Committee for Risk Assessment

RAC

Opinion

proposing harmonised classification and labelling
at EU level of

difenoconazole (ISO); 1-({2-[2-chloro-4-(4-chlorophenoxy)phenyl]-4-methyl-1,3-dioxolan-2-yl}methyl)-1H-1,2,4-triazole; 3-chloro-4-[(2RS,4RS;2RS,4SR)-4-methyl-2-(1H-1,2,4-triazol-1-ylmethyl)-1,3-dioxolan-2-yl]phenyl 4-chlorophenyl ether

EC Number: -
CAS Number: 119446-68-3

CLH-O-0000007004-85-01/F

Adopted

10 June 2021

10 June 2021

CLH-O-0000007004-85-01/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: **difenoconazole (ISO); 1-({2-[2-chloro-4-(4-chlorophenoxy)phenyl]-4-methyl-1,3-dioxolan-2-yl}methyl)-1H-1,2,4-triazole; 3-chloro-4-[(2RS,4RS;2RS,4SR)-4-methyl-2-(1H-1,2,4-triazol-1-ylmethyl)-1,3-dioxolan-2-yl]phenyl 4-chlorophenyl ether**

EC Number: **-**

CAS Number: **119446-68-3**

The proposal was submitted by **Spain** and received by RAC on **19 March 2020.**

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

PROCESS FOR ADOPTION OF THE OPINION

Spain 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 **31 March 2020**. Concerned parties and Member State Competent Authorities (MSCA) were invited to submit comments and contributions by **1 June 2020**.

ADOPTION OF THE OPINION OF RAC

Rapporteur, appointed by RAC: **Miguel A. Sogorb**

Co-Rapporteur, appointed by RAC: **Zilvinas Uzomeckas**

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 **10 June 2021** by **consensus**.

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

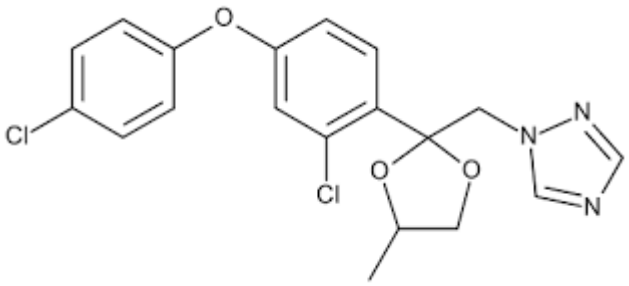
	Index No	Chemical name	EC No	CAS No	Classification		Labelling			Specific Conc. Limits, M-factors and ATE	Notes
					Hazard Class and Category Code(s)	Hazard statement Code(s)	Pictogram, Signal Word Code(s)	Hazard statement Code(s)	Suppl. Hazard statement Code(s)		
Current Annex VI entry	No current Annex VI entry										
Dossier submitters proposal	TBD	difenoconazole (ISO); 1-({2-[2-chloro-4-(4-chlorophenoxy)phenyl]-4-methyl-1,3-dioxolan-2-yl}methyl)-1H-1,2,4-triazole; 3-chloro-4-[(2RS,4RS;2RS,4SR)-4-methyl-2-(1H-1,2,4-triazol-1-ylmethyl)-1,3-dioxolan-2-yl]phenyl 4-chlorophenyl ether		119446-68-3	Acute Tox. 4 Eye Irrit. 2 Aquatic Acute 1 Aquatic Chronic 1	H302 H319 H400 H410	GHS07 GHS09 Wng	H302 H319 H410		oral: ATE = 1453 mg/kg bw M=10 M=10	
RAC opinion	TBD	difenoconazole (ISO); 1-({2-[2-chloro-4-(4-chlorophenoxy)phenyl]-4-methyl-1,3-dioxolan-2-yl}methyl)-1H-1,2,4-triazole; 3-chloro-4-[(2RS,4RS;2RS,4SR)-4-methyl-2-(1H-1,2,4-triazol-1-ylmethyl)-1,3-dioxolan-2-yl]phenyl 4-chlorophenyl ether		119446-68-3	Carc. 2 Acute Tox. 4 Eye Irrit. 2 Aquatic Acute 1 Aquatic Chronic 1	H351 H302 H319 H400 H410	GHS07 GHS08 GHS09 Wng	H351 H302 H319 H410		oral: ATE = 1450 mg/kg bw M=10 M=10	
Resulting Annex VI entry if agreed by COM	TBD	difenoconazole (ISO); 1-({2-[2-chloro-4-(4-chlorophenoxy)phenyl]-4-methyl-1,3-dioxolan-2-yl}methyl)-1H-1,2,4-triazole; 3-chloro-4-[(2RS,4RS;2RS,4SR)-4-methyl-2-(1H-1,2,4-triazol-1-ylmethyl)-1,3-dioxolan-2-yl]phenyl 4-chlorophenyl ether		119446-68-3	Carc. 2 Acute Tox. 4 Eye Irrit. 2 Aquatic Acute 1 Aquatic Chronic 1	H351 H302 H319 H400 H410	GHS07 GHS08 GHS09 Wng	H351 H302 H319 H410		oral: ATE = 1450 mg/kg bw M=10 M=10	

GROUNDS FOR ADOPTION OF THE OPINION

RAC general comment

Difenoconazole is an active substance of a plant protection products used as a fungicide. It is not currently listed in Annex VI of Regulation (EC) 1272/2008, although in the past an EFSA peer review of the pesticide risk assessment proposed a classification of difenoconazole as Xn, R22; R53.

The chemical structure is shown below:



The CLH report was prepared by the Dossier Submitter (DS) mainly based on the available data from the Renewal Assessment Report developed in accordance with the Commission Regulation (EC) No. 844/2012. At the time of submission of the CLH report, difenoconazole had not been registered under REACH.

RAC evaluation of physical hazards

Summary of the Dossier Submitter's proposal

Based on the available information, the DS proposed no classification of difenoconazole for the following hazards: flammable solid, phyrophoric solid, self-heating substance, substance that in contact with water emit flammable gases, oxidising solid and corrosive to metals. DS proposed no classification of difenoconazole for explosivity and self-reactive substance due to data lacking.

Comments received during consultation

The applicant acknowledged the data gaps identified for some physical hazards (explosivity and self-reactive substances) and confirmed that the missing studies (according to the CLP Regulation UN RTDG methods) would be conducted to fulfil the data requirement by October 2020. RAC noted that these studies were not finally submitted.

Assessment and comparison with the classification criteria

Difenoconazole contains a triazole substituent, i.e. contiguous nitrogen atoms, which structure is associated with explosive properties. In addition, the exothermic decomposition energy is not available and therefore it is not possible to know whether the criteria for classification is fulfilled. However, RAC notes that, based on the negative result of one study with method EC A.14 and the handling experience, the concern for the explosive properties of difenoconazole is low. Overall,

RAC supports the DS proposal for **no classification of difenoconazole for explosive properties based on lack of data.**

An EC A.10 study showed as difenoconazole did not ignite on contact with the ignition source. However, as reflected in the CLP Guidance and ECHA Guidance on Information Requirements and Chemical Safety Assessment (R.7.1.10.3), if the result of an A.10 method indicates that a substance is not flammable, no more testing is necessary. Overall, RAC supports the DS's proposal for **no classification of difenoconazole as flammable solid.**

As commented above, difenoconazole contains a chemical structure structurally associated with explosive properties. However, no data on thermal stability are available. Thus, RAC supports the DS's proposal for **no classification of difenoconazole as a self-reactive substance based on lack of data.**

There are no reports demonstrating that difenoconazole spontaneously ignites upon coming into contact with air at normal temperatures. In addition, RAC notes that if a substance does not ignite upon contact with a very hot flame (as in an EU A.10 test) or upon heating, it will not ignite spontaneously at room temperature. Thus, RAC supports the DS's proposal for **no classification of difenoconazole as a pyrophoric solid.**

According to CLP criteria substances with melting point lower than 160 °C should not be considered for classification for self-heating substances. Difenoconazole has a melting point of 82-83 °C. Moreover, an EC A.16 test showed no auto-ignition below the melting point. Overall, RAC supports the DS's proposal for **no classification of difenoconazole for self-heating substances.**

Difenoconazole does not contain metals or metalloids. Moreover, the experience in production and handling shows that the substance does not react with water and is soluble in water forming a stable solution. Thus, RAC supports the DS's proposal for **no classification of difenoconazole for substances in contact with water emit flammable gases.**

Difenoconazole is not an oxidizing substance in an EC A.17 test. However, this method is not considered in the CLP criteria for the classification of oxidising solids. According to the CLP screening procedure, if the substance contains oxygen, fluorine or chlorine and these elements are chemically bonded only to carbon or hydrogen the classification is not warranted. Thus, RAC supports the DS's proposal for **no classification of difenoconazole for oxidising solids.**

There is no data derived in accordance with recommended methods for setting classification for substances corrosive to metals. However, only solids with a melting point below 55°C need to be tested, see CLP guidance 2.16.4.1., in addition, RAC notes that based on handling experience difenoconazole is not corrosive to metals. Overall, RAC support the DS's proposal for **no classification of difenoconazole as corrosive to metals.**

HUMAN HEALTH HAZARD EVALUATION

RAC evaluation of acute toxicity

Summary of the Dossier Submitter's proposal

The CLH report contains two acute oral toxicity studies in rats and mice reporting LD_{50} of 1453 mg/kg bw and greater than 2000 mg/kg bw; respectively. The CLH report also contains one acute dermal toxicity study in rabbits with an LD_{50} greater than 2010 mg/kg bw. The only acute inhalation toxicity study yielded a LC_{50} greater than 3.3 mg/L. Based on the result of these studies,

the DS proposed no classification of difenoconazole for acute dermal and inhalation toxicity and classification for acute oral toxicity category 4 (H302) with and ATE of 1453 mg/kg bw.

Comments received during consultation

One Member State Competent Authority (MSCA) supported the classification of difenoconazole as Acute Tox. 4; H302 and the oral ATE of 1453 mg/kg bw.

Assessment and comparison with the classification criteria

The table below summarises all the available studies for assessment of acute toxicity of difenoconazole.

Table: Summary of animal studies on acute toxicity with difenoconazole.

Study	Dose level	Results	Reference
Acute oral toxicity study in rats OECD TG 401 GLP: Yes Sprague-Dawley rats Oral (gavage) 5 rats/sex/group 14-day observation period	Vehicle: 3% corn starch with 1% polysorbate 80 Doses: 0, 1000, 2000, 3000 mg/kg bw Purity: Not specified	Clinical signs: hypoactivity, stains around the mouth, perineal staining, ataxia, lacrimation, soft faeces, hypothermia, salivation, spasms, prostration, chromodacryorrhea, and prostration in both males and females. Necropsy: solid red clot and/or red stomach in males and females of the 2000 mg/kg bw dose group. Body weight: slight decrease in body weight in both males and females in the 2000 mg/kg bw dose group. DOSE (mg/kg bw) / MORTALITY Males / Females: 0: 0/5 / 0/5; 1000: 2/5 / 2/5; 2000: 2/5 / 2/5; 3000: 5/5 / 5/5 **LD_{50} = 1453 mg/kg bw for both sexes**	Anonymous 7, 1987 B.6.2.1-01 (AS)
Acute oral toxicity study in mice OECD TG 401 (1987) GLP: Yes Tif: MAG f (SPF) mouse Oral (gavage) 5 mice/sex/group 14-day observation period	Vehicle: Arachis oil Purity: Not specified Doses: 1000, 2000 mg/kg bw	Clinical signs: piloerection, abnormal body positions, dyspnoea, reduced locomotor activity and ataxia. Necropsy: no treatment-related effects. Body weight: no treatment-related effects. DOSE (mg/kg bw) / MORTALITY Males / Females: 1000: 0/5 / 1/5; 2000: 1/5 / 2/5 **LD_{50} >2000 mg/kg bw for both sexes**	Anonymous 8, 1990 B.6.2.1-02 (AS)
Acute dermal toxicity Study OECD TG 402 (1981)	Vehicle: Ethanol Purity: Not specified Dose: 2010 mg/kg bw (limit test)	Clinical signs: mild cutaneous effects including erythema (2 males and 1 female) and desquamation of the skin in all animals on day 7 and all males and 2 females on day 14. Necropsy: no treatment-related effects	Anonymous 9, 1987a B.6.2.2 (AS)

GLP: Yes New Zealand White rabbits Occlusive dressing 5 rabbits/sex/dose 14-day observation period	24h exposure	Body weight: no treatment-related effects. Mortality: none **LD_{50} > 2010 mg/kg bw for both sexes**	
Acute inhalation toxicity study OECD 403 (1981) GLP: Yes Tif: RAI f (SPF) rats 5 rats/sex/dose Nose-only 14-day observation period	Exposure for 4 h Purity: 96.2% Suspension of difenoconazole with 5% Sipernat 50 S (inert silica) (maximum achievable concentration) Nominal concentration: 3967 mg/m^3 Gravimetric concentration: 3458 ± 137 mg/m^3 Particle size MMAD: 1.1 - 1.5 µm Particles < 3 µm: 73-81%	Clinical signs: piloerection, hunched posture, dyspnoea and reduced locomotor activity. Necropsy: no treatment-related effects. Body weight: lower body weight gain in males on the first week after exposure. Mortality: none **LC_{50} > 3.3 mg/L**	Anonymous 10, 1991 B.6.2.3 (AS)

Comparison with the criteria

Two acute oral toxicity studies performed following OECD Guidelines and GLP compliant yielded LD_{50} of 1453 mg/kg bw (rats) and greater than 2000 mg/kg bw (mice). The LD_{50} estimated for rats fulfils the criteria for classification within category 4 (LD_{50} greater than 300 mg/kg bw and lower than 2000 mg/kg bw). Thus, classification of difenoconazole for acute oral toxicity is warranted. RAC supports the DS's proposal for **classification of difenoconazole as Acute Tox. 4; H302** via the oral route with the ATE derived from the LD_{50} in rats rounded to 1450 mg/kg bw.

One acute dermal toxicity study performed following OECD Guideline and GLP compliant yielded a LD_{50} greater than 2000 mg/kg bw. This LD_{50} is above the threshold value of 2000 mg/kg bw considered for triggering classification. Thus, RAC supports the DS's proposal for **no classification of difenoconazole for acute dermal toxicity.**

One acute inhalation toxicity study performed following OECD Guideline and GLP compliant yielded a LC_{50} greater than the maximum attainable concentration of 3.3 mg/L. The threshold for triggering classification for acute inhalation is 5.0 mg/L for dust and mist. However, considering that 3.3 mg/L caused no mortalities, RAC notes that it is very unlikely that the LC_{50} could be below 5.0 mg/L. Overall, RAC supports the DS's proposal for **no classification of difenoconazole for acute inhalation toxicity.**

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

Summary of the Dossier Submitter's proposal

DS proposed no classification of difenoconazole for STOT SE based on the lack of non-lethal target organ toxicity, narcotic effects or respiratory tract irritation found in the acute toxicity studies and in the acute neurotoxicity study in rats.

Comments received during consultation

No comments were received.

Assessment and comparison with the classification criteria

The CLH report contains information about four different acute toxicity studies; which are summarised in the table above and the acute neurotoxicity study summarised in the table below.

Table: Summary of acute neurotoxicity study in rat with difenoconazole.

Study	Dose level	Results			Reference
Acute neurotoxicity study OECD 424 (1997) EEC B.43 GLP: Yes Alpk:APfSD rats 10 rats/sex/dose Oral (gavage)	Purity: 94.3% Doses of 0, 25, 200 and 2000 mg/kg bw	No mortalities occurred during the study 2000 mg/kg bw			Anonymous 28, 2006 B.6.7.1.1. (AS)
			males	females	
		Clinical signs (statistical significance not available)			
		Reduced splay reflex (day 1)	1/10	1/10	
		Reduced splay reflex (day 7)	0/10	2/10	
		Upward curvature of spine (day 1)	8/10	9/10	
		Decreased activity (day 1)	6/10	7/10	
		Piloerection (day 1)	3/10	5/10	
		Sides pinched (day 1)	3/10	7/10	
		Abnormal gait (day 1)	3/10	8/10	
		Body weight and food consumption			
		Body weight (day 1)	↓7%	↓7%	
		Body weight (day 8)	↓5%	-	
		Body weight (day 15)	↓4%	-	
		Food consumption (week 1)	↓19%	-	
		Functional Observational Battery			
		Time to tail flick (day 1)	-	5.6 vs 4.1 controls	
		Fore-limb grip strength (day 1)	↓26%	-	
		Fore-limb grip strength (day 15)	-	↑22%	
		Hind limb grip strength (day 8)	-	↑17%	
		Motor activity			
		Day 1	↑55%	↓37%	
		Day 8	-	↓31%	
		200 mg/kg bw			
			males	females	
		Body weight (day 1)	↓2%	↓2%	
		Fore-limb grip strength (day 1)	↓23%	-	

Hind limb grip strength (day 15)	↓23%	-
Motor activity		
Day 1	↑50%	-

The effects noted in the range warranting classification as STOT SE 1 (≤ 300 mg/kg bw) were observed in the acute neurotoxicity study at 200 mg/kg bw and included increased motor activity on day 1, decreased fore-limb strength on day 1 and decrease in hind-limb strength on day 15 (table above). Both effects observed on day 1 were reverted at the end of the study. The reduction in hind limb grip strength reported on day 15 was not noted at 2000 mg/kg bw; which suggests that this effect could be incidental.

