Committee for Risk Assessment

RAC

Opinion

proposing harmonised classification and labelling
at EU level of

(5-chloro-2-methoxy-4-methyl-3-pyridyl)(4,5,6-trimethoxy-*o*-tolyl)methanone; pyriofenone

EC Number: 692-456-8
CAS Number: 688046-61-9

CLH-O-0000001412-86-287/F

Adopted

13 June 2019

13 June 2019

CLH-O-0000001412-86-287/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: **(5-chloro-2-methoxy-4-methyl-3-pyridyl)(4,5,6-trimethoxy-*o*-tolyl)methanone; pyriofenone**

EC Number: **692-456-8**

CAS Number: **688046-61-9**

The proposal was submitted by the **United Kingdom** and received by RAC on **22 February 2018.**

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

PROCESS FOR ADOPTION OF THE OPINION

The **United Kingdom** has submitted a CLH dossier containing a proposal together with the justification and background information documented in a CLH report. The CLH report was made publicly available in accordance with the requirements of the CLP Regulation at ***http://echa.europa.eu/harmonised-classification-and-labelling-consultation/*** on **9 April 2018**. Concerned parties and Member State Competent Authorities (MSCA) were invited to submit comments and contributions by **8 June 2018**.

ADOPTION OF THE OPINION OF RAC

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

Co-Rapporteur, appointed by RAC: **Žilvinas Užomeckas**

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 **13 June 2019** by **consensus**.

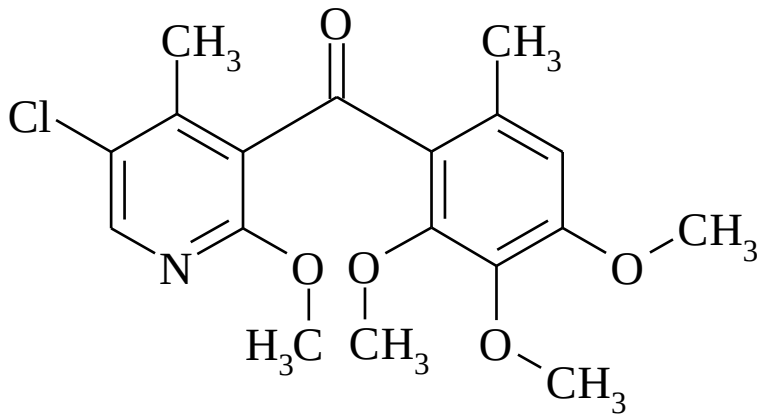
Classification and labelling in accordance with the CLP Regulation (Regulation (EC) 1272/2008)

	Index No	International Chemical Identification	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	(5-chloro-2-methoxy-4-methyl-3-pyridyl)(4,5,6-trimethoxy-*o*-tolyl)methanone; pyriofenone	692-456-8	688046-61-9	Carc. 2 Aquatic Chronic 1	H351 H410	GHS08 GHS09 Wng	H351 H410		M=1	
RAC opinion	TBD	(5-chloro-2-methoxy-4-methyl-3-pyridyl)(4,5,6-trimethoxy-*o*-tolyl)methanone; pyriofenone	692-456-8	688046-61-9	Carc. 2 Aquatic Chronic 1	H351 H410	GHS08 GHS09 Wng	H351 H410		M=1	
Resulting Annex VI entry if agreed by COM	TBD	(5-chloro-2-methoxy-4-methyl-3-pyridyl)(4,5,6-trimethoxy-*o*-tolyl)methanone; pyriofenone	692-456-8	688046-61-9	Carc. 2 Aquatic Chronic 1	H351 H410	GHS08 GHS09 Wng	H351 H410		M=1	

GROUNDS FOR ADOPTION OF THE OPINION

RAC general comment

Pyriofenone is a new pesticidal active substance in the scope of Regulation 1107/2009. It is an aryl phenyl ketone fungicide designed for the control of powdery mildew (*Blumeria graminis*) on cereals (wheat, rye, barley, spelt, oats, and triticale) and for controlling mildew on grapes. At the time of submission, there were no registrations for this substance under REACH. There is no existing entry on Annex VI of CLP. The mode of action is inhibition of the formation of fungal appressoria and failure of infection due to lack of subsequent penetration of the hyphae into the host plant cells.



A number of confidential impurities are present, however none of these are considered relevant for the classification of the substance.

An initial evaluation in the Draft Assessment Report (DAR) provided by the UK as Rapporteur Member State (RMS) was submitted to EFSA in 2012 and EFSA concluded its review in 2013. All hazard classes were open for assessment in this opinion document.

Administration of pyriofenone at the high dose led to increased rate and extent of exposure to radioactivity in rats when compared to the low dose. However, they were not proportionate to the size of the dose increase, indicating non-linear kinetics. These increases were greater in males than in females. Pyriofenone was well absorbed and completely eliminated. There was no evidence of tissue accumulation or the presence of any unidentified or toxic metabolites.

RAC evaluation of physical hazards

Summary of the Dossier Submitter's proposal

The Dossier Submitter (DS) did not propose classification of pyriofenone for physical hazards on the basis of the following results:

- Pyriofenone was tested using EEC Method A.14 and was found not to be explosive (*Turner, 2009c*);
- Pyriofenone was assessed for auto-flammability using EEC Method A.15 - 'Auto-Ignition Temperature (liquids and gases)'. This method is more suitable for materials with melting temperatures of less than 100°C. The traditional method for solids (EEC Method A.16 - 'Relative Self-Ignition Temperature for Solids') was not performed based on practical considerations. If this method was applied to pyriofenone then it

would melt at a test temperature of approximately 92-95°C. The resulting melt would then seep through the test vessel and therefore any subsequent thermal effects would not be noted. The result using EEC Method A.15 showed no self-ignition up to the temperature of the melting point (*Turner, 2009c*);

- Pyriofenone was tested for oxidising properties using EEC Method A.17. Burn rates for mixtures of pyriofenone:cellulose were found to be significantly less than the reference mixture. Therefore, pyriofenone is considered not oxidising (*Turner, 2009c*);
- In a standard study (EEC Method A.10), a preliminary test showed that pyriofenone burned locally with a yellow flame, which extinguished two seconds after removal of the heat source. Pyriofenone does not meet the criteria for classification as a flammable solid (*Turner, 2009c*).

Pyriofenone is an odourless, opaque white solid in the form of a fine powder. Pyriofenone melted at 93-95°C. No decomposition occurred below the melting point. The DS considered pyriofenone was not flammable, not auto-flammable, nor explosive and had no oxidising properties.

Comments received during public consultation

No comments were received.

Assessment and comparison with the classification criteria

The criteria for classification of physical hazards have not been satisfied based on the data obtained from several key studies. RAC agrees with the DS, that **no classification for physical hazards is warranted**, including no classification as a self-heating substance based on the available but limited data from method EEC A.15.

HUMAN HEALTH HAZARD EVALUATION

RAC evaluation of acute toxicity

Summary of the Dossier Submitter's proposal

The DS proposed no classification of pyriofenone with acute oral toxicity based on one negative study performed at a single limit dose of 2000 mg/kg bw by oral gavage in aqueous methyl cellulose (1% w/v) (10 mL/kg bw) with six CD (Crl:CD SD) female rats according to GLP and OECD TG 423 (Anonymous, 2008a). LD_{50} > 2000 mg/kg bw. Clinical signs were minimal in nature (two animals with abnormal body position) and recovery was complete by five hours post dosing. There were no mortalities and no gross abnormalities were found at necropsy.

The DS proposed no classification of pyriofenone for acute dermal toxicity based on no lethalities at the limit dose (2000 mg/kg bw) in a GLP and OECD TG 402 study (Anonymous, 2008b), semi occlusive, 24-hour exposure to 5 male and 5 female CD (Crl:CD SD) rats. Clinical signs were confined to slight erythema observed in all animals, which had resolved by day 7, scabbing in 1 female on day 7 and 1 female from day 4 until the end of the study at day 15 may suggest some irritation potential. There were no internal findings at necropsy.

The DS proposed no classification for acute inhalation toxicity. In a GLP and OECD TG 403 guideline compliant acute inhalation study (Anonymous, 2008c), groups of 5 Sprague Dawley strain rats/sex were nose-only exposed for 4 h to an aerosol of pyriofenone at a concentration of 5.18 ± 0.24 mg/L (gravimetrically determined). The MMAD was 3.9 µm and a GSD of 2.25 with

at least 40% of the particles in the respirable range. Clear nasal discharge was observed in only 3 rats immediately following exposure, however, all rats appeared active and healthy during the course of the study. No rats died and there were no macroscopic findings at necropsy. The LC_{50} was > 5.18 mg/L.

Comments received during public consultation

No comments were received.

Assessment and comparison with the classification criteria

(1) Acute oral toxicity: In order to be classified with acute toxicity category 4 (oral), the lowest category for this endpoint, the LD_{50} must fall between the following range: 300 < LD_{50} ≤ 2000 mg/kg bw. The oral LD_{50} of > 2000 mg/kg bw for rats is above the value for classification according to CLP Regulation. The substance is not classified.

(2) Acute dermal toxicity: In order to be classified with acute toxicity category 4 (dermal), the LD_{50} should be between 1000 < LD_{50} ≤ 2000 mg/kg bw. The dermal LD_{50} of > 2000 mg/kg bw for rats is above the value for classification according to CLP Regulation. The substance is not classified.

(3) Acute inhalation toxicity: In order to be classified with acute toxicity category 4 (inhalation), the LC_{50} should lie between 1.0 < LC_{50} ≤ 5.0 mg/L (dusts and mists). The 4 h inhalation LC_{50} of > 5.18 mg/L for rats is above the value for classification in the CLP Regulation and thus there is no requirement to classify for acute inhalation toxicity.

Overall, RAC agrees with the DS' proposal for **no classification for acute toxicity (all routes of exposure)**.

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

Summary of the Dossier Submitter's proposal

Pyriofenone was investigated in a number of acute toxicity studies by the oral, dermal and inhalation routes using a limit dose of up to 2000 mg/kg bw. In all studies, there were few clinical signs and no macroscopic abnormalities. There was no evidence of any effects that might support specific target organ toxicity. An acute neurotoxicity study (Anonymous, 2010a; DAR B.6.7.2) in Crl:CD(SD) rats using 10 animals/sex/dose up to 2000 mg/kg bw was similarly devoid of evidence for STOT SE.

Comments received during public consultation

No comments were received.

Assessment and comparison with the classification criteria

RAC supports the conclusions of the DS; pyriofenone did not cause specific organ toxicity following a single exposure in rats by the oral, inhalation or dermal routes. **Classification for specific target organ toxicity single exposure is not supporte**d by the available data.

RAC evaluation of skin corrosion/irritation

Summary of the Dossier Submitter's proposal

The DS described a primary dermal irritation study (GLP, OECD TG 404, Anonymous, 2008d) where 3 young female adult New Zealand White rabbits were exposed to 0.5 g pyriofenone, applied to the intact shaved flank under a semi-occlusive dressing, for 4 hours. Skin reactions were scored at 1, 24, 48 and 72 hours after removal of the dressings. No clinical signs were observed in the animals during the study and no mortality occurred. No local dermal signs were observed in the treated animals throughout the study. The mean scores / animal (at 24, 48 and 72 hours) for erythema and oedema were 0. Hence the DS did not propose classification for skin corrosion/ irritation.

Comments received during public consultation

No comments were received during the public consultation.

Assessment and comparison with the classification criteria

There was no evidence of a skin reaction in any of the treated animals (mean scores for erythema and oedema were 0), therefore the data do not meet the criteria for classification and labelling. Therefore, RAC supports the DS proposal for **no classification for skin corrosion/irritation.**

RAC evaluation of serious eye damage/irritation

Summary of the Dossier Submitter's proposal

In a single GLP and OECD TG 405 compliant primary eye irritation study (Anonymous, 2008e), minor and transient signs of conjunctival irritation were observed. There were no effects on the cornea or iris. There was no mortality or clinical signs of systemic toxicity observed in the animals during the study.

Redness of the conjunctiva was observed in 3/3 animals 1 h post installation (grade 1) and in 1/3 animals at 24 h post installation (grade 1). The redness disappeared in all animals by 48 h after application.

Conjunctival chemosis was observed in 2/3 animals (grade 1) at 1 h after application but this was shown to be fully reversible within 24 h.

Mean scores for corneal opacity, iritis and chemosis were 0 in all animals. The mean score for conjunctival redness (after 24 to 72 hours) was 0.33 in two rabbits and 0 in one rabbit.

The DS did not propose classification.

Comments received during public consultation

No comments were received.

Assessment and comparison with the classification criteria

The mean scores in all animals were negative for category 2 classification (corneal opacity < 1; iritis < 1; conjunctival redness < 2; chemosis < 2). The data do not meet the criteria for classification according to CLP.

RAC agrees with the DS proposal for **no classification for eye irritation**.

RAC evaluation of skin sensitisation

Summary of the Dossier Submitter's proposal

Pyriofenone was tested for skin sensitisation in a GLP and guideline (OECD TG 429) compliant Local Lymph Node Assay (LLNA) using CBA/JNCrlj mice (Anonymous, 2009a). In this study, 5 females per group were given 3 consecutive daily topical applications of pyriofenone to the dorsal surface of both ears. The doses used were selected following a preliminary study and were 0, 5, 10 or 25% (w/v) dissolved in acetone/olive oil (4:1 v/v). The criterion for a positive response is one or more of the concentrations tested should elicit a 3-fold or greater increase in isotope incorporation relative to the vehicle control group. A positive control group received 25% α-Hexylcinnamaldehyde (HCA) in the same vehicle mixture.

No mortality or signs of systemic toxicity were observed during the study. There were no indications of any irritancy at the site of application.

Stimulation index values of the test item were:

Test concentration: 5% -- 0.78,
10% -- 1.04 and,
25% -- 0.57

Pyriofenone was shown to be a non-sensitiser in the LLNA.

In the positive control group, α-Hexylcinnamaldehyde induced a positive response with a stimulation index of 7.74, confirming the validity of the protocol used in this study.

A preliminary dose-range finding test was conducted with 3 female mice per dose group. The dose levels initially tested were 0, 5, 10, 25 and 50% for 3 days. No general toxicity or skin irritation was observed. Good solubility was noted up to 25% but the 50% dose was noted as being a suspension or paste with some difficulty presented regarding the consistent application to the auricles.

Pyriofenone has poor water solubility (1.56 mg/L at 20 °C and pH 6.6, Turner, 2007) but it is soluble in organic solvents (> 250 g/L acetone; Turner, 2009) such that the vehicle used in the sensitisation test was an appropriate one.

The DS did not propose classification for skin sensitisation.

Comments received during public consultation

No comments received.

Assessment and comparison with the classification criteria

The initial pre-screen test included a 50% test concentration in a suitable vehicle and this was described as having a paste-like consistency. There was little detail in the original study report

justifying the final testing concentrations, the implication being that 50% was considered an insoluble paste. The chemical properties of pyriofenone indicated that it is soluble in acetone at > 250 g/L (maximum concentration not stated), so a 25% concentration in acetone: olive oil; 4:1, v/v may be a valid top dose to test. In principle, the 50% paste could have been tested, though not an ideal dose selection according to the study authors. It is unlikely that 25% may be considered to meet the requirement in OECD TG 429 where it is stated that "*the maximum dose level tested should be 100% of the test substance for liquids or the maximum possible concentration for solids or suspensions*". The 50% paste may be considered a suspension and was not associated with excessive local skin irritation according to the initial pre-screen test.

As no evidence of skin sensitisation was observed in the LLNA in the mouse (i.e. the SI value was < 3 in all cases), the criteria for classification according to CLP were not met. However, this the no classification conclusion is based on the available data, and it is possible that the maximum dose level according to OECD TG 429 may not have been achieved. RAC recommends **no classification for skin sensitization but based on limited data**.

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

Summary of the Dossier Submitter's proposal

The DS did not propose classification for STOT RE. Table 28 in the Background Document summarises the repeat dose studies on pyriofenone.

The repeated dose oral toxicity of pyriofenone was investigated in 28-day, 90-day and 1-year studies in rats and dogs, a 90-day study in mice and in chronic/carcinogenicity studies in rats and mice. Its dermal repeated dose toxicity has been investigated in a 28-day study in rats. No adverse effects were reported in the dermal study when pyriofenone was applied at doses up to 1000 mg/kg bw/day. The 28-day dog study was considered as a sighting study for the 90-day study and therefore was not optimal for hazard assessment because only 1 animal per sex per dose was used. The liver (rat, mouse, dog), kidney (rat, mouse) and caecum (rat) were identified as the main target organs.

Liver: Effects on the liver comprised of treatment related increases in absolute and relative weights, clinical chemistry alterations and histopathology findings (hepatocyte hypertrophy and darkening in colour). Clear liver toxicity (including liver foci and hepatocellular necrosis) was reported in the carcinogenicity studies in rats and mice.

Kidney: The main effects to the kidney included increased absolute and relative weight and increased hyaline droplet deposition in proximal tubule cells. In the chronic/carcinogenicity studies, increased incidences of chronic nephropathy were observed in rats and cortical tubular basophilia was observed in mice.

GI tract: Effects on the large intestine were observed in rats. The effects on the caecum were described as distention with contents. The study report suggested that these effects might be a substance related effect on the intestine microflora.

Other adverse changes included a prolongation of activated partial thromboplastin time observed in most of the rat studies but always at doses much greater than those relevant for classification.

The DS concluded that most of the adverse effects observed occurred at doses much higher than the guidance values for classification with STOT RE 2 and there were no studies in which adverse effects occurred at doses relevant for classification with STOT RE 1.

Comments received during public consultation

No comments were received.

Assessment and comparison with the classification criteria

***Table**: Summary of studies with effects for consideration of STOT RE 2 at doses relevant for classification.*

Study	Relevant effect level	Cat. 1 mg/kg bw/day	Cat. 2 mg/kg bw/day	Significant & Potentially Relevant Effects	Reference
Rat, 28 day oral dietary **Not sufficient for classification**	3000 ppm **M**: 251 mg/kg bw/day **F**: 261 mg/kg bw/day	≤ 30 No relevant effects	**≤ 300**	**Males**: (control vs 3000 ppm) ↑ Rel. liver wt. (+10%) ↑ hyaline droplet deposition in proximal tubule cells of the kidney: 6/6 males** (versus 0 in controls) - Distended caecum with contents: 6/6 males** (versus 0 in controls) ↓ Alkaline phosphatase 10%** **Females**: (control vs 3000 ppm) - Distended caecum with contents: 6/6 females** (versus 0 in controls) ↓ Total bilirubin 50%**	DAR B.6.3.1a Anonymous, 2010b
Rat, 90 day oral dietary **Not sufficient for classification**	1000 ppm **M**: 60.5 mg/kg bw/day **F**: 69 mg/kg bw/day	≤ 10 No relevant effects	**≤ 100**	**Males**: (control vs 1000 ppm) ↓ Alanine aminotransferase 10%** ↓ Total bilirubin 25% **Females**: (control vs 1000 ppm) ↓ Total bilirubin 25%**	DAR B.6.3.1b Anonymous, 2010c
Two generation reproduction Rat, oral dietary **Not sufficient for classification**	1000 ppm **M**: 64.1 mg/kg bw/day **F**: 62 - 138 mg/kg bw/day	≤ 10 No relevant effects	**≤ 100**	**Females**: (control vs 1000 ppm) ↑ Abs. liver wt. (+11%**) ↑ Rel. liver wt. (+9%**)	DAR B.6.6.1b Anonymous, 2019d
Dog, 90 day oral dietary **Not sufficient for classification**	3000 ppm **M**: 90.3 mg/kg bw/day **F**: 89.9 mg/kg bw/day	≤ 10 No relevant effects	**≤ 100**	**Females**: (control vs 3000 ppm) ↑ ALP x 2.2 fold in females	DAR B.6.3.3b Anonymous, 2010d

Dog, 12 month oral dietary **Not sufficient for classification**	500 ppm **M**: 13.7 mg/kg bw/day **F**: 14.1 mg/kg bw/day	≤ 2.5 No relevant effects	**≤ 25**	**Males**: (control vs 500 mg/kg bw/day) ↓ Body weight gain 44%	DAR B.6.3.3c Anonymous, 2010e

** significantly different from control, p ≤ 0.01.

In several oral repeated dose toxicity studies in rats, mice and dogs, the clear target organs were the liver, kidney and caecum. The effects occurring at doses relevant for classification are summarised in the table above. The majority of the adverse effects observed occurred at doses much higher than the guidance values for classification with STOT RE 2 and there were no studies in which adverse effects occurred at doses relevant for classification as STOT RE 1.

STOT RE is assigned on the basis of a substance demonstrating evidence of significant or severe toxicity, generally at or below the oral guidance value of 100 mg/kg bw/d (for a classification in category 2) obtained in a 90-day rat study. In a 28-day oral rat study and a 2-generation reproduction study in rats, increased relative liver weight was observed in one sex only, however, there was no associated histopathology and the weight change may be considered an adaptive effect rather than an adverse one. Changes to the proximal cells of the kidney were observed in males of the rat 28-day study, however the weighting given to this effect is low in consideration of the absence of such changes occurring at doses relevant for classification in the rat 90-day study. Other findings at doses relevant for classification were changes in clinical chemistry in rats and dogs and a decrease in body weight gain in male dogs following a year of treatment. The changes in clinical chemistry were not consistent or significantly adverse. Whilst they are considered treatment related, RAC agrees with the DS that they are not considered sufficiently adverse and thus do not support classification.

RAC concludes that repeated dosing with pyriofenone produced no effects that were indicative of organ dysfunction or significant toxicity at dose levels below the guidance value for classification. Overall, RAC considers that **no classification as STOT RE is warranted**.

RAC evaluation of germ cell mutagenicity

Summary of the Dossier Submitter's proposal

The DS reported that pyriofenone was tested in a range of GLP and OECD guideline compliant *in vitro* and *in vivo* genotoxicity assays (see the table below; for further details see table 17 of the CLH report).

***Table**: Summary of genotoxicity tests with pyriofenone.*

Study	Result	Test System	Reference
***In vitro* studies:**			
Bacterial mutagenicity	negative	GLP, OECD TG 471 *Salmonella* Strains: TA1535, TA1537, TA98, TA100 *E. coli* strain : WP2*uvr*A	May, 2007
Mammalian cell mutagenicity	negative	GLP, OECD TG 476	Hynes, 2008

		Mouse Lymphoma L5178Y Cells (thymidine kinase locus)	
Clastogenicity	positive, weak clastogenic activity	GLP, OECD TG 473 cultured Chinese Hamster Lung cells	Pritchard, 2008
***In vivo* studies:**			
Micronucleus	negative	GLP, OECD TG 474 (1997) Male and female CD-1 mouse; bone marrow (single oral gavage, short term assay)	Anonymous, 2008f
UDS	negative	GLP, OECD TG 486 (1997) Male Crl:CD(SD) rat hepatocytes	Anonymous, 2010f
Comet Assay in mice	negative	Non-GLP, non-guideline; used ICR (Crlj:CD1) male mice; 500, 1000, 2000 mg/kg bw	Anonymous, 2011
Comet Assay in rat	negative	GLP, OECD TG 489 (2016) Fischer F344 rat; 500, 1000, 2000 mg/kg bw	Anonymous, 2017

***In vitro* results**

(1) In bacteria, an Ames assay was performed using histidine–dependent auxotrophic mutants of *Salmonella typhimurium*, strains TA1535, TA1537, TA98 and TAI00, and a tryptophan-dependent mutant of *Escherichia coli*, strain WP2 *uvr*A. No signs of toxicity were observed towards the tester strains in the presence and absence of rat liver S9 mix. Precipitate was observed on all plates containing pyriofenone at 1500 and 5000 µg/plate. There was no evidence of mutagenic activity seen for any level of pyriofenone (May, 2007).

(2) Pyriofenone was assessed for its mutagenic potential in mouse lymphoma L5178Y cells, there were no increases in mean mutant frequency at the tk locus in treated cells (Hynes, 2008).

(3) The clastogenic effect of pyriofenone was tested in an *in vitro* chromosome aberration study in Chinese Hamster Lung (CHL) cells (Pritchard, 2008).

Metaphase analysis:

Test 1:

- Without S9 mix – 3 h treatment, 12 h recovery: 60, 65 and 70 µg/mL
- Plus S9 mix – 3 h treatment, 12 h recovery: 90, 110 and 120 µg/mL

Test 2:

- Without S9 mix – 15 h treatment: 20, 30 and 40 µg/mL
- Plus S9 mix – 3 h treatment, 12 hr recovery: 100, 110 and 130 µg/mL

There was no evidence of chromosome aberrations in Chinese Hamster Lung (CHL) cells in the presence of S9 mix. However, in the absence of S9, when CHL cells were treated for 3 hours up to a concentration of 70 µg/mL, followed by a 12 hour recovery period (test 1), the number of aberrant cells both excluding gaps and including gaps increased in a concentration-dependent manner, reaching statistical significance ($p < 0.01$) at 70 µg/mL (excluding gaps). Given that the mitotic index was reduced by only approximately 18% at the 70 µg/mL dose level (table 3, original study report), this was not considered sufficiently cytotoxic to discard the result, indeed higher concentrations could have been selected for metaphase analysis (typically a maximum concentration is based on cytotoxicity, the highest concentration should aim to achieve 55 ± 5% cytotoxicity).

In contrast, in a second test without S9, involving 15 h continuous exposure up to a concentration of 40 µg/mL, (test 2), there was no increase in the number of aberrant cells (excluding gaps). The top dose produced a 50% reduction in mitotic index and was considered by the DS to have been sufficiently high. An increase in aberration frequency including gaps was seen at the top dose, but this finding was not statistically significant. All positive controls behaved as expected. The DS considered that the positive result in the first test could not be dismissed and considered pyriofenone to have the potential to display weak clastogenic activity in cultured mammalian cells, in the absence of exogenous metabolic activation.

***In vivo* results**

(1) In a micronucleus test in male and female CD-1 mice (5/dose), pyriofenone was administered in a single oral dose of 500, 1000 or 2000 mg/kg bw in aqueous methyl cellulose (1% w/v) by oral gavage. The vehicle served as the negative control and mitomycin C (12 mg/kg bw) as the positive control. There was no increase in the number of polychromatic erythrocytes that contained micronuclei and the rate of micronuclei was similar to that observed in the concurrent negative control data. The positive control, mitomycin C, led to a substantial increase in the rate of polychromatic erythrocytes that contained micronuclei (60-150 x fold relative to the concurrent controls, 24 h data).

(2) In an *in vivo-in vitro* unscheduled DNA synthesis test in male Sprague Dawley rats (3/dose), pyriofenone was administered at 0 (vehicle control), 500, 1000 or 2000 mg/kg bw in aqueous methyl cellulose (1% w/v). Positive control groups were administered dimethylnitrosamine or 2-acetylaminofluorene at 10 or 50 mg/kg bw, respectively. Hepatocytes were isolated from the livers at 2- and 16-hour post-administration of pyriofenone. There were no increases in net nuclear grains or percentage of cells in repair. Results were comparable with the negative control data. In contrast, the positive controls exhibited a substantial increase in UDS activity, typically increases of between 10- to 20-fold relative to the concurrent controls. Under the conditions of this study, pyriofenone did not induce UDS in rat hepatocytes.

(3) Comet assays available were a recent, well-performed guideline test in rats (Anonymous, 2017) and a non-guideline assay in mice (Anonymous, 2011). In both studies, only the liver was investigated.

Fischer rats (5 males/dose) received two doses of pyriofenone (21 h interval) by gavage (0, 500, 1000 and 2000 mg/kg bw). Three hours after the final dose, the rats were sacrificed and the liver was removed. The results of this study were negative with no change in the range of mean % tail DNA in any of the treatment groups.

In an older non-GLP and non-guideline study carried out in mice, male CD-1 mice (5/dose) were treated with pyriofenone (oral gavage) for 48 h (0, 500, 1000 or 2000 mg/kg bw/day). Three hours after the final dose, the mice were sacrificed and the liver was removed. The results of the study showed no evidence of single-strand DNA damage in mouse liver following administration of pyriofenone. All % tail DNA values were comparable to the vehicle control and within the historical control data provided by the laboratory.

Conclusion

According to the DS, in consideration of all the data, pyriofenone did not present a genotoxic hazard. There were no studies in germ cells. The DS did not propose to classify pyriofenone as mutagenic.

Comments received during public consultation

No comments were received.

Assessment and comparison with the classification criteria

The exposure of *S. typhimurium* and *E. coli* tester strains to pyriofenone up to and including the limit concentration of 5000 µg/plate did not produce an increased number of reversions, either with or without metabolic activation.

In an *in vitro* assay for chromosomal aberrations in CHL cells, there was evidence of an increase in cells containing chromosomal aberrations when incubated in the absence of metabolic activation. This result may be considered positive in the absence of significant cytotoxicity and at most potentially weakly clastogenic compared with the result of the positive control (see the table below, taken from table 4 in the original study report).

***Table**, taken from table 4 in the original study report:*

Without S9 mix, 3 hours treatment and 12 hours recovery

Nominal concentration of IKF-309 Technical (µg/mL)	No. cells examined	Aberrations						No. of aberrant cells				Relative Mitotic Index %
		Chromatid type		Chromosome type		Others	Gaps	Exc. gaps	Mean %	Inc. gaps	Mean %	
		ctb	cte	csb	cse		ctg csg					
0	100	1					2	1	1.0	3	3.0	100
(DMSO)	100	1					2	1		3		
60	100	1		2			1	2	2.5	3	4.0	95
	100	3	1	3			2	3		5		
65	100	2	1	2			2	4	4.5	6	5.5	80
	100	3	1	1				5		5		
70	100	4	1	1			2	6	5.5	6	7.0	82
	100	4				1	3	5	**	8		
0.1	100	37	4	4			15 1	24	22.5	30	28.5	110
Mitomycin C	100	26	5	3			7	21	***	27	***	

*** p < 0.001
** p < 0.01

There was no evidence of mutagenic potential with pyriofenone in a mouse lymphoma cell mutation assay.

Positive results from *in vitro* studies alone are not enough to classify for germ cell mutagenicity. There were four *in vivo* tests also available: (i) a micronucleus test in male and female CD-1 mice; (ii) an *in vivo-in vitro* unscheduled DNA synthesis test in male Sprague Dawley rats; (iii) a recent, well-performed guideline Comet test in rats (Anonymous, 2017) and (iv) an older non-guideline assay in mice (Anonymous, 2011). All *in vivo* tests were negative for mutagenicity. There were no studies in germ cells.

Conclusion for germ cell mutagenicity

No human data are available for pyriofenone, therefore a classification with Muta. 1A is not supported. Data are not available illustrating the induction of mutagenic effects in germ cells (a criterion for Category 1B). RAC does not support classification with Muta. 1A or 1B.

Pyriofenone may have the ability to damage chromosomes *in vitro*. However, in accordance with the CLP regulation, positive results from *in vitro* studies alone are not enough to classify for germ cell mutagenicity. As there was no evidence of mutagenicity in *in vivo* tests including a micronucleus test in mice, an unscheduled DNA synthesis test in rats or in Comet assays in rats and mice, pyriofenone does not meet the requirements for classification for germ cell mutagenicity. Therefore, classification in category 2 is not warranted.

The overall weight of evidence for pyriofenone supports no potential for genotoxicity in somatic cells from a selection of *in vivo* and *in vitro* GLP and guideline compliant studies.

RAC agrees with the DS and concludes that **no classification for germ cell mutagenicity is warranted**.

RAC evaluation of carcinogenicity

Summary of the Dossier Submitter's proposal

Two guideline and GLP compliant long-term oral (dietary) toxicity/carcinogenicity studies were available to the DS: a 2-year carcinogenicity study in the Fischer F344 rat (Anonymous, 2010g) and an 18-month carcinogenicity study in the CD-1 mouse (Anonymous, 2010h). Study details were summarised in table 19 in the CLH report. Pyriofenone produced a weak carcinogenic response in the liver of male rats (not statistically significant). The incidences observed were above that of the concurrent control and above the contemporary historical control data (HCD) provided. No response was seen in female rats. Only small increases in liver adenoma, carcinoma and adenoma/carcinoma combined were observed in male mice. Females were unaffected. In contrast to the rat, in mice the relationship to dose was weak and the findings in all treatment groups were within the HCD provided.

Several additional studies were conducted to investigate the Mode of Action (MoA) and human health relevance of the rodent tumours. These included an assessment of cytochrome P450 (CYP450) gene expression and replicative DNA synthesis in isolated rat and human hepatocytes and enzyme induction and cell proliferation in rats and mice. Also available was a mechanistic study comparing CAR-knock-out rats to wild-type rats. The main findings are summarised in tables 20-23 of the CLH report. The DS concluded from this data that the evidence for pyriofenone acting via a non-genotoxic pathway solely involving CAR-activation had too many areas of uncertainty to discount the relevance of the tumour findings for humans. The DS proposed a category 2 classification for carcinogenicity.

1*. In-vivo animal studies*

1.1 Rat 2-year dietary toxicity/oncogenicity study

In a rat GLP and OECD TG 451 compliant carcinogenicity dietary study (Anonymous, 2010g), treatment with pyriofenone was found to reduce the survival of male rats at the top dose during the last 3 weeks of the study. In week 101, cumulative mortality was 28% (vs 10%); in the final week (104), cumulative mortality was 34% (vs 14%). Fischer rats (50/sex/dose) were

administered pyriofenone in the diet for 104 weeks at doses of 0, 200, 1000 or 5000 ppm. The table below shows the mean dose received by each group.

***Table**: Mean dose received (mg/kg bw/day)*

Dietary concentration of pyriofenone (ppm)	0	200	1000	5000
Males	0	7.25	36.4	197
Females	0	9.13	46.5	254

The main target organs for non-neoplastic effects were the liver, kidneys and large intestines, with most effects occurring in animals of the top dose group. General toxicity was displayed at the top dose in males and females; liver weights were increased (males 30% (relative) and females 32% (relative) and 13% (absolute) when compared to controls). Associated histopathology included an increased incidence in liver necrosis (8/50 males versus 0 in controls), fatty changes (23/50 males versus 2/50 in controls and 33/50 females versus 7/50 in controls), hypertrophy (24/50 males and 37/50 females versus 0 in controls) and focal congestion (13/50 females versus 1/50 in controls).

1.1.1 Neoplastic findings

Neoplastic findings were only observed in the liver of male rats. There was no link seen between increased mortality and the incidence of liver tumours. There was a dose related but non-significant increase in the incidence of hepatocellular adenoma (2%, 4% and 12% in the low, mid and high dose groups respectively). However, the biological significance of these findings is uncertain because hepatocellular adenoma was also seen in 8% of control males (and this was greater than the HCD range). The incidence of hepatocellular carcinoma was also increased marginally at all doses (0, 2%, 2% and 4% in the control, low, mid and high dose groups respectively).

The DS gave the HCD from the performing laboratory the greatest weight in its assessment of HCD from several sources. The DS revised the supplied rat HCD from the performing laboratory. The DS noted that according to CLP, the HCD should be contemporary to the study being evaluated (e.g. within a period of up to 5 years of the main study) and data older than this should be used with caution, acknowledging its lower relevance and reliability. Thus, utilising only the studies within a 5-year time period of the concurrent study, the incidence of adenoma ranged from 0 – 4% while the carcinoma incidence was 0. The DS also noted that further examples of HCD for spontaneous hepatocellular adenoma and carcinoma in male F344 rats were supplied by the applicant. These included a paper by the US National Toxicology Program that indicated maximum incidences of adenoma and carcinoma in this strain of male rats of 10% and 6%, respectively (Haseman *et al.*, 1998) and a report by Charles River showing incidences of hepatocellular adenoma and carcinoma of 4.3% and 3.3%, respectively (Lang, 1990).

A 1-year chronic toxicity study in Fischer F344 rats (Anonymous, 2010h), showed no evidence of hepatic tumours and no dose response relationship associated with foci of cell alteration (either eosinophilic or basophilic) in males. There was a significant decrease in the incidences of foci of basophilic cellular alteration in females. In the recent 2 year rat study, there was also no significant dose response relationship associated with incidences of foci of cell alteration in males (eosinophilic: 13, 14, 14, 17 out of 50 animals for 0, 7.25, 36.4 and 197 mg/kg bw/day, respectively, and basophilic: 43, 42, 45, 38 out of 50 animals for 0, 7.25, 36.4 and 197 mg/kg bw/day, respectively). Females did not show a significant decrease in foci of basophilic cellular alteration as they did in the 1-year study, but neither was there any significant increase with dose (30, 25, 34, 35 out of 50 animals for 0, 9.13, 46.5 and 254 mg/kg bw/day, respectively). There was a small non-statistically significant increase in foci of eosinophilic cellular alteration in

females (14, 8, 13 and 20 out of 50 animals for 0, 9.13, 46.5 and 254 mg/kg bw/day, respectively).

1.2 Mouse 18-month dietary carcinogenicity study

In a mouse GLP and OECD TG 451 compliant carcinogenicity dietary study (Anonymous, 2010i), treatment with pyriofenone did not reduce the survival of mice up to the highest doses tested. Groups of 52 male and 52 female CD-1 mice were fed diets containing 0, 600/300, 1800/1000 or 5400/3000 ppm of pyriofenone respectively for a period of at least 78 weeks.

Table: Mean dose received (mg/kg bw/day)

Dietary concentration of pyriofenone (ppm)	0	600/300	1800/1000	5400/3000
Males	0	77.6	237	716
Females	0	49.4	167	486

Non-neoplastic findings in this study were generally limited to the male liver and the kidneys. Liver hypertrophy was observed (13/52, 16/52 and 12/52 in the low, mid and high dose groups respectively versus 0 in controls) and minimal to slight necrosis of individual hepatocytes was seen without a clear dose response relationship (6/52, 8/52 and 7/52 in the low, mid and high dose groups respectively versus 1/52 in controls). There were only slight findings in female livers. In top dose males, kidneys were observed to be granular in an increased number of animals and cortical scarring was also seen. There was an increase in cortical tubular basophilia in males of the mid and top dose groups.

1.2.1 Neoplastic findings

No significant neoplastic findings were observed in female mice.

A higher incidence of hepatocellular carcinomas and adenomas was observed in males administered pyriofenone at > 77.6 mg/kg bw/day (> 600 ppm) when compared with the control group.

In males, there was an increase in hepatocellular adenoma incidence at all doses when compared to controls (6%, 13%, 11% and 17% in the control, low, mid and high dose groups, respectively). However, the relationship to dose was weak: 13% adenoma incidence at 78 mg/kg bw/day, yet only 17% at 716 mg/kg bw/day, an almost 10-fold increase in dose. There was also a small increase in the incidence of liver cell carcinoma at all doses when compared to controls (2%, 4%, 6%, 6%) but statistical significance was only reached when combined incidences of adenoma and carcinoma were compared. Furthermore, the incidence rates of these tumours were well within the HCD provided for the performing laboratory (combined rate: 9.8 – 36%).

The DS concluded that small increases in liver adenoma, carcinoma and adenoma/carcinoma combined were observed in male mice only. However, these findings were well within the HCD such that they were not of concern. The DS considered pyriofenone had no carcinogenic potential in the mouse.

2. *Mechanism of action and supporting data relevant for findings in the rat liver*

2.1 Description and results from the mechanistic studies

The DS briefly described several mechanistic studies (tables 20, 21, 22 and 23 of the CLH report) that were conducted to elucidate the MoA for the liver hepatocellular carcinomas and adenomas observed in male rats (table below).

***Table**: mechanistic studies presented in the Pyriofenone CLH report*

Study	Details	Reference
1. *In vitro* rat hepatocytes (male F344 rat hepatocytes)	Expression of CYP genes – mRNA expression (CYP2B1 and CYP1A2).	Shikama, 2013a
2. *In vitro* rat hepatocytes (male F344 rat hepatocytes)	Effect of pyriofenone on DNA replication (BrdU incorportation). Epidermal growth factor (EGF), PB and pyriofenone tested.	Shikama, 2013b
3. *In vivo* rat 3- and 7-day dietary study. (male F344 Rats)	Effect of pyriofenone on DNA replication (BrdU incorportation) at 3- and 7-days.	Anonymous, 2009b (DAR: B.6.8.3b)
4. *In vivo* 14-day feeding mechanistic hepatotoxicity study (male F344 Rats)	Hepatic enzyme induction in rats. Liver wt., PROD (CYP2B1) and ECOD (CYP1A2) activity, immunoblot densitometry of enzyme protein.	Anonymous, 2011a (DAR: B.6.8.3a)
5. *In vivo* 7-day dietary mechanistic study in CAR-knock-out (KO) rats (male Sprague Dawley rats)	CYP2B1 gene expression (mRNA), CYP2B1 enzyme activity, hepatocyte proliferation (Ki-67).	Anonymous, 2017a
6. *In vivo* 28-day dietary mechanistic study (male CD-1 mice)	EROD enzyme activity, (a marker for CYP1A1 and 1A2); testosterone hydroxylase and dehydrogenase activities (a monitor for CYP2A, 2B, 2C and 3A); lauric acid hydroxylase activities (a monitor for CYP2E and 4A); proliferating cell nuclear antigen (PCNA).	Anonymous, 2010j (DAR B.6.8.4)
7. *In vitro* human hepatocytes (male cryopreserved cells)	Expression of CYP genes – mRNA expression (CYP2B6 and CYP1A2) Number of donors and health status unknown.	Shikama, 2013c
8. *In vitro* human hepatocytes (male cryopreserved cells)	Effect of pyriofenone on DNA replication (BrdU incorportation). EGF, PB and pyriofenone tested.	Shikama, 2013d

2.1.1 In vitro studies in rat hepatocytes

Study 1. In vitro rat hepatocytes: Expression of CYP genes – mRNA expression (CYP2B1 and CYP1A2)

This study (Shikama, 2013a), investigated the effects of pyriofenone on the expression of the CYP genes *CYP2B1* and *CYP1A2* in rat hepatocytes through the mRNA transcript level. Comparisons were made to phenobarbital (PB). The DS noted several limitations of the study such as inadequate reporting and no statistical analysis and a single replicate. The test concentrations used in the main study were PB: 3, 30 and 300 ppm and pyriofenone: 1.25, 2.5 and 5 ppm, and the test exposure was for 24 hours. There was no information on cell cytotoxicity such as cellular ATP levels.

Results indicated pyriofenone increased levels of *CYP2B1* and *CYP1A2* expression in a dose dependent manner in contrast to PB, which affected *CYP2B1* expression only. The study may only be viewed as indicative (table 20 CLH report).

Study 2. In vitro rat hepatocytes: Effect of pyriofenone on DNA replication (BrdU incorporation)

This study (Shikama, 2013b), investigated the effects of pyriofenone on DNA replication (a measurement of cell proliferation) in isolated male F344 rat hepatocytes, by measurement of incorporation of the DNA precursor 5-bromo-2'-deoxyuridine (BrdU). Both PB and the positive control, EGF (a weak response was noted, usually a much stronger signal is expected) caused DNA replication. This study also had serious limitations and very low levels of pyriofenone were tested. There was insufficient reporting, no statistical analysis and the results are inconclusive with respect to the ability of pyriofenone to induce increased DNA replication. This study is also best interpreted as indicative only (table 20, CLH report).

2.1.2 In vivo studies in rats

Study 3. In vivo rat 3- and 7-day dietary study: Effect of pyriofenone on DNA replication (BrdU incorporation) at 3- and 7-days

An *in vivo* study (Anonymous, 2009b), carried out in male F344 rats, investigated the effect of pyriofenone on hepatic cell proliferation. Rats (5/group) received either pyriofenone (0, 200 or 20000 ppm) for 3 or 7 days in their diet or the positive control, chloroform (1000 mg/kg bw/day) for 2 days. Two hours before necropsy, each animal received a dose of the DNA precursor 5-bromo-2'-deoxyuridine (BrdU).

No deaths or treatment related clinical signs occurred during the study. Significant reductions in body weight, food consumption and food efficiency were seen at the top dose tested, 20000 ppm (Group 3 – high dose for 3 days [849 mg/kg bw/day] and Group 6 – high dose for 7 days [1109 mg/kg bw/day]) and in the positive controls (Group 7 – chloroform at 1000 mg/kg bw/day for 2 days).

The replicative DNA synthesis (RDS) incidences were calculated as percentages of BrdU-incorporating cells.

Results:

Effects were seen at the top dose only (20000 ppm) equivalent to 849 mg/kg bw/day for animals in the 3-day feeding group and 1109 mg/kg bw/day for animals in the 7-day feeding group.

1. Increased liver weight was only seen for the 7-day animals on the top dose; +16% (absolute organ weight) and +27% (relative organ weight) relative to controls.

2. Increased RDS (replicative DNA Synthesis) index was observed at both 3-days and 7-days in the top dose groups only along with the positive control:

Table 9*: results for replicative DNA Synthesis*

Group (G): dose (ppm)	Mean total number of hepatocyte count	Mean RDS index %
3-day treatment		
G1: 0 Control	2199	**1.23**
G2: 200 pyriofenone	2423	1.21
G3: 20000 pyriofenone	2687	**2.98**
7 day treatment		
G4: 0 Control	2265	1.42
G5: 200 pyriofenone	2425	1.38

G6: 20000 pyriofenone	2399	**3.91****
Positive control (2 day treatment period)		
G7: 1000 mg/kg bw/d chloroform	2735	**[a]14.3 ****

[a] treatment period was 2 days and the values were compared to 3 day non-treatment group.
** $p<0.01$.

The DS concluded that pyriofenone can cause an increase in replicative proliferation in Fischer rats at high doses.

Study 4. In vivo rat 14-day feeding mechanistic hepatotoxicity study: Hepatic enzyme induction in F344 rats

In this study (Anonymous, 2011a), the rats were divided into two groups (table below); group 1 (G1) received pyriofenone in the diet at either 0, 200 or 20000 ppm and phenobarbital (PB) at 500 ppm for 14 days and group 2 (G2) received pyriofenone in the diet at either 0 or 20000 for 14 days with a 14 day recovery period to assess the reversibility of any effects observed.

***Table**: Mean dose received (mg/kg bw/day)*

Dietary concentration of pyriofenone (ppm)	0	G1: 200	G1: 20000	G2: 20000
Male F344 rats	0	14.3	1300	1289

Results:

1. Liver weight was increased at the top dose (relative organ weight: pyriofenone +43% and PB +35% with respect to controls). The extent of the absolute liver weight changes were similar to the relative liver weight changes.

2. Total CYP450 content was increased with PB treatment but not with pyriofenone (*not in accordance with points 3 and 4, below*).

3. PROD and ECOD activities were increased with pyriofenone and PB treatment. The relative increase in PROD compared with ECOD activity following pyriofenone treatment was dramatic (for PROD) at 20-fold and 1.5-fold of controls respectively. PB treatment had a much stronger effect.

4. Treatment with pyriofenone caused a selective increase in CYP2B1 protein content (8-fold) and only a small increase in CYP1A2 (1.6-fold). This is a more definitive measure of gene transcription than enzyme activity because it relied on the measurement of the actual gene product produced (via immunoblot densitometry of enzyme protein using monoclonal antibodies). PB had a greater increase in CYP2B1 protein content but a weaker effect on CYP1A2 content when compared with pyriofenone.

5. The results from group 2 indicated that these effects were reversible.

The DS concluded pyriofenone caused enzyme induction *in vivo* in Fischer F344 rats.

Study 5. In vivo 7-day dietary mechanistic study in CAR-knock-out (KO) rats: Hepatic enzyme induction in F344 rats

An *in vivo* mechanistic study *(Anonymous*, 2017a) compared the effects of pyriofenone in CAR-knock out (KO) and wild-type (WT) Sprague Dawley rats (5/males/dose). Animals received pyriofenone in the diet at a concentration of either 0 or 5000 ppm (357.2/363.6 mg/kg bw/day KO/WT, same dietary dose as the top dose in the carcinogenicity bioassay) for 7 days. The most

pertinent measurements for MoA analysis were liver weight, CYP2B1 gene expression, CYP2B1 activity and hepatocyte proliferation (Ki-67). No deaths occurred during the dosing period and there were no abnormal clinical signs. Body weight in the 5000 ppm groups remained comparable to controls.

Results:

1. Liver weight increased with treatment. In WT rats the increase was +16% greater than controls (absolute and relative) and in KO rats the increase was +20% and +19% greater than controls (absolute and relative respectively).

2. CYP2B1 (mRNA) gene expression was increased in both WT (130-fold) and KO (120-fold) groups relative to controls.

3. Pyriofenone did not increase CYP2B1 activity in the CAR-KO rats, whilst in WT rats CYP2B1 activity was found to increase by 8-fold relative to controls (CYP2B1 activity was undefined in the CLH report).

4. Measurement of the Ki-67 positive ratio in hepatocytes did not show any increase in either WT or KO rats.

The results are somewhat contradictory. The DS noted some effects were consistent with CAR activation (increased CYP2B1 activity in WT) but others were not (increased CYP2B1 gene expression in both WT and KO animals, lack of increased Ki-67 in both WT and KO animals). Overall the results were inconclusive; the study did not rule out other mechanisms of action which could explain how pyriofenone affects the liver.

Study 6. In vivo mouse 28-day dietary mechanistic study: Hepatic enzyme induction and cell proliferation.

This study was performed as a mechanistic study to explain a small increase in hepatocellular tumours observed in male mice at the highest dietary concentration (5400 ppm) in the 18-month carcinogenicity study. CD-1(ICR) male mice (12/dose) were administered pyriofenone in the diet at concentrations of 0, 5000 (854 mg/kg bw/day) or 10000 ppm (1714 mg/kg bw/day) for 4 weeks (Anonymous, 2010j). There were no clinical signs of toxicity at any dose level. There were no treatment related effects on bodyweight.

1. Relative liver weight increased following both doses of pyriofenone (+12% and +14% at 5000 and 10000 ppm respectively).

2. There was an increase (42-57%) in cytochrome P450 in both dose groups, indicating Phase I enzyme induction.

3. CYP1A: There was a small (approximately 40-50%, statistically significant) increase in EROD activity (approximately 1.4 fold).

4. CYP2A, 2B, 2C and 3A: little to no evidence of induction after assessment of testosterone hydroxylase and dehydrogenase activities as general markers for these CYPs.

5. CYP2E and 4A: No evidence of induction following the assessment of lauric acid 11- or 12-hydroxylase (CYP2E and CYP4A marker activity, respectively) activities.

6. No evidence from the PCNA results that pyriofenone caused any increase in the rate of hepatocyte proliferation.

The DS concluded that pyriofenone had little impact on hepatic phase I enzyme induction or cell proliferation in mice.

2.1.3 In vitro studies in human hepatocytes

Study 7. In vitro human hepatocytes: Hepatic CYP2B6 and CYP1A2 gene expression

Pyriofenone was tested in human hepatocytes for its ability to cause changes in expression of the CYP2B6 and CYP1A2 genes (Shikama, 2013c). Once again serious deficiencies were noted by the DS such as a lack of sufficient detail (sex, number of donors, age and health status unknown). The study can only be considered as indicative. Cells were exposed to concentrations of PB at 30 and 300 ppm and pyriofenone at 2.5 and 5 ppm for 24 hours. Both pyriofenone and PB increased CYP2B6 gene expression with no effect on CYP1A2 consistent with a CAR MoA.

Study 8. In vitro human hepatocytes: effect of pyriofenone on DNA replication

Pyriofenone was tested for its ability to affect DNA replication in human hepatocytes by measurement of incorporation of BrdU (Shikama, 2013d). Once again serious deficiencies were noted by the DS (as above). There was no evidence of DNA replication following treatment with pyriofenone or PB. However, an increase of +48% was seen in cultures treated with EGF, confirming the potential of the cells to undergo S-phase DNA synthesis (however, a much stronger response is expected with this level of EGF). The DS concluded this was also consistent with a CAR MoA.

2.1.4 Weight of Evidence

The long-term studies in rats and mice showed that the liver was the primary target organ with the development of hepatic adenomas and carcinomas observed in male F344 rats. The incidences of liver tumours observed in the CD-1 mouse, though apparently dose dependent, fell within the HCD for the performing laboratory.

Mechanistic studies were described to try to explain the borderline responses seen in male rats upon exposure to pyriofenone. The DS outlined that the most plausible modes of action that could account for the weak carcinogenic response appeared to be non-genotoxic and involved either cytotoxicity or activation of the constitutive androstane receptor (CAR). Evidence to show that this carcinogenic response was driven by the hepatotoxicity of pyriofenone is limited. A clear link between exposure to pyriofenone, toxicity and the formation of pre-neoplastic lesions in the liver was lacking. The DS summarised the evidence and how it related to key and associative events involved in the CAR-mediated formation of liver tumours in rodents (table 25, CLH report).

However, several alternative possible mechanistic explanations were not investigated in detail. The available investigations focused on providing evidence in support of one mode of action, i.e. on a non-genotoxic mode of action involving hepatocyte proliferation, induced via CAR activation. In several instances, the data appeared to be conflicting and major limitations were associated with some of the studies. The *in-vivo* study with CAR-knock out rats was limited to investigating changes in expression to CYP2B, liver effects and hepatocyte proliferation without regard to other gene/enzyme markers. The data here was somewhat unclear. Liver weight increases and increases in CYP2B expression in the CAR KO rats were seen without showing increases in CYP2B enzyme activity. AhR involvement was shown to be small but present. There were limited investigations into changes in the expression of CYP4A so that any involvement of PPARα activation and hepatic peroxisomal β-oxidation remain unresolved. Inhibition of apoptosis and other associative events in the CAR associated tumour model have not been investigated (e.g. altered epigenetic changes, gap junctional intercellular communication and oxidative stress).

The available mechanistic data indicated that the MoA for liver tumours in rats may be hepatocellular proliferation induced by activation of the CAR.

2.2 Conclusions

The DS' evaluation of the MoA studies demonstrated a CAR activation MoA for rat liver tumours was a plausible explanation but given certain study limitations and data gaps they were not sufficiently convincing to propose no classification. The DS considered there was insufficient evidence to underpin the proposed species-specific mechanism of action. There was sufficient uncertainty to support a category 2 classification for carcinogenicity.

Comments received during public consultation

There were two comments received during public consultation.

(1) Company-Manufacturer:

Industry commented that they disagreed with the proposed classification and supplied a comprehensive white paper (RSA/ISK005_4160_001) which reviewed the available data along with several helpful reference publications and HCD for the F344 rat.

(2) Member State Competent Authority:

The proposed classification with Carc. 2 was supported. A clarification regarding statistical analysis was provided by the DS and a minor typographical error in the CLH report was noted.

Additional key elements

It has come to the attention of the RAC, that three additional mechanistic studies were available that were not assessed by the DS in the CLH report and were not presented during public consultation. They were not in the original DAR but were included in the DAR addendum of November 2012. They are:

i. Nagaike (2012) Medium term liver carcinogenesis bioassay in F344 rats. Report no. AN-2902 (a promotion and initiation study using diethyl nitrosamine, PB and pyriofenone on partially hepatectomised F344 rats), study 9.

ii. Ohtsuka (2012) IKF-309 Technical: Mechanistic study of CAR function in rat liver. Report no. IET 12-0007, study 10.

iii. Takeda (2012) IKF-309 Technical: Additional testing of Hepatic enzyme induction Study in rats. Report no. IET 12-0005, study 11.

Additional mechanistic studies

Study 9. Medium-term liver carcinogenesis bioassay in F344 rats.

This was a non-guideline, non GLP mechanistic study (Nagaike, 2012) designed to detect promotion or initiation effects on hepatocytes by exposure of animals to pyriofenone in their diet. All animals, male Fischer rats (F344/DuCrlCrlj), were distributed into 5 test groups as follows (table below):

- Group 1: initiation with diethyl nitrosamine (DEN) alone.
- Group 2: initiation with DEN and 500 ppm phenobarbital (PB).
- Group 3: initiation with DEN and 200 ppm pyriofenone.
- Group 4: initiation with DEN and 10000 ppm pyriofenone.
- Group 5: treatment with 10000 ppm pyriofenone alone.

***Table**: experimental design of the medium term carcinogenesis bioassay*

Group	DEN	Treatment	Number of animals
1	+	Basal diet	10
2	+	PB 500 ppm	10
3	+	Pyriofenone 200 ppm	12
4	+	Pyriofenone 10000 ppm	13
5	-	Pyriofenone 10000 ppm	10

The dosing schedule was as follows: all rats in groups 1 to 4 were administered a single intraperitoneal (i.p.) injection of DEN (200 mg/kg bw) dissolved in saline at 5 weeks of age to initiate hepatocarcinogenesis on the first day of the experiment. Rats of group 5 received the saline vehicle instead. After 2 weeks, animals were administered with pyriofenone at a concentration of 200 (group 3), 10000 ppm (group 4 and 5) or PB at a concentration of 500 ppm (group 2) for the following 6 weeks. All animals were subjected to two-thirds partial hepatectomy (PH) at the end of week 3.

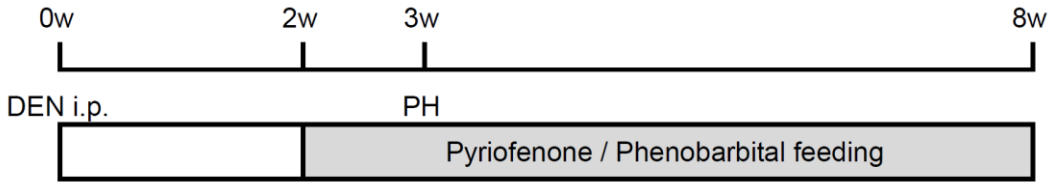


***Figure:** Design of the dosing schedule for the medium term carcinogenesis bioassay.*

Pyriofenone or PB were administered by feeding for 6 weeks. There were no test substance related effects on mortality, clinical signs, body weight, and food consumption in any group. The study lacks an overall negative control group, i.e. one with no initiation by DEN and no exposure to pyriofenone.

***Table**: Mean dose received (mg/kg bw/day)*

Dietary concentration of PB/pyriofenone (ppm)	**0**	**G2: 500**	**G3: 200**	**G4: 10000**	**G5: 10000**
Male F344 rats	0	34.2	13.3	667	644

Results:

1. Liver weight (absolute) was statistically significantly increased relative to controls in the PB and 10000 ppm pyriofenone treated groups (groups 2, 4 and 5 by +47%, +41% and +42% respectively). Increases in relative weight were very similar.

2. The areas and numbers of GST-P positive liver foci (indicative of pre-neoplastic lesions) were increased for all DEN treated groups.

3. The areas and numbers of GST-P positive liver foci in DEN+PB or DEN+10000 ppm pyriofenone-treated animals were significantly increased over the DEN controls. Pyriofenone treatment without DEN initiation did not promote the development of GST-P positive foci.

***Table**: Results of medium-term carcinogenesis assay.*

Group	DEN	Test Chemicals	Number of rats	Body weight (g)	Liver weights		GST-P foci	
					Absolute (g)	Relative (mg/g BW)	Area (mm^2/cm^2)	Number ($No./cm^2$)
1	+	Basal diet	8	269	8446	31.37	0.528	0.187
2	+	PB 200 ppm	7	280	12397**	44.68**	1.392**	0.299**
3	+	Pyriofenone 200 ppm	12	276	8997*	32.65	0.565	0.163
4	+	Pyriofenone 10000 ppm	9	274	11894**	43.28**	1.642**	0.224*
5	-	Pyriofenone 10000 ppm	3	274	12025**	43.92**	0.001**	0.001**

Significantly different from control: * $p \leq 0.05$; ** $p \leq 0.01$

The results indicated that pyriofenone acted in a similar manner to PB with regard to liver effects. Pyriofenone did not show any initiation of carcinogenesis in the way classical genotoxic agents such as DEN do. Pyriofenone acted like a tumour promoter in the same way that phenobarbital acts in rats and strongly promoted the development of GST-P positive foci in DEN-treated rats.

Study 10. Mechanistic study of CAR function in rat liver.

This was a non-guideline, non GLP mechanistic 14-day dietary study (Ohtsuka, 2012) designed to localise and quantify the CAR receptor in hepatic cells following exposure to PB sodium salt and pyriofenone. Pyriofenone was administered in feed at 0, 200 and 20000 ppm to 5 male Fischer rats (F344/DuCrlCrlj) per dose for 2 weeks. Phenobarbital (PB) administered at 500 ppm served as a positive control.

There were no significant changes in mortality, general clinical signs, and food consumption. There was a significant but slight increase in body weight detected in animals treated with PB compared to the negative controls.

***Table**: Mean dose received (mg/kg bw/day)*

Dietary concentration of PB/pyriofenone (ppm)	**0**	**G2: 200**	**G3: 20000**	**G4: PB 500**
Male F344 rats	0	13.7	1246	34.3

Results:

1. Liver weight (absolute) was statistically increased relative to controls in the PB and 20000 ppm pyriofenone groups (+34% and +37% respectively). Increases in relative weight were nearly identical.

2. Immunohistochemistry of hepatocytes for CAR: Very subtle differences between pyriofenone and PB were observed but the results were not conclusive. The immunohistochemistry indicated CAR was being upregulated and showed greater staining in both the cytoplasm and the nucleus. It was not a definitive illustration of the translocation of activated CAR into the nucleus.

In the pyriofenone 20000 ppm and PB groups, both nuclei and cytoplasms were moderately positive for CAR staining in the centrilobular region. In the periportal region, nuclei and cytoplasms of hepatocytes were slightly positive in the PB group, while nuclei of hepatocytes were moderately positive and cytoplasms were negative in the pyriofenone 20000 ppm group. Evidence of nuclear translocation of CAR was clearer in the periportal region of the liver with the pyriofenone 20000 ppm group. The controls and low dose pyriofenone livers showed weaker staining in both the cytoplasm and nuclear compartments.

3. Western blot analysis was carried out for CYP2B1 and CAR using different subcellular fractions: nuclear, soluble (cytosol) and microsomal. The ratio of CAR protein level in the nuclear fraction to that in the soluble (cytosolic) fraction was calculated. The hepatic microsomal fraction was used to measure CYP2B1. The western blot results give a clearer view than the immunohistochemistry and confirm CAR translocation into the nucleus as a result of pyriofenone and PB exposure.

Western blot analysis for CYP2B1 confirmed the induction of this isoform. In the pyriofenone 20000 ppm and PB groups, CYP2B1 was significantly increased in both groups when compared to the negative control group. The ratio of CAR protein level in the nuclear fraction to that in the soluble (cytosolic) fraction were significantly higher in the 20000 ppm and PB groups compared to the negative controls. This result of a higher nuclear-cytoplasmic ratio supports the occurrence of CAR translocation into the nucleus in these two groups. There were no treatment related changes in the pyriofenone 200 ppm group.

***Table**: Western blot analysis - Group mean values for male F344 rats*

Dose (ppm)		CYP2B1 (pmol/mg protein)	CAR ratio (Nuclear/Cytosol)
0	Mean	9.1	0.10
200	Mean	10.2	0.11
20000	Mean	40.7 **	0.18 *
PB500	Mean	94.8 **	0.28 **

PB500: Phenobarbital sodium at 500 ppm
Significantly different from control: * $p \leq 0.05$; ** $p \leq 0.01$

This study revealed that pyriofenone acