Committee for Risk Assessment

RAC

Opinion

proposing harmonised classification and labelling
at EU level of

benfluralin (ISO);
N-butyl-*N*-ethyl-*α*,*α*,*α*-trifluoro-2,6-dinitro-*p*-toluidine

EC Number: 217-465-2
CAS Number: 1861-40-1

CLH-O-0000006963-64-01/F

Adopted

18 March 2021

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CLH-O-0000006963-64-01/F

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

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

Chemical name: **benfluralin (ISO);**
***N*-butyl-*N*-ethyl-*α*,*α*,*α*-trifluoro-2,6-dinitro-*p*-toluidine**

EC Number: **217-465-2**

CAS Number: **1861-40-1**

The proposal was submitted by **Norway** and received by RAC on **18 November 2019.**

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

PROCESS FOR ADOPTION OF THE OPINION

Norway 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 **9 December 2019**. Concerned parties and Member State Competent Authorities (MSCA) were invited to submit comments and contributions by **7 February 2020**.

ADOPTION OF THE OPINION OF RAC

Rapporteur, appointed by RAC: **Brendan Murray**

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 **18 March2021** by **consensus**.

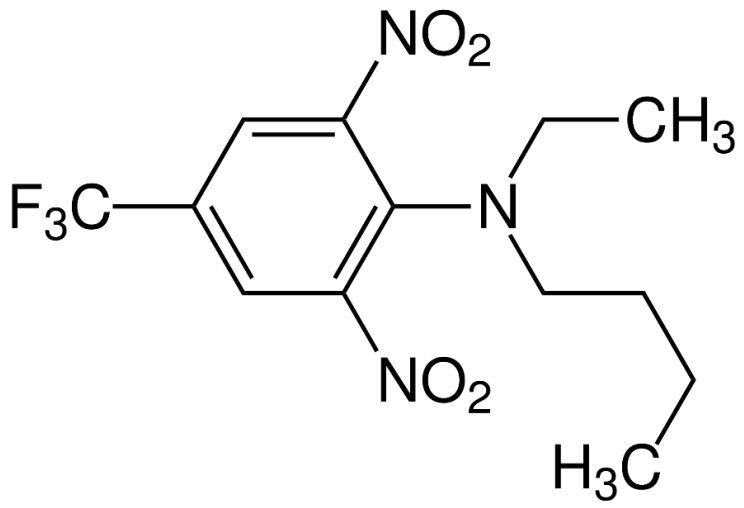
Classification and labelling in accordance with the CLP Regulation (Regulation (EC) 1272/2008)

	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	benfluralin (ISO); *N*-butyl-*N*-ethyl-*α,α,α*-trifluoro-2,6-dinitro-*p*-toluidine	217-465-2	1861-40-1	Carc. 2 Repr. 2 Lact. STOT SE 2 Skin Irrit. 2 Eye Irrit. 2 Skin Sens. 1 Aquatic Acute 1 Aquatic Chronic 1	H351 H361d H362 H371 H315 H319 H317 H400 H410	GHS08 GHS07 GHS09 Wng	H351 H361d H362 H371 H315 H319 H317 H410		M=10 M=10	
RAC opinion	TBD	benfluralin (ISO); *N*-butyl-*N*-ethyl-*α,α,α*-trifluoro-2,6-dinitro-*p*-toluidine	217-465-2	1861-40-1	Carc. 2 Repr. 2 Skin Irrit. 2 Eye Irrit. 2 Skin Sens. 1 Aquatic Acute 1 Aquatic Chronic 1	H351 H361d H315 H319 H317 H400 H410	GHS08 GHS07 GHS09 Wng	H351 H361d H315 H319 H317 H410		M=10 M=10	
Resulting Annex VI entry if agreed by COM	TBD	benfluralin (ISO); *N*-butyl-*N*-ethyl-*α,α,α*-trifluoro-2,6-dinitro-*p*-toluidine	217-465-2	1861-40-1	Carc. 2 Repr. 2 Skin Irrit. 2 Eye Irrit. 2 Skin Sens. 1 Aquatic Acute 1 Aquatic Chronic 1	H351 H361d H315 H319 H317 H400 H410	GHS08 GHS07 GHS09 Wng	H351 H361d H315 H319 H317 H410		M=10 M=10	

GROUNDS FOR ADOPTION OF THE OPINION

RAC general comment

Benfluralin is a pre-emergent dinitroaniline herbicide used to control grasses and other weed species. It is registered for uses against weeds in chicory and lettuce crops which have been evaluated in the context of the Plant Protection Products Regulation EC 1107/2009 (EFSA, 2019). Benfluralin affects seed germination and prevents weed growth by inhibition of root and shoot development.



Benfluralin is not listed in Annex VI of CLP and has not been considered for harmonised classification and labelling previously. All hazard endpoints were open for consideration. The proposed classification by the dossier submitter (DS) covers several endpoints; Skin Irrit. 2, Eye Irrit. 2, Skin Sens. 1, STOT SE 2, Carc. 2, Repr. 2 (development), Lact., Aquatic Acute 1 (M=10) and Aquatic Chronic 1 (M=10).

Benfluralin was discussed at the EFSA Pesticides Peer Review Expert's Meeting 182 in September 2018 and at the Pesticide Peer Review Meeting 05 (joint Mammalian toxicology–Ecotoxicology meeting) in May 2019. The final peer review report was published in 2019 (EFSA Journal 2019;17(11):5842).

The oral absorption of benfluralin is limited, around 20%. The active substance is widely distributed, showing some affinity for fat, extensively metabolised and rapidly eliminated, mainly via faeces.

RAC evaluation of physical hazards

Summary of the Dossier Submitter's proposal

Explosive

The screening procedure (EC method A.14) was used to derive no thermal sensitivity (effect of flame), no mechanical sensitivity (shock, Fall Hammer test) and no mechanical sensitivity (friction, Friction test). Despite the negative results, the DS stated that the acceptance procedure should have been performed, as benfluralin contains groups associated with explosive properties

(nitro compounds). Therefore, it cannot be conclusively concluded that benfluralin is not explosive. The overall DS conclusion was that no classification for explosive is warranted due to lack of data.

Flammable solids

Based on the outcome of EC method A.10, the test substance did not ignite but melted, and combustion did not propagate along the test pile. The DS stated that a substance (non-metal) is classified as a flammable solid when the burning time is < 45 seconds or the burning rate is > 2.2 mm/s. Benfluralin melted but did not ignite. Therefore, the criteria for classification as a flammable solid are not met. The overall DS conclusion was that no classification for flammable solid is warranted based on conclusive but not sufficient for classification data.

Self-reactive substances

Benfluralin contains groups associated with explosive properties (nitro compounds) and none of the test series E (preliminary procedure) or H was performed. In a study, conducted in accordance with OECD TG 103, an endothermic reaction corresponding to a boiling point was not observed with temperatures ≤ 400ºC (highest test temperature). Decomposition or volatilisation began at 205ºC. The overall DS conclusion was that no classification for self-reactive substance is warranted due to lack of data.

Pyrophoric solids

No studies are available. No incidences of self-ignition when exposed to air have been reported. The substance is known to be stable at room temperature for prolonged periods of time (days). The overall DS conclusion was that, based on the CLP criteria, no classification for pyrophoric solids is warranted based on conclusive but not sufficient for classification data.

Self-heating substances

An available study, conducted according to EC method A.15, resulted in a self-ignition temperature of 304°C (250 mg sample, 38 s ignition delay time, 997 mbar pressure). The DS also stated that since benfluralin presents a low melting point (66.4°C), method A.15 for liquids was preferred instead of method A.16 used for solids. The DS acknowledged that studies conducted according to EC method A.15 are generally inappropriate for a sound assessment and the findings do not lead to a classification. However, substances or mixtures with a low melting point, i.e. < 160°C, should not be considered for classification in this class since the melting process is endothermic and the substance-air surface is drastically reduced.

The overall DS conclusion was that, based on the CLP criteria, no classification for self-heating substances is warranted based on conclusive but not sufficient for classification data.

Substances which in contact with water emit flammable gases

No data derived in accordance with the recommended test method in CLP have been provided. Benfluralin has, however, been handled in water in other studies conducted and referred to in the CLH dossier, where no incidences of violent reaction and emission of flammable gases have been reported. The DS stated that according to Section 2.12.4.1 of Annex I of CLP, the classification procedure for this hazard class need not be applied if the chemical structure does not contain metals or metalloids, or if experience in production or handling shows that the substance does not react with water or if the substance is known to be soluble in water to form stable solution. Thus, the overall DS conclusion was that, based on the CLP criteria, no classification for this hazard class is warranted based on conclusive but not sufficient for classification data.

Oxidising solids

In a study conducted according to EC method A.17, the maximum burning rate of the test substance/cellulose mixture was determined to be 3.08 mm/s (test substance/cellulose 60/40% w/w). Maximum burning rate of reference mixture was 0.72 mm/s (barium nitrate/cellulose 40/60% w/w). However, when conducted with inert kieselguhr, burning rates were even faster than those observed with combustible cellulose; max = 4.76 mm/s (test substance/kieselguhr 70/30% w/w and 60/40% w/w). It was concluded that presence of cellulose was thus not important for burning of mixtures. Instead, "wick effect" was observed, i.e. sample melted when ignition source was applied and became flammable in the presence of a solid support.

Benfluralin contains oxygen chemically bounded to nitrogen and should therefore be regarded as potentially oxidising. Test O.1 in Part III, subsection 34.4.1 of the UN Recommendations on the Transport of Dangerous Goods, Manual of Tests and Criteria should have been used for classification. Studies on oxidising properties of benfluralin, however, followed EC method A.17. Because the results generated from the EC method A.17 are not directly comparable with the CLP criteria, the results can be regarded as inconclusive.

Thus, the overall DS conclusion was that no classification for oxidising solids is warranted due to lack of data.

Corrosive to metals

No studies are available, and the corrosiveness of benfluralin to metals has not been considered. However, according to the DS, the classification criteria (UN Test C.1; test temperature of 55°C) exclude solids having a melting temperature > 55°C. Benfluralin is a crystalline solid, and has a melting point of 66.4°C. Further, the water solubility of benfluralin is low, i.e. 0.064 mg/L at 20°C. Therefore, benfluralin is not expected to materially damage metals.

Thus, the overall DS conclusion was that, no classification for corrosive to metals is warranted based on conclusive but not sufficient for classification data.

Comments received during consultation

A company/manufacturer proposed that the available data should take precedence over the DS's observations that benfluralin contains groups associated with explosive properties. The DS responded that negative results of the EU method A.14 is not sufficient to conclusively exclude explosive properties, and the full screening procedure (Annex I, 2.1.4.2) should be used instead.

Assessment and comparison with the classification criteria

Explosive

RAC agrees with the DS conclusion for **no classification as an explosive due to lack of data.**

Flammable solids

RAC agrees with the DS conclusion **for no classification as flammable solid due to conclusive data but not sufficient for classification.**

Self-reactive substances

RAC agrees with the DS conclusion for **no classification as self-reactive substance due to lack of data.**

Pyrophoric solids

RAC agrees with the DS conclusion for **no classification as pyrophoric solid due to conclusive data.**

Self-heating substances

RAC agrees with the DS conclusion for **no classification as self-heating substance due to conclusive data.**

Substances which in contact with water emit flammable gases

RAC agrees with the DS conclusion for **no classification as flammable gas due to conclusive data.**

Oxidising solids

RAC agrees with the DS conclusion for **no classification as oxidising solid due to lack of data.**

Corrosive to metals

RAC agrees with the DS conclusion for **no classification as corrosive to metals due to conclusive data.**

HUMAN HEALTH HAZARD EVALUATION

RAC evaluation of acute toxicity

Summary of the Dossier Submitter's proposal

Acute Oral Toxicity

The DS did not propose classification. There was one guideline compliant (OECD TG 401, 1987) acute oral study available as shown in table below:

Table: *Summary of the Acute oral toxicity studies*

Study, guideline, animal strain	**Test substance,**	**Dose levels, duration of exposure**	**Value LD_{50}**	**Reference**
OECD TG 401 (1987) GLP: Yes Fischer 344	Benfluralin (95.8%) Vehicle: 0.5% w/v hydroxy-propyl methyl cellulose	5/sex/dose (5000 mg/kg bw)	LD_{50} > 5000 mg/kg bw	B.6.2.1/ RAR, 2019 Anonymous, 1996

All animals at the highest dose of 5000 mg/kg bw survived except for 2 females where the cause of death was determined to be as the result of a gavage error.

Animals showed a number of clinical signs including hypoactivity (5/5 males, 3/4 females, day 1), perineal soiling with urine (1/5 males, 4/4 females, day 1-3) and lachrymation (1/5 males, 3/4 females, day 1-2). All animals appeared symptom-free at the end of the 14-day observation period.

No significant effect on bodyweight was recorded for either sex, and there were no treatment-related gross pathological observations in any of the surviving rats.

Acute Dermal Toxicity

The DS proposed no classification. Benfluralin was investigated in two reliable, guideline- (OECD TG 402, 1987) and GLP-compliant dermal toxicity studies, conducted in rabbits. Benfluralin was either administrated in solid form (purity 95.64%) or moistened with aqueous methylcellulose (95.8% purity) and was tested in 5 animals/sex at 5000 mg/kg bw.

Study 1: Anonymous, 1990. Benfluralin applied in solid form (not moistened with water)

The dose, under a damp semi-occlusive wrap, covered 10% of the body surface and treatment was maintained for 24 hours. Rabbits were observed for clinical signs one hour after removal of the wrap, and daily for the subsequent 28 days. None of the treated rabbits died and no overt signs of toxicity were noted during the 28-day post-exposure observation period. The test material stained the treated sites yellow for up to 23 days. Desquamation and signs of skin irritation (moderate to severe erythema and oedema), were observed in these animals and cleared in all animals within 28 days. No treatment-related pathological changes were evident during necropsy.

Study 2: Anonymous, 1997. Benfluralin applied moistened with aqueous methylcellulose

The test substance was moistened with 5.0 mL of 0.5% aqueous methylcellulose and placed on the dorsum under a semi-occlusive gauze/cotton patch. Rabbits were observed for clinical signs and/or dermal reactions on the day of dosing and then daily (on workdays) for the remainder of the 15-day observation period. All rabbits survived the 5000 mg/kg bw dose level. Some degree of erythema and oedema (males and females), burns and fissures at the site (males) and scale formation and scabs (males and females) was observed. The scab formation was not reversed during the study as only one of the ten rabbits had normal skin by day 15. Macroscopic abnormalities were limited to observation of a moderate crust present at the dermal test site of several rabbits.

According to the DS, there was no evidence for treatment related lethality and the acute lethal dermal dose LD_{50} of benfluralin was greater than 5000 mg/kg bw. No classification was proposed.

Acute Inhalation Toxicity

The DS proposed no classification for acute toxicity via inhalation. One reliable, acute inhalation toxicity study was available, which was conducted in Fisher 344 rats in accordance with OECD TG 403 (1981), with 10 animals/sex/dose (vs. 5 animals/sex/dose recommended according to the most current guideline). The 10 males and 10 females were exposed in a 4-hour, nose only exposure study (Anonymous, 1986), to solid particulate aerosols of technical benfluralin containing up to 2.16 mg/L air benfluralin. This was the highest technically achievable concentration for benfluralin (97.3%). The Mass Median Aerodynamic Diameter (MMAD) was 25.88 ± 4.06 µm and 23.72 ± 3.59 µm at target concentrations of 1.12 or 2.16 mg/L air. A significant respirable fraction (with particles that can reach all regions of the respiratory tract) was not achievable. The DS concluded that benfluralin did not meet the criteria for acute inhalation toxicity classification. However, two males and one female exposed to 2.16 mg/L died on day 1 and on the basis of necropsy findings, the DS proposed STOT SE classification (see further down).

The acute inhalation LC_{50} (4h, aerosol) for male and female rats in the study was determined by the DS to be > 2.16 mg benfluralin/L air.

Comments received during consultation

There was one comment from a Member State Competent Authority (MSCA). They agreed that the study results do not support classification of benfluralin for acute toxicity. However, it was

noted that the two dermal studies in rabbits support classification of the test substance as a skin irritant. They also highlighted concerns with respect to the acute inhalation study, suggesting that the data did not support classification because a respirable fraction of the tested substance was not generated.

Assessment and comparison with the classification criteria

Acute Oral Toxicity

According to the CLP criteria, classification for acute oral toxicity is warranted if the LD_{50} of a substance is ≤ 2000 mg/kg bw. The acute oral LD_{50} value for male and female rats was > 5000 mg/kg bw. Overall, the available data on benfluralin does not meet the criteria for classification. The ATE is considered > 5000 mg/kg bw. RAC agrees with the DS proposal of **no classification for acute oral toxicity**.

Acute Dermal Toxicity

The LD_{50} of benfluralin was greater than 5000 mg/kg bw. RAC agrees with the DS that **no classification for acute dermal toxicity is warranted.**

Acute Inhalation Toxicity

According to ECHA guidance on the application of the CLP criteria, v.5.0 (CLP guidance), results from studies in which substances show particle sizes with a MMAD > 4 µm can generally not be used for classification while at the same time recognising that expert judgement is required where there are indications of high toxicity.

In the available acute inhalation study, industry brought to attention the technical difficulty with generating an inhalable dust fraction from a wet cake of benfluralin. The highest technically attainable concentration of benfluralin was 2.16 mg/L air but the MMAD was 25.88 ± 4.06 µm. A respirable fraction was not achieved at any tested concentration of benfluralin. The number of animals used was satisfactory according to the guidelines in force at the time of the study (OECD TG 403, 1981 stipulated at least 5 animals of each sex but did not indicate an upper limit to the number of animals that may be used). There were three animal deaths in total at the highest dose tested; two males (2/10) and one female (1/10); necropsy revealed hepatic and pulmonary congestion, and this is considered further under classification for STOT SE. The three deaths occurred during the exposure period on day 1. There were no indications of high toxicity. The effects following inhalation exposure were not severe enough to result in mortality in one half or more of the animals. The study data may be considered inconclusive for classification purposes due to the difficulties in generating a significant respirable particle size fraction (e.g. MMAD ≤ 2.6 µm accounted for 5.7% of the total particles) for the solid aerosol.

The LC_{50} for male and female rats in the study could not be determined, but it is reasoned to be > 2.16 mg/L. A respirable fraction was not achieved at any tested concentration. RAC agrees with the DS that **no classification is warranted due to inconclusive data.**

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

Summary of the Dossier Submitter's proposal

The DS considered the acute rat inhalation study (Anonymous, 1985) in the context of proposing classification for STOT SE. Groups of ten male and ten female fasted Fischer 344 rats were

exposed nose-only for a single 4-hour period to solid test atmospheres of technical benfluralin at target concentrations of 1.12 or 2.16 mg/L air. Two males and one female exposed to 2.16 mg/L died during the exposure on day 1. Necropsy revealed hepatic and pulmonary congestion in these three animals. The remaining high dose and all low dose animals survived treatment. Clinical signs in the high dose group included dyspnoea, hypoactivity, poor grooming and body weight loss with recovery apparent by day 6. There were no abnormalities evident in the surviving animals at necropsy following scheduled termination at the end of the study.

The DS proposed classification as STOT SE 2 based on:

1. Hepatic and pulmonary congestion observed in two males and one female which died during exposure to 2.16 mg/L; this exposure was compared with the guidance values (GV) for category 2 via the inhalation route for dusts (GV: 5.0 ≥ C > 1.0 mg/L).
2. Clinical signs of the survivors (dyspnoea, hypoactivity, poor grooming and body weight loss with recovery apparent by day 6).

Comments received during consultation

Two comments were received: one from a MSCA and the other from Industry. In both cases there was no support for the DS proposal for STOT SE based on hepatic and pulmonary congestion observed in two males and one female which died during exposure in the high dose group.

The MSCA noted that congestion of the lungs or the liver was the most remarkable pathological finding in the 3 deceased animals of the top dose group. The MSCA expressed doubts over the approach taken by the DS and concluded with no support for STOT SE. They based this on several points:

1. STOT SE is intended to cover non-lethal target organ toxicity. What is not known is if the hepatic and pulmonary congestion had directly contributed to death.
2. Common transient effects in the high dose group were also used to support the classification proposal. This was not supported by the MSCA. These findings, generic in nature, do not represent specific target organ toxicity.
3. The high MMAD raises serious doubts as to whether a respirable fraction could be achieved; this makes the study unsuitable for classification purposes.

