Committee for Risk Assessment

RAC

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

Adopted

11 June 2020

11 June 2020

CLH-O-0000006819-59-01/F

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

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

Chemical name: **piperonyl butoxide (ISO); 2-(2-butoxyethoxy)ethyl 6-propylpiperonyl ether**

EC Number: **200-076-7**

CAS Number: **51-03-6**

The proposal was submitted by **Greece** and received by RAC on **30 May 2019**.

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

PROCESS FOR ADOPTION OF THE OPINION

Greece has submitted a CLH dossier containing a proposal together with the justification and background information documented in a CLH report. The CLH report was made publicly available in accordance with the requirements of the CLP Regulation at ***http://echa.europa.eu/harmonised-classification-and-labelling-consultation/*** on **1 July 2019**. Concerned parties and Member State Competent Authorities (MSCA) were invited to submit comments and contributions by **30 August 2019**.

ADOPTION OF THE OPINION OF RAC

Rapporteur, appointed by RAC: **Ralf Stahlmann (adviser: Anna Sonnenburg)**

Co-Rapporteur, appointed by RAC: **Irina Karadjova**

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

The RAC opinion on the proposed harmonised classification and labelling was adopted on **11 June 2020** by **consensus**.

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

	Index No	Chemical name	EC No	CAS No	Classification		Labelling			Specific Conc. Limits, M-factors and ATE	Notes
					Hazard Class and Category Code(s)	Hazard statement Code(s)	Pictogram, Signal Word Code(s)	Hazard statement Code(s)	Suppl. Hazard statement Code(s)		
Current Annex VI entry	No current Annex VI entry										
Dossier submitters proposal	TBD	piperonyl butoxide (ISO); 2-(2-butoxyethoxy)ethyl 6-propylpiperonyl ether	200-076-7	51-03-6	STOT SE 3 Aquatic Acute 1 Aquatic Chronic 1	H335 H400 H410	GHS07 GHS09 Wng	H335 H410	EUH066	M=1 M=1	
RAC opinion	TBD	piperonyl butoxide (ISO); 2-(2-butoxyethoxy)ethyl 6-propylpiperonyl ether	200-076-7	51-03-6	Eye Irrit. 2 STOT SE 3 Aquatic Acute 1 Aquatic Chronic 1	H319 H335 H400 H410	GHS07 GHS09 Wng	H319 H335 H410	EUH066	M=1 M=1	
Resulting Annex VI entry if agreed by COM	TBD	piperonyl butoxide (ISO); 2-(2-butoxyethoxy)ethyl 6-propylpiperonyl ether	200-076-7	51-03-6	Eye Irrit. 2 STOT SE 3 Aquatic Acute 1 Aquatic Chronic 1	H319 H335 H400 H410	GHS07 GHS09 Wng	H319 H335 H410	EUH066	M=1 M=1	

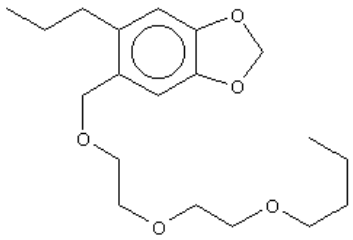
GROUNDS FOR ADOPTION OF THE OPINION

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:



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**.

Oxidising liquids

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.**

HUMAN HEALTH HAZARD EVALUATION

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.**

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.**

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.**

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 the classification criteria.

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.

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.**

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),	adjusted GV for Cat 2: 16 < C ≤ 160 mg/kg bw/d Deviations from guideline: 1. only 8-weeks exposure period.

Method	Results	Remarks
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]	- hepatocellular hypertrophy in all four animals 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)	2. only 2 animals/sex/dose 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)	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)	GV for Cat 2: 10 < C ≤ 100 mg/kg bw/d -> LOAEL slightly above upper limit

Method	Results	Remarks
[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]	- 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)	
Rats: 13-week (91 days), feeding **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]	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 for Cat 2: 10 < C ≤ 100 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 the guidance values were tested.. Therefore, RAC concludes that **no classification for STOT RE is warranted.**

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 <u>due to insufficient data.</u>**

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	low relevance gastric and caecal haemorrhage at all dose levels; MTD exceeded at mid and high doses; high dose > limit dose

Method	Results	Remarks
	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)	
Mouse (Charles River CD-1): 18-months, feeding OECD TG 451 Doses: 0, 30, 100, 300 mg /kg bw/d Anon. 11	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) 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)	reliable
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.**

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.**

ENVIRONMENTAL HAZARD EVALUATION

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)**	**LC_{50} (mg/L)**	**LC_{100} (mg/L)**	Results were based on mean measured concentrations Klimisch score: 1	Anonymous - 1 (1992b) CAR Doc III A7.4.1.1/01
	2.97	**3.94**	≥ 5.24		
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)**	**LC_{50} (mg/L)**	**LC_{100} (mg/L)**	Results were based on mean measured concentrations Klimisch score: 1	Anonymous - 2 (1992c) CAR Doc III A7.4.1.1/02
	2.34	5.37	≥ 6.94		
Fish: Rainbow trout *Oncorhynchus mykiss* (96 hours; flow-through	**LC_0 (mg/L)**	**LC_{50} (mg/L)**	**LC_{100} (mg/L)**	Results were based on mean measured concentrations	**Error! Reference source not found.**
	3.71	6.12	≥ 8.00		

Method	Results			Remarks	Reference
system) EPA, Subdivision E, Series 72; consistent with the OECD Testing Guideline 203; GLP study				Klimisch score: 1	(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)** 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

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	**Error! Reference source not found.** (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).

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.

ANNEXES:

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

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