The effects reported in the acute oral and inhalation toxicity studies (table summary of animal studies on acute toxicity with difenoconazole) could be considered for classification as STOT SE 2. However, the effects in oral toxicity were noted at doses causing mortalities and therefore should not be used for setting STOT SE classification in order to avoid double classification. The effects noted in the acute inhalation toxicity study seems to be unspecific rather than organ specific and thus could not be used for warranting STOT SE classification (table summary of animal studies on acute toxicity with difenoconazole).

The top dose (2000 mg/kg bw) in the acute neurotoxicity study is borderline for warranting classification as STOT SE 2. At this dose level, the effects observed were treatment-related motor activity and clinical signs observed on day 1 (table summary of acute neurotoxicity study in rat with difenoconazole) and that were fully reverted by day 5 (males) and on day 7 (females). These effects are not considered by RAC severe enough for supporting a classification. Moreover, at this borderline dose also an increment in fore-limb grip strength in females on day 15 was noted. However, this difference was only seen in females and at one time-point and therefore, this observation is considered by RAC as incidental to treatment with difenoconazole.

No narcotic effects or respiratory tract irritation were observed in the available studies (tables above). Thus, classification as STOT SE 3 is not warranted.

Overall, RAC supports the DS's proposal for **no classification of difenoconazole for STOT SE.**

RAC evaluation of skin corrosion/irritation

Summary of the Dossier Submitter's proposal

The DS proposed no classification of difenoconazole for skin irritation/corrosion based on the result of an irritation study in rabbits showing no erythema and no oedema.

Comments received during consultation

No comments were received.

Assessment and comparison with the classification criteria

The table below summarises the available study for assessment of dermal irritation/corrosion of difenoconazole.

Table: Summary of the animal study on skin corrosion/irritation with difenoconazole.

Study	Dose level	Results	Reference
Primary dermal irritation study in rabbits Method comparable to OECD TG 404 (2002) GLP: Yes Deviations: Skin reactions were scored at 30 min instead of 60 min after patch removal Hra: (New Zealand White) SPF rabbits 3 rabbits/sex/dose	Purity: 91.5% 0.5 g moistened with 0.9% saline Test item applied under semi-occluded conditions Exposure: 4 hours	One female rabbit was found with a grade 1 erythema at 30 min post patch removal This effect was totally reversible at 24 h The average irritation scores observed at 24h, 48h and 72h for both erythema and oedema were 0	Anonymous 11, 1991a Anonymous 12, 1992 Supplemental information irritation study B.6.2.4. (AS)

RAC supports the DS's proposal for **no classification of difenoconazole for dermal irritation or corrosion** since no erythema or oedema were observed.

RAC evaluation of serious eye damage/irritation

Summary of the Dossier Submitter's proposal

The DS proposed the classification of difenoconazole as Eye Irrit. 2; H319 based on the results of one assay comparable to OECD TG 405 showing conjunctival erythema in four out of six rabbits with scores ≥ 2 (mean score calculated at 24, 48 and 72h).

Comments received during consultation

One MSCA supported the classification of difenoconazole as Eye Irrit. 2; H319.

Assessment and comparison with the classification criteria

The table below summarises the available study for assessment of serious eye damage/eye irritation of difenoconazole.

Table: Summary of the animal study on eye irritation/corrosion with difenoconazole.

Study	Dose level	Results	Reference
Primary eye irritation study in rabbits Method comparable to OECD TG 405 (2002) GLP: Yes Hra: (New Zealand White) SPF rabbits	Purity: 91.5% Washout group: test item was washed off 30 seconds after instillation Dose: 0.05 g test item (0.1 mL weight equivalent) undiluted was	Mean scores calculated at 24h, 48h and 72h:	Anonymous 13, 1991b B.6.2.5. (AS)

			Conjunctiva	
	Cornea	Iris	Redness	Chemosis
Unwashed	0.6	0.6	2	1
	0.3	0.6	2.6	0.6
	0.3	0.3	2	0.6
	1.3	0.6	2.6	1.6
	0	0.3	1	0.3
	0	0	1.6	0.6

3 rabbits/sex/unwashed group 2 male and 1 female rabbit/washout group	instilled into the right eye. Observations after 1h, 24h, 48h, 72h and 96h	Washed	0	0.3	1	0.6
			0	0	1.3	0.3
			0	0	1.3	0
		Signs of irritation were reversible by day 4				

Comparison with the criteria

According to the classification criteria, a reversible conjunctival redness ≥ 2 in 2 of 3 animals warrants classification. The table above shows as four of six rabbits showed a reversible redness score ranging between 2 and 2.6. Thus, the criteria are met and RAC supports the DS's proposal for classification of difenoconazole as **Eye Irrit. 2; H319 (causes serious eye irritation).**

RAC evaluation of respiratory sensitisation

Summary of the Dossier Submitter's proposal

The DS proposed no classification of difenoconazole for respiratory sensitisation based on lack of data.

Comments received during consultation

No comments were received.

Assessment and comparison with the classification criteria

RAC notes that: i) there are no data indicating evidence of respiratory tract irritation with difenoconazole; ii) the acute inhalation study showed no evidence of respiratory system impairment; and iii) rabbit dermal and eye irritation studies indicated lack of irritant potential on skin and mucosal membranes. Overall, RAC agrees the DS's assessment that **no classification is warranted for difenoconazole for respiratory sensitisation based on lack of data**. However, RAC notes that due to an omission, this hazard class was not open for consultation, although the DS proposal was made available.

RAC evaluation of skin sensitisation

Summary of the Dossier Submitter's proposal

The DS proposed no classification of difenoconazole for skin sensitisation based on negative results of a modified Buehler test in guinea pigs.

Comments received during consultation

No comments were received.

Assessment and comparison with the classification criteria

The table below summarises the available study for assessment of skin sensitisation of difenoconazole.

Table: Summary of the animal study on skin sensitisation with difenoconazole.

Study	Dose level	Results	Reference
Modified Buehler Test in guinea pigs Guideline: OECD TG 406 (1981) Deviations: 1) The number of animals used in the study (10) is less than the recommended (20); 2) Information on the size of the pads is not provided. GLP: Yes Hartley guinea pig Females	Purity: Not specified Topical induction: 6h occlusive exposures to 0.5 g difenoconazole or 0.05% positive control on days 1, 3, 6, 8, 10, 13, 15, 17, 20 and 22. Topical challenge: 6h occlusive exposures to 0.5 g difenoconazole or 0.05% positive control on days 36.	No erythema was observed at induction in the difenoconazole-treated group 1 (0/10). No positive reactions were observed after challenge with difenoconazole (0/10).	Anonymous 14, 1987b B.6.2.6 (AS)

No response was observed in the sensitised animals. However, RAC notes that in the modified Buhler test no erythema was observed at induction, and according to the CLP Guidance section 3.4.2.2.4, the Buhler test should be conducted at the highest induction dose causing mild skin irritation. Moreover, since difenoconazole is a solid it should have been applied in a vehicle rather than directly in solid state. Thus, due to these uncertainties**,** RAC supports **no classification of difenoconazole in this case, due to inconclusive data.**

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

Summary of the Dossier Submitter's proposal

The DS identified liver and eye as targets of repeated dose toxicity studies. However, according to DS, liver effects are an adaptive response rather than a toxic effect or do not appear at dose levels below the guidance values for STOT RE classification. On the other hand, DS considered that the capability of difenoconazole to induce cataracts are not toxicologically relevant or are above the guidance values for STOT RE classification. Overall, DS proposed no classification of difenoconazole for STOT RE.

Comments received during consultation

No comments were received.

Assessment and comparison with the classification criteria

The table contained in Annex III summarises the repeated dose toxicity studies with difenoconazole.

The battery of repeated dose toxicity studies showed the following effects: i) alterations in body weight and body weight gain; ii) alterations in blood and clinical chemistry; iii) alterations in organ weights; iv) clinical signs; v) mortalities; vi) eye alterations; and, vii) liver alterations.

The Guidance on the Application of the CLP Criteria establishes that clinical observations or small changes in bodyweight gain, food consumption as well as small changes in clinical biochemistry or haematology, when such changes or effects are of doubtful or minimal toxicological importance as shown above cannot be used for supporting a classification as STOT RE. RAC also notes that the alterations in organs weight other than liver were not accompanied with histopathological alterations or organ dysfunction and therefore these effects were neither considered for classification.

Clinical signs and mortalities noted in the table above were considered a consequence of general toxicity, without a specific target organ and therefore these clinical effects do not warrant classification, especially considering that in all cases these clinical signs appeared at dose levels above the guideline values for supporting classification.

The 28 weeks repeated dose toxicity study in dogs reported lenticular aberrations (cataracts), although at dose levels above the limit for warranting classification. These ocular alterations were also confirmed, also at doses above the guideline limit value, in a 56-day cataractogenicity study in chicken. However, an 18-week toxicity study in dogs for assessing the cataractogenic potential of difenoconazole failed in the detection of these effects in the treated animals. Moreover, it is also noted that the formation of cataracts in the 1-year repeated dose toxicity study in dogs was not observed. Overall, RAC does not consider that the eye alterations reported above were enough for supporting a STOT RE classification.

The table above shows that liver is the target organ of difenoconazole. Difenoconazole caused liver alterations (mainly increases in absolute and relative weights with histopathological alterations as individual cell necrosis, focal/multifocal necrosis, hepatocyte hypertrophy, liver fatty change and bile stasis) in major or minor extension in the following studies: 28-days dietary study in rats; two 90-days dietary studies in rats; 13-weeks oral neurotoxicity study in rats; 2-year long-term toxicity and carcinogenicity study in rats; 90-days dietary study in mice; 78-week carcinogenicity study in mice; and, 28 weeks dietary study in dogs.

The assessment of the hepatic toxicity presented above shows that the relevant effects are below the guideline values for supporting classification:

- Increases in absolute and relative liver weights between 15-22% at 156-166 mg/kg bw/day in the 28-day oral toxicity study in rat (STOT RE 2 ≤ 300 mg/kg bw/day)
- Increases in relative liver weights between 11-19% at 20-21 mg/kg bw/day in the 13-week oral toxicity study in rat (STOT RE 2 ≤ 100 mg/kg bw/day)
- Increases in absolute and relative liver weights between 21-28% at 51-66 mg/kg bw/day in the 13-week oral toxicity study in rat (STOT RE 2 ≤ 100 mg/kg bw/day)
- Increases in relative liver weights of 16-21% at 13-17 mg/kg bw/day in the 13-week oral toxicity study in rat (STOT RE 2 ≤ 100 mg/kg bw/day)
- Increases in relative liver weights of 15% together with diffuse hepatocyte enlargement (1/10 males and 2/8 females) and centrilobular hepatocellular enlargement in 9/10 males at 34-45 mg/kg bw/day in the 13-week oral toxicity study in mouse (STOT RE 2 ≤ 100 mg/kg bw/day)

In most of the cases reported above, increases in absolute and relative liver weight were not accompanied by histopathological findings or clinical chemistry and, at these concentrations, are considered by RAC as adaptive rather than adverse effects and therefore do not warrant classification. Among all the hepatotoxicity effects observed below the guideline levels for warranting classification only one case was accompanied by histopathological alterations (8 cases of minimum and 1 case of slight centrilobular hepatocellular enlargement). This is not considered

by RAC robust enough for supporting classification. Overall, RAC supports the DS's proposal for **no classification of difenoconazole for STOT RE.**

RAC evaluation of germ cell mutagenicity

Summary of the Dossier Submitter's proposal

DS proposed no classification of difenoconazole for germ cell mutagenicity based on negative *in vitro* results in the following tests: *in vitro* tests for gene mutation (Ames test, mouse lymphoma cell assay), chromosomal aberration (cytogenetic assay in CHO cells and human lymphocytes) and DNA damage (unscheduled DNA synthesis); and also based on negative *in vivo* tests for chromosomal aberration (mouse micronucleus test).

Comments received during consultation

One company-manufacturer supported the proposal for no classification of difenoconazole for germ cell mutagenicity.

Assessment and comparison with the classification criteria

The tables below summarise the *in vitro* and *in vivo* studies found in the CLH report for assessing the difenoconazole potential for inducing germ cell mutagenicity.

Table: Summary of mutagenicity/genotoxicity *in vitro* studies with difenoconazole.

Method	Tested concentrations	Results	Reference
Bacterial gene mutation (Ames test) Comparable to OECD TG 471 (1983) and OECD TG 472 (1983) GLP: Yes *S. typhimurium:* TA1535, TA1537, TA98, TA100 *E. coli:* WP2uvrA S9 from livers of rats induced with Aroclor 1254	Purity: 91.8% Solvent: DMSO Exp. 1 and 2: 340, 681, 1362, 2723, 5447 µg/plate (±S9) with all strains Exp. 3: 85, 170, 340, 681, 1362 µg/plate (±S9) with TA1537 & TA98	**Negative**	Ogorek, 1990 B.6.4.1.1 (AS)
Mammalian cell gene mutation test Comparable to OECD TG 476 (1984) GLP: Yes Mouse lymphoma L5178Y TK+/- cells S9 from livers of rats induced with Aroclor 1254	Purity: 94.5% Solvent: DMSO Exp. 1 (4h): 8, 16, 32, 48, 64, 72, 80 µg/mL (-S9); 5, 10, 20, 30, 40, 45, 50 µg/mL (+S9) Exp. 2 (4h): 15, 30, 60, 90, 120, 135, 150 µg/mL (-S9); 3, 6, 12, 18, 24, 27, 30 µg/mL (+S9) Exp. 3 (4h): 12, 24, 48, 72, 96, 108, 120 µg/mL (-S9)	Exp. 1: No toxicity (-S9) and toxicity above 20 µg/mL (+S9). Exp. 2: Toxicity from 120 µg/mL (-S9) and at 30 µg/mL (+S9). Exp. 3: Toxicity from 72 µg/mL (-S9) **Negative**	Dollenmeier, 1986a B.6.4.1.2 (AS)

Mammalian cell chromosome aberration test OECD TG 473 (1997) GLP: Yes Chinese hamster ovary (CHO) cells S9 from livers of rats induced with Aroclor1254	Purity: 94.3% Solvent: DMSO <u>Exp. 1 (3h):</u> 21.99, 27.49, 34.36 µg/mL (-S9); 34.36, 53.69, 67.11 µg/mL (+S9) <u>Exp. 2. (3h):</u> 21.99, 27.49, 34.36 µg/mL (-S9); 34.36, 53.69, 67.11, 83.89 µg/mL (+S9)	The frequency of chromosomal aberrations exceeds the historical negative control range at 67.11 µg/mL, in Exp. 1 (+S9), might be due to cytotoxicity (59% reduction in MI), but this effect was not repeated at 67.11 µg/mL (45% reduction in MI) in Exp. 2 (+S9) **Negative (-S9)** **Equivocal (+S9)**	Lloyd, 2001 B.6.4.1.3.1.1 (AS)
Mammalian cell chromosome aberration test OECD TG 473 (1997) GLP: Yes Chinese hamster ovary (CHO) cells S9 from livers of rats induced with Aroclor 1254	Purity: 94.3% Solvent: DMSO <u>Exp. 1 (3h):</u> 26.3, 39.5, 59.3 µg/mL (-S9) <u>Exp. 2 (3h):</u> 11.7, 17.6 µg/mL (+S9) <u>Exp. 3 (21h):</u> 2.3, 5.2, 11.7 µg/mL (-S9) <u>Exp. 4 (3h):</u> 7.8, 11.7, 17.6 µg/mL (+S9)	In exp. 4 (+S9), at 17.6 µg/mL, the frequency of chromosomal aberrations exceeds the historical negative control range, but this effect was not repeated in exp. 2 (+S9), and there was no sign of cytotoxicity at this concentration. **Negative (-S9)** **Equivocal (+S9)**	Ogorek, 2001 B.6.4.1.3.1.2 (AS)
Mammalian cell chromosome aberration test Comparable to OECD TG 473 (1983) GLP: Yes Human lymphocytes S9 from livers of rats induced with Aroclor 1254	Purity: 94.5% Solvent: DMSO <u>Exp.1 (3h):</u> 2.5, 5, 10, 20, 40 µg/mL (-S9) <u>Exp. 2 (3h):</u> 2.5, 5, 10, 20, 40 µg/mL (+S9)	Cytotoxicity at the two highest dose levels in both experiments (±S9) **Negative**	Strasser, 1985 B.6.4.1.3.1.3 (AS)
Mammalian cell chromosome aberration test OECD TG 473 (1997) GLP: Yes Human lymphocytes S9 from livers of rats induced with phenobarbital and β-naphthoflavone	Purity: 94.3% Solvent: DMSO <u>Exp. 1 (3h):</u> 5, 30, 75 µg/mL (-S9); 5, 30, 62 µg/mL (+S9) <u>Exp. 2:</u> 1, 5, 10 µg/mL (20h, -S9); 5, 30, 50 µg/mL (3h, +S9)	Cytotoxicity at the highest dose levels in both experiments (±S9) **Negative**	Fox, 2001 B. 6.4.1.3.1.4 (AS)

DNA damage (UDS test) OECD TG 482 (1987) GLP: Yes Primary hepatocytes from male TIF: RAIf (SPF) rats	Purity: 91.8% Solvent: DMSO <u>Exp 1 and 2:</u> 0.46, 1.39, 4.17, 12.5, 25, 50 µg/mL	**Negative**	Hertner, 1992 B.6.4.1.4 (AS)

Difenoconazole was negative in both bacterial and mammalian cell assays for gene mutation, negative for chromosomal aberration in both cytogenetic assays using isolated human lymphocytes and negative for DNA damage in the unscheduled DNA synthesis assay. Increases in chromosomal aberrations were reported in both cytogenetic assays using CHO cells, but only at high concentrations, sometimes concurrently with cytotoxicity. These chromosomal aberrations were not clearly reproducible between experiments or across studies. Therefore, these observations are not considered of significance by RAC, especially considering the negative results of other *in vitro* genotoxicity assays.

Table: Summary of mutagenicity/genotoxicity *in vitro* studies with difenoconazole.

Method	Tested concentrations	Results	Reference
Micronucleus test OECD TG 474 (1983) GLP: Yes Tif: MAGf (SPF) mice	Purity: 91.8% Vehicle: Arachis oil Dosing by oral gavage <u>1st part:</u> 1600 mg/kg bw with sampling times at 16, 24 and 48h after treatment <u>2nd part:</u> 400, 800, 1600 mg/kg bw with a single sampling time at 24h after treatment	At 1600, 800 and 400 mg/kg bw, mice showed clinical symptoms of piloerection, laterocumbency and ataxia, but no mortality No cytotoxicity on blood forming cells **Negative**	Anonymous 15, 1991 B.6.4.2.1 (AS)

Difenoconazole was negative in the *in vivo* micronucleus test. Deviations from the current OECD TG 474 (2016) requirements were noted on the acclimatisation period, the number of polychromatic erythrocytes scored for micronuclei and the proof of exposure of the bone marrow. The toxicokinetic studies do not provide evidence that difenoconazole was able to reach bone marrow, although the clinical signs reported in the micronucleus test and the systemic toxicity reported in the 3-month feeding study in mouse, suggest systemic bioavailability.

In addition to the micronucleus test summarised in the table above, an additional *in vivo* micronucleus test without the deficiencies stated above (see below the section Supplemental information - In depth analyses by RAC) was provided. This study demonstrated that, up to the maximum tolerable dose of 320 mg/kg bw/day in male mice, difenoconazole is not able to induce clastogenicity or aneugenicity.

Overall, RAC does not consider difenoconazole a substance of genotoxic concern and supports the DS's proposal for **no classification of difenoconazole for germ cell mutagenicity.**

RAC evaluation of carcinogenicity

Summary of the Dossier Submitter's proposal

The DS proposed no classification of difenoconazole based on: i) a 2-year long-term toxicity and carcinogenicity study in rats showing no relevant neoplastic changes; ii) a 78-week

carcinogenicity study in mice showing adenomas and carcinomas in liver: iii) a number of mechanistic studies suggesting that the liver adenomas and carcinomas in mice are induced through a phenobarbital-like mode of action (MoA) based on constitutive androstane receptor (CAR) activation.

Comments received during consultation

One MSCA questioned that difenoconazole was able to activate the rat CAR. The DS replied recognizing the uncertainties as regard the CAR activation in rats and noting that this issue has no final impact in the proposal of classification since liver tumours were not observed in rats.

One company-manufacturer supported the proposal for no classification of difenoconazole for carcinogenicity.

Assessment and comparison with the classification criteria

2-year long-term toxicity and carcinogenicity study in rats

Difenoconazole was tested at dose levels of 0, 10, 20, 500 and 2500 ppm, equivalent to 0, 0.5, 1, 24 and 124 mg/kg bw/day for males and 0, 0.6, 1.3, 33 and 170 mg/kg bw/day for females. The table above (summary of repeated dose toxicity studies in rats with difenoconazole) shows the main non-neoplastic findings of this study.

No mortality or clinical signs were associated to the treatment. In general, bodyweights at the top dose tended to be lower than those of control animals. Minor alterations in clinical chemistry and haematology were also noted. Nevertheless, these differences were considered not relevant due to the low magnitude of the change, inconsistency across study intervals and/or the lack of a dose response. Macroscopic examinations did not reveal any treatment-related findings. Absolute liver weights were unaffected among the groups during the study. However, the relative liver weights for the 2500 ppm animals were higher than control values at weeks 53 (↑14% in males and 48% in females) and 104 (↑18% no significant in males and ↑ 44% in females) but were similar to control values following the 4 weeks recovery period. Other alterations in other organ weights (adrenals, ovaries, spleen) were not considered as toxicologically relevant.

Non-neoplastic changes revealed by histopathological examinations consisted in an increased incidence and severity of hepatocellular hypertrophy in 500 and 2500 ppm animals at study termination. For males, the incidence was 65 and 89% in the 500 and 2500 ppm dose groups, respectively, compared to 17.5% in the control group. Corresponding values for females were 34 and 84%, compared to 12.5% in the control group.

No neoplastic changes were considered relevant, due to the lack of a dose response and/or the low incidence and there were no increases in neoplasia in treated animals.

78-week carcinogenicity study in mice

Difenoconazole was tested at dose levels of 0, 10, 30, 300, 3000-2500 and 4500 ppm, equivalent to 0, 1.5, 4.7, 46, 508-423 and 819 mg/kg bw/day for males and 0, 1.9, 5.6, 58, 616-513 and 983 mg/kg bw/day for females. In the fifth group, the original dose of 3000 ppm was reduced to 2500 ppm at the beginning of week 2, due to early mortality. Table summary of repeated dose toxicity studies in rats with difenoconazole shows the main non-neoplastic findings of this study.

All (70) females in the 4500 ppm dose group died or were sacrificed in a moribund condition during the first 2 weeks. Eleven males (out of 70) in the 4500 ppm dose group died or were sacrificed for the same reason during the first 3 weeks of the study. At the next lower dose, 3000 ppm, 15 (out of 70) females died or were sacrificed during the first week, which led to a reduction to 2500 ppm for both sexes of this dose group, beginning at week 2 of the study. After the

lowering of dose, one additional female died during the week 2 of experiment. At the beginning of week 3, ten females of the control group were moved to 2500 ppm group to maintain an adequate sample size of this last group for the duration of the study (replacement animals); 3 of these 10 females were sacrificed due to moribund condition during their first week of exposure to 2500 ppm. After the initial mortality in females of 2500 ppm group during the first 3 weeks of the study, there was no remarkable effect on survival.

Clinical signs observed in this study included higher incidence of thinness, hunched appearance and rough haircoat in females of 2500 ppm group and in males of 4500 ppm group, compared to controls. The incidence of reduced motor activity was increased for the 4500 ppm males when compared with control.

There was a dose-dependent reduction in body weight of treated animals. Males of the two highest dose groups (2500 and 4500 ppm) had significantly lower body weights (↓6% and 7%, respectively) than controls from week 1 throughout to week 56. Females of the 2500 ppm group had significantly lower body weight (< 10%) throughout the study period. Mean body weight gains were significantly decreased through 76 weeks for the groups of 4500 ppm in males and 2500 ppm in females.

Haematological analysis showed some alterations of unclear biological significance. Clinical chemistry revealed statistically significant increases in liver enzyme values. However, these changes were at least in part reversible.

Macroscopic examinations noted for unscheduled deaths included an increased overall incidence of liver findings (enlargement, pale areas and masses) in males and females of 2500 ppm group and in males of 4500 ppm group. Remarkable observations recorded for the liver at terminal sacrifice included enlargement, pale areas and masses. At terminal sacrifice in males at 4500 ppm the incidences of liver enlargement, pale areas and masses were 50%, 56% and 44%, respectively, whereas in females at 2500 ppm were 45%, 41% and 28%, respectively. Mean absolute and relative liver weight values were significantly higher than control values for males of 2500 ppm and 4500 ppm groups and females of 2500 ppm group. Liver weights in males of recovery group (week 57) were lower than the weights for the animals at week 53 (interim sacrifice), indicating reversibility.

Non-neoplastic changes found at study termination were observed in liver. The following hepatocellular findings were significantly increased in males of 2500 ppm and 4500 ppm groups and in females of 2500 ppm group, focal/multifocal necrosis (males only), individual cell necrosis, fatty change, hepatocyte hypertrophy and bile stasis. A statistically significantly increased incidence was also noted for individual cell necrosis and hypertrophy in males of 300 ppm group. The incidences of individual cell necrosis, hepatocyte hypertrophy, fatty change and bile stasis in the liver of males of 4500 ppm group were lower after the 4-week recovery period than that observed after 53 weeks of treatment, indicative of partial recovery.

Neoplastic changes detected in mice are summarised in the tables below for males and females, respectively. Statistical analysis of liver adenomas and carcinomas revealed significant increases for males of 2500 and 4500 ppm groups, and for females of 2500 ppm group. The incidence of adenomas and/or carcinomas was already elevated in the 4500 ppm males at the interim and recovery sacrifices.

Table: Incidences of hepatocellular adenoma and carcinoma in male mice. * = Statistically significant for p ≤ 0.05 ** = Statistically significant for p ≤ 0.01.

ppm	**0**	**10**	**30**	**300**	**3000-2500**	**4500**
mg/kg bw/day	**0**	**1.5**	**4.7**	**46**	**508-423**	**819**
Adenoma						
Unscheduled deaths	0/20	3/17	1/23	2/26	9/16	6/34
Interim sacrifice	0/10	1/10	2/10	0/10	1/10	2/10
Recovery sacrifice week 57	0/9	-	-	-	0/10	3/10
Terminal	4/31	6/32	5/27	7/24	3/34	9/16
Total	4/70 (6%)	10/59 (17%)	8/60 (13%)	9/60 (15%)	**13/70*** (19%)	**20/70**** (29%)
Carcinoma						
Unscheduled deaths	0/20	0/17	1/23	0/26	1/16	4/34
Interim sacrifice	0/10	0/10	0/10	0/10	0/10	2/10
Recovery sacrifice week 57	0/9	-	-	-	1/10	1/10
Terminal	1/31	0/32	0/27	0/24	3/34	6/16
Total	1/70 (1%)	0/59 (0%)	1/60 (2%)	9/60 (0%)	5/70 (7%)	**13/70**** (19%)

Table: Incidences of hepatocellular adenoma and carcinoma in female mice. * = Statistically significant for p ≤ 0.05 ** = Statistically significant for p ≤ 0.01.

ppm	**0**	**10**	**30**	**300**	**3000-2500**
mg/kg bw/day	**0**	**1.9**	**5.6**	**58**	**616-513**
Adenoma					
Unscheduled deaths	0/26	0/14	0/21	0/15	5/21
Interim sacrifice	0/10	0/10	0/10	0/10	1/10
Recovery sacrifice week 57	-	-	-	-	0/10
Terminal	0/24	0/35	0/29	1/35	10/29
Total	0/60 (0%)	0/59 (0%)	0/60 (0%)	1/60 (2%)	**16/70**** (23%)
Carcinoma					
Unscheduled deaths	0/26	0/14	0/21	0/15	2/21
Interim sacrifice	0/10	0/10	0/10	0/10	0/10
Recovery sacrifice week 57	-	-	-	-	0/10
Terminal	0/24	0/35	1/29	0/35	2/29
Total	0/60 (0%)	0/59 (0%)	1/60 (2%)	0/60 (0%)	4/70 (6%)

Mechanistic study: Oral study of 14-days in mice

Mice were orally dosed with difenoconazole (purity 91.8%) at 0, 1, 10, 100 and 400 mg/kg bw/day for 14 days. The number of animals per each experimental group was nine. Forty mg/kg bw/day of phenobarbital (PB) were intraperitoneally administered to six mice for 4 days. Eighty mg/kg bw/day of 3-methylcholanthrene (3-MC) were intraperitoneally administered to six mice for two days. One hundred mg/kg bw/day of nafenopin (NAF) were intraperitoneally administered to three mice for six days. The mice strain was Tif:MAGf (SPF). The effects of each treatment on bodyweight, liver weight protein content of different P450 isoforms, P450 enzymatic activities, testosterone hydroxylation, peroxisomal fatty acid oxidation and glutathione S-transferase were determined. The results are summarised in the table below.

Table: Effect of difenoconazole and other reference substances on hepatic function of mice. ns= Non-significant.

		Difenoconazole			**PB**	**3-MC**	**NAF**
mg/kg bw/day	**1**	**10**	**100**	**400**	**40**	**80**	**100**
Bodyweight	ns	ns	↓11%	ns	ns	ns	↑17%
Liver weight	ns	ns	ns	↑79%	ns	↑28%	↑88%
Cytochrome P-450	↑5% ns	↑8% ns	↑161%	↑323%	↑80%	↑147%	↑40% ns
CYP1A	↑5%	↑9%	↑23%	↑35%	↑43%	↑4432%	↓39%
CYP2B	↑12%	↑38%	↑49%	↑43%	↑5%	↓34%	↑28%

CYP3A	↑20%	↑37%	↑115%	↑316%	↑287%	↓33%	↑227%
CYP4A	↑37%	↑51%	↑53%	↑17%	↑33%	↓50%	↑810%
MEH	↑19% ns	↑5% ns	↑245%	↑245%	↑115%	↑15% ns	↑233%
Microsomal morphine UDPGT	↑16% ns	↑5% ns	↑50% ns	↑59% ns	↑49% ns	↑42% ns	↑45% ns
Microsomal 1-naphtol UDPGT	↑10% ns	↑19% ns	↑28% ns	↑20% ns	↑81% ns	↑52%	↑119%
EROD	↑31% ns	↑56% ns	↑285%	↑231%	↑237%	↑4525%	↑56% ns
PROD	↑124%	↑140%	↑1839%	↑3246%	↑1522%	↑162%	↑65% ns
Lauric acid 11-hydroxylase	↑7% ns	↑8% ns	↑78%	↑130%	↑101%	↑16% ns	↑247%
Lauric acid 12-hydroxylase	↓48%	↓67%	↓38% ns	↓17% ns	↓4% ns	↓49%	↑753%

MEH: microsomal poxide hydrolase UDPGT uridine 5'-diphospho-glucuronosyltransferase: EROD: ethoxyresorufin-*O*-deethylase PROD: pentoxyreso-rufin-*O*-depentylase

The level of total testosterone hydroxylation was induced 6-fold in a dose-related manner, in mice at 400 mg/kg bw/day difenoconazole. Except for 7α-hydroxy-testosterone, all testosterone metabolites were increased between 3- and 12.5-fold in mice at 400 mg/kg bw/day of difenoconazole. PB caused increases of 6β- , 15β-, 6α- and 16α-hydroxy-testosterone.

A slight, dose-dependent and not statistically significant decrease of peroxisomal fatty acid β-oxidation was observed in mice treated with difenoconazole. PB and 3-MC did not affect the peroxisomal fatty acid β-oxidation whereas the treatment with NAF produced an increase of 4.4-fold in this process.

The activity of cytosolic glutathione S-transferase in mice treated with 400 mg/kg bw/day difenoconazole was increased 1.4-fold. A similar level of induction was seen with PB, and to a lesser extent with NAF. 3-MC reduced this enzyme activity to 1.3-fold. All these changes in cytosolic GST activity were not statistically significant.

In conclusion, the treatment with difenoconazole at 400 mg/kg bw/day in mice produced changes that were similar to those with PB, as increases of microsomal proteins (microsomal morphine UDPGT and microsomal 1-naphtol UDPGT) activities, increases of cytochrome P450 content, changes in CYP isoenzymes levels, increases of liver enzymes (MEH, EROD, PROD) activities and increases of testosterone hydroxylation.

Mechanistic study: In vitro study with CD-1 mice hepatocytes

Primary monolayer cultures of hepatocytes were cultured in Leibowitz CL15 medium for 4 hours to allow adherence. The medium was changed and the hepatocytes exposed to PB at 10, 100 and 1000 µM, to Epidermal Growth Factor (EGF, positive control for replicative DNA synthesis) at 25 ng/mL, or to difenoconazole (purity 93.9%) at 0.5, 1, 2, 4, 8 and 12.5 µM or to 0.5% DMSO (vehicle) for 96h.

There were no cytotoxicity at the tested doses since 25 µM difenoconazole caused around 3% ATP depletion. Treatment of isolated male CD-1 mouse hepatocyte cultures with difenoconazole at concentrations up to 12.5 µM resulted in increases in replicative DNA-synthesis as determined by the S-phase labelling index. However, difenoconazole did not increase either Cyp2b10 or Cyp3a11 mRNA levels. PROD and BROD (benzyloxyresorufin-*O*-debenzyl-ase) activities were also unchanged, but difenoconazole did increase BQ activity (benzyloxyquinoline-*O*-debenzylation; indicative of CYP3A activity) in concentration-dependent manner. Treatment with the positive controls PB and EGF gave the expected set of responses, indicating the suitability of the system.

Mechanistic study: In vitro study with human hepatocytes

Primary male human hepatocytes in Cryopreserved Hepatocytes Plating Medium were cultured for up to 6 hours to allow adherence. Then the medium was changed to Leibowitz HCL15 medium

and the hepatocytes exposed to PB at 10, 100 and 1000 µM, to difenoconazole at 0.5, 1, 2, 4, 6 and 8 µM, to EGF at 25 ng/mL or to 0.5% DMSO (vehicle) for 96h.

Treatment with 6 µM and 8 µM difenoconazole resulted in hepatocellular cytotoxicity with ATP levels being reduced to 75% and 49% of control, respectively. Treatment with PB did not cause a statistically significant decrease in ATP levels. Treatment of isolated male human hepatocyte cultures with difenoconazole at concentrations up to 8 µM had no effect on replicative DNA-synthesis, as determined by the S-phase labelling index. However, difenoconazole led to PROD and BROD activities induction, which are mainly representative of CYP2B and CYP2B/3A, respectively. In contrast, BQ activity was unaffected by treatment with difenoconazole at the lower concentrations, and then reduced at the higher concentrations assessed in the presence of cytotoxicity.

Mechanistic study: CAR transactivation study

Expression vectors for CAR variants of mouse, rat and human with a CYP2B6 response element-luciferase reporter were transfected into COS-1 cells along with necessary cofactors. After an expression time (16-18h) cells were incubated during 24h with difenoconazole (purity 93.9%) at 1, 3, 10 and 30 µM and with the following CAR ligands (positive controls): CITCO (substrate for human CAR) at 5 µM; TBPOBOP (substrate for mouse CAR) at 0.5 µM; and clotrinazole (substrate for rat CAR) at 10 µM.

Under conditions of this assay, difenoconazole was a direct activator of mouse CAR and not an activator of human CAR. A small increase in activation of rat CAR (1.6-fold) was observed at 30 µM difenoconazole, but this difference was not statistically significant, indicating that difenoconazole was at most a low potency activator of rat CAR.

Mechanistic study: PXR transactivation study

Expression vectors were constructed with the ligand binding domains of pregnane X receptor (PXR) variants of mouse, rat and human fused to the DNA binding domain of the transcription factor Gal4 and with a Gal4 response luciferase reporter. These vectors were transfected into HEK cells (human embryonic kidney). After 16-18h of expression, cells were incubated during 24h with difenoconazole (purity 93.9%) at 13.7, 41.2, 123, 370, 1111, 3333, 10000, 30000 nM and with pregnenolone-16α-carbonitrile and TO901317 at appropriate ranges of concentrations.

Based on the results of these luciferase reporter assays, difenoconazole is not an activator of human, rat or mouse PXR. Positive controls gave the expected results.

Mechanistic study: Acute toxicity and toxicokinetic study (1- and 7-day) in CD-1 and C57BL/6J mice

Male Charles River CD-1 and Envigo C57BL/6J mice were treated in following three different conditions as follows. Condition 1 (five animals/group/strain): Treatment for 1 day by oral gavage at 0, 15, 45, 150 and 400 mg/kg bw/day CD-1 mice or 0, 15 and 150 mg/kg bw/day C57BL/6J mice. Condition 2 (five animals/group/strain): Treatment for 5 days by oral gavage at 0, 15, 45, 150 and 400 mg/kg bw/day CD-1 mice or 0, 15 and 150 mg/kg bw/day C57BL/6J mice. Condition 3 (five animals/group/strain): Intravenous treatment for 1 day at 1 mg/kg bw/day both strains.

Difenoconazole treatment produced increased liver weights accompanied by centrilobular hypertrophy and decreased glycogen content in the liver at doses ≥ 150 mg/kg bw/day in both strains of mouse and an increased incidence of cytoplasmic vacuolation at 400 mg/kg bw/day in CD-1 mice. Furthermore, there were dose-related increases in hepatic total cytochrome P450 content and on PROD, BROD and BQ activities (markers for CYP2B, CYP2B/3A and CYP3A, respectively) along with increases of Cyp2b10 and Cyp3a11 mRNAs levels in both strains of

mouse. Overall, difenoconazole administration resulted in similar effects in male CD-1 and C57BL/6 mice at dose levels tested.

Mechanistic study: Acute toxicity and toxicokinetic study (1- and 7-day) in CAR/PXR double KO and hCAR/hPXR mice

Taconic Biosciences Inc. CAR/PXR double KO, hCAR/hPXR and C57BL/6NTAc (WT) mice were treated in following three different conditions as follows. Condition 1 (five animals/group/strain): Treatment for 1 day by oral gavage at 0, 15 and 150 mg/kg bw/day (all strains). Condition 2 (five animals/group/strain): Treatment for 5 days by oral gavage at 0, 15 and 150 mg/kg bw/day (all strains). Condition 3 (five animals/group/strain): Intravenous treatment for 1 day at 1 mg/kg bw/day (all stains).

Absolute and relative liver weight were unaffected in CAR/PXR double KO but both weights were significantly increased in hCAR/hPXR mice. Histopathological analysis showed a dose-related mild centrilobular hypertrophy only in hCAR/hPXR mice livers, whereas CAR/PXR double KO mice livers showed no treatment related changes. The analysis of liver enzyme activities and of corresponding mRNA expression levels in the liver showed no response at any dose level in male CAR/PXR double KO mice. However, in hCAR/hPXR mice at 150 mg/kg bw/day, BROD and BQ enzyme activities (markers for CYP2B/3A and CYP3A, respectively) showed statistically significant increases and Cyp2b10 and Cyp3a11 mRNA levels were higher than controls. In conclusion, these data suggest that the hepatic effects of difenoconazole are dependent on the presence of a functional CAR and/or PXR.

Mechanistic study: Hepatocellular proliferation and liver enzymatic induction study (7-day) in CAR/PXR double KO, C57BL/6NTAc WT and CD-1 WT mice

Charles River UK CD-1 WT, Taconic Biosciences Inc. C57BL/6NTAc WT, CAR/PXR double KO and CD-1 WT mice were treated (12 animals/group/strain) by oral gavage for 7 days with either 150 mg/kg bw/day difenoconazole or 80 mg/kg bw/day PB.

In CD-1 WT and C57BL/6NTAc WT mice, difenoconazole caused increases in absolute and relative liver weight, hepatocellular proliferation and centrilobular hypertrophy, accompanied by liver enzymes (EROD, PROD, BROD and BQ) induction, suggesting activation of CAR and possibly PXR nuclear hormone receptors. In the CAR/PXR double KO mice, there was not increase in absolute and relative liver weight, nor in centrilobular hypertrophy in liver enzymes induction, suggesting these were all CAR-mediated events. However, there was hepatocellular proliferation (albeit much reduced), that may be a consequence of the low BrdU labelling index for control (CMC) mice. Additionally, the formation of 12-OH lauric acid was also higher than the control response in both the wild type and CAR/PXR double KO mice treated with difenoconazole. PB caused the expected results in all three-mouse strains evaluated, except in CD-1 WT mice by a slight increase in lauric acid 12-hydroxylase (LAH).

Proposed mode of action

The applicant proposes a MoA consistent with the Adverse Outcome Pathway 107 developed by the OECD (AOPwiki, http://aopwiki.org/aops/107) entitled "*Constitutive androstane receptor activation leading to hepatocellular adenomas and carcinomas in the mouse and the rat*".

The proposed MoA for difenoconazole liver tumours consists of the activation of the CAR in the liver. CAR activation conduces to increased expression of pro-proliferative and anti-apoptotic genes in the liver and an early, transient, increase in hepatocellular proliferation. Over time, the increased hepatocellular foci because of clonal expansion of spontaneously mutated cells in the mouse results in slight increases in liver tumour incidence compared to concurrent controls. In a review article (Elcombe *et al.*, 2014), it is analysed the evidence that mouse or rat liver tumours

that occur via a CAR MoA are not relevant to humans based on qualitative differences between the species. The table below shows the key and associative events of the CAR activation MoA.

Table: Key events and associative events in the mode of action based on AOP number 107.

Key events	Key events Associative events
Key event 1: CAR nuclear receptor activation	
Key event 2: Altered gene expression specific to CAR activation	Enzyme induction (CYP2B and CYP3A) Hepatocellular hypertrophy Liver weight increase
Key event 3: Increased cell proliferation	
Key event 4: Clonal expansion leading to foci/areas of altered hepatocytes (eosinophilic)	
Key event 5: Liver adenomas/carcinomas	

The tables below show the experimental evidence for the key and associative events of a CAR mediated induction of liver tumours in rats, mice, dogs and humans studies with difenoconazole.

Table: Evidence for the key events in rats, mice, dogs and humans.

Key events	Rats	Mice	Dogs	Humans
CAR activation	YES: Difenoconazole produced a slight trend no significant toward direct activation of rat CAR in the transactivation study Omiecinski, 2016	YES: Difenoconazole was a direct activator of mouse CAR in the transactivation study. Increases in absolute and relative liver weight, hepatocellular proliferation, centrilobular hypertrophy and hepatic enzymes induction are not observed in CAR/PXR double KO mice, therefore they are CAR-dependent effects. Omiecinski, 2016 Anonymous 21, 2017b Anonymous 22, 2017	Not determined	NO: Difenoconazole was not a direct activator of human CAR in the transactivation study. In hCAR/hPXR mice, there were CAR-dependent effects as increases in absolute and relative liver weight, centrilobular hypertrophy and hepatic enzymes induction. Omiecinski, 2016 Anonymous 21, 2017b
Altered gene expression specific to CAR activation	Not determined	YES: Increases in Cyp2b10 and Cyp3a11 mRNAs levels. The liver mRNA expression levels showed no response at any dose in male CAR/PXR double KO. However, in hCAR/hPXR mice, increases in Cyp2b10 and Cyp3a11 mRNA levels were observed. Anonymous 20, 2017a Anonymous 21, 2017b	Not determined	Not determined
Increased cell proliferation	*In vitro:* Not determined *In vivo:* Not observed/ reported	*In vitro:* YES *In vivo:* YES Vardy, 2016a Anonymous 22, 2017	Not determined	*In vitro*: NO Vardy, 2016b
Clonal expansion leading to altered foci	Not observed/ reported	YES: Increase in inflammatory cell foci in C57BL/6NTAc WT mice.	Not observed/ reported	Not determined

		Anonymous 22, 2017		
Liver adenomas/ carcinomas	Not observed/ reported	YES: Hepatocellular adenomas and carcinomas were observed in liver in male mice at 4500 ppm (819 mg/kg bw/day) ($p < 0.01$) and male and female mice at 2500 ppm (♂ $p < 0.05$ and ♀ < 0.01) (423/513 mg/kg bw/day for males/females, respectively). Anonymous 18, 1989b	Not observed/ reported	Not determined

Table: Evidence for the associative events in rats, mice, dogs and humans.

Associative events	**Rats**	**Mice**	**Dogs**	**Humans**
Enzyme induction (CYP2B and CYP3A)	Not determined	*In vitro:* YES Increases in hepatic enzymes levels, especially CYP3A. *In vivo:* YES Increases in hepatic enzymes levels, especially CYP1A, CYP2B*, CYP3A and UDPGT. Vardy, 2016a Anonymous 19, 1992 (*In this study CYP2B levels decrease) Anonymous 20, 2017a Anonymous 21, 2017b Anonymous 22, 2017	Not determined	*In vitro:* YES Increases in hepatic enzymes levels, especially, CYP2B/3A. *In vivo:* Not determined Vardy, 2016b
Hepatocellular hypertrophy	YES Anonymous 36, 2000 Anonymous 16, 1989a and 17, 1992	YES Anonymous 18, 1989b Anonymous 20, 2017a Anonymous 21, 2017b Anonymous 22, 2017	NO Anonymous 34, 1987 Anonymous 35, 1988	Not determined
Increased liver weight	YES Anonymous 29, 1986a Anonymous 30, 1986b Anonymous 31, 1987a Anonymous 36, 2000 Anonymous 16, 1989a and 17, 1992	YES Anonymous 33, 1987b Anonymous 18, 1989b Anonymous 20, 2017a Anonymous 21, 2017b Anonymous 22, 2017	YES Anonymous 34, 1987	Not determined

The whole database, including the toxicity and mechanistic studies, meets the key events of this CAR activation mechanism, including altered gene expression, increased cell proliferation, clonal expansion leading to altered foci and liver tumours. This same database also provides evidence for the associative events such as enzyme induction, hepatocellular hypertrophy and increased liver weight.

Uncertainties, inconsistencies and data gaps in the proposed mode of action

The following uncertainties were found:

- The applicant explained that double CAR/PXR knockout mice were utilised instead of single CAR knockout animals, as it is almost impossible to split the two nuclear receptors because of shared ligands, co-activators and response elements. Furthermore, in the PXR transactivation assay (Korrapati & Sherf, 2016), a non-dose related increment of PXR activation was observed after difenoconazole treatment in mouse. It is also noted that PXR activation was tested with the PXR ligand-binding domain (of each species) fused to the Gal4 protein and not with the actual receptor. The presence of positive controls reduces the level of uncertainty from this experiment.
- In humans, there are several CAR proteins derived from alternative splicing (e.g. hCAR1,hCAR2, hCAR3), no details were given about which hCAR version was inserted in the mice employed in the 1 and 7-day investigative *in vivo* assay (Anonymous 21, 2017b).
- It is unclear if the lack of information about the presence of altered hepatocytes foci in the carcinogenicity study in mice is because this parameter was not considered in the experimental design or if foci were investigated but they were not found in the liver. These foci were observed only in C57BL/6NTAc mice in the 7 days study (Anonymous 22, 2017), although according to this MoA this should not be an early stage event. Nevertheless, it is noticed that, according to AOP number 107 from AOPwiki, no data for the key event of increased altered foci are available in CD-1 mice treated with PB whereas studies in male C57BL/10J mice also treated with PB show a clear increase in altered foci.
- No tumours were observed in the rat carcinogenesis study (Anonymous 16, 1989a and 17, 1992), although the results from the *in vitro* assay (Omiecinski, 2016) suggested that CAR was also activated by difenoconazole in this species. However, this could be explained as a delay in the response of this test system.

The following points were considered as inconsistencies:

- The reason for the lack of increase of Cyp2b10 and Cyp3a11 mRNA levels with difenoconazole treatment in mouse primary hepatocytes *in vitro* (Vardy, 2016c) is unknown. If CAR activation is proposed as the MoA for the tumourigenic effect of difenoconazole in mice, an increment of the transcription levels in these two genes should occur. Moreover, it would have been desirable that the same information could be available for the expression of the orthologous genes in human hepatocytes.
- After *in vitro* difenoconazole treatment of mouse cells (Vardy, 2016a) only BQ activity increased in concentration-dependent manner but unexpectedly neither PROD nor BROD activities changed, while in human cells the three activities were incremented (Vardy, 2016b). Accordingly, in the oral 14-day study (Anonymous 19, 1992), CYP2B protein level (whose markers are PROD and BROD) did not increase as expected from the proposed MoA. Unexpectedly, in the same study PROD enzyme activity was increased and this pattern was similar with PB tested in this study. This CYP2B-independent activation of PROD after difenoconazole treatment was not justified. Moreover, using liver microsomes prepared from PB-induced male CD-1 mice, difenoconazole inhibited both CYP2B and CYP3A induction in an in vitro assay when the enzymatic activities were assessed (Vardy, 2016c). A similar *in vitro* PB pre-treatment was not tested in human cells. This unexpected inhibition was not justified and the potential consequences for the proposed MoA were not addressed.

- Contrary to the proposed MoA, an increase in hepatocellular proliferation was also reported in the CAR/PXR double KO mice treated with difenoconazole, although it was much reduced compared to the proliferation observed in wild-type mice (CD-1 and C57BL/6NTAc) with the same treatment. In contrast to this, in the case of the CAR/PXR double KO mice treated with PB there was not an increase in hepatocellular proliferation, as would be expected (Anonymous 22, 2017).
- The formation of 12-OH lauric acid was higher in both wild-type and CAR/PXR double KO mice treated with difenoconazole than in untreated controls (Anonymous 22, 2017). In this study it was suggested that these results might be indicative of a small PPARα nuclear hormone receptor induction caused by difenoconazole, and that this induction could explain the residual hepatocellular proliferation in the CAR/PXR KO mice through a CAR-dependent block of PPARα transcription. However, the presence of 12-OH lauric acid also in wild-type mice indicates that this alternative explanation should be dismissed, since if an active CAR suppresses the PPARα transcription, and PPARα transcription was observed in the wild-type where CAR activation was observed, it could mean that difenoconazole can also activate PPARα to such an extend to overcome the transcription block from CAR.

The following data gap was detected:

- The lack of an HMG-CoA reductase activity study to rule out that difenoconazole could be related to a statin-like MoA.

Other potential modes of action

To define a MoA in liver, it is critical to ensure that other modes of action do not contribute significantly to hepatocarcinogenesis. In addition to CAR activation, other mechanisms may be involved in difenoconazole-induced tumorigenesis in mice liver. The plausibility of other possible modes of action is discussed in the table below.

Table: Assessment of alternative MoA for difenoconazole induced liver tumours.