Industry's position was that the highlighted findings were considered a post-mortem artefact. The animals were not autopsied immediately after death. The animals were found dead following the inhalation exposure period (4h). Following death, the lungs and liver are vascular soft tissues particularly prone to stagnation of blood and localised pooling. With the cessation of blood flow, *in situ* clotting is eventually observed. Under normal procedures of necropsy, immediately following sacrifice, there is no time for blood to pool in tissues because the animal is immediately exsanguinated. In the case of animals found dead, blood that has clotted *in situ* in the lungs and liver is generally responsible for acute passive congestion. According to Industry, these observations were concluded to be due to the sequence of post-mortem changes naturally occurring following sudden death and should therefore not be interpreted as being a consequence of a pathological condition in the absence of other supporting data.

Assessment and comparison with the classification criteria

STOT SE 2 was proposed by the DS based on two points:

1. Three deaths (3/20; 2/10 males and 1/10 females) in the acute inhalation study in rats with congestion of the lungs or the liver being the most remarkable pathological finding.

2. Reversible clinical signs in survivors were used to further support the proposal.

STOT SE is intended to cover non-lethal target organ effects and it should be considered where there is clear evidence of toxicity to a specific organ. In the present case, the DS proposed classification of benfluralin based on a presumed impairment of liver and lungs which led to lethality. However, this interpretation can be disputed.

True congestion of the liver parenchyma is a frequent finding in chronic injury of the organ such as the case with cirrhosis. Other features would accompany the finding of congestion if it had a true pathological origin, such as atrophy of centrilobular hepatocytes and notable necrosis or fibrosis in the case of chronic conditions. In the present case, the time frame is too short for such an advanced pathological feature to be expressed. Acute passive congestion of the liver is a very common finding at autopsy in humans caused by the presence of right-sided heart failure before death causing retrograde venous congestion of the liver.

Similarly, true congestion of the lungs is a frequent finding in humans suffering from congestive heart failure with other features such as intra-alveolar haemosiderin deposition. Again, the time frames here are indicative of an underlying pathology (e.g. it takes about 2 days for haemoglobin to be converted to haemosiderin). No such features were reported in the present study. As in the case of the liver findings, acute passive congestion of the lungs seems most likely due to the sequence of post-mortem changes naturally occurring following sudden death.

The original study report was brief and did not include any in-depth discussion of the necropsy findings. There was no information regarding the basis for the morphological changes observed in the liver and lungs of the three animals found dead following exposure. No comment can be made regarding cytotoxicity, inflammation or any other pathology.

Suggesting that the congestive changes observed in the lungs and the liver was the cause of death for the two males and one female in the top dose group is speculative without evidence of an underlying pathology due to treatment. The transient clinical signs observed in surviving rats were generic in nature and not indicative of any type of single organ toxicity.

In addition, it could be argued that STOT SE classification for narcosis (i.e. category 3) may be warranted on the basis of specific transient effects. Hypoactivity was observed in the acute studies and could potentially indicate narcosis. On the other hand, it is commonly accepted as a generic clinical sign of toxicity and without further evidence of more severe effects that impact on the overall function of an organism, such as a state of deep stupor, unconsciousness or anaesthesia, or lethargy, lack of coordination righting reflex and ataxia, hypoactivity alone is not sufficient to indicate a state of narcosis. It is reasonable to assume that if narcosis was truly present then it would have been described as such on the basis of strong evidence (i.e. accompanied by some of the signs indicated above), in the original study report. No such description was reported.

RAC does not consider the evidence indicative of an effect that supports classification with STOT SE. Hence, **classification for STOT SE is not considered to be warranted**.

RAC evaluation of skin corrosion/irritation

Summary of the Dossier Submitter's proposal

Two skin irritation studies were performed in New Zealand White (NZW) rabbits according to GLP and OECD TG 404 (1981). Both were acceptable to the DS.

Anonymous, 1990

The skin irritation potential of benfluralin (purity: 95.64%, batch # 231EF4) was assessed in 6 animals in total (3 male and 3 female NZW rabbits). Benfluralin (not moistened with water) was applied topically as a 0.5 g dose under a damp semi-occlusive wrap, approximately 6 cm^2 in area, to the clipped dorsum of each rabbit. The dose was maintained *in situ* for 4 hours by an elastic sleeve. After removing the semi-occlusive wrap, the treatment sites were rinsed with warm water. Animals were observed for signs of toxicity 1 hour after removal of the semi-occlusive wrap and daily for the subsequent 14 days.

Anonymous, 1997

The skin irritation potential of benfluralin (purity: 95.8%, batch # ACD13683) was assessed in 6 animals in total (3 male and 3 female NZW rabbits). Benfluralin (moistened with 0.5 mL of 0.5% aqueous methylcellulose) was applied topically as a 0.5 g dose to the clipped dorsum of each rabbit and covered with a gauze patch with cotton backing. The dose was maintained *in situ* for 4 hours by an elastic jacket. After removing the patches, the treatment sites were rinsed with warm water. Animals were observed for signs of toxicity within 30 minutes, and 24, 48 and 72 hours after removal of the patches and on test days 7, 8 and 9. Animals were sacrificed on day 9.

Results

In both studies, mean gradings for severity of damage, i.e. erythema/eschar, or for oedema in at least 4 of 6 tested animals at the 24, 48- and 72-hour time points (after patch removal) were not sufficient to support a classification for skin irritation.

The DS outlined that the criteria for classification according to CLP Regulation (Annex I: 3.2.2.8.2) were in fact met when consideration was given to reversibility of skin lesions, in particular the persistence of inflammation to the end of each study period. In Anonymous (1990), erythema persisted in 3 out of 6 rabbits at the end of the observation period (15 days). In Anonymous (1997), the erythema and oedema were unremarkable but scaliness was observed in 5/6 animals at the end of the observation period (9 days).

The DS also noted that EFSA (2008) supported skin irritation classification previously based on results from two 21-day dermal subchronic toxicity studies in rabbits (Anonymous, 1986; Anonymous, 1993). Both studies were GLP and OECD TG 410 (1981) compliant with treatment groups comprised of 5 rabbits/sex/dose. The dermal application of benfluralin to the rabbit over a period of 21 days caused dermatitis at all doses, and an associated inflammatory increase of leukocytes and thrombocytes at 500 mg/kg bw/d and higher. Dermal irritation indices were calculated for the different treatment groups, and it appeared that a dose- and time-related increase of very significant dermal irritation was observed. Corrosivity was not supported by the data because animals did not generally show fissuring, scabs (crusts) or necrosis.

The DS also described significant and severe dermal irritation in two acute dermal toxicity tests. Desquamation and signs of skin irritation (moderate to severe erythema and oedema), were observed and cleared in all animals within 28 days in the Anonymous (1990) study. In the Anonymous (1997) study, erythema and oedema (males and females), burns and fissures at the site (males) and scale formation and scabs (males and females) were observed. The scab formation was not reversed during the study as only one of the ten rabbits had normal skin by day 15. The DS proposed Skin Irrit. 2; H315 (Causes skin irritation).

Comments received during consultation

There was a single comment from an MSCA supporting classification for skin irritation (Skin Irrit. 2; H315).

Assessment and comparison with the classification criteria

According to the CLP criteria, a substance is irritant to the skin under a number of conditions including inflammation that persists to the end of the study observation period, in at least 2 animals exhibiting alopecia (limited area), hyperkeratosis, hyperplasia, and scaling of the skin.

Both studies presented for the assessment of skin irritation appear to support skin irritation classification based on this aspect of the criteria rather than on the standard reliance on severity of damage as indicated by the degree of erythema and/or oedema. The persistence of clear signs of skin irritation to the end of each study observation period is clear. What is not so clear from the CLP guidance are:

1. Does the observation period adhere strictly to 14 days? Study observation periods for skin irritation studies are normally referred to as being 14 days in length but individual studies can differ in this respect. There is no further explanation in the guidance text if the end of the study observation period applies to all skin irritation studies regardless of the length of their observation period following substance removal from the skin, i.e. is a 9-day observation period as valid as a 14-day one?
2. The guidance specifies effects seen to persist in at least two animals. The text, following on from a brief description of erythema and oedema scores in a 3-animal study would therefore presume persistent effects in two animals out of three. However, there is no explicit confirmation that this is indeed the case. Does the guidance actually mean 2 animals out of 3 in a study or 2 animals regardless of the number of animals used in the study? The two dermal irritancy tests were conducted with 6 animals.

Regardless of these uncertainties, there is ample evidence for supporting a classification for skin irritancy; see table below.

***Table**: Summary of the evidence for skin irritation*

Test guideline / GLP	**Animals**	**Purity**	**Dose**	**Results**	**Reference**
Skin irritation studies					
OECD TG 404 / GLP: Yes	Rabbit NZW 3M/3F	95.64%	[1]0.5 g active	Erythema persisted in **3/6** rabbits at the end of the study (day 15).	Anonymous, 1990 Report No. B09690
OECD TG 404 / GLP: Yes	Rabbit NZW 3M/3F	95.8%	[2]0.5 g active	Scaliness observed in **5/6** rabbits at the end of the study (day 9).	Anonymous, 1997 Report No. 971153
Acute dermal toxicity studies					
OECD TG 402 / GLP: Yes	Rabbit NZW 5M/5F	95.64%	[3]5000 mg/kg bw	28-day post-exposure observation period. By day 5 or 6, a moderate to severe erythematous and oedematous response was evident. Desquamation was present from day 6, and 2 females developed coriaceous or cracked dermal treatment sites from day 7 or 10. Irritation cleared in all animals by 28 days.	Anonymous, 1990 Report No. B04990
OECD TG 402 / GLP: Yes	Rabbit NZW 5M/5F	95.8%	[4]5000 mg/kg bw	15-day post-exposure observation period. Evidence of erythema and oedema present from day 2 to day 15. Females showed some scale formation and scabs developed from day 7. The males had burns and fissures at the site from day 2 to 4 with more fissures developing on day 10/11; scaling was seen	Anonymous, 1997 Report No. 971155

				from day 7 and scab formation from day 10. Nine of the 10 rabbits showed persistent skin irritation up to day 15.	
Subchronic (21-day) dermal toxicity studies					
OECD TG 410 / GLP: Yes	Rabbit NZW 5M/5F	97.3%	[5]0, 100, 325 or 1000 mg/kg bw	Exposure was for 6h/d for 21 days. A dose- and time-related persistent increase of dermal irritation was observed. At 325 mg/kg bw/d: moderate irritation progressed to severe erythema and slight oedema to severe oedema. At 1000 mg/kg bw/d: slight irritation progressed to severe erythema and moderate oedema progressed to severe oedema. In all treatment groups desquamation occurred within 5-20 days of treatment, followed by epithelisation (without scar tissue or other indication of corrosive effects). The skin exhibited a coriaceous, cracking and bleeding appearance.	Anonymous, 1986 Report No. 02185
OECD TG 410 / GLP: Yes	Rabbit NZW 5M/5F	95.8%	[6]0, 100, 500 or 1000 mg/kg bw	Exposure was for 6 h/d for 5 d/week for 21 days. A dose-dependent increase of erythema, eschar, oedema and scaling was observed from the lowest dose in both the males and the females. At the highest doses, necrosis, ulcers and suppurative lesions occurred. Underlying tissues were also affected, as inflammation and oedema of the dermis, and sebaceous gland hyperplasia was observed at all doses.	Anonymous, 1993 Report No. DR-0097-3397-002

[1] not moistened, but covered with moistened dressing.
[2] moistened with 0.5 mL of 0.5% aqueous methylcellulose.
[3] not moistened, but applied in solid form under a damp semi-occlusive wrap.
[4] moistened with 5.0 mL of 0.5% aqueous methylcellulose.
[5] not moistened, but covered with a moistened gauze pad.
[6] moistened with 1 mL of water per gram of test material.

The data from several studies indicates that the inflammation persists to the end of the study period. This fulfils one of the main CLP criteria for classification of skin irritation, that of a substance which is irritant to the skin and produces reversible damage to the skin following its application for a period up to 4 hours. Based on a weight of evidence assessment, RAC considers that there is sufficient and clear evidence and that **classification as Skin Irrit. 2; H315 is warranted**.

RAC evaluation of serious eye damage/irritation

Summary of the Dossier Submitter's proposal

The DS proposed to classify benfluralin as Eye Irrit. 2; H319 based on a single GLP and guideline compliant study (OECD TG 405, 1987), in three male and three female NZW rabbits (Anonymous, 1997, RAR B.6.2.5, 2018). Benfluralin (95.8%) was instilled as a finely ground 0.1 g aliquot into

the conjunctival sac of the right eye of each rabbit. The left eye remained untreated and served as a control. An ocular anaesthetic was used for all rabbits (both eyes) after discomfort was observed in the first dosed rabbit. All eyes were examined 1, 24, 48 and 72 hours after dosing and again on days 7 and 14. The eyes of all rabbits remained unwashed post treatment. Results are presented in the table below.

Table: *Summary of rabbit ocular data*

Mean values for ocular lesions 24, 48 and 72 hours after instillation

Animals	**Corneal opacity**	**Iridial lesions**	**Conjunctival**	
			Redness	**Chemosis**
1. male 97A22-868	**1**	0	**2.3**	**2**
2. female 97A22-871	**1**	0.7	**2.3**	1.7
3. male 97A22-869	**1**	**1**	**2**	1.3
4. female 97A22-872	**1**	0.3	**2.7**	**2.3**
5. male 97A22-870	**1**	0.7	**2**	**3**
6. female 97A22-873	**1.3**	**1**	**2.7**	**4**
CLP Criteria: Eye Irrit. (Cat. 2)	≥ 1	≥ 1	≥ 2	≥ 2
CLP Criteria: Eye Dam. (Cat. 1)	≥ 3	> 1.5	na	na

According to the criteria defined in the CLP Regulation, the mean scores for corneal opacity, conjunctival redness and conjunctival chemosis (oedema), following grading at 24, 48 and 72 hours after installation of the test material, are above the trigger for classification as a category 2 eye irritant. All six animals are positive for at least two indicators supporting classification. The DS therefore proposed classification of benfluralin as Eye Irrit. 2; H319.

Comments received during consultation

There was a single comment from an MSCA supporting classification for eye irritation (Eye Irrit. 2; H319).

Assessment and comparison with the classification criteria

According to the CLP criteria, substances that have the potential to induce reversible eye irritation shall be classified in Category 2. In the single available study (Anonymous, 1997), scores were equal or above 1 in 6/6 animals for corneal opacity, in 2/6 animals for iris lesion, and equal or above 2 in 6/6 animals for conjunctival redness, and in 4/6 animals for chemosis. The criteria specifying at least 4 animals out of 6 with scores in indicators of reversible irritation above the trigger values was fulfilled. RAC agrees with the DS that **benfluralin should be classified as Eye Irrit. 2; H319**.

RAC evaluation of respiratory sensitisation

Summary of the Dossier Submitter's proposal

Not assessed, as no study data was available. No classification is thus warranted, due to lack of data.

RAC evaluation of skin sensitisation

Summary of the Dossier Submitter's proposal

The skin sensitisation potential of benfluralin (95.8%) was investigated in two GLP-compliant studies according to OECD TG 406 (one modified Buehler method, Anonymous, 1984; and one Magnusson and Kligman Maximisation test, Anonymous, 1998).

The Buehler study was considered acceptable but only as supporting data by the DS due to two main shortcomings: (1) the use of 12 test and 6 control animals, instead of at least 20 test and ten control animals, and (2) there were no results for preliminary testing/dose ranging investigations to justify the dose selection. Both the dermal induction and the challenge doses were 5% benfluralin. As consistent positive results were observed throughout the observation period, and the sensitisation rate was 75%, the DS considered this test highly indicative of sensitising potential. The positive control, dinitrochlorobenzene, responded as expected (100% sensitisation rate).

The maximisation test was the key study and performed according to guidelines with one small caveat; pre-treatment with sodium lauryl sulphate prior to topical induction was not required, the selected dose concentration was already moderately irritating.

A pretest identified a 5% w/v benfluralin formulation in mineral oil as suitable for intradermal injection; a 25% w/v paste in petrolatum as suitable for the topical phase of the induction period and 10% w/v benfluralin in petrolatum as the maximum non-irritating concentration for use in the challenge phase. There was a typo in the CLH report, all 20 animals exposed to 5% w/v benfluralin intradermal induction were positive at both the 24-hour and 48-hour time points following challenge. At 24h, 20/20 animals exhibited erythema score 2 (moderate and diffuse redness) or 3 (intense redness and swelling), and at 48h, 1/20 animals exhibited score 1, while 19/20 exhibited score 2 or 3. Only 2 control group animals responded to application of benfluralin and the reactions did not exceed transient moderate erythema.

According to the criteria defined in CLP Regulation, the DS considered that benfluralin is a skin sensitiser and proposed classification as Skin Sens. 1; H317 with no sub-categorisation.

Comments received during consultation

There was a single comment from an MSCA supporting classification for skin sensitisation (Skin Sens. 1; H317) with no sub-categorisation.

Assessment and comparison with the classification criteria

In the two available studies, strong sensitisation reactions were elicited. According to the CLP criteria, a substance may be classified as a skin sensitiser on the basis of positive test results in either the mouse LLNA, Guinea pig Buehler assay or Guinea pig maximisation test. The data for benfluralin supporting classification for skin sensitisation was available from both a Buehler and Maximisation study. The Maximisation study was the key study. Using an intradermal induction with 5% w/v benfluralin resulted in a 100% sensitisation incidence (20/20 for test animals, 2/20 in controls) at both the 24-hour and 48-hour time points following challenge.

Based on the Maximisation study results benfluralin can be categorised as a moderate sensitiser. The Buehler or occluded patch test used less than the recommended number of test animals, which could lead to a potential reduction in accuracy. However, the results from the Buehler test are sufficiently robust to support the results from the Maximisation test. Potency on the basis of

the Guinea pig maximisation test was determined by comparison with the criteria for a moderate sensitiser:

- A sensitisation incidence ≥ 30%
- An intradermal induction using > 1.0% (w/v) test substance

The results of the Maximisation test (5% intradermal induction concentration with a 100% nt sensitisation incidence) support that benfluralin is a moderate sensitiser with a predicted sub-category of 1B.

The concentration of benfluralin used for intradermal induction was high, 5%. This makes it impossible to exclude the possibility of the substance being a Category 1A sensitiser since that would require to test benfluralin in the low concentration range of > 0.1 - ≤ 1.0%. On this basis RAC cannot propose sub-categorisation for benfluralin.

Specific concentration limits for sensitisation generally apply for the most potent skin sensitisers classified in Category 1A. There was no adequate or reliable scientific information available showing that skin sensitisation warranted deviations from the GCL for classification.

RAC supports the classification proposal by the DS, that benfluralin should be **classified as Skin Sens. 1; H317 without sub categorisation**.

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

Summary of the Dossier Submitter's proposal

The DS included the following repeat dose studies with benfluralin in the assessment of STOT RE:

1. 3 × 90-day rat dietary studies (Anonymous, 1996)
2. 1 × 90-day mouse dietary study (Anonymous, 1988)
3. 1 × 90-day dog dietary study (Anonymous, 1993)
4. 1 × 1-year dog dietary study (Anonymous, 1995)
5. 2 × 21-day rabbit dermal application studies (Anonymous, 1986; 1993)

All studies were guideline- and GLP compliant.

Rat 90-day studies

The rat studies tested several doses from 50 ppm up to 7500 ppm (3-522/604 mg/kg bw/d) in both males and females. Both liver and kidneys were the primary affected organs; several other generic effects on body weight and blood parameters suggesting slight anaemia were also noted. Clinical chemistry changes indicated liver and kidney involvement. Increased liver weight and hepatocyte hypertrophy were significant at high dose levels and increases in alpha 2µ-globulin in male rats at high doses contributed to nephropathy. Ovary cysts were observed at 20 (2/15), 88 (7/15) and 395 (1/15) mg/kg bw/d, but not at 604 mg/kg bw/d. The lack of a dose response does not support this effect as a substance mediated one. None of these effects were considered relevant for classification.

Mouse 90-day study

Groups of 15 male and 15 female B6C3F1/CrlBr mice were fed a diet containing 0, 100, 300, 1000, 3000 and 10000 ppm of benfluralin (equivalent to 0, 13.5, 40.3, 132.8, 420.8 or 1364.4 mg/kg bw/d for males and 0, 17.4, 51.1, 168.2, 506.6 or 1730.2 mg/kg bw/d for females) for 13 weeks

/90 days. The liver and blood were target organs, but effects were only of toxicological relevance at very high doses. The NOAEL was set at 133 mg/kg bw/d, above the criterion for STOT RE 2. The DS found no evidence to support adverse effects at or below the guidance values for classification.

Dog 90-day and 1-year studies

In the dog studies, 4 animals/sex/dose (Beagle, purebred) were fed gelatine capsules with benfluralin at dose levels of 0, 5, 25 or 125 mg/kg bw/d for a period of 90 days or a period of 12 months. There was evidence of increased red blood cell turnover, slight increases in alkaline phosphatase activity and liver effects were also noted and considered adverse only at the top dose of 125 mg/kg bw/d. There were no effects considered to be toxicologically relevant at the lower doses. The DS did not propose classification based on a lack of effects in the dog studies.

Rabbit 21-day dermal studies

The subchronic toxicity of benfluralin was also studied by the dermal route in rabbit. In the first dermal study (1986), 5 rabbits/sex/dose (NZW) were exposed to benfluralin, at dose levels of 0, 100, 325 or 1000 mg/kg bw during 6h/d for 21 days. The test article was not moistened. In the second dermal study (1993), 5 rabbits/sex/dose (NZW) were exposed to benfluralin, at dose levels of 0, 100, 500 or 1000 mg/kg bw during 6h/d for 21 days (except weekends). The neat test material was moistened with 1 mL distilled water per gram of test material and applied to the back of the rabbits under a gauze patch. Benfluralin caused dermatitis at all doses. Both studies showed clear, local irritation effects and treatment-related lesions in the skin. In the second study, there were some indications of liver necrosis with accompanying inflammation, but the lesions were not consistent and did not follow a clear dose response and lacked a clear mode of action (MoA). These dermal studies were also used to support the proposal for classification for Skin Irrit. 2; H315 (Causes skin irritation).

The DS did not consider any other studies in its assessment of repeated dose toxicity. The DS concluded there were no observations to support classification and did not propose STOT RE classification for benfluralin.

Comments received during consultation

There was a single comment from an MSCA. Classification for STOT RE was not supported. Adverse effects that might warrant consideration were confined to dose levels above the guidance values or were species-specific with regard to kidney effects.

Assessment and comparison with the classification criteria

The DS concluded that no adverse effects were observed at or below the adjusted guidance values that fulfilled the criteria for classification. The DS did not assess data from other repeated dose studies for benfluralin; these included studies on carcinogenicity and fertility and development.

Assessment of data from 90-day studies, 1-year dog and 28-day dermal toxicity studies

A set of standard, well conducted oral toxicity studies were available in a range of species (rats, mice and dogs), as well as two dermal toxicity studies (rabbits).

In rats, renal findings included nephrosis in males with hyaline droplets positive for α2μ-globulin (specific for males), and an increased incidence of golden-brown pigment deposition in the females. Nephrotoxicity characterised by pigment deposition in the female rat was minimal and not considered adverse at the relevant doses for classification. Effects on the liver were indicated

by clinical chemistry changes, enlarged and heavier livers as well as increased CYP1A activities with incidences of hepatocellular hypertrophy. These were considered to be indicative of an adaptive rather than an adverse response. A similar adaptive response in the liver was also observed in the 90-day mouse study.

Prominent effects in dogs included increases in alkaline phosphatase activities, liver weight and increased sinusoidal spleen pigmentation at the top-dose (125 mg/kg bw/d). The spleen haemosiderosis at 25 mg/kg bw/d was associated with an adaptive physiological response.

The dermal application of benfluralin to the rabbit in the 21-day studies caused dermatitis at all doses, and an associated inflammatory increase of leukocytes and thrombocytes at 500 mg/kg bw/d and higher. Liver necrosis with accompanying inflammation in the rabbits lacked a clear MoA. The finding was not consistent with lesions in the rat or dog and it was confined to one study only. The second rabbit study did not show this effect.

In summary, the effects observed following administration of benfluralin showed no evidence of organ dysfunction at the cut-off levels where classification could be considered. Marked effects generally occurred at levels above the guidance values for classification.

Assessment of data from other repeat dose studies, rodent carcinogenicity, rat 2-generation and rat and rabbit developmental toxicity studies

In the rat 2-year carcinogenicity study many renal lesions occurred at ≥ 5/7 mg/kg bw/d (100ppm) benfluralin (a level which could in principle, support classification of STOT RE 2 for renal toxicity). The effects exhibiting a clear increase in a dose-dependent response included:

- Hyaline droplets in the renal tubule lining cells,
- Tubule cell karyomegaly,
- Transitional cell hyperplasia of the renal papilla,
- Large pelvic calculi,
- Small calculi in the pelvic epithelium.

However, at low levels of benfluralin these effects did not occur with sufficient adversity to cause significant toxicity or organ dysfunction.

Overall, RAC considers that **classification for STOT RE is not warranted**.

RAC evaluation of germ cell mutagenicity

Summary of the Dossier Submitter's proposal

The genotoxicity of benfluralin was investigated in several guideline- and GLP-compliant *in vitro* tests and *in vivo* bone marrow micronucleus tests (see table 21 and 22 of the CLH report). No germ cell mutagenicity tests were conducted. The DS reported on the following:

In vitro assays:

- 4 × Ames tests (reverse mutation assay with *Salmonella typhimurium* and *Escherichia coli*) (Rexroat, 1985; Deparade, 2002; Lloyd, 2017a, 2017b),
- 3 × mammalian cell gene mutation tests (mouse lymphoma L5178Y cells) (Bewsey, 1985; Lloyd, 2017c, 2017d),
- 2 × chromosome aberration tests (mammalian cell micronucleus tests, human lymphocytes) (Lloyd, 2017e, 2017f),
- 1 × UDS test (Hill, 1985).

In vivo assays:

- 2 × micronucleus assays in rat bone marrow (Anonymous, 2017a, 2017b),
- 1 × micronucleus assay in mouse bone marrow (Anonymous, 2004).

In vitro results

Benfluralin was found to be negative in studies investigating gene mutations *in vitro* in bacteria and in mammalian cells either with or without metabolic activation. The newer *in vitro* genotoxicity studies were clearly negative except for the *in vitro* gene mutation assay at the *hprt* locus (Lloyd, 2017c), tested with benfluralin containing the highest specification of the genotoxic impurity ethyl-butyl-nitrosamine (EBNA, Batch 2228, 0.085 mg/kg). The mean mutation frequency (10.04 M/F), at the highest concentration tested (200 µg/mL) in the presence of S9, was not within the distribution of the historical control data (HCD, 1.29-9.08 M/F). The result was difficult to interpret due to the observed precipitation at this concentration and the DS deferred to the conclusions of the EFSA peer review and final assessment report. Here experts noted that within the OECD TG 476 it states that positive effects observed in the presence of precipitation *could* be artefactual. On this basis the DS agreed with the final EFSA conclusion that the overall result for the Lloyd (2017c) study should be considered negative. Benfluralin showed no evidence of clastogenic potential, the *in vitro* human lymphocyte micronucleus studies were negative both in the presence and absence of S9 mix. An unscheduled DNA synthesis test *in vitro* in rat hepatocytes was negative.

In vivo results

The clastogenic effect of benfluralin was further investigated *in vivo* in three bone marrow micronucleus tests (2 in Sprague Dawley rats and 1 in CD-1 mice) conducted with three different batches. An extra study available in the RAR (2018) was considered supplementary but not assessed by the DS (Female Chinese hamster bone-marrow Sister Chromatid exchange test, Anonymous, 1985).

In the *in vivo* mouse bone marrow micronucleus study (Anonymous, 2004), six mice/dose/sex were exposed by gavage (dosing volume 10 mL/kg bw), to benfluralin dissolved in 0.5% w/v methylcellulose, at a dose level of 2000 mg/kg bw. Animals were sacrificed 24h and 48h after dosing. There was no evidence of bone marrow toxicity, the PCE/NCE ratio was not reduced. In males, the mean value of micronucleated PCE (MN PCE) showed a non-statistically significant increase in the treatment group, however, this was within the historical control range. The positive control induced a marked statistically significant increase confirming the sensitivity of the assay. The DS noted that this study was conducted with a benfluralin batch containing a lower content of EBNA (0.04 mg/kg) than the current specification for the active substance (0.1 mg/kg). The DS also noted some concern over actual bone marrow exposure by benfluralin.

In the two rat bone marrow micronucleus tests, doses of 500, 1000 and 2000 mg/kg bw were administrated twice (0 and 24 hours) by gavage to male rats (six rats/dose), before bone marrow was sampled at 48 hours. There was no evidence of any test article-induced toxicity to the bone marrow in either of the tests (no decrease in %PCE values compared to the vehicle control group). Results from ADME studies in rats provided evidence that the bone marrow was exposed.

In the first rat bone marrow micronucleus study (Anonymous, 2017a, substance batch 2228, EBNA content 0.085 mg/kg), the mean micronucleated PCE frequencies of treatment groups were increased compared to the vehicle control. The increases were statistically significant ($p \leq 0.05$) at 500 and 2000 mg/kg bw (but not at 1000 mg/kg bw) and did not follow a dose response. The DS also noted that all individual animal and group mean micronucleus frequencies fell within the laboratory's historical vehicle control 95% reference range and that the small and variable increases in MN PCE were not treatment related.

In the second study (Anonymous, 2017b, substance batch 2614, EBNA content 0.058 mg/kg) all doses exhibited MN PCE frequencies that were similar to the concurrent vehicle control group and that fell within the laboratory's historical vehicle control 95% reference range.

Conclusion

The DS noted that the batches of benfluralin used in the genotoxicity studies had a lower content of the genotoxic impurity EBNA than the current approved specification (0.1 mg /kg). The highest level of EBNA tested was 0.085 mg/kg. According to the DS, there was no evidence from the available data set that benfluralin is a somatic cell mutagen. The DS did not propose to classify benfluralin as mutagenic.

Comments received during consultation

There was a single comment from an MSCA that supported the DS conclusion that benfluralin was unlikely to be genotoxic. Despite some deficiencies, they accepted the study database for benfluralin.

Assessment and comparison with the classification criteria

Summary

The genotoxicity of benfluralin was investigated in several guideline- and GLP-compliant *in vitro* tests and three *in vivo* bone marrow micronucleus tests using different batches of benfluralin (table below). Benfluralin generally gave negative responses in studies investigating gene mutations *in vitro* in bacteria and in mammalian cells. However, in one case a negative conclusion is not as certain as described by the DS. The studies presented by the RMS in the RAR (2019), section B.6.4 Genotoxicity, table B.6.4-01 "Summary of studies on genotoxicity" detail the complete genotoxicity database. There are extra studies incorporated in this table not described by the DS and this is supported by RAC since the original RMS concluded in these cases that the studies for various reasons were not acceptable from a regulatory point of view. However, one of the newer *in vitro* mammalian gene mutation assays at the hprt locus (Lloyd, 2017c), and tested with benfluralin containing the highest specification of the genotoxic impurity EBNA (Batch 2228, 0.085 mg/kg), should be best described as equivocal rather than negative for genotoxicity as it showed a weak positive result in the presence of precipitation.

Along with three *in vivo* bone marrow micronucleus studies there was also a Chinese Hamster, bone marrow Sister Chromatid Exchange test described by the RMS in the RAR (2019) under section B.6.4.2.1 (Anonymous, 1985). This was briefly noted by the DS as a supplementary study but not assessed. RAC agrees with the RMS that deficiencies in the study (use of only 3 animals/dose, all female; GLP but not an OECD guideline study and is not included in the recommended test battery according to regulation (EC) 283/2013), relegate it as a supplementary study, but one with a clear but limited result; benfluralin did not increase the frequency of chromosome aberrations (sister chromatid exchange).

The DS described in detail the *in vivo* mouse bone marrow micronucleus study (Anonymous, 2004) and concluded the results were equivocal. The DS included a statement speculating on whether the data from the toxicokinetic studies in rats could be used as evidence for bone marrow exposure in the mouse micronucleus test. The concern was raised because no ADME studies on the mouse were available to prove bioavailability. OECD TG 474 (1997) states that the test is unsuitable only if there is evidence of the substance not reaching the target tissue. The mouse 90-day study, using a different strain but dosed at near comparable levels, showed clear systemic effects illustrating that the limit dose approach was correct for the *in vivo* mouse MN test. In

principle, data from ADME and toxicokinetic studies in an animal species such as the rat are applied to human risk assessment scenarios. This indicates it is a reasonable assumption that ADME data from the rat can also be used to justify bioavailability in the mouse as long as there are no substantial differences in the delivery or route of administration of the tested substance.

The CD-1 mouse study was GLP- and guideline- (OECD TG 474, 1997) compliant. A single limit dose (2000 mg/kg bw) was tested and no effects on body weight or other indications of toxicity were noted during the in-life portion of the micronucleus test. The vehicle used was 0.5% methyl cellulose, the same as used in the two rat *in vivo* MN tests. The original rat ADME studies used a 10% acacia solution (gum Arabic) as vehicle which is comparable to methyl cellulose and compatible with oral toxicity studies as a suitable test vehicle.

In contrast to the position of the DS, RAC considers that bone marrow exposure in the mouse was highly probable, taking into account all the available data including that from the rat metabolism studies. According to the available studies, benfluralin, while poorly absorbed (approximately 20% of an oral dose), does exhibit extensive distribution and is found in the blood which perfuses the bone marrow.

According to the DS, the results of the mouse micronucleus assay were described as equivocal based purely on the mean value of the treated group (2.6‰ MN PCE) being outside the range of the mean values of the HCD (0.3-2.2‰ MN PCE). However, the mean of the treatment group (2000 mg/kg bw) was not statistically significantly different from the control group and in no case did any of the individual animal results (range 2.5-3.5‰ MN PCE) exceed the range of the laboratory HCD (range 0-6‰ MN PCE). A significant biological effect is not supported. There was no reduction of the PCE:NCE ratio. The positive control induced a marked increase confirming the sensitivity of the assay. RAC considers this study to be valid and negative for the induction of micronuclei.

In vitro tests

Several well conducted studies investigating gene mutations *in vitro* in bacteria and in mammalian cells were available for assessment of mutagenicity (table below). RAC concludes that the available data does not support a mutagenic potential for benfluralin.

Table: *Summary of genotoxicity tests with benfulralin (and content of EBNA) adapted from table 21 and 22 in the CLH report, and table B.6.4-01 in the RAR (2019).*

Study	Result	Test System	Reference
***In vitro* studies**			
Bacterial mutagenicity *[EBNA] = 0.31 mg/kg*	**negative**	GLP, non-guideline *Salmonella* Strains: TA98, TA100, TA1535, TA1537, TA1538	Rexroat, 1985 Supplementary
Bacterial mutagenicity *[EBNA] = 0.04 mg/kg*	**negative**	GLP, OECD TG 471 (1997) *Salmonella* Strains: TA98, TA100, TA1535, TA1537 *E. coli* strain WP2uvrA$^-$	Deparade, 2002 Acceptable
Bacterial mutagenicity *[EBNA] = 0.085 mg/kg*	**negative**	GLP, OECD TG 471 (1997) *Salmonella* Strains: TA98, TA100, TA102, TA1535, TA1537	Lloyd, 2017a Acceptable
Bacterial mutagenicity *[EBNA] = 0.058 mg/kg*	**negative**	GLP, OECD TG 471 (1997) *Salmonella* Strains: TA98, TA100, TA102, TA1535, TA1537	Lloyd, 2017b Acceptable
Mammalian cell mutagenicity *[EBNA] = 0.31 mg/kg*	**negative**	GLP, Guideline not stated. Mouse Lymphoma L5178Y Cells (Thymidine Kinase locus)	Bewsey, 1985 Acceptable
Mammalian cell mutagenicity *[EBNA] = 0.085 mg/kg*	**equivocal**	GLP, OECD TG 476 (2016) Mouse Lymphoma L5178Y Cells (*hprt* locus)	Lloyd, 2017c Acceptable

Mammalian cell mutagenicity *[EBNA] = 0.058 mg/kg*	**negative**	GLP, OECD TG 476 (2016) Mouse Lymphoma L5178Y Cells (*hprt* locus)	Lloyd, 2017d Acceptable
Clastogenicity *[EBNA] = 0.085 mg/kg*	**negative**	GLP, OECD TG 487 (2016) Human Lymphocyte Micronucleus Assay	Lloyd, 2017e Acceptable
Clastogenicity *[EBNA] = 0.058 mg/kg*	**negative**	GLP, OECD TG 487 (2016) Human Lymphocyte Micronucleus Assay	Lloyd, 2017f Acceptable
Primary DNA damage *[EBNA] = 0.31 mg/kg*	**negative**	GLP, Guideline not stated. rat hepatocyte (*ex vivo*) UDS	Hill, 1985 Supplementary
***In vivo* studies**			
Biomarker exposure/repair *[EBNA] = 0.31 mg/kg*	**negative**	GLP, non-guideline bone-marrow Sister Chromatid exchange test	Anonymous, 1985 Supplementary
Clastogenicity *[EBNA] = 0.04 mg/kg*	**negative**	GLP, OECD TG 474 (1997) Mouse [CD-1], bone marrow micronucleus	Anonymous, 2004 Acceptable
Clastogenicity *[EBNA] = 0.085 mg/kg*	**negative**	GLP, OECD TG 474 (2016) Rat [SD], bone marrow micronucleus	Anonymous, 2017a Acceptable
Clastogenicity *[EBNA] = 0.058 mg/kg*	**negative**	GLP, OECD TG 474 (2016) Rat [SD], bone marrow micronucleus	Anonymous, 2017b Acceptable

In vivo tests

The genotoxicity of benfluralin was tested *in vivo* in three acceptable bone marrow micronucleus tests conducted in rodents. In addition, a non-standard but acceptable Sister Chromatid Exchange (SCE) test was also available from the RAR (2019). There was no evidence from the available data set that benfluralin is a somatic cell mutagen.

Classification Assessment

No human data are available for benfluralin, therefore a classification with Muta. 1A is not supported. Benfluralin is negative in acceptable *in vitro* tests and negative in *in vivo* somatic cell mutagenicity guideline tests in mammals. Data are not available illustrating the induction of mutagenic effects in germ cells (a criterion for Category 1B). RAC does not support classification with Muta. 1A or B.

The overall weight of evidence for benfluralin supports no potential for genotoxicity in bacterial or somatic cells from a battery of *in vivo* and *in vitro* GLP and guideline compliant studies. Therefore, no classification in Category 2 is warranted. RAC notes that the genotoxic impurity EBNA, was tested up to a level of 0.31 mg/kg in older studies, all negative, but mainly supplementary by regulatory standards. In newer studies, EBNA was tested up to a level of 0.085 mg/kg.

RAC agrees with the DS that **classification of benfluralin for germ cell mutagenicity is not warranted**.

RAC evaluation of carcinogenicity

Summary of the Dossier Submitter's proposal

Two guideline- and GLP compliant long-term oral (dietary) toxicity/carcinogenicity studies were reported by the DS: a 2-year combined chronic toxicity/carcinogenicity study in the Fischer 344 rat (Anonymous, 1996) and a 2-year carcinogenicity study in the B6C3F1 mouse (Anonymous, 1988). Study details were summarised in Table 23 in the CLH report. Benfluralin had

treatment-related neoplastic findings in rats, the most significant being liver (males only) and thyroid tumours (both sexes, along with a slight increase of kidney adenoma (males) and Leydig cell tumours). An increased incidence of hepatocellular adenoma and carcinoma was observed in female mice. An additional study was conducted to investigate the MoA and human health relevance of the rodent tumours. This was also part of the RAR (2019) and described by the DS. Several additional and new studies were conducted to further investigate the MoA and human health relevance of the rodent tumours. These were made available after the EFSA peer review of the active substance and after completion of the CLH report. They have not been previously assessed by either the RMS or the DS. Please refer to the section on Additional key elements for the RAC assessment.

In vivo animal studies

Rat 2-year dietary toxicity/oncogenicity study

Benfluralin was assessed in a rat GLP- and OECD TG 453 (1981) compliant carcinogenicity dietary study (Anonymous, 1996). Significantly reduced survival rates were apparent in the males at ≥ 5.4 mg/kg bw/d (81%, 84%, 62%*, 66%*, and 64%* in controls, 0.5, 5.4, 136 and 275 mg/kg bw/d dose groups respectively). Signs of poor antemortem condition including hunched posture, thin appearance, prostration or entire body paleness were noted more frequently. Fischer 344 strain rats were divided into treatment groups and scheduled kills were conducted after 12 months treatment for 10 animals/sex/group and at study termination after 24 months treatment for the remaining nominal 50 animals/sex/group.

Table: *Mean dose of benfluralin (purity 95.8%) received (mg/kg bw/d)*

Dietary concentration of benfluralin (M/F ppm)	0	10	100	2500	5000
Males	0	0.5	5.4	136.3	274.8
Females	0	0.7	6.8	167.9	331.3

The liver, the thyroid and the kidneys were the target organs after long-term administration of benfluralin to the F344 rat. The maximum tolerated dose (MTD) was achieved. Significant treatment-related effects were observed. General toxicity was evident as significantly lower body weight gain compared to controls which amounted to 15% (males) and 32% (females) at 136/168 mg/kg bw/d, and to 30% (males) and 50% (females) at the top-dose of 275/331 mg/kg bw/d (in males and females respectively). Several other treatment effects were particularly noticeable at the mid to top dose groups and affected haematological parameters, clinical chemistry and urinalysis, but not to an extent that could support classification for STOT RE category 1 or 2.

Dose-related increased liver weights (males, females) and adrenal weights (females) at 136/168 mg/kg bw/d and higher were observed both at the interim and terminal sacrifice, while thyroid weight increase from this dose level was obvious at interim sacrifice. At terminal sacrifice there was still a strong response, i.e. substance related, but in males a dose response between the two top dose levels was not so clear and lacked statistical significance. In the thyroid, follicular hyperplasia was increased at 136/168 mg/kg bw/d (2500 ppm) and above and this was considered the first stage for neoplastic change.

The Fischer 344 rat is naturally susceptible to chronic progressive nephropathy (CPN) with high background incidences common in this strain making the interpretation of the condition difficult. While the incidence was similar across all groups, the severity of lesions associated with CPN were increased with the dose of benfluralin, both at the interim sacrifice and at the final sacrifice at levels of ≥ 5/7 mg/kg bw/d (100 ppm). The presence of hyaline and/or fine granular casts in the urine of animals at doses ≥ 136/331 (M/F) mg/kg bw/d correlated with the treatment-related

increase in severity of CPN. Several other lesions were noted (such as hyaline droplets in the renal tubule cells; tubule cell karyomegaly; transitional cell hyperplasia of the renal papilla) but may be associated with exacerbation of CPN by benfluralin and/or the significant presence of calculi in the renal pelvis. The DS postulated that the kidney calculi were the primary cause of both the exacerbation of nephropathy and of transitional cell hyperplasia, possibly leading to tubular cell adenoma in two males at the top-dose of 275 mg/kg bw/d.

Neoplastic findings

According to the CLH report there were three main tumour types of concern following benfluralin treatment:

1. Thyroid follicular adenoma and carcinoma in both sexes
2. Hepatocellular adenoma and carcinoma in males
3. Renal tubular cell adenomas in 2 males from the top dose group

The liver, the thyroid and the kidneys were the target organs after long-term administration of benfluralin to the F344 rat.

Thyroid follicular cell adenoma and carcinoma were increased at 136/168 (M/F) mg/kg bw/d (2500 ppm) and the high dose (table below 'RAC Overview of thyroid tumours in the long-term rat study'). A significant trend in neoplastic incidence was demonstrated for the males (adenoma) and for males and females combined.

At 136 mg/kg bw/d (2500 ppm) and above, the individual and combined incidences of hepatocellular adenoma and carcinoma were increased in males only (table below 'RAC Overview of liver tumours in the long-term rat study'). Both benign and malignant tumours occurred at greater than or equal to this level.

In the kidney the DS noted tubule cell adenoma (table below 'RAC overview of kidney tumours in the long-term rat study'), at the top dose of 275 mg/kg bw/d (5000 ppm), also confined to males (2/50, 4%). This was considered secondary to the observed CPN and associated tubule cell hyperplasia arising from irritation by pelvic calculi.

There were no HCD provided by the DS. Leydig cell tumours were also noted by the DS. At the top dose, the incidence of these tumours was slightly elevated, but only in the 1-year sacrifice group. These tumours occur with a naturally high incidence in F344 rats and cannot be considered to arise from a treatment related effect.

There were some minor deviations from OECD TG 453, but the DS considered the study acceptable for regulatory purposes. The DS noted the rat study was conducted with a batch of benfluralin containing a lower level of the impurity EBNA (0.04 mg/kg) than that tested in the mutagenicity studies (0.085 mg/kg) and concluded that benfluralin was unlikely to be genotoxic. However, the DS supported the RMS conclusion that benfluralin was to be considered carcinogenic in the rat liver and thyroid.

***Table**: RAC overview of thyroid tumours in the long-term rat study (Anonymous, 1996)*

Males					
Dose (mg/kg bw/d)	**0**	**0.5**	**5.4**	**136.3**	**274.8**
N. of animals week 104)	48	50	48	50	50
Follicular cell adenoma Follicular cell carcinoma	1 0	1 0	1 0	3 4	5 3
Combined	1	1	1	7	8

Females					
Dose (mg/kg bw/d)	**0**	**0.7**	**6.8**	**167.9**	**331.3**
N. of animals (week 104)	50	50	50	50	50
Follicular cell adenoma Follicular cell carcinoma	0 0	0 0	0 1	3 2	2 2
Combined	0	0	1	5	4

***Table**: RAC overview of liver tumours in the long-term rat study (Anonymous, 1996)*

Males					
Dose (mg/kg bw/d)	**0**	**0.5**	**5.4**	**136.3**	**274.8**
N. of animals (week 104)	48	50	48	50	50
Hepatocellular adenoma Hepatocellular carcinoma	1 1	2 0	1 0	3 2	9 2
Combined	2	2	1	5	11
Females					
Dose (mg/kg bw/d)	**0**	**0.7**	**6.8**	**167.9**	**331.3**
N. of animals (week 104)	50	50	50	50	50
Hepatocellular adenoma Hepatocellular carcinoma	2 0	0 0	1 0	1 0	2 0
Combined	2	0	1	1	2

***Table**: RAC overview of kidney tumours in the long-term rat study (Anonymous, 1996)*

Males					
Dose (mg/kg bw/d)	**0**	**0.5**	**5.4**	**136.3**	**274.8**
N. of animals (week 104)	48	50	50	50	50
Tubule cell adenoma	0	0	0	0	**2**
Large free pelvic calculi Small renal pelvic epithelial calculi	3 16	1 23	4 22	37 5	47 1
Females					
Dose (mg/kg bw/d)	**0**	**0.7**	**6.8**	**167.9**	**331.3**
N. of animals (week 104)	50	50	50	50	50
Tubule cell adenoma	0	0	0	0	0
Large free pelvic calculi Small renal pelvic epithelial calculi	6 39	7 31	8 38	30 18	26 15

B6C3F1 Mouse 2-year dietary toxicity/oncogenicity study

Benfluralin was also assessed in a mouse GLP- and OECD TG 451 (1981) partially compliant but acceptable chronic toxicity and carcinogenicity dietary study (Anonymous, 1988). For logistic reasons, the study was conducted as two replicates (30 mice/sex/dose in each replicate), with initiation dates separated by a 15-day interval. The replicate results were combined for the final report.

Unlike the animals in the rat study, survival was not significantly impacted by treatment. Apart from colouration of the urine (chromaturia), a common finding in animals given high doses of

dinitroanaline compounds, no other clinical signs of significance were noted. Small statistically significant decreases in body weight were observed from the lowest dose on throughout the study. At termination, results indicated only slight changes at 36 mg/kg bw/d (males) and above (males, females).

***Table**: Mean dose of benfluralin (purity 95.25-96.15%) received (mg/kg bw/d)*

Dietary concentration of benfluralin (M/F ppm)	0	50	300	1500
Males	0	6.0	36.4	184.7
Females	0	6.9	41.8	223.5

The target organs in the mouse were the kidney and the liver. Haematology was unremarkable at termination. Clinical chemistry showed increases in alkaline phosphatase (top dose) and alanine aminotransferase along with blood urea nitrogen levels (42 mg/kg bw/d and above) in females. No other renal involvement was noted. Treatment-related non-neoplastic lesions were essentially confined to the liver. At the top dose, liver weights were increased in both sexes (mean relative liver weight in males was approximately +9.5% and in females about +30% greater than in controls) and the incidence of hepatocellular hyperplasia was notably increased in males and females in the top dose group. The increased incidence of focal hyperplasia was accompanied by an increase in severity of the plastic response and a more frequent occurrence of multiple foci of hyperplasia (following a dose response) within affected livers of both sexes.

Neoplastic findings

According to the CLH report there was one main tumour type of concern following benfluralin treatment:

1. Hepatocellular adenoma and carcinoma in males and females

Increased incidences of adenoma (224 mg/kg bw/d) and of carcinoma (≥ 7 mg/kg bw/d) were especially noted in females due to low or no incidence in concurrent controls (table below). The combined incidence was also increased at 42 mg/kg bw/d onwards (females), and a statistically significant trend was detected when the Peto's survival adjusted trend test was applied ($p = 0.018$). These incidences were compared to the in-house contemporary historical control incidence of liver neoplasia in females, based on studies conducted after 1980 (the Anonymous, 1988 report was based on an in-life study period from 1985–1987). RAC notes that the individual incidences of adenomas and carcinomas (5.1%), and the combined incidence (10%), in the top dose group were outside the in-house HCD range for adenomas and combined adenomas & carcinomas only (0-3.4% and 0-6.9%, respectively). HCD were only reported for females. In the males, a high number of combined adenomas and carcinomas was observed (12/60, top dose group, 185 mg/kg bw/d), but this was not statistically increased relative to the concurrent controls due to the high number of hepatocellular carcinomas in the control group (7/60 animals).

***Table**: RAC overview of liver tumours in the long-term mouse study (Anonymous, 1988)*

Males				
Dose (mg/kg bw/d)	**0**	**6.0**	**36.4**	**184.7**
N. of animals	60	60	60	60
Hepatocellular adenoma: Hepatocellular carcinoma:	2 7	1 7	3 5	5 8
Combined	9	8	8	13

Females				
Dose (mg/kg bw/d)	**0**	**6.9**	**41.8**	**223.5**
N. of animals	60	60	60	59
Hepatocellular adenoma: Hepatocellular carcinoma:	1 0	1 2	1 3	3 3
Combined	1	3	4	**6**

There were some deviations from OECD TG 451 (1981) but the DS considered the study acceptable for regulatory purposes. The DS noted the mouse study was conducted with a batch of benfluralin containing a higher level of the impurity EBNA (0.31 mg/kg) than that tested in the mutagenicity studies (0.085 mg/kg). The DS concluded that benfluralin should be considered carcinogenic in the mouse liver based on the incidence of hepatocellular carcinomas in female mice.

MoA for benfluralin-induced tumours in F344/DuCrl rats

The DS presented a summary of a 14-day dietary study (Anonymous, 2010; RAR, section B.6.8.2), designed to examine the possible MoA for benfluralin-induced tumours in F344/DuCrl rats. Male rats (6/dose/time period) were exposed to 0 and a mean of 440 mg/kg bw/d (5000 ppm) benfluralin in the diet for 7 or 14 days. The dose levels were above the known tumorigenic dose following data from the 2-year dietary rat study.

The main endpoints examined provided support for liver and thyroid tumour induction via the constitutive androstane receptor (CAR) and UDP-glucuronyltransferase (UGT)-mediated MoAs:

i. Thyroid function analysis (T_4, T_3, TSH, histopathology)
ii. Liver and thyroid organ weight increases
iii. Liver and thyroid follicular cell proliferation
iv. Targeted gene-expression
v. Liver metabolic enzyme activities

i. Thyroid function analysis

According to the DS, the available mechanistic data proposes a rodent specific explanation for the observed tumours in the thyroid. A decrease in T4 was demonstrated in the MoA study (table below). However, the DS did not take note of the compensatory increase in TSH at 7 days, instead proposing no effect on TSH based on the 14-day result alone. The DS provided evidence from further studies with benfluralin that strengthened the argument that the decrease in T4 stimulates the pituitary to release more TSH that drives the thyroid to make more T4. In the SD rat, 14-day oral gavage pubertal assays conducted with benfluralin (RAR, section B.6.8.3.4), decreased T4 levels corresponded with increases in TSH levels. These studies were GLP- and USEPA OPPTS 890.1450 (2009) guideline compliant. In the female pubertal assay (Anonymous, 2012), decreased T4 levels were demonstrated in the mid (75 mg/kg bw/d) and high-dose (300 mg/kg bw/d) groups with corresponding increases in TSH levels in the highest dose group. In males, T4 levels were reduced in all dose groups (25, 100 and 400 mg/kg bw/d) with corresponding increases in TSH levels at the two highest dose groups and significantly increased thyroid weights and altered thyroid histopathology in the top dose group. The DS outlined a conservative opinion stating that the MoA for thyroid tumours had not been clearly demonstrated.

***Table**: RAC overview of thyroid function and organ weights (Anonymous, 2010)*

Exposure length	**7 days**		**14 days**	
Dose (mg/kg bw/d)	**0**	**449**	**0**	**436**
N. of animals	6	6	6	6
T3 (ng/dL) T4 (µg/dL) TSH	115 5.6 3.6	**94*** (- 18.3%) **2.2***(- 60.7%) 5.3 (+ 47%)	118 5.0 4.8	106 (- 10%) **2.6 (- 48%)*** 4.6
Liver weight absolute relative		 + 25%* + 30%*		 +30%* +35%*
Thyroid weight absolute relative		 + 17%* + 21%*		 +16%* +22%*

ii. Liver and thyroid organ weights

All repeat dose studies show that benfluralin is a strong growth promoter of the liver. At 7 and 14 days, absolute and relative liver weights were elevated. A similar effect was noted for the thyroid (table above).

iii. Liver and thyroid follicular cell proliferation

Histopathological changes were observed in the liver and thyroid. Benfluralin-induced liver effects consisted of very slight hypertrophy of hepatocytes in the centrilobular and midzonal regions of the hepatic lobule. In the thyroid gland, treatment-related microscopic changes were minimal. Very slight follicular cell hypertrophy was seen in 3 out of 6 treated males after 7 days of benfluralin exposure and 4 out of 6 treated males following 14 days of exposure.

Incorporation of 5-bromo-2'-deoxyuridine (BrdU; a structural analogue of thymidine) into nuclear DNA was used as a surrogate marker for cell proliferation. Rats were continuously infused with BrdU via implanted osmotic pumps. At 7 days, hepatic proliferation was significantly elevated only in the periportal region (1.7-fold higher), whereas by 14 days, proliferation was significantly elevated in all regions of the liver (centrilobular, midzonal, and periportal; 1.4-fold, 1.2-fold and 1.6-fold, respectively). Thyroid follicular cell proliferation was significantly elevated at 7 days (1.6-fold), however, this returned to near control levels by day 14 (1.2-fold, non-significant).

iv. Targeted gene-expression

A summary of targeted gene expression is presented in the table below. The CAR-related transcripts, Cyp2b1 and Cyp2b2 were significantly elevated at both 7 and 14 days. The PXR-related transcript, Cyp3a3 was also significantly elevated at these two time points. The PPARα-related transcript, Cyp4a1 was not significantly altered at 7 or 14 days. The two T4-specific UGT genes, Ugt1a6 and Ugt2b17, were significantly elevated at both 7 and 14 days.

***Table**: RAC overview of targeted gene expression (at the RNA level) expressed as fold-change relative to concurrent controls (Anonymous, 2010)*

Exposure length	**7 days**		**14 days**	
Dose (mg/kg bw/d)	**0**	**449**	**0**	**436**
N. of animals	6	6	6	6
CAR: Cyp2b1 Cyp2b2	1 1	**475*** **11***	1 1	**344*** **12***
PXR: Cyp3a3	1	**9.4***	1	**7.5***

PPARα: Cyp4a1	1	0.9	1	1
CAR (T4 specific): Ugt1a6 Ugt2b17	 1 1	 **7.4*** **7.3***	 1 1	 **6.9*** **8.3***

* Criteria for substantial induction of the gene: significantly different at p = 0.05 and ≥ 1.5-fold-change in mRNA expression

v. Liver metabolic enzyme activities

Liver EROD (AhR), PROD (CAR), and UGT-related enzyme activities were all significantly elevated at 7 and 14 days (table below).

***Table**: RAC overview of liver enzyme activity (at the protein level) expressed as fold-change relative to concurrent controls (Anonymous, 2010)*

Exposure length		**7 days**		**14 days**	
Dose (mg/kg bw/d)		**0**	**449**	**0**	**436**
N. of animals		6	6	6	6
AhR/CAR:	EROD	1	**3.5***	1	**3.3***
CAR:	PROD	1	**6.6***	1	**4.5***
	UGT	1	**2.5***	1	**2.7***

* Criteria for substantial induction of the gene: significantly different at p = 0.05 and ≥ 1.5-fold-change in mRNA expression

Summary

There were important indicators that CAR may be involved as a mechanism for benfluralin-induced liver tumours. There were elevated levels of the CAR-related gene transcripts Cyp2b1, Cyp2b2, and the two T4-specific UGT genes, Ugt1a6 and Ugt2b17. There were elevated activities of the hepatic enzymes PROD, UGT, and crosstalk from CAR that may explain the elevated EROD activity (rather than activation of the Ah-receptor), as well as clear evidence for hepatocellular proliferation. The PPARα-related transcript, Cyp4a1 was not altered at 7 or 14 days.The DS concluded that the available mechanistic data supported a rodent specific explanation for the observed tumours (CAR) but due to a lack of further investigation (i.e. stimulation of hepatocyte cell division and use of CAR-knock out models) the DS remained unconvinced that the relevance to humans had been sufficiently explored.

With respect to the possible mechanism for thyroid tumours, greater expression of UGT mRNA transcripts and increased UGT enzymatic activity along with a decrease in T4 was demonstrated. Decreased T4 levels corresponded with increases in TSH levels in the pubertal assays conducted with benfluralin, but this was not corroborated by other studies. The effects on thyroid hormone homeostasis could be due secondary to liver enzyme induction, and may be responsible for the thyroid tumours in male Fischer 344 rats following chronic exposure to benfluralin. However, no testing strategies for alternative MoAs were employed in the Anonymous (2010) study. The DS concluded that there were no effect on TSH from this study while at the same time providing evidence for such an effect in other studies (pubertal assays, Anonymous, 2012).

Overall, the DS considered there was enough evidence to establish a positive association between exposure and tumours and to suspect that benfluralin was carcinogenic and proposed classification for benfluralin as Carc. 2; H351.

Comments received during consultation

In the response to comments document following the consultation of the CLH report, there were two comments provided, one from an MSCA supporting the DS with classification for carcinogenicity (Carc. 2; H351) and one comment from industry supporting no classification.

The MSCA outlined similar reasons to those given by the DS in supporting Carc. 2:

- Increased incidence of (mostly benign) liver and (benign as well as malignant) thyroid tumours in the long-term study in the rat.
- Increase in liver cell adenoma and carcinoma in female mice.
- Mechanistic studies do not exclude human relevance.
- Concluded there was a multi-site response in two species from which human relevance could not be excluded.
- Acknowledged that effects occurred at high dose levels resulting in significant non-neoplastic pathological findings, organ weight changes, reduced survival, and a flat dose response in mice for the liver tumours and supported a Category 2 classification.

