n=5 per group. A Student's t-test (two-tailed) was performed on the results; statistically different from control *p<0.05; ** p<0.01; *** p<0.001.
ND – Not determined due to high cytotoxicity at this concentration.
[#] Some globules (hypothesised to be Test Item crystals) observed on the base of the plate. Hepatocytes, although sparse, were morphologically normal.
[##] Large globules observed on the base of the plate. Hepatocytes, sparse and appeared damaged from a morphological perspective.

Treatment with 5 µM PBO resulted in a statistically significant increase in Cyp3a11 mRNA levels, whereas the treatment of male CD-1 mouse hepatocytes with ≥20 µM PBO resulted in a marked statistically significant reduction in Cyp2b10 and Cyp3a11 mRNA levels (Table 43).

Treatment with 5 – 200 µM PBO produced significant increases in CYP2B6 mRNA levels in the male human hepatocytes but small increases in the female human hepatocytes. Statistically-significant increases in CYP3A4 mRNA levels were observed both in the male and the female human hepatocytes following PBO administration (Table 43).

Table 43: Expression of CYP2B and CYP3A mRNA Following NaPB or Piperonyl Butoxide Administration in Mouse and Human Hepatocytes.

Test/Control Item	Mouse		Human - male		Human - female	
	Cyp2b10 mRNA	Cyp3a11 mRNA	CYP2B6 mRNA	CYP3A4 mRNA	CYP2B6 mRNA	CYP3A4 mRNA
Vehicle control	1.00±0.19	1.00±0.09	1.00±0.18	1.00±0.17	1.00±0.20	1.00±0.13
10 µM NaPB	1.03±0.03	0.98±0.06	2.03±1.28	1.22±0.48	0.81±0.29	0.95±0.33
100 µM NaPB	1.72±0.08**	1.14±0.21	2.90±0.89*	1.71±0.36*	1.16±0.45	1.20±0.20
1000 µM NaPB	2.33±0.17***	1.63±0.26*	9.92±1.95**	6.98±1.60**	2.80±0.35**	3.62±0.62**
5 µM PBO	1.58±0.35	1.40±0.10**	3.52±0.19***	2.20±0.36**	2.02±1.65	2.05±1.53
10 µM PBO	0.79±0.06	1.31±0.29	3.29±1.09*	1.74±0.61	1.36±0.70	1.39±0.50
20 µM PBO	0.04±0.03***	0.23±0.09***	3.83±1.37*	2.58±0.88*	1.36±0.25	1.65±0.47
50 µM PBO	0.01±0.01***	0.10±0.02***	2.92±0.32***	2.29±0.44**	1.18±0.25	1.92±0.46*
200 µM PBO	0.08±0.06**	0.17±0.08***	3.55±1.00*	2.30±1.02	2.95±1.50	3.63±0.54**
500 µM PBO	0.11±0.17**	0.02±0.04***	ND		1.76±0.40*	1.94±0.55*

Values are Mean ± SD; n=3 per group. Results are expressed as fold change relative to control, where control values were normalised to 1.00. β-actin was employed as the internal control. A Student's t-test (two-tailed) was performed on the results; statistically different from control *p<0.05; ** p<0.01 ;***p<0.001.

ND – Not determined due to high cytotoxicity at this concentration.

Overall, despite clear evidence that Piperonyl Butoxide, like NaPB, activates both human CAR and PXR nuclear hormone receptors as seen in mouse, however it does not cause cell proliferation in cultured human liver cells. This is in strong contrast to the situation observed in the mouse study and is considered to reflect a key difference in CAR activation and mitogenic response between the species.

MoA analysis of Piperonyl Butoxide

The weight of evidence supports a MoA for liver tumour formation in mouse, where altered gene expression in response to Piperonyl Butoxide-induced CAR activation leads to increased replicative DNA synthesis and hepatocellular proliferation resulting in liver adenomas.

This is a well-known and accepted MoA for mouse and rat liver tumour formation, which lack relevance to humans due to a key species difference. This can be explained with reference to the key events and results from Phase I, II and III studies, which are consistent with the scientific literature and which is briefly summarised below.

An increase in DNA replicative synthesis occurred in vitro, in cultured mouse hepatocytes and also in CD1 mice dosed for 7 days with PBO. However, no increase in replicative DNA synthesis was observed in cultured human hepatocytes, although treatment with the positive control EGF produced a marked mitogenic response, indicating the functional viability of the *in vitro* test system (Table 42). However, CAR activation does not stimulate replicative DNA synthesis in human hepatocytes (Table 42) and this is considered to be the key difference between the two species.

A recent *in vivo* study describes the hepatic effects of NaPB in wild-type mice (CD-1) and rats (Wistar) and in mice and chimeric mice with humanised livers. In contrast to the effects in wild-type mice and rats, treatment with NaPB did not stimulate replicative DNA synthesis and did not increase the expression of cell proliferation related genes in the humanised hepatocytes of the chimeric mice (Yamada *et al*., 2014[4]). A number of studies performed in mice lacking CAR have shown the important role of increased cell proliferation in a CAR activator MoA for rodent liver carcinogenicity (Elcombe *et al*, 2014[5]). In CAR knockout mice, NaPB does not stimulate hepatocellular proliferation and does not promote liver tumours after treatment with a genotoxic carcinogen (Huang *et al*., 2005; Wei *et al*., 2000; Yamamoto *et al*., 2004). Similarly, a plethora of studies have shown that while NaPB induces replicative DNA synthesis in cultured rat hepatocytes, this is not observed in human hepatocytes (Parzefall *et al*., 1991, Hirose *et al*., 2009, Lake, 2009 and Yamada *et al*., 2015).

As Phase I, II and III studies show that PBO is NaPB-like, it may be concluded that the MoA in mouse, where CAR activation leads to liver tumours at high doses of PBO, has no relevance to humans, due to the discussed qualitative differences.

The conclusion that the MoA for Piperonyl Butoxide-induced mouse liver tumour formation is qualitatively not relevant to humans is further supported by available epidemiological studies with NaPB (La Vecchia and Negri, 2014[6]). In such studies, NaPB administration for many years at doses which produced similar plasma levels to those known to be carcinogenic in mice (Monro, 1993[7]), did not result in increased cancer risk. As PBO is NaPB-like, this observation further supports the lack of human relevance of mouse liver tumours.

Overall Conclusion

A robust MoA for Piperonyl Butoxide-induced mouse liver tumour formation is established and confirmed. This MoA involves CAR activation and induction of replicative DNA synthesis in mouse hepatocytes. However, the MoA of PBO in mouse carcinogenicity is not relevant to humans due to qualitative differences between the two species. Specifically, CAR receptor activation does not result in a proliferative response in cultured human hepatocytes, as it is observed in mouse. Therefore, it can be concluded that the occurrence of hepatic adenomas in mouse at high doses following life-time administration of PBO does not consist a cancer hazard for humans.

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[4] Yamada T, Okuda Y, Kushida M, Sumida K, Takeuchi H, Nagahori H, Fukuda T, Lake BG, Cohen SM, Kawamura S. Human hepatocytes support the hypertrophic but not the hyperplastic response to the murine nongenotoxic hepatocarcinogen sodium phenobarbital in an in vivo study using a chimeric mouse with humanized liver. Toxicol Sci. 2014 Nov;142(1):137-57. doi: 10.1093/toxsci/kfu173. Epub 2014 Aug 21. PubMed PMID: 25145657.

[5] Elcombe CR, Peffer RC, Wolf DC, Bailey J, Bars R, Bell D, Cattley RC, Ferguson SS, Geter D, Goetz A, Goodman JI, Hester S, Jacobs A, Omiecinski CJ, Schoeny R, Xie W, Lake BG. Mode of action and human relevance analysis for nuclear receptor-mediated liver toxicity: A case study with phenobarbital as a model constitutive androstane receptor (CAR) activator. Crit Rev Toxicol. 2014 Jan;44(1):64-82. doi: 10.3109/10408444.2013.835786. Epub 2013 Nov 4. Review. PubMed PMID: 24180433; PubMed Central PMCID: PMC4019974.

[6] La Vecchia C. and Negri E. (2014). A review of epidemiological data on epilepsy, phenobarbital, and risk of liver cancer. European Journal of Cancer Prevention. 23(1):1–7, JAN 2014. DOI: 10.1097/CEJ.0b013e32836014c8, Publication Date: 2014/01/01

[7] Monro A. (1993). How useful are chronic (life-span) toxicology studies in rodents in identifying pharmaceuticals that pose a carcinogenic risk to humans? Adverse Drug Reactions and Toxicological Reviews [01 Jan 1993, 12(1):5-34], PMID:8513076.

Carcinogenicity: inhalation

No data.

Carcinogenicity: dermal

No data.

4.10.1.2 Human information

No chronic toxicity and carcinogenicity data available.

4.10.1.3 Other relevant information

None available.

4.10.1.4 Summary and discussion of carcinogenicity

Chronic toxicity and oncogenicity of Piperonyl Butoxide has been assessed in rats and mice.

In rats, Piperonyl Butoxide was not found to be carcinogenic in a two-year dietary study (Anonymous - 10, 1987) at doses up to 500 mg/kg bw/day.

In mice, a positive dose related trend in the incidence of adenomas and the incidence of combined adenomas and carcinomas with statistical increases in the middle and high doses was observed in males from the dose of 100 mg/kg bw/day. No statistical evaluation of the results for female mice was performed by the Industry. These findings were confirmed in an open literature study in mice (Takahashi 1994b), where Piperonyl Butoxide induced hepatocellular carcinomas in all treated groups in a dose-dependent manner when administered orally in the diet at daily doses of 6000, 12000 ppm.

However, based on the results of the Phase I, II and III studies, that were performed to elucidate the MoA of Piperonyl butoxide in mouse carcinogenicity, the mouse liver tumours are not considered relevant to humans due to qualitative differences between the two species. This MoA involves CAR activation and induction of replicative DNA synthesis in mouse hepatocytes. However, the MoA of PBO-induced mouse liver formation is qualitatively not plausible for humans. Specifically, Piperonyl butoxide does not stimulate a proliferative response in cultured human hepatocytes, as it is observed in mouse. Therefore, it can be concluded that the occurrence of hepatic adenomas in mouse at high doses following life-time administration of PBO does not consist a cancer hazard for humans.

4.10.2 Comparison with criteria

The criteria under CLP specify that classification as carcinogen category 1A is largely based on human evidence. There is no evidence of Piperonyl Butoxide having caused cancer in humans, therefore Carc. 1A is not warranted.

The criteria under CLP specify that classification as carcinogen category 1B is largely based on animal evidence, i.e. there should be sufficient evidence for carcinogenicity in animal experiment. This latter is defined in CLP Annex I 3.6.2.2.3. (b) Carcinogenicity in experimental animals:

Sufficient evidence of carcinogenicity: a causal relationship has been established between the agent and an increased incidence of malignant neoplasms or of an appropriate combination of benign and

malignant neoplasms in (a) two or more species of animals or (b) two or more independent studies in one species carried out at different times or in different laboratories or under different protocols. An increased incidence of tumours in both sexes of a single species in a well-conducted study, ideally conducted under Good Laboratory Practices, can also provide sufficient evidence. A single study in one species and sex might be considered to provide sufficient evidence of carcinogenicity when malignant neoplasms occur to an unusual degree with regard to incidence, site, type of tumour or age at onset, or when there are strong findings of tumours at multiple sites;

In animal studies, tumour incidence was observed in only one species (mouse), in one tissue (liver), no evidence of mutagenic MOA. A carcinogenic effect was observed in a second species (rat) but at dose levels exceeding the MTD, so the findings were not considered reliable.

Based on the results of the Phase I, II and III studies, that were performed to elucidate the MoA of Piperonyl butoxide in mouse carcinogenicity, the mouse liver tumours are not considered to have relevance to humans due to qualitative differences between the two species. This MoA involves CAR activation and induction of replicative DNA synthesis in mouse hepatocytes. However, the MoA of PBO-induced mouse liver formation is qualitatively not plausible for humans. Specifically, Piperonyl butoxide does not stimulate a proliferative response in cultured human hepatocytes, as it is observed in mouse. Therefore, it can be concluded that the occurrence of hepatic adenomas in mouse at high doses following life-time administration of PBO does not consist a cancer hazard for humans. Therefore, classification under category 2 is not warranted.

4.10.3 Conclusions on classification and labelling for carcinogenicity

CLP: No classification based on available data.

RAC evaluation of carcinogenicity

Summary of the Dossier Submitter's proposal

Carcinogenicity studies

Carcinogenicity was assessed in rats and mice.

In an OECD TG 453 study with deviations (no satellite groups, some haematology parameters missing, highest dose level exceeded MTD) in rats, liver and kidney were target organs but only single incidences of adenomas or carcinomas were observed up to the highest dose. Another non-guideline oral carcinogenicity study in rats was deemed not acceptable as the MTD was exceeded in both the mid and high dose. At the lowest dose level of approximately 547 and 537 mg/kg bw/d (males and females, respectively) toxicity evidenced by caecal haemorrhages, altered haematology, and hepatotoxicity occurred. However no hepatocellular adenomas or carcinomas were observed.

The DS concluded that there was no indication of carcinogenicity of PBO in rats.

In mice, an OECD TG 451 study is available. Relative and absolute liver weights increased in both sexes from the mid dose. Hepatocellular adenomas were observed in males from the lowest dose of 30 mg/kg bw/d (at this dose the findings are within the historical control data (HCD) range). Hepatocellular carcinomas occurred at the highest dose of 300 mg/kg bw/d in 5/60 males. Low

incidences for carcinomas of 2 or 3 out of 60 occurred in the mid and low dose males. There was disagreement concerning the first diagnosis of carcinomas in this study. Therefore, two independent reviews of the findings were performed. The results are shown in the table below.

Table: *Results of the initial diagnosis of carcinomas in male mice, and of two peer reviews regarding incidences of carcinomas in male mice treated with PBO. Table 33 of the CLH report*.

Dose level (mg/kg bw/d)	**Control**	**Control**	**30**	**100**	**300**
Male mice examined	60	60	60	60	60
Male mice with carcinoma					
BRRC first diagnosis	1	0	3	2	5
BRRC 1st peer review	2	2	3	2	7
Majority opinion in 2nd peer review	3	3	2	2	5

The DS followed the majority opinion in the second peer review and presented a statistical analysis of these incidences. They concluded that a positive dose-related trend in the incidence of adenomas and the incidence of combined adenomas and carcinomas with statistical increases in the mid and high doses was observed in male mice. Furthermore, incidences of hepatocellular adenomas in male mice in mid and high dose groups, and of hepatocellular carcinomas in male mice of the high dose group were outside the respective HCD range. The Joint FAO/WHO Meeting on Pesticide Residues (JMPR) concluded that hepatocellular adenomas observed in PBO-treated CD-1 mice were histologically different from spontaneous adenomas found in this strain.

In a second non-guideline study, male Crj:CD-1 mice were dosed with approximately 900 or 1800 mg PBO/kg bw/d via feed for one year. Terminal body weights were decreased by 29% compared to controls in the higher dose. Thus, MTD was exceeded. In the lower dose, hepatocellular adenomas and carcinomas were observed. The DS considered the study to be of low reliability due to the high dose levels and shorter exposure duration (12 months instead of 18 months).

Overall, the DS concluded that PBO was carcinogenic in mice. They included several studies on a putative MoA for the formation of hepatic tumours via CAR/PXR activation to show that findings in mice were not relevant for humans. These are summarised below.

Mode of action

Industry provided a test strategy to elucidate the postulated MoA. It comprised three phases.

Phase I: As the MoA proposed is the same as for phenobarbital, hepatic effects of PBO and sodium phenobarbital (NaPB) were compared in CD-1 mice.

Phase II: Hepatic effects of PBO administration were compared in wild type and CAR/PXR double knockout mice to investigate whether effects are mediated through CAR.

Phase III: An *in vitro* comparison of PBO- and NaPB-induced effects was conducted in human and mouse hepatocytes concerning CYP activation and induction of replicative DNA synthesis.

- Phase I study

Groups of 7 or 8 male CD-1 mice were administered 0, 30, 100, or 300 mg PBO/kg bw/d via the diet for 14 days. A positive control group received 100 mg NaPB/kg bw/d. Recovery groups received 300 mg PBO/kg bw/d or 100 mg NaPB/kg bw/d for 14 days followed by a 28-day

recovery period. Hepatic CYP expression was altered in PBO exposed mice in a dose-dependent manner.

PBO and NaPB treatment resulted in the induction of *Cyps 1a2*, *2b10*, and *3a11*. Induction was reversible in the recovery period. The study authors concluded that hepatic effects of PBO were similar to those of NaPB, which suggests that PBO is a constitutive androstane receptor (CAR) activator in mice.

- Phase II study

In this study, groups of 8 male C57BL/6J wild-type and CAR/PXR double knockout mice were administered diets containing 0 or 1243-1354 ppm PBO for 14 days. The calculated mean intakes for the C57BL/6J wild-type and CAR/PXR double knockout mice were 291 and 236 mg/kg bw/d, respectively. Liver homogenates were assayed for cyanide-insensitive palmitoyl-CoA oxidation activity to assess the induction of hepatic peroxisome proliferation. Hepatic CYP mRNA levels and microsomal CYP protein content were determined.

In contrast to C57BL/6J WT mice, the treatment of CAR/PXR KO mice with PBO resulted in a statistically significant reduction in hepatic *Cyp2b10* mRNA levels and produced only small statistically significant increases in microsomal CYP2B protein levels. The treatment of CAR/PXR KO mice with PBO also induced statistically significant increases of markers of *Cyp3a* induction. While PBO was shown to produce some increase of the markers of hepatic peroxisome proliferation, the effects were more marked in CAR/PXR KO mice than in WT mice.

The study authors concluded that results support the proposed MoA.

- Phase III studies

To investigate the response of human hepatocytes to PBO, primary human hepatocytes from two donors (one male, one female, both Caucasian, 51 and 52 years old, respectively) were cultured and incubated with increasing concentrations of PBO. Cultures incubated with increasing concentrations of NaPB served as positive controls. For comparison, primary male mouse hepatocytes were cultured similarly. Cytotoxicity was determined by ATP-depletion and replicative DNA synthesis by S-phase labelling. Furthermore, mRNA levels were measured of *Cyp2b10* and *Cyp3a11* in mouse hepatocytes, and *Cyp2b6* and *Cyp3a4* in human cultures. From a concentration of 200 µM PBO cell viability was markedly decreased in both cultures (to 1.6% of controls in mice, 5.2% of controls in male human hepatocytes, and 11.4% of controls in female human hepatocytes).

The positive control, 1000 µM NaPB induced significant increases in *Cyp2b* and *Cyp3a* mRNA levels in mouse and human hepatocytes: 2.33- and 1.63-fold in mouse hepatocytes, respectively, 9.92- and 6.98-fold in male hepatocytes, respectively, and 2.8- and 3.62-fold in female hepatocytes, respectively. *Cyp2b* mRNA was also statistically significantly induced 2- to 3.5-fold without a clear dose-response in male human hepatocytes treated with 5 to 50 µM PBO, but neither in mouse nor human female hepatocytes. In mouse hepatocytes levels were decreased to 1% of control levels in cultures treated with 50 µM PBO. Statistically significant increases in *Cyp3a* levels were measured in mouse and male human hepatocytes at 5 µM PBO (1.4- and 2.2-fold, respectively), in male human hepatocytes at 20 µM PBO (2.58-fold), and in male and female human hepatocytes at 50 µM (2.29- and 1.92-fold, respectively). In contrast, *Cyp2b10* and *Cyp3a11* mRNA levels decreased in mouse hepatocytes in a dose dependent manner down to 10% of control levels at 50 µM PBO. It was not clear why CYP2B6 and CYP3A enzymes were not induced in the mouse hepatocyte culture. The study authors assumed this may be due to the late time point of the measurement (96 hours post treatment), but in a parallel experiment with Na

PB the expected increase was observed. Another explanation for the observation was, that PBO is an inhibitor of CYP enzyme activities, however, this should only have an impact on enzyme activities not on the mRNA levels of the respective enzymes.

PBO, like NaPB, activated both human CAR and PXR nuclear hormone receptors, however it did not cause cell proliferation in cultured human liver cells. Positive control EGF did induce increased labelling indices, indicating the functional viability of the test cultures. The different responses to NaPB and PBO in human hepatocytes in contrast to the situation observed in the mouse studies were considered by the DS to reflect a key difference in CAR activation and mitogenic response between the species.

The DS concluded that a robust rodent specific MoA for PBO-induced mouse liver tumour formation was established and confirmed, and that the occurrence of hepatic adenomas in mouse at high doses following lifetime administration of PBO does not constitute a cancer hazard for humans.

Conclusion on classification

Overall, tumour incidence was observed in only one species (mouse), in one tissue (liver), and there was no evidence of mutagenic MoA. A carcinogenic effect was observed in a second species (rat) but at dose levels exceeding the MTD, so the findings were considered not relevant for assessment. The established MoA was deemed rodent specific and not relevant for humans. Therefore, the DS concluded that **no classification** for Carcinogenicity is warranted.

Comments received during public consultation

One IND comment supported no classification for Carcinogenicity.

One MSCA commented that there are the following arguments for Carc. 2 classification:

- In the non-guideline rat study mortality related to caecal haemorrhage was only reported for mid dose males at weeks 45 to 58, but not in females or high dose group. Adenoma occurred from the low dose (~500 mg/kg bw/d), additional carcinoma from the mid dose of ~1000 mg/kg bw/d. Thus, hepatic neoplasia was observed in two species. Incidences should have been reported.
- Significantly increased size and number of GST-P positive foci were observed in gpt delta rats when administered 12000 ppm PBO in feed over 4 weeks (Matsushida *et al.* 2013).
- While CAR involvement was shown in mechanistic studies, AhR related pathways were not ruled out. *Cyp1a* induction was shown in WT and CAR/PXR KO mice. This induction is supported by findings by Kawai *et al.* (2009). Therefore, the MSCA considered the involvement of an AhR pathway a plausible alternative MoA which is relevant for humans.

The DS replied that the CAR/PXR MoA is also valid for rats. They questioned the validity of the non-guideline study, since neoplastic changes occurred only at the mid and high dose, which caused a marked decrease in body weights by 14.5% and 30% for males and females, respectively, at mid dose, and 22.8% and 50% for males and females, respectively, at high dose. Furthermore, the study authors noted that contamination with safrole compounds might have been responsible for the observed tumours. In the second rat study (Anon. 10), body weights were also decreased at the high dose of 500 mg/kg bw/d by up to 20% in males and 27% in females compared to controls. Adenomas were observed in only 2 out of 60 males in the high dose. Overall, the DS considered findings in the rat not robust enough for classification.

Concerning the MoA, the DS noted the following:

- Although *Cyp1a* was induced by PBO in CAR/PXR KO mice, EROD enzyme activity compared to controls was reduced compared to WT mice (646% vs 141% in WT vs KO compared to controls). This indicates no activation of AhR.
- *Cyp1a* induction was very low compared to the induction of *Cyp2b10*.
- Although increases of cells in S-phase were of the same magnitude in WT and KO mice, these findings were not accompanied by hepatocellular hypertrophy or increases in liver weights in KO mice, while 10/10 WT mice showed hepatocellular hypertrophy and increased relative liver weights (up to 24%). (RAC notes that liver weights or histological effects observed in this study were not reported in the CLH report and that group size was reported to be 8, not 10.)
- Replicative DNA synthesis was increased in mouse hepatocytes but not in human hepatocytes treated with PBO.
- Both *Cyp1a* and AhR mRNA are also induced by CAR activation.

Overall, the DS concluded that carcinogenesis was observed only in one species, and the proposed MoA is plausible and not relevant for humans.

Assessment and comparison with the classification criteria

Carcinogenicity studies

No human data are available. Four experimental carcinogenicity studies are available, two in rats and two in mice. They are summarised in the table below.

***Table**: Available carcinogenicity studies in rats and mice. All effects mentioned exhibited statistical significance, unless noted otherwise.*

Method	Results	Remarks
Rat (Sprague-Dawley): 2-year, feeding OECD TG 453 Purity: 87.67-89.71% Doses: 0, 30, 100, 500 mg /kg bw/d Anon. 10	500 mg/kg bw/d: ↓ body weight (males: -22%, females: -26% compared to controls) ➔ MTD exceeded, findings not relevant for assessment 100 mg/kg bw/d: ↓ testes weights, bilateral testes atrophy (equivocal biological significance) ↑ relative liver weights ↑ kidney weights (females only) glomerulonephritis (females only) focal mixed cells in liver (females only) hyperplasia of thyroid follicles (females only) 30 mg/kg bw/d: bilateral testes atrophy (equivocal biological significance) ↑ relative brain weights (males only) ↑ liver weights (females only) ↑ kidney weights (females only) hyperplasia of thyroid follicles (males only)	Deviations from guideline: 1. no satellite groups for evaluation of pathology other than tumours 2. additional blood sampling should have been performed at approximately 12 weeks. 3. no measurement of packed cell volume. 4. highest dose level depressed body weight gain by more than 10%.

Rat (Fisher 344/DuCrj): 2-year feeding non-guideline Doses: 0, 6000, 12000, 24000 ppm males: 0, 547, 1052, 1877 mg/kg/d females: 0, 537, 1061, 2002 mg/kg/d (values calculated based on food consumption in a preliminary trial) Takahashi 1994a	1877/2002 mg/kg bw/d: ↓ body weight (males: -48%, females: -50%) survival: Males: 25/33 (ctrl: 25/30) Females: 26/33 (ctrl: 24/30) Hepatocellular carcinoma: Males: 20/25 (ctrl: 0/25) Females: 15/26 (ctrl: 0/24) ➔ MTD exceeded, findings not relevant for assessment 1052/1061 mg/kg bw/d: ↓ body weight (males: -15%, females: -23%) survival (↓ in males) Males: 15/30 (ctrl: 25/30) Females: 25/30 (ctrl: 24/30) Hepatocellular carcinoma: Males: 4/15 (ctrl: 0/25) Females: 0/25 (ctrl: 0/24) ➔ MTD exceeded, findings not relevant for assessment 547/537 mg/kg bw/d: ↓ body weight (males: -4.4%, females: -9.7%) no hepatocellular adenomas or carcinomas ↑ (relative) liver weights (females only) caecal enlargement caecal haemorrhages (significantly increased incidences only in dead males) erosion of caecal mucosa black coloured kidneys (significantly increased incidences in surviving females only) thrombocythemia (males only) ↑ number of rats with macroscopic nodules of the liver (males only) hepatocellular basophilic or clear cell foci (males only)	low relevance gastric and caecal haemorrhage at all dose levels; MTD exceeded at mid and high doses; high dose > limit dose
Mouse (Charles River CD-1): 18-months, feeding OECD TG 451 Doses: 0, 30, 100, 300 mg /kg bw/d	300 mg/kg bw/d: ↑ (relative) liver weights liver hypertrophy hepatocellular adenomas hepatocellular carcinomas (5/60 males; ctrl: 3/60) hepatocellular necrosis (males only, high incidences also in control males)	reliable

Anon. 11	100 mg /kg bw/d: ↑ (relative) liver weights liver hypertrophy (males only) hepatocellular adenomas (males only) hepatocellular necrosis (females only) 30 mg/kg bw/d: hepatocellular adenomas (13/60 males vs 8/60 and 7/60 in control groups, upper border of HCD range)	
Mouse (Crj:CD-1): 1-year feeding non-guideline males only Doses: 0, 6000, 12000 ppm 0, 900 and 1800 mg/kg bw/d (doses in mg/kg bw/d calculated by DS based on default values for food consumption) Takahashi 1994b	1800 mg/kg bw/d: ↓ body weight (-29%) ↓ survival (81% vs 94% in controls) ➔ MTD exceeded, findings not relevant for assessment 900 mg/kg bw/d: hepatocellular adenomas and carcinomas (combined: 24.5% vs 1.9% in control)	low relevance MTD exceeded at high dose exposure for 12 instead of 18 months two dose levels only only one sex

In a 2-year oral carcinogenicity study in rats performed according to OECD TG 453 with some deviations, and with doses up to 500 mg/kg bw/d, no treatment related adenomas or carcinomas were observed in the livers of treated animals. The high dose produced marked systemic toxicity indicated by decreased terminal body weights in both sexes by 22 and 26% as compared to controls. However, kidneys and liver were the target organs as shown by increased organ weights in females of all doses. At the mid dose, focal mixed cells were observed in the livers of females.

In a second, non-guideline study, rats received higher doses (males: 0, 547, 1052, 1877 mg/kg bw/d). Both the mid and high dose led to markedly decreased terminal body weights. In the lowest dose group, no hepatocellular adenomas or carcinomas were observed, but some males had macroscopic nodules of the liver and hepatocellular basophilic or clear cell foci. At this dose – as well as in higher dose groups - signs of toxicity consisted of enlarged caecum, erosion of the caecal mucosa, caecal haemorrhages, black coloured kidneys, and haematological changes. Due to the pronounced toxicity in this study, its relevance is considered to be low.

In mice, one relevant guideline conforming study is available. Hepatocellular adenomas were observed in males starting from the lowest dose of 30 mg/kg bw/d. Incidence at this dose was at the upper border of the HCD range for the performing laboratory. Liver weights were statistically significantly increased in both males and females in the mid and high dose groups. Liver hypertrophy in the mid dose group was limited to males, in the high dose group it was observed in both males and females. Both sexes had statistically significantly increased incidences of hepatocellular adenomas at the high dose, accompanied by carcinomas of the liver in 5 of 60 males (control: 3/60).

Table: *Statistical analysis of incidences of liver adenoma and carcinoma in male mice in the guideline conforming carcinogenicity study as determined in a peer review. Modified from table 34 of the CLH report, n = 60 per group.*

Fisher Exact Test						
Dose (mg/kg bw/d)	**Control A**	**30**	**100**	**300**	**Control E**	**Trend**
Carcinoma, n	**3**	**2**	**2**	**5**	**3**	
%	5.00	3.33	3.33	8.33	5.00	
P	C	0.9318	0.9318	0.5683	C	0.2917
Adenoma, n	**8**	**13**	**21**	**25**	**7**	
%	13.33	21.67	35.00	41.67	11.67	
P	C	0.1706	0.0010	0.0000	C	0.0000

These results are supported by results of a non-guideline oral carcinogenicity study in mice. This study with two doses was performed in males only for 12 months. The high dose exceeded the MTD as shown by decreased survival and body weights. Hepatocellular adenomas and carcinomas were observed in male mice that received a dose of approximately 900 mg/kg bw/d at rates of 7/52 for adenomas (control: 1/49) and 6/52 (11.5%, control: 0/49) for carcinomas. Terminal body weights were also decreased in this dose group by 16.6% as compared to controls but survival was not affected.

Mode of Action

A mode of action via CAR/PXR activation was established in mice in three phases. First, it was shown in a 14-d feeding study in CD-1 mice, that administration of PBO produced about the same induction of CYP enzymes as administration of the CAR activator NaPB, this effect was most pronounced for *Cyp2b*. Second, in comparison of C57BL/6J wild type and CAR/PXR double knockout mice, KO mice lacked the induction of Cyp2b when treated with PBO for 14 days via the diet. Third, cultured human hepatocytes did not react with replicative DNA synthesis to treatment with PBO, while male mouse hepatocytes did. *Cyp2b* induction in mouse hepatocytes failed *in vitro* with PBO treatment but was shown in male human hepatocytes for both NaPB and PBO.

RAC notes some limitations in the presented mechanistic studies.

For the phase I and II studies RAC notes the following:

- Different strains of mice were used in the mechanistic studies. This may have had practical reasons (i.e. availability of the KO model) , but hampers comparison.
- Knockout mice had a lower mean intake of PBO than WT mice (236 vs. 291 mg/kg bw/d, respectively) which may have had an impact on results given that CYP induction in the phase I study was dose dependent. *Cyp2b10* induction was 4.3-fold at a dose of 100 mg PBO/kg bw/d and 17-fold at 300 mg PBO/kg bw/d, suggesting a steep dose-response curve.
- Although all CYP protein levels and *Cyp1a*, *3a*, and *4a* mRNA levels measured in the phase I and II studies were quite similar for CD-1 and WT C57BL/6J mice, respectively, *Cyp2b10* mRNA levels were 76-fold higher in the latter at a similar dose of PBO. This suggests that quantitative differences occur also within one species.

For the phase III studies RAC notes:

- Only one male and one female donor where used for human hepatocyte cultures.
- Only male hepatocytes were used for mouse cultures, data on effects of PBO in female mouse hepatocytes are missing.
- Precipitation of the test substance (as hypothesised by the study authors) was observed in human hepatocyte cultures from concentrations of 50 µM PBO in the S-phase labelling experiments but not in mouse hepatocytes cultures of the same concentrations.
- PBO treatment resulted in a dose dependent reduction in Cyp2B10 and Cyp3A11 mRNA levels at doses ≥ 20µM, while NaPB treatment showed the expected increase.
- Statistically significant cytotoxicity was observed in all PBO and NaPB concentrations of the mouse hepatocyte culture. In contrast, statistically significant cytotoxicity in human hepatocyte cultures was observed only from concentrations of 50 µM PBO onwards.

Overall, relevance and comparability of the *in vitro* cultures with mouse and human hepatocytes may be questioned at least for the presented studies.

RAC notes that other MoAs were not explicitly ruled out in the CLH report. Other possible MoAs are evaluated below.

Mutagenicity: PBO was not genotoxic in the presented studies *in vitro* and *in vivo*.

Cytotoxicity: PBO was cytotoxic in mouse hepatocytes *in vitro* and induced a slight increase of the incidence of hepatocellular necrosis in male mice of the highest dose group in the carcinogenicity study. Cytotoxicity was also observed in human hepatocytes *in vitro* but at higher PBO concentrations. Alterations in necrosis parameters (alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase) were not reported in rodents. However, increased alkaline phosphatase levels were reported in dogs in both repeated dose toxicity studies.

Oestrogen activity: PBO is not structurally related to oestrogens, and no treatment related effects indicative of oestrogen activity were reported in any of the studies.

AhR: In the public consultation the DS noted the following regarding possible AhR induction:

- Although *Cyp1a* was induced by PBO in CAR/PXR KO mice, EROD enzyme activity compared to controls was reduced compared to WT mice (646% vs 141% in WT vs KO compared to controls). This indicates no activation of AhR.
- *Cyp1a* induction was very low compared to the induction of *Cyp2b10*.
- Both *Cyp1a* and AhR mRNA are also induced by CAR activation and are thus not specifically indicative of the AhR pathway.

PPAR-alpha: This possible pathway was not discussed neither in the CLH report nor the public consultation. CYP4B protein levels were significantly induced in both WT and CAR/PXR KO mice but induction was more pronounced in KO mice (1.37-fold induction in WT vs. 2.12-fold induction in KO mice compared to controls). On the mRNA level, induction was even more pronounced in KO mice (4.5-fold vs 1.33-fold in WT mice). *Cyp4a* levels were not determined in the *in vitro* comparison of mice and human hepatocytes. Thus, no conclusion can be drawn about the relevance of the PPAR-alpha pathway.

Conclusion on classification

As no human data are available, Cat. 1A is not appropriate. For Cat. 1B the CLP guidance demands carcinogenic responses in two or more animal species, or two or more independent studies in one species, or in one well conducted GLP study in both sexes of one species, or in one sex of one species, when malignant neoplasms occur with an unusual degree or tumour type, at an early time point or at multiple sites. None of these criteria are met.

If there is limited evidence from animal studies, Cat. 2 should be considered.

RAC concurs with the DS that there is evidence from one species (mouse) and mainly one sex (male) and that studies performed in rats are not informative due to excessive toxicity as evidenced by markedly decreased terminal body weights and reduced survival in the high doses used. Furthermore, RAC considers the established MoA plausible, although some data gaps remain and limitations were seen in the mechanistic studies. Nevertheless, the concern for human relevance is lowered by the data provided, which is further substantiated by the relatively low number of carcinomas observed in one sex only. Therefore, **no classification for carcinogenicity is warranted.**

4.11 Reproductive toxicity

4.11.1 Short summary and overall relevance for the provided information on adverse effects on sexual function and fertility

4.11.1.1 Non-human information

Effects of Piperonyl Butoxide on fertility were assessed in a two-generation reproduction study in rats (Anonymous - 14, 1986). Summarised data are presented in **Table 44**.

Table 44: Summary table of relevant reproductive toxicity studies

Method	Results	Remarks	Reference
Rats: Two generation reproduction, feeding EPA F, 83-4, OECD 416 Purity: 90% Batch No.: FEG-32 Doses: 0, 300, 1000, 5000 ppm (0, 30, 100, 500 mg/kg bw/day)	**Parental** LOAEL: 500 mg/kg bw/day - significantly decreased body weight and body weight gain (> ↓ 20%) NOAEL: 100 mg/kg bw/day **Reproductive** LOAEL > 500 mg/kg bw/day - No adverse effects NOAEL: 500 mg/kg bw/day **Offspring** LOAEL: 500 mg/kg bw/day - significantly decreased body weight and body weight gain	CLP: No classification Klimisch score: 1	**Anonymous - 14** 1986 (Piperonyl Butoxide CAR Doc IIIA6.8.2/01)

Method	Results	Remarks	Reference
	NOAEL: 100 mg/kg bw/day		

In the two-generation reproduction study, groups of 26 male and 26 female Charles River rats received diets containing Piperonyl Butoxide at concentrations of 0, 300, 1000, or 5000 ppm equivalent to 0, 30, 100, and 500 mg/kg bw per day for two consecutive generations, including two breeding trials/generation.

Parental toxicity was evidenced as significantly decreased body weight and body weight gain (↓20%) at the top dose. Body weight changes of F0 generation animals are summarised in Table 45. There was no dose-related increase in the incidence of gross and/or histopathological findings among males and females in the Piperonyl Butoxide treated groups in comparison to control group animals. Histopathology of reproductive organs (testes and ovaries) is in line with observations in the chronic toxicity studies in rats (see Section 4.10.1.1) in which effects in ovaries (hyperplasia of Sertoli-like cells) were attributed to aging of the animals and testicular atrophy (grade: severe) was only observed at a dose above the MTD (500 mg/kg bw/day).

Table 45: Summary of parental body weight values, F0 generation (Anonymous - 14, 1986)

Study period	Sex	Body weight (g)	Dose (mg/kg bw/day)			
			0	30	100	500
F0 generation						
Pre-mating	Males	week 4	355.0	351.4	353.5	341.1
		week 8	456.9	450.2	451.8	424.8***
		week 12	502.7	495.7	495.9	464.2 **
		week 27	636.3	624.8	625.3	567.4 **
	Females	week 4	218.4	212.4	215.6	204.7 ***
		week 8	266.2	255.4	260.4	240.9 **
		week 11	273.5	269.4	271.2	262.4 **
		week 22	322.8	314.3	319.4	294.6 **
Gestation	Females (1st littering phase)	Day 0	288.2	317.9	347.2	414.8
		Day 7	270.4 *	297.3 *	328.6	388.5 *
		Day 15	283.6	308.8	343.4	409.7
		Day 21	258.8 **	283.3 **	316.4 **	379.7 **
	Females (2nd littering phase)	Day 0	323.4	311.3	323.7	290.6 **
		Day 7	347.6	334.1	344.6	312.8 **
		Day 15	380.5	363.3	381.1	344.5 **
		Day 21	446.2	438.5	457.7	403.1 ***
Lactation	Females (1st littering phase)	Day 0	310.5	302.1	317.8	291.4 *
		Day 4	320.3	305.8	320.8	295.6 **
		Day 7	320.8	302.5	323.1	305.7
		Day 14	332.0	310.3 ***	319.1	303.9 ***

Study period	Sex	Body weight (g)	Dose (mg/kg bw/day)			
			0	30	100	500
		Day 21	317.5	304.8	319.5	306.0
	Females (2^{nd} littering phase)	Day 0	353.7	335.6	345.9	315.7 **
		Day 4	353.0	344.7	354.9	326.1 ***
		Day 7	361.7	347.7	357.9	337.1 ***
		Day 14	362.3	351.1	361.8	344.6
		Day 21	342.8	329.7	343.7	335.3

Significant differences from control at * 0.01-0.05, ** < 0.001, *** 0.001-0.01 (Student's)

There were no effects on all reproductive parameters assessed. Pup body weights were also significantly lower in the 5000 ppm group in pups of both breeding trials of both generations, compared to the respective controls.

Further details on selected reproductive, gestation and offspring parameters are included in Table 46 and Table 47.

Table 46: Summary table on selected reproductive and gestation parameters (Anonymous - 14, 1986)

Parameter	Dose (mg/kg bw/day)			
	0	30	100	500
First reproductive phase				
No. animals/sex	26	26	26	26
No. of females failing to mate	0	1	0	1
Mean no. of days to mating (± SD)	5.7 ± 5.87	3.2 ± 2.70	4.5 ± 3.58	4.0 ± 3.54
No. of pregnant females	22	19	24	22
Mating Index (%)	100.0	96.2	100.0	96.2
Fertility Index (%)	84.6	73.1	92.3	84.6
Conception Rate (%)	84.6	76.0	92.3	88.0
Gestation Index (%)	100.0	100.0	100.0	100.0
Length of gestation (days) ± SD	21.7 ± 0.46	21.8 ± 0.69	21.6 ± 0.59	21.5 ± 0.51
Duration of parturition (hours)	2.42 ± 1.071	2.48 ± 0.412	2.10 ± 0.628	2.43 ± 1.081
No. of pups at birth – live	13.5 ± 2.74	11.4 ± 3.79	13.3 ± 3.09	13.1 ± 2.51
– dead	0.32 ± 0.477	0.47 ± 0.964	0.25 ± 0.608	0.41 ± 0.908
Sex ratio (% males)	49.34 ± 11.287	44.45 ± 21.677	48.23 ± 14.232	49.25 ± 14.699
Second reproductive phase				
No. animals/sex	26	26	26	26
No. of females failing to mate	1	2	1	3
Mean no. of days to mating (± SD)	3.5 ± 4.62	4.0 ± 4.51	4.1 ± 4.51	2.1 ± 2.07
No. of pregnant females	19	16	17	18
Mating Index (%)	96.2	92.3	96.2	88.5

Parameter	Dose (mg/kg bw/day)			
	0	30	100	500
Fertility Index (%)	73.1	61.5	65.4	69.2
Conception Rate (%)	76.0	66.7	68.0	78.3
Gestation Index (%)	94.7	93.8	100.0	100.0
Length of gestation (days) ± SD	21.9 ± 0.49	21.7 ± 0.49	21.8 ± 0.44	21.8 ± 0.73
Duration of parturition (hours)	3.3 ± 0.91	2.5 ± 1.03	3.9 ± 0.53	9.5 ±11.75
No. of pups at birth – live	13.0 ± 3.83	13.5 ± 3.00	14.6 ± 2.45	11.8 ± 5.61
– dead	0.39 ± 0.608	0.53 ± 0.743	0.29 ± 0.588	0.61 ± 2.118
Sex ratio (% males)	51.77 ± 13.011	49.81 ± 10.089	47.25 ± 13.829	52.94 ± 16.260

Table 47: Summary table on selected offspring parameters (Anonymous - 14, 1986)

Parameter	Dose (mg/kg bw/day)			
	0	30	100	500
F1a generation				
Viability Index (%) – Day 4 *p.p.*	96.1 ± 7.90	94.7 ± 22.94	98.0 ± 3.67	98.3 ± 3.43
Survival Index (%) – Day 7 *p.p.*	100.0 ± 0.00	100.0 ± 0.00	100.0 ± 0.00	98.9 ± 3.68
Survival Index (%) – Day 14 *p.p.*	93.8 ± 22.41	100.0 ± 0.00	100.0 ± 0.00	98.9 ± 3.68
Survival Index (%) – Day 21 *p.p.*	93.8 ± 22.41	100.0 ± 0.00	99.5 ± 2.55	98.3 ± 4.39
Pup body weight (g) – Day 0 *p.p.*	5.9 ± 0.48	6.2 ± 0.55	5.9 ± 0.55	5.8 ± 0.51
– Day 4 *p.p.*	9.4 ± 1.03	9.9 ± 1.30	9.5 ± 1.35	8.7 ± 0.96 *
– Day 7 *p.p.*	14.8 ± 1.91	15.4 ± 1.40	15.2 ± 1.57	13.8 ± 1.13 *
– Day 14 *p.p.*	31.2 ± 3.24	29.7 ± 3.97	28.8 ± 3.30 *	26.3 ± 2.68 **
– Day 21 *p.p.*	46.9 ± 6.09	45.6 ± 6.15	44.3 ± 5.39	38.2 ± 3.70 **
F1b generation				
Viability Index (%) – Day 4 *p.p.*	99.5 ± 1.96	96.6 ± 7.22	97.8 ± 4.19	91.5 ± 23.18
Survival Index (%) – Day 7 *p.p.*	100.0 ± 0.00	100.0 ± 0.00	98.5 ± 4.15	99.3 ± 3.03
Survival Index (%) – Day 14 *p.p.*	100.0 ± 0.00	99.2 ± 3.23	98.5 ± 4.15	92.6 ± 24.23
Survival Index (%) – Day 21 *p.p.*	100.0 ± 0.00	98.3 ± 4.40	98.5 ± 4.15	91.9 ± 24.58
Pup body weight (g) – Day 0 *p.p.*	6.1 ± 0.84	6.8 ± 0.64	5.9 ± 0.42	5.9 ± 1.19
– Day 4 *p.p.*	9.7 ± 1.67	9.7 ± 1.29	9.4 ± 1.36	8.9 ± 2.28
– Day 7 *p.p.*	15.3 ± 2.08	15.6 ± 1.92	15.2 ± 2.67	13.2 ± 3.37*
– Day 14 *p.p.*	31.4 ± 3.15	31.7 ± 2.22	30.8 ± 3.22	27.5 ± 4.05 ***
– Day 21 *p.p.*	50.1 ± 5.52	49.3 ± 3.33	48.1 ± 4.75	43.2 ± 6.09 ***
F2a generation				
Viability Index (%) – Day 4 *p.p.*	99.0 ± 2.40	99.3 ± 2.10	98.3 ± 3.72	99.3 ± 2.22
Survival Index (%) – Day 7 *p.p.*	100.0 ± 0.00	100.0 ± 0.00	98.8 ± 5.46	100.0 ± 0.00
Survival Index (%) – Day 14 *p.p.*	100.0 ± 0.00	100.0 ± 0.00	95.6 ± 19.57	99.5 ± 2.61
Survival Index (%) – Day 21 *p.p.*	99.4 ± 2.80	100.0 ± 0.00	94.7 ± 20.12	99.5 ± 2.61
Pup body weight (g) – Day 0 *p.p.*	6.0 ± 0.54	6.1 ± 0.48	6.1 ± 0.53	6.1 ± 0.48

Parameter	Dose (mg/kg bw/day)			
	0	30	100	500
– Day 4 *p.p.*	9.5 ± 1.25	9.4 ± 1.20	9.8 ± 1.57	9.0 ± 1.13
– Day 7 *p.p.*	14.6 ± 1.25	14.3 ± 2.32	14.7 ± 2.43	13.6 ± 1.62
– Day 14 *p.p.*	28.5 ± 2.74	28.4 ± 3.55	29.3 ± 4.94	25.5 ± 3.81 ***
– Day 21 *p.p.*	44.4 ± 5.49	42.7 ± 5.29	43.8 ± 7.50	38.6 ± 6.79 ***

Significant differences from control at * 0.01-0.05, ** < 0.001, *** 0.001-0.01 (Student's)

The study NOAEL was set at 5000 ppm (equivalent to 500 mg Piperonyl Butoxide/Kg bw/day) with regard to reproductive parameters and at 1000 ppm (equivalent to 100 mg Piperonyl Butoxide/kg bw/day) with regard to parental and offspring toxicity.

Overall, Piperonyl Butoxide did not show toxic effects on fertility in a two-generation reproductive toxicity study in the rat at dietary doses up to 500 mg Piperonyl Butoxide/kg bw/day.

4.11.1.2 Human information

No relevant data.

4.11.2 Short summary and overall relevance for the provided information on adverse effects on development

4.11.2.1 Non-human information

Developmental toxicity of Piperonyl Butoxide was assessed in rats and rabbits. Summarised data are presented in **Table 48**.

Table 48: Summary table of relevant reproductive toxicity studies

Method	Results	Remarks	Reference
Rats (Sprague-Dawley)**:** Teratology, gavage EPA F, 83-3, OECD 414 Purity: 90.78% Batch No.: FEP-100 Doses: 0, 200, 500, 1000 mg/kg bw/day	**Maternal** LOAEL: 500 mg/kg bw/day - Clinical signs (1 dam with perinasal encrustation, 2 dams with red urogenital discharge), - decreased food consumption, - decreased body weight (↓4%) and body weight gain (↓18%). NOAEL: 200 mg/kg bw/day **Developmental** LOAEL >1000 mg/kg bw/day - No adverse effects NOAEL: 1000 mg/kg bw/day	Klimisch score: 1	**Anonymous - 15** , 1991 (Piperonyl Butoxide CAR Doc IIIA6.8.1/01)
Rabbits (NZW): Teratology, gavage	**Maternal** LOAEL: 200 mg/kg bw/day	CLP: No classification	**Anonymous - 16** , 1986

Method	Results	Remarks	Reference
EPA F, 83-3, OECD 414 Purity: 87.67 – 89.71% Batch No.: FEG-32 Doses: 0, 50, 100, 200 mg/kg bw/day	- Decreased body weight gain (↓4%) NOAEL: 100 mg/kg bw/day **Developmental** LOAEL > 200 mg/kg bw/day - No adverse effects NOAEL: 200 mg/kg bw/day	Klimisch score: 2 Deviations from testing protocol: - fewer animals with implantations in the mid dose group - lack of measurements of the following parameters: gravid uterine weight data, food consumption, uterine weight, resorption incidence and the number and percent of live offspring	(Piperonyl Butoxide CAR Doc IIIA6.8.1/02)

In a developmental toxicity study in the Sprague Dawley rat, groups of 25 mated females were given orally by gavage 0, 200, 500, and 1000 mg/kg bw/day on days 6 through 15 of gestation (Anonymous - 15, 1991). Maternal toxicity was evidenced from the dose of 500 mg/kg bw/day as clinical signs including perinasal encrustation (1 dam) and red urogenital discharge (2 dams) and significantly decreased food consumption, body weight (↓4%) and body weight gain (↓18% & 21% at 500 and 1000 mg/kg bw/day) during the entire treatment period. Relative liver weight was significantly increased in the high dose group dams. There was no evidence of embyotoxicity, teratogenicity of foetotoxicity at any dose tested. The NOAEL for maternal toxicity was set at 200 mg/kg bw/day, and that for developmental toxicity was 1000 mg/kg bw/day, the highest dose tested. Further summarised data on the study are presented in **Table 49** - **Table 50** -**Table 51**.

Table 49: Corrected final body weights (body weight- gravid uterine weight), absolute and relative liver weights of rats treated with Piperonyl Butoxide (Anonymous - 15, 1991)

Dose level (mg PBO/kg bw/day)	0	200	500	1000
Mean final corrected body weight (g)	319.9	314.7	308.6	311.9
Body weight on gestation days 6-15 (treatment)	52.38	47.75	42.80**	41.04**
Mean absolute liver weight (g)	16.3	16.3	16.4	17.6
Mean relative liver weight (%)	5.10	5.18	5.31	5.63**

** Significantly different from control group ($p < 0.01$)

Table 50: Caesarean section observations in rat developmental toxicity study (Anonymous - 15, 1991)

Observations	Dose Level [mg PBO/kg/day]			
	0	**200**	**500**	**1000**
No. Assigned	25	25	25	25
Females gravid	24	21	23	24
Maternal wastage				
# died	0	0	0	0
# sacrificed	0	0	0	0
# aborted	0	0	0	0
# early delivery	0	0	0	0
# non pregnant	1	4	2	1
Corpora lutea/dam	15.3	15.5	15.7	15.0
Total implants	15.3	14.4	14.5	13.7
Viable implants	14.8	14.1	13.8	13.2
Early resorptions	0.4	0.2	0.8	0.5
Late resorptions	0	0	0	0
Dead fetuses	0	0	0	0
Sex ratio (% male fetuses)	50.7	46.5	47.8	50.6
Fetal weight [g]	5.5	5.5	5.4	5.6

Table 51: Summary of fetal external, visceral and skeletal malformations in rat developmental toxicity study (Anonymous - 15, 1991)

	Fetuses/Litters			
Dose Level [mg PBO/kg/day]	**0**	**200**	**500**	**1000**
No. Examined Externally	355	311	331	317
External Malformations	7/1	0/0	0/0	0/0
No. Examined Viscerally	184	162	170	165
Soft tissue malformations	12/8	2/2	9/5	7/6
No. Examined Skeletally	171	149	161	152
Skeletal malformations	3/1	0/0	2/2	0/0
Total fetuses/litters with malformations	15/8	2/2	11/6	7/6

In a developmental toxicity study in the New Zealand white SPF rabbit, groups of 16 inseminated females received Piperonyl Butoxide orally by gavage at doses of 0, 50, 100 or 200 mg/kg bw/day on days 7-19 of gestation (Anonymous - 16, 1986). Maternal body weight loss or reduced body weight gain during the treatment period was observed at the top dose group animals (**Table 52**). No treatment-related effects on gestation (Table 53) and foetal development were observed (Table 54). It is noted that the liver that appears to be a target organ in rats and mice, was not evaluated in rabbits in line with the OECD TG 414 (2001).

Limitations of the study included fewer animals with implantations in the mid dose group as well as lack of measurements of the following parameters: gravid uterine weight data, food consumption, uterine weight, resorption incidence and the number and percent of live offspring. The NOAEL for maternal toxicity was 100 mg actual Piperonyl Butoxide /kg bw/day and that for developmental toxicity was 200 mg/kg bw/day, the highest dose tested.

Table 52: Effect of Piperonyl Butoxide on group mean maternal body weight in a rabbit teratology study (Anonymous - 16, 1986)

Day of gestation	Mean body weights (g) at dose levels (mg/kg b.w./d)			
	0	50	100	200
0	3685	3541	3709	3597
7	3813	3637	**3864**	**3679**
13	3864	3706	**3858**	**3528**
20	3852	3748	**3845**	**3521**
24	3902	3817	3954	3731
29	3890	3841	3979	3814

Table 53: Effect of Piperonyl Butoxide on maternal and fetal parameters in a rabbit teratology study (**Anonymous - 16**, 1986)

Parameter	Observation at dose level (mg/kg b.w./d)											
	0 (Control)			50			100			200		
	No.	%	±SD	No.	%	±SD	No.	%	±SD	No.	%	± SD
Animals on study	16	-	-	16	-	-	16	-	-	16	-	-
Animals that were gravid	16	-	-	16	-	-	14	-	-	16	-	-
Animals that died	0	-	-	0	-	-	0	-	-	0	-	-
Animals that delivered near term	0	-	-	0	-	-	1	-	-	1	-	-
Animals examined at Cesarean section	16	-	-	16	-	-	15	-	-	16	-	-
Non-gravid	0	-	-	0	-	-	2	-	-	0	-	-
Gravid	16	-	-	16	-	-	14	-	-	16	-	-
Does with resorption only	0	-	-	0	-	-	0	-	-	1	-	-
Does with viable fetuses	16	-	-	16	-	-	13	-	-	16	-	-
Viable fetuses/doe	8.1		2.82	8.1		2.43	7.5		2.88	8.3		2.33
Corpora lutea/doe	11.4		2.96	11.8		2.35	11.2		2.28	12.3		2.55
Group mean pre implantation loss (%) [1)]		24.7			25.0			24.0			27.4[1)]	
Group mean post implantation loss (%) [2)]		5.8			8.5			11.7			9.5	
Mean fetal body weight (grams)	38.9		6.12	41.2		6.09	39.2		3.84	39.4		5.14
Fetal sex distribution - male		58.1			40.3*			53.1			53.0	
- female		41.9			59.7			46.9			47.0	

1) Value does not include doe with fewer corporea lutea than total implantations
* significantly different from control group ($p<0.01$)

Table 54: Summary of the incidence of fetal malformations in the rabbit teratology study (**Anonymous - 16**, 1986)

Parameter	No of fetuses (No. of litters) at dose level (mg/kg b.w./d)			
	0	**50**	**100**	**200**
No. of fetuses (litters) examined	123 (16)	129 (16)	107 (14)	134 (16)
Cleft palate	-	-	1 (1)	-
Cleft lip	-	-	1 (1)	-
Hindlimbs malpositioned and curved	1 (1)	-	-	-
Small ventricle	1 (1)	-	-	-
Bulbous aortic arch with vestigial pulmonary trunk	1 (1)	-	-	-
Kidney and ureter absent	-	1 (1)	-	-
Spherical enlargement of ribs	-	1 (1)	-	1 (1)
Total fetuses (litters) with malformations	2 (2)	2 (2)	1 (1)	1 (1)

The (-) indicates zero incidence

Overall, under the conditions of the developmental toxicity studies performed in rats and rabbits with Piperonyl Butoxide there was no evidence of embyotoxicity, teratogenicity and/or foetotoxicity at all doses tested (i.e. up to 1000 mg/kg bw/day in rats and up to 200 mg/kg bw/day in rabbits).

4.11.2.2 Human information

No relevant data.

4.11.3 Comparison with criteria

The criteria under CLP specify that classification as reproductive toxicant category 1A is largely based on human evidence. There is no evidence of Piperonyl Butoxide having caused effects on fertility and/or development in humans, therefore Repr. 1A is not warranted.

The criteria under CLP specify that classification as reproductive toxicant category 1B is largely based on animal evidence, i.e. *"there should be 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. 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."*

Piperonyl Butoxide did not show toxic effects on fertility in a two-generation reproductive toxicity study in the rat at dietary doses up to 500 mg Piperonyl Butoxide/kg bw/day. Under the conditions of the developmental toxicity studies performed in rats and rabbits with Piperonyl Butoxide there was no evidence of embyotoxicity, teratogenicity and/or foetotoxicity at all doses tested (i.e. up to 1000 mg/kg bw/day in rats and up to 200 mg/kg bw/day in rabbits). Moreover, there are no mechanistic data available that would question the results of the animal studies.

The criteria under CLP specify that classification as reproductive toxicant category 2 is relevant for substances *"when there is some evidence from humans or experimental animals, possibly supplemented with other information, of an adverse effect on sexual function and fertility, or on development, and where the evidence is not sufficiently convincing to place the substance in Category 1".*
In the absence of such human or animal evidence it may be concluded that the criteria for Repr. 2 classification are also not fulfilled.

4.11.4 Conclusions on classification and labelling for reproductive toxicity

CLP: No classification based on available data.

RAC evaluation of reproductive toxicity

Summary of the Dossier Submitter's proposal

Fertility

The DS evaluated one 2-generation reproductive toxicity studies with two matings per generation. Groups of 26 male and 26 female rats were administered PBO at doses of 0, 30, 100, and 500 mg/kg bw/d for two consecutive generations. At the highest dose group, significantly decreased parental body weights in males and females were observed starting from weeks 6 and 3 of treatment, respectively. Pup body weights in the highest dose group were also statistically significantly decreased in both offspring generations starting from day 4 of lactation. No treatment related statistically significant effects on reproductive parameters (mating index, fertility index, gestation index, length of gestation, numbers of live pups, survival and viability indices) were observed in any of the generations.

The DS concluded that there were no indications for an effect on fertility mediated by PBO intake. Thus, **no classification** for Fertility was proposed.

Development

The DS presented two developmental toxicity studies, one in rats and one in rabbits.

In an OECD TG 414 study, groups of 25 mated female rats were administered PBO at doses of 0, 200, 500, and 1000 mg/kg bw/d via gavage at gestation days 6-15. Maternal toxicity occurred from doses of 500 mg/kg bw/d, as evidenced by clinical signs in three dams, and decreased food consumption and body weight gain. In the high dose group, dams had increased relative liver weights as compared to controls. No treatment related embryo- and foetotoxic or teratogenic effects were observed.

In an OECD TG 414 study in rabbits, females received 0, 50, 100, or 200 mg PBO/kg bw/d via gavage from day 7 to day 19 of gestation. The study protocol deviated from the guideline: in the mid dose group fewer animals with implantations were used, and some parameters were not measured (gravid uterus weight, food consumption, resorption incidence, percent of live pups). Only single incidences of malformations without dose response were observed in all groups including controls.

Based on these data, the DS concluded that **no classification** for Development is warranted.

The DS did not include an assessment of the available data for effects on or via lactation.

Overall, the DS proposed **no classification** for Reproductive Toxicity.

Comments received during public consultation

Two MSCAs commented on this endpoint. One agreed that no classification for Reproductive Toxicity was triggered by available data. The second noted that findings from the 2-year feeding study in mice (Anon. 10) should also have been considered for male fertility:

- Statistically significantly increased dose-dependent incidence of smaller seminal vesicles in 5%, 6.67%, 15%, 16.67%, and 20% for the control group 1, control group 2, 30, 100, and 500 mg/kg bw/d groups, respectively;
- Statistically significantly increased incidences of bilateral testicular atrophy in 18%, 15%, 33.3%, 46.67%, and 43.33% for the control group 1, control group 2, 30, 100, and 500 mg/kg bw/d groups, respectively.

Furthermore, they noted that no assessment for effects on or via lactation was made in the CLH report.

The DS clarified that smaller seminal vesicles were only observed in males found dead or sacrificed for ethical reasons during the study, and that testicular atrophy was considered an age-related effect. Moreover, analysis of the study report revealed incidences for surviving animals: 3/18, 8/22, 3/13, 8/18, 7/22 at 0, 0, 30, 100, 500 mg/kg bw/d, respectively. These were not considered robust enough for classification purposes.

In addition, the DS clarified that since no observations referring to lactation or analytical measurements of PBO residues in milk were available, and the only effect seen during lactation was decreased body weight of pups from both breeding trials and both generations at the highest dose accompanied by slightly decreased maternal body weights, no classification for effect on or via lactation is justified.

Assessment and comparison with the classification criteria

Fertility

One 2-generation reproductive toxicity study in rats is available. It was performed according to OECD TG 416. Groups of male and female rats were administered doses up to 500 mg PBO/kg bw/d via the diet for 12 weeks prior to the first mating period. A second mating period was performed in each generation. Selected body weights of the F0 generation were presented in the CLH report. (Note: some of the numbers were interchanged in the corresponding table 45 of the CLH report, see Supplemental Information).

Body weight development of the following generations was similar with statistically significant decreases in the highest dose group. RAC notes that in the study report obtained from Industry, the tables 42 to 45 are missing which presumably show body weights of the F1b females before and during gestation. No effects on any of the fertility parameters in any generation were observed.

During public consultation, one MSCA noted that in the 2-year carcinogenicity study in rats, smaller seminal vesicles and testicular atrophy were observed. However, smaller seminal vesicles and testicular atrophy were observed mainly in rats that were found dead or were sacrificed

moribund during the study. Incidences of testicular atrophy in surviving males were: 16.7%, 36.4%, 23.1%, 44.4%, and 31.8% at 0, 0, 30, 100, 500 mg/kg bw/d.

RAC concurs with the DS that data from the carcinogenicity study are not robust enough to trigger classification and no effects of concern were observed in the 2-generation study nor in any of the repeated dose toxicity studies. Decreased testicular weights in the 8-week repeated dose toxicity study in dogs were not accompanied by microscopic changes. Therefore, **no classification** for Fertility is warranted.

Development

There are two developmental toxicity studies available that were conducted according to OECD guidelines. One study in rats (OECD TG 414) and one in rabbits (OECD TG 414 with deviations). Females were dosed via gavage in both studies.

In rats, mated females received 0, 200, 500, or 1000 mg PBO/kg bw/d on days 6 through 15 of gestation. Body weight gains of dams were statistically significantly reduced in mid and high doses by 18 and 22%, respectively, as compared to controls. Mean final corrected body weights were also slightly lower in treated groups but without statistical significance (by 3.5% and 2.5% compared to controls in mid and high dose, respectively). In the highest dose group, mean relative liver weights of dams were statistically significantly increased. In this dose group the number of viable implants was decreased but without statistical significance. A summary of malformation incidences is presented in the table below.

Table: *Malformation incidences the rat developmental toxicity study. Modified from table 51 of the CLH report.*

	Foetuses/Litters			
Dose [mg/kg bw/d]:	**0**	**200**	**500**	**1000**
No. examined externally	355	311	331	317
External malformations	**7/1**	**0/0**	**0/0**	**0/0**
No. examined viscerally	184	162	170	165
Soft tissue malformations	**12/8**	**2/2**	**9/5**	**7/6**
No. examined skeletally	171	149	161	152
Skeletal malformations	**3/1**	**0/0**	**2/2**	**0/0**
Total foetuses/litters with malformations	**15/8**	**2/2**	**11/6**	**7/6**

Malformations were observed in controls as well in treated rats, without dose-response relationship. RAC notes that control incidences were higher than incidences in treated groups.

In rabbits treated with 0, 50, 100, or 200 mg PBO/kg bw/d at gestation days 7 through 19 only a slight reduction of maternal body weights was observed in treated groups on some days of gestation. The effect lacked a dose response. RAC notes that the absence of maternal toxicity indicates that the chosen dose levels were too low. No statistically significant effects on the number of viable foetuses per dam, percentage of pre- or post-implantation losses were observed, although numbers were smaller in the mid dose group. Single incidences of cleft palate (one foetus in the mid dose), malpositioned hind limbs, small ventricle, bulbous aortic arch (one each in control group), absent kidney and ureter (one in low dose), spherical enlargement of ribs

(one each in low and high dose dose) were observed. These were not considered treatment related.

Effects on or via lactation

In the 2-generation reproductive toxicity study, pup body weights were statistically significantly decreased in the highest dose groups of both generations. Decreased pup body weights compared to controls were observed from day 4 of the lactation period in the F1a generation, from day 7 in the F1b generation, and from day 14 in the F2a generation. These changes were accompanied by reduced maternal weights. No other effects were observed. No information is available concerning PBO concentrations in the milk or the substance's transfer to it.

Conclusion on classification

No effects on fertility were observed in a 2-generation reproductive toxicity study in rats. Testes atrophy and smaller seminal vesicles observed in a carcinogenicity study, occurred mainly in animals that were found dead or were sacrificed during the study period. These effects are considered not sufficient for classification.

No treatment related developmental effects were observed in developmental toxicity studies in rats and rabbits.

Some effects on pup body weights during lactation were observed in the 2-generation study, which were accompanied by reduced maternal body weights as compared to controls. Since no information on PBO residues in the milk is available, RAC considers classification for effects on or via lactation not warranted.