Mode of Action	Data relating to difenoconazole	Conclusion
Mutagenicity	DNA reactivity and mutagenicity can be excluded since the genotoxicity testing *in vivo* and *in vitro* of difenoconazole gave no evidence of a genotoxic potential.	Unlikely
Cytotoxicity and regenerative hyperplasia	In the oral 90-day study in mice at 7500 ppm and 15000 ppm, hepatotoxicity was observed by hepatocellular enlargement and necrosis of individual hepatocytes (table 'summary of repeated dose toxicity studies in rats with difenoconazole'). Furthermore, in the carcinogenicity study in mice necrosis of individual hepatocytes, focal/multifocal necrosis of hepatocytes, bile stasis and fatty change were observed (table 'summary of repeated dose toxicity studies in rats with difenoconazole'). Since these findings occurred accompanied of increases in liver weights, they are considered a secondary effect to excessively large increases in liver weight and liver size, since literature has shown late-onset necrosis will occur as a secondary effect to very large increases in liver weight and liver size (Maronpot *et al.*, 2010; Hall *et al.*, 2012). A small increase in the incidence of mild to moderate single-cell necrosis can sometimes occur, particularly after long-term treatment of mice with CAR activators. However, more severe/diffuse necrosis in the liver suggests that an alternative MoA via cytotoxicity might be operative (Hall *et al.*, 2012). The limited amount of hepatic necrosis (single cell or focal) observed in the *in vivo* mouse treated with difenoconazole studies is in contrast with the pattern of effects seen with classic cytotoxic carcinogens that cause a diffuse necrosis (widespread multifocal hepatocyte death) in the liver that progressed to a sustained regenerative hyperplasia, as is the case of chloroform and carbon tetrachloride. In the mice study in which hepatic tumours were observed only localized areas of hepatic necrosis were seen in both sexes.	Unlikely

	Therefore, it is not considered that cytotoxicity is an additional MoA involved in the hepatocellular tumour formation.	
Estrogenic activity	Difenoconazole does not present structural similarity with oestrogens. The *in silico* mechanistic data indicated that the probability of binding of difenoconazole on oestrogen receptors is low and ToxCast ER model data showed negative results for oestrogens (B.6.8.3).	Unlikely
Statin-like activity	It has not been experimentally shown that difenoconazole has not activity as an HMG-CoA reductase inhibitor.	Plausible
Aryl Hydrocarbon Receptor (AhR) activation	Difenoconazole produces increases in EROD activity and increases in CYP1A protein levels in liver microsomes of treated mice and these markers are greatly increased by AhR activators. However, these increases are clearly lower than the increases produced in these markers by the reference substance 3-MC that is an AhR agonist (Anonymous 19, 1992).	Unlikely
Peroxisome proliferator activated receptor alpha (PPARα) activation	Difenoconazole produced a slight decrease in LAH activity and in CYP4A levels of protein in liver fractions of treated mice and did not increase peroxisomal lipid beta-oxidation (Anonymous 19, 1992). Each of these markers are greatly increased by peroxisome proliferators. However, in the Anonymous 22, 2017 study an increase in LAH has been observed in different strains of mice treated with difenoconazole. No reference substance (like the PPARα agonist nafenopin, NAF) was tested in the same study and therefore the relevancy of such increase is unclear. The plausibility of this MoA cannot be rejected, but the low repetitiveness of the result suggests it is not probable.	Plausible, but not probable
Immunosuppression	No changes in the immune system or immune cells were detected in difenoconazole studies.	Unlikely

Comparison with criteria

Category 2 is reserved for substances with evidence of carcinogenicity not sufficiently convincing to place the substance in Category 1A or 1B and can be set if the evidence of carcinogenicity is restricted to a single experiment, as is the case of difenoconazole. However, a full range of investigative studies was performed to determine the MoA of difenoconazole in the mouse. These experiments provide experimental evidence suggesting that liver carcinogenicity is induced through a PB-like MoA (CAR activation → increase of replicative DNA synthesis → hypertrophy → carcinogenesis); which can be considered as not relevant for humans. However, RAC notes that some uncertainties and inconsistences remain (see the assessment above) and this, considering that alternative modes of action have not been fully ruled-out, is insufficient evidence to support the non-relevance of the observed liver tumours for humans. Therefore, RAC considers that **classification of difenoconazole as Carc. 2; H351 (Suspected of causing cancer)** is warranted.

RAC evaluation of reproductive toxicity

Summary of the Dossier Submitter's proposal

The DS proposed no classification of difenoconazole for sexual function and fertility based on a 2-generation reproductive toxicity study showing no alterations in reproductive parameters and no significant offspring toxicity.

The DS proposed no classification of difenoconazole for developmental toxicity based on one teratogenicity study in rats and one teratogenicity study in rabbits showing minor developmental changes that were not statistically significant or dose-dependent and that could be associated to maternal toxicity.

The DS proposed no classification of difenoconazole for adverse effects on or via lactation since the reproductive study does not provide evidence of adverse effects in the offspring due to transfer in the milk or adverse effect on the quality of the milk.

Comments received during consultation

One company-manufacturer supported the proposal for no classification of difenoconazole for reproductive toxicity.

Assessment and comparison with the classification criteria

2-generation reproductive toxicity study

The table below summarises the 2-generation reproductive toxicity study with difenoconazole. This study is deficient in some endpoints including oestrus cyclicity, landmarks of sexual development, sperm analysis and ovarian follicle counts. The histopathology undertaken is limited in adults and offspring and, in particular, the target organ (liver) toxicity has not been evaluated.

Toxicity in the parental animals (F0 and F1) were observed at 2500 ppm. It included reduced body weight, body weight gain and food consumption. In F0 parents during the pre-mating period the reduction of body weight as regard control group was 8% for males and 15% for females, the reduction of body weight gain was 12 to 14% for males and 30% for females and during mating the reduction of body weight as regard the control was approximately 9% for males and persisted into gestation (body weight was 12% lower and body weight gain was 34%) and lactation (body weight was 13% lower and body weight gain was 52% lower) for females. F1 animals also showed reductions in mean body weight and weight gain during the pre-mating period (body weight was more than 15% lower for males and females; body weight gain was 10% lower for males and 22% for females), this reduction persisted in females into gestation and lactation (body weight was more than 20% lower and body weight gain was 30% lower during the first week of gestation).

No adverse effect of difenoconazole on sexual function or the fertility of the rat was identified at dose levels which induced some parental toxicity. Furthermore, there were no effects of difenoconazole on the development of the offspring other than lower body weights at birth. In F1 offspring generation there was a slight decrease, in percentage survival of male pups from days 0 to 4 at 2500 ppm.

Treatment-related reductions in pup weights of F1 and F2 offspring generations were observed at 2500 ppm for both sexes through lactation period. These reductions were statistically significant on all assessment occasions except for females on lactation day 0 of F1 offspring generation.

Table: Summary of animal studies on adverse effects on sexual function and fertility with difenoconazole.

Method	Results	Reference
2-generation reproductive toxicity study OECD TG 416 (1981) GLP: compliant Sprague Dawley rats	**PARENTAL TOXICITY** See table summary of repeated dose toxicity studies in rats with difenoconazole **REPRODUCTIVE TOXICITY** There were no treatment-related effects	Anonymous 23, 1988 B.6.6.1.1 (AS)

F0 and F1: 30 rats/sex/dose

Deviations: No oestrus cyclicity, landmarks of sexual development, no sperm analysis and ovarian follicle counts

Purity: 97.4 %

Oral (diet)

0, 25, 250 or 2500 ppm

F0 and F1 (mean): males: 0, 1.7, 17, or 171 mg/kg bw/day females: 0, 1.9, 19, 189 mg/kg bw/day

Pre-mating treatment: F0 (77 days); F1 (98 days); Treatment continued in F0 and F1 throughout gestation and lactation

OFFSPRING TOXICITY

2500 ppm (171/189 mg/kg bw/day)

	F1 offspring		F2 offspring	
	males	females	males	females
Survival (days 0 to 4 pre-cull)	95.2% vs 98.7%	-	-	-
Pup weight (birth)	↓6%	-	↓8.2%	↓7.4%
Pup weight (day 4 pre-cull)	↓13%	↓11%	↓14%	↓13%
Pup weight (day 4 post-cull)	↓14%	↓11%	↓15%	↓14%
Pup weight (day 7)	↓23%	↓20%	↓21%	↓20%
Pup weight (day 14)	↓27%	↓26%	↓26%	↓26%
Pup weight (day 21)	↓30%	↓29%	↓33%	↓32%

250 ppm (17/19 mg/kg bw/day)

	F1 offspring		F2 offspring	
	males	females	males	females
Pup weight (birth)	-	-	-	-
Pup weight (day 4 pre-cull)	-	-	-	-
Pup weight (day 4 post-cull)	-	-	-	-
Pup weight (day 7)	-	-	-	-
Pup weight (day 14)	-	-	-	-
Pup weight (day 21)	↓7%	-	-	-

Developmental toxicity study in rats

The study was performed following the OECD TG 414 guideline in compliance with GLP. Crl rats were dosed by gavage with 0, 2, 16, 100 and 200 mg/kg bw/day of difenoconazole (purity 95.7%) during gestation days 6-15. Table summary of repeated dose toxicity studies in rats with difenoconazole summarises the maternal toxicity. At the highest dose of 200 mg/kg bw/day the onset of dosing was associated with a 14% loss of body weight (days 6-8) as regard the control group. For the dosing period (days 6-15) the body weight gain was reduced by approximately 56% and the overall reduction (GD 0-20) was 12% lower as regard controls. Excess salivation was significantly increased in 19 out of 25 (76%) dams. There was one female at this dose level with severe weight loss that totally resorbed its litter.

At the intermediate dose, 100 mg/kg bw/day, the onset of dosing was associated with an overall statistically significant reduction in body weight gain of 23% (days 6-15) compared to control. The incidence of excess salivation was significantly increased in 14 out of 23 (61%) dams. Full recovery was made in the post-dosing period and hence there was no effect on foetal body weight or ossification.

The table below summarises the foetal alterations. At the top dose, there were statistically significant alterations in the foetal ossification sites (an increase in the average number of ossified hyoid, number of thoracic vertebrae and mean number of ribs and a decrease in the average number of lumbar vertebrae and sternal sternum). In addition, there was an increased incidence of some skeletal alterations in litters (thoracic central bifid). At 100 mg/kg bw/day there was an increased incidence of some skeletal alterations in litters (thoracic central bifid). Although these differences were not statistically significant, they were dose-dependent (at this dose level and

higher doses) and out of the historical control data (8.7 vs 7.26 HCD). No treatment-related effects on foetus were noted at 16 and 2 mg/kg bw/day.

Table: Foetal alterations detected in the teratogenicity study in rats with difenoconazole. * = Statistically different form control for p ≤ 0.05 * = Statistically different form control for p ≤ 0.01.

		Control	200 mg/kg bw/day	100 mg/kg bw/day	HCD
Examined foetuses		**182**	**160**	**160**	**3417**
Examined litters		**25**	**24**	**24**	**413**
Thoracic central bifid	foetus	0/182	5/160 (3.1%)**	2/168 (1.2%)	31/3417 (0.91%)
Thoracic central bifid	litter	0/25	4/24 (17%)	2/23 (8.7%)	30/413 (7.3%)
Thoracic central unilateral ossification	foetus	0/182	3/160 (1.9%)**	-	2/3417 (0.06%)
Thoracic central unilateral ossification	litter	0/25	1/24 (4.2%)	-	2/413 (0.48%)
No. of hyoid ossification sites	foetus/litter	0.72	0.95*	-	-
No. of thoracic ossification sites	foetus/litter	13.0	13.2**	-	-
No. of lumbar ossification sites	foetus/litter	6.0	5.8*	-	-
No. of ribs	foetus/litter	13.0	13.2**	-	-
No. of sternal ossification sites	foetus/litter	3.7	3.4*	-	-

Developmental toxicity study in rabbits

The study was performed following OECD TG 414 guideline and observing GLP procedures. New Zealand White rabbits were dosed by gavage with 0, 1, 25 and 75 mg/kg bw/day of difenoconazole (purity 95.7%) during gestation days 7-19. Table summary of repeated dose toxicity studies in rats with difenoconazole summarises the maternal toxicity. At the highest dose the onset of dosing was associated with loss body weight gain (days 7-10; 10-14 and 0-29), abortion in two rabbits and death following anorexia in another rabbit.

There were no significant differences in pregnancy or litter parameters among the groups. There was an increase in the number and percent of resorptions (mainly early, 0.6 vs 0.3 control), increase in post implantation loss (12.9%), and prenatal death (0.13 vs 0.07 control). Although these differences were not statistically significant the increase was noteworthy and, given the absence of HCD, it cannot be ruled out that they are related to the treatment. No treatment-related external, visceral or skeletal abnormalities were seen. No effects were seen in dams or in foetuses from dams treated with 1 or 25 mg/kg bw/day.

Comparison with criteria

Difenoconazole caused no adverse effects on sexual function or fertility at dose levels causing parental toxicity. Overall, RAC supports the DS's proposal for **no classification of difenoconazole for sexual function and fertility.**

According to the results of submitted studies, no irreversible effects such as structural malformations, foetal embryo/lethality, and significant postnatal functional deficiencies were observed. The effects observed were minor developmental changes that could be associated with maternal toxicity (excess salivation in 19/25 dams, ↓56% body weight gain days 6-15 and ↓12% body weight gain on days 0-20). A slight decrease in the percentage survival of male pups from days 0 to 4 pre-cull at 2500 ppm was noted. However, it was slight and not sufficient for supporting a classification. Treatment-related reductions in pup weights of F1 and F2 offspring generations in both sexes were noted at the top dose through lactation period. However, these reductions could be associated with reductions in body weight and body weight gain in F0 and F1 female parents during this period at this dose. Overall, RAC supports the DS's proposal for **no classification of difenoconazole for developmental toxicity.**

The classification is intended to indicate when a substance may cause harm due to its effects on or via lactation and is independent of consideration of the reproductive or developmental toxicity of the substance. This can be due to the substance being absorbed by women and adversely affecting milk production or quality, or due to the substance (or its metabolites) being present in breast milk in amounts sufficient to cause concern for the health of a breastfed child.

The available reproductive study does not provide evidence of adverse effects in the offspring due to transfer in the milk or adverse effect on the quality of the milk. Toxicokinetic studies do not indicate the likelihood that the substance can be potentially present in breast milk. Thus, there were no effects to warrant classification of difenoconazole for effects on or via lactation. RAC supports the DS's proposal for **no classification of difenoconazole for adverse effects on or via lactation.**

RAC evaluation of aspiration toxicity

Summary of the Dossier Submitter's proposal

DS considered that this hazard is not applicable for difenoconazole.

Comments received during consultation

No comments were received.

Assessment and comparison with the classification criteria

RAC notes that the hazard aspiration toxicity is not relevant for fine crystalline powders and therefore concurs with the DS assessment. However, RAC does not propose a conclusion on this hazard class as it was not open for consultation and thus is outside of RAC's mandate.

ENVIRONMENTAL HAZARD EVALUATION

RAC evaluation of aquatic hazards (acute and chronic)

Summary of the Dossier Submitter's proposal

Regarding the renewal of difenoconazole as an active substance in the context of the PPP regulation, a Renewal Assessment Report has been developed and the CLH report also relied on data submitted in the context of the application for approval as an active substance under Regulation (EC) No 1107/2009.

Overall, the DS concluded that difenoconazole is not rapidly degradable, has a low potential for bioaccumulation and proposed classification based on aquatic acute toxicity to algae and aquatic chronic toxicity to invertebrates:

Aquatic Acute 1 with an M-factor of 10, based on the lowest measured 72h E_rC_{50} value of 0.0876 mg/L for *Scenedesmus subspicatus* and Aquatic Chronic 1 with an M-factor of 10, based on the lowest measured 28-d NOEC of 0.0023 mg/L for *Americamysis bahia*.

Degradation

A ready biodegradability test (OECD TG 301B) showed that 0% biodegradation of difenoconazole was observed after 29 days (Baumann, 1993). Therefore, difenoconazole was considered as not readily biodegradable.

The results of a hydrolysis study (OECD TG 111, GLP) showed that difenoconazole is hydrolytically stable in solutions at pH 4 to 9 at 25ºC over a period of 30 days (Atkins, 1991).

Difenoconazole is stable to direct photolysis in aqueous systems at pH 7 at 25ºC over a period of 15 days (Gaauw, 2002a). Direct photolysis is assessed to be an insignificant process for degradation of difenoconazole in surface waters (Hennecke, 2002a).

In an aerobic mineralisation study (OECD TG 309, GLP), difenoconazole degraded with DT_{50} values of 104.7 and 146.7 days depending on test concentration (10 µg/L and 95 µg/L respectively) to the major metabolites CGA205375 and CGA142856 (Gartner and Herrechen, 2016).

Regarding the water/sediment system, four studies (Gonzalez-Valero, 1993; Ulbrich, 1997; Lin, 2006; Yeomans and Mould, 2018) were submitted. A kinetics assessment (Terry, 2015c) was performed in accordance with FOCUS degradation kinetics guidance. Although short DT_{50} and DT_{90} values were registered for the water phases (DT_{50} between 2.13 and 5.52 days and DT_{90} between 7.08 and 18.3 days), difenoconazole disappears via dissipation, binding to sediment. At the end of the above studies, the maximum carbon dioxide increased to 3.9% AR, indicating minimal mineralization. Thus, difenoconazole can be considered as not rapidly degradable in the aquatic environment from the water/sediment system studies carried out.

Overall, due to the results summarised above, the DS concluded that degradation information does not provide sufficient data to show that difenoconazole is ultimately degraded to a level equal to or greater than 70% within 28 days (equivalent to a half-life of less than 16 days) or is degraded to non-classifiable products. Therefore, difenoconazole was considered by the DS as being not rapidly degradable, according to the CLP criteria.

Aquatic Bioaccumulation

Two 28 days studies (US EPA FIFRA 72-6) on bioconcentration by bluegill sunfish (*Lepomis macrochirus*) were carried out. Only one concentration was tested in each study, while the test guidelines require at least two exposure levels. The two available studies examined together were considered to fulfil the requirement of more than one exposure concentration.

The studies were conducted in a flow-through system to nominal concentrations of 0.02 mg/L (Anonymous, 1987) and 1.0 µg/L (Anonymous, 1992) for a period of 28 days followed by a 14 days period of depuration in fresh water.

For the whole fish, the steady-state bioconcentration factor (BCF) was calculated to be 320 L/kg and 330 L/kg for the treatment levels of 0.02 mg/L and 1.0 µg/L, respectively.

The determined log K_{OW} of 4.36 ± 0.02 at pH 8 and 25^0C (OECD TG 107) meets the CLP trigger value of ≥ 4 indicating a potential for bioaccumulation. The log K_{OW} was not pH dependent as difenoconazole did not dissociate at environmentally relevant pHs (Kettner, 1999b).

Consequently, as preference in CLP is given to experimentally derived BCF values (in this case equal to 330 L/kg, which is below the CLP criterion of ≥ 500), the DS concluded that difenoconazole can be considered as having low potential for bioaccumulation.

Aquatic Toxicity

The aquatic toxicity test results from available acute and chronic studies for all trophic levels of difenoconazole are summarised in the following table and sections. Acute and chronic aquatic

toxicity data on difenoconazole are available for fish, invertebrates, algae and aquatic plants. Algae is the most acutely sensitive trophic group. Invertebrates are the most chronically sensitive trophic group. All provided studies were considered as acceptable and reliable by the DS.

Table: Acute Aquatic toxicity

Test organism	Guideline, test method	Short-term result (endpoint)	Reference / Test item
Fish			
Salmo gairdneri	US EPA FIFRA 72-1 / GLP	96h LC_{50} = 1.1 mg/L (mm)	Anonymous, 1990a / Difenoconazole (96.1%)
Lepomis macrochirus	US EPA FIFRA 72-1 / GLP	96h LC_{50} = 1.21 mg/L (mm)	Anonymous, 1988 / Difenoconazole (96.1%)
Cyprinodon variegates	US EPA FIFRA 72-3 / GLP	96h LC_{50} = 1.16 mg/L (mm)	Anonymous, 1993 / Difenoconazole (96%)
Pimephales promelas	OECD TG 203; OPPTS Draft Guideline 850.1075 / GLP	96h LC_{50} = 1.9 mg/L (mm)	Anonymous, 2011 / Difenoconazole (97.3%)
Salmo gairdneri	OECD TG 203 / GLP	96h LC_{50} = 0.66 mg/L (mm)	Anonymous, 2001a / CGA205375 (triazolylalcohol 99%)
Aquatic invertebrates			
Daphnia magna	US EPA FIFRA 72-2 / GLP	48h LC_{50} = 0.77 mg/L (mm)	Forbis, 1988a / Difenoconazole (96.1%)
Mysidopsis bahia	US EPA FIFRA 72-3 / GLP	48h LC_{50} = 0.15 mg/L (mm)	Surprenant, 1990c / Difenoconazole (95%)
Crassostrea virginica	US EPA FIFRA 72-3 / GLP	48h LC_{50} > 0.3 mg/L (mm)	Surprenant, 1990d. / Difenoconazole (95%)
Algae / other aquatic plants			
Scenedesmus subspicatus	OECD TG 201 / GLP	72h E_bC_{50} = 0.032 mg/L (mm)	Grade, 1993b / Difenoconazole (91.8%)
Scenedesmus subspicatus	Statistical re-analysis of data from the previous study (Grade, 1993b)	**72h E_rC_{50} = 0.0876 mg/L (mm)**	Taylor and Pickering / 2016b / Difenoconazole (91.8%)
Pseudokirchneriella subcapitata	OECD TG 201 / GLP	72h E_rC_{50} = 1.2 mg/L (mm)	Hoberg, 2006 / Difenoconazole (94.4%)
Lemna gibba	US EPA FIFRA 122-2 / GLP	14d EC_{50} = 18.5 mg/L (frond number) (nom) 14d EC_{50} = 9.9 mg/L (dry weight) (nom)	Drottar, 1986 / Difenoconazole (96.1%)
Lemna gibba	OECD TG 221; US EPA, OPPTS 850.4400 / GLP	7d E_bC_{50} = 1.8 mg/L 7d E_rC_{50} > 6.5 mg/L (frond number) (mm)	Hoberg, 2006d / Difenoconazole (94.4%)

mm: mean measured concentration, nom: nominal concentration

Four studies have been submitted on the acute toxicity of difenoconazole to fish. The reported 96h LC_{50} values of difenoconazole in all studies with fish were above 1 mg/L based on mean measured concentration. In addition, toxicity data of metabolite CGA205375 have been submitted as well. The reported 96h LC_{50} values of metabolite CGA 205375 (triazolylalcohol) was 0.66 mg/L based on mean measured concentration.

Three studies have been submitted on the acute toxicity of difenoconazole to aquatic invertebrates. The reported 48h LC_{50} values of difenoconazole in all studies with invertebrates varied between 0.15 – 0.77 mg/L, based on mean measured concentration.

Two original studies have been submitted on the acute toxicity of difenoconazole to algae. The indicated acute toxicity value were 72h E_bC_{50} = 0.032 mg/L with *Scenedesmus subspicatus* (Grade, 1993b) and 72h E_rC_{50} = 1.2 mg/L with *Pseudokirchneriella subcapitata* (Hoberg, 2006), based on mean measured concentration. However, the reported value of 0.032 mg/L from the study with *Scenedesmus subspicatus* (Grade, 1993b) was below the limit of detection of the active substance (0.04 mg/L). Therefore, statistical re-analysis (Taylor and Pickering, 2016b) of

data from this study (Grade, 1993b) was presented as well. The re-calculated acute toxicity value 72h E_rC_{50} = 0.0876 mg/L was derived.

Two studies have been submitted on the acute toxicity of difenoconazole to aquatic macrophytes. The reported 14 and 7- day EC_{50} values of difenoconazole in both studies were above 1 mg/L.

Overall, the DS proposed to classify difenoconazole as Aquatic Acute 1 based on the 72h E_rC_{50} for *Scenedesmus subspicatus* of 0.0876 mg/L mean measured concentration based on the provided statistical re-analysis. As this acute toxicity value falls within the $0.01 < L(E)C_{50} \leq 0.1$ mg/L range, the acute M-factor proposed by the DS was 10.

Table: Aquatic Chronic toxicity

Test organism	Guideline, test method	Long-term result (endpoint)	Reference / Test item
Fish			
Fathead minnow *(Pimephales promelas)*	Fish early life stage US EPA FIFRA 72-4 / GLP	32d NOEC = 0.0076 mg/L (mm)	Anonymous, 1987b / Difenoconazole (96.1%)
Fathead minnow *(Pimephales promelas)*	Statistical re-analysis of data from the previous study (Anonymous, 1987b)	34d NOEC = 0.0076 mg/L 34d EC_{10} = 0.0129 mg/L (mm)	Anonymous, 2016a / Difenoconazole (96.1%)
Fathead minnow *(Pimephales promelas)*	Fish early life stage US EPA FIFRA 72-4 / GLP	30d NOEC $_{(post\ hatch)}$ = 0.0087 mg/L (mm)	Anonymous, 1990b / Difenoconazole (95%)
Fathead minnow *(Pimephales promelas)*	Fish full life-cycle OPPTS Draft Guideline 850.1500 / GLP	90d $NOEC_{(post\ hatch)}$ = 0.0036 mg/L	Anonymous, 2009 / Difenoconazole (97.4%)
Fathead minnow *(Pimephales promelas)*	Statistical re-analysis of data from the previous study (Anonymous, 2009)	90d $NOEC_{(post\ hatch)}$ = 0.0036 mg/L 90d $EC_{10\ (90d\ post\ hatch)}$ = 0.02151 mg/L (mm)	Anonymous, 2016 / Difenoconazole (97.4%)
Aquatic invertebrates			
Water flea *(Daphnia magna)*	US EPA FIFRA 72-4 / GLP	21d NOEC 0.0056 mg/L (mm)	Forbis 1988b / Difenoconazole (96.1%)
Water flea *(Daphnia magna)*	Statistical re-analysis of data from the previous study (Forbis, 1988b)	21d NOEC 0.0056 mg/L (mm) 21d EC_{10} = 0.0046 mg/L (mm)	Taylor and Pickering, 2016a / Difenoconazole (96.1%)
Mysids *(Americamysis bahia)*	OPPTS Guideline 850.1350 and FIFRA Guideline 72-4 / GLP	28d NOEC 0.0046 mg/L (mm)	Lee, 2009 / Difenoconazole (94.4%)
Mysids *(Americamysis bahia)*	Statistical re-analysis of data from the previous study (Lee, 2009)	28d NOEC 0.0046 mg/L (mm)	Taylor and Allen, 2016b / Difenoconazole (94.4%)
Mysids *(Americamysis bahia)*	OPPTS Guideline 850.1350 and ASTME Guideline 1191-03a / GLP	**28d NOEC = 0.0023 mg/L (mm)**	Sayers, 2014 / Difenoconazole (94.4%)
Mysids *(Americamysis bahia)*	Statistical Re-analysis of data from the previous study (Sayers, 2014)	28d NOEC = 0.0023 mg/L (mm)	Taylor and Allen, 2016c / Difenoconazole (94.4%)
Algae /other aquatic plants			
Green algae *(Scenedesmus subspicatus)*	OECD TG 201 / GLP	72h NOEC = 0.0086 mg/L (mm)	Grade, 1993b / Difenoconazole (91.8%)
Green algae *(Scenedesmus subspicatus)*	Statistical re-analysis of data from the previous study (Grade, 1993b)	72h NOEC = 0.0086 mg/L (mm) 72h E_rC_{10} = 0.015 mg/L	Taylor and Pickering, 2016b / Difenoconazole (91.8%)

Freshwater green algae *(Pseudokirchneriella subcapitata)*	OECD TG 201 / GLP	72h NOEC = 0.36 mg/L (mm)	Hoberg, 2006 / Difenoconazole (94.4%)
Duckweed *(Lemna gibba)*	OECD TG 221; US EPA, OPPTS 850.4400 / GLP	7d NOEC = 0.11 mg/L (mm)	Hoberg, 2006d / Difenoconazole (94.4%)
Sediment dwelling organisms			
Midge larvae *(Chironomus riparius)*	ASTM E1706 / GLP	Water phase: 28d NOEC = 0.015 mg/L (mm) Sediment phase: 28d NOEC = 0.00525 mg/kg (mm)	Van der Kolk, 1999 / Difenoconazole (91%)
Midge larvae *(Chironomus riparius)*	OECD TG 218 / GLP	28d NOEC $_{emergence}$ = 14 mg/kg dry sediment (corresponding to 0.038 mg/L) 28d NOEC $_{developmental}$ = 8.2 mg/kg dry sediment (corresponding to 0.018 mg/L) (mm)	Eckenstein, 2014 / Difenoconazole (96.6%)
Midge larvae *(Chironomus riparius)*	OECD proposed guideline for toxicity test with Chironomidae; BBA Guideline proposal	26d NOEC = 0.4 mg/L (water column) 28d NOEC = 10 mg/kg (sediment)	Grade, 2001 / CGA205375 (triazolylalcohol 99%)

mm: mean measured concentration, nom: nominal concentration

Three original studies and a re-assessment of two studies have been submitted on the chronic toxicity of difenoconazole to fish. The original and re-assessed NOEC values of difenoconazole fall in the range above 0.001 to below 0.01 mg/L, based on mean measured concentration. The Probit analysis with linear maximum likelihood regression was used to determine the concentration response function in the re-assessment. Although EC_{10} values of difenoconazole originally not were estimated they have been estimated during re-analysis.

Three original studies have been submitted on the chronic toxicity of difenoconazole to invertebrates. All of them have been re-assessed by the Dossier Submitter and the originally obtained chronic toxicity values were confirmed. The reported chronic toxicity values of difenoconazole on invertebrates slightly differ but were within the same range of above 0.001 to below 0.01 mg/L. The lowest chronic toxicity value was 28-day NOEC = 0.0023 mg/L for *Americamysis bahia*, based on mean measured concentration.

Three original studies and the re-assessment of one study have been submitted on the chronic toxicity of difenoconazole to algae / other aquatic plants. The statistical re-analyses confirmed the obtained NOEC and provided estimated EC_{10} values. In addition, two studies on the chronic toxicity of difenoconazole and one study on the chronic toxicity of metabolite (CGA205375) with non-biting midge (*Chironomus riparius*) in a water/sediment system have been submitted.

Overall, the DS proposed to classify difenoconazole as Aquatic Chronic 1 based on the 28-day NOEC for *Americamysis bahia* of 0.0023 mg/L, based on mean measured concentration. As the substance was considered not rapidly degradable and chronic toxicity falls within the 0.001 < NOEC ≤ 0.01 mg/L range, the chronic M-factor proposed by the DS was 10.

Comments received during consultation

Two MSCAs submitted comments on the environmental part of the DS's CLH proposal. Both of them agreed with the proposed classification by the DS without further comments.

Assessment and comparison with the classification criteria

Degradation

A ready biodegradation study (OECD TG 301B) with difenoconazole indicated 0% degradation after 29 days, indicating that difenoconazole is not readily biodegradable.

No hydrolysis of difenoconazole was observed and substance was stable in solutions at pH 4 to 9 at 25°C over a period of 30 days (EPA, 540/9-82-021). Calculated half-lives for difenoconazole were above 1000 days.

Difenoconazole was stable to direct photolysis in aqueous systems at pH 7 at 25°C over a period of 15 days (EPA, OPPTS 835.2210). After 15 days of continuous irradiation, difenoconazole represented 91% of the AR. Detected radioactive fractions do not exceeded 6.3% AR. The calculated half-lives of difenoconazole at 52°N were between 11.8 years and above 10000 years, depending on the season. Thus, difenoconazole is stable to direct photolysis in aqueous systems and direct photolysis is an insignificant process for degradation of difenoconazole in surface water.

In an aerobic mineralisation study (OECD TG 309), max mineralisation after 61 days was 16.3% AR. Difenoconazole degraded with DT_{50} values of 104.7 and 146.7 depending on the test concentration (10 µg/L and 95 µg/L respectively) to the major metabolites CGA205375 and CGA142856. For the non-sterilised, viable test systems Difenoconazole decreased to a mean of 61.9 - 71.1% AR (10 µg/L) and 71.6 - 78.9% AR (95 µg/L) after 61 days. For the sterilised samples, difenoconazole was found to be stable, with 92% AR remaining at 61 day. Thus, degradation of difenoconazole in natural water is microbially mediated.

Water/sediment studies suggest that difenoconazole mainly dissipates from aquatic systems by physical-chemical processes. Partitioning to sediment is the main route of dissipation of difenoconazole in water sediment systems primarily via binding to sediment. For the water phase, DT_{50} values were 2.16 days (pond system) and 5.52 days (river system). DT_{90}s were 7.16 days (pond system) and 18.3 days (river system). The whole system DT_{50} values were 318 days (pond system), 300 days (river system) and DT_{90} > 1000 days (pond system), > 998 days (river system). Based on the other study results, the DT_{50} of water phase was 3.2 days and DT_{90} – 10.6 days. However, for whole system the DT_{50} was 1113 days and the DT_{90} – 2300 days. At the end of the above studies, the maximum carbon dioxide increased to 3.9% AR indicating minimal mineralization.

Overall, due to the results summarised above, RAC agrees with the assessment of the DS that difenoconazole is not ultimately degraded to ≥ 70% within 28 days (equivalent to a half-life < 16 days), or rapidly transformed to non-classifiable products. Consequently, RAC agrees that difenoconazole should be considered as **not rapidly degradable**.

Aquatic Bioaccumulation

In the two available experimental studies to determine the bioconcentration potential, the determined whole fish BCF values of 320 and 330 L/kg for difenoconazole is below the CLP trigger value of ≥ 500. Although only one concentration was tested in each study while the test guidelines require at least two exposure levels, RAC agrees with the DS that two available studies together could be considered as fulfilling the requirement of more than one exposure concentration. Therefore, the whole fish calculated steady-state bioconcentration factor (BCF) is 320 L/kg and 330 L/kg for the treatment levels of 0.02 mg/L and 1.0 µg/L, respectively.

The derived Log K_{OW} value of 4.36 (pH 8 at 25°C) meets the CLP trigger value for indication of bioaccumulation (Log K_{OW} ≥ 4). However, following the CLP regulation (section 4.1.2.8.1), the available, reliable experimental BCF determined in fish is taken in preference to the Log K_{OW}. Therefore, based on the BCF_{fish} below 500, RAC agrees with the DS that difenoconazole is **not bioaccumulative** according to the CLP criteria.

Aquatic Toxicity

RAC notes that there are reliable acute and chronic aquatic toxicity data for all trophic levels. RAC agrees that the provided studies are acceptable and reliable. The most acutely sensitive trophic group is algae and most chronically sensitive trophic group is aquatic invertebrates. Chronic toxicity values for aquatic invertebrates slightly varied, but within the same order of magnitude. In addition, most of the chronic toxicity values for fish and algae were also within the same order of magnitude as chronic toxicity values for invertebrates.

Consequently, RAC agrees that the lowest acute toxicity endpoint for aquatic acute classification is the 72h E_rC_{50} value for *Scenedesmus subspicatus* of 0.0876 mg/L, based on re-assessment of the study results. The lowest chronic endpoint for aquatic chronic classification is the 28d NOEC value for *Americamysis bahia* of 0.0023 mg/L, based on mean measured concentration and confirmed by the re-assessment of the study.

Conclusion on classification

Difenoconazole is considered as not rapidly degradable and does not fulfil the criteria for bioaccumulation. Based on the available and reliable information, RAC agrees with the DS that difenoconazole warrants classification as:

Aquatic Acute 1 based on E_rC_{50} = 0.0876 mg/L for *Scenedesmus subspicatus.* As this acute toxicity value falls within the $0.01 < L(E)C_{50} \leq 0.1$ mg/L range, the **acute M-factor is 10**.

Aquatic Chronic 1 based on NOEC = 0.0023 mg/L for *Americamysis bahia*. As this chronic toxicity value falls within the $0.001 < NOEC \leq 0.01$ mg/L range, the **chronic M-factor is 10**.

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).

Annex 3 Summary of the repeated dose toxicity studies with difenoconazole

Annex 3 – Summary of the repeated dose toxicity studies with difenoconazole

Table: Summary of repeated dose toxicity studies in rats with difenoconazole. HCD: historical control data

Method	Results			Reference
28-day toxicity study OECD TG 407 (1981) GLP: No Wistar rats 10 rats/sex/dose Purity: 95% Oral (diet) 0, 250, 1500 or 10000 ppm 0, 27, 156 and 914 mg/kg bw/day (males) 0, 27, 166 and 841 mg/kg bw/day (females) *Guideline value for classification:* *STOT RE 2 ≤ 300 mg/kg bw/day* *STOT RE 1 ≤ 30 mg/kg bw/day*	10000 ppm (914/841 mg/kg bw/day)			Anonymous 29, 1986a B.6.3.1.1 (AS)
		males	females	
	Body weight (statistics not available):			
	Week 1	↓30%	↓29%	
	Week 2	↓39%	↓36%	
	Week 3	↓40%	↓36%	
	Week 4	↓42%	↓36%	
	Haematology (out HCD, statistics not available):			
	Thromboplastin time	↓8%	-	
	Clinical chemistry (out HCD, statistics not available):			
	Cholesterol	↑230%	↑231%	
	γ-Glutamyl transferase	↑75%	↑73%	
	Albumin	↑15%	↑9%	
	α1-Globulin	↓29%	↓10%	
	β-globulins	↓29%	↓27%	
	A/G ratio	↑58%	↑13%	
	Organ weights:			
	Terminal body weights	↓44%	↓40%	
	Liver (absolute)	↓14%	-	
	Liver (relative)	↑54%	↑74%	
	Kidneys (absolute)	↓37%	↓28%	
	Kidneys (relative)	↑13%	↑20%	
	Brain (absolute)	↓14%	↓11%	
	Brain (relative)	↑55%	↑48%	
	Heart (absolute)	↓39%	↓35%	
	Heart (relative)	↑38%	↑42%	
	Spleen (absolute)	↓38%	↓42%	
	Thymus (absolute)	↓49%	↓47%	
	Testes (absolute)	↓18%	-	
	Testes (relative)	↑49%	-	
	Ovaries (absolute)	-	↓56%	
	Ovaries (relative)	-	↓40%	
	Adrenals (absolute)	-	↓29%	
	Adrenals (relative)	↑50%	-	
	1500 ppm (156/166 mg/kg bw/day)			
		males	females	
	Haematology (out HCD, statistics not available):			
	Thromboplastin time	↓6%	↓4%	
	Clinical chemistry (out HCD, statistics not available):			
	Albumin	↑8%	-	
	β-globulins	↓22%	-	
	A/G ratio	↑26%	-	
	Organ weights:			
	Terminal body weights	-	↓6%	
	Liver (absolute)	↑17%	-	
	Liver (relative)	↑22%	↑15%	
	Kidneys (absolute)	-	↓8%	
	Kidneys (relative)	↑10%	-	

250 ppm (27/27 mg/kg bw/day)

	males	females
Haematology (out HCD, statistics not available):		
Thromboplastin time	-	↓8%
Organ weights:		
Terminal body weights	↓14%	-
Liver (absolute)	↓20%	-
Spleen (absolute)	↓25%	-

13-week toxicity study

OECD TG 408 (1981)

GLP: No

Wistar rats

10 rats/sex/dose

Purity: 94.5%

Oral (diet)

Doses of 0, 40, 250 or 1500 ppm

0, 3.3, 20 and 121 mg/kg bw/day (males)

0, 3.5, 21 and 129 mg/kg bw/day (females)

Guideline value for classification:
STOT RE 2 ≤ 100 mg/kg bw/day
STOT RE 1 ≤ 10 mg/kg bw/day

Anonymous 30, 1986b

B.6.3.2.1.1 (AS)

1500 ppm (121/129 mg/kg bw/day)

	males	females
Body weight (statistics not available):		
Week 9	↓12%	↓9%
Week 13	↓13%	↓10%
Week 17 (after recovery period)	No significant differences	
Organ weights:		
Terminal body weights	↓14%	↓13%
Liver (absolute, week 13)	↑18%	↑22%
Liver (relative, week 13)	↑33%	↑39%
Brain (relative, week 13)	↑20%	↑25%
Brain (relative, week 17)	↑20%	↑13%
Testes (relative, week 13)	↑11%	-
Ovaries (relative, week 13)	-	↑25%

250 ppm (20/21 mg/kg bw/day)

	males	females
Relative liver weight (week 13)	↑11%	↑19%

13-week toxicity (feeding)

OECD TG 408 (1981)

GLP: Yes

Wistar rats

15 rats/sex/dose

10 rats/sex as control group

No historical control data provided

Purity: 94.5%

Oral (diet)

Anonymous 31, 1987a

B.6.3.2.1.2 (AS)

Mortality: 1 female at 1500 ppm considered incidental and 1 male at 200 ppm regarded accidental. 7 females after blood sampling on day 90: 1 at 0 ppm, 1 at 200 ppm, 2 at 750 ppm, 1 at 1500 ppm and 2 at 3000 ppm.

Clinical signs: Discomfort and hunched appearance, alopecia, lacrimation, swollen or enlarged ears, thinness, scores, chromodacryorrhea, soft faeces and exophthalmos. No dose pattern observed with these effects.

3000 ppm (121/129 mg/kg bw/day)

	males	females
Body weight:		
Week 1	↓12%	-
Week 4	-	↓15%
Week 8	-	↓18%
Week 13	↓10%	↓20%
Bodyweight gain (weeks 0-13)	↓50%	↓57%
Clinical chemistry:		
Blood urea nitrogen (week 13)	↑38%	-
Haematology		

Doses of 0, 20, 200, 750, 1500 or 3000 ppm 0, 1.3, 13, 51, 105 and 214 mg/kg bw/day (males) 0, 1.7, 17, 66, 131 and 275 mg/kg bw/day (females) *Guideline value for classification:* *STOT RE 2 ≤ 100 mg/kg bw/day* *STOT RE 1 ≤ 10 mg/kg bw/day*	Red Blood Cell count (week 13) Haematocrit (week 13) Haemoglobin (week 13) *Organ weights:* Terminal body weights Liver (absolute) Liver (relative) Adrenals (absolute) Brain (relative) Diffuse hepatocyte enlargement	↓7% ↓8% ↓8% ↓17% ↑22% ↑40% ↓17% - 10/10 vs 1/10	↓11% ↓14% - - ↑32% ↑67% - ↑16% 10/10 vs 0/10	

1500 ppm (105/131 mg/kg bw/day)

	males	females
Body weight:		
Week 4	-	↓10%
Week 8	-	↓11%
Week 13	-	↓11%
Bodyweight gain (weeks 0-13)	-	↓34%
Clinical chemistry:		
Blood urea nitrogen (week 13)	↑23%	-
Haematology		
Red Blood Cell count (week 13)	↓10%	↓9%
Haematocrit (week 13)	↓11%	↓12%
Haemoglobin (week 13)	↓5%	-
Organ weights:		
Terminal body weights	-	↓6%
Liver (absolute)	↑31%	↑27%
Diffuse hepatocyte enlargement	10/10 vs 1/10	4/10 vs 0/10

750 ppm (51/66 mg/kg bw/day)

	males	females
Body weight (week 13)	-	↓7%
Bodyweight gain (weeks 0-13)	-	↓18%
Haematology:		
Red Blood Cell count (week 13)	↓8%	-
Haematocrit (week 13)	↓9%	-
Organ weights:		
Liver (absolute)	↑26%	↑24%
Liver (relative)	↑21%	↑28%

200 ppm (13/17 mg/kg bw/day)

	males	females
Bodyweight gain (weeks 0-13)	-	↓16%
Relative liver weight	-	↑21%

90-day neurotoxicity study OECD TG 424 (1997) GLP: Yes Alpk:APfSD rats 10 rats/sex/dose Purity: 94.3%	1500 ppm (107/120 mg/kg bw/day) *Body weight:* Week 2 Week 6 Week 8 Week 12 Week 13 Food consumption (week 13) *Functional observational battery:* Time to tail flick (week 5)	males ↓4% ↓7% ↓7% ↓9% ↓9% - -	females - ↓5% ↓4% ↓4% ↓6% ↓7% 6.7 vs 4.8 control	Anonymous 32, 2006a B.6.7.1.2. (AS)

Oral (diet) Doses of 0, 40, 250 and 1500 ppm 0, 2.8, 17 and 107 mg/kg bw/day (males) 0, 3.2, 20 and 120 mg/kg bw/day (females) *Guideline value for classification:* *STOT RE 2 ≤ 100 mg/kg bw/day* *STOT RE 1 ≤ 10 mg/kg bw/day*	Fore-limb grip strength in female (week 9) Hind limb grip strength (week 2) Hind limb grip strength (week 9) Hind limb grip strength (week 14) *Organ weights:* Liver (absolute) Liver (relative) <u>250 ppm (17/20 mg/kg bw/day)</u>	- ↓23% ↓18% ↓27% ↑28% ↑38%	↓24% - - - ↑36% ↑45%	

	males	females
Food consumption (week 13)	-	↓7%
Hind limb grip strength (week 14)	↓20%	-

13-week toxicity OECD TG 408 (1981) GLP: Yes CD-1 mouse 15 mice/sex/dose 20 mice/sex/control Purity: 94.5% Oral (diet) 0, 20, 200, 2500, 7500 and 15000 ppm 0, 3.3, 34, 440, 1320 and 2640 mg/kg bw/day (males) 0, 4.6, 45, 639, 1917 and 3834 mg/kg bw/day (females) *Guideline value for classification:* *STOT RE 2 ≤ 100 mg/kg bw/day* *STOT RE 1 ≤ 10 mg/kg bw/day*	<u>Mortality:</u> There was 100% mortality within the first 3 weeks of the study in the 7500 and 15000 ppm dose groups. Additionally: 0 ppm (1 male), 20 ppm (1 male), 200 ppm (2 females one of them incidental) and 2500 ppm (1 female). Deaths below 7500 ppm can be regarded as incidental. <u>Clinical signs:</u> Thinness, hunched posture, languor and tremor observed for early deaths. Clinical signs in the remaining 4 groups did not show a dose pattern. Females in the 2500 ppm dose group showed polypnea during the first week of the study. Other observations in more than one animal included alopecia, thinness, lacrimation, opaque, small or ulcerated eye and swollen abdomen. <u>2500 ppm (440/639 mg/kg bw/day)</u>			Anonymous 33, 1987b B.6.3.2.2.1 (AS)

	males	females
<u>Bodyweight gain (weeks 0-13)</u>	-	↓12%
Organ weights:		
Terminal body weights	-	↓17%
Liver (absolute)	↑82%	↑70%
Liver (relative)	↑94%	↑86%
Heart (absolute)	-	↓8%
Brain (relative)	-	↑16%
<u>Ovaries (absolute)</u>	-	↑25%
Macro pathology:		
Liver enlargement	6/10 vs 0/9	7/9 vs 0/10
Liver pale area	1/10 vs 0/9	1/9 vs 0/10
Liver prominent reticular pattern	4/10 vs 0/9	-
Histopathology:		
Diffuse hepatocyte enlargement	10/10 vs 0/9	9/9 vs 0/10
Hepatic vacuolization	7/10 vs 1/9	7/9 vs 1/9
Sinusoidal cell pigmentation	3/10 vs 0/9	-
Coagulative necrosis	-	4/9 vs 0/10

200 ppm (34/45 mg/kg bw/day)

	males	females
Relative liver weight	↑94%	↑86%
Histopathology:		
Diffuse hepatocyte enlargement	1/10 vs 0/9	2/8 vs 0/10
Centrilobular hepatocellular enlargement	9/10 vs 2/9	-

28-week oral toxicity

OECD TG 452 (1981)

GLP: Yes

Beagle dogs

3 animals/sex/dose

Purity: 96.1%

Oral (diet)

0, 100, 1000, 3000 and 6000 ppm

0, 6, 31, 97 and 158 mg/kg bw/day (males)

0, 3, 35, 111 and 204 mg/kg bw/day (females)

Guideline value for classification:
STOT RE 2 ≤ 50 mg/kg bw/day
STOT RE 1 ≤ 5 mg/kg bw/day

Clinical signs: Lenticular opacity in one female at 3000 ppm and all animals at 6000 ppm during weeks 20-29.

Anonymous 34, 1987

B.6.3.2.3.1 (AS)

6000 ppm (158/204 mg/kg bw/day)

	males	females
Absolute body weight (week 28)	↓30%	↓32%
Food consumption (week 1-28)	↓35-87%	-
Food consumption (week 1-4)	-	↓40-78%
Platelet count (week 14)	↑60%	-
Platelet count (week 28)	↑121%	-
Total protein in (week 28)	-	↓15%
Calcium (week 28)	-	↓14%
Alkaline phosphatase (week 17)	↑136%	-
Alkaline phosphatase (week 28)	-	↑287%
Organ weights:		
Terminal body weights	↓31%	↓31%
Liver (absolute)	↑44%	-
Liver (relative)	-	↑65%
Kidneys (relative)	-	↑50%
Brain (relative)	↑34%	↑33%
Heart (absolute)	↓30%	-
Prostate (absolute)	↓61%	-
Prostate (relative)	↓45%	-
Salivary gland (absolute)	↓28%	-

Ocular findings: Bilateral subcapsular, equatorial, anterior cortical and posterior cortical lenticular aberrations (cataracts) in all dogs. Subsequent examinations revealed slight to marked progression of the lenticular aberration

	males	females
Bilateral ocular opacity	1/3	1/3
Eyeball-ciliary body: minimal acute purulent inflammation	1/3	-
Eyeball-ciliary body: minimal cysts	1/3	-
Moderate cataract left eye	2/3	1/3
Minimal cataract right eye	1/3	1/3
Severe cataract right eye	-	2/3

3000 ppm (97/111 mg/kg bw/day)

	males	females
Food consumption (week 1)	↓50%	-
Food consumption (week 2)	↓23%	-
Food consumption (week 4)	↓16%	-
Alkaline phosphatase (week 17)	-	↑243%
Alkaline phosphatase (week 28)	-	↑455%
Liver (relative)	-	↑27%
Liver (relative)	-	↑41%
Brain (relative)	-	↓14%

Ocular findings: Bilateral subcapsular, equatorial, anterior cortical and posterior cortical lenticular aberrations (cataracts)

	in 1 male and one female. Subsequent examinations revealed slight to marked progression of the lenticular aberration	

	males	females
Bilateral ocular opacity	-	1/3
Minimal cataract left eye	-	1/3
Moderate cataract left eye	1/3	1/3
Minimal cataract right eye	1/3	-
Severe cataract right eye	-	1/3

1000 ppm (31/35 mg/kg bw/day)

	males	females
Food consumption (week 2)	↓28%	-
Food consumption (week 3)	↓28%	-
Food consumption (week 4)	↓26%	-

1-year study OECD TG 452 (1981) GLP: Yes Beagle dogs 4 dogs/sex/dose Purity: 96.1% Oral (diet) 0, 20, 100, 500 and 1500 ppm 0, 0.7, 3.4, 16 and 51 mg/kg bw/day (males) 0, 0.6, 3.7, 19.4 and 44 mg/kg bw/day (females) *Guideline value for classification:* *STOT RE 2 ≤ 25 mg/kg bw/day* *STOT RE 1 ≤ 2.5 mg/kg bw/day*	Clinical signs: Minor faecal changes and emesis observed in one or two animals in each group and swollen appendage in one female at 20 ppm (considered not treatment-related). Corneal opacity was seen in one female of the control group.	Anonymous 35, 1988 B.6.3.3.1.1 (AS)

1500 ppm (51/44 mg/kg bw/day)

	males	females
Body weight gain (week 1)	-	↓5%
Food consumption (week 1)	-	↓23%
Food consumption (week 5)	-	↓19%
Food consumption (week 10)		↓24%
Food consumption (week 51)		↓29%
Reticulocyte count (week 52)		↓63%
Blood urea nitrogen (week 52)	↓27%	-
Alkaline phosphatase (week 13)	↑42%	-
Alkaline phosphatase (week 26)	↑98%	-
Alkaline phosphatase (week 52)	↑143	-
Sodium (week 52)	-	↑5%

500 ppm (16/19 mg/kg bw/day)

	males	females
Alkaline phosphatase (week 52)	↑55%	-
Relative adrenals weight	↓23%	-

100 ppm (3.4/3.7 mg/kg bw/day)

	males	females
Blood urea nitrogen (week 52)	↓29%	-

28-day repeated dose toxicity study OECD TG 410 (1981) GLP: Yes HanIbm: Wist rats 10 rats/sex/dose Purity: 91.8% Dermal suspended in 1% carboxymethyl	1000 mg/kg bw/day	Anonymous 36, 2000 B.6.3.4.2.1 (AS)

	males	females
Food consumption (week 1)	↓10%	-
Total bilirubin	↓37%	-
Globulin	↓8%	-
Calcium	↓4%	-
Albumin	↑11%	-
Absolute liver weight	↑12%	-
Relative liver weight	↑16%	↑11%
Skin hyperkeratosis at application site	6/10 vs 2/10	10 vs 4/10
Minimal hepatocellular hypertrophy	7/10 vs 2/10	7/10 vs 1/10

cellulose in 0.1% Tween 80 and distilled water. Doses of 0, 10, 100 and 1000 mg/kg bw/day (males/females) Application for 6 h/day for 5 days/week for the first 3 weeks and every day thereafter *Guideline value for classification:* *STOT RE 2 ≤ 600 mg/kg bw/day* *STOT RE 1 ≤ 60 mg/kg bw/day*	Thyroid - hypertrophy of follicular epithelium	8/10 (severity 2.0) vs 8/10 (severity 1.56)	9/10 (severity 1.7) vs 7/10 (severity 1.3)	
56-day cataractogenicity in young chicken No test method available GLP: No Hisex chickens 5 chickens/sex in treated groups 3 chickens/sex in negative and positive control groups Purity not stated Oral (diet) 0, 5000 ppm 0, 318 mg/kg bw/day (males and females) Positive control: 2,4-dinitrophenol (158.9 mg/kg bw/day) Eye examinations and histopathological examinations carried out on day 57 *Guideline value for classification:* *STOT RE 2 ≤ 161 mg/kg bw/day* *STOT RE 1 ≤ 16 mg/kg bw/day*	<u>Difenoconazole</u> <u>Mortality:</u> 1 female on day 36. <u>Clinical signs:</u> Ruffled feathers in all animals from day 7 until termination. Slight reduced locomotor activity on days 9 and 10 and between day 13 and 23. <u>Body weight</u>*:* ↓ in males and females (days 28 and 56) (percentage not reported). <u>Food consumption:</u> ↓ in males and females throughout the study (percentage not reported). <u>Eye examinations in treated group:</u> Lens alterations observed throughout the study in males (5/5) and females (2/5) of which 4/5 males and 1/5 females were irreversible by day 56. <u>Histopathology in treated group:</u> Initial changes in the lens, indicative of cataract, in 3/5 males and 1/5 females. The lesions comprised slight swelling of the epithelial cells either at the equator or anteriorly, and/or necrosis of the lens fibres posteriorly, under the capsule or in the outer cortex. <u>2,4-dinitrophenol</u> <u>Eye examinations in treated group:</u> Positive control group: marked lens opacities on days 3 and 7 and became slight alterations until study termination (except for one female, which had no findings after day 38). <u>Histopathology in treated group:</u> 2/3 males developed changes indicative of cataract and one female showed a slight swelling of the lens epithelium at the equator.			Anonymous 37, 1987 B.6.8.2.1-01 (AS)

18-week toxicity study for assessment of cataractogenic potential No test method available GLP: Yes Beagle dogs Group 1 (G1) (18-week treatment): 1 dog/sex Group 2 (G2) (3-week treatment and 15-week recovery): 2 dogs/sex Purity: 96.1% Oral (diet) G1 doses: 6000 ppm (214 mg/kg bw/day) (days 1-8); 3000 ppm (107 mg/kg bw/day) (days 9-63); 4000 ppm (143 mg/kg bw/day) (days 64-127). G2 doses: 6000 ppm (214 mg/kg bw/day) (days 1-8); 3000 ppm (107 mg/kg bw/day) (days 9-21); 0 ppm (days 22-127) *Guideline value for classification G1: STOT RE 2 ≤ 72 mg/kg bw/day* *STOT RE 1 ≤ 7.2 mg/kg bw/day* *Guideline value for classification G2: STOT RE 2 ≤ 428 mg/kg bw/day* *STOT RE 1 ≤ 42.8 mg/kg bw/day*	Clinical signs: Vomiting observed in 1 male (G1) and 1 female (G2). Faecal changes (mucus and worms/red areas) in 1 female (G1) during weeks 6-9 and 1 male (G2) during week 10. Diarrhoea in 1 female (G2) during week 14. Eye examinations: No signs of cataractogenic potential of the test and no histological alterations observed in the eye. G1 **males / females** Slight interstitial pneumonia: 1/1 / 1/1 Small intestine severe follicular hyperplasia: 1/1 / 1/1 Large intestine moderate follicular hyperplasia: 1/1 / 1/1 Severe spleen congestion: 1/1 / 1/1 Moderate cervical lymph node (erythrophagocytosis): 1/1 / - Moderate cervical lymph node (sinus oedema): 1/1 / - Ovaries cyst: - / 1/1 G2 **males / females** Slight interstitial pneumonia: 1/2 / 1/2 Moderate bronchopneumonia: - / 1/2 Small intestine severe follicular hyperplasia: 1/2 / 2/2 Small intestine moderate follicular hyperplasia: 1/2 / - Large intestine severe follicular hyperplasia: 2/2 / 2/2 Severe spleen congestion: 2/2 / 2/2 Slight cervical lymph node (erythrophagocytosis): 1/2 / - Moderate cervical lymph node (sinus oedema): 1/2 / - Severe cervical lymph node (sinus oedema): 1/2 / - Slight cervical lymphoid (hyperplasia): 1/2 / - Moderate cervical lymphoid (hyperplasia): - / 1/2 Ovaries cyst: - / 1/2	Anonymous 38, 1989 B.6.8.2.1-02 (AS)
2-years toxicity and carcinogenicity Study OECD TG 453 (1981) GLP: Yes Sprague-Dawley rats 80/sex/dose group	2500 ppm (124/170 mg/kg bw/day) **males / females** *Body weight:* Week 52: ↓11% / ↓23% Week 76: ↓8% / ↓23% Week 104: - / ↓22% Body weight gain (week 13 until the end): ↓11-22% / ↓32-40% *Haematology:* Red blood cell count (week 28): - / ↓28%	Anonymous 16, 1989a Anonymous 17, 1992 (HCD) B.6.5.1 (AS)

Recovery animals: 10/sex/supplementary group (control or high dose groups) Purity: 94.5% weeks 1-20; and 95% weeks 21-104 Oral (diet) 0, 10, 20, 500 and 2500 ppm 0, 0.5, 1, 24 and 124 mg/kg bw/day (males) 0, 0.6, 1.3, 33 and 170 mg/kg bw/day (females) *Guideline value for classification:* STOT RE 2 ≤ 12.5 mg/kg bw/day STOT RE 1 ≤ 1.25 mg/kg bw/day	Haemoglobin (week 28/week 53)	-	↓8/7%
	Haematocrit	↓-/10%	↓13/13%
	Mean corpuscular volume (week 28/week 53/week 79)	↓-/3/-	↓4/4/3%
	Mean corpuscular haemoglobin (week 28/week 53)	↑4/5%	-
	Mean corpuscular haemoglobin concentration	↑3/8/10%	↑5/8/7%
	Platelets (week 28/week 53/week 79/week 104)	↓17/24/22/19%	-
	White blood count (week 104)	↓30%	↓36%
	Total segmented neutrophils (week 104)	↓28%	↓49%
	Total lymphocytes (week 104)	↓31%	-
	Clinical chemistry:		
	Albumin (week 28/week 53)	↑5/5%	↑7/-%
	Globulin (weeks 53/week 104)	↓10/17%	-
	Albumin/globulin ratio (week 53/week 104)	↑18/48%	-
	Alanine aminotransferase (weeks 28/week 53)	↑-/32%	↑59/32%
	Glucose (week 28)	↑12%	↑8%
	Cholesterol (week 28/week 104)	↑23/48%	↑28/-%
	Total bilirubin (week 28/week 53/week 79)	↓44/-/-%	↓67/73/79%
	Organ weights:		
	Absolute carcass (week 53/week 104)	↓11/-%	↓21/22%
	Relative liver (week 53/week 104)	↑14	↑48/43%
	Absolute adrenals (week 53)	↓29%	-
	Absolute spleen (week 57)	-	↓18%
	Absolute ovaries (week 104)	-	↑90%
	Relative ovaries (week 104)	-	↑132%
	Hepatocellular hypertrophy	89% vs 17.5%	84% vs 12.5%

500 ppm (24/33 mg/kg bw/day)

	males	females
Body weight (week 52)	-	↓6%
Body weight gain (week 13, 24, 52)	↓6-7%	↓10-11%
Haematology:		
Haemoglobin (week 28)	-	↓5%
Mean corpuscular volume (week 79)	↓5%	-
Platelets (week 28/week 53)	↓9/11%	-
Clinical chemistry:		

Albumin (week 28/week 53)	↑5/5%	↑7/-%
Alanine aminotransferase (weeks 28)	-	↑41%
Alanine aminotransferase (weeks 53)	↓42%	-
Relative ovary weight	-	↑109%
↑ Hepatocellular hypertrophy	65% vs 18%	34% vs 13%

20 ppm (1/1.3 mg/kg bw/day)

	males	females
Body weight (week 13)	↓5%	-
Haematology:		
Haemoglobin (week 28)	↑8%	↓4%
Haematocrit (week 28)	↑7%	-

10 ppm (0.5/0.6 mg/kg bw/day)

	males	females
Mean corpuscular volume (week 79)	↑4%	-
Relative weight ovaries	-	↑41%

78-weeks carcinogenicity study

OECD TG 451 (1981)

GLP: Yes

CD-1® (ICR) mouse

60/sex/dose group

Purity: 94.5% weeks 1-20; and 95% weeks 21-80

Oral (diet)

0, 10, 30, 300, 3000-2500* and 4500 Ppm

0, 1.5, 4.7, 46, 508-423 and 819 mg/kg bw/day (males)

0, 1.9, 5.6, 58, 616-513 and 983 mg/kg bw/day (females)

Guideline value for classification:
STOT RE 2 ≤ 17 mg/kg bw/day
STOT RE 1 ≤ 1.75 mg/kg bw/day

Mortality at 4500 ppm: All (70) the females dose group died or were humanely sacrificed during the first 2 weeks. Survival for the male group was significantly lower than control.

Mortality at 3000 ppm: 15 females died/were sacrificed on week 1, which led to a reduction in dose to 2500 ppm for males and females during week 2. An additional female died during week 2. On week 3, 10 control females were moved to 2500 group; 3 of these females were humanely scarified during their first week of treatment.

Clinical signs: Thinness, hunched appearance and rough hair coat were noted more frequently in 2500 ppm female group and in 4500 ppm male group when compared to controls. In 4500 ppm male group, also ↑ in the incidence of reduced motor activity was observed.

4500 ppm (819/983 mg/kg bw/day)

	males
Body weight (week 56)	↓7%
Body weight gain (week 0-76)	↓34%
Clinical chemistry:	
Alanine aminotransferase (week 53/week 79)	↑280/311%
Alkaline phosphatase (week 79)	↑444%
Sorbitol dehydrogenase (week 53/week 79)	↑378/353%
Organ weights:	
Absolute carcass (week 57)	↓14%
Absolute liver/gall bladder (week 53/week 79)	↑63/112%
Relative liver/gall bladder (week 53/week 79)	↓77/121%
Relative brain (week 53)	↓6%
Gross pathology (liver) (week 79):	
Liver enlargement	50% vs 16%
Pale areas	56% vs 3%
Masses	44% vs 10%
Histopathology (liver):	

Anonymous 18, 1989b

B.6.5.2 (AS)

Individual cell necrosis	76% vs 7%
Focal/multifocal necrosis	23% vs 6%
Hepatocyte hypertrophy	81% vs 24%
Fatty change	44% vs 1%
Bile stasis	71% vs 1%

3000-2500 ppm (508-423/615-513 mg/kg bw/day)

	males	females
Body weight (week 52, week 60, week 72, week 76)	↓6/-/-/-%	↓6/7/8/8%
Body weight gain (week 0-76)	-	↓22%
Body weight gain (week 0-52)	↓21%	-
Haematology:		
Segmented neutrophils	-	↑19%
Lymphocytes	-	↓38%
Clinical chemistry		
Alanine aminotransferase (week 53/week 79)	↑247/-%	↑-/528%
Sorbitol dehydrogenase (week 53/week 79)	↑298/125%	↑-/160%
Organ weights:		
Absolute carcass (week 57/week 79)	↓10/-%	↓-/8%
Absolute liver/gall bladder (week 53/week 79)	↑34/-%	↑41/82%
Relative liver/gall bladder (week 53/week 79)	↑38/-%	↑46/99%
Gross pathology (liver) (week 79):		
Enlargement	24% vs 16%	45% vs 0%
Pale areas	35% vs 3%	41% vs 0%
Masses	15% vs 10%	28% vs 0%
Histopathology (liver):		
Individual cell necrosis	74% vs 7%	39% vs 5%
Focal/multifocal necrosis	16% vs 6%	
Hypertrophy	87% vs 24%	76% vs 3%
Fatty change	19% vs 1%	11% vs 0%
Bile stasis	80% vs 1%	71% vs 0%

300 (46/58 mg/kg bw/day)

	males	females
Body weight gain (week 0-13)	-	↓16%
Body weight gain (week 0-52)	↓15%	-
Sorbitol dehydrogenase (week 53)	↑98	-
Organ weights:		
Absolute liver/gall bladder (week 53)	-	↑20%
Relative liver/gall bladder (week 53)	-	↑17%
Histopathology (liver):		
Individual cell necrosis	22% vs 7%	-
Hypertrophy	43% vs 24%	-

Multigeneration reproductive toxicity study

OECD TG 416 (1981)

GLP: compliant

Sprague Dawley CRCD rats

30 rats/sex/dose.

Purity: 97.4 %

Oral (diet)

Doses: 0, 25, 250 or 2500 ppm

F0 and F1 (mean) males: 0, 1.7, 17, or 171 mg/kg bw/day females: 0, 1.9, 19, 189 mg/kg bw/day

Exposure: Pre-mating treatment: F0 (77 days) F1 (98 days); Treatment continued in F0 and F1 throughout gestation and lactation

PARENTAL TOXICITY

2500 ppm (171/189 mg/kg bw/day)

F0 adults

	males	females
Body weight:		
Premating and mating	↓4-8%	↓4-15%
Gestation and lactation	-	↓13%
Bodyweight gain:		
Premating (days 0-77)	↓12%	↓30%
Gestation (days 0-7)	-	↓34%
Gestation (days 0-20)	-	↓10%
Lactation (days 0-7)	-	↓52%
Lactation (days 0-20)	-	↓26%
Food consumption:		
Premating	↓9%	↓11%
Gestation	-	↓13%
Relative testes weight	↑9%	-

F1 adults

	males	females
Body weight:		
Premating and mating	↓29-16%	↓22-19%
Gestation	-	↓22-19%
Lactation	-	↓22-18%
Bodyweight gain:		
Premating (days 0-98)	↓10%	↓22%
Gestation (days 0-7)	-	↓30%
Gestation (days 0-20)	-	↓7%
Food consumption:		
Premating	↓11%	↓17%
Gestation	-	↓16-22%
Relative testes weight	↑14%	-
Relative ovaries weight	-	↑33%

250 ppm (17/19 mg/kg bw/day)

F0 adults

	males	females
Bodyweight gain	-	↓17%

F1 adults

	males	females
Premating body weight (days 0-7)	↓8%	-

Anonymous 23, 1988

B.6.6.1.1 (AS)

Rat developmental toxicity study

US EPA 83-3 comparable to OECD TG 414 (1981)

GLP: Yes

Crl:COBS®CD®(SD)BR rats

25 females/group

MATERNAL TOXICITY

200 mg/kg bw/day

Clinical signs: Excess salivation in 19/25 dams vs 0/25 controls; red vaginal exudate in 3/25 dams vs 0/25 controls.

Body weight: ↓14% on day 8 and ↓4-7% on days 10, 15 and 19.

Body weight gain: ↓ 56% days 6-15 and ↓12% on days 0-20.

Food consumption: ↓ 10-44% days 6-16, ↑12 days 17-20.

Anonymous 25, 1992

Supplemental information Teratology study

B.6.6.2.1 (AS)

Purity: 95.7% Oral (gavage) 0, 2, 16, 100 and 200 mg/kg bw/day Exposure: days 6-15 (gestation period) *Guideline value for classification:* *STOT RE 2 ≤ 900 mg/kg bw/day* *STOT RE 1 ≤ 90 mg/kg bw/day*	<u>100 mg/kg bw/day</u> <u>Clinical signs:</u> Excess salivation in 14/25 dams vs 0/25 controls; red vaginal exudate in 3/25 dams vs 0/25 controls. <u>Body weight gain:</u> ↓23% days 6-15. <u>Food consumption:</u> ↓11-17% days 6-12.	
Rabbit developmental toxicity study US EPA FIFRA 83-3 comparable to OECD TG 414 (1981) GLP: Yes New Zealand White rabbits 20 females/group Purity: 95.7% 0, 1, 25 and 75 mg/kg bw/day Exposure: days 7 to 19 *Guideline value for classification:* *STOT RE 2 ≤ 692 mg/kg bw/day* *STOT RE 1 ≤ 69 mg/kg bw/day*	**MATERNAL TOXICITY** <u>75 mg/kg bw/day</u> <u>Mortality:</u> 1/19 animal died on gestational day 18 following a period of apparent treatment-related anorexia. <u>Abortions:</u> 2/19 abortions on gestational days 18 and 24. <u>Clinical signs:</u> ↑ stool variations 12/19 vs 2/19 controls (secondary to variation in food consumption) included the two dams with abortions. <u>Body weight gain:</u> ↓34% days 0-29. <u>Food consumption:</u> ↓49% days 9-10, ↓48% days 13-14, ↓35% days 17-18 and ↓26% days 18-19. <u>25 mg/kg bw/day</u> <u>Clinical signs:</u> ↑ stool variations 7/19 vs 2/19 controls (secondary to variation in food consumption). <u>Body weight gain:</u> ↓34% days 0-29. <u>Food consumption:</u> ↓30% days 26-27.	Anonymous 26, 1987 Anonymous 27, 1992 Report addendum B.6.6.2.2 (AS)