Industry, however, provided new mechanistic data to address the concerns over the human relevance of the tumours in liver and thyroid. A set of new studies were provided that have not been assessed prior to this opinion document and were not available at the time of drafting the RAR or the CLH report. The DS has not evaluated these studies. Industry concluded that a CAR/PXR driven MoA for liver tumours was demonstrated and that this is not relevant for humans. Similarly, further studies on the thyroid were provided to support the rodent-specific UDPGT-driven thyroid tumorigenesis observed in the chronic rat study.

Additional studies

Introduction

New data from a series of additional studies or published reports were made available to address the human relevance of liver and thyroid tumours seen in long-term studies with benfluralin in rats and mice. The new information may be broadly defined into two categories:

1. *In vitro* hepatocellular proliferation studies comprising;
    i. cultured hepatocytes from wildtype Sprague-Dawley rats,
    ii. cultured hepatocytes from CAR/PXR-double knockout SD rats,
    iii. cultured hepatocytes from F344 rats,
    iv. cultured hepatocytes from B6C3F1 mice,
    v. cultured hepatocytes from human donors.

2. Thyroid MoA studies comprising;
    i. an *in vivo* 90-day study in male and female F344 rats with oral administration of benfluralin via diet (Anonymous, 2019),
    ii. an investigation on a direct effect on the thyroid by inhibition of the Thyroid Peroxidase enzyme in rat thyroid microsomes (Anonymous, 2019),
    iii. peer reviewed publication from ToxCast data with information about the lack of benfluralin inhibition on the sodium iodide symporter.

Points of note:

- In the *in vitro* studies there was precipitation of compound at both 100 µM (slight) and 300 µM benfluralin.
- Batch 1919 used in the *in vitro* studies had a high content of ethyl-butyl nitrosamine (0.18 mg/kg EBNA).

- The number of animals used in the cultured hepatocyte tests was not stated in the original study reports whereas a single animal was used to harvest cells for the cytotoxicity range-finding studies.
- Independent tests for hepatocellular proliferation were conducted on hepatocytes cultured from 3 human donors (2 males, 1 female).
- A 4th donor (male) was also tested but categorised the results as abnormal.

In vitro studies

The objective of these studies was to investigate the potential of benfluralin to stimulate cell proliferation in the hepatocytes of wildtype rats and mice in comparison with human hepatocytes to assess the relevance of the liver effects in the rodents to humans. Furthermore, proliferation was also measured in CAR^{KO}/PXR^{KO} (Constitutive androstane receptor-knockout/ pregnane X receptor-knockout) rat hepatocytes to evaluate the relevance of CAR and PXR for the liver tumour MoA.

Prior to conducting the proliferation studies, cytotoxicity was determined by measuring adenosine 5´-triphosphate (ATP) levels relative to those levels in concurrent control samples. Concentrations up to the limit of cytotoxicity were used in the main investigations. Epidermal growth factor (EGF) and phenobarbital (PB) served as positive mitogenic controls.

In vitro mouse studies

Treatment with benfluralin stimulated cell proliferation in cultures of primary hepatocytes isolated from male and female B6C3F1 mice in a dose dependent manner to a maximum of 2.6-fold at 100 µM (table below). This was similar to or greater than the effect produced by the positive control PB.

***Table**: summary of the mouse in vitro studies*

Study	Result	Test System	Reference
***In vitro* mouse studies:**			
#1. Benfluralin - cytotoxicity range finding study in cultured female B6C3F1 mouse hepatocytes	Benfluralin concentrations in the main study were 3, 10, 30 and 100 µM. Exposure to 300 µM led to an ATP reduction (~ 33%).	GLP, non-guideline 1° hepatocytes → one female B6C3F1 mouse Batch 1919; 96.4% [EBNA] = 0.18 mg/kg	Chatham, 2019
#2. Benfluralin - induction of DNA-synthesis in cultured female B6C3F1 mouse hepatocytes	Benfluralin was tested at 3, 10, 30 and 100 µM. PB at 1 mM and EGF at 25 ng/mL. No cytotoxicity. Treatment of cultured B6C3F1 hepatocytes with benfluralin resulted in increases in cell proliferation (BrdU) at ≥ 10 µM. Increases above controls of 1.2; **1.2, 1.9, 1.8, 2.6** and 4.1-fold were observed for PB, **benfluralin** and EGF, respectively. PB and EGF positive controls performed well.	GLP, non-guideline 1° hepatocytes → number of female B6C3F1 mice not reported Batch 1919; 96.4% [EBNA] = 0.18 mg/kg	Chatham, 2019
#3. Benfluralin - cytotoxicity range finding study in cultured male B6C3F1 mouse hepatocytes	Benfluralin concentration used in the main study were set at 10, 30, 100 and 300 µM. Exposure to 300 µM led to an ATP reduction (~ 13%).	GLP, non-guideline 1° hepatocytes → one male B6C3F1 mouse Batch 1919; 96.4% [EBNA] = 0.18 mg/kg	Chatham, 2019
#4. Benfluralin - induction of DNA-synthesis in cultured male B6C3F1 mouse hepatocytes	Benfluralin was tested at 10, 30, 100 and 300 µM. PB at 1 mM and EGF at 25 ng/mL. Significant **cytotoxicity** at ≥ 100 µM benfluralin. Treatment of cultured B6C3F1 hepatocytes with benfluralin resulted in increases in cell proliferation (BrdU) at 10 µM and 30 µM (max concentration tested).	GLP, non-guideline 1° hepatocytes → number of male B6C3F1 mice not reported Batch 1919; 96.4% [EBNA] = 0.18 mg/kg	Chatham, 2019

	Observed increase above controls were 1.5, **1.7 to 2.4,** and 9.4-fold for PB, **benfluralin** and EGF respectively. PB and EGF positive controls performed well.		

In vitro rat studies

Wild-type Sprague Dawley Rats

Treatment with benfluralin stimulated cell proliferation in cultures of primary hepatocytes isolated from Sprague Dawley rats (strain from whose genetic background CAR/PXR knockout rats are available), in a dose dependent manner to a maximum of 1.8-fold at 300 µM (table below). This was similar to or greater than the effect produced by the positive control PB.

Wild-type Fischer 344 Rats

Treatment with benfluralin tested at 3, 10, 30 and 100 µM was not very convincing from a dose response point of view. Benfluralin stimulated cell proliferation in cultures of primary hepatocytes isolated from F344 rats at 10 µM only with a maximum induction of 1.4-fold. Significant cytotoxicity was evident at 100 µM.

***Table**: summary of the wild-type rat in vitro studies*

Study	Result	Test System	Reference
***In vitro* rat studies**			
#5. Benfluralin - cytotoxicity range finding study in cultured male SD rat hepatocytes	Benfluralin concertation used in the main study were set at 10, 30, 100 and 300 µM. Exposure to 300 µM led to an ATP reduction relative to controls (~ 13%).	GLP, non-guideline 1° hepatocytes → one male SD rat Batch 1919; 96.4% [EBNA] = 0.18 mg/kg	Chatham, 2019
#6. Benfluralin - induction of DNA-synthesis in cultured male SD rat hepatocytes	Benfluralin was tested at 10, 30, 100 and 300 µM. PB at 1 mM and EGF at 25 ng/mL. Some cytotoxicity at 300 µM benfluralin (ATP reduction ~ 20%). Treatment of cultured SD rat hepatocytes with benfluralin resulted in dose related increases in cell proliferation (BrdU) at < 300 µM. Observed increase above controls were 1.4; **1.1, 1.4, 1.8, 1.8** and 3.4-fold for for PB, **benfluralin** and EGF. PB and EGF positive controls performed well.	GLP, non-guideline 1° hepatocytes → number of male SD rat donors not reported. Batch 1919; 96.4% [EBNA] = 0.18 mg/kg	Chatham, 2019
#7. Benfluralin - cytotoxicity range finding study in cultured male F344 rat hepatocytes	Benfluralin concentration in the main study were set at 3, 10, 30, and 100 µM. Exposure to 300 µM led to an ATP reduction relative to controls (~ 36%).	GLP, non-guideline 1° hepatocytes → one male F344 rat Batch 1919; 96.4% [EBNA] = 0.18 mg/kg	Chatham, 2019
#8. Benfluralin - induction of DNA-synthesis in cultured male F344 rat hepatocytes	Benfluralin was tested at 3, **10**, 30 and 100 µM. PB at 1 mM and EGF at 25 ng/mL. Significant **cytotoxicity** at 100 µM benfluralin (ATP reduction ~ 31%). Treatment of cultured F344 rat hepatocytes with benfluralin (max 100 µM) did not result in a clear proliferative response (BrdU) with dose. Observed increase above controls were 1.3, **1.1, 1.4, 1.1, 1.1** and, 4.8-fold increase for PB, **benfluralin** and EGF respectively. PB and EGF positive controls performed well.	GLP, non-guideline 1° hepatocytes → number of male F344 rat donors not reported. Batch 1919; 96.4% [EBNA] = 0.18 mg/kg	Chatham, 2019

CAR^{KO}/PXR^{KO} double knockout rats

This study investigated the potential effect of benfluralin to stimulate cell proliferation in cultures of primary hepatocytes isolated from male Sprague Dawley rats lacking a functional constitutive androstane receptor and pregnane X receptor (CAR^{KO}/PXR^{KO} rats). Benfluralin concentrations ≤ 300 µM did not result in cell proliferation in cultured hepatocytes from CAR^{KO}/PXR^{KO} rats which supports the proposed liver tumour MoA via CAR/PXR induction (table below).

***Table**: summary of the transgenic rat in vitro studies*

Study	Result	Test System	Reference
***In vitro* CAR^{KO}/PXR^{KO} double knockout rat studies:**			
#9. Benfluralin - cytotoxicity range finding study in cultured male CAR^{KO}/PXR^{KO} SD rat hepatocytes	Benfluralin concentration were 10, 30, 100 and 300 µM. Exposure up to 300 µM → no cytotoxicity → no ATP reduction.	GLP, non-guideline 1° hepatocytes → one male CAR^{KO}/PXR^{KO} SD rat Batch 1919; 96.4% [EBNA] = 0.18 mg/kg	Chatham, 2019
#10. Benfluralin - induction of DNA-synthesis in cultured male CAR^{KO}/PXR^{KO} SD rat hepatocytes	Benfluralin was tested at 10, 30, 100 and 300 µM. PB at 1 mM and EGF at 25 ng/mL. No evidence of cytotoxicity at any concentration of benfluralin. Treatment of CAR/PXR double knockout rat hepatocytes with benfluralin or PB resulted in **no** increases in cell proliferation. A change/increase of 0.9, **0.9, 0.9, 0.9, 0.8** and 3.5-fold, relative to control were observed for PB, **benfluralin** and EGF respectively. PB → no cell proliferation EGF → enhanced cell proliferation.	GLP, non-guideline 1° hepatocytes → number of male CAR/PXR double knockout SD rat donors not reported. Batch 1919; 96.4% [EBNA] = 0.18 mg/kg	Chatham, 2019

In vitro cultured human hepatocyte studies

In human hepatocytes no proliferation occurred after treatment with benfluralin at concentrations of up to 30 µM, based on the data from three donor hepatocyte studies (table below). A 4th donor (male, 385), was described as an outlier by the Notifier because of an increase in the relative S-phase labelling index. What fails to be noticed is that reliance on the relative change of hepatocyte proliferation as a general index of the mitogenic strength of a substance ignores the absolute measure of the S-phase labelling index itself. The results from all 4 donors indicate just how low the basal rate of hepatocyte proliferation is in humans (0.1-0.2) relative to wild-type rats (5.5-6.0). The mitogenic stimulus is far stronger in all the rat studies compared with the response by any of the hepatocytes from the human donors. The basal rate of hepatocyte proliferation in the double knockout rats is also very high (4.5) but they are refractory to a mitogenic stimulus from benfluralin.

***Table**: summary of the human donor hepatocyte in vitro studies*

Study	Result	Test System	Reference
***In vitro* Human hepatocyte studies:**			
#11. Benfluralin - cytotoxicity range finding study in cultured human hepatocytes	Benfluralin concentration used in the main study were set at 1, 3, 10, 30 and up to 100 µM for 1 donor. Cytotoxicity was evident at 100 µM for 2 donors (ATP ↓55%) and at 300 µM for 1 male donor (ATP ↓24%).	GLP, non-guideline 1° hepatocytes → 3 human donors (2M, 1F) Batch 1919; 96.4% [EBNA] = 0.18 mg/kg	Chatham, 2019
#12. Benfluralin - induction of DNA-synthesis in cultured human hepatocytes	Benfluralin was tested at 1, 3, 10, 30 and 100 µM. PB at 1 mM and EGF at 25 ng/mL.	GLP, non-guideline 1° hepatocytes → 3 human	Chatham, 2019

	No evidence of cytotoxicity at ≤ 10 µM benfluralin. Donor 8210 (M) cytotoxicity at 30 µM benfluralin (ATP ↓40%). Donor 8219 (M) cytotoxicity at 100 µM benfluralin (ATP ↓45%). Donor 8239 (F) no significant cytotoxicity up to 30 µM benfluralin (ATP ↓15%). Treatment of human hepatocytes with benfluralin resulted in **no** increases in cell proliferation (tested up to 30 µM). PB → no cell proliferation in 2 donors (8210, 8219). PB → proliferation in 1 donor (8239, highly variable). EGF → enhanced cell proliferation of 4.5, 5.4 and 8.9-fold increase relative to controls for donors 8210, 8219 and 8239 respectively.	donors (2M, 1F) Batch 1919; 96.4% [EBNA] = 0.18 mg/kg --- *Note:* a fourth donor (#385) was also tested in an independent 2018 study. The results were categorised as outliers because increases in cell proliferation of 2.1, 1.5 and 2.3-fold relative to controls wre observed at 30, 100 and 300 µM benfluralin. There was little to no cytotoxicity up to 300 µM benfluralin. PB → no cell proliferation EGF → enhanced cell proliferation (5.3-fold induction).	

Summary of the *in vitro* cultured hepatocyte studies

No proliferation was seen in human hepatocytes from 3 donors. An increase in the proliferation of hepatocytes was only observed in hepatocytes from wild-type rats and mice, but not in CAR/PXR double knockout rats which provides support for a CAR/PXR-mediated mechanism responsible for the liver tumour induction of benfluralin. There were four main weaknesses associated with these studies:

1. It is unclear how many rats were used to harvest hepatocytes for each study. A single animal would not constitute a representative sample of the rat strain population.
2. The results from the Fischer 344 rat study were not particularly convincing from a dose response point of view. They indicate a mitogenic response at 10 µM benfluralin only but not at higher concentrations (30 or 100 µM). Cytotoxicity may explain the low response at 100 µM but not at 30 µM. Essentially this is weak support for a similarity of response between the F344 rat and the SD rat strains. The responses by the positive controls were in line with expected results.
3. The results of a test in another (4^{th}), human donor (male, 385), which was performed separately from the other 3 donors were not in line with the toxicity and proliferation data of the other donors, i.e. there was evidence of hepatocyte proliferation at 30, 100 and 300 µM benfluralin albeit with a large degree of variability. The original study report noted that the control S-phase fold-induction data for PB and EGF were consistent with the long-term HCD for male human hepatocyte cultures in the test facility. Yet, it must be noted that this response in terms of the absolute S-phase labelling index remains greatly reduced relative to the responses in the rat. The PB stimulus was minimal as expected and the EGF response was moderately strong, also as expected. It is uncertain why the results for this individual should have any less biological relevance than those of the other donors and it may indicate that a large number of donors is crucial for interpreting these kinds of *in vitro* assays to allow for normal variation in the tested population.
4. It is unclear why the B6C3F1 mouse strain was used: this strain has a very high and variable background incidence in liver adenomas and carcinomas in both sexes that makes it unsuitable as a platform for testing liver tumour susceptibility to exogenous compounds. In any case, the results indicate that hepatocyte proliferation may be stimulated by benfluralin, PB and EGF.

Thyroid mode of action studies

Since in rats and mice the liver was a main target, increased metabolic enzyme induction as an underlying mechanism for the thyroid tumours may be likely. The data made available to RAC sought to provide clarity with respect to perturbations in T4 metabolism due to induction of liver phase II UDPGT conjugating enzymes and also test if there were any direct actions on the thyroid itself that could explain changes to this gland.

90-day study in male and female F344 rats with oral administration of benfluralin via diet (Anonymous, 2019)

One group of Fischer 344 rats (10/sex) was administered the test item, benfluralin (batch 16/2018, purity 96.4%, [EBNA] not stated), via a dietary admixture at a concentration of 10000 ppm for 13 weeks (table below). Another group of 10 males and 10 females received only the untreated diet under the same experimental conditions and acted as a control group.

Thyroid hormone measurements were performed on day 29 and at the end of the treatment period (day 89). At necropsy, liver, thyroids with parathyroids, and pituitary gland were weighed and preserved for microscopic examination.

There were no unscheduled deaths. Several clinical signs such as thin appearance, piloerection, soiled urogenital region and hunched posture were reported. No behavioural or neurological abnormalities were observed during the functional observation battery tests for any animal. Small decreases in body weight were observed at the end of the treatment period (-5% and -9% compared to controls for males and females, respectively).

***Table**: Mean dose of benfluralin received (mg/kg bw/d)*

Dietary concentration of benfluralin (ppm)	**0**	**10000 (M)**	**10000 (F)**
Achieved dose (mg/kg bw/d)	0	557	595

Increased liver weights in animals given the top dose of benfluralin were noted in both sexes and correlated with a centrilobular or diffuse hepatocellular hypertrophy in both sexes. Significantly increased thyroid weights were noted in males and a similar trend was noted in females. These changes correlated with the minimal to slight follicular cell hypertrophy/hyperplasia noted in the thyroid gland. Significantly lower mean T4 concentrations ranging from -57 to -77% compared to control for the males and from -52 to -65% compared to control for the females after 4 and 13 weeks of treatment were reported concomitantly to an increase in TSH levels (1.9/1.5- and 1.75/2.8-fold induction in week 4 and 13 for males and females respectively compared to control values) while T3 hormone levels were slightly increased (table below).

***Table**: Overview of thyroid hormone and organ weight changes with treatment (Anonymous, 2019)*

Exposure length	**Day 29**				**Day 89**			
Dose (mg/kg bw/d)	**0**	**M (557)**	**0**	**F (595)**	**0**	**M (557)**	**0**	**F (595)**
N. of animals	10	10	10	10	10	10	10	10
T3 (ng/mL) T4 (ng/mL) TSH (pg/mL)	0.27 35.8 2534	**0.38**** **8.1**** **4923****	0.52 23.9 1058	0.52 **8.3**** **1585***	0.44 39.4 2425	**0.58**** **16.7**** **4252****	0.47 25.2 713	**0.60**** **12.2**** **2016****
Liver weight absolute relative						**+53%**** **+61%****		**+45%**** **+61%****
Thyroid weight absolute relative						**+20%*** **+27%****		+1% +12%

Statistically significantly different: * $p < 0.05$, ** $p < 0.01$

Conclusion

These results support the proposed liver enzyme induction MoA on the thyroid via increased T4 metabolism and clearance with subsequently lowered T4 levels and a corresponding feedback-initiated mechanism that increases TSH levels. After prolonged exposure in rodents, this leads to follicular cell hypertrophy/hyperplasia and finally could give rise to thyroid tumours.

Inhibition of rat microsomal thyroid peroxidase by benfluralin (Anonymous *et al*., 2019)

To investigate whether a direct effect on the thyroid could have been involved in the promotion of tumours, the potential of benfluralin (batch 16/2018, purity 96.4%, [EBNA] not stated), to inhibit the thyroid peroxidase (TPO) enzyme was investigated in rat thyroid microsomes. Thyroid homogenates were prepared from 5 rats, strain not specified. The thyroid peroxidase activity determination was performed using the fluorogenic substrate Amplex UltraRed (AUR) which is converted to fluorescent Amplex UltroRex by TPO in the presence of excess H_2O_2. A convincing inhibition curve was generated for 6-N-Propyl-2-Thiouracil (PTU).

TPO was inhibited 76% by the positive control PTU with an IC_{50} of 0.79 µM. Benfluralin did not show any inhibition of TPO activity up to its highest soluble concentration in the incubation medium of 100 µM.

Conclusion

These results suggest the promotion of thyroid tumours by benfluralin is not via the mechanism of TPO inhibition.

Other studies investigating thyroid effects

Thyroid uptake of iodide via the sodium-iodide symporter (NIS) is the first step in the biosynthesis of thyroid hormones that are critical for health and development. Studies on NIS inhibition performed with a variety of compounds, including benfluralin, were described in a publication (Wang *et al.*, 2018)[1]. This study applied a previously validated high-throughput approach to screen for NIS inhibitors in the ToxCast phase I library. Benfluralin was negative in this test so, based on this result, no evidence of a direct thyroid effect via NIS inhibition was detected.

Assessment and comparison with the classification criteria

Introduction

There were increased incidences of 4 main types of tumours seen in long-term studies with benfluralin in rats and mice:

1. Hepatocellular adenoma and carcinoma in male Fischer 344 rats.
2. Thyroid follicular adenoma and carcinoma in both sexes in Fischer 344 rats.
3. Renal tubular cell adenomas in 2 males from the top dose group in Fischer 344 rats.

[1] High-Throughput Screening and Quantitative Chemical Ranking for Sodium-Iodide Symporter Inhibitors in ToxCast Phase I Chemical Library. Jun Wang, Daniel R. Hallinger, Ashley S. Murr, Angela R. Buckalew, Steven O. Simmons, Susan C. Laws, and Tammy E. Stoker. *Environmental Science & Technology* **2018** *52* (9), 5417-5426

4. Hepatocellular adenomas and carcinomas at the highest dose in both sexes of B6C3F1 mice.

Fischer 344 rat - Hepatocellular adenoma and carcinoma

The benign and malignant liver tumours in the male F344 rat (above table 'RAC overview of liver tumours […]') are the key reasons for consideration of classification of benfluralin as a carcinogenic substance.

The liver is a key target organ for benfluralin. Dose-related increased liver weights (males, females) at 136/168 mg/kg bw/d and higher were observed both at the interim and terminal sacrifice. Hepatocellular hypertrophy was also seen within 14 days (prepubertal studies, Anonymous, 2012). In the subchronic toxicity studies in rats, hepatocellular hypertrophy was seen at the end of the 90-day studies (including the new 90-day thyroid toxicity study, Anonymous, 2019). Neoplastic liver findings were only seen in the long-term studies, there was no indication of reduced tumour latency.

Proposed MoA for the liver tumours

The postulated MoA is that the activation of CAR and PXR nuclear receptors in male rats results in the altered expression of several genes as well as an increase in hepatic cell proliferation leading to hepatocellular tumours.

The human relevance framework has been used to assess the human relevance of the rodent tumours. The DS could not exclude the relevance of these tumours and, based on insufficient data from existing mechanistic studies and a lack of more in-depth mechanistic studies geared towards investigating the involvement of CAR as a MoA, concluded benfluralin was carcinogenic in the rat liver.

Is the weight of evidence (WoE) provided sufficient to establish the MoA in animals in the case of benfluralin?

In addition to the studies already presented in both the RAR and CLH report, new *in vitro* mechanistic data has become available since 2019. Both phenobarbital and EGF were utilised as positive control mitogens. This data is considered in this assessment.

Three key events have been considered: the activation of CAR/PXR nuclear receptors, hepatocellular proliferation and expression of key genes and enhanced transcription of liver enzymes.

Activation of CAR and PXR nuclear receptors

Note: there were no *in vivo* mechanistic studies that utilised CAR/PXR knockout animals. Hepatocellular proliferation was briefly investigated over 7 and 14 days in the Anonymous (2010) mechanistic study. Several CAR/PXR-related effects were observed:

- All repeat dose studies show that benfluralin is a strong growth promoter of the liver. At 7 and 14 days, absolute and relative liver weights were elevated (Anonymous, 2010), similarly after 90 days (Anonymous, 2019) and 2-years (Anonymous, 1996).
- *In vivo* hepatic proliferation was significantly elevated throughout all areas of the liver after 14 days of treatment with benfluralin (Anonymous, 2010).
- Elevated CAR/PXR-related mRNA transcripts from target genes, table 'RAC overview of liver enzyme activity […]' (Cyp2b1, Cyp2b2, Cyp3a3, Ugt1a6, Ugt2b17).
- Elevated CAR/PXR-related liver enzyme activities, table 'RAC overview of liver enzyme activity […]' (EROD, PROD, UGT).

- New *in vitro* mechanistic studies using rat hepatocytes showed increased hepatic proliferation in both SD and F344 strains though the response in the F344 rat was not robust nor especially convincing. In addition, a robust response was observed in mice hepatocytes.
- *In vitro* mechanistic studies with CAR^{KO}/PXR^{KO} double knock out SD rats do not show hepatic proliferation upon treatment with benfluralin.
- *In vitro* mechanistic studies with human hepatocytes from several donors show (1) low basal levels of proliferation relative to rats, and (2) no enhanced proliferation with benfluralin treatment. An extra 4^{th} donor gave some curious results, but the levels of proliferation remained much lower than in the rat.

Enzyme markers for CAR mediated gene induction were increased as shown by enhanced activity of PROD and UGT. The increase in EROD activity must be interpreted with care as there can be significant overlap between CAR and AhR mediated induction of this enzymatic activity. The liver induction profile of benfluralin was thus consistent with CAR/PXR activation. Although no comparison with a positive control was performed in the Anonymous (2010) study, Cyp2b1 induction was higher than Cyp3a3 induction as observed with a primary CAR rather than a PXR activator. Hepatocellular proliferation as shown by BrDU labelling of hepatocytes was statistically significantly increased in both *in vivo* and *in vitro* studies. Hepatocellular proliferation was not investigated in longer term studies.

Exclusion of alternative MoA

- Genotoxicity as a potential MoA for liver tumours can be excluded based on a complete data package *in vitro* and *in vivo* addressing mutagenicity and clastogenicity.
- The presence of the genotoxic impurity EBNA has been documented in most studies and the levels in the rat long term study (0.04 mg/kg), were much lower than in many other studies (including those in the mutagenicity data pack). Therefore, it is unlikely to have contributed to the tumorigenic profile of the rat.
- No evidence of activation of PPARα was noted in the 14-day study. Therefore, peroxisomal proliferation can be ruled out (Anonymous, 2010).
- The *in vitro* rat CAR^{KO}/PXR^{KO} double knockout study (Anonymous, 2019) showed that the presence of functional CAR and/or PXR appeared essential for the initial hepatic proliferative response from both benfluralin and phenobarbital. Indeed, in contrast with the results observed in the *in vitro* study performed with wild-type rat hepatocytes, no cell proliferation was observed at non-cytotoxic concentrations either with benfluralin or phenobarbital.
- Cyp1a1 was not tested, thus AhR activation cannot be ruled out even though EROD activity was increased and there can be some overlap in the expression profiles from CAR and AhR ligands.
- There was some evidence of liver cytotoxicity, an increase in the incidence of single cell necrosis was observed in a dose related manner in males from the 2-year rat study [2, 6, 5, 15*, 27* at 0, 0.5, 5.4, 136 and 275 mg/kg bw/d respectively]. Cytotoxicity may not be ruled out completely, but it does not appear to constitute a major MoA for rat liver tumours in this case. See further discussion below.
- There is no data suggesting that other MoA such as porphyria, statins/altered cholesterol synthesis, oestrogenic activity and immunosuppression could be responsible.

Cytotoxicity

Cytotoxicity, followed by regenerative cell proliferation, is a widely recognised, well characterised nongenotoxic MoA. In the case of benfluralin, some cytotoxicity was evident, but the effects were not convincing to support this as a major MoA for the observed liver tumours. The hepatocellular necrosis was described as predominantly individual cell necrosis with occasional areas of coagulation necrosis (table 'RAC overview of liver histopathology […]' above). There was no evidence of a substance related increase in preneoplastic lesions (cellular alteration, clear/eosinophilic/basophilic) or inflammation. No centrilobular degeneration was noted.

Evidence for cytotoxicity:

- There was a treatment related increase in individual cell necrosis.
- There was a treatment related increase in vacuolization.
- There was a treatment related increase in hepatocellular pigment (unknown relevance).

Evidence against cytotoxicity as a major MoA:

- Clinical chemistry does not support a cytotoxic MoA. Significant decreases were measured in serum alanine aminotransferase, aspartate aminotransferase, and alkaline phosphatase activities. Substantial liver cytotoxicity is associated with increases in these enzymes.
- No evidence of liver atrophy. Absolute increases in mean liver weights for the mid upper dose group (136/168 mg/kg bw/d M/F) and the top dose group (275/331 mg/kg bw/d M/F) were 19 and 27% for males and 22 and 43% for females respectively. There was a treatment related increase in hypertrophy but not hyperplasia.
- Hepatocellular necrosis was not observed by week 53, only after 2 years, it was increased in males (where it was graded minimal to slight), but not in females.

***Table**: RAC overview of liver histopathology in the long-term rat study (Anonymous, 1996, unscheduled deaths and terminal sacrifice)*

Males					
Dose (mg/kg bw/d)	**0**	**0.5**	**5.4**	**136.3**	**274.8**
N. of animals (week 104)	48	50	48	50	50
Hepatocellular necrosis Vacuolisation Hepatocellular pigment	2 4 0	6 2 0	5 2 0	15 3 18	25 10 30
Females					
Dose (mg/kg bw/d)	**0**	**0.7**	**6.8**	**167.9**	**331.3**
N. of animals (week 104)	50	50	50	50	50
Hepatocellular necrosis Vacuolisation Hepatocellular pigment	7 4 5	5 2 6	11 3 24	5 0 44	9 1 41

On balance and considering all the available data, RAC agrees that the proposed MoA is plausible in male rats. Nevertheless, significant uncertainties remain:

1. A general absence of dose-response data for surrogates of CAR/PXR activation
2. No data for apoptosis because of alterations in gene expression

3. No *in vivo* studies using CAR/PXR knock out or humanised CAR/PXR animals were performed to confirm the *in vitro* results
4. No exclusion of AhR activation or investigation into the contribution of cytotoxicity in the liver to the development of tumours
5. Sex differences in tumour induction have not been investigated
6. Similarities in response between the two main rat strains were not adequately explored. *In vivo* studies were not performed. Details of the numbers of animals used to harvest hepatocytes in *in vitro* studies were not reported

The new *in vitro* studies (Anonymous, 2019) showed that there were quantitative differences in the activation of CAR by benfluralin in rat and human hepatocytes *in vitro*. Indeed, the background activation of human CAR is lower than rat CAR, and cell proliferation was not detected in human hepatocytes in 3 out of 4 donors while the opposite was true in the rat. These two differences are broadly consistent with the lack of relevance of the CAR activation mechanism in humans as observed in the rat which leads to carcinogenesis in this species. RAC agrees with the DS that the sum total of the data supports the plausibility of the CAR/PXR MoA; however, there are several key uncertainties (as explained above), that prevent RAC from assuming human relevance can be excluded. The most important ones are the lack of *in vivo* investigations and that similarities in response between the two main rat strains (SD and F344) were not adequately explored. Accordingly, RAC supports the proposal of the DS to classify as Carc. 2 based on liver tumours in this case.

<u>Fischer 344 Rat - Thyroid follicular adenoma and carcinoma in both sexes</u>

In rats treated for 2 years with benfluralin, both males and females (treated with ≥ 136/168 mg/kg bw/d), had higher incidences of follicular cell adenoma and carcinoma of the thyroid (table 'RAC overview of thyroid tumours […]' above). There was a dose-related increase of the combined incidence of benign and malignant tumours on study conclusion. The findings were supported by an increased occurrence of focal hyperplasia at these doses.

Proposed MoA for the thyroid tumours:

The postulated MoA is that the activation of CAR and PXR nuclear receptors enables a liver enzyme-mediated mechanism to act on the thyroid via increased T4 metabolism due to induction of UDPGT. The subsequently lower T4 levels trigger a corresponding increase of TSH levels which lead to follicular cell hypertrophy/hyperplasia and finally thyroid tumours in rodents after lifetime exposure to benfluralin.

The human relevance framework has been used to assess the human relevance of these rodent tumours. The DS could not exclude the relevance of these tumours to humans and supported classification in Category 2 for carcinogenicity.

Is the WoE provided sufficient to establish the MoA in animals in the case of benfluralin?

In addition to the studies already presented in both the RAR and CLH report, new *in vivo and in vitro* mechanistic data has become available since 2019. This data is considered in this assessment.

Several key events have been considered: there is no direct effect on the thyroid; there is liver enzyme induction that is specific to the metabolism of thyroxine (T4) which results in enhanced clearance from the blood; there is a compensatory increase of TSH levels resulting in thyroid stimulation, follicular hypertrophy, hyperplasia, and growth; continued stimulation leads to tumours.

What is the weight of evidence for this thyroid MoA?

A new 90-day study in male and female F344 rats with oral administration of benfluralin via diet with a focus on thyroid function and hormonal measurements was conducted (Anonymous, 2019). In addition, the potential of benfluralin to inhibit the thyroid peroxidase (TPO) enzyme was investigated in rat thyroid microsomes. A recent published study on compounds that might interfere with the sodium iodide symporter (NIS) was also provided (Wang *et al.*, 2018).

- Thyroid findings were limited to rodents; no comparable effects were observed in dogs.
- An amphibian metamorphosis assay was also available in the RAR (B.9.2.3.1/01 Anonymous, 2011). This was GLP and OECD TG 231 (2009) compliant. Following 21 days flow through with a mean measured concentration up to 74.4 µg/L there was no effect on thyroid activity in benfluralin-exposed tadpoles. This lends further support to the specificity of the thyroid effects in rodents and a general lack of a direct effect on the thyroid during metamorphosis.
- The dietary 14-day mechanistic study in rats (Anonymous, 2010) indicated increased thyroid weight with treatment, increased expression of T4-specific genes (Ugt1a6, Ugt2b17) responsible for glucuronide conjugation and increased UGT enzyme activity in the liver.
- In the male and female pubertal assays, a decrease of T4 along with a corresponding increase of TSH was observed following 14 days treatment with benfluralin (Anonymous, 2012).
- In the new 90-day study (Anonymous, 2019), significantly lower mean T4 concentrations (already observed in the existing 14-day study), were recorded after 4 and 13 weeks of treatment. In addition, TSH levels were increased by factors of 1.5 up to 2.8.
- Significantly increased thyroid weights were noted in males with a similar trend in females which correlated with the minimal to slight follicular cell hypertrophy/hyperplasia noted in the thyroid gland.
- An investigation into a direct effect on the thyroid by inhibition of the TPO enzyme in rat thyroid microsomes did not show any inhibition of TPO activity by benfluralin (Anonymous, 2019).
- According to a publication investigating many compounds, including benfluralin on NIS inhibition, benfluralin was found to be negative (Wang, 2018).
- Genotoxicity as a MoA could be excluded since the genotoxicity studies did not provide evidence of a genotoxic potential of benfluralin.

On balance and considering all the available data, RAC agrees that the proposed MoA is plausible and likely responsible for the thyroid tumours in F344 rats. In the CLP guidance one of the mechanisms of tumour formation considered not relevant for humans is thyroid tumours in rodents mediated by UGT induction (see CLP guidance, v5 (2017) section 3.6.2.3.2). Accordingly, RAC does not propose classification based on these tumours in this case.

Fischer 344 Rat - Renal tubular cell adenomas

In the kidney the DS noted a low incidence of tubule cell adenoma (table 'RAC overview of kidney tumours […]' above), in males only at the top dose of 275 mg/kg bw/d (2/50, 4%). The kidney was also a clear target for benfluralin. Many renal lesions occurred at ≥ 5/7 mg/kg bw/d (100ppm) benfluralin (a level which could in principle, support classification of STOT RE 2 for renal toxicity). The effects exhibiting a clear increase in a dose-dependent response included:

- Hyaline droplets in the renal tubule lining cells.

- Tubule cell karyomegaly.
- Transitional cell hyperplasia of the renal papilla.
- Large pelvic calculi.
- Small calculi in the pelvic epithelium.

At 5.4 mg/kg bw/d (100 ppm) and above, large, free kidney calculi were observed, which were believed to be the primary cause of both an exacerbation of nephropathy (CPN) and of transitional cell hyperplasia, possibly leading to the tubular cell adenoma (males) at the top-dose (275 mg/kg bw/d). The development of these kidney tumours in association with chemically induced α2u globulin nephropathy was not shown in this study. RAC agrees with the DS that these tumours were of low relevance to humans and most likely secondary to the observed CPN and associated tubule cell hyperplasia. RAC does not propose classification for carcinogenicity based on these renal adenomas.

B6C3F1 mouse - Hepatocellular adenomas and carcinomas

Increased incidences of adenoma (224 mg/kg bw/d) and of carcinoma (≥ 7 mg/kg bw/d) were especially noted in females due to low or no incidence in concurrent controls (table 'RAC overview of liver tumours […]' above). The DS concluded that benfluralin should be considered carcinogenic in the mouse liver based on the incidence of hepatocellular carcinomas in female mice. RAC disagrees with this assessment based on the strain of mouse used in this study.

In the early 1960's the National Cancer Institute adopted the B6C3F1 mouse, the F1 hybrid of the C57BL/6 female and C3H male, as the mouse for use in the cancer bioassay program. When using specifically susceptible mouse strains such as the B6C3F1 hybrid, relatively high and variable incidences of liver tumours can occur in the untreated or vehicle control mice. In such cases the tumour incidence in the treated group may be significantly above the concurrent control but could still be within the historical incidence range for that tumour type in that species and therefore may not be providing reliable evidence of treatment related carcinogenicity.

Usually, for assessment of liver tumour data in cancer bioassays, laboratory specific HCD are extremely valuable in putting unusual high or low tumour responses into perspective. However, in this case there are some limited HCD with very low incidence of liver tumours. This is not the situation for the majority of published data where the B6C3F1 mouse strain is notorious for high background levels of liver tumours, both benign and malignant in both sexes. RAC notes that in the CLP guidance, liver tumours in B6C3F1 mice are recognised to occur with a high frequency. On this premise, RAC does not consider the tumour profile of benfluralin in this particular strain of mouse to present sufficient or robust evidence for classification.

Comparison with the criteria

Based on the mechanistic data available, a CAR/PXR mediated effect for the development of tumours is plausible although uncertainties have been noted by RAC.

Classification into category 1A

There is no information from epidemiological studies available to inform on carcinogenic potential and so classification in category 1A is not supported.

Classification into category 1B

Category 1B depends on strength of evidence, which consists of animal experiments for which there is sufficient evidence to demonstrate animal carcinogenicity together with additional considerations (part of an extended weight of evidence approach). This means a causal relationship has been established between the chemical 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* in two or more independent studies in one species carried out at different times (or in different laboratories or under different protocols);

(b) a single, well conducted study involving both sexes of a single species;

(c) a single study in one species and sex with the occurrence of malignant neoplasms to an unusual degree with regard to the incidence, site, type of tumour or age at onset, or when there are strong findings of tumours at multiple sites.

The criteria may initially seem to be fulfilled for benfluralin. Two species are affected by chemically induced tumours; rats have treatment-related malignant tumours of the liver (one sex) and thyroid (both sexes); mice have malignant tumours of the liver in females. However, liver tumours in the mouse are set against a very high background level in the particular strain that was used in the 2-year bioassay. There are convincing reasons to consider the thyroid tumours in both sexes of the rat to arise as a consequence of a secondary effect mediated by increased liver enzyme induction.

In conclusion, these criteria are not fully met with benfluralin. Classification as Category 1B is not supported, since there are significant additional considerations that lessen the strength of evidence.

Classification into category 2 or no classification

There is evidence of carcinogenicity in rats and mice. RAC recognises that the B6C3F1 hybrid is a specifically susceptible mouse strain for both benign and malignant neoplasms in the liver and for this reason cautions against interpreting the results in the Anonymous (1988) as being only treatment related. RAC considers the results from the B6C3F1 mouse study do not provide reliable evidence of treatment related carcinogenicity.

RAC initially considered the liver and thyroid tumours in the rat to be of primary concern for the purposes of classification (positive dose-response, incidences above historical controls, both sexes affected, statistical significance).

However, there are significant additional considerations that must be evaluated in a weight of evidence approach in order to decide if classification in Category 2 or no classification is appropriate for benfluralin.

***Table**: Weight of Evidence – consideration of main points*

Key factors to consider	Overall level of concern Classification
#01. Tumour type and background.	**Rat:** Benign and malignant liver/thyroid tumours → high concern. **Mouse:** High background liver tumours → no concern. **Classification:** **Cat. 1**
#02. Multi-site responses.	**Rat:** Yes, liver, thyroid → high concern. **Mouse:** No, liver only → no concern. **Classification:** **Cat. 2**
#03. Progression of lesions to malignancy.	**Rat:** Yes, liver (limited), thyroid → mild concern. **Mouse:** Yes, but high background incidence → no concern. **Classification:** **Cat. 2**
#04. Reduced tumour latency.	No reduced latency → no concern. **Classification:** **none**
#05. Responses in single or several species?	Two species → rat (high concern), mouse (low concern).

	Classification: Cat. 2
#06. Responses in single or both sexes.	**Rat:** liver → single sex (M), thyroid → both sexes → high concern. **Classification:** → **Cat. 2**
#07. Structural similarity to carcinogenic substance.	Insufficient data → no conclusions can be drawn. **Classification: none**
#08. Routes of exposure.	Directly relevant for humans → high concern.
#09. Comparison of ADME between animals and humans.	Very similar metabolic profile → high concern.
#10. Confounding effect of excessive toxicity at test doses.	Limited evidence, some histopathology but not supported with clinical chemistry, AhR response genes not investigated → low concern. **Classification: borderline**
#11. MoA of tumour formation and its relevance to humans.	Non-genotoxic. **Thyroid:** secondary to T4 metabolism → no concern. **Liver:** CAR/PXR activation → overall data package → mild concern. **Classification:** relevance for humans not fully explored → **Cat. 2**.

Tumours in experimental animal cancer studies that are associated with species-specific mechanisms or modes of action may not be considered predictive of a similar hazard to humans. If a MoA can be demonstrated to not operate in humans, experimental animal responses are not considered relevant for humans.

The weight of evidence for benfluralin suggests that the thyroid tumours in the F344 rat arise as a secondary effect rather than a direct effect on the thyroid. They are a consequence of liver enzyme induction, especially of UGT, which in rodents leads to increased thyroid hormone metabolism with subsequently lower T4 levels and increased TSH feedback. This leads to thyroid tumour induction after lifetime exposure. In the CLP guidance, one of the mechanisms of tumour formation considered not relevant for humans is thyroid tumours in rodents mediated by UGT induction (see CLP guidance, v5 (2017) section 3.6.2.3.2).

The liver tumours are considered to be the prime evidence on which to base classification. Based on the complete data package for benfluralin, RAC recognises that there are certain data deficiencies that would have made the assessment more robust such as *in vivo* studies with CAR^{KO}/PXR^{KO} double knock out animals or a wider scope of investigation into mRNA transcripts and liver enzyme activities with an emphasis on eliminating other possible MoAs. RAC concludes that while there is sufficient evidence to presume the CAR/PXR MoA is plausible in this case, significant uncertainties and a lack of *in vivo* investigations promote caution. The human relevance of the rat liver tumours cannot be excluded. **RAC considers that classification as Carc. 2; H351, is warranted.**

RAC evaluation of reproductive toxicity

Summary of the Dossier Submitter's proposal

Adverse effects on sexual function and fertility

The DS described two studies in its evaluation of benfluralin for effects on sexual function and fertility. The first study was an old (Anonymous, 1973), non-guideline, non-GLP-compliant generation study that was included for supportive information only. However, the study was

considered unacceptable by the DS for regulatory purposes and is not considered further in this opinion.

The DS based its evaluation on a 2-generation reproductive toxicity study in SD rats (GLP-compliant, based on OECD TG 416) from 1995. The DS noted that several critical reproductive endpoints were missing (table 40, CLH report) and this made a reliable assessment of reproductive toxicity with regard to classification and labelling difficult. Developmental landmarks were not assessed.

The MTD was exceeded, clear maternal toxicity was evident in both generations from significant reductions in food consumption (-10-34% relative to controls), body weight (-10-22%) and body weight gain (-4-54%) at the top dose. There were two unscheduled female deaths in the F0 high dose group and 1 in the F1 high dose group. Liver weights were significantly and grossly increased at the top dose (absolute weight: ↑40%/↑39% for F0/F1; relative weight: ↑63%/↑76% for F0/F1). There was also an increase in the incidence of animals observed with CPN in the top dose group (17/29 for F0/F1 compared with controls 2/1 for F0/F1).

The calculated intake of benfluralin (purity 95.8% [EBNA] = 0.04 mg/kg) was poorly described. A range of values were presented for each phase of the study. The most notable effects occurred in the 1000 ppm and 5000 ppm dose groups.

***Table**: F0 intake of benfluralin in mg/kg bw/d throughout the study*

Females				
Dose (ppm)		**100**	**1000**	**5000**
Study Phase:	Premating: Gestation: Lactation:	11-7 7-6 9-20	97-70 74-65 91-198	502-334 339-311 394-854

F1 intakes were similar.

The most severe reproductive effects were seen at the top dose level:

- The number of females with ≥ 23-day gestation period increased substantially at the top dose in both generations [6/28 and 7/28 in F0 and F1, respectively].
- Significant reductions were observed on pup survival following birth and weaning for the top dose group in both generations with an aggravated effect for the second generation [PND0-28 pup loss, not culled: 58/75 in F0/F1, respectively vs. 13/20 observed in the control group].
- Failure to thrive: pup bw (covariate adjusted mean) was significantly and highly impacted from LD4 to LD21 in the top dose group in both generations [↓29-41% / ↓27-40% for F0/F1 pups, respectively].

The overall gestation index was unaffected. Information on implantation sites (useful in interpreting the reduced litter size and the lower number of liveborn pups) was missing, as was the timing of pubertal landmarks. It is not known if the reduced survival of the pups was caused by effects on the pups *in utero*, effects via the milk, or effects on the rearing of the pups.

The DS did not propose classification for adverse effects on sexual function and fertility.

Developmental toxicity

The DS described two studies in its evaluation of benfluralin for effects on development.

Rat developmental toxicity

In a GLP and guideline-compliant (US EPA 40 CFR 158) study from 1985, 25 mated female SD rats/dose group received benfluralin (purity 97.3%, [EBNA] = 0.09 mg/kg) dissolved in a 10%

w/v acacia oil by gavage, at dose levels of 0, 50, 225, 475 and 1000 mg/kg bw/d, from gestation day (GD)6 through to GD15. There was no maternal mortality. Clinical signs were minor.

There was no foetal mortality, and the number of corpora lutea and implantations was unaffected by the treatment. The number of resorptions was not increased by treatment, and foetal weights were unaltered.

Treatment-related findings included a slightly increased incidence of vertebral anomalies or ossification delays, and unossified sternebrae at the highest dose. The centra abnormalities of the vertebrae and ossification delays were considered in the context of reversible growth retardation. There was no evidence of an increase in the incidence of malformations.

Rabbit developmental toxicity

In a GLP and guideline-compliant (US EPA 40 CFR 158) study from 1991, 20 artificially inseminated rabbits/dose group (NZW) received benfluralin (purity 97.3%, [EBNA] = 0.31 mg/kg) dissolved in 10% w/v aqueous acacia oil by gavage at dose levels of 0, 25, 50, 100 or 225 mg/kg bw/d from GD6 to GD19 inclusive.

With 3 abortions (dams were visibly anorexic) and one maternal death (intubation error), the number of viable litters was decreased at the top dose. Other parameters were unaffected by treatment. Maternal toxicity was limited to isolated effects, reduced food consumption and reduced body weight gain.

There were no treatment related increases in malformations. Various skeletal variations and rib anomalies were present but did not always follow a dose response. The only possible meaningful change was a dose-dependent increased incidence of skull variations (accessory bones in the suture line).

The DS concluded that benfluralin did not give rise to adverse developmental effects in rabbits.

Effects on or via lactation

The DS described a metabolism study with a single Holstein dairy cow. The cow was administered gelatine capsules containing [U-phenyl-14C] and unlabelled benfluralin (both dissolved in dichloromethane) at a nominal dose level of 10 mg/kg bw for 3 days.

This was the only evidence available with data confirming the presence of benfluralin residues in milk. Milk production was unaffected by the treatment. Low levels of total radioactive residues in tissues and milk indicated that the parent compound was poorly absorbed by the ruminant. This is in agreement with metabolism studies with rats. No parent compound was recovered in any of the matrices, suggesting that benfluralin was rapidly and extensively metabolised. RAC considers this study of limited use since there is no indication that parent benfluralin is available to neonates via milk. Instead, various residues at low levels may be available but there is no data with respect to their identification or toxicity.

The DS proposed classification for effects on or via lactation citing the effects seen in the rat 2-generation study on pup survival and body weight in the first days of lactation before the pups started eating the pelleted feed.

Conclusion

The DS proposed classification of Repr. 2 for development and classification for effects on or via lactation. The DS relied on results from the rat 2-generation study which included decreased body weight and survival of offspring (weaning index) during the lactation phase, a decrease in litter size and the increased incidence of vertebral centra change (reduced ossification) in foetuses of the developmental toxicity study in rats.

Comments received during consultation

In the response to comments document following the consultation of the CLH report, there were two comments provided from Industry supporting no classification, and one from an MSCA supporting the DS with classification for effects on or via lactation only.

Industry provided a position paper in which they explained that the effects were due to a combination of excessive maternal toxicity and direct toxicity of the substance toward juveniles.

The MSCA did not support classification for development and disagreed with the DS point of view that the effects should be considered as effects on development rather than fertility. The MSCA outlined its support for classification for effects on or via lactation.

Assessment and comparison with the classification criteria

There are no relevant data on adverse effect on development in humans; hence, classification in Category 1A is not proposed.

Classification in Category 1B is largely based on data from animal studies. According to the CLP criteria, such data shall provide clear evidence on fertility/development in the absence of other toxic effects. The criteria for Category 1B are not fulfilled; the study data for benfluralin suggests there may be reproductive effects but the studies either do not show any evidence (as is the case for the rat and rabbit developmental studies) or lack the detail and robustness (2-generation study).

Adverse effects on sexual function and fertility

The main effects of concern from the rat 2-generation study and pubertal developmental studies were:

1. ↑ frequency of dams with an elongation of the gestation period (≥ 23-day) in the top dose group over 2 generations [6/28 and 7/28 for the F0 and F1 dams, respectively, compared with controls; 1/26 and 1/24 for the F0 and F1 dams, respectively].
2. Neonatal survival was adversely affected (table 'Pup loss following birth for both the F0 and F1 parents' below). Significant reductions were observed on pup survival following birth and weaning for the top dose group in both generations → support classification for developmental toxicity according to the CLP Regulation.
3. Failure to thrive: pup bw (table 'Pup bw reductions […]' below) was significantly and grossly impacted from Lactation day (LD)4 to LD21 in the top dose group in both generations → support classification for developmental toxicity according to the CLP Regulation.
4. Mean age at vaginal opening was significantly increased (↑2.9 days at 300 mg/kg bw/d) → Pubertal development study (Anonymous, 2012; RAR B.6.8.3.4).
5. Serum testosterone was significantly decreased (↓69% at 400 mg/kg bw/d) relative to control testosterone levels → Pubertal development study (Anonymous, 2012; RAR table B.6.8.3.4-10).

Only two of the main effects (pup survival and pup body weight), support classification according to the CLP Regulation, Annex I, 3.7.1.4, Adverse effects on development of the offspring; these are detailed under *7.3 Development*.

The remaining effects were not considered sufficient to support classification for sexual function and fertility. RAC considers that no classification is warranted for adverse effects on sexual function and fertility.

Adverse effects on or via lactation

No data are available for the concentration of benfluralin and/or its metabolites in milk. ADME data showed poor oral absorption and a wide distribution of benfluralin including fat suggesting that transfer to milk is plausible. Nevertheless, the reduced mean weight and the mortality in post-weaning (PND 22-28) pups demonstrates that direct toxicity to pups from the diet during weaning is also plausible along with the toxicity of benfluralin or its residues in milk. The lethality among these offspring may thus be a combination of events involving maternal toxicity impacting on nurturing behaviour, exposure to benfluralin and/or residues in the mother's milk and a potential consequence of the food intake to body weight ratio and poor xenobiotic clearance mechanisms increasing systemic exposure in the immature juvenile. Cage-side observations showed an increase in effects at the top dose: weak appearance, pups felt cold to the touch or showed a pale appearance in addition, a lack of milk in the stomach suggesting inadequate nursing. RAC considers that no classification is warranted for effects on or via lactation.

Development

In both rat and rabbit developmental studies, no treatment-related malformation was induced, and benfluralin was not considered teratogenic. The increased incidence of reduced ossification sites in the vertebrae (rat) or accessory skull bones (rabbit) were not considered adverse, and the developmental NOAELs were established at the top-dose. In the rat, foetal body weight or litter parameters were not impaired. Maternal toxicity (decreased body weight change) occurred at 475 mg/kg bw/d and above. In the rabbit, the maternal toxicity was established at 50 mg/kg bw/d, based upon decreased food consumption and a concomitant decreased body weight gain at the next dose. Litter parameters or foetal weight were not affected.

It is noteworthy that the prenatal development study in rats which had less maternal toxicity after dosing up to 1000 mg/kg bw/d (approximately 7-fold higher than the 2-generational study) had no effect on embryo-foetal survival at any dose level (which would translate postnatally to smaller litter sizes). This lack of consistency in effect across studies decreases the concern for a direct effect on the maintenance of the pregnancy but does not provide an explanation for the changes in post-natal survival or subsequent growth retardation.

RAC considers the 2-generation rat study to be the key study for reproductive classification purposes. Though several deficiencies were noted, very clear effects on the retardation of pup growth (table 'Pup bw reductions […]' below) and increased pup loss (table 'Pup loss following birth for both the F0 and F1 parents' below) were observed.

With reference to ***CLP Regulation, Annex I, 3.7.1.4.*** *Adverse effects on development of the offspring:*

"Developmental toxicity… any effect which interferes with normal development... either before or after birth… resulting from exposure… of the developing offspring during prenatal development, or ***postnatally, to the time of sexual maturation****.* [Furthermore]*… These effects can be manifested at any point in the life span of the organism. The major manifestations of developmental toxicity include* ***(1) death of the developing organism,*** *(2) structural abnormality,* ***(3) altered growth****, and (4) functional deficiency."*

The CLP Regulation states that any death/growth retardation following birth up to sexual maturation is a development effect.

***Table**: Pup bw reductions relative to controls following birth for both the F0 and F1 parents. Comparison of raw mean data*

Mean dose (mg/kg bw/d)		0	15.4 / 15.3	149 / 156	651 / 664
PND F0/F1:	0	0 / 0		-- / --	↓5% / --
	4 (pre cull)	0 / 0		↓11% / ↓3%	↓24% / ↓22%
	7	0 / 0	*no treatment effect	↓12% / ↓6%	↓37% / ↓34%
	14	0 / 0		↓11% / ↓10%	↓39% / ↓42%
	21	0 / 0		↓8% / ↓10%	↓40% / ↓42%

Males and females combined. – no change or no reduction in bw
*Mean pup weights were similar or slightly greater than controls for the 15 mg/kg bw/d group, no treatment effect.

Benfluralin may be considered a reproductive toxicant. The decreased pup weights and increased post-natal mortality at >650 mg/kg bw/d (5000 ppm) were treatment-related, and maybe regarded as a failure or reduction in reproductive performance or fecundity for the rat population because of retarded growth and decreased neonatal survival over 2 generations. The reductions in body weight occurred throughout the lactation period up to PND 21 (table 'Pup bw reductions […]' below).

Benfluralin treatment resulted in a statistically significant increase in age at vaginal opening (+2.9 days) and age at first oestrus (37.2 days vs 35.4 days in controls) together with decreased weights in oestrogen-sensitive tissues (ovaries, ↓18% and blotted uterus, ↓21%). The decrease in uterine weights was not due to differences in stages of oestrous at termination.

Age and body weight at preputial separation were not affected by benfluralin treatment. Serum testosterone was significantly decreased (↓69% at 400 mg/kg bw/d) relative to control testosterone levels. However, this was secondary to enhanced liver metabolism resulting in enhanced clearance (Anonymous, 2012; Hershberger assay, ^{14}C-testosterone clearance study, RAR Table B.6.8.3.3-10). There were no effects on male or female reproductive indices in the 2-generation rat study.

***Table**: Pup loss following birth for both the F0 and F1 parents*

Mean dose (mg/kg bw/d)		0	15.4 / 15.3	149 / 156	651 / 664
PND F0/F1	0-4	10 / 16	7 / 2	21 / 8	26 / 28
	5-21	3 / 4	1 / 1	9 / 0	13 / 45
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	22-28	0 / 0	2 / 0	0 / 0	19 / 2

Pup survival was adversely affected throughout the lactation period and beyond (table above). However, RAC notes that the effects in the pups were compounded by significant maternal toxicity (decreased body weight, liver and kidney toxicity). Lower pup body weights and survival are well recognised as a common consequence of poor maternal nurturing and care as a secondary consequence of excessive maternal toxicity. It has already been demonstrated by the DS that the body weights and food consumption of dams at the top dose was severely impacted by treatment, constituting significant maternal toxicity. Clinical signs for the dams, however, were sparce, nothing of note was documented during lactation and mating behaviour was unaffected by benfluralin treatment. It is not possible to determine the extent to which maternal toxicity contributed to the effects in the offspring. RAC concluded that the criteria for classification as developmental toxicant are fulfilled.

Conclusion

RAC considers the 2-generation rat study to be the key study for reproductive classification purposes. Though several deficiencies were noted, very clear effects on the retardation of pup growth and increased pup loss were observed. In addition, several other effects with the potential to impact on fertility were also noted; these included a significant delay in vaginal opening, significant reductions in serum testosterone (albeit as a secondary effect to liver enzyme induction), and an increase in the frequency of dams with an elongation of the gestation period. In conclusion, RAC considers that **classification is warranted for developmental toxicity, as Repr. 2; H361d**.

ENVIRONMENTAL HAZARD EVALUATION

RAC evaluation of aquatic hazards (acute and chronic)

Summary of the Dossier Submitter's proposal

DS proposal: Aquatic Acute 1; H400with an M-factor of 10 based on the acute toxicity to Mysid shrimp *Mysidopsis bahia* (96h EC_{50} of 0.043 mg/L), and Aquatic Chronic 1; H410 with an M-factor of 10, based a 49-day NOEC of 0.0019 mg/L to *Oncorhynchus mykiss* (Rainbow trout) and substance not rapidly degradable.

Degradation

Ready biodegradability

The DS considered the substance not readily biodegradable based on a GLP ready biodegradation study performed according to the OECD TG 301D (Closed bottle test). A low degradation of 5% was observed for test item at concentration 1.6 mg/L after 28 days. The validation criteria were met: the control item sodium benzoate degraded by 82% after 28 days (threshold for ready biodegradability was ≥ 60% after 7 days) and the degradation of control and benfluralin samples indicated that the presence of the test item did not hinder the effectiveness of the inoculum used.

Hydrolysis

DS accepted as key a GLP aqueous hydrolysis study based on OECD TG 111. Hydrolysis of 0.03 mg/L radiolabelled [Phenyl-U-^{14}C]-benfluralin (radiochemical purity ≥99%) was examined in sterile buffer solutions at pH 4, 7 and 9. Samples were incubated at 50 ℃ in the dark for 5 days and the results indicated that benfluralin was stable at all tested pH values. In a second, non-GLP study, benfluralin was stable to hydrolysis at 26 ºC. However, the reporting of the study was very brief without details therefore this study can be used as supporting information only.

Water, water-sediment and soil degradation data (including simulation studies)

The aerobic degradation of benfluralin was investigated according to OECD TG 309 at two concentration levels 0.00294 (low dose) and 0.0326 mg/L (high dose) in surface waters from two different locations (pH 8.2, TOC 3.6, 6.5 or 14.9 mg/L). Samples were incubated in the dark for a period of 17 days (high dose) and 16 days (low dose), respectively, at approx. 20.5 °C. The experimental set-up was specifically adapted due to the critical properties (i.e. low water solubility and high volatility) of the test item. Benfluralin did not degrade during the test period of 16-17 days but was mainly volatilized from the test system due to its relatively high Henry's constant and medium vapour pressure. AR remained in the water phase after 16-17 days was between 7.2 – 17.5%. The test system according to OECD TG 309 is not entirely suitable for the investigation

of the biodegradation of benfluralin in surface water due to its volatility and its strong adsorption to particles, which would lead to a rapid partitioning to the sediment.
Aerobic mineralisation of benfluralin (phenyl-label) was studied also in two natural water/sediment systems (2 cm sediment covered with 6 cm deep water) following the guideline BBA Part IV, Section 5-1. The ^{14}C-benfluralin was applied to the surface water in each vessel to give a nominal initial concentration of 0.03 mg/L in the water phase. Flasks were incubated at 20 °C in the dark for up to 100 days. Results at the end of the incubation (100 days) showed that the mineralisation was low (1.7% to 2.0% AR). The dissipation of benfluralin from the water phase in the water sediment systems was mainly via volatilization (64.9% to 63.2% AR measured as volatile benfluralin at study end) and dissipation to the sediment (26% to 31.4% AR after 100 days as bound residue). At the end of the study (100 days) benfluralin was not detected in the water phase of the two systems (< 0.1% AR). As the major part of radioactivity was evaporated from the water phase, it was considered that no significant degradation occurred in the water phase. The major part of degradation occurred in sediment only. The geometric mean DT_{50} of the total system was estimated to be 3.1 days. No degradation rate could be calculated for benfluralin in the water phase. One significant degradation product was observed, benfluralin diamine, which was observed almost exclusively in the sediment layer and reached a maximum of 8.7% AR (day 2). There were not presented any acceptable degradation kinetics for this metabolite and the degradation rate of this metabolite could therefore not be determined. DS concluded that although the primary degradation of benfluralin in the test water/sediment systems is rapid and benfluralin quickly dissipates through volatilization or adsorption to sediments, higher concentrations in water phase could not be excluded due to variations of environmental conditions. DS considered studies on hydrolysis, rapid biodegradability and mineralisation in water are indicative of classification of benfluralin as not rapidly degradable.

Bioaccumulation

Benfluralin has a log K_{OW} of 5.27 ± 0.11 at 20 °C, pH 6.0-7.0 which is experimentally determined according to the OECT TG 107, (shake flask method and analysis by GC-FID). The potential for aquatic bioaccumulation could not be excluded.

Only one experimental bioaccumulation study was available that was considered by the DS as not valid and the calculated BCF values were deemed of low quality, not recommended to be used for classification purposes. The bioaccumulation study with the bluegill sunfish, *Lepomis macrochirus* had a 28 days exposure period and 14 days depuration period in a flow-through system and a maximum whole fish bioconcentration factor (BCF) of 1740.8 was derived L/kg. The study did not fulfil all of the validity criteria in OECD TG 305 (2012); i.e. the concentration of the test substance in the chambers was not maintained within ± 20% of the mean of the measured concentration during the uptake phase, the growth of the juvenile fish and the lipid content were not reported. Based on the experimentally determined log Kow of 5.27, DS concluded that benfluralin has the potential to bioaccumulate, according to the CLP-criteria.

Acute toxicity

Valid acute toxicity studies were available for all three trophic levels with benfluralin and with main degradation products TR-15 and TR-6, summarised in table below.

***Table**: Summary of valid studies for acute toxicity of benfluralin and metabolites TR-15 and TR-6*

Method	Species	Endpoint	Exposure		Results		Reference
			Design	Duration	Endpoint	Toxicity	
Acute toxicity to fish, ASTM E729 80 (1980), evaluated according to OECD TG 203 , GLP	*Oncorhynchus mykiss* (Rainbow trout)	Mortality	Semi-static	96 hours	LC_{50}	0.081 mg a.s./L (mm)	Anonymous,1985 Report No. F00185/ CA 8.2.1/01
Acute toxicity to fish, OECD TG 203, GLP	*Oncorhynchus mykiss* (Rainbow trout)	Mortality	Static	96 hours	LC_{50}	1.00 mg TR-6/L (mm)[1]	Anonymous,2001 a Report No. 011092/ CA 8.2.1/06
Acute toxicity to fish, OECD TG 203, GLP	*Oncorhynchus mykiss* (Rainbow trout)	Mortality	Static	96 hours	LC_{50}	5.46 mg TR-15/L (mm)[2]	Anonymous,2001 b Report No. 011106/ CA 8.2.1/07
Acute toxicity to fish, OCSPP Draft Guideline 850.1075, evaluated according to OECD TG 203, GLP	*Oncorhynchus mykiss* (Rainbow trout)	Mortality	Flow-through	96 hours	LC_{50}	> 0.048 mg a.s./L (mm)	Anonymous,2014 a Report No. 14050.6160/ CA 8.2.1/08
Acute toxicity to fish, OCSPP Draft Guideline 850.1075, evaluated according to OECD TG 203 GLP	*Lepomis macrochirus* (Bluegill sunfish)	Mortality	Flow-through	96 hours	LC_{50}	> 0.042 mg a.s./L (mm)	Anonymous,2013 a Report No. 14050.6125/ CA 8.2.1/09
Acute toxicity to fish, OCSPP Draft Guideline 850.1075, evaluated according to OECD TG 203, GLP	*Cyprinodon variegatus* (Sheepshead Minnow)	Mortality	Flow-through	96 hours	LC_{50}	> 0.027 mg a.s./L (mm)	Anonymous,2013 b Report No. 14050.6161/ CA 8.2.1/10
Acute toxicity to fish, OECD TG 203, GLP	*Cyprinus carpio* (carp)	Mortality	Flow-through	96 hours	LC_{50}	> 0.029 mg a.s./L (mm)	Anonymous, 2004 Report No. 12550.6332/ CA 8.2.1/11

Daphnia sp., Acute Immobilisation Test, OECD TG 202, GLP	*Daphnia magna*	Immobility	Static	48 hours	EC_{50}	3.52 mg TR-6/L (mm)[1]	Marino *et al*., 2001c Report No. 011093/ CA 8.2.4.1/02
Daphnia sp., Acute Immobilisation Test, OECD TG 202, GLP	*Daphnia magna*	Immobility	Static	48 hours	EC_{50}	9.36 mg TR-15/L (mm)[2]	Marino *et al*., 2001d Report No. 011105/ CA 8.2.4.1/03
Daphnia sp., Acute Immobilisation Test, OCSPP Guideline 850.1010, evaluated according to OECD 202, GLP	*Daphnia magna*	Immobility	Flow-through	48 hours	EC_{50}	> 0.034 mg a.s./L (mm)	Urann, 2014b Report No. 14050.6154/ CA 8.2.4.1/04
Mysid Acute Toxicity Test, US EPA FIFRA 72 3, GLP	*Mysidopsis bahia* (Mysid shrimp)	Mortality	Flow-through	96 hours	LC_{50}	**0.043 mg a.s./L (mm)**	Sousa, 1990b Report No. 90-6-3343/ CA 8.2.4.2/01
Freshwater Alga and Cyanobacteria, Growth Inhibition Test, US EPA FIFRA 123 2 evaluated according to OECD TG 201, GLP	*Pseudokirchneriella subcapitata* (Green algae)	Growth-rate	Static	72 hours	E_rC_{50} E_yC_{50}	> 5.56 mg TR-6/L (mm) 4.09 mg TR-6/L (mm)[1]	Henry et al., 2002 Report No. 011101/ CA 8.2.6.1/02
Freshwater Alga and Cyanobacteria, Growth Inhibition Test, OECD TG 201, GLP	*Pseudokirchneriella subcapitata* (Green algae)	Growth-rate	Static	72 hours	E_rC_{50} E_yC_{50}	> 9.15 mg TR-15/L (mm) 3.82 mg TR-15/L (mm)[2]	Marino *et al.,* 2001 Report No. 011102/ CA 8.2.6.1/03
Freshwater Alga and Cyanobacteria, Growth Inhibition Test, OECD TG 201, GLP	*Pseudokirchneriella subcapitata* (Green algae)	Growth-rate	Static	96 hours	E_rC_{50} E_yC_{50}	> 0.0132 mg a.s./L (mm) > 0.0132 mg a.s./L (mm)	Softcheck, 2015a Report No. 14050.6228/ CA 8.2.6.1/04

Lemna sp. Growth Inhibition Test, OECD TG 221, GLP	*Lemna gibba* (Duck weed)	Growth-rate	Semi-static	7 days	E_rC_{50} E_yC_{50}	> 0.032 mg a.s./L (mm) 0.017 mg a.s./L (mm)	Hoberg, 2007 Report No. 12550.6485/ CA 8.2.7/01

[1] Tested with the metabolite Trifluralin Metabolite TR-6
[2] Tested with the metabolite Trifluralin Metabolite TR-15
mm: mean measured

As can be seen both metabolites TR-15 and TR-6 are with much lower toxicity than parent compound benfluralin for all three trophic levels and will not be discussed any longer.

Short term toxicity to fish

Acute toxicity of benfluralin to fish was investigated in 5 studies, which were considered valid by the DS and equivalent to OECD TG 203.

96-hour fish toxicity was estimated to be LC_{50} = 0.081 mg/L in one study with *Oncorhynchus mykiss* (Rainbow trout) exposed to benfluralin under semi-static conditions was above the water solubility of 0.064 mg/L.

Four different fish species *Oncorhynchus mykiss* (Rainbow trout) *Lepomis macrochirus* (Bluegill sunfish) *Cyprinodon variegatus* (Sheepshead Minnow) *and Cyprinus carpio* (carp) were exposed to benfluralin under flow-through conditions for 96 h. The lowest endpoint value expressed as mean measured concentration was the 96-hour LC_{50} > 0.027 mg/L found for *Cyprinodon variegatus*. The fish species were exposed to benfluralin (nominal exposure range of 0.031, 0.063, 0.012, 0.025, and 0.050 mg/L.) under flow-through conditions for 96 h. Test design included a saturator column made of glass and Teflon packed with glass wool to dose the substance. The column was designed to provide a constant flow of saturated aqueous solution without the use of a carrier solvent. A slightly different design was used in the 96-hour flow-through study conducted with *Cyprinus carpio* (carp) exposed to benfluralin, The nominal exposure range is 0.0063, 0.013, 0.025, 0.050 and 0.100 mg/L and exposure solutions were prepared with the aid of the solvent acetone, a solvent control was included. The 96-hour endpoint was estimated to be: LC_{50} > 0.029 mg a.s./L.

Short term toxicity to invertebrates

There were two valid acute toxicity studies available for invertebrates, both conducted in a flow-through system, under analytical control of benfluralin concentrations.

The acute toxicity study to *Daphnia magna* was conducted following the procedures described and the recommendations provided in the OECD TG 202 with a nominal exposure range of 0.0031, 0.0063, 0.012, 0.025, and 0.050 mg/L benfluralin. The test substance was dosed with a saturator column made of glass and Teflon packed with glass wool. The column was designed to provide a constant flow of saturated aqueous solution without the use of a carrier solvent. The 48-hour endpoint was estimated to be: EC_{50} > 0.034 mg benfluralin/L.

The acute toxicity of benfluralin to the Mysid shrimp *Mysidopsis bahia* was tested in 96-hour flow-through study at a nominal exposure range of 0.019, 0.032, 0.054, 0.090 and 0.150 mg/L. Exposure solutions were prepared with the aid of the solvent acetone and a solvent control was included. Although some unclear effects (lethargic species with darkened pigmentation at the first 48 hours and 72 hours sampling time point) observed in two replicates of solvent control, the study was accepted as valid because these effects were absent after 96 hours exposure, no effects

were observed in the other solvent control replicate or in the two lowest exposure groups, in addition the solvent concentration was below the maximum recommended concentration of 0.1 mL/L. The 96-hour endpoint was estimated to be: LC_{50} = 0.043 mg/L benfluralin.

Short-term toxicity to algae

Two algae tests are given for benfluralin with *Pseudokirchneriella subcapitata* and *Lemna gibba.*

Toxicity of benfluralin to *Pseudokirchneriella subcapitata* was studied under static conditions as an algal growth inhibition test following GLP and OECD TG 201. The nominal exposure range was 0.0041, 0.0081, 0.016, 0.033, and 0.065 mg/L with exposure solutions prepared in solvent dimethylformamide (DMF) (solvent control was included). The measured 96h concentrations were ≈ 0 to 9.2% of nominal and the endpoints for *Pseudokirchneriella subcapitata* were re-calculated to the geometric mean of the initial measured concentrations (0h) and the 96h measured concentrations. No inhibitory effects on yield or growth rate were observed up to the highest concentration tested of 0.0132 mg/L. The concentrations falling below the LOQ at the two lowest exposure groups does not invalidate the establishment of the endpoint, which was calculated from measured concentration in the upper exposure group.
The 96-hour acute endpoints based on mean measured concentrations were presented as:
E_rC_{50} > 0.0132 mg/L and E_yC_{50} > 0.0132 mg/L

A valid and reliable toxicity test to *Lemna gibba* was conducted for 7 days under semi-static conditions. The nominal exposure range was 0.0042, 0.0083, 0.017, 0.034 and 0.066 mg a.s./L and the endpoints were reported as geometric mean measured concentrations. The 7-day acute endpoints were: E_rC_{50} > 0.032 mg/L and E_yC_{50} = 0.017 mg/L (based on frond density).

Based on these, the DS proposed a classification of Category Acute 1: ≤ 1 mg/L, based on the lowest available acute toxicity value for crustacea (LC_{50} = 0.043 mg a.s./L) and an appropriate M-factor of 10, since the toxicity is within the range 0.01 < $L(E)C_{50}$ ≤ 0.1.

Chronic toxicity

Valid and reliable long-term toxicity studies are summarised in table below.

***Table**: Summary of valid studies for chronic toxicity of benfluralin*

Method	Species	Endpoint	Exposure		Results		Reference
			Design	Duration	Endpoint	Toxicity	
Fish Early Life-Stage (ELS) toxicity test, US EPA FIFRA 72-4, evaluated according to OECD TG 210, GLP	*Oncorhynchus mykiss* (Rainbow trout)	Growth	Flow-through	49 days	NOEC	**0.0019 mg a.s./L (mm)**	Anonymous, 1990 Report No. F00690/ CA 8.2.2.1/01
Daphnia magna Reproduction test, OCSPP Draft Guideline 850.1300, evaluated according to OECD TG 211, GLP	*Daphnia magna*	Reproduction, survival and growth, NOEC	Flow-through	21 days	NOEC	0.046 mg a.s./L (mm)	Urann, 2013c Report No. 14050.6156 / CA 8.2.5.1/02

Freshwater Alga and Cyanobacteria, Growth Inhibition Test, OECD TG 201, GLP	*Pseudokirchneriella subcapitata* (Green algae)	Growth-rate	Static	96 hours	NOE_rC NOE_yC	0.0132 mg a.s./L (mm)	Softcheck, 2015a Report No. 14050.6228 / CA 8.2.6.1/04
Lemna sp. Growth Inhibition Test, OECD TG 221, GLP	*Lemna gibba* (Duck weed)	Growth-rate	Semi-static	7 days	NOE_rC	0.0069 mg a.s./L (mm)	Hoberg, 2007 Report No. 12550.6485 / CA 8.2.7/01

mm: mean measured

Long-term toxicity to fish

A 49-day flow-through chronic toxicity study following GLP and OECD TG 210 with *Oncorhynchus mykiss* (rainbow trout) exposed to benfluralin (prepared in solvent acetone and a solvent control was included) in the nominal exposure range 0.0012, 0.0037, 0.011, 0.033 and 0.100 mg/L was conducted. The following endpoints based on mean measured concentrations were recorded: larval survival at complete hatch, larval survival at test termination, hatchability, time to hatch and growth (length and weight). The chronic endpoint relevant for the hazard classification of benfluralin is considered to be: NOEC = 0.0019 mg/L (based on length).

Long-term toxicity to invertebrates

One GLP chronic toxicity study was available for aquatic invertebrate *Daphnia magna* conducted under flow-through conditions, for 21 days in accordance with OECD testing guideline 211. The test substance with a nominal exposure range of 0.0031, 0.0063, 0.012, 0.02525, and 0.050 mg a.s./L. was dosed with a saturator column made of glass and Teflon packed with glass wool ensuring constant flow of saturated aqueous solution of tested substance without the use of a carrier solvent. No effects were observed for endpoints considered: survival, cumulative offspring per female and growth (length and dry weight). Consequently, the mean measured long-term endpoint for survival, offspring per female, body length and dry weight was determined as NOEC = 0.046 mg/L.

Long-term toxicity to algae

Valid toxicity studies available for two algae species *Pseudokirchneriella subcapitata* and *Lemna gibba* have been already described in a previous section of this ODD. For *Pseudokirchneriella subcapitata* no inhibitory effects on yield or growth rate were observed up to the highest concentration tested – 0.0132 mg/L (mean measured). The chronic endpoints for *Pseudokirchneriella subcapitata* exposed to benfluralin as mean measured concentrations were NOE_rC = 0.0132 mg a.s./L and NOE_yC = 0.0132 mg a.s./L.

The calculated chronic endpoints for *Lemna gibba* exposed to benfluralin were: E_rC_{10} - 0.012 mg/L (based on frond density) and E_yC_{10} - 0.0085 mg/L (based on frond density);
NOE_rC = 0.0069 mg/L (based on frond density) and NOE_yC = 0.0069 mg/L (based on frond density).

Based on these, the DS proposed a classification of Aquatic Chronic 1 based on a lowest available toxicity value of 0.0019 mg a.s./L, with an M-factor of 10 since the toxicity is within the range of $0.001 < NOEC \leq 0.01$ and the substance is non-rapidly degradable.

Comments received during consultation

One comment was received during the consultation, supporting the proposed environmental classification Aquatic Acute 1; H400 (M=10) and Aquatic Chronic 1; H410 (M=10).

Assessment and comparison with the classification criteria

Degradation

RAC agrees with the DS to consider benfluralin as 'not rapidly degradable' due to a 5% degradation in 28 days in a ready biodegradability test (OECD TG 301D, Closed bottle test) and the substance being hydrolytically stable in sterile buffer solutions at pH 4, 7 and 9.

Bioaccumulation

Based on the value of log Kow of 5.27 ± 0.11 at 20 °C bioaccumulation could not be excluded. An experimentally determined BCF of 1740.8 L/kg was available, however the study did not meet the relevant validity criteria. RAC agrees with the DS that given the experimentally determined log Kow of 5.27, it can be concluded that benfluralin has the potential to bioaccumulate according to the CLP-criteria.

Acute aquatic toxicity

There were acute toxicity data available for three trophic levels.

The lowest available acute toxicity value was obtained with Mysid shrimp *Mysidopsis bahia* (LC_{50} = 0.043 mg/L). This endpoint is lower than the classification criterion for Category Acute 1 ≤ 1 mg/L. The appropriate M-factor is 10, since the toxicity is within the range 0.01 < $L(E)C_{50}$ ≤ 0.1.

Chronic aquatic toxicity

There were chronic toxicity data available for all three trophic levels.

The lowest available chronic toxicity value was observed in a fish study *Oncorhynchus mykiss* (Rainbow trout) (NOEC = 0.0019 mg/L). This endpoint is lower than the classification criterion for Category Chronic 1 ≤ 0.1 mg/L. The appropriate M-factor is 10, since the toxicity is within the range of 0.001 < NOEC ≤ 0.01 and the substance is non-rapidly degradable.

In conclusion, RAC agrees with the DS that Benfluralin warrants classification as:

- **Aquatic Acute 1; H400, M = 10 and**
- **Aquatic Chronic 1; H410, M = 10.**

RAC evaluation of hazards to the ozone layer

Summary of the Dossier Submitter's proposal

An Ozone Depleting Potential is not reported for benfluralin and it is not listed in Annex I to Regulation (EC) No. 1005/2009. This hazard was therefore not considered further in this report. The overall DS conclusion was that no classification for hazards to the ozone layer is warranted in the presence of conclusive but not sufficient for classification data.

Comments received during consultation

No comments have been received.

Assessment and comparison with the classification criteria

RAC agrees with the DS conclusion for no classification for hazards to the ozone layer due to conclusive but not sufficient for classification data.

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).