Thus, RAC concurs with the DS that based on the available data **no classification for Reproductive Toxicity is warranted.**

Supplemental information - In depth analyses by RAC

Table: *Body weights of the F0 generation in the 2-generation reproductive toxicity study in rats (Anon. 14), corrected and amended from table 45 of the CLH report.*

Study period	**Sex**	**Dose: mg/kg bw/d**	**0**	**30**	**100**	**500**
		Time point	**Body weight (g)**			
Pre-mating	Males	week 4	355.0	351.4	353.5	341.1
		week 8	456.9	450.2	451.8	424.8*
		week 12 #	502.7	495.7	495.9	464.2*
		week 22 ##	605.5	597.7	599.0	545.0*
	Females	week 4	218.4	212.4	215.6	204.7*
		week 8	266.2	255.4	260.4	240.9*
		week 12 #	287.9	277.3	284.3	261.3*
		week 22 ##	322.8	314.3	319.4	294.6*
Gestation	Females	Day 0	288.2	270.4*	283.6	258.8*
		Day 7	317.9	297.3*	308.8	283.3*

	1st littering phase	Day 15	347.2	328.6	343.4	316.4*
		Day 21	414.8	388.5*	409.7	379.7*
	Females	Day 0	323.4	311.3	323.7	290.6*
		Day 7	347.6	334.1	344.6	312.8*
	2nd littering phase	Day 15	380.5	363.3	381.1	344.5*
		Day 21	446.2	438.5	457.7	403.1*
Lactation	Females	Day 0 +	310.5	302.1	317.8	291.4*
	1st littering phase	Day 4	320.3	305.8	320.8	295.6*
		Day 7	320.8	302.5	323.1	305.7
		Day 14	332.0	310.3*	319.1	303.9*
		Day 21	317.5	304.8	319.5	306.0
	Females	Day 0 +	353.7	335.6	345.9	315.7*
	2nd littering phase	Day 4	353.0	344.7	354.9	326.1*
		Day 7	361.7	347.7	357.9	337.1*
		Day 14	362.3	351.1	361.8	344.6
		Day 21	342.8	329.7	343.7	335.3
* values statistically significantly different from control # start of mating period 1, ## presumed start of mating period 2 + corrected body weights						

4.12 Specific target organ toxicity (CLP Regulation) – single exposure (STOT SE)

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

Piperonyl butoxide has a vapour pressure of $<10^{-2}$ Pa (

Table 8) therefore it cannot be considered as volatile; however due to its intended use as a synergist, that is a chemical expected to enhance the effect of the active substance in the product (Guidance on the BPR: Volume III Human Health, Assessment & Evaluation (Parts B+C), version 4.0, December 2017), toxicity *via* the inhalation route was assessed and was therefore included in the report.

Two inhalation toxicity studies in rats (one acute and one short-term), are reported. Additionally, epidemiological data of individuals exposed to products containing pyrethrins are considered.

Table 55: Summary table of relevant inhalation toxicity studies

Method	Results	Remarks	Reference
Acute inhalation Rat US EPA 81-3 Batch No. FEP-100 Task Force II Blend Purity: 90.78%, GLP	Male & Female: LC_{50} > 5.9 mg/L	-	**Anonymous - 5** (1991) (Piperonyl Butoxide CAR Doc IIIA6.1.3/01)
Rat: 3-month, inhalation OECD 413 Piperonyl Butoxide purity 90.78% Batch No. FEP-100 Task Force II Blend Exposure pattern: whole-body exposure, 6h/day, 5 days/week Doses: 0.015, 0.074, 0.155, 0.512 mg/L	**Systemic toxicity:** LOAEL: 0.512 mg/L - hepatotoxicity (decreased serum liver enzymes activity and increased liver weight), - increased kidneys weight. NOAEL: 0.155 mg/L **Local toxicity:** LOAEL: 0.512 mg/L - red nasal discharge, - histopathological alterations in the larynx including slight squamous metaplasia with hyperkeratosis (minimal) and inflammation (moderate). NOAEL: 0.155 mg/L	CLP: no classification GV: 0.2 mg/L/6h/day (mist) Klimisch score: 2 The following deviations from OECD 413 are indicated: 1. Temperature range should have been 19-25 ºC instead of 17-29 ºC. 2. Humidity range should have been 30-70% instead of 26-74%. 3. In Clinical Chemistry, there was no determination of γ-GT and ODC activities. 4. Heart weight was not recorded.	**Anonymous - 17** , 1992 (Piperonyl Butoxide CAR Doc IIIA6.4.3)

GV: Guidance value for classification

Epidemiological data of individuals exposed to products containing pyrethrins have revealed that respiratory symptoms such as bronchospasm, cough/choke, and dyspnea were more likely if the exposure included piperonyl butoxide (US-EPA, Memorandum, Review of Piperonyl butoxide Incident Reports, 2004). These symptoms are likely the reason for increased risk of moderate effects which typically would require medical attention. Other literature suggests that pyrethrin-based products may pose a hazard to asthmatics (Ellenhorn *et al.* 1997, Reigart and Roberts 1999, Wagner 2000).

Moreover, slight respiratory tract irritation evidenced as nasal discharge and laboured breathing accompanied by red foci in the lungs of 2/5 females was noted in the acute toxicity study by inhalation in rats (Anonymous - 5, 1991). In addition, in the 3-month inhalation study in rats red nasal discharge and histopathological alterations in the larynx including slight squamous metaplasia with minimal hyperkeratosis and moderate inflammation were noted at 0.512 mg/L (Anonymous - 17, 1992). These findings are considered relevant as part of weight of evidence evaluation of the potential of Piperonyl butoxide to cause respiratory tract irritation.

4.12.1.1 Acute toxicity: inhalation

No mortalities occurred after acute inhalation exposure to Piperonyl Butoxide as an aerosol to rats. LC_{50} exceeded 5.9 mg/L Piperonyl Butoxide.

Slight or no weight losses were noted on the day following exposure. Normal gains were noted thereafter and all animal were in excess of their initial body weight by day 5.

Table 56: The acute inhalation toxicity of Piperonyl Butoxide to the rat (mean body weights)

	Day 1	Day 2	Day 3	Day 5	Day 8	Day 15
Male	228 g	228 g	235 g	255 g	289 g	349 g
Female	213 g	206 g	212 g	225 g	242 g	254 g

During exposure nasal discharge, excessive salivation, eye closure and decreased activity were noted.

On day one and during the first week excessive lacrimation and salivation nasal discharge and laboured breathing were noted. Most of these responses abated during the second week of observation.

Red foci were noted in lungs of 2/5 females. Other post mortem findings occurred sporadically and were considered unrelated to the test substance.

4.12.1.2 Repeated dose toxicity: inhalation

A subchronic 3-month inhalation toxicity was conducted in Sprague Dawley rats (Anonymous - 17, 1992) at dose levels of 0, 0.015, 0.074, 0.155, and 0.512 mg Piperonyl Butoxide/L (whole-body exposures, 6h/day, 5 days/week).

In order to allow comparison of the inhalation to the oral toxicity studies, a conversion of the doses tested from mg/m^3 to mg/kg bw/day is performed as indicated in Table R.8-2 of the relevant REACH Guidance document [8]:

$$NOAELoral = \frac{\text{Breathing rate} \times NOAEC_{inhalation}}{ABS_{oral} / ABS_{inhalation}}$$

where,

- ABS_{oral} = 100% (see Section 4.1.1 of this report)
- The default breathing rate of 0.8 L/min/kg bw in rats was adjusted for the 6-hour duration of daily exposure in the study, as follows:
  0.8 L/min/kg bw x (6h/day x 60 min/h) = 288 L/kg bw/day or 0.288 m^3/kg bw/day.

The results of conversion of doses tested from mg/m^3 to mg/kg b.w./day are summarised below:

[8] ECHA, Guidance on information requirements and chemical safety assessment. Chapter R.8: Characterisation of dose [concentration]-response for human health, Version: 2.1 November 2012

Table 57: Conversion of doses tested from mg/m³ to mg/kg b.w./day

mg/m³	15	74	155	512
mg/kg bw/day	4.32	21.31	44.64	147.46

Regarding systemic toxicity the effects of Piperonyl Butoxide exposure included decreased serum liver enzymes activity and increased relative liver and kidney weight at the top dose of 0.512 mg/L. The target organs identified were the liver and kidneys and the NOAEL for systemic toxicity was set at 0.155 mg/L.

Regarding local toxicity the effects of Piperonyl Butoxide exposure consisted of red nasal discharge of slight/moderate severity evidenced from the dose of 0.155 mg/L in females (14/15 animals) and at 0.512 mg/L in males (15/15 animals) (Table 58). The severity of the finding increased from slight to slight/moderate at the top dose for males and from 0.155 mg/L for females.

Other local effects considered to be adverse at 0.512 mg/L were histopathological alterations in the larynx including slight squamous metaplasia with hyperkeratosis (minimal) and inflammation (moderate). The incidence of microscopic findings in the larynx in male and female rats is summarised in Table 59 and Table 60, respectively.

Squamous/squamoid metaplasia of the pseudostratified ciliated/nonciliated columnar epithelium was seen in animals of all treated groups with increasing severity from minimal (≤ 0.155 mg/L) to slight (0.512 mg/L).

Squamous/squamoid metaplasia/hyperplasia of the ventral diverticulum (columnar epithelium) and hyperplasia and hyperkeratosis of the stratified squamous epithelium were only observed at the top dose. The severity of the effects was minimal or slight.

According to Kaufmann *et al.* (2009) focal squamous metaplasia graded minimal or slight is only considered to be adverse if keratinisation is observed, since only then a dysfunction may be assumed. Thus, in this study mucosal metaplasia was considered to be adverse at 0.512 mg/L.

Mucosal inflammation had an overall incidence equivalent in all groups, including the controls, but the severity was higher in both males and females treated with 0.512 mg/L Piperonyl Butoxide (moderate inflammation). According to the pathology report in the study, this increase in severity was considered to be indicative of a response to irritation rather than systemic toxicity.

The presence of inflammatory lesions graded as moderate at the top dose is supportive to the adversity of the histological effects at 0.512 mg/L.

Granulomatous inflammation was observed in a few animals of all treatment groups. The severity of the effect was minimal, slight or moderate but did not exhibit a clear dose-related increase and was thus considered of unclear toxicological significance.

Overall, the histological effects of the larynx at the top dose of 0.512 mg/L were considered to be adverse. This conclusion is in agreement with an additional pathology report recently submitted by the Industry (Gopinath, 2014).

Additional publications (Lewis, 1991 and Burger *et al.*, 1989) indicate that the larynx is a major site of induced changes in rats exposed by inhalation to chemicals. The most commonly induced laryngeal lesions in rats involve degeneration of the original epithelial cells with subsequent hyperplasia and squamous metaplasia. The metaplastic epithelium may show superficial keratinisation.

Table 58: Incidence of dried red nasal discharge in male and female rats (13-weeks inhalation exposure to Piperonyl Butoxide)

Parameter	n = 15/sex	0 control	0.015 (mg/L)	0.074 (mg/L)	0.155 (mg/L)	0.512 (mg/L)
dried red nasal discharge	M	0	6	6	11	15
	F	3	8	14	14	15

Table 59: Incidence of microscopic findings in the larynx in male rats (13-weeks inhalation exposure to Piperonyl Butoxide)

Parameter	0 control	0.015 (mg/L)	0.074 (mg/L)	0.155 (mg/L)	0.512 (mg/L)
Number of animals examined	**15**	**15**	**15**	**15**	**15**
Mucosa: Pseudostratified ciliated/nonciliated columnar epithelium - squamous/squamoid metaplasia/hyperplasia					
Total	0	7	14	14	15
Minimal	0	7	14	13	1
Slight	0	0	0	1	14
Ventral diverticulum: columnar epithelium – squamous/squamoid metaplasia/hyperplasia					
Total	0	0	0	0	12
Minimal	0	0	0	0	12
Mucosa: stratified squamous epithelium – hyperplasia					
Total	0	0	0	0	1
Minimal	0	0	0	0	1
Slight	0	0	0	0	0
Mucosa: stratified squamous epithelium – hyperkeratosis					
Total	0	0	0	0	1
Minimal	0	0	0	0	1
Slight	0	0	0	0	0
Mucosa: subacute (chronic active) / chronic inflammation					
Total	15	15	15	15	15
Minimal	1	6	3	3	0
Slight	12	9	12	12	7
Moderate	2	0	0	0	8
Mucosa: granulomatous inflammation/ granulomas(s) (associated with cartilage in the area of the ventral diverticulum)					
Total	1	0	2	0	1
Minimal	0	0	1	0	0
Slight	1	0	0	0	1
Moderate	0	0	1	0	0

Table 60: Incidence of microscopic findings in the larynx in female rats (13-weeks inhalation exposure to Piperonyl Butoxide)

Parameter	0 control	0.015 (mg/L)	0.074 (mg/L)	0.155 (mg/L)	0.512 (mg/L)
Number of animals examined	**15**	**15**	**15**	**15**	**15**
Mucosa: Pseudostratified ciliated/nonciliated columnar epithelium - squamous/squamoid metaplasia/hyperplasia					
Total	1	13	14	15	15
Minimal	1	13	14	14	7
Slight	0	0	0	1	8
Ventral diverticulum: columnar epithelium – squamous/squamoid metaplasia/hyperplasia					
Total	0	1	0	0	13
Minimal	0	1	0	0	13
Mucosa: stratified squamous epithelium – hyperplasia					
Total	0	0	0	0	3
Minimal	0	0	0	0	1
Slight	0	0	0	0	2
Mucosa: stratified squamous epithelium – hyperkeratosis					
Total	0	0	0	0	3
Minimal	0	0	0	0	1
Slight	0	0	0	0	2
Mucosa: subacute (chronic active) / chronic inflammation					
Total	15	15	15	15	15
Minimal	0	3	4	1	0
Slight	13	12	11	13	7
Moderate	2	0	0	1	8
Mucosa: granulomatous inflammation/ granulomas(s) (associated with cartilage in the area of the ventral diverticulum)					
Total	2	2	0	6	6
Minimal	0	0	0	3	1
Slight	2	2	0	2	3
Moderate	0	0	0	1	2

4.12.2 Comparison with criteria of repeated dose toxicity findings relevant for classification as STOT SE

In the CLP Regulation, paragraph 3.8.2.2.1 (a) it is stated that: *"Respiratory irritant effects (characterised by localised redness, oedema, pruritis and/or pain) that impair function with symptoms such as cough, pain, choking, and breathing difficulties are included. This evaluation will be based primarily on human data"*. Epidemiological data of individuals exposed to products containing pyrethrins have revealed that respiratory symptoms such as bronchospasm, cough/choke,

and dyspnea were more likely if the exposure included piperonyl butoxide (US-EPA, Memorandum, Review of Piperonyl butoxide Incident Reports, 2004). These symptoms are likely the reason for increased risk of moderate effects which typically would require medical attention. Other literature suggests that pyrethrin-based products may pose a hazard to asthmatics (Ellenhorn *et al.* 1997, Reigart and Roberts 1999, Wagner 2000). These findings are probably related to the synergistic properties of Piperonyl Butoxide, being a chemical expected to enhance the effect of the active substance in the product (Guidance on the BPR: Volume III Human Health, Assessment & Evaluation (Parts B+C), version 4.0, December 2017).

In the CLP Regulation, paragraph 3.8.2.2.1 (d) it is stated that: *"There are currently no validated animal tests that deal specifically with RTI, however, useful information may be obtained from the single and repeated inhalation toxicity tests. For example, animal studies may provide useful information in terms of clinical signs of toxicity (dyspnoea, rhinitis etc) and histopathology (e.g. hyperemia, edema, minimal inflammation, thickened mucous layer) which are reversible and may be reflective of the characteristic clinical symptoms described above. Such animal studies can be used as part of weight of evidence evaluation".* Slight respiratory tract irritation evidenced as nasal discharge and laboured breathing accompanied by red foci in the lungs of 2/5 females was noted in the acute toxicity study by inhalation in rats (Anonymous - 5, 1991). In addition, in the 3-month inhalation study in rats red nasal discharge and histopathological alterations in the larynx including slight squamous metaplasia with minimal hyperkeratosis and moderate inflammation were noted at 0.512 mg/L (Anonymous - 17, 1992). These findings are considered relevant as part of weight of evidence evaluation of the potential of Piperonyl butoxide to cause respiratory tract irritation.

Respiratory irritation observed both in animal studies and human epidemiological data only occur in the absence of other more severe effects in the respiratory system, fulfilling the CLP criterion described in paragraph 3.8.2.2.1 (e): *"this special classification would occur only when more severe organ effects including in the respiratory system are not observed"*.

Considering all the available data, it is considered that criteria (a), (d) and (e) described in paragraph 3.8.2.2.1 of the CLP for respiratory tract irritation are fulfilled.

4.12.3 Conclusions on classification and labelling of repeated dose toxicity findings relevant for classification as STOT SE

Classification for specific target organ toxicity after single exposure (STOT SE) with H335 "May cause respiratory irritation" is justified.

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

Summary of the Dossier Submitter's proposal

The DS summarised inhalation data and focussed on their evaluation for STOT SE 3, H335.

Human Data

The DS briefly summarised a US-EPA Review of Piperonyl butoxide Incident Reports from 2004, which found that respiratory symptoms such as bronchospasm, cough/choke, and dyspnoea were more likely if the exposure to pyrethrins included piperonyl butoxide.

Animal Data

In the acute inhalation toxicity study assessed by the DS in this section, nasal discharge and laboured breathing accompanied by red foci in the lungs of 2/5 females were observed at 5.9 mg/L.

In the sub chronic study, groups of 15 rats per sex and dose were exposed whole body to 0, 0.015, 0.074, 0.155, or 0.512 mg PBO/L air for 6 hours a day, 5 days a week. Systemic toxicity consisted of decreased serum liver enzyme activity and increased relative liver and kidney weights at the highest dose. Local toxicity in the respiratory tract included red nasal discharge from 0.155 mg/L.

Overall, the DS considered effects seen in humans and rats adverse and indicative for respiratory tract irritation and proposed classification of PBO as **STOT SE 3, H335**.

Comments received during public consultation

Three Member State Competent Authorities (MSCAs) and one Company-Manufacturer commented. All of them supported the proposed classification. One of the MSCAs noted that the proposed classification is in agreement with the outcome of the discussion in the biocide review procedure.

Assessment and comparison with the classification criteria

Human Data

The DS mentioned data from the 2004 US-EPA memorandum on the Review of piperonyl butoxide Incident Reports. The data derive from experience with moderate, major and fatal cases, 479 cases for pyrethrins plus PBO and 760 for pyrethrins alone. RAC notes that this is a compilation of case reports and thus significance of the results is limited.

Besides bronchospasm, cough/choke and dyspnoea, chest pain, erythema, dermal irritation or pain, pruritus, rash, nausea, vomiting, dizziness/vertigo, and headache were symptoms observed more likely when pyrethrins were combined with PBO with similar odds ratios. However, for most odds ratios numbers of patients were small (< 50) and due to methodological flaws results must be interpreted with caution.

Animal Data

In the acute inhalation toxicity studies the following effects were noted at 5.9 mg/L and 5.2 mg/L, respectively: excessive salivation, eye closure, and decreased activity during exposure, excessive lacrimation and salivation, nasal discharge, and laboured breathing during the first week of observation in the first study. Respiratory tract irritation in this study was confirmed by red foci in the lungs of 2 of 5 females. In the second study, slightly reduced motility, slight ataxia, and slight dyspnoea were recorded.

In a sub chronic inhalation toxicity study in rats, red nasal discharge and histopathological changes in the larynx, especially at the highest dose of 0.512 mg/L/d were observed. However, RAC notes that all animals of all groups including controls showed subacute or chronic inflammation of the laryngeal mucosa. Moreover, effects were recorded after three month of exposure and no details were provided in the CLH report as to acute effects after single exposure. Therefore, RAC considers this study as supportive.

Further supportive evidence is provided by two eye irritation studies in which PBO was found to be slightly irritating to the eye of rabbits.

Conclusion on classification

Criteria for classification for respiratory tract irritation according to CLP guidance include:

"(a) respiratory irritant effects (characterized by localized redness, oedema, pruritis and/or pain) that impair function with symptoms such as cough, pain, choking, and breathing difficulties are included. This evaluation will be based primarily on human data.

(d) animal studies may provide useful information in terms of clinical signs of toxicity (dyspnoea, rhinitis etc.) and histopathology (e.g. hyperaemia, oedema, minimal inflammation, thickened mucous layer) which are reversible and may be reflective of the characteristic clinical symptoms described above. Such animal studies can be used as part of weight of evidence evaluation."

Based on these criteria with symptoms of respiratory tract irritation in humans and rats, which were confirmed in rats by histopathological changes, and in absence of more severe organ effects, RAC concurs with the DS that **classification as STOT SE 3, H335 (may cause respiratory irritation) is warranted.**

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

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

The repeated dose toxicity of Piperonyl butoxide was assessed in four short-term studies, including three oral studies in the mouse, rat and dog and one inhalation study in the rat. It is noted that Piperonyl butoxide has a vapour pressure of $<10^{-2}$ Pa (

Table 8) therefore it cannot be considered as volatile; however due to its intended use as a synergist, that is a chemical expected to enhance the effect of the active substance in the product (Guidance on the BPR: Volume III Human Health, Assessment & Evaluation (Parts B+C), version 4.0, December 2017) toxicity *via* the inhalation route was assessed and was therefore included in the report.

Additionally, two short-term preliminary studies, i.e. one oral in the dog and one dermal in the rabbit, are reported.

Table 61: Summary table of relevant repeated dose toxicity studies

Method	Results	Remarks	Reference
Dog: 8-week, feeding * OECD 409 Piperonyl Butoxide purity 90.78% Batch No.: FEP-100 Task Force II Blend <u>Doses:</u> 0, 500, 1000, 2000, 3000 ppm [M: 0, 14.7, 32, 63, 90 mg/kg bw/day F: 0, 14.8, 37, 61, 85 mg/kg bw/day]	LOAEL: 2000 ppm (63/61 mg/kg bw/d) - reduced body weight gain, - increased alkaline phosphatase activity, - slightly decreased cholesterol levels, - increased liver weight (absolute and relative), - hepatocellular hypertrophy - decreased testicular weight (no associated microscopic changes). NOAEL: 1000 ppm (32/37 mg/kg bw/day)	CLP: no classification Klimisch score:: 2 Acceptable, as a preliminary study. Deviations from OECD 409: 1. Range-finding study with only 8-weeks exposure period. 2. No weight determination for epididymides, uterus and thymus.	**Anonymous - 18** , 1993a (Piperonyl Butoxide CAR Doc IIIA6.3.1)
Dog: 1-year, feeding OECD 452 Piperonyl Butoxide purity 90.78% Batch No. FEP-100 Task Force II Blend Doses: 0, 100, 600, 2000 ppm [M: 0, 2.9, 15.5, 53 mg/kg bw/day F: 0, 2.7, 16.3, 71 mg/kg bw/day]	LOAEL: 2000 ppm (53/71 mg/kg bw/d) - reduction (not statistically significant) in body weight gain & food consumption, - decreased (not statistically significant) cholesterol levels, - statistically significant increased alkaline phosphatase activity, - statistically significant increased relative-to-body liver/gallbladder weight values, consistent with the hepatocyte hypertrophy observed by the microscopic pathology NOAEL: 1000 ppm (15.5/16.3 mg/kg bw/d)	CLP: no classification Klimisch score: 1 Deficiencies: none	**Anonymous - 19** , 1993b (Piperonyl Butoxide CAR Doc IIIA6.4.1/02)
Mouse: 90-day, feeding OECD 408 Piperonyl Butoxide purity 90.78% Batch No. FEP-100 Task Force II Blend Doses: 52 – 5804 ppm (male mice) and 36 – 4275 ppm (female mice) [M: 0, 10.3, 30.3, 102.6, 308.9, 1127.1 mg/kg bw/day F: 0, 10.3, 30.8, 103.5, 317.7, 1053.6 mg/kg bw/day]	LOAEL: 100 ppm (102.6/103.5 mg/kg bw/d) - statistically significant increased relative liver weight (males), - statistically significant increased incidence of hepatocellular hypertrophy (males and females). NOAEL: 30 ppm (30.3/30.8 mg/kg bw/d)	CLP: no classification GV: 100 (this is the GV for rats which is considered to be relevant for mice as well due to comparable lifespan of these rodents) Klimisch score: 1 Deficiencies: none	**Anonymous - 20** , 1993 (Piperonyl Butoxide CAR Doc IIIA6.4.1/01)

Method	Results	Remarks	Reference
Rats: 13-week (91 days), feeding ** No guideline Piperonyl Butoxide technical No details on purity/batch No. Doses: 6000, 12000, 24000 ppm [M/F: 0, 300, 600, 1200 mg/kg bw/day]	LOAEL: 6000 ppm (300 mg/kg bw/d) - reduced body weight (8% in males and 7.5% in females), - increased relative kidney weight (11% in males and 7.7% in females), - increased relative liver weight (not statistically significant; was however enlarged by 26% in males and 28% in females) NOAEL: < 6000 ppm (< 300 mg/kg bw/d)	GV: 100 CLP: no classification	Fujitani T. et al., Toxicology, 72 (1992) 291-298
Rabbits: 21-day, dermal Piperonyl Butoxide purity 90.78% Batch No. FEP-100 Task Force II Blend Doses: 0, 100, 300, 1000 mg/kg bw/day	**Systemic toxicity:** NOAEL: 1000 mg/kg bw/day **Local toxicity:** LOAEL: 100 mg/kg bw/day - dermal observations (erythema, edema, desquamation, fissuring and red raised areas) in both sexes; no indication of reversibility, no statistical significance NOAEL: <100 mg/kg bw/day	CLP: EUH066 GV: skin dryness, flaking or cracking, but not irritant after acute exposure Klimisch score: 2 Acceptable as a preliminary study. Deviation from OECD 410: The duration of exposure was 21 instead of 28 days.	**Anonymous - 21**, 1992 (Piperonyl Butoxide CAR Doc IIIA6.3.2)
Rat: 3-month, inhalation OECD 413 Piperonyl Butoxide purity 90.78% Batch No. FEP-100 Task Force II Blend Exposure pattern: whole-body exposure, 6h/day, 5 days/week Doses: 0.015, 0.074, 0.155, 0.512 mg/L	**Systemic toxicity:** LOAEL: 0.512 mg/L - hepatotoxicity (significantly decreased serum liver enzymes activity and increased relative-to-body liver weight), - increased kidneys weight (not statistically significant). NOAEL: 0.155 mg/L **Local toxicity:** LOAEL: 0.512 mg/L - red nasal discharge (all animals), - histopathological alterations in the larynx including slight squamous metaplasia with hyperkeratosis (minimal) and inflammation (moderate).*** NOAEL: 0.155 mg/L	CLP: no classification GV: 0.2 mg/L/6h/day (mist) Klimisch score: 2 The following deviations from OECD 413 are indicated: 1. Temperature range should have been 19-25 ºC instead of 17-29 ºC. 2. Humidity range should have been 30-70% instead of 26-74%. 3. In Clinical Chemistry, there was no determination of γ-GT and ODC activities. 4. Heart weight was not recorded.	**Anonymous - 17**, 1992 (Piperonyl Butoxide CAR Doc IIIA6.4.3)

GV: Guidance value for classification
* Due to the small number of animals in each group (2 animals/sex/dose group), statistical analyses were not performed.
** No information on statistical analysis; the top dose of 1200 mg/Kg bw/day exceeded the MTD (reduction in body weight by 36% and 23% in males and females, respectively) therefore the findings in this group were excluded from the evaluation.
*** Although histopathological alterations in the larynx were observed in animals of all treated groups with increasing severity, the presence of inflammatory lesions graded as moderate at the top dose is supportive to the adversity of the histological effects at 512 mg/m^3.

It is noted that in the REACH registration dossier one further subacute feeding study in rats (1981) is included. No details regarding the batch number of the technical Piperonyl Butoxide use in this study (IUCLID 1) is provided and thus its relevance to the impurity profile stated in the identity section to this report (Table 1), is unknown. Moreover, this study is considered by the registrants as supporting data. The following is concluded by the registrants:

28-31 day feeding study in Sprague Dawley rats, 1981 (Exp Supporting Repeated dose toxicity: oral. 002)

"Fours week's daily administration via the diet of Piperonyl Butoxide at dose levels of 62.5, 125, 250, 500, 1000, 2000 mg/kg/day to groups of 10 male and 10 female Sprague-Dawley rats showed that the test compound caused histologically detectable adaptive liver changes at all dose levels. Signs of possible degenerative liver changes were observed only at dose level of 1000 and 2000 mg/kg/day. Changes in liver weight (relative to body weight) were detectable at dose levels of 250 mg/kg/day or above for males and 500 mg/kg/day or above for females. Dose levels of 1000 or 2000 mg/kg/day for males and 500, 1000, 2000 mg/kg/day for females caused marked and dose related reduction in body weight gain. Other groups treated with Piperonyl Butoxide shoved minimal reductions in body weight gain. Hematology and biochemistry parameters did not reveal marked signs of toxic effect.
Within the limits of this study where Piperonyl Butoxide was administrated to rats over relatively short period of time, it would appear that dose levels of more than 500 mg/kg/day would be unsuitable for prolonged administration."

Overall, it is not considered necessary to request the original study report of this study in order to include it in the CLH dossier. This in the not a key study. It is a subacute (28-31 days feeding study in rats) considered by the registrant as supporting data. Since a reliable 13-week feeding study in rats is included (Fujitani, 1992), additional consideration of preliminary data is not necessary.

4.13.1.1 Repeated dose toxicity: oral

In Beagle dogs, piperonyl butoxide oral administration was tested in two feeding studies. The target organ in both studies was the liver.

The first study was an 8-week preliminary study (Anonymous - 18, 1993a) at dose levels of 500, 1000, 2000 and 3000 ppm. The NOAEL was set at 1000 ppm (equivalent to 32 mg/kg bw/day in males and 37 mg/kg bw/day in females) based on reduced body weight gain, liver toxicity (evidenced as increased alkaline phosphatase activity, slightly decreased cholesterol levels, increased liver weight and hepatocellular hypertrophy) and decreased testicular weight with no associated microscopic changes. There was no weight determination for epididymides, uterus and thymus in the study report.

Table 62: Mean body weights (Anonymous - 18, 1993a)

Dose level (ppm)	Group Mean Body Weights (kg) (% difference from pretest)			
	Male		Female	
	pretest	week 8	pretest	week 8
0	12.1	14.2 (+17.4)	8.4	9.6 (+14.3)
500	12.7	14.4 (+13.4)	8.1	9.5 (+17.3)
1000	12.1	13.8 (+14.0)	8.2	8.9 (+8.5)
2000	12.8	13.6 (+6.3)	9.6	10.3 (+7.3)
3000	12.3	11.4 (-7.3)	9.2	8.8 (-4.3)

Table 63: Clinical chemistry-selected parameters (Anonymous - 18, 1993a)

Parameter	n = 2 / sex	0 ppm control	500 ppm	1000 ppm	2000 ppm	3000 ppm
Alkaline phosphatase (IU/l)	M	91	63	93	178	178
	F	83	66	97	136	130
Cholesterol (mg/dL)	M	149	145	152	104	110
	F	142	134	158	129	95

Table 64: Selected organ weights and pathologies (Anonymous - 18, 1993a)

ppm Piperonyl butoxide	sex (n = 2)	body weight (kg)	Liver (g)	Relative liver weight (%)	Testis (g)*)	Relative testis weight (%)
0	m	13.6	319.6	2.37	11.5	8.49
	f	9.3	242.8	2.62		
500	m	13.9	381.7	2.75	11.3	8.1
	f	9.1	269.3	2.96		
1000	m	13.4	399.2	3.01	10.45	7.98
	f	8.4	252.2	3.00		
2000	m	13.1	450.1	3.44	7.82	5.95
	f	9.6	355.1	3.78		
3000	m	11.2	455.0	4.09	8.32	7.46
	f	8.4	380.6	4.61		

Table 65: Hypertrophy of hepatocytes (Anonymous - 18, 1993a)

Parameter	n = 2 / sex	0 ppm control	500 ppm	1000 ppm	2000 ppm	3000 ppm
Number of animals with hepatocellular hypertrophy	M	0	1	1	2	2
	F	0	0	0	1	2

The second study was a 52-week study (Anonymous - 19, 1993b) at dose levels of 100, 600 and 2000 ppm. The NOAEL was set at 600 ppm (equivalent to 15.50 mg/kg bw/day in males and 16.30 mg/Kg bw/day in females), based on reduction in body weight gain and food consumption and liver toxicity indicated by decreased cholesterol levels, increased alkaline phosphatase activity and increased liver/gallbladder weight values consistent with the hepatocyte hypertrophy observed in microscopic pathology. Additionally, there was a non-statistically significant increase in the absolute thyroid weight (>20%) in all piperonyl butoxide treated female groups. The relative-to-body thyroid weight was only significantly increased at the top dose females. However, there were no microscopic changes in the thyroid, and the effect on thyroid weight was not considered to be adverse. There were no treatment-related thyroid effects in males.

Table 66: Mean body weight pretest and at week 52 in a 52-week dog feeding study (Anonymous - 19, 1993b)

Dosage level (ppm)	Group Mean Body Weights (kg)			
	Male		Female	
	pretest	week 52	pretest	week 52
0	13.3	16.6	11.0	13.4
100	13.2	16.0	10.9	13.6
600	13.1	15.2	10.6	12.7
2000	13.1	13.5	10.2	10.0

Table 67: Clinical chemistry-selected parameters in a 52 week dog feeding study at 0, 6 and 12 months (Anonymous - 19, 1993b)

Parameter	Sex	0 ppm			100 ppm			600 ppm			2000 ppm		
		0	6	12	0	6	12	0	6	12	0	6	12
Alkaline Phosphatase (IU/L)	M	93	45	36	98	55	47	112	72	59	114	**152***	**194***
	F	108	60	49	133	90	71	98	46	44	99	**221***	**300***
Aspartate Aminotransferase (IU/L)	M	27	29	21	22	27	23	23	23	22	24	22	19
	F	24	23	19	23	23	20	27	24	18	29	24	22
Alanine Aminotransferase (IU/L)	M	33	39	36	32	39	41	29	33	34	27	29	35
	F	30	30	27	32	38	44	33	34	32	37	32	40
Cholesterol (mg/dL)	M	183	162	161	171	153	134	186	154	136	189	143	129
	F	171	192	206	170	160	174	138	158	129	168	126	100

* significantly different from the control group: $p < 0.05$

Table 68: Selected organ weights and pathologies in a 52 week dog feeding study (Anonymous - 19, 1993b)

ppm Piperonyl butoxide	Sex	Body weight (kg)	Liver/Gall -bladder (g)	Relative Liver/Gall-bladder (%)	Thyroid left (mg)	Relative Thyroid left (mg))	Thyroid right (g)	Relative Thyroid right (%)
0	m	15.9	362	2.29	1,0	6.21	0.85	5.18
	f	13.0	293	2.26	0.59	4.63	0.63	4.90
100	m	15.5	354	2.34	0.82	5.27	0.76	4.82
	f	13.2	286	2.22	0.72	5.35	0.67	5.06
600	m	14.5	375	2.58	0.85	5.86	0.81	5.56
	f	12.2	329	2.69	0.75	6.08	0.68	5.56
2000	m	12.9	442	**3.49***	0.87	6.83	0.83	6.53
	f	9.6	397	**4.21****	0.79	**8.22****	0.85	**8.99****

* significantly different from the control group: $p < 0.05$
** significantly different from the control group: $p < 0.01$

A subchronic 3-month (90 days) feeding toxicity was conducted in **CD-1 mice** at dose levels of 0, 10, 30, 100, 300 and 1000 ppm (Anonymous - 20, 1993). The liver was the target organ. The NOAEL was set at 30 ppm (equivalent to 30.3 mg/kg bw/day in males and 30.8 mg/kg bw/day in females), based on increased relative liver weight (males), and hepatocellular hypertrophy (both sexes). There is no evidence of increased mortality and/or body weight changes.

Table 69: Effects of Piperonyl Butoxide oral administration in mice for 90 days

Parameter	Sex	0	10	30	100	300	1000
		mg/Kg bw/day					
Number examined	M	15	15	15	15	15	15
	F	15	15	15	15	15	15
Mortality	M	0	0	0	0	0	0
	F	0	0	0	0	1	1
Clinical signs	M	0	0	0	0	0	0
	F	0	0	0	0	0	0
Body weight (g)	M	35.6	36.8	37.0	35.4	35.7	33.7*
	F	29.3	28.9	29.5	29.6	28.8	28.1
Liver weight							
Liver weight, absolute (g)	M	1.88	2.03*	2.04*	2.07*	2.60**	3.50**
	F	1.56	1.47	1.53	1.63	1.84**	2.70**
Liver weight, relative-to-body (%)	M	5.25	5.56*	5.48	5.83**	7.22**	10.42**
	F	5.26	5.09	5.13	5.52	6.34**	9.55**
Liver histopathology							
Necrosis	M	1	1	1	5	2	10
	F	1	2	6	3	1	3
Hypertrophy (minimal or mild)	M	2	4	6	8	3	0
	F	2	3	6	5	12	3
Hypertrophy (moderate or marked)	M	0	1	0	1	11	15
	F	0	0	0	1	2	11
Total hypertrophy	M	2	5	6	9*	14**	15**
	F	2	3	6	6	14**	14**

* statistically significant different from the control group $p < 0.05$
** statistically significant different from the control group $p < 0.01$

Piperonyl butoxide oral administration in **F344 rats** was studied by Fujitani *et al*. (Toxicology, 72 (1992) 291-298) in a subchronic (13 weeks) study at dose levels of 0, 0.6, 1.2 and 2.4% in food (equivalent to 0, 6000, 12000, 24000 ppm in food). Although the study report does not include food consumption data, an arbitrary calculation of the amount of test substance ingested is estimated considering that an adult rat consumes approximately 20 g food/day (WHO, Environmental health criteria, 70 (1987)). Thus, the doses of 6000, 12000 24000 ppm correspond to 300, 600, 1200 mg/kg bw/day, respectively. All treatment groups exhibited effects of systemic toxicity including reduced body weight (8% in males and 7.5% in females), increased relative kidney weight (11% in males and 7.7% in females) and increased relative liver weight (not statistically significant; was however enlarged by 26% in males and 28% in females); therefore a NOAEL could not be established (LOAEL = 300 mg/kg bw/day). Moreover, the top dose of 1200 mg/kg bw/day exceeded the MTD (reduction in body weight by 36% and 23% in males and females, respectively) therefore the findings in this group were excluded from the evaluation. Males seemed to be more sensitive to the piperonyl butoxide oral administration and the target organs were the liver and kidney.

4.13.1.2 Repeated dose toxicity: inhalation

A subchronic 3-month inhalation toxicity was conducted in Sprague Dawley rats (Anonymous - 17, 1992) at dose levels of 0, 0.015, 0.074, 0.155, and 0.512 mg Piperonyl Butoxide/L (whole-body exposures, 6h/day, 5 days/week).

In order to allow comparison of the inhalation to the oral toxicity studies, a conversion of the doses tested from mg/m^3 to mg/kg bw/day is performed as indicated in Table R.8-2 of the relevant REACH Guidance document [9]:

$$NOAELoral = \frac{\text{Breathing rate} \times NOAEC_{inhalation}}{ABS_{oral} / ABS_{inhalation}}$$

where,

- ABS_{oral} = 100% (see Section 4.1.1 of this report)
- The default breathing rate of 0.8 L/min/kg bw in rats was adjusted for the 6-hour duration of daily exposure in the study, as follows:
  0.8 L/min/kg bw x (6h/day x 60 min/h) = 288 L/kg bw/day or 0.288 m^3/kg bw/day.

The results of conversion of doses tested from mg/m^3 to mg/kg b.w./day are summarised below:

Table 70: Conversion of doses tested from mg/m^3 to mg/kg b.w./day

mg/m^3	15	74	155	512
mg/kg bw/day	4.32	21.31	44.64	147.46

Regarding systemic toxicity the effects of Piperonyl Butoxide exposure included decreased serum liver enzymes activity and increased relative liver and kidney weight at the top dose of 0.512 mg/L. The target organs identified were the liver and kidneys and the NOAEL for systemic toxicity was set at 0.155 mg/L.

Regarding local toxicity the effects of Piperonyl Butoxide exposure consisted of red nasal discharge of slight/moderate severity evidenced from the dose of 0.155 mg/L in females (14/15 animals) and at 0.512 mg/L in males (15/15 animals) (Table 71). The severity of the finding increased from slight to slight/moderate at the top dose for males and from 0.155 mg/L for females. Based on the nature and severity of the effect no classification for specific target organ toxicity after repeated exposure (STOT RE) is warranted.

Other local effects considered to be adverse at 0.512 mg/L were histopathological alterations in the larynx including slight squamous metaplasia with hyperkeratosis (minimal) and inflammation (moderate). The incidence of microscopic findings in the larynx in male and female rats is summarised in Table 72 and Table 73, respectively.

Squamous/squamoid metaplasia of the pseudostratified ciliated/nonciliated columnar epithelium was seen in animals of all treated groups with increasing severity from minimal (≤ 0.155 mg/L) to slight (0.512 mg/L).

[9] ECHA, Guidance on information requirements and chemical safety assessment. Chapter R.8: Characterisation of dose [concentration]-response for human health, Version: 2.1 November 2012

Squamous/squamoid metaplasia/hyperplasia of the ventral diverticulum (columnar epithelium) and hyperplasia and hyperkeratosis of the stratified squamous epithelium were only observed at the top dose. The severity of the effects was minimal or slight.

According to Kaufmann *et al.* (2009) focal squamous metaplasia graded minimal or slight is only considered to be adverse if keratinisation is observed, since only then a dysfunction may be assumed. Thus, in this study mucosal metaplasia was considered to be adverse at 0.512 mg/L.

Mucosal inflammation had an overall incidence equivalent in all groups, including the controls, but the severity was higher in both males and females treated with 0.512 mg/L Piperonyl Butoxide (moderate inflammation). According to the pathology report in the study, this increase in severity was considered to be indicative of a response to irritation rather than systemic toxicity.

The presence of inflammatory lesions graded as moderate at the top dose is supportive to the adversity of the histological effects at 0.512 mg/L.

Granulomatous inflammation was observed in a few animals of all treatment groups. The severity of the effect was minimal, slight or moderate but did not exhibit a clear dose-related increase and was thus considered of unclear toxicological significance.

Overall, the histological effects of the larynx at the top dose of 0.512 mg/L were considered to be adverse. This conclusion is in agreement with an additional pathology report recently submitted by the Industry (Gopinath, 2014).

Additional publications (Lewis, 1991 and Burger *et al.*, 1989) indicate that the larynx is a major site of induced changes in rats exposed by inhalation to chemicals. The most commonly induced laryngeal lesions in rats involve degeneration of the original epithelial cells with subsequent hyperplasia and squamous metaplasia. The metaplastic epithelium may show superficial keratinisation.

Table 71: Incidence of dried red nasal discharge in male and female rats (13-weeks inhalation exposure to Piperonyl Butoxide)

Parameter	n = 15/sex	0 control	0.015 (mg/L)	0.074 (mg/L)	0.155 (mg/L)	0.512 (mg/L)
dried red nasal discharge	M	0	6	6	11	15
	F	3	8	14	14	15

Table 72: Incidence of microscopic findings in the larynx in male rats (13-weeks inhalation exposure to Piperonyl Butoxide)

Parameter	0 control	0.015 (mg/L)	0.074 (mg/L)	0.155 (mg/L)	0.512 (mg/L)
Number of animals examined	**15**	**15**	**15**	**15**	**15**
Mucosa: Pseudostratified ciliated/nonciliated columnar epithelium - squamous/squamoid metaplasia/hyperplasia					
Total	0	7	14	14	15
Minimal	0	7	14	13	1
Slight	0	0	0	1	14
Ventral diverticulum: columnar epithelium – squamous/squamoid metaplasia/hyperplasia					
Total	0	0	0	0	12
Minimal	0	0	0	0	12
Mucosa: stratified squamous epithelium – hyperplasia					
Total	0	0	0	0	1
Minimal	0	0	0	0	1
Slight	0	0	0	0	0
Mucosa: stratified squamous epithelium – hyperkeratosis					
Total	0	0	0	0	1
Minimal	0	0	0	0	1
Slight	0	0	0	0	0
Mucosa: subacute (chronic active) / chronic inflammation					
Total	15	15	15	15	15
Minimal	1	6	3	3	0
Slight	12	9	12	12	7
Moderate	2	0	0	0	8
Mucosa: granulomatous inflammation/ granulomas(s) (associated with cartilage in the area of the ventral diverticulum)					
Total	1	0	2	0	1
Minimal	0	0	1	0	0
Slight	1	0	0	0	1
Moderate	0	0	1	0	0

Table 73: Incidence of microscopic findings in the larynx in female rats (13-weeks inhalation exposure to Piperonyl Butoxide)

Parameter	0 control	0.015 (mg/L)	0.074 (mg/L)	0.155 (mg/L)	0.512 (mg/L)
Number of animals examined	**15**	**15**	**15**	**15**	**15**
Mucosa: Pseudostratified ciliated/nonciliated columnar epithelium - squamous/squamoid metaplasia/hyperplasia					
Total	1	13	14	15	15
Minimal	1	13	14	14	7
Slight	0	0	0	1	8

Parameter	0 control	0.015 (mg/L)	0.074 (mg/L)	0.155 (mg/L)	0.512 (mg/L)
Ventral diverticulum: columnar epithelium – squamous/squamoid metaplasia/hyperplasia					
Total	0	1	0	0	13
Minimal	0	1	0	0	13
Mucosa: stratified squamous epithelium – hyperplasia					
Total	0	0	0	0	3
Minimal	0	0	0	0	1
Slight	0	0	0	0	2
Mucosa: stratified squamous epithelium – hyperkeratosis					
Total	0	0	0	0	3
Minimal	0	0	0	0	1
Slight	0	0	0	0	2
Mucosa: subacute (chronic active) / chronic inflammation					
Total	15	15	15	15	15
Minimal	0	3	4	1	0
Slight	13	12	11	13	7
Moderate	2	0	0	1	8
Mucosa: granulomatous inflammation/ granulomas(s) (associated with cartilage in the area of the ventral diverticulum)					
Total	2	2	0	6	6
Minimal	0	0	0	3	1
Slight	2	2	0	2	3
Moderate	0	0	0	1	2

No classification for laryngeal effects is also warranted since the dose of 0.512 mg/L exceeds the guidance value of 0.2 mg/L/6h/day for STOR RE Category 2 classification of a dust/mist/fume.

4.13.1.3 Repeated dose toxicity: dermal

A repeated dose dermal preliminary toxicity study was conducted in New Zealand White rabbits (Anonymous - 21, 1992). Piperonyl Butoxide was administered dermally, undiluted as received, at dose levels of 100, 300 and 1000 mg/kg bw/day, 5 days/week for three weeks (21 days) to 5 rabbits/sex/group. Piperonyl Butoxide dermal application did not cause systemic toxicity to the rabbit, therefore the NOAEL was set at 1000 mg/kg bw/day. Nevertheless, Piperonyl Butoxide dermal application caused effects of local toxicity, such as dermal observations (erythema, oedema, desquamation, fissuring and red raised areas) in both sexes. There is no indication of reversibility of dermal observations of treated animals during exposure with Piperonyl Butoxide and the study design did not include a recovery period. Since all tested doses exhibited local effects, a definite NOAEL could not be established ($NOAEL_{local\ effects}$<100 mg/kg bw/day).

The following tables depicting dermal observations, incidence of macroscopic and microscopic observations and organ weight changes are included to assist evaluation of the results:

Table 74: Dermal observations (combined sexes) – rabbits: 21-day dermal exposure

Dermal Observations		**No. of animals (no. of days observed*)**			
Dose (mg/kg bw/day)		**0**	**100**	**300**	**1000**
No. of animals examined		**10**	**10**	**10**	**10**
Erythema	total	0 (0)	5 (14)	10 (13)	10 (13)
	very slight	0 (0)	5 (14)	8 (13)	7 (13)
	slight	0 (0)	0 (0)	2 (13)	3 (13)
Oedema		0 (0)	1 (14)	5 (14)	6 (14)
Desquamation		0 (0)	3 (7)	6 (7)	8 (7)
Fissuring		0 (0)	0 (0)	1 (6)	3 (6)
Red raised areas		0 (0)	0 (0)	1 (4)	1 (4)

**Note that dermal observations were not made during weekends, i.e. days 3-4, 10-11 and 17-18 of the 21 study days. Thus, both exposure with Piperonyl Butoxide and dermal observations were made for 5-days per study week for 3-weeks.*

There is no indication of reversibility of dermal observations of treated animals during exposure with Piperonyl Butoxide and the study design did not include a recovery period.

Table 75: Incidence of local Macroscopic Observations

Skin, treated (mg/kg bw/day)		**0**		**100**		**300**		**1000**	
Sex		M	F	M	F	M	F	M	F
No. of animals examined		5	5	5	5	5	5	5	5
Within normal limits		3	1	1	0	0	1	0	1
Red area/s	trace	1	2	0	5	0	1	1	0
	mild	1	2	4	0	5	3	4	3
Scabs	trace	0	0	1	0	0	1	0	0
	mild	0	0	0	0	0	0	1	1
Thick	trace	0	0	1	0	1	1	1	0
	mild	0	0	0	0	3	1	1	2
	moderate	0	0	0	0	0	0	1	0

Table 76: Incidence of Microscopic Observations

Effect	**Severity**	**0**		**100**		**300**		**1000**	
		M	F	M	F	M	F	M	F
Kidney									
Haemorrhage	mild	0	0	1	0	0	0	0	0
Mineralization	trace	2	2	1	3	4	3	4	2
	mild	1	0	1	1	0	2	0	0
Liver									
Necrosis	mild	0	0	1	0	0	0	0	1
	moderate	0	0	1	1	0	0	0	0
Inflammation	trace	0	0	0	0	0	1	0	1
	mild	0	0	0	0	0	0	0	1
	moderate	0	0	0	1	0	0	0	0

It is proposed that based on the skin effects (erythema, edema, desquamation, fissuring, red raised areas), piperonyl butoxide should be assigned the additional hazard statement **EUH066 – 'Repeated exposure may cause skin dryness or cracking'.** There is no indication of reversibility of dermal

observations of treated animals during exposure with Piperonyl Butoxide and the study design did not include a recovery period.

4.13.1.4 Human information

Medical surveillance on manufacturing plant personnel

Industry stated *that "air monitoring of the workplace is regularly performed. No exposure at significant levels were detected inside the production buildings and in the breathing zones of the workers. All workers involved in the manufacture (chemical synthesis) or handling (drumming, filling) of Piperonyl Butoxide underwent a regular health check (twice per year) and health records have been maintained for over 20 years. On a yearly basis blood chemistry and urinalysis are conducted. The workers in manufacture (chemical synthesis) or handling (drumming, filling etc.) were found to be clinically healthy. No substance related effects have been detected among the entire workforce. In particular, no cases of sensitization and/or allergenic response and/or hypersensitivity of the workers exposed to the substance have occurred."*

Although the health records of the Plant personnel over the period of 20 years have not been provided by the Industry, the results of a surveillance programme performed in 2005 (Savron, 2005) confirmed their statement. There was no indication of any anomalies or medical situations to be kept under control or cases in which alterations could be considered strictly related to exposure to substances used in the Industrial Plant.

Report on clinical cases and poisoning accidents

Industry stated that *"There are no reports or publications on incidental or suicidal poisoning with Piperonyl Butoxide in line with the lack of acute toxicity observed in experimental animal studies investigating acute toxicity of Piperonyl Butoxide. Poisoning of consumers with Piperonyl Butoxide containing products, such as pyrethrins and Piperonyl Butoxide containing pesticides have not been reported. Based on more than 80.000 calls, the Report to the American Association of Poison Control Centers (AAPCC) concludes "that products containing Pyrethrins or pyrethroids can be used with the expectation of no undue risk" (Anonymous, 2001). A more recent report of AAPCC is presented in the Appendix "Open Literature Review of Piperonyl Butoxide". No increased health risk resulted from the use of piperonyl butoxides was identified by this report as well."* It is noted that the aforementioned report of AAPCC is actually a review paper funded by the Piperonyl Butoxide Task Force II (Osimitz *et al*., 2009). Notably, its conclusion contradicts the conclusions and recommendations of the US-EPA, Memorandum, Review of Piperonyl butoxide Incident Reports, 2004 (See APPENDIX).

Industry has also provided a brief summary of an unpublished report (Tagliani, 2004) on human volunteers. According to Industry's conclusion, *"no adverse effects have been reported upon oral exposure of male volunteers towards 50 mg Piperonyl Butoxide"*. However, the original report of the medical incidence has not been provided by the Industry, no evaluation of the severity of the medical incidences can be made, and no conclusion can be drawn. Industry claimed that they can generate these data. In this report by Tagliani (2004), an accident is also mentioned which occurred in a production plant in South Africa. Following eye contamination with Piperonyl Butoxide, the eye was washed with water and a non-specified cream was applied. Burning sensation and redness of the eye was reversible within a few days.

4.13.1.5 Other relevant information

No data.

4.13.1.6 Summary and discussion of repeated dose toxicity

Piperonyl Butoxide is a potent inhibitor of cytochrome P450 enzymes (and of esterases). This is the proposed mechanism of acting as a synergist to pyrethrins and synthetic pyrethroids, by inhibition of the enzymatic degradation. Upon repeated exposure, Piperonyl Butoxide induces hepatic cytochrome P450 enzymes, resulting at high dose levels, in hepatocellular hypertrophy, cell proliferation and hepatotoxicity as it has been demonstrated in mechanistic studies in rodents. In section 4.10.1.1 it is explained in detail that a robust MoA for Piperonyl Butoxide-induced mouse liver tumour formation is established and confirmed. This MoA involves CAR activation and induction of replicative DNA synthesis in mouse hepatocytes. This MoA is rodent-specifc and it is not relevant to humans due to qualitative differences between species. Specifically, in the same section *in vitro* data demonstrated that CAR receptor activation does not result in a proliferative response in cultured human hepatocytes. Therefore, it can be concluded that liver effects in rodents in repeated dose toxicity studies do not constitute a hazard for humans.

Subchronic oral administration studies were conducted in mice, rats and dogs. The dog was the most sensitive species with an overall NOAEL of 16 mg/kg bw/day (1-year study). Target organs were the liver (mouse, rat, dog) and kidneys (rat).

Dermal application of Piperonyl Butoxide at doses up to 1000 mg/kg bw/day for 21 days caused no systemic toxicity in rabbits. However, dermal effects (erythema, oedema, desquamation, fissuring and red raised areas) were noted from the lowest dose of 100 mg/kg bw/day. There is no indication of reversibility of dermal observations of treated animals during exposure with Piperonyl Butoxide and the study design did not include a recovery period. It is proposed that based on the skin effects (erythema, edema, desquamation, fissuring, red raised areas), piperonyl butoxide should be assigned the additional hazard statement **EUH066 – 'Repeated exposure may cause skin dryness or cracking'.** There is no indication of reversibility of dermal observations of treated animals during exposure with Piperonyl Butoxide and the study design did not include a recovery period.

In a subchronic 90-day inhalation study in the rat, hepatotoxicity evidenced as decreased serum liver enzyme activity and increased relative liver weight, as well as kidney toxicity indicated by increased relative kidneys weight, were observed at the top dose of 0.512 mg/L. The target organs identified were the liver and kidneys. The NOAEL for systemic toxicity was set at 0.155 mg/L. Local effects included red nasal discharge of slight/moderate severity evidenced from the dose of 0.155 mg/L in females (14/15 animals) and at 0.512 mg/L in males (15/15 animals). Based on the nature and severity of the effect no classification for specific target organ toxicity (STOT RE) is warranted. Other local effects considered to be adverse at 0.512 mg/L were histopathological alterations in the larynx including slight squamous metaplasia with hyperkeratosis (minimal) and inflammation (moderate). No classification for laryngeal effects is also warranted since the dose of 0.512 mg/L exceeds the guidance value of 0.2 mg/L for STOT RE Category 2 classification for dust/ mist.

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

- Oral

Subchronic oral administration studies were conducted in mice, rats and dogs. The dog was the most sensitive species with an overall NOAEL of 16 mg/kg bw/day (1-year study). Target organs were the liver (mouse, rat, dog) and kidneys (rat).

- **Mouse** 90-day feeding study (Anonymous - 20, 1993): liver effects at 100 ppm (102.6/103.5 mg/kg bw/d)
- **Rat** 91-day feeding study (Fujitani T. et al., 1992): body weight, liver and kidney effects at 6000 ppm (300 mg/kg bw/d).
- **Dog** 1-year feeding study (Anonymous - 19, 1993b): body weight and liver effects at 2000 ppm (53/71 mg/kg bw/d).

As described in detail in section 4.13.2, liver effects in rodents in repeated dose toxicity studies do not constitute a hazard for humans due to the underlined mode of action. Kidney effects in rats are observed at doses that exceed the guidance value range of $10 < C \leq 100$ mg/kg bw/day indicated in Table 3.9.3 to Regulation (EC) No. 1272/2008 for specific target organ toxicity category 2 classification.

Liver effects in dogs were also observed at doses exceeding the upper limit of 25 mg/kg bw/day for STOT RE category 2 classification (the upper is estimated considering the guidance value of 100 mg/kg b.w./day for 90-day study and adjusted for study duration).

Thus, although it cannot be excluded that the observed effects in dogs are relevant to humans, the DS proposed no classification for STOT RE in category 2 since the effects are observed above the respective guidance values.

- Inhalation (rat) mist

In a subchronic 90-day inhalation study in the rat, hepatotoxicity evidenced as decreased serum liver enzyme activity and increased relative liver weight, as well as kidney toxicity indicated by increased relative kidneys weight, were observed at the top dose of 0.512 mg/L. The target organs identified were the liver and kidneys. The NOAEL for systemic toxicity was set at 0.155 mg/L. Local effects included red nasal discharge of slight/moderate severity evidenced from the dose of 0.155 mg/L in females (14/15 animals) and at 0.512 mg/L in males (15/15 animals). Based on the nature and severity of the effect no classification for specific target organ toxicity (STOT RE) is warranted. Other local effects considered to be adverse at 0.512 mg/L were histopathological alterations in the larynx including slight squamous metaplasia with hyperkeratosis (minimal) and inflammation (moderate). No classification for laryngeal effects is also warranted since the dose of 0.512 mg/L exceeds the guidance value of 0.2 mg/L for STOT RE Category 2 classification for dust/ mist.

- Dermal (rat or rabbit)

Dermal application of Piperonyl Butoxide at doses up to 1000 mg/kg bw/day for 21 days caused no systemic toxicity in rabbits. However, dermal effects including erythema, oedema, desquamation,

fissuring and red raised areas, were noted from the lowest dose of 100 mg/kg bw/day. There is no indication of reversibility of dermal observations of treated animals during exposure with Piperonyl Butoxide and the study design did not include a recovery period. Moreover, since all tested doses exhibited local effects, a definite NOAEL could not be established ($NOAEL_{local\ effects}$<100 mg/kg bw/day).

The criteria for assigning the phase EUH066 – 'Repeated exposure may cause skin dryness or cracking' are the following and are considered fulfilled:

… for substances and mixtures which may cause concern as a result of skin dryness, flaking or cracking but which do not meet the criteria for skin irritancy in section 3.2 of Annex I, based on … relevant evidence concerning their predicted effects on the skin.

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

No STOT RE proposal is justified.

Based on the skin effects (erythema, oedema, desquamation, fissuring, red raised areas), Piperonyl Butoxide should be assigned the additional hazard statement EUH066 – 'Repeated exposure may cause skin dryness or cracking'.

RAC evaluation of specific target organ toxicity – repeated exposure (STOT RE)

Summary of the Dossier Submitter's proposal

Human Data

The DS provided a citation from an industry report on air monitoring and health checks including blood chemistry and urinalysis for workers of an unspecified plant over 20 years. Industry claimed that no significant exposure levels have been measured in the air of the production zone. Biomonitoring was performed on a yearly basis and found no substance related effects among the workers over the whole time span. In addition, no cases of sensitisation/ allergy/ hypersensitivity were observed. These statements were confirmed by a surveillance programme in 2005.

Industry provided a short summary of an unpublished report that stated that no adverse effects have been reported upon oral exposure of male volunteers towards 50 mg of PBO. No details or clinical data were provided.

Animal Data

The DS summarised several oral repeated dose toxicity studies in dogs, rats, and mice, as well as one 21-day dermal toxicity study in rabbits, and the 3-month inhalation toxicity study in rats. Furthermore, the DS mentioned a four-week feeding study in rats from the registration dossier but did not consider its results since it was not a guideline study and an acceptable 13-week feeding study in rats was available. Target organs were liver and kidney (in rats only) consistently in all oral studies and in the inhalation study.

The DS dismissed effects seen in rodents as not relevant for humans because of the proposed mode of action (MoA) via CYP induction and CAR activation (for details see Carcinogenicity section). Although enzyme activation was also seen, PBO did not induce a proliferative response in human hepatocytes *in vitro.* One study in dogs was deemed a preliminary study due to short duration (8 weeks) and small number of animals tested (2/sex/dose), and results were not considered by the DS. In the second dog study, effects occurred at doses above the adjusted guidance values for Cat. 2. Thus, the DS considered classification for liver and kidney effects not warranted.

In the dermal study in rabbits (similar to OECD TG 410, but with only 21 instead of 28 days of exposure), PBO did not induce systemic toxicity up to the top dose of 1000 mg/kg bw/d. It did, however, induce local effects consisting of erythema, oedema, desquamation, fissuring, and red raised areas at the application site in both sexes from the lowest dose of 100 mg/kg bw/d. The DS concluded that additional labelling with EUH066 "Repeated exposure my cause skin dryness or cracking" was justified.

Comments received during public consultation

One Industry (IND) comment noted that although based on the results of the sub-acute dermal study EUH066 labelling may be justified, the findings were observed after repeated exposure under semi-occlusive dressing and that these conditions are not relevant for real-life exposure. The DS pointed out that EUH066 according to CLP guidance shall apply to substances with the intrinsic property to cause skin effects that are not covered by Acute Tox. classification.

One MSCA commented that EUH066 labelling is in accordance with the discussion in the biocide review procedure and supported the proposal.

Assessment and comparison with the classification criteria

Human Data

According to an industry report, no PBO related health issues were observed in biomonitored workers of a production plant over 20 years. Available human data do not warrant classification for STOT RE.

Animal Data

The available oral repeated dose toxicity studies including the oral study in rats from the registration dossier are summarised in the table below. The inhalation study is also included since it provides supporting evidence.

Table: *Repeated dose toxicity studies in dogs, mice and rats with oral and inhalative exposure to PBO.*

Method	Results	Remarks
Dog: 8-week, feeding OECD TG 409 Batch No.: FEP-100 Task Force II Blend purity 90.78%	3000 ppm (90/85 mg/kg bw/d): - reduced body weight compared to pre-test (-7.3% males, -4.3% females) - increased liver weight (absolute and relative), - hepatocellular hypertrophy in all four animals	adjusted GV for Cat 2: 16 < C ≤ 160 mg/kg bw/d Deviations from guideline: 1. only 8-weeks exposure period. 2. only 2 animals/sex/dose

Groups: 2/sex/dose Doses: 0, 500, 1000, 2000, 3000 ppm [M: 0, 14.7, 32, 63, 90 mg/kg bw/d F: 0, 14.8, 37, 61, 85 mg/kg bw/d]	LOAEL: 2000 ppm (63/61 mg/kg bw/d) - reduced body weight gain - increased alkaline phosphatase activity, - slightly decreased cholesterol levels, - increased liver weight (absolute and relative), - hepatocellular hypertrophy - decreased testicular weight (no associated microscopic changes). NOAEL: 1000 ppm (32/37 mg/kg bw/d)	3. no weight determination for epididymides, uterus and thymus.
Dog: 1-year, feeding OECD TG 452 Batch No. FEP-100 Task Force II Blend purity 90.78% Groups: not reported in CLH report Doses: 0, 100, 600, 2000 ppm [M: 0, 2.9, 15.5, 53 mg/kg bw/d F: 0, 2.7, 16.3, 71 mg/kg bw/d]	LOAEL: 2000 ppm (53/71 mg/kg bw/d) - reduction (not statistically significant) in body weight gain & food consumption, - decreased (not statistically significant) cholesterol levels, - statistically significant increased alkaline phosphatase activity, - statistically significant increased relative liver/gallbladder weight values, consistent with hepatocyte hypertrophy observed by the microscopic pathology NOAEL: 600 ppm (15.5/16.3 mg/kg bw/d)	adjusted GV for Cat 2: 2.5 < C ≤ 25 mg/kg bw/d -> LOAEL above upper limit
Mouse: 90-day, feeding OECD TG 408 Batch No. FEP-100 Task Force II Blend purity 90.78% Groups: 15/sex/dose Doses: 52 – 5804 ppm (males) 36 – 4275 ppm (females) [M: 0, 10.3, 30.3, 102.6, 308.9, 1127.1 mg/kg bw/d F: 0, 10.3, 30.8, 103.5, 317.7, 1053.6 mg/kg bw/d]	1127.1/1053.6 mg/kg bw/d - statistically significantly increased relative and absolute liver weight, - total liver hypertrophy in 15/15 males and 14/15 females 308.9/317.7 mg/kg bw/d - statistically significantly increased relative and absolute liver weight, - total liver hypertrophy in 14/15 males and 14/15 females LOAEL: 100 ppm (102.6/103.5 mg/kg bw/d) - statistically significant increased relative liver weight (males), - statistically significantly increased incidence of hepatocellular hypertrophy (males and females). NOAEL: 30 ppm (30.3/30.8 mg/kg bw/d)	GV for Cat 2: 10 < C ≤ 100 mg/kg bw/d -> LOAEL slightly above upper limit
Rats: 13-week (91 days), feeding	LOAEL: 6000 ppm (300 mg/kg bw/d)	GV for Cat 2: 10 < C ≤ 100 mg/kg bw/d

No guideline Piperonyl butoxide technical Groups: 10/sex/dose Doses: 6000, 12000, 24000 ppm [M/F: 0, 300, 600, 1200 mg/kg bw/d – arbitrary calculation by DS]	- reduced body weight (8% in males and 7.5% in females), - increased relative kidney weight (11% in males and 7.7% in females), - increased relative liver weight (not statistically significant; was however enlarged by 26% in males and 28% in females) NOAEL: < 6000 ppm (< 300 mg/kg bw/d)	-> LOAEL above upper limit, but no lower doses tested
Rats: 4-week (28 days), feeding **No guideline** Piperonyl butoxide technical Groups: 10/sex/dose Doses: 0, 62.5, 125, 250, 500, 1000, 2000 mg/kg bw/d	from 250 (males) or 500 (females) mg/kg bw/d: - increased relative liver weight LOAEL: 62.5 mg/kg bw/d - histologically detectable liver changes NOAEL: < 62.5 mg/kg bw/d	adjusted GV for Cat 2: 30 < C ≤ 300 mg/kg bw/d
Rat: 3-month, inhalation OECD TG 413 Batch No. FEP-100 Task Force II Blend purity 90.78% Doses: 0.015, 0.074, 0.155, 0.512 mg/L	LOAEL: 0.512 mg/L - significantly decreased serum liver enzymes activity - increased liver weight, - increased kidneys weight (not statistically significant). NOAEL: 0.155 mg/L	GV for Cat 2: 0.2 mg/L/6h/day (mist) -> LOAEL above upper limit supportive: whole-body exposure -> oral exposure through grooming cannot be excluded Deviations from guideline: 1. Temperature range should have been 19-25 ºC instead of 17-29 ºC. 2. Humidity range should have been 30-70% instead of 26-74%. 3. In Clinical Chemistry, there was no determination of γ-GT and ODC activities. 4. Heart weight was not recorded.

In the first range finding study in dogs, two animals per sex and dose were exposed to PBO via feed for 8 weeks. At a dose of 63 or 61 mg/kg bw/d (males and females, respectively) reduced body weight gain (at 8 weeks compared to pre-test), increased alkaline phosphatase levels, decreased cholesterol levels, increased absolute and relative liver weights accompanied by hepatocellular hypertrophy, and decreased testicular weight without histopathological changes, were observed. RAC notes that body weight of females in the 2000 ppm group at the pre-test measurement was already as high as in controls at the 8-week measurement (pre-test 8.4 kg in controls, and 9.6 kg in the 2000 ppm group, at 8 weeks 9.6 and 10.3 kg in controls and 2000 ppm group, respectively).

Similar effects on liver and liver enzymes were observed in the 1-year dog feeding study starting from doses of 53 and 71 mg/kg bw/d in males and females, respectively. Results were statistically significant. The NOAEL in this study was 15.5 or 16.3 mg/kg bw/d for males and females,

respectively. In the first study, effects on the liver were seen at doses within the adjusted guidance values for Cat. 2, in the second study above the upper limit of adjusted guidance values (25 mg/kg bw/d for a one-year study).

Statistically significant increases in relative liver weight (in males) and incidence of hepatocellular hypertrophy (in males and females) were also observed in a 90-day feeding study in mice at a dose only slightly above the upper guidance value for Cat. 2 (102.6 and 103.5 mg/kg bw/d for males and females, respectively). The next lower dose and NOAEL in this study was 30.3 and 30.8 mg/kg bw/d for males and females, respectively. It is likely that effects would also have been observed at doses slightly below the upper guidance value. RAC considers the results borderline.

In rats, two non-guideline feeding studies are available. In the 90-day study increased relative kidney and liver weights, and decreased body weights were observed from 300 mg/kg bw/d (dose calculated with standard values for food consumption by DS), but lower doses were not tested. In the 28-day study, histologically detectable liver changes (no details provided) were observed from a dose of 62.5 mg/kg bw/d. Increased liver weights were reported from 250 mg/kg bw/d in males and 500 mg/kg bw/d in females. No conclusion can be drawn from the first study since doses within the guidance values were not tested. In the second study, histological changes and increased liver weights in males were observed within the borders of the adjusted guidance values (30 to 300 mg/kg bw/d) for Cat. 2.

Decreased serum liver enzyme activity and increased liver weights in the 3-month rat inhalation study were observed at concentrations above the upper limit for dusts and mists (0.2 mg/L) at 0.512 mg/L. However, since exposure was whole-body in this study, oral exposure due to grooming activities cannot be excluded.

In a dermal study in New Zealand White rabbits, PBO was applied undiluted to the skin of 5 rabbits/sex/dose for 5 days a week for three weeks with a semi-occlusive dressing. Doses were 100, 300, and 1000 mg/kg bw/d. No systemic effects were observed. Dermal symptoms were erythema, oedema, desquamation, fissuring, and red raised areas. These symptoms were observed in all dose groups and were not reversible during the study, which did not include a recovery period.

In the carcinogenicity studies, liver was also a target organ in rats and mice but only doses above the adjusted upper guidance value for Cat. 2 (12.5 mg/kg bw/d) were tested. The lowest dose tested was 30 mg/kg bw/d in both rats and mice. This dose produced increased liver weights in female rats and hepatocellular adenomas in male mice.

In a developmental toxicity study in rats, dams had statistically significantly increased mean relative liver weights at a dose of 1000 mg/kg bw/d (upper adjusted guidance value for a 10-day exposure). RAC notes that increases observed in pregnant rats were small (10% compared to controls) and are therefore not sufficiently adverse to justify classification for STOT RE.

Conclusion on classification

RAC concurs with the DS that **additional labelling with EUH066 is justified** based on the results of the dermal study in rabbits. Increased kidney weights seen in rats occurred only at doses above guidance values and increases were not statistically significant and not relevant for classification. As for hepatotoxicity, RAC considers results from studies with longer exposure periods more relevant for classification purposes. No effects on the liver were observed at doses within guidance values in the 1-year study in dogs and the 90-day study in mice. In rats, no

doses within guidance values were tested.. Therefore, RAC concludes that **no classification for STOT RE is warranted.**

4.14 Other effects

4.14.1 Short summary and overall relevance of the provided information on neurotoxicity

4.14.1.1 Non-human data

In the submitted subchronic, chronic and reproductive toxicity studies there are no indications for a neurotoxic activity of Piperonyl Butoxide.

4.14.1.2 Human information

In the context of the evaluation of Piperonyl Butoxide in the frame of Reg. (EC) 528/2012 an "Open Literature Review of Piperonyl Butoxide" on toxicological aspects (i.e. immunotoxicity, neurotoxicity and human biomonitoring) in included in the CAR. This review which is not exhaustive of the available data in the open literature is presented in the Appendix.

Among the data reviewed, the following study is identified as key study:

Megan *et al.*; PEDIATRICS, March 2011; "Impact of Prenatal Exposure to Piperonyl Butoxide and Permethrin on 36-Month Neurodevelopment" (Columbia Center for Children's Environmental Health, Mailman School of Public Health; Columbia University, New York).

The objective of the study was to explore the association between pre-natal exposure to permethrin (a common pyrethroid) and piperonyl butoxide (a pyrethroid synergist) and 36-month neurodevelopment.

METHODS: Participants is this study were part of a prospective cohort of black and Dominican mothers and newborns living in low-income neighborhoods in New York City. We examined 36-month cognitive and motor development (using the Bayley Scales of Infant Development, second edition) as a function of permethrin levels measured in maternal and umbilical cord plasma collected on delivery, and permethrin and piperonyl butoxide levels measured in personal air collected during pregnancy. All models were controlled for gender, gestational age, ethnicity, maternal education, maternal intelligence, quality of the home environment, and prenatal exposure to environmental tobacco smoke and chlorpyrifos.

RESULTS: Prenatal exposure to permethrin in personal air and/or plasma was not associated with performance scores for the Bayley Mental Developmental Index or the Psychomotor Developmental Index.

After data adjustment, children more highly exposed to piperonyl butoxide in personal air samples (4.34 ng/m^3) scored 3.9 points lower on the Mental Developmental Index than those with lower exposures (95% confidence interval: - 0.25 to -7.49). However, co-exposure to permethrin and other chemicals cannot be excluded and direct association of Piperonyl Butoxide exposure and neurodevelopment cannot be made.

CONCLUSIONS: Prenatal exposure to piperonyl butoxide was negatively associated with 36-month neurodevelopment.

The Industry commented the following on the above publication:

The deficiencies in the paper fall into several categories:

- Factual errors;
- Lack of critical review of cited literature that is used to justify causality;
- Unreliable characterization of Piperonyl Butoxide exposure;
- Over emphasis on what at best is a very minor association.

In view of the results of the above study and taking into account also the effects on neurodevelopment and potentiation of neurotoxicity presented in Appendix "Open Literature Review of Piperonyl Butoxide" the eCA for the evaluation of Piperonyl Butoxide in the frame of Reg. (EC) 528/2012 **originally proposed this issue and the need for further investigation of the effects of Piperonyl Butoxide on the nervous system during development to be discussed in a meeting of experts.**

During trilateral discussions of Piperonyl Butoxide (February, 2016), the applicant responded that *"An acute neurotoxicity study and a waiver argument on Subchronic neurotoxicity of PBO is available and can be submitted. It was accepted by USEPA."* The eCA accepted to evaluate these data in view of the WG-II-2016 and the following were concluded:

a) Anonymous - 22

Acute Neurotoxicity Study of Piperonyl Butoxide in Rats by Gavage Administration (2014)

The acute neurotoxicity study was performed in line with the OECD 424 and it was considered to be acceptable. Male and female Crl:CD(SD) rats (10/sex/dose) received a single gavage dose of Piperonyl Butoxide in corn oil, i.e. 100, 500, or 1000 mg/kg bw. Body weight, food consumption, clinical signs, functional observational battery (FOB) and motor activity assessments were performed.

No neurotoxic effects were observed among treated animals at doses up to 500 mg/kg bw/day, where systemic toxicity was evident in the form of decreased body weight gain and feed consumption.

Neurotoxic signs were observed on study day (DS) 1 (one day after dosing) in both male and females rats treated with 1000 mg/kg bw These effects included decreased motor activity and forelimb grip in both sexes and open field observations such as unusual posture, ataxia, splayed or dragging limbs, tip-toe walk, and decreased body temperature in females only.

The effects were not evident on DSs 8 or 15 and were therefore considered as transient. Moreover, there were no neurotoxic changes in any examined tissue, including the central or peripheral nervous system, eyes with retinas, optic nerves and skeletal muscle.

Overall, in agreement with the author of the original study report, these findings at or close to the established MTD for Piperonyl Butoxide are indicative of a transient functional effect on the day of dosing rather than evidence for structural nerve damage, indicating a low neurotoxic potential for PBO.

a) <u>Subchronic neurotoxicity</u>

The applicant submitted the following justification for waiving a subchronic neurotoxicity study:

1. In the acute neurotoxicity study (Anonymous - 22, 2014), the high dose level (limit dose of 1000 mg/g bw) was the only dose at which evidence of potential neurotoxicity (FOB findings and effects on motor activity) was observed;
2. The fact that indications of potential neurotoxicity were observed in the presence of systemic toxicity (body weight gain suppression) at 1000 mg/kg bw for all endpoints affected but only on day 1 of dosing;
3. The comparatively lower dose levels used for the current short-term, intermediate-term and chronic risk assessments; and
4. Based on the results of the studies used for short-term, intermediate-term and chronic risk assessments, it is unlikely that neurotoxicity will be observed in a subchronic neurotoxicity study for which a dose of not much greater than 500 mg/kg/day can be tested without causing excessive toxicity. This will not affect the relevant existing risk assessments.

The WG-II-2016 of the BPC agreed by consensus that there is no concern for neurotoxicity and no further information is requested. The DS complies with this conclusion.

4.14.1.3 Summary and discussion on neurotoxicity

In the submitted repeated dose toxicity studies there are no indications for neurotoxic activity from Piperonyl Butoxide. In an acute neurotoxicity study, there was no evidence of neurotoxic effects among treated animals at doses up to 500 mg/kg bw/day. Data in the open literature are inconclusive and of low reliability. The overall weight of evidence suggests that there is no concern for neurotoxicity.

4.14.2 Short summary and overall relevance of the provided information on immunotoxicity

4.14.2.1 Non-human data

In the submitted subchronic, chronic and reproductive toxicity studies there are no indications for an immunotoxic activity of Piperonyl Butoxide. The effects of open literature studies (See Appendix) – Open Literature Review) report immunosuppressive effects of Piperonyl Butoxide which include: depletion of T-cells in the spleen and thymus, induction of hypoplasia of the bone marrow, inhibition of T-cells proliferation in lymphoid tissues, increase in thymocyte apoptosis in vitro, marked inhibition of sheep red blood cells (SRBC)-specific IgM production in mice, aggravation of allergic airway inflammation and atopic dermatitis in experimental animal models by prior exposure to Piperonyl Butoxide.

During the trilateral discussions of Piperonyl Butoxide (February, 2016) in the frames of Regulation (EC) 528/2012, the Industry submitted: "*A waiver argument on immunotoxicity of PBO is available and can be submitted. It was accepted by USEPA."* The applicant submitted the following justification for waiving an immunotoxicity study:

"A stand-alone immunotoxicity study will require dose levels of testing that will likely result in liver and kidney toxicity that would confound any evaluation of potential immunotoxicity. In light of this information, we submit that further immunotoxicity studies are not warranted at this time and respectfully request consideration of a waiver from the immunotoxicity testing requirement for piperonyl butoxide."

Overall it may be concluded that based on the available studies Piperonyl Butoxide exhibits no immunotoxic potential. Data in the open literature might imply some uncertainty, especially at high doses, but no convincing evidence. The WG-II-2016 of the BPC agreed that there is no concern for immunotoxicity and no further information is requested. The DS complies with this conclusion.

4.14.2.2 Human information

No data.

4.14.2.3 Summary and discussion on immunotoxicity

In the submitted repeated dose toxicity studies there are no indications for immunotoxic activity from Piperonyl Butoxide. The overall weight of evidence suggests that there is no concern for immunotoxicity.

4.14.3 Comparison with criteria

Not relevant.

4.14.4 Conclusions on classification and labelling

Not relevant.

5 ENVIRONMENTAL HAZARD ASSESSMENT

5.1 Degradation

Table 77: Summary of relevant information on degradation

Method	Results	Remarks	Reference
Hydrolysis			
Hydrolysis of Piperonyl Butoxide, labelled with carbon-14 in its phenyl ring. *US EPA Subdivision N Guideline 161-1*	Piperonyl Butoxide is hydrolytically stable in solution in the dark at 25 °C at pH 5, 7 and 9 and its half-life under these conditions is greater than 500 days.	**Acceptable** Klimisch score: 1 GLP study	Kirkpatrick D. (1995): Piperonyl Butoxide – Hydrolysis as a function of pH at 25 °C; Huntington Research Centre Ltd., Cambridgeshire, England; report number PBT 4/943285 **CAR IIIA 7.1.1.1.1**
Photolysis			
Aqueous photolysis of ring labelled ^{14}C-Piperonyl Butoxide *US EPA Pesticide Assessment Guideline, Subdivision N Section 161-2*	^{14}C-Piperonyl Butoxide was found to rapidly photolyze in an aqueous buffer at pH 7 and 25 °C when exposed to natural sunlight. Within 36 hours of exposure to sunlight less than 10% of the total radioactivity in the exposed sample was ^{14}C-Piperonyl Butoxide. The calculated $t_{1/2}$ of the aqueous photolysis rate of Piperonyl Butoxide was 8.4 hours of sunlight and the photolysis of Piperonyl Butoxide followed first order kinetic. Two major degradates were observed at concentrations greater than 10% of the total radioactivity. One degradate was identified as an alcohol degradate of Piperonyl Butoxide, the other was identified as the corresponding aldehyde of the alcohol degradate.	Further information should be provided about the degradate G (RT:28.3 min) which is appeared with maximum of 6.3% of applied radioactivity in the exposed sample. Since sunlight was used, tabular values of solar photon irradiances for the season and latitude should be provided. In addition the presence of clouds during the irradiation period should be reported. **Acceptable** Klimisch score: 2 GLP study	Selim S. (1995): Isolation and identification of major degradates of Piperonyl butoxide following aqueous photolysis; BTC-Biological Test Center, Irvine, CA, report number P0594010 **CAR IIIA 7.1.1.1.2**
Photo-transformation in air			
No guideline available	The photochemical degradation of Piperonyl butoxide in air was estimated using the model	GLP is not compulsory for calculations.	Bosse D. (1999): Substance Piperonyl butoxide – Calculation of the indirect photolysis reaction using the

	AOPWIN (version 1.80). The constants for reaction on different adsorption systems and mechanisms of binding to hydroxyl radicals were estimated to be 51.273 x 10^{-12} cm³/molec. sec for hydrogen abstraction and 56.3119 x 10^{-12} cm³/molec. sec for addition to aromatic rings. Half-life in the troposphere was calculated to be 3.579 hours for overall OH rate constant.	incremental method of Atkinson and the program AOPWIN, version 1.80; InfraServ GmbH Höchst KG, Frankfurt, Germany, report number not stated. **CAR IIIA 7.3**

5.1.1 Stability

Hydrolysis

The hydrolytic stability of Piperonyl Butoxide was studied in aqueous buffer solutions at pH 5, 7 and 9 and incubated at 25 °C under aseptic conditions in the dark. Piperonyl Butoxide was labelled with carbon-14 in its phenyl ring. The nominal initial concentration was 1 mg/L. At each pH, samples were analysed at zero time and at six additional times, up to 30 days. Incubated solutions were analysed by RP-HPLC to determine the relative proportions of Piperonyl Butoxide and any degradation products.

Piperonyl Butoxide was found to be hydrolytically stable at the tested pH (5, 7, 9) under test conditions.

The remaining amount of Piperonyl Butoxide, using two HPLC methods, was 98.6% and 97.2% at pH 5, 97.6% and 97.0% at pH 7 and 96.1% and 97.6%, at the end of the study.

The calculated DT_{50} of hydrolysis is greater than 500 days.

Photo transformation in water

^{14}C-Piperonyl Butoxide was found to rapidly photolyse in an aqueous buffer at pH 7 and 25 °C when exposed to natural sunlight. Within 36 hours of exposure to sunlight less than 10% of the total radioactivity in the exposed sample was ^{14}C-Piperonyl Butoxide. The calculated $t_{1/2}$ of the aqueous photolysis rate of Piperonyl Butoxide was 8.4 hours of sunlight and the photolysis of Piperonyl Butoxide followed first order kinetic. Two major degradates were observed at concentrations greater than 10% of the total radioactivity. One degradate was identified as an alcohol degradate of Piperonyl Butoxide, the other was identified as the corresponding aldehyde of the alcohol degradate.

Photo transformation in air

The photochemical degradation of Piperonyl butoxide in air was estimated using the model AOPWIN (version 1.80). The constants for reaction on different adsorption systems and mechanisms of binding to hydroxyl radicals were estimated to be 51.273 x 10^{-12} cm³/molec. sec for hydrogen abstraction and 56.3119 x 10^{-12} cm³/molec. sec for addition to aromatic rings.

Half-life in the troposphere was calculated to be 3.579 hours for overall OH rate constant.

5.1.2 Biodegradation

Table 78: Biodegradation

Method	Results	Remarks	Reference
Aerobic degradation in soil			
The degradation of Piperonyl Butoxide has been studied in soil (sandy loam) under aerobic conditions for 285 days. Piperonyl Butoxide labelled with ^{14}C in the phenyl ring had a radiochemical purity of 98% and was applied at an application rate of 10.1 µg/ g soil. Radioactivity of the extracts of soil samples was analysed by LSC and TLC. Possible degradation products were analysed by HPLC (detection limit of < 0.3%) and EI-MS for identification. The DT_{50} and DT_{90} values of Piperonyl Butoxide were calculated by means of non-linear regression analysis. *EPA Guideline, Subdivision N, Section 162-1*	^{14}C-Piperonyl Butoxide was mineralised to more than 50 % after 242 days. The level of ^{14}C-Piperonyl Butoxide in soil accounted for 98% AR at zero time, for 53% at 14 days and for 1% at 210 days. A degradation product identified as 3,4-methylenedioxy-6-propylbenzoic acid amounted to 17 % at day 30. The DT_{50} and DT_{90} value of Piperonyl Butoxide under aerobic conditions were calculated to be 14 days and 50 days, respectively.	**Acceptable** Klimisch score: 2 GLP	Mayo, B.C. (1995): Piperonyl Butoxide aerobic soil metabolism. Huntingdon Research Centre Ltd., Cambridgeshire, England; unpublished report no. PBT 7/951484 (October 2, 1995) Experimental phase: October 13, 1994 to September 28, 1995 **CAR 7.2.1/01**

Degradation rates of ^{14}C-labelled Piperonyl Butoxide and of selected metabolites were investigated onto three soils according to the SETAC-Guideline and the OECD-Guideline 307. ^{14}C-labelled Piperonyl Butoxide was applied at 7.6 mg/kg. The incubation of soil was performed under dark aerobic conditions at 20°C in the dark. The soil extracts were analysed for the test item and metabolites by radio-TLC, reversed phase radio-HPLC with UV-detection and radioactive monitoring and liquid scintillation counting (LSC). The obtained data sets were analysed using the program ModelMaker 4.0. The kinetic model considered for the analysis was single 1st order. *OECD guideline 307*	In the two out of three soils, EN 1-93/3 has been identified as major metabolite reaching up to 16.1% and 19.4% of AR. In the third soil (LUFA 3A) EN 1-93/3, was detected in lower amounts of max. 7.5 %. However, the decline was not finished at the end of the study. In LUFA 3A, the main metabolite was M2 amounted up to 14.4% after 70 days. M2 was detected in the other two soils as well. Further metabolites were M1 (max. 5.9% AR) and EN 1-101/4 (max. 6.6% AR). The DT_{50} (geometric mean) of Piperonyl Butoxide in the three different soils calculated by the computer program ModelMaker was 34.9 days.	Volatile radioactivity have been ranged from 29.5 to 33.8 percent of applied dose (for all soils at the end of the study) a more extent investigation should be performed in order to identify possible individual compounds that account for more than 10% of applied radioactivity. Incubation period should be continued since the decline of metabolite EN 1-93/3 is not finished after 120 days in two soils. **Acceptable** Klimisch score: 1 GLP	Derz, K. (2006): Degradation rates of Piperonyl Butoxide and selected metabolites in soil under aerobic conditions. Fraunhofer Institute for Molecular Biology and Applied Ecology, Schmallenberg, Germany; unpublished report no. GAB-011/7-90 Derz, K. (2011): 1st Final Report Amendment: Degradation rates of Piperonyl Butoxide and selected metabolites in soil under aerobic conditions. Fraunhofer Institute for Molecular Biology and Applied Ecology, Schmallenberg, Germany; unpubl. report no. GAB-011/7-90 **CAR 7.2.2.1/02**
Anaerobic degradation in soil			
An anaerobic soil metabolism study with ^{14}C-Piperonyl Butoxide (purity = 99.1 %) applied to a sandy loam soil at a nominal 10 µg/g rate was conducted. The study lasted 70 days in the dark at 25 ± 1°C. Soil extracts were characterized for parent compound by TLC. The level of bound ^{14}C-residues was determined by combustion radioanalysis of the post-extracted samples. Possible degradation products were analysed by HPLC (detection limit of < 0.3 %) and EI-MS for identification. The half-life of Piperonyl Butoxide for the aerobic and anaerobic incubation period was calculated by means of linear regression analysis.	One metabolite (Metabolite F) accounted for 27.9% of the applied radioactivity after 60 days (anaerobic phase). The level of Piperonyl Butoxide in soil accounted for 99.8% AR at zero time, for 81.3% at 10 days and for 29.6% after 60 days. DT_{50}=144 days.	The chemical structure of Metabolite F is not stated unequivocally **Acceptable** Klimisch score: 2 GLP	Williams, M.D. (1991): Anaerobic soil metabolism of Piperonyl Butoxide. ABC Laboratories, Inc., Columbia, Missouri, USA; unpublished report no. 38585 (August 22, 1991) Experimental phase: May 29, 1990 – August 31, 1990 **CAR 7.2.2.4/01**

EPA Guideline, Subdivision N, Section 162-2

Water/sediment study			
Aerobic degradation of Piperonyl Butoxide has been tested in two different water/sediment systems (pond and creek) according to OECD guideline 308. The incubation was performed under aerobic conditions at 20^0C in the dark. Samples were taken for analysis at days 0, 1, 3, 7, 14, 30, 62, 100 and 120 after application. After sampling, the radioactivity in the water and sediment (i.e. extractables) was separately worked-up and analysed by TLC and HPLC.	Piperonyl Butoxide detected in the water phase decreased continuously with increasing incubation time down to values of 0.1% (pond) and 0.7% (creek) at 120 days after application. This decrease was compensated by an increase of Piperonyl Butoxide in the sediment extract. At the end of experiments 23.7 % (pond) and 17.2 % (creek) system were identified as Piperonyl Butoxide. One main metabolite (M2) was detected, amounted to up to 21.4 % (entire pond system) and in the entire creek system, metabolite M2 reached a maximum value of 40.7 % (100 days), which decreased to 36.1 % at the end of experiment. Further metabolites were M1 reached up a 7.6 % maximum (pond), and EN 1-93/3 which was detected in amounts of up to 6.1 % (pond). Metabolite EN 1-101/4 was detected only in minor extent, never exceeding 4.4 % (in the entire system). Metabolite M1 was characterized after further identification analyses, but M2 could not finally be characterized. The mean disappearance time of Piperonyl Butoxide in the whole water/sediment system was determined to be 55 days (DT_{50}) and 181 days (DT_{90}), respectively.	A justification for the big difference in mean disappearance time of Piperonyl Butoxide in water and sediment of each system must be provided. **Acceptable** Klimisch score: 1 GLP	Derz, K. (2006): Aerobic transformation of Piperonyl Butoxide in water/sediment systems (OECD 308). Fraunhofer Institute for Molecular Biology and Applied Ecology, Schmallenberg, Germany; unpublished report no. GAB-011/7-92 (July 31, 2006) Experimental phase: November 21, 2005 to April 28, 2006 *Derz, K. (2011):* 1st Final Report Amendment: *Aerobic transformation of Piperonyl Butoxide in water/sediment systems (OECD 308). Fraunhofer Institute for Molecular Biology and Applied Ecology, Schmallenberg, Germany; unpublished report no. GAB-011/7-92.* **CAR 7.1.2.2.2**

5.1.2.1 Biodegradation estimation

5.1.2.2 Screening tests

Table 79: Screening test

Method	Results	Remarks	Reference
Ready biodegradability			
The biological degradability of Piperonyl Butoxide was investigated over a period of 28 days in the CO_2 evolution test. The test material was the sole source of carbon and energy, it was suspended in a buffered mineral salts medium at a concentration of ca 22 mg/L. Microorganisms derived from activated sludge not previously exposed to the test substance. Vessels were incubated in darkness at 22 ± 2°C. The extent of biodegradation was determined by expressing the cumulative recovered yield as a percentage of the theoretical (165 mg CO_2), calculated from the carbon content of the test substance. *OECD Guideline 301 B*	The total CO_2 production from each blank vessel (mean = 26.3 mg/L CO_2) had not exceeded 40 mg/L CO_2 after 28 days. The biological degradation rate as percentage of the theoretical CO_2 yield was calculated to be 24 % after 28 days. The value of Replicate II seems to be an outlier. The degradation of sodium benzoate as reference substance was determined to be > 60 % after 6 days (>90% at the end of the study). Pass levels were not reached within a 10-d window.	More details should be provided in order to explain the 50% difference of the two replicate values at the end of the test. **Acceptable** Klimisch score:2 GLP	Bealing, D.J. (2002): Piperonyl Butoxide: Assessment of ready biodegradability by measurement of carbon dioxide evolution. Covance Laboratories Ltd., North Yorkshire, UK; unpublished report no. 2145/3-D2149 (May 24, 2002) **CAR 7.1.1.2.1**

5.1.2.3 Simulation tests

5.1.3 Summary and discussion of degradation

Piperonyl Butoxide degrades with a half-life of 109 days for the entire system when applied to an aerobic aquatic environment. A half-life of 55 days for the entire system has been resulted in a second study.

However, it shows a short photolytic half-life of aqueous systems of 8.4 hours and a rapid photochemical degradation in air with a half-life of 3.579 hours. In an aqueous hydrolysis study, Piperonyl Butoxide was found to be hydrolytically stable at pH values 5, 7 and 9. Piperonyl Butoxide was neither readily biodegradable under the conditions in the modified Sturm test performed.

It could be shown that Piperonyl Butoxide rapidly degrades in soil under aerobic conditions in the laboratory with a half-life of 14 days at 25 °C. In a second study (three different soils) an average half-life of 34.9 days has been resulted.

Conclusion

Based on the abovementioned key studies Mayo, 1995 (reliability score: 2) and Derz K., 2006a,b (reliability score: 1) and Williams, 1991 (supporting data, reliability score: 2), PBO should be considered as not rapidly degradable based on weight-of-evidence approach.

5.2 Environmental distribution

5.2.1 Adsorption/Desorption

Table 80: Adsorption/desorption

Method	Results	Remarks	Reference
Adsorption/Desorption			
Adsorption/desorption properties of Piperonyl Butoxide on four different soil types were characterised by determining the adsorption isotherms and Freundlich constants. Four aqueous solutions of ^{14}C-Piperonyl Butoxide (4.0, 3.0, 2.0 and 0.4 mg/L) were prepared. The analysis has been performed by LSC. *EPA Guideline, Subdivision N, Section 163-1*	Mean percent of compound adsorbed to the test soils was 55.5 %, 69.3 %, 81.5 % and 71.6 % for sand, clay loam, sandy loam and silt loam, respectively. Piperonyl Butoxide has a low mobility in clay loam soil and a low mobility in sandy loam and silt loam soil.	The selected soil types did not cover a wide range of pH and Organic Matter (a soil with Organic matter 4-6% should have been tested). Only one soil/solution ratio was tested. Please justify the selection of this specific ratio. **Acceptable** Klimisch score: 2 GLP	Daly, D. (1991): Soil/Sediment Adsorption-Desorption of Piperonyl Butoxide. ABC Laboratories, Inc., Columbia, Missouri, USA; unpublished report no. 38360 (January 4, 1991) Experimental phase: January 22 to January 31, 1990
Soil column leaching			
Leaching behaviour of Piperonyl Butoxide and its aged residues was studied using the column leaching method according to EPA Guideline, Subdivision N, Section 163-1. 4 soil types were used (sand, clay loam, sandy loam, silt loam). ^{14}C-Piperonyl Butoxide was applied to the tops of 30 cm soil columns at a rate equivalent to 5 kg/ha. Extracts were analysed by means of HPLC. *EPA Guideline, Subdivision N, Section 163-1*	^{14}C-Piperonyl Butoxide showed a low mobility in 3 of 4 soil types used (clay loam, silt loam, sandy loam). The compound was very mobile on the sand. Furthermore, Piperonyl Butoxide degraded rapidly during 18 days aging under aerobic conditions on sandy loam soil.	**Acceptable** Klimisch score: 1 GLP	Elsom, L.F. (1995): Soil column leaching of non-aged and aged residues of ^{14}C-Piperonyl Butoxide. Huntingdon Research Centre Ltd., Cambridgeshire, England; unpublished report no. PBT 10B/950899 (June 5, 1995) Experimental phase: January 24 – April 25, 1995

5.2.2 Volatilisation

The volatility of Piperonyl Butoxide is moderate (Henry constant: 1.648 x 10^{-4} Pa*m³*mol^{-1} at 25 °C) (Tiemann, J, 2006, GLP).

5.2.3 Distribution modelling

Not relevant for this dossier.

5.3 Aquatic Bioaccumulation

5.3.1 Aquatic bioaccumulation

The potential for bioaccumulation is an important criterion to determine whether a chemical substance has a potential hazard to the environment. Bioaccumulation of a substance into an organism is not a hazard in itself, but should be considered in relation to potential long-term effects.

A first indication of the potential bioaccumulation of a substance can be provided by the surrogate measure of octanol:water partition coefficient (log K_{OW}). It is accepted that values of log K_{OW} equal to or greater than 3 indicate that the substance may bioaccumulate in terrestrial and aquatic biota. Since Piperonyl Butoxide has a log K_{OW} of 4.8 the potential for aquatic bioaccumulation has been explored.

5.3.1.1 Bioaccumulation estimation

As a study investigating the bioaccumulation potential of Piperonyl Butoxide in aquatic organisms (i.e. fish) is available, no theoretical BCF_{fish} estimation is required.

5.3.1.2 Measured bioaccumulation data

The aquatic bioaccumulation of Piperonyl Butoxide was experimentally investigated using Bluegill sunfish (*Lepomis macrochirus)* (Anonymous - 23 1992).

The test fish were maintained under flow-through conditions and exposed to a mixture of radiolabelled and non-radiolabelled Piperonyl Butoxide at a nominal concentration of 0.1 ppm for 28 days followed by a 14 days depuration phase. Observations on behaviour of the fish and the physical appearance of the test substance were made daily. The concentration of [^{14}C] residues in the fish and the bioconcentration factors (BCFs) were quantified by collecting and sacrificing five fish from exposure and control aquariums on days 0, 3, 7, 14, 21 and 28 of exposure and days 1, 3, 7, 10 and 14 of depuration for analysis. The levels and identities of the parent product and degradates in water and tissues were determined by extraction, chromatographic methods, and liquid scintillation counting (LSC). The test was in compliance with GLP standards and conducted following to the OECD guideline 305.

Steady state BCF (BCF_{SS}) values in edible tissues (191 L/kg), non-edible tissues (380 L/kg) and whole body (260 L/kg) were calculated based on residue data in fish and water obtained on days 0, 3, 7, 14 and 21 after test initiation. The data obtained on day 28 were excluded from the calculations of mean BCF_{SS} since, according to the original study report, the tissue concentrations of Piperonyl Butoxide obtained on day 28 were considered aberrant. In addition, kinetic (mean) BCF values in edible (99 L/kg), non-edible (450 L/kg) and whole fish (290 L/kg) were calculated from the uptake and depuration rate constants using the BIOFAC© computer model. The estimated times to reach 50% depuration were 0.67, 1.6 and 1.3 days for edible tissues, non-edible tissues and whole fish, while

estimated times to reach 90% depuration were 2.2, 5.2 and 4.2 for edible tissues, non-edible tissues and whole fish.

According to the OECD testing guideline 305 (2011), steady-state fish bioconcentration factors could be calculated when a steady-state has been reached by the end of the exposure phase, i.e. when in the plot of test substance in fish (C_f) against time the curve becomes parallel to the time axis and three successive analyses of C_f made on samples taken at intervals of at least two days are within ± 20% of each other. If steady-state has not been reached by the end of the exposure period (28 days), either the BCF is calculated using only the kinetic approach, which is not reliant on steady-state being reached, or the uptake phase can be extended, taking further measurements, until steady-state is reached or for 60 days, whichever is shorter. Based on the measured residue data in fish during the uptake fish and the aforementioned recommendations, no steady state has been reached by day 28 of exposure. Thus, the estimated kinetic BCF values were considered more reliable to address the bioaccumulation potential of Piperonyl Butoxide in fish.

Further, according to the OECD testing guideline 305 (2011), biological factors such as fish lipid content and growth dilution can have a strong impact on the test results and need to be taken into account. No fish lipid content measurement was conducted during the test and thus the calculated BCF values were not lipid normalized. Although growth data (length and weight) for the test fish were submitted by the applicant following DS's request, no calculation of the growth rate constant and thus no growth dilution correction of the kinetic BCF value was performed. Taking into account the above deviations, a Klimisch score of 2 was assigned to the study. The study results are presented in the following table.

Table 81: Measured aquatic bioaccumulation of Piperonyl Butoxide

Test method	Species	Endpoint(s)	Exposure		Results	Remarks	Reference
			Design	Duration			
EPA, Subdivision N, Series 165-4; consistent with the OECD Testing Guideline 203; GLP study	Bluegill sunfish (*Lepomis macrochirus*)	Bioconcentration factor/uptake and depuration rate constants	Flow-through	28 d uptake phase 14 d depuration phase	BCF_K 99 L/kg (edible tissue) BCF_K 450 L/kg (non edible tissue) BCF_K 290 L/kg (whole fish) DT_{50} (whole fish) = 1.3 days DT_{90} (whole fish) = 4.2 days	Klimisch score: 2	**Anonymous - 23** (1992) CAR Doc IIIA 7.4.3.3.1

5.3.2 Summary and discussion of aquatic bioaccumulation

The BCF of Piperonyl Butoxide in fish was experimentally determined to be 290 L/kg (whole fish).

In accordance with the Regulation (EC) No 1272/2008 and the ECHA Guidance on the Application of the CLP Criteria (Version 5.0; July, 2017), since the measured BCF is lower than the cut-off value of 500 L/kg, Piperonyl Butoxide is unlikely to bioaccumulate in fish or other aquatic food webs and can be regarded as non bioaccumulative substance.

5.4 Aquatic toxicity

Table 82: Summary of relevant information on aquatic toxicity

Method	Results			Remarks	Reference
Acute aquatic toxicity					
Fish: Sheepshead minnow *Cyprinodon variegatus* (96 hours; flow-through system) EPA, Subdivision E, Series 72-3; consistent with the OECD Testing Guideline 203; GLP study	**LC_0 (mg/L)** 2.97	**LC_{50} (mg/L)** **3.94**	**LC_{100} (mg/L)** ≥ 5.24	Results were based on mean measured concentrations Klimisch score: 1	**Anonymous - 24** (1992b) CAR Doc III A7.4.1.1/01
Fish: Bluegill sunfish *Lepomis macrochirus* (96 hours; flow-through system) EPA, Subdivision E, Series 72-1; consistent with the OECD Testing Guideline 203; GLP study	**LC_0 (mg/L)** 2.34	**LC_{50} (mg/L)** 5.37	**LC_{100} (mg/L)** ≥ 6.94	Results were based on mean measured concentrations Klimisch score: 1	Anonymous - 25 (1992c) CAR Doc III A7.4.1.1/02
Fish: Rainbow trout *Oncorhynchus mykiss* (96 hours; flow-through system) EPA, Subdivision E, Series 72; consistent with the OECD Testing Guideline 203; GLP study	**LC_0 (mg/L)** 3.71	**LC_{50} (mg/L)** 6.12	**LC_{100} (mg/L)** ≥ 8.00	Results were based on mean measured concentrations Klimisch score: 1	Anonymous - 26 (1992a) CAR Doc III A7.4.1.1/03
Aquatic invertebrates: *Daphnia magna* (48 hours; flow-through system) EPA, Subdivision E, 72-2; consistent with the OECD Testing Guideline 202; GLP study	**EC_0 (mg/L)** 0.15	**EC_{50} (mg/L)** 0.51	**EC_{100} (mg/L)** > 0.74	Results were based on mean measured concentrations Klimisch score: 1	Holmes and Smith (1992d) CAR Doc III A7.4.1.2/01
Aquatic invertebrates: *Mysidopsis bahia* (96 hours; flow-through system) EPA, Subdivision E, Series 72-3; consistent with the OPPTS 850.1035 testing guideline; GLP study	**LC_0 (mg/L)** 0.05	**LC_{50} (mg/L)** 0.32	**LC_{100} (mg/L)** > 0.34	Results were based on mean measured concentrations Klimisch score: 1	Holmes and Smith (1992f) CAR Doc III A7.4.1.2/02

Method	Results			Remarks	Reference
Aquatic invertebrates: *Mysidopsis bahia* (96 hours; flow-through system) EPA, Subdivision E, Series 72; consistent with the OPPTS 850.1035 testing guideline; GLP study	**LC_0 (mg/L)** 0.16	**LC_{50} (mg/L)** 0.49	**LC_{100} (mg/L)** > 0.73	Results were based on mean measured concentrations Klimisch score: 1	Roberts and Swigert (1995) A7.4.1.2/03
Aquatic invertebrates: *Crassostrea virginica* (96 hours; flow-through system) EPA, Subdivision E, Series 72; consistent with the OPPTS 850.1025 testing guideline; GLP study	**EC_0 (mg/L)** < 0.04	**EC_{50} (mg/L)** **0.23**	**EC_{100} (mg/L)** > 0.57	Results were based on mean measured concentrations Klimisch score: 1	Holmes and Smith (1992) CAR Doc III A7.4.1.2/04
Algae: *Selenastrum capricornutum* (72 hours; static system) OECD Testing Guideline 201; GLP study	**E_rC_{50} (mg/L)** **3.89**	**E_bC_{50} (mg/L)** 2.09	**NOE_rC (mg/L)** **0.824**	Results were based on mean measured concentrations Klimisch score: 1	Mattock (2002) CAR Doc III A7.4.1.3/01
Chronic aquatic toxicity					
Fish: Fathead minnow *Pimephales promelas* (4-day incubation & 31-day post-hatch exposure period; flow-through system) EPA, Subdivision E, Series 72-4; consistent with the OECD testing guideline 210; GLP study	**EC_{50} (mg/L)** -	**LOEC (mg/L)** 0.42	**NOEC (mg/L)** **0.18**	Results were based on mean measured concentrations (although they were satisfactorily maintained, e.g. ± 20% of nominal) Klimisch score: 1	Anonymous - 27 (1994) CAR Doc III A7.4.3.2
Aquatic invertebrates: *Daphnia magna* (21 days; flow-through system) EPA, Subdivision E, Series 72-4; consistent with the OECD testing guideline 211; GLP study	**EC_{50} (mg/L)** > 0.65 (parents) 0.21 (offspring)	**LOEC (mg/L)** 0.047	**NOEC (mg/L)** **0.030**	Results were based on mean measured concentrations Klimisch score: 1	Putt (1994) CAR Doc III A7.4.3.4/01

Method	Results				Remarks	Reference
Aquatic invertebrates: *Daphnia magna* (21 days; flow-through system) EPA, Subdivision E, Series 72-4; consistent with the OECD testing guideline 211; GLP study	**EC_{50} (mg/L)**	**LOEC (mg/L)**	**NOEC (mg/L)**		Results were based on mean measured concentrations Klimisch score: 1	Holmes and Smith (1992) CAR Doc III A7.4.3.4/02
	-	0.12	0.066			
Sediment dwelling-organisms: *Chironomus riparius* (28 days; water spiking exposure scenario) OECD Testing Guideline 219; GLP study	**EC_{50} (mg/L)**	**EC_{100} (mg/L)**	**NOEC**		Results were based on geomean measured concentrations Klimisch score: 1	Stäbler (2006) CAR Doc III A7.4.3.5.1
			mg/L	**mg/kg**		
	-	-	**0.0148**	**0.0933**		

5.4.1 Fish

5.4.1.1 Short-term toxicity to fish

Three acute (short-term) toxicity studies of Piperonyl Butoxide to freshwater and marine fish species were performed with *Cyprinodon variegatus* (Anonymous - 24, 1992b), *Lepomis macrochirus* (Anonymous - 25, 1992c) and *Oncorhynchus mykiss* (Anonymous - 26, 1992a).

The three studies were conducted under flow through conditions in accordance with the OECD testing guideline 203 and fulfilled the corresponding validity criteria. Therefore, they are all considered acceptable for use in the aquatic hazard assessment and risk characterization.

The test principles, test design and test performance were similar in the three tests. Ten fish per test concentration were exposed to a mixture of radiolabelled and non-radiolabelled Piperonyl Butoxide for 96 hours. The nominal concentrations of Piperonyl Butoxide tested were: 1.3, 2.2, 3.6, 6.0 and 10 mg a.s./L for the acute toxicity test with the marine species *Cyprinodon variegatus*; 1.8, 3.0, 5.0, 8.4 and 14.0 mg a.s./L for the acute toxicity test with the freshwater species *Lepomis macrochirus* and 1.6, 2.6, 4.3, 7.2 and 12 mg a.s./L for the acute toxicity test with the freshwater species *Oncorhynchus mykiss*. Observations of mortality and other signs of toxicity (treatment related effects) were made at test start and every 24 hours thereafter. In addition, analytical determinations of Piperonyl Butoxide concentration were conducted at each treatment group every 24 hours. Radioactivity determination was conducted by liquid scintillation spectrometry (LSC), while total Piperonyl Butoxide concentrations were determined by high performance liquid chromatography (HPLC).

In all three tests, measured concentrations in the test treatments were not maintained within ± 20% of nominal throughout the test and therefore test results were based on mean measured concentrations. Statistical analysis of the study results, demonstrated that the 96-hour LC_{50} of Piperonyl Butoxide for the sheepshead minnow *Cyprinodon variegatus* was 3.94 mg a.s./L, for the bluegill sunfish *Lepomis macrochirus* was 5.37 mg a.s./L and for the rainbow trout *Oncorhynchus mykiss* was 6.12 mg a.s./L. The three studies' results are presented in the following table.

Table 83: Acute toxicity of Piperonyl Butoxide to fish

Guideline / Test method/ GLP status	Species	Endpoint / Type of test	Exposure		Results mg a.i./L			Remarks	Reference
			Design	Dura-tion	LC_0	LC_{50}	LC_{100}		
EPA, Subdivision E, Series 72-3; consistent with the OECD Testing Guideline 203; GLP study	Sheepshead minnow (*Cyprinodon variegatus*)	Mortality; acute toxicity test	Flow-through	96 hours	2.97	3.94	≥5.24	Results were based on mean measured concentrations Klimisch score: 1	Anonymous - 24 (1992b) CAR Doc IIIA 7.4.1.1/01
EPA, Subdivision E, Series 72-1; consistent with the OECD Testing Guideline 203; GLP study	Bluegill sunfish (*Lepomis macrochirus)*	Mortality; acute toxicity test	Flow-through	96 hours	2.34	5.37	≥6.94	Results were based on mean measured concentrations Klimisch score: 1	Anonymous - 25 (1992c) CAR Doc III A7.4.1.1/02
EPA, Subdivision E, Series 72; consistent with the OECD Testing Guideline 203; GLP study	Rainbow trout (*Oncorhynchus mykiss*)	Mortality / acute toxicity test	Flow-through	96 hours	3.71	6.12	≥8.00	Results were based on mean measured concentrations Klimisch score: 1	Anonymous - 26 (1992a) CAR Doc III A7.4.1.1/03

5.4.1.2 Long-term toxicity to fish

The effects of Piperonyl Butoxide on reproduction and growth of fish were examined in a 34-day chronic toxicity test with *Pimephales promelas* (Anonymous - 27, 1994) conducted according to the procedures described and the recommendations provided in the OECD testing guideline 210. The test was initiated by exposing fathead minnow eggs to Piperonyl Butoxide under flow-through conditions at the nominal concentrations of 0.047, 0.094, 0.19, 0.38 and 0.75 mg/L. Following a 4-day pre-hatching period, 80 live larvae from each treatment group were placed into their respective exposure aquaria (e.g. 40 larvae per replicate/80 larvae per treatment level) and remained for 31 days. Observations on embryo hatching as well as on larval survival, growth and behaviour were recorded on a daily basis. Analytical determinations for Piperonyl Butoxide concentration were conducted at each treatment group at the start and thereafter on days 4, 11, 18, 21, 25, 28, 31 and 35. For the treated groups with nominal Piperonyl Butoxide concentrations of 0.047, 0.094, 0.19, 0.38 and 0.75 mg/L the mean measured concentrations were 0.040, 0.11, 0.18, 0.42 and 0.80 mg/L, respectively. Statistical analysis of the study results demonstrated that the 35-day no-observed effect concentration (NOEC) of Piperonyl Butoxide for *Pimephales promelas* was 0.18 mg/L based on mean measured concentrations. The study results are presented in the following table.

Table 84: Chronic toxicity of Piperonyl Butoxide to fish

Guideline / Test method/ GLP status	Species	Endpoint / Type of test	Exposure		Results mg a.i./L		Remarks	Reference
			Design	Duration	NOEC	LOEC		
EPA, Subdivision E, Series 72-4; consistent with the OECD testing guideline 210; GLP study	Fathead minnow (*Pimephales promelas*)	Embryo hatch, survival and growth of larvae/chronic toxicity test	Flow-through	4-day incubation period and 31-day post-hatch exposure period	0.18	0.42	Results were based on mean measured concentrations (although they were satisfactorily maintained, e.g. ± 20% of nominal) Klimisch score: 1	**Anonymous - 27 (1994)** CAR Doc III A7.4.3.2

5.4.2 Aquatic invertebrates

5.4.2.1 Short-term toxicity to aquatic invertebrates

The acute toxicity of Piperonyl Butoxide to aquatic invertebrates was investigated with three aquatic invertebrate species, inhabiting freshwater or marine environment. The tested species were the cladoceran freshwater flea *Daphnia magna* (Holmes and Smith, 1992d), the shrimp-like marine crustacean *Americamysis bahia* (formerly *Mysidopsis bahia*) (Holmes and Smith (1992f), Roberts and Swigert (1995)) and the eastern oyster *Crassostrea virginica* which inhabits marine environments (Holmes and Smith, 1992).

The study on acute toxicity of Piperonyl Butoxide to *Daphnia magna* (Holmes and Smith, 1992d) was conducted by following the procedures described and the recommendations provided in the OECD testing guideline 202. Twenty daphnids divided into 2 groups of 10 animals each were exposed to a mixture of radiolabelled and non-radiolabelled Piperonyl Butoxide for 48 hours under flow-through conditions. Five nominal concentrations of Piperonyl Butoxide were tested: 0.13, 0.22, 0.36, 0.6 and 1.0 mg a.s./L. Observations on immobilization and other signs of toxicity (treatment related effects) were made 1.5 h after the test start and every 24 hours thereafter. Analytical determinations of Piperonyl Butoxide concentration were conducted at each treatment group at the start and at the end of the test. Radioactivity determination was conducted with liquid scintillation spectrometry (LSC), while total Piperonyl Butoxide concentrations were determined with high performance liquid chromatography (HPLC). For the treated groups with nominal Piperonyl Butoxide concentrations of 0.13, 0.22, 0.36, 0.6 and 1.0 mg a.s./L the mean measured concentrations of radioactivity were 0.08, 0.15, 0.25, 0.4 and 0.74 mg a.s./L, respectively. Statistical analysis of the study results, demonstrated that the 48-hour EC_{50} value of Piperonyl Butoxide for *Daphnia magna* was 0.51 mg/L (ppm), while the no effect concentration was 0.15 mg a.s./L after 48 h exposure. Based on the 48-hour EC_{50} of 0.51 mg a.s./L and the criteria outlined in the CLP Regulation 1272/2008, Piperonyl Butoxide is classified as very toxic to *Daphnia magna*.

The study of Holmes and Smith (1992f) on acute toxicity of Piperonyl Butoxide to the mysid shrimp *Americamysis bahia* (formerly *Mysidopsis bahia*) was conducted according to the OPPTS testing guideline 850.1035 (Mysid Acute Toxicity Test). Twenty mysids divided into 2 replicates of 10 animals each were exposed to a mixture of radiolabelled and non-radiolabelled Piperonyl Butoxide

for 96 hours under flow-through conditions. Five nominal concentrations of Piperonyl Butoxide were tested: 0.06, 0.11, 0.18, 0.30 and 0.50 mg a.s./L. All mysid shrimps were observed approximately 2.5, 24, 48, 72 and 96 h after the start of the test to evaluate the number of mortalities and the number of individuals exhibiting clinical signs of toxicity or abnormal behaviour. Analytical determinations for Piperonyl Butoxide concentration were conducted at each treatment group at approximately 0, 24, 48, 72 and 96 hours after the test initiation. For the treated groups with nominal Piperonyl Butoxide concentrations of 0.06, 0.11, 0.18, 0.30 and 0.50 mg a.s./L the mean measured concentrations of radioactivity were 0.05, 0.08, 0.10, 0.21 and 0.34 mg a.s./L, respectively. Statistical analysis of the study results, demonstrated that the 96-hour EC_{50} value of Piperonyl Butoxide for *Americamysis bahia* (formerly *Mysidopsis bahia*) was 0.32 mg/L, while the no effect (mortality) concentration was 0.05 mg a.s./L after 96 h exposure. Based on the 96-hour EC_{50} of 0.32 mg a.s./L and the criteria outlined in the CLP Regulation 1272/2008, Piperonyl Butoxide is classified as very toxic to the saltwater mysid shrimp *Americamysis bahia* (formerly *Mysidopsis bahia*).

Besides the study of Holmes and Smith (1992f), the acute toxicity of Piperonyl Butoxide to the mysid shrimp *Americamysis bahia* (formerly *Mysidopsis bahia*) was investigated in an additional GLP study by Roberts and Swigert (1995). The study followed the procedures described in the OPPTS testing guideline 850.1035 (Mysid Acute Toxicity Test) and fulfilled the corresponding validity criteria. In addition, the quality criteria of the OECD testing guideline 202 were met. The objective of the study was to evaluate the acute toxicity of Piperonyl Butoxide to the saltwater mysid shrimp *Americamysis bahia* (formerly *Mysidpopsis bahia*) during a 96-hour exposure period under flow-through conditions. Twenty mysids were exposed to Piperonyl Butoxide at nominal concentrations of 0.11, 0.18, 0.30, 0.50 and 0.83 mg a.s./L. Two replicates with 10 mysids of a negative (saltwater) control, a solvent control and each test concentration were run in parallel. Observations of mortality and other signs of toxicity (treatment related effects) were made at test start, and every 24 hours thereafter. Statistical analysis of the study results demonstrated that the 96-hour LC_{50} of Piperonyl Butoxide for *Americamysis bahia* (formerly *Mysidopsis Bahia*) was 0.49 mg a.s./L while the no mortality concentration was 0.16 mg a.s./L, based on mean measured concentrations.

The study of Holmes and Smith (1992) on the acute toxicity of Piperonyl Butoxide to the eastern oyster *Crassostrea virginica* was conducted according to the OPPTS 850.1025 testing guideline (Oyster Acute Toxicity Test (Shell Deposition)) and fulfilled the corresponding validity criteria. The objective of the study was to evaluate the acute toxicity of Piperonyl Butoxide to the eastern oyster *Crassostrea virginica* during a 96-hour exposure period under flow-through conditions. More specifically, the aim of the study was to determine the Piperonyl Butoxide concentration inducing 50% reduction in shell growth. Twenty oysters were exposed to a mixture of radiolabelled and non-radiolabelled Piperonyl Butoxide at nominal concentrations of 0.06, 0.10, 0.17, 0.29, 0.48 and 0.80 mg a.s./L. Methanol was used as solvent and one test chamber of solvent control was run in parallel to the treated groups. Observations of mortality and other clinical signs of toxicity were made at 24-hour intervals throughout the test. Shell growth increments, measured after the 96-hour exposure, was the primary criterion used to evaluate the acute toxicity of the active substance to the exposed eastern oysters. Analytical determinations for Piperonyl Butoxide concentration were conducted in each treatment group at approximately 0, 24, 48, 72 and 96 hours after the test initiation. For the treated groups with nominal Piperonyl Butoxide concentrations of 0.06, 0.10, 0.17, 0.29, 0.48 and 0.80 mg a.s./L the mean measured concentrations of radioactivity were 0.04, 0.07, 0.11, 0.17, 0.34 and 0.57 mg a.s./L, respectively. Statistical analysis of the study results demonstrated that the 96-hour EC_{50} of Piperonyl Butoxide for *Crassostrea virginica* was 0.23 mg a.s./L, while the no effect concentration was 0.04 mg a.s./L based on mean measured concentrations.

The available studies on acute effects of Piperonyl Butoxide to aquatic invertebrates are summarized in the following table.

Table 85: Acute toxicity of Piperonyl Butoxide to aquatic invertebrates

Guideline / Test method / GLP status	Species	Endpoint / Type of test	Exposure		Results mg a.i./L			Remarks	Reference
			Design	Duration	$E(L)C_0$	$E(L)C_{50}$	$E(L)C_{100}$		
EPA, Subdivision E, 72-2; consistent with the OECD Testing Guideline 203; GLP study	*Daphnia magna*	Mortality/ Immobility; acute toxicity test	Flow-through	48-hours	0.15	0.51	> 0.74	Results were based on mean measured concentration Klimisch score: 1	Holmes and Smith (1992d) CAR Doc III A7.4.1.2/01
EPA, Subdivision E, Series 72-3; consistent with the OPPTS 850.1035 testing guideline; GLP study	*Americamysis bahia* (formerly *Mysidopsis bahia*)	Mortality; acute toxicity test	Flow-through	96-hours	0.05	0.32	> 0.34	Results were based on mean measured concentrations Klimisch score: 1	Holmes and Smith (1992f) CAR Doc III A7.4.1.2/02
EPA, Subdivision E, Series 72; consistent with the OPPTS 850.1035 testing guideline; GLP study	*Americamysis bahia* (formerly *Mysidopsis bahia*)	Mortality; acute toxicity test	Flow-through	96-hours	0.16	0.49	> 0.73	Results were based on mean measured concentrations Klimisch score: 1	Roberts and Swigert (1995) CAR Doc III A7.4.1.2/03
EPA, Subdivision E, Series 72; consistent with the OPPTS 850.1025 testing guideline; GLP study	*Crassostrea virginica*	Mortality/ Shell growth; acute toxicity test	Flow-through	96-hours	< 0.04	0.23	> 0.57	Results were based on mean measured concentrations Klimisch score: 1	Holmes and Smith (1992) A7.4.1.2/04

5.4.2.2 Long-term toxicity to aquatic invertebrates

The effects of Piperonyl Butoxide on reproduction and growth of aquatic invertebrates were investigated with two GLP chronic toxicity tests with *Daphnia magna*, i.e. Putt (1994) and Holmes and Smith (1992). The two studies were conducted under flow through conditions for 21 days in

accordance with the procedures described and the recommendations provided in the OECD testing guideline 211.

In the study of Putt (1994), forty daphnids divided into 4 replicates of 10 animals each were exposed to Piperonyl Butoxide at nominal concentrations of 47, 94, 190, 380 and 750 µg/L. Survival (mortality) of adult daphnids was determined at least every three days while offspring production was assessed on day 7 and at least three times per week through test termination. At test termination, the length and weight of each surviving adult daphnid was also measured. Analytical determinations of Piperonyl Butoxide concentration were conducted at each treatment group on days 0, 2, 7, 14 and 21. For the treated groups with nominal Piperonyl Butoxide concentrations of 47, 94, 190, 380 and 750 µg/L the mean measured concentrations were 30, 47, 95, 210 and 650 µg/L, respectively. Statistical analysis of the study results demonstrated that the 21-day NOEC of Piperonyl Butoxide for *Daphnia magna* was 30 µg/L based on mean measured concentrations.

In the study of Holmes and Smith (1992), seven parent daphnids were maintained individually (one per test vessel) at each test concentration for survival, reproduction and growth observations. In addition, three replicates of five daphnids at each treatment level were observed for survival only. The Piperonyl Butoxide nominal concentrations used in the study were 25, 50, 100, 200 and 400 µg/L. Biological observations were performed three times per week, while analytical determinations of the test substance concentration were made at least weekly over the 21-day test period. Statistical analysis of the survival, reproduction, growth and clinical observation data collected demonstrated that the 21-day NOEC of Piperonyl Butoxide for *Daphnia magna* was 66 µg/L based on mean measured concentrations.

The available studies on chronic effects of Piperonyl Butoxide to aquatic invertebrates (*Daphnia magna*) are summarized in the following table.

Table 86: Chronic toxicity of Piperonyl Butoxide to aquatic invertebrates

Guideline/ Test method/ GLP status	Species	Endpoint / Type of test	Exposure		Results µg a.i./L			Remarks	Reference
			Design	Duration	EC_{50}	NOEC	LOEC		
EPA, Subdivision E, Series 72-4; consistent with the OECD testing guideline 211; GLP study	*Daphnia magna*	Reproduction, survival and growth/ chronic toxicity test	Flow-through	21 days	> 650 (par) 210 (offspr)	30	47	Results were based on mean measured concentrations Klimisch score: 1	Putt (1994) CAR Doc III A7.4.3.4/01
EPA, Subdivision E, Series 72-4; consistent with the OECD testing guideline 211; GLP study	*Daphnia magna*	Reproduction, survival and growth/ chronic toxicity test	Flow-through	21 days	-	66	120	Results were based on mean measured concentrations Klimisch score: 1	Holmes and Smith (1992) CAR Doc III A7.4.3.4/02

5.4.3 Algae and aquatic plants

5.4.3.1 Short- and long- term toxicity to algae

In a growth inhibition test, cultures of the green algae *Selenastrum capricornotum* were exposed to Piperonyl Butoxide for 72 hours under static conditions (Mattock, 2002). An initial concentration of 10^4 algal cells/mL from a semi-continuous liquid stock culture was exposed to Piperonyl Butoxide at nominal concentrations of 0.45, 0.90, 1.8, 3.5, 7.0 and 14.3 mg a.s./L. Algal biomass determinations were conducted at approximately 24-hour intervals after the start of incubation through cell counting by using an electronic particle counter. Analytical determinations of Piperonyl Butoxide concentrations were conducted at the beginning (0h) and at the end (72h) of the test. For the treated groups of nominal Piperonyl Butoxide concentrations of 0.45, 0.90, 1.8, 3.5, 7.0 and 14.3 mg a.s./L the mean measured exposure concentrations were 0.166, 0.379, 0.824, 1.78, 4.40 and 6.87 mg Piperonyl Butoxide/L, respectively. Since the mean measured concentrations were not maintained within ±20% of nominal concentrations, effect data were calculated on the basis of mean measured concentrations. Statistical analysis of the study results demonstrated that the 72-hour E_rC_{50} of Piperonyl Butoxide to *Selenastrum capricornotum* was 3.89 mg a.s./L, while the E_bC_{50} was 2.09 mg a.s./L. Based on the growth rate inhibition, the no observed effect concentration (NOEC) of Piperonyl Butoxide to *Selenastrum capricornotum* was 0.824 mg a.s./L. The study results are presented in the following table.

It is noted that an additional algae growth inhibition test with Piperonyl Butoxide was available (Voigt, 1990). Although the study followed in general the procedures described in the OECD testing guideline 201 and fulfilled the corresponding performance validity criteria, a number of deviations have been identified. More specifically, i) no analytical determination of test substance during the test was performed; therefore effect endpoints were based on nominal concentrations, ii) test conditions (temperature, pH, dissolved oxygen) were not reported, iii) test medium composition was not described, iv) test substance specification and purity was not stated. Based on the above-mentioned deviations, the study was invalidated by the DS and the respective results were not considered in the classification of Piperonyl Butoxide.

Table 87: Growth inhibition of Piperonyl Butoxide on algae

Guideline/ Test method/ GLP status	Species	Endpoint / Type of test	Exposure		Results mg a.i./L			Remarks	Reference
			Design	Duration	NOEC	E_bC_{50}[1]	E_rC_{50}[2]		
OECD Testing Guideline 201; GLP study	*Selenastrum capricornutum*	Growth and biomass inhibition	static	72 hours	0.824	2.09	3.89	Results were based on mean measured concentrations Klimisch score: 1	Mattock (2002) CAR Doc IIIA A7.4.1.3/01

[1] calculated from the area under the growth curve; [2] calculated from growth rate

5.4.3.2 Short- and long- term toxicity to aquatic plants

Not data available.

5.4.4 Other aquatic organisms (including sediment)

5.4.4.1 Short-term toxicity to other aquatic organisms (including sediment)

Not data available.

5.4.4.2 Long-term toxicity to other aquatic organisms (including sediment)

In addition to the acute and long-term toxicity studies summarized above, the toxicity of Piperonyl Butoxide to aquatic organisms was investigated in a GLP water spiked study with *Chironomus riparius* (Stäbler, 2006).

Chironomus riparius first-instar larvae were exposed for 28 days to Piperonyl Butoxide at nominal concentrations of 0.063, 0.100, 0.160, 0.256, 0.410, 0.655, and 1.05 mg/L. One hundred first instar larvae divided into 4 replicates of 25 animals each were used for each treatment level and the control group. Effects on emergence rate and development rate were observed daily (during the period of expected emergence) or at least three times per week. Test item concentrations were verified by analysis of overlying water, sediment and pore water 0, 7 and 28 days after start initiation in the lowest (0.063 mg/L) and highest (1.05 mg/L) treatment levels.

The total amount (mg) of Piperonyl Butoxide in the whole test system (water column + sediment + pore-water) exposed to 0.063 mg a.s./L was 117%, 88.4% and 68.8% of nominal 0, 7 and 28 DAA, respectively. The total amount (mg) of Piperonyl Butoxide in the whole test system (water column + sediment + pore-water) exposed to 1.05 mg a.s./L was 97.8%, 87.7% and 75.4% of nominal 0, 7 and 28 DAA, respectively. The analytical data obtained show a transfer of the test item to the sediment and some degradation during the test. In fact, the test item concentrations were not maintained satisfactorily (i.e. ±20% of nominal) throughout the test duration (28 days) in both treatment levels analysed. Thus, in line with the recommendations given in the OECD Guidance Document No. 23 (2000) on aquatic toxicity testing of difficult substances and mixtures, the effect endpoints were calculated and expressed relative to the time-weighted geometric mean of measured concentrations.

The duration of the *Chironomus riparius* test was long enough in order to enable an assessment of all potential routes of exposure (water, sediment and food in the sediment) to Piperonyl Butoxide. The relative importance of each exposure route, and the time taken for each to contribute to the overall toxic effects, was dependent on the physical-chemical properties of the test substance. Taking into account that the concentration of Piperonyl Butoxide was measured in the sediment during the study and that the test substance was relocated in the sediment over time, the calculation of a sediment-based NOEC in addition to the water-based NOEC endpoint is acceptable.

Statistical analysis of the study results demonstrated that the 28-day NOEC of Piperonyl Butoxide for *Chironomus riparius* should be set at the nominal concentration of 0.063 mg/L based on statistically significant effects on the development rate of *C. riparius* midges exposed to higher treatment levels. Thus, the 28-day water-based and 28-day sediment-based NOEC were calculated to be 0.0148 mg/L and 0.0933 mg/kg dwt, respectively, based on time-weighted geometric mean measured concentrations. The study is summarized in the following table.

Table 88: Effects of Piperonyl Butoxide to sediment-dwelling organisms exposed via spiked water

Guideline/ Test method/ GLP status	Species	Endpoint / Type of test	Exposure Design	Exposure Duration	NOEC mg/L	NOEC mg/kg	Remarks	Reference
OECD Testing Guideline 219; GLP study	*Chironomus riparius*	Development and emergence rate of midges / long-term toxicity test	Spiked water; static	28 days	0.0148	0.0933	Results were based on TWA geometric mean measured concentrations Klimisch score: 1	Stäbler (2006) CAR Doc III A7.4.3.5.1

5.5 Comparison with criteria for environmental hazards (sections 5.1 – 5.4)

The acute (short-term) toxicity of Piperonyl Butoxide to aquatic organisms was investigated with fish, aquatic invertebrates (including daphnia, mysid shrimp and eastern oyster) and algae. The most sensitive species to Piperonyl Butoxide under acute exposure conditions was the eastern oyster *Crassostrea virginica* with a 96-hour EC_{50} of 0.23 mg Piperonyl Butoxide/L.

According to CLP criteria, classification as Acute 1 is warranted when the $L(E)C_{50}$ of most sensitive trophic level is below 1 mg/L. Since Piperonyl Butoxide $L(E)C_{50}$ for the most sensitive trophic level is in the range 0.1 to 1 mg/L, the appropriate multiplying factor (M-factor) is 1.

The long-term toxicity of Piperonyl Butoxide to aquatic organisms was investigated with fish, aquatic invertebrates (*Daphnia magna*), aquatic insects (*Chironomus riparius*) and algae. The most sensitive species to Piperonyl Butoxide under long-term exposure conditions was *Daphnia magna* with a 21-day NOEC of 0.030 mg Piperonyl Butoxide/L 28-day. *Chironomus riparius* NOEC of 0.0148 mg Piperonyl Butoxide/L is not taken into consideration for classification since in the Guidance on the application of CLP criteria (version 5.0 – July 2017) it is stated that "The classification scheme is limited in scope in that it does not, as yet, include aquatic sediments, nor higher organisms at the top end of the aquatic food-chain, although these may to some extent be covered by the criteria selected".

According to CLP criteria, classification as Chronic 1 is warranted for non-rapidly degradable substances when the NOEC or EC_x of most sensitive trophic level is below 0.1 mg/L. Since Piperonyl Butoxide is not readily biodegradable and the NOEC for the most sensitive trophic level is in the range 0.01 to 0.1 mg/L, the appropriate multiplying factor (M-factors) is 1.

5.6 Conclusions on classification and labelling for environmental hazards (sections 5.1 – 5.4)

In accordance with the classification criteria outlined in the Regulation (EC) No 1272/2008 and taking into account the available information (e.g. critical acute and chronic toxicity endpoints, low bioaccumulation potential in fish and other aquatic organisms, the fact that the active substance is not readily biodegradable), Piperonyl Butoxide should be classified as follows:

Acute (short-term) aquatic hazard: Aquatic Acute Category 1 (H400: Very toxic to aquatic life) with an M-factor of 1

Chronic (long-term) aquatic hazard: Aquatic Chronic Category 1 (H410: Vey toxic to aquatic life with long lasting effects) with an M-factor of 1

In addition, the environmental hazard pictogram GHS09, the signal word "Warning" and the precautionary statements P273 (prevention), P391 (response) and P501 (disposal) are required.

RAC evaluation of aquatic hazards (acute and chronic)

Summary of the Dossier Submitter's proposal

Piperonyl butoxide (PBO) is a synergist and biocidal-active substance in the scope of the Biocidal Product Regulation (EC 528/2012). PBO has not previously been reviewed for harmonised classification and labelling and does not have an existing entry in Annex VI of Table 3.1 of CLP.

DS proposal: **Aquatic Acute 1 (H400) with an M-factor of 1** based on the acute toxicity to eastern oyster *Crassostrea virginica* (**96 h EC50 of 0.23 mg/L**), and **Aquatic Chronic 1 (H410) with an M-factor of 1**, based on the **21-day NOEC of 0.030 mg/L** to *Daphnia Magna* and available information for low potential for bioaccumulation and not rapidly biodegradable.

Degradation

Abiotic degradation

Hydrolysis

The hydrolytic stability of PBO was studied in aqueous buffer solutions at pH 5, 7 and 9 under aseptic conditions, in the dark. PBO with nominal initial concentration of 1 mg/L was labelled with ^{14}C in its phenyl ring and incubated at 25 °C. The degradation products were determined by HPLC. The remaining amount of PBO was 98.6% and 97.2% at pH 5, 97.6% and 97.0% at pH 7 and 96.1% and 97.6% at pH 9, at the end of the study (as determined by HPLC). The calculated DT_{50} of hydrolysis was greater than 500 days.

Photo transformation in water

Direct photochemical degradation of radiolabelled ^{14}C-PBO, exposed to a natural sunlight was investigated at 25 °C and pH 7. After 36 hours of exposure to sunlight less than 10% of the total radioactivity in the sample was ^{14}C-PBO. The photolysis of PBO followed first order kinetic and two major degradants were observed at concentrations greater than 10% of the total radioactivity. One degradant was identified as an alcohol degradant of PBO, the other was identified as the corresponding aldehyde of the alcohol degradant.

Photo transformation in air

The photochemical degradation of PBO in air was modelled using the model AOPWIN (version 1.80). Half-life in the troposphere was calculated to be 3.6 hours for overall OH rate constant.

Biodegradation

The ready biodegradability of PBO was investigated over a period of 28 days in the CO_2 evolution test, according to OECD TG 301. The extent of biodegradation was determined by expressing the cumulative recovered yield as a percentage of the theoretical (165 mg CO_2), calculated from the carbon content of the test substance. The CO_2 yield found after 28 days was 24%. DS concluded that pass levels (60% of theoretical oxygen demand) were not reached within a 10-d window.

Biodegradation of PBO has also been tested in two different water/sediment systems (pond and creek) under aerobic conditions, at 20 ºC, in the dark, according to OECD TG 308. The concentration of PBO decreased in the water phase and increased in sediment. Several metabolites were identified using HPLC. The mean disappearance time of PBO in the whole water/sediment system was determined to be 55 days (DT_{50}) and 181 days (DT_{90}), respectively.

Furthermore, aerobic soil metabolism of PBO studied by radioactivity measurements, HPLC and ESI MS identified several degradation products and showed DT_{50} values between 14 and 34.9 days.

Overall, the DS considered the substance as not rapidly degradable, based on a weight-of-evidence approach.

Aquatic Bioaccumulation

PBO has a log K_{OW} of 4.8, thus potential for aquatic bioaccumulation cannot be excluded.

Results of a GLP bioaccumulation study (OECD TG 305) with Bluegill sunfish (*Lepomis macrochirus*) at an average exposure concentration of 0.1 ppm of radiolabelled and non-radiolabelled PBO have been presented. The test fish were maintained under flow-through conditions and exposed to the above-mentioned nominal concentration for 28 days, followed by a 14 days depuration phase. The levels of PBO and degradants in water and fish tissues (sacrificed five fish) were determined by HPLC and liquid scintillation counting on days 0, 3, 7, 14, 21 and 28 of exposure and days 1, 3, 7, 10 and 14 of depuration phase. Steady state BCF (BCFSS) values in edible tissues (191 L/kg), non-edible tissues (380 L/kg) and whole body (260 L/kg) were calculated based on residue data in fish and water.

In addition, kinetic (mean) BCF values in edible (99 L/kg), non-edible (450 L/kg) and whole fish (290 L/kg) were calculated from the uptake and depuration rate constants using the BIOFAC© computer model. The estimated times to reach 50% depuration were 0.67, 1.6 and 1.3 days for edible tissues, non-edible tissues and whole fish respectively, while estimated times to reach 90% depuration were 2.2, 5.2 and 4.2 for edible tissues, non-edible tissues and whole fish respectively. The steady state has not been reached by day 28 of exposure, hence the estimated kinetic BCF values were considered more reliable to address the bioaccumulation potential of PBO in fish. The calculated BCF values were not lipid-normalized and growth dilution correction of the kinetic BCF value was not performed. The experimentally determined kinetic BCF of 290 L/kg (whole fish) is lower than the trigger value of 500 L/kg (criterion for bioaccumulation potential, Regulation EC 1272/2008).

The DS considered PBO as unlikely to bioaccumulate in fish or other aquatic food webs and that it can be regarded as a non bioaccumulative substance.

Aquatic toxicity

Acute aquatic toxicity

Table: *Summary of relevant information on acute aquatic toxicity of PBO*

Method	Results			Remarks	Reference
Fish: Sheepshead minnow *Cyprinodon variegatus* (96 hours; flow-through system) EPA, Subdivision E, Series 72-3; consistent with the OECD Testing Guideline 203; GLP study	**LC_0 (mg/L)** 2.97	**LC_{50} (mg/L)** **3.94**	**LC_{100} (mg/L)** ≥ 5.24	Results were based on mean measured concentrations Klimisch score: 1	Anonymous - 28 (1992b) CAR Doc III A7.4.1.1/01
Fish: Bluegill sunfish *Lepomis macrochirus* (96 hours; flow-through system) EPA, Subdivision E, Series 72-1; consistent with the OECD Testing Guideline 203; GLP study	**LC_0 (mg/L)** 2.34	**LC_{50} (mg/L)** 5.37	**LC_{100} (mg/L)** ≥ 6.94	Results were based on mean measured concentrations Klimisch score: 1	Anonymous - 29 (1992c) CAR Doc III A7.4.1.1/02
Fish: Rainbow trout *Oncorhynchus mykiss* (96 hours; flow-through system) EPA, Subdivision E, Series 72; consistent with the OECD Testing Guideline 203; GLP study	**LC_0 (mg/L)** 3.71	**LC_{50} (mg/L)** 6.12	**LC_{100} (mg/L)** ≥ 8.00	Results were based on mean measured concentrations Klimisch score: 1	Anonymous - 26 (1992a) CAR Doc III A7.4.1.1/03
Aquatic invertebrates: *Daphnia magna* (48 hours; flow-through system) EPA, Subdivision E, 72-2; consistent with the OECD Testing Guideline 202; GLP study	**EC_0 (mg/L)** 0.15	**EC_{50} (mg/L)** 0.51	**EC_{100} (mg/L)** > 0.74	Results were based on mean measured concentrations Klimisch score: 1	Holmes and Smith (1992d) CAR Doc III A7.4.1.2/01
Aquatic invertebrates: *Mysidopsis bahia* (96 hours; flow-through system) EPA, Subdivision E, Series 72-3; consistent with the OPPTS 850.1035 testing guideline; GLP study	**LC_0 (mg/L)** 0.05	**LC_{50} (mg/L)** 0.32	**LC_{100} (mg/L)** > 0.34	Results were based on mean measured concentrations Klimisch score: 1	Holmes and Smith (1992f) CAR Doc III A7.4.1.2/02

Aquatic invertebrates: *Mysidopsis bahia* (96 hours; flow-through system) EPA, Subdivision E, Series 72; consistent with the OPPTS 850.1035 testing guideline; GLP study	**LC_0 (mg/L)**	**LC_{50} (mg/L)**	**LC_{100} (mg/L)**	Results were based on mean measured concentrations Klimisch score: 1	Roberts and Swigert (1995) A7.4.1.2/03
	0.16	0.49	> 0.73		
Aquatic invertebrates: *Crassostrea virginica* (96 hours; flow-through system) EPA, Subdivision E, Series 72; consistent with the OPPTS 850.1025 testing guideline; GLP study	**EC_0 (mg/L)**	**EC_{50} (mg/L)**	**EC_{100} (mg/L)**	Results were based on mean measured concentrations Klimisch score: 1	Holmes and Smith (1992) CAR Doc III A7.4.1.2/04
	< 0.04	**0.23**	> 0.57		

There were three acute toxicity studies available for fish, four for aquatic invertebrates and one for algae. The lowest acute toxicity value for fish was a 96-h LC_{50} value of 3.94 mg/L for *Cyprinodon variegatus*; lowest LC_{50} value for invertebrates was a 96-h EC_{50} value of 0.23 mg/L for *Crassostrea virginica* based on shell growth and, for alga, the lowest E_rC_{50} value was 3.89 mg/L for *Selenastrum capricornutum*.

Based on the lowest value of 0.23 mg/L for *Crassostrea virginica*, the DS proposed a classification of Aquatic Acute category 1, with an M-factor of 1.

- Short-term toxicity to fish

Acute toxicity of PBO to fish was investigated in 3 studies, which can be considered valid and equivalent to OECD TG 203. Three different fish species *Cyprinodon variegatus*, *Lepomis macrochirus*, and *Oncorhynchus mykiss* were exposed to PBO (different nominal concentrations) under flow-through conditions for 96 h. Test design and test performance were similar in the three tests. Ten fish per test concentration level were exposed to a mixture of radiolabelled and non-radiolabelled PBO. The analytical determinations of PBO concentration were conducted at each treatment group every 24 hours using liquid scintillation spectrometry and HPLC. The test results were based on mean measured concentration. The lowest LC_{50} value was 3.94 mg/L for Cyprinodon variegatus.

- Short-term toxicity to aquatic invertebrates

There were four valid acute toxicity studies available for invertebrates, all conducted in a flow-through system, under analytical control of PBO concentrations. The acute toxicity study to *Daphnia magna* was conducted following the procedures described and the recommendations provided in the OECD TG 202 using radiolabelled and non-radiolabelled PBO. The acute toxicity of PBO to the eastern oyster *Crassostrea virginica* was tested in line with the OPPTS 850.1025 testing guideline (Oyster Acute Toxicity Test (Shell Deposition)), fulfilling the corresponding validity criteria. The aim of the study was to determine the PBO concentration inducing 50% reduction in shell growth. Observations of mortality and other clinical signs of toxicity were

made at 24-hour intervals throughout the test. The lowest LC_{50} value was 0.23 mg/L for *Crassostrea virginica* (mean measured) based on reduction in shell growth.

It is noted that toxicity to algae will be presented later on in the ODD.

Chronic aquatic toxicity

Table: *Summary of relevant information on chronic aquatic toxicity of PBO*

Method	Results				Remarks	Reference
Fish: Fathead minnow *Pimephales promelas* (4-day incubation & 31-day post-hatch exposure period; flow-through system) EPA, Subdivision E, Series 72-4; consistent with the OECD testing guideline 210; GLP study	**EC_{50} (mg/L)**	**LOEC (mg/L)**	**NOEC (mg/L)**		Results were based on mean measured concentrations (although they were satisfactorily maintained, e.g. ± 20% of nominal) Klimisch score: 1	Anonymous - 27 (1994) CAR Doc III A7.4.3.2
	-	0.42	**0.18**			
Aquatic invertebrates: *Daphnia magna* (21 days; flow-through system) EPA, Subdivision E, Series 72-4; consistent with the OECD testing guideline 211; GLP study	**EC_{50} (mg/L)**	**LOEC (mg/L)**	**NOEC (mg/L)**		Results were based on mean measured concentrations Klimisch score: 1	Putt (1994) CAR Doc III A7.4.3.4/01
	> 0.65 (parents) 0.21 (offspring)	0.047	**0.030**			
Aquatic invertebrates: *Daphnia magna* (21 days; flow-through system) EPA, Subdivision E, Series 72-4; consistent with the OECD testing guideline 211; GLP study	**EC_{50} (mg/L)**	**LOEC (mg/L)**	**NOEC (mg/L)**		Results were based on mean measured concentrations Klimisch score: 1	Holmes and Smith (1992) CAR Doc III A7.4.3.4/02
	-	0.12	0.066			
Sediment dwelling-organisms: *Chironomus riparius* (28 days; water spiking exposure scenario) OECD Testing Guideline 219; GLP study	**EC_{50} (mg/L)**	**EC_{100} (mg/L)**	**NOEC**		Results were based on geomean measured concentrations Klimisch score: 1	Stäbler (2006) CAR Doc III A7.4.3.5.1
			mg/L	**mg/kg**		
	-	-	**0.0148**	**0.0933**		

There was one chronic aquatic toxicity study available for fish, two for aquatic invertebrates and one for algae. The NOEC found for *Pimephales promelas* was 0.18 mg/L based on mean measured concentration; the lowest chronic toxicity value for invertebrates was a 21-day NOEC (mean measured) of 0.030 mg/L for *Daphnia magna*; the NOEC of PBO to *Selenastrum capricornutum* was 0.824 mg/L.

Based on the lowest value of 0.030 mg/L for *Daphnia magna*, the DS proposed a classification of Aquatic chronic category 1 and an M-factor of 1, for a non-rapidly degrading substance.

- Long-term toxicity to fish

One chronic study was available for fish (*Pimephales promelas*) conducted according to a flow-through test procedure, considered to be equivalent to OECD TG 210. Eggs of fathead minnow were exposed to 0.047, 0.094, 0.19, 0.38 and 0.75 mg/L PBO for 35 days. Observations on embryo hatching as well as on larval survival, growth and behaviour were recorded on a daily basis. Statistical analysis of the study results demonstrated that the 35-day no-observed effect concentration (NOEC) of PBO for *Pimephales promelas* was 0.18 mg/L, based on mean measured concentration.

- Long-term toxicity to aquatic invertebrates

Two GLP, chronic, toxicity studies were available for the aquatic invertebrate *Daphnia magna*. Both studies were conducted under flow-through conditions for 21 days, in accordance with the procedures described and the recommendations provided in the OECD TG 211. Analytical control of PBO concentrations was carried out at least weekly over the test duration period. In the study, survival (mortality) of adult daphnids was determined at least every three days, whilst offspring production was assessed on day 7 and at least three times per week until the test termination. At test termination, the length and weight of each surviving adult daphnid was also measured. The mean measured 21-day NOEC was found to be 0.030 mg/L.

- Short- and long- term toxicity to algae

Table: *Growth inhibition of PBO on algae*

Guideline/ Test method/ GLP status	Species	Endpoint / Type of test	Exposure		Results mg/L			Remarks	Reference
			Design	Duration	NOEC	E_bC_{50}[1]	E_rC_{50}[2]		
OECD TG 201; GLP study	*Selenastrum capricornutum*	Growth and biomass inhibition	static	72 hours	0.82	2.09	3.89	Results were based on mean measured concentrations Klimisch score: 1	Mattock (2002) CAR Doc IIIA A7.4.1.3/01

[1] calculated from the area under the growth curve; [2] calculated from growth rate

One valid study was available for PBO toxicity to algae, in line with OECD TG 201. In this growth inhibition test, cultures of the green algae *Selenastrum capricornutum* (104 algal cells/mL as initial density) were exposed for 72 hours under static conditions to PBO. Algal

biomass determinations were conducted at approximately 24-hour intervals after the start of incubation, while analytical determinations of PBO concentrations were conducted at the beginning (0h) and at the end (72h) of the test. Effect data were calculated on the basis of mean measured concentrations. Statistical analysis of the study results demonstrated that the 72-hour E_rC_{50} of PBO to *Selenastrum capricornutum* was 3.89 mg /L, while the E_bC_{50} was 2.09 mg/L. Based on the growth rate inhibition, the no observed effect concentration (NOEC) of PBO to *Selenastrum capricornutum* was 0.824 mg/L.

Comments received during public consultation

One Member State supported the proposed environmental classification Aquatic Acute 1, H400 (M=1) and Aquatic Chronic 1, (M=1) based on the available data for the most sensitive species.

An Industrial commentator also agreed that the available information justified the proposed classification as Aquatic Acute 1 (H400), acute M-factor = 1, as well as Aquatic Chronic 1 (H410), with a Chronic M-factor of 1.

Assessment and comparison with the classification criteria

Degradation

RAC agrees with the Dossier Submitter to consider PBO as 'not rapidly degradable', due to 24% degradation in 28 days in a ready biodegradability test (OECD TG 301B). Furthermore, biodegradation of PBO has also been tested in two different water/sediment systems (pond and creek) under aerobic conditions, at 20 ºC, in the dark, according to OECD TG 308, with mean disappearance time of PBO in the whole water/sediment system determined to be 55 days (DT_{50}) and 181 days (DT_{90}), respectively.

Bioaccumulation

Based on the value of log Kow of 4.8, bioaccumulation could not be excluded by the DS. However, CLP sets out that an experimentally-determined BCF value provides a better measure for bioaccumulation and shall be used in preference to the Kow, if available. In this case, an experimentally determined whole-fish BCF value of 290 L/kg exists. Hence, RAC agrees with the DS to consider PBO not to possess a potential to bioaccumulate.

Acute aquatic toxicity

There were acute toxicity data available for all three trophic levels. The lowest acute toxicity value was a 96-h LC_{50} of 0.23 mg/L (mean measured) for eastern oyster *Crassostrea virginica*.

Chronic aquatic toxicity

There were chronic toxicity data available for all three trophic levels. The lowest chronic toxicity value was from a 21-day NOEC (mean measured) of 0.030 mg/L for *Daphnia magna*.

In conclusion, RAC agrees with the DS that PBO warrants classification as:

- **Aquatic Acute 1; H400,** with **M = 1** (0,1 < L(E) C50 ≤ 1) **and;**
- **Aquatic Chronic 1; H410, M = 1** (0,01 < NOEC ≤ 0,1).

6 OTHER INFORMATION

No data

7 REFERENCES

Physical and chemical properties and methods of analysis

Author(s)	Year	Title Testing Facility Owner / Source (where different from owner) Report No GLP or GEP status (where relevant) Published or not	Data protection claimed yes/no	Owner
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Mazza, M., Di Blasi, G.	2000a	PHYSICAL-CHEMICAL TESTING WITH PIPERONYL BUTOXIDE - PHYSICAL STATE COLOUR Endura, Fine Chemicals, Bologna, Italy Endura S.p.A Report-no. 01/2000 GLP: no Published: no	yes	END

Author(s)	**Year**	**Title** **Testing Facility** **Owner / Source (where different from owner)** **Report No** **GLP or GEP status (where relevant)** **Published or not**	**Data protection claimed** **yes/no**	**Owner**
Mazza, M., Di Blasi, G.	2000b	PHYSICAL-CHEMICAL TESTING WITH PIPERONYL BUTOXIDE - ODOUR Endura, Fine Chemicals, Bologna, Italy Endura S.p.A Report-no. 02/2000 GLP: no Published: no	yes	END
Mazza, M., Di Blasi, G.	2000c	PHYSICAL-CHEMICAL PROPERTIES OF PIPERONYL BUTOXIDE - SPECTRA OF PURIFIED SUBSTANCE SPECTRA OF POTENTIAL IMPURITIES OF TOXICOLOGICAL CONCERN Endura, Fine Chemicals, Bologna, Italy Endura S.p.A Report-no. 04/2000 GLP: no Published: no	yes	END
Bär, C.	2006a	WATER SOLUBILITY OF PIPERONYL BUTOXIDE GAB Biotechnologie GmbH, Niefern-Öschelbronn, Germany Endura S.p.A Report-no. 20051476/01-PCSB GLP: yes Published: no	yes	END
Bär, C.	2006b	PARTITION COEFFICIENT OF PIPERONYL BUTOXIDE (HPLC METHOD) GAB Biotechnologie GmbH, Niefern-Öschelbronn, Germany Endura S.p.A Report-no. 20051476/01-PCPC GLP: yes Published: no	yes	END
Flack, I.	2000	PIPERONYL BUTOXIDE BOILING TEMPERATURE Huntingdon Life Sciences Limited, Huntingdon, UK Endura S.p.A Report-no. RTS 022/002381 GLP: yes Published: no	yes	END
Smeykal, H.	2004a	PIPERONYL BUTOXIDE - AUTOFLAMMABILITY (DETERMINATION OF THE TEMPERATURE OF SELF-IGNITION OF VOLATILE LIQUIDS AND OF GASES) Sicherheitstechnik Siemens Axiva GmbH & Co. KG, Frankfurt Endura S.p.A Report-no. 20040491.02 GLP: no Published: no	yes	END
Forster, J.	1999	DETERMINATION OF FLASH POINT OF PIPERONYL BUTOXIDE Chemex International plc, Bar Hill, UK Endura S.p.A Report-no. ENV4386 GLP: yes Published: no	yes	END
Forster, J.	2000	DETERMINATION OF THE SURFACE TENSION AND RELATIVE DENSITIY PIPERONYL BUTOXIDE Chemex International plc, Bar Hill, UK	yes	END

Author(s)	**Year**	**Title** **Testing Facility** **Owner / Source (where different from owner)** **Report No** **GLP or GEP status (where relevant)** **Published or not**	**Data protection claimed** **yes/no**	**Owner**
		Endura S.p.A Report-no. ENV4934 GLP: yes Published: no **Submitted in: A3.1.3/01**		
Bär, C.	2006c	VISCOSITY OF PIPERONYL BUTOXIDE GAB Biotechnologie GmbH, Niefern-Öschelbronn, Germany Endura S.p.A Report-no. 20051476/01-PCVC GLP: yes Published: no	yes	END
Smeykal, H.	2004b	PIPERONYL BUTOXIDE - EXPLOSIVE PROPERTIES Sicherheitstechnik Siemens Axiva GmbH & Co.KG Endura S.p.A Report-no. 20040491.01 GLP: no Published: no	yes	END
Tiemann, J.	2004	PIPERONYL BUTOXIDE - POINT 2.15 OXIDIZING PROPERTIES OF THE ACTIVE SUBSTANCE GAB Consulting GmbH, Lamstedt, Germany Endura S.p.A Report-no. 135174-A2-0215-01 GLP: no Published: no	yes	END

END = Endura

Human Health Risk Assessment active substance

Author(s) sanitised	**Year**	**Title** **Testing Facility** **Owner / Source (where different from owner)** **Report No** **GLP or GEP status (where relevant)** **Published or not**	**Data protection claimed** **yes/no**	**Owner**
Fujitani, T., Ando, H., Fujitani, K., Ikeda, T., Kojima, A., Kubo, Y., Ogata, A., Oishi, S., Takahshi, H., Takahashi, O., Yoneyama, M.	1992	SUB-ACUTE TOXICITY OF PIPERONYL BUTOXIDE IN F344 RATS - Toxicology, 1992 (72), 291 - 298 Report-no. not applicable GLP: no Published: yes	no	-
James, J.A.	1995	PIPERONYL BUTOXIDE (PIPERONYL BUTOXIDE) (EU) A REVIEW OF TOXICOLOGY Hoechst Schering AgrEvo GmbH, Frankfurt am Main, Germany Endura S.p.A Report-no. A94952 GLP: no Published: no	yes	END

Author(s) sanitised	**Year**	**Title** **Testing Facility** **Owner / Source (where different from owner)** **Report No** **GLP or GEP status (where relevant)** **Published or not**	**Data protection claimed** **yes/no**	**Owner**
Lake, B.G.	1996	AN INVESTIGATION OF THE EFFECT OF PIPERONYL BUTOXIDE ON UNSCHEDULED DNA SYNTHESIS IN CULTURED HUMAN LIVER SLICES BIBRA International, Surrey, UK Endura S.p.A Report-no. 1501/1/2/96 GLP: yes Published: no	yes	END
Lawlor, T.E.	1991	PIPERONY BUTOXIDE IN THE SALMONELLA/MAMMALIAN-MICROSOME REVERSE MUTATION ASSAY (AMES TEST) WITH A CONFIRMATORY ASSAY Hazleton Laboratories, Kensington, Maryland, USA Endura S.p.A Report-no. 14413-0-401R GLP: yes Published: no	yes	END
McFarlane, M., Cockburn, A.	1998	A WEIGHT OF EVIDENCE EXPERT REPORT REGARDING MOUSE BENIGN HEOATIC PROLIFERATIVE LESION AgrEvo, Hoechts and Schering, Marburg, Germany Endura S.p.A Report-no. A97917 GLP: no Published: no	yes	END
McKeon, M.E.	1991	GENOTOXICITY TEST ON PIPERONYL BUTOXIDE IN THE ASSAY FOR UNSCHEDULED DNA SYNTHESIS IN RAT LIVER PRIMARY CELL CULTURES WITH A CONFIRMATORY ASSAY Hazleton Laboratories, Kensington, Maryland, USA Endura S.p.A Report-no. 14413-0-447R GLP: yes Published: no	yes	END
Moretto, A.	1995	PIPERONYL BUTOXIDE - A MONOGRAPH PREPARED BY THE JOINT FAO/WHO MEETING ON PESTICIDES RESIDUES (GENEVA 1995) Istituto di Medicina del Lavoro, Padua, Italy Joint FAO/WHO Meeting on Pesticides Residues (Geneva 1995) Report-no. not applicable GLP: no Published: yes	no	-
Murli, H.	1991	MEASURING CHROMOSOMAL ABERRATIONS IN CHINESE HAMSTER OVARY (CHO) CELLS WITH MULTIPLE HARVESTS UNDER CONDITIONS OF METABOLIC ACTIVATION Hazleton Laboratories, Kensington, Maryland, USA Endura S.p.A Report-no. 14413-0-437c GLP: yes Published: no	yes	END

Author(s) sanitised	**Year**	**Title** **Testing Facility** **Owner / Source (where different from owner)** **Report No** **GLP or GEP status (where relevant)** **Published or not**	**Data protection claimed** **yes/no**	**Owner**
Phillips, J.C., Price, R.J., Morag, E. Cunninghame, M.E., Osimitz, T.G., Cockburn, A., Gabriel, K.L., Preiss, F.J., Butler W.H., Lake, B.G.	1997	EFFECT OF PIPERONYL BUTOXIDE ON CELL REPLICATION AND XENOBIOTIC METABOLISM IN THE LIVERS OF CD-1 MICE AND F344 RATS - Fundam Appl Toxicol, 38, 64-74 Report-no. not applicable GLP: no Published: yes	no	-
Tagliani, A.	2004	CLINICAL SIGNS AND SYMPTOMS OF POISONING AND DETAILS OF CLINICAL TESTS not applicable Endura S.p.A Report-no. not applicable GLP: no Published: no	yes	END
Takahashi O, Oishi S, Fujitani T, Tanaka T, Yoneyama M.	1994a	CHRONIC TOXICITY STUDY OF PIPERONYL BUTOXIDE IN F344 RATS INDUCTION OF HEPATOCARCINOMA Fundam Appl Toxicol. 1994 Feb;22(2):293-303 GLP: no Published: yes	no	END
Takahashi O, Oishi S, Fujitani T, Tanaka T, Yoneyama M.	1994b	PIPERONYL BUTOXIDE INDUCES HEPATOCELLULAR CARCINOMA IN MALE CD-1 MICE Arch Toxicol. 1994;68(7):467-9 GLP: no Published: yes	no	END
Tu, A.S.	1986	EVALUATION OF PIPERONYL BUTOXIDE IN THE CHO/HGPRT MUTATION ASSAY WITH AND WITHOUT METABOLIC ACTIVATION Arthur D. Little, Inc. Cambridge Endura S.p.A Report-no. 53906 GLP: yes Published: no	yes	END
Wester, R.C., Bucks D.A.W., Maibach H.I.	1994	HUMAN IN VIVO PERCUTANEOUS ABSORPTION OF PYRETHRIN AND PIPERONYL BUTOXIDE - Food Chem Toxicol, 32, 51 - 53 Report-no. Not applicable GLP: no Published: yes	no	-
Anonymous - 1	1991	ABSORPTION, DISTRIBUTION, METABOLISM AND EXCRETION (ADME) STUDIES OF PIPERONYL BUTOXIDE IN THE RAT Biological Test Center, Irvine, CA 92713-9791-USA Endura S.p.A Report-no. P01825 GLP: yes Published: no	yes	END

ANNEX 1 - BACKGROUND DOCUMENT TO RAC OPINION ON PIPERONYL BUTOXIDE (ISO); 2-(2-BUTOXYETHOXY)ETHYL 6-PROPYLPIPERONYL ETHER

Author(s) sanitised	**Year**	**Title** **Testing Facility** **Owner / Source (where different from owner)** **Report No** **GLP or GEP status (where relevant)** **Published or not**	**Data protection claimed** **yes/no**	**Owner**
Anonymous - 2	1999	PIPERONYL BUTOXIDE-[^{14}C]: RAT – METABOLISM FOLLOWING ORAL DOSING AT 50 AND 500 MG/KG BODYWEIGHT Hoechst Schering AgrEvo GmbH, Frankfurt am Main, Germany Endura S.p.A Report-no. TOX/99/280-4 GLP: Yes Published: no	yes	END
Anonymous - 3	1991a	ACUTE ORAL TOXICITY, LD50 - RATS Biosearch Incorporated, Philadelphia, PA 19134 Endura S.p.A Report-no. 91-7317A GLP: yes Published: no	yes	END
Anonymous - 4	1991b	ACUTE DERMAL TOXICITY, SINGLE LEVEL - RABBITS Biosearch Incorporated, Philadelphia, PA 19134 Endura S.p.A Report-no. 91-7317A GLP: yes Published: no	yes	END
Anonymous - 5	1991	AN ACUTE INHALATION TOXICITY STUDY OF PIPERONYL BUTOXIDE IN THE RAT Bio/Dynamics, Inc., New Jersey, USA Endura S.p.A Report-no. 91-8330 GLP: yes Published: no	yes	END
Anonymous - 6	1991a	PRIMARY SKIN IRRITATION - RABBITS Biosearch Incorporated, Philadelphia, PA 19134 , USA Endura S.p.A Report No. 91-7317A GLP: Yes Published: no	yes	END
Anonymous - 7	1991	PRIMARY EYE IRRITATION - RABBITS Biosearch Incorporated, Philadelphia, PA 19134 , USA Endura S.p.A Report No. 91-7317A GLP: Yes Published: no	yes	END
Anonymous - 8	1991b	GUINEA PIG DERMAL SENSITIZATION - MODIFIED BUEHLER METHOD Biosearch Incorporated, Philadelphia, PA 19134 , USA Endura S.p.A Report No. 91-7317A GLP: Yes Published: no	yes	END
Anonymous - 9	1989	MUTAGENICITY TESTS ON PIPERONYL BUTOXIDE Istituto Di Farmacologia Farmacocinetica E Tossicologia Endura S.p.A Report-no. not applicable GLP: no Published: no	yes	END

ANNEX 1 - BACKGROUND DOCUMENT TO RAC OPINION ON PIPERONYL BUTOXIDE (ISO); 2-(2-BUTOXYETHOXY)ETHYL 6-PROPYLPIPERONYL ETHER

Author(s) sanitised	**Year**	**Title** **Testing Facility** **Owner / Source (where different from owner)** **Report No** **GLP or GEP status (where relevant)** **Published or not**	**Data protection claimed** **yes/no**	**Owner**
Anonymous - 10	1987	A 24-MONTH DIETARY TOXICITY AND CARCINOGENICITY STUDY OF PIPERONYL BUTOXIDE IN THE ALBINO RAT Bio Research Laboratories LTD. Montréal, Canada Endura S.p.A Report-no. 81690 GLP: yes Published: no	yes	END
Anonymous - 11	1993	CHRONIC DIETARY ONCOGENICITY STUDY WITH PIPERONYL BUTOXIDE IN CD-1 MICE Bushy Run Research Center (BRRC) Endura S.p.A Report-no. 91N0134 GLP: yes Published: no	yes	END
Anonymous - 12	2012a	PHASE I STUDY: A 14-DAY DIETARY STUDY COMPARING THE HEPATIC EFFECTS OF PIPERONYL BUTOXIDE AND SODIUM PHENOBARBITAL IN MALE CD-1 MICE. Leatherhead Food Research, Surrey, UK. Ref, No: 5491/1. Published: no	yes	END
Anonymous - 13	2012b	PHASE II STUDY: COMPARISON OF THE HEPATIC EFFECT OF PIPERONYL BUTOXIDE IN MALE CONSTITUTIVE ANDROSTANE RECEPTOR (CAR)/PREGNANE X RECEPTOR (PXR) DOUBLE KNOCKOUT AND WILD TYPE C57BL/6J MICE. Leatherhead Food Research, Surrey, UK. Ref, No: 5507/1. Published: no	yes	END
Anonymous - 14	1986	A TWO GENERATION (TWO LITTER)REPRODUCTION STUDY OF PIPERONYL BUTOXIDE ADMINISTERED IN THE DIET TO THE RAT Biosearch Incorporated, Philadelphia, PA 19134 , USA Endura S.p.A Report No. 91689 GLP: Yes Published: no	yes	END
Anonymous - 15	1991	DEVELOPMENTAL TOXICITY EVALUATION OF PIPERONYL BUTOXIDE ADMINISTERED BY GAVAGE TO CD (SPRAGUE-DAWLEY) RATS Endura S.p.A Report No. 54-586 GLP: Yes Published: no	yes	END
Anonymous - 16	1986	TERATOLOGY STUDY IN RABBITS International Research and Development Corp. Mattawan, Michigan 49071, USA Endura S.p.A Report No. 542-002 GLP: Yes Published: no	yes	END

ANNEX 1 - BACKGROUND DOCUMENT TO RAC OPINION ON PIPERONYL BUTOXIDE (ISO); 2-(2-BUTOXYETHOXY)ETHYL 6-PROPYLPIPERONYL ETHER

Author(s) sanitised	**Year**	**Title** **Testing Facility** **Owner / Source (where different from owner)** **Report No** **GLP or GEP status (where relevant)** **Published or not**	**Data protection claimed** **yes/no**	**Owner**
Anonymous - 17	1992	A SUBCHRONIC (3-MONTH) INHALATION TOXICITY STUDY OF PIPERONYL BUTOXIDE IN THE RAT VIA WHOLE-BODY EXPOSURES Bio/Dynamics, Inc., New Jersey, USA Endura S.p.A Report-no. 91-8333 GLP: yes Published: no	yes	END
Anonymous - 18	1993a	EVALUATION OF PIPERONYL BUTOXIDE IN AN EIGHT WEEK TOXICITY STUDY IN DOGS Int. Res.and Develop.Corp. Mattawan, Michigan 49071, USA Endura S.p.A Report-no. 542-004 GLP: yes Published: no	yes	END
Anonymous - 19	1993b	EVALUATION OF PIPERONYL BUTOXIDE IN A ONE YEAR CHRONIC DIETARY TOXICITY STUDY IN DOGS Int. Res.and Develop.Corp. Mattawan, Michigan 49071, USA Endura S.p.A Report-no. 542-005 GLP: yes Published: no	yes	END
Anonymous - 20	1993	NINETY DAY DIETARY RANGE FINDING STUDY WITH PIPERONYL BUTOXIDE IN MICE Bushy Run Research Center, Union Carbide, Export, Pennsylvania, USA Endura S.p.A Report No. 91N0052 GLP: Yes Published: no	yes	END
Anonymous - 21	1992	21-DAY REPEATED DOSE DERMAL TOXICITY STUDY WITH PIPERONYL BUTOXIDE IN RABBITS Int. Res.and Develop.Corp. Mattawan, Michigan 49071, USA Endura S.p.A Report-no. 542-007 GLP: yes Published: no	yes	END
Anonymous - 22	2014	AN ACUTE NEUROTOXICITY STUDY OF PIPERONYL BUTOXIDE IN RATS BY ORAL (GAVAGE) ADMINISTRATION. Charles River Laboratories, Horsham, PA, USA Endura S.p.A Report No. 20046982. GLP: Yes Published: no	yes	END

END = Endura

Environmental Risk Assessment

Author(s)	Year	Title Testing Facility Owner / Source (where different from owner) Report No GLP or GEP status (where relevant) Published or not	Data protection claimed yes/no	Owner
Kirkpatrick, D.	1995	Piperonyl Butoxide Hydrolysis as a Function of pH at 25°C Huntingdon Research Centre Ltd., Huntingdon, UK Endura S.p.A Report-no. PBT 4/943285 GLP: no Published: no	yes	END
Selim, S.	1995	Isolation and Identification of Major Degradates of Piperonyl Butoxide (Piperonyl Butoxide) following aqueous photolysis Biological Test Center, Irvine, CA 92713-9791-USA Endura S.p.A Report-no. P0594010 GLP: no Published: no	no	END
Derz, K.		Aerobic transformation of Piperonylbutoxide (Piperonyl Butoxide) in water/sediment systems (OECD 308) Fraunhofer Institut, Schmallenberg-Grafschaft, Germany Endura S.p.A Report-no. GAB-011/7-92 GLP: yes Published: no	yes	END
Williams, M.D.	1991c	Aerobic Aquatic Metabolism of Piperonyl Butoxide ABC Laboratories, Columbia, Missouri, USA Endura S.p.A Report-no. 38549 GLP: yes Published: no	yes	END
Williams, M.D.	1991d	Anaerobic Aquatic Metabolism of Piperonyl Butoxide ABC Laboratories, Columbia, Missouri, USA Endura S.p.A Report-no. 38507 GLP: yes Published: no	yes	END
Daly, D.	1991	Soil/Sediment Adsorption-Desorption of Piperonyl Butoxide ABC Laboratories, Columbia, Missouri, USA Endura S.p.A Report-no. 38360 GLP: yes Published: no	yes	END
Elsom, L.F.	1995b	14C-Piperonyl Butoxide Adsorption/Desorption on Soil Huntingdon Life Sciences Limited, Huntingdon, UK Endura S.p.A Report-no. PBT 10A/950775 GLP: yes Published: no	yes	END
Mayo, B.C.	1995a	Piperonyl Butoxide Aerobic Soil Metabolism Huntingdon Life Sciences Limited, Huntingdon, UK Endura S.p.A	yes	END

		Report-no. PBT 7/951484 GLP: yes Published: no		
Derz, K.		Degradation rates of Piperonyl Butoxide and selected metabolites in soil under aerobic conditions Fraunhofer Institut, Schmallenberg-Grafschaft, Germany Endura S.p.A Report-no. GAB-011/7-90 GLP: yes Published: no	yes	END
Williams, M.D.	1991f	Anaerobic Soil Metabolism of Piperonyl Butoxide ABC Laboratories, Columbia, Missouri, USA Endura S.p.A Report-no. 38585 GLP: yes Published: no	yes	END
Elsom, L.F.	1995c	14C-Piperonyl Butoxide Soil Column Leaching of non-Aged and Aged Residues of 14C-Piperonyl Butoxide Huntingdon Life Sciences Limited, Huntingdon, UK Endura S.p.A Report-no. PBT 10B/950899 GLP: yes Published: no	yes	END
Bosse, D.	1999	Substance Piperonyl Butoxide - Calculation of the Indirect Photolysis Reaction Using the Incremental Method af Atkinson and the Program AOPWIN, Version 1.80 Hoechst AG, Frankfurt am Main, Germany Endura S.p.A Report-no. 99.0001 GLP: no Published: no	yes	END
Bealing, D.J.	2002	Piperonyl Butoxide: Determination of inhibition of respiration of activated sludge Covance Laboratories Ltd., Harrogate, UK Endura S.p.A Report-no. 2145/2-D2149 GLP: no Published: no	yes	END
Holmes, C.M., Smith, G.J.	1992d	A 48-hour flow-through acute toxicity test with Piperonyl Butoxide in daphnids (*Daphnia magna*). Wildlife International, Ltd., Easton, Maryland, USA Endura S.p.A Report-no. 306A-105A GLP: yes Published: no	yes	END
Holmes, C.M., Smith, G.J.	1992f	A 96-hour flow-through acute toxicity test with Piperonyl Butoxide in mysid shrimp (*Mysidopsis bahia*). Wildlife International, Ltd., Easton, Maryland, USA Endura S.p.A Report-no. 306A-109 GLP: yes Published: no	no	END
Roberts, C.A., Swigert, J.P.	1995	Piperonyl Butoxide : A 96-hour flow-through acute toxicity test with the saltwater mysid (*Mysidopsis bahia*). Wildlife International, Ltd., Easton, Maryland, USA Endura S.p.A Report-no. 306A-111A GLP: yes Published: no	To be confirmed by the Industry	END

Holmes, C.M., Smith, G.J.	1992	A 96-hour shell deposition test with Piperonyl Butoxide in the eastern oyster (*Crassostrea virginica*). Wildlife International, Ltd., Easton, Maryland, USA Endura S.p.A Report-no. 306A-110A GLP: yes Published: no	To be confirmed by the Industry	END
Mattock S.D.	2002	Piperonyl Butoxide: inhibition of growth the alga *Selenastrum capricornutum.* Covance Laboratories Ltd., Harrogate, UK Endura S.p.A Report-no. 2145/1-D2149 GLP: yes Published: no	yes	END
Putt, A.E.	1994	Piperonyl Butoxide technical task force blend PB200 - the chronic toxicity to *Daphnia magna* under flow-through conditions Spingborn Lab. Inc., Wareham, Massachusetts 02571, USA Endura S.p.A Report-no. 94-5-5270 GLP: yes Published: no	yes	END
Holmes, C.M., Smith, G.J.	1992	A flow-through life-cycle toxicity test with Piperonyl Butoxide in daphnids (*Daphnia magna*). Wildlife International, Ltd., Easton, Maryland, USA Endura S.p.A Report-no.: 306A-104 GLP: yes Published: no	To be confirmed by the Industry	END
Stäbler, D.	2006	Assessment of side effects of Piperonyl Butoxide on the larvae of the midge, *Chironomus riparius* with the laboratory test method GAB Biotechnologie GmbH, Niefern-Öschelbronn, Germany Endura S.p.A Report-no. 20051329/01-ASCr GLP: yes Published: no	yes	END
Anonymous - 23	1992	A bioconcentration study with Piperonyl Butoxide in the bluegill (Leponis macrochirus) Wildlife International, Ltd., Easton, Maryland, USA Endura S.p.A Report-no. 306A-102 GLP: yes Published: no	yes	END
Anonymous - 24	1992b	A 96-hour flow-through acute toxicity test with Piperonyl Butoxide in sheepshead minnow (Cyprinodon variegatus). Wildlife International, Ltd., Easton, Maryland, USA Endura S.p.A Report-no. 306A-108A GLP: yes Published: no	yes	END
Anonymous - 25	1992c	A 96-hour flow-through acute toxicity test with Piperonyl Butoxide in the bluegill (Lepomis macrochirus). Wildlife International, Ltd., Easton, Maryland, USA Endura S.p.A Report-no. 306A-101 GLP: yes Published: no	yes	END

Anonymous - 26.	1992a	A 96-hour flow-through acute toxicity test with Piperonyl Butoxide in the rainbow trout (Oncorhynchus mykiss). Wildlife International, Ltd., Easton, Maryland, USA Endura S.p.A Report-no. 306A-106 GLP: yes Published: no	To be confirmed by the Industry	END
Anonymous - 27	1994	Piperonyl Butoxide technical, task force blend pb200 - the toxicity to fathead minnow (Pimephales promelas) during an early life stage exposure. Spingborn Lab. Inc., Wareham, Massachusetts 02571, USA Endura S.p.A Report-no. 94-5-5264 GLP: yes Published: no	yes	END

Additional references

Kawai *et al.* Mechanistic study on hepatocarcinogenesis of piperonyl butoxide in mice. Toxicol Pathol. 2009 Oct;37(6):761-9. doi: 10.1177/0192623309344087

Matsushita *et al.* Development of a Medium-term Animal Model Using gpt Delta Rats to Evaluate Chemical Carcinogenicity and Genotoxicity J Toxicol Pathol. 2013 Mar;26(1):19-27. doi: 10.1293/tox.26.19.

8 APPENDIX

Piperonyl Butoxide Literature Review on Human Health Risk Assessment

(conducted by the eCA in the role of the evaluating Competent Authority for Piperonyl Butoxide in the frame of Reg. (EC) No. 528/2012)

This review is not exhaustive; it includes studies on toxicological aspects (i.e. immunotoxicity, neurotoxicity and human biomonitoring) not addressed in the CAR and for which there are open literature data available.

Immunotoxicity

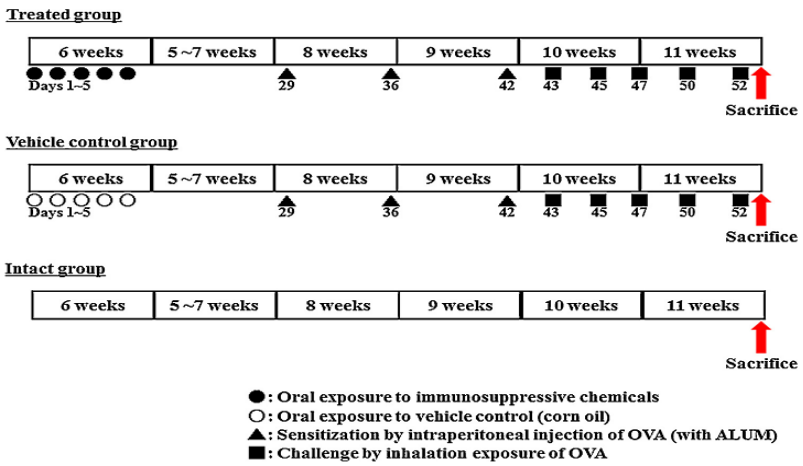
Nishino et al, Toxicology; 2013 Jul 5; 309: 1-8. "Prior oral exposure to environmental immunosuppressive chemicals methoxychlor, parathion, or piperonyl butoxide aggravates allergic airway inflammation in NC/Nga mice".

The association between environmental immunosuppressive chemicals (among which Piperonyl Butoxide is reported) and the allergic airway inflammation development was investigated. The prior information upon which the characterization of Piperonyl Butoxide as immunosuppressive substance was based is the following:

- piperonyl butoxide administration depletes T-cells in the spleen and thymus, induces hypoplasia of the bone marrow, and inhibits T-cells proliferation in lymphoid tissues (Mitsumori *et al*., 1996; Diel *et al*., 1999; Battaglia *et al*., 2010).
- piperonyl butoxide exposure results in an increase in thymocyte apoptosis in vitro, and markedly inhibits sheep red blood cells (SRBC)-specific IgM production in mice (study conducted by the same research group as the present study; Fukuyama *et al*., 2013; See presentation of the study at point 3 below).

STUDY DESIGN: The study used a mouse model of ovalbumin (OVA)-induced allergic airway inflammation. NC/Nga mice were exposed orally to pesticides parathion (an organophosphate compound) or methoxychlor (an organochlorine compound), or to an insecticide synergist piperonyl butoxide, prior to OVA intraperitoneal sensitization and inhalation challenge. The following parameters were assessed: Serum IgE levels, B-cell counts, cytokine production, IgE production in hilar lymph nodes; eosinophil counts, chemokine levels in bronchoalveolar lavage fluid; and cytokine gene expression in the lung.

The detailed experimental protocol is illustrated below:



OVA, Ovalbumin; ALUM, aluminum hydroxide hydrate gel suspension.

RESULTS: Exposure to environmental immunosuppressive chemicals (including Piperonyl Butoxide at dose of 300mg/kg bw/day) markedly increased serum IgE - IgE-positive B-cells, IgE and cytokines in lymph nodes - eosinophils and chemokines in BALF - IL-10a and IL-17 in the lung.

Conclusion: Allergic airway inflammation can be aggravated by prior exposure to Piperonyl Butoxide.

Fukuyama et al, "Role of regulatory T cells in the induction of atopic dermatitis by immunosuppressive chemicals".Toxicol Lett. 2012 Sep 18;213(3):392-401.

Based on the abstract of the study, the effects of the immunosuppressive environmental chemicals (including Piperonyl Butoxide) on picryl-chloride-induced AD (atopic dermatitis) in NC/Nga mice, was examined. Mice were orally exposed (age: 5 weeks) to these chemicals; during their sensitization and challenge (age: 8-12 weeks) with picryl chloride, ear thickness and scored skin dryness, erythema, edema, and wounding were measured. After the challenge, the following parameters were analyzed: dermatitis severity and cytokine gene expression in the pinna, serum levels of IgE and IgG2a, T- and B-cell numbers and cytokine production in auricular lymph nodes, and counted splenic regulatory T cells. Exposure to Piperonyl Butoxide markedly increased dermatitis severity and gene expression in the pinna; serum IgE and IgG2a levels; and numbers of helper T cells and IgE-positive B cells, production of Th1 and Th2 cytokines, and production of IgE in auricular lymph-node cells and markedly decreased the numbers of splenic regulatory T cells.

Conclusion: Prior exposure to Piperonyl Butoxide aggravates AD; a decrease in the numbers of regulatory T cells may influence this process.

Fukuyama T et al, J Immunotoxicol. 2013 Apr-Jun;10(2):150-9. "Immunotoxicity in mice induced by short-term exposure to methoxychlor, parathion, or piperonyl butoxide".

Based on the abstract, in order to assess the immunosuppressive response to short-term exposure to some commonly used pesticides, the authors were focused on the investigation of the response of mice after exposures to the organochlorine pesticide methoxychlor, the organophosphorus pesticide parathion, or the agricultural insecticide synergist Piperonyl Butoxide. According to the study design, 7-week-old mice were orally administered (by gavage) methoxychlor, parathion, or Piperonyl Butoxide daily for five consecutive days. On Day 2, all mice in each group were immunized with sheep red blood cells (SRBC), and their SRBC-specific IgM responses were subsequently assessed. In addition, levels of B-cells in the spleen of each mouse were also analyzed via surface antigen expression. The results of these studies on Piperonyl Butoxide indicated that treatments with Piperonyl Butoxide induced marked decreases in the production of SRBC-specific IgM antibodies as well as in the expression of surface antigens in IgM- and germinal center-positive B-cells. Based on these outcomes, it is concluded that the short-term exposure protocol was able to detect potential immunosuppressive responses to piperonyl butoxide *in situ*.

Inflamm Res. 2003 Apr;52(4):154-63; Pyrethroid insecticides influence the signal transduction in T helper lymphocytes from atopic and nonatopic subjects; Diel F, Horr B, Borck H, Irman-Florjanc T.

OBJECTIVE AND DESIGN: Pyrethroids are claimed to have a low human toxicity with some neuro- and immunotoxicity. The objective of this study was to investigate the immunotoxicological

properties of six commercially used pyrethroids, including natural pyrethrum and synergist Piperonyl Butoxide.

MATERIAL AND METHODS: PHA-stimulated cultures of T-helper lymphocytes and blood basophil incubates from non-atopic and atopic patients (IgE > 1000 IU) provided cytokine and histamine determination. Western blot analysis was used for the measurement of Th2-specific signal transducer and activator of transcription-6 (STAT6). Pyrethroids and xenobiotics were added 4 h post-plating.

RESULTS: Regarding the Piperonyl Butoxide effects, the pyrethroids fenvalerate and S-bioallethrin combined with 10-fold Piperonyl Butoxide in the atopic-enriched blood basophil incubates, caused a weak but significant increase in histamine release.

Neurotoxicity

A. Neurobehavioral effects during development

Toxicol Ind Health. 2009 Aug;25(7):489-97; Effects of piperonyl butoxide on spontaneous behavior in F1-generation mice; Tanaka T, Takahashi O, Oishi S, Ogata A. (Department of Environmental Health and Toxicology, Tokyo Metropolitan Institute of Public Health).

Piperonyl Butoxide was given in the diet to provide levels of 0 (control), 0.02%, 0.06%, and 0.18% from 5 weeks of age of the F(0) generation to 12 weeks of age of the F(1) generation in mice. Select reproductive and neurobehavioral parameters were then measured. In exploratory behaviour in the F(0) generation, vertical time of adult females increased significantly in a dose-related manner. In behavioural developmental parameters, cliff avoidance was delayed significantly in the high-dose group in male offspring, and this effect was significantly dose-related. In female offspring, surface righting was significantly delayed in the high-dose group, and this effect was significantly dose-related. In spontaneous behaviour in the F(1) generation, females showed more activities in some variables in the high-dose group. Conclusion: Dose levels of piperonyl butoxide used in the present study produced several adverse effects in neurobehavioral parameters in mice.

Food Addit Contam. 2003 Mar;20(3):207-14; Reproductive and neurobehavioural effects of piperonyl butoxide administered to mice in the diet; Tanaka T (Department of Toxicology, Tokyo Metropolitan Research Laboratory of Public Health).

Piperonyl Butoxide was given in the diet to mice to provide levels of 0 (control), 0.01, 0.03 and 0.09% from 5 weeks of age of the F(0) generation to 9 weeks of age of the F(1) generation, and selected reproductive and neurobehavioural parameters were measured. There were no adverse effects of piperonyl butoxide on either litter size, litter weight or sex ratio at birth. The average body weight of male offspring was significantly increased in the middle-dose group at post-natal days 4 and 7 during lactation. That of female offspring was significantly increased in the middle-dose group at post-natal days 7 and 14 during lactation. In behavioural developmental parameters, surface righting at post-natal day 7 was significantly delayed in the higher-dose groups in male offspring, and those effects were significantly dose related ($p < 0.01$). Olfactory orientation at post-natal day 14 was significantly depressed in the higher-dose groups in male offspring, and those effects were significantly dose related ($p < 0.01$). For movement activity of exploratory behaviour at 9 weeks of age of the F(1) generation, the total distance of males was significantly increased in the higher-dose groups, and those effects showed a dose-related manner ($p < 0.01$). Average distance and speed were significantly increased in the high-dose group, and those effects showed a dose-related manner ($p < 0.01$ in each). Conclusion: The dose levels of piperonyl butoxide in the present study produced some adverse effects in reproductive and neurobehavioural parameters in mice.

Food Chem Toxicol. 1992 Dec;30(12):1015-9; Reproductive and neurobehavioural effects in three-generation toxicity study of piperonyl butoxide administered to mice; Tanaka T, Takahashi O, Oishi S (Department of Toxicology, Tokyo Metropolitan Research Laboratory of Public Health, Japan).

Piperonyl Butoxide was administered continuously to mice from 5 weeks of age in the F0 generation to weaning of the F2 generation. Piperonyl Butoxide was administered in the diet at levels of 0 (control), 0.1, 0.2, 0.4 and 0.8%. Selected reproductive, developmental and behavioural parameters were measured. Litter size and litter weight were reduced in higher-dosed groups, and the body weight of the pups in the lactation period was reduced in dosed pups in each generation. The survival index at postnatal day 21 of the group receiving 0.8% Piperonyl Butoxide was reduced in each generation. The developmental and behavioural parameters in the lactation period were little different from those of the controls, apart from olfactory orientation in the F1 generation. However, in the F2 generation mice, surface righting, cliff avoidance and olfactory orientation were adversely affected in treatment groups. The results suggest that Piperonyl Butoxide had adverse effects on reproductive, developmental and behavioural parameters of mice, with increasing effects in subsequent generations of offspring.

PEDIATRICS Volume 127, Number 3, March 2011; Impact of Prenatal Exposure to Piperonyl Butoxide and Permethrin on 36-Month Neurodevelopment; Megan K. Horton, Andrew Rundle, David E. Camann, Dana Boyd Barr, Virginia A. Rauh and Robin M. Whyatt

The objective of the study was to explore the association between pre-natal exposure to permethrin (common pyrethroid) and Piperonyl Butoxide (pyrethroid synergist) and 36-month neurodevelopment.

METHODS: Participants is this study were part of a prospective cohort of black and Dominican mothers and newborns living in low-income neighborhoods in New York City. 36-month cognitive and motor development (using the Bayley Scales of Infant Development, second edition) were examined as a function of permethrin levels measured in maternal and umbilical cord plasma collected on delivery and permethrin and piperonyl butoxide levels measured in personal air collected during pregnancy. All models were controlled for gender, gestational age, ethnicity, maternal education, maternal intelligence, quality of the home environment, and prenatal exposure to environmental tobacco smoke and chlorpyrifos.

RESULTS: After data adjustment, children more highly exposed to piperonyl butoxide in personal air samples (4.34 ng/m^3) scored 3.9 points lower on the Mental Developmental Index than those with lower exposures (95% confidence interval:- 0.25 to -7.49).

CONCLUSIONS: Prenatal exposure to piperonyl butoxide was negatively associated with 36-month neurodevelopment.

B. Neurotoxicity in adult experimental animals

Toxicol Lett. 1993 Aug;69(2):155-61; Behavioural effects of piperonyl butoxide in male mice; Tanaka T; Department of Toxicology, Tokyo Metropolitan Research Laboratory of Public Health, Japan.

Piperonyl Butoxide was administered to male mice from 5 to 12 weeks of age in the diet at levels of 0 (control), 0.15, 0.30, and 0.60%, and some behavioural parameters were measured. The animals performed three trials in multiple water T-maze at 10 weeks of age, and the number of errors was significantly decreased in treatment groups on the 3rd trial, while there was no biologically significant

effect of piperonyl butoxide on maze learning. The motor activity of the exploratory behaviour was measured by ANIMATE AT-420 at 8 and 11 weeks of age. At 8 weeks of age, some parameters were increased in the 0.30% group, while there was no consistent compound- or dose-related effect. At 11 weeks of age, some parameters were different in treatment groups, and there were biologically consistent significant effects; i.e., number of movements, movement time, total distance, average speed, and number of turnings increased. From these results, piperonyl butoxide showed adverse effects on the motor activity of the exploratory behaviour in male mice.

Epilepsia. 1984 Oct;25(5):551-5; Anticonvulsant activity and neurotoxicity of piperonyl butoxide in mice. Ater SB, Swinyard EA, Tolman KG, Franklin MR.

Piperonyl Butoxide, a microsomal monooxygenase inhibitor, administered intraperitoneally to mice exerts peak anti-maximal electroshock activity and peak neurotoxicity at 5 and 7h, respectively. The median neurotoxic dose is 1,690 mg/kg. In the maximal electroshock seizure test, the median effective dose (ED50) is 457 mg/kg and the protective index (PI) is 3.69. In the subcutaneous pentylenetetrazol test, the ED50 is 443 mg/kg and the PI is 3.81. Piperonyl Butoxide prevents seizure spread and elevates seizure threshold. Its PI compares favorably with PIs of clinically useful anticonvulsants.

Bull Environ Contam Toxicol. 1978 Jul;20(1):9-16; Potentiation of methylmercury toxicity by piperonyl butoxide; Friedman MA, Eaton LR.

Methylmercury (MeHg) is an extremely potent neurotoxin about 25% of which is degraded in vivo to inorganic mercury. Piperonyl butoxide (PB) is a widely used pesticidal synergist which inhibits many mammalian detoxification reactions. In a preliminary experiment with the high doses of PB and MeHg, PB induced a 12% decrease in mean survival time and a 20% decrease in mean latency time to neurotoxicity. The weight loss in PB-MeHg group was far greater than the control MeHg group. In a dose response experiment, mean survival times in rats fed 40 ppm MeHg-C1 were 5.75, 5.3, and 5.0 weeks at 0, 0.5, and 1% PB, respectively. By the nineth week 25% of rats fed 20 ppm MeHg-C1 showed neurotoxicity and 63% of the 0.5% PB fed showed neurotoxicity with some mortality. In experiments at 20 ppm MeHg-C1 both PB fed groups weighted considerably less than corresponding controls.

C. HUMAN MONITORING STUDIES

Whyatt et al. Within- and Between-Home Variability in Indoor-Air Insecticide Levels during Pregnancy among an Inner-City Cohort from New York City. Environmental Health Perspectives, Vol 115 (3), 2007

BACKGROUND: Residential insecticide use is widespread in the United States, but few data are available on the persistence and variability in levels in the indoor environment.

OBJECTIVE: The study aim was to assess within- and between-home variability in indoor-air insecticides over the final 2 months of pregnancy among a cohort of African-American and Dominican women from New York City.

METHODS: Women not employed outside the home were enrolled between February 2001 and May 2004 (n= 102); 9 insecticides and an adjuvant were measured in 48-hr personal air samples and 2-week integrated indoor air samples collected sequentially for 7.0 ± 2.3 weeks (n= 337 air samples).

RESULTS: Sixty-one percent of the women reported using pest control during the air samplings. Piperonyl Butoxide was detected in 45.5–68.5% (0.2–608 ng/m^3). There was little within-home variability and no significant difference in air concentrations within homes over time ($p \geq 0.2$); between-home variability accounted for 62% of the variance in the indoor air levels of Piperonyl Butoxide ($p < 0.001$). Indoor and maternal personal air insecticide levels were highly correlated ($r = 0.7–0.9$, $p < 0.001$).

CONCLUSION: Results showed that Piperonyl Butoxide was persistent in the home with little variability in air concentrations over the 2 months and contributed to chronic maternal inhalation exposures during pregnancy.

Environmental Health Perspectives • VOLUME110 | NUMBER5 | May 2002; Residential Pesticide Use during Pregnancy among a Cohort of Urban Minority Women

Whyatt et al

Columbia Center for Children's Environmental Health, Mailman School of Public Health, Columbia University, New York, New York, USA; Southwest Research Institute, San Antonio, Texas, USA.

The authors have gathered questionnaire data on pesticide use in the home during pregnancy from 316 African-American and Dominican women residing in northern Manhattan and the South Bronx. Additionally, 72 women underwent personal air monitoring for 48 hr during their third trimester of pregnancy to determine exposure levels to 21 pesticides (19 insecticides and 2 fungicides). Of the women questioned, 266 of 314 (85%) reported that pest control measures were used in the home during pregnancy; 111 of 314 (35%) reported that their homes were sprayed by an exterminator, and of those, 45% said the spraying was done more than once per month. Most (≥90%) of the pesticide was used for cockroach control. Use of pest control measures increased significantly with the level of housing disrepair reported. Of the women monitored, 47–83% had detectable levels of the following four insecticides: the pyrethroid trans-permethrin, Piperonyl Butoxide and the organochlorines 1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane and chlordane. Exposures were generally higher among African Americans than among Dominicans. Results show widespread prenatal pesticide use among minority women in this cohort.

Changes in Pest Infestation Levels, Self-Reported Pesticide Use and Permethrin Exposure during Pregnancy after the 2000–2001 U.S. Environmental Protection Agency Restriction of Organophosphates

Williams et al

Columbia Center for Children's Environmental Health, Mailman School of Public Health, Columbia University, New York, New York; Centers for Disease Control and Prevention, Atlanta, Georgia, USA; Southwest Research Institute, San Antonio, Texas, USA.

METHODOLOGY: 511 pregnant women from inner-city New York were enrolled between 2000 and 2006. Permethrin, a pyrethroid insecticide; Piperonyl Butoxide, a pyrethroid synergist; chlorpyrifos; and diazinon were measured in 48-hr prenatal personal air samples. Data on pest infestation and pesticide use were collected via questionnaire.

RESULTS (concering PBOPiperonyl Butoxide): Eighty-eight percent of women reported using pesticides during pregnancy; 55% reported using higher-exposure pesticide applications (spray cans, pest bombs and/or professional pesticide applicators). Self-reported pest sightings and use of higher-exposure applications increased significantly after the regulations were implemented ($p < 0.001$). Piperonyl Butoxide, cis-, and trans-permethrin were detected in 75, 19, and 18% of personal air

samples, respectively. Detection frequencies of Piperonyl Butoxide increased significantly over time ($p < 0.05$ controlling for potential confounders). Levels and/or detection frequencies of Piperonyl Butoxide were significantly higher among mothers reporting use of high exposure pesticide applications ($p \leq 0.05$).

In the current study, Piperonyl Butoxide was detected in 75% of personal air samples collected during the third trimester of pregnancy between 2000 and 2006 from African-American and Dominican women residing in New York City, and exposures were generally low (0.19–104.86 ng/m^3). Given the evidence of widespread exposure to Piperonyl Butoxide during pregnancy, further investigation into the potential of Piperonyl Butoxide to alter the metabolism of other xenobiotic compounds appears warranted.

CONCLUSION: This is one of the first studies to document widespread residential exposure to Piperonyl Butoxide.

Human exposure to insecticide products containing pyrethrins and piperonyl butoxide (2001–2003); Food and Chemical Toxicology 47 (2009) 1406–1415

Osimitz et al

[work funded by the Pyrethrin Joint Venture (PJV) and the Piperonyl Butoxide Task Force II (PBTFII)]

According to the abstract of the study, the authors investigated human incidents reported through the American Association of Poison Control Centers (AAPCC) Toxic Exposure Surveillance System (TESS) associated with regulated insecticides containing pyrethrins and piperonyl butoxide (PY/PBOPiperonyl Butoxide) from 2001 to 2003. Special attention was paid to dermal and respiratory effects. Although there are limitations associated with TESS data, it was observed that despite extensive use, incidents with reports of moderate or major adverse effects were relatively rare (717 moderate and 23 major outcomes out of 17,873 calls). Following label-directed use of the products, adverse dermal or respiratory reactions were very rare; (dermal – 17 moderate, 1 major; respiratory – 18 moderate, 0 major). The data suggest that asthmatics and people sensitive to ragweed (Ambrosia artisifolia) are not unusually sensitive to PY/PBOPiperonyl Butoxide.

In view of their widespread use, the data indicates that PY/PBOPiperonyl Butoxide products can be used with a relatively low risk of adverse effects. Moreover, the data suggest that they are not likely to cause reactions in people with asthma or allergies.

The eCA notes that the conclusions of the present study contradict the conclusions and recommendations of the US-EPA, Memorandum, Review of Piperonyl Butoxide Incident Reports, 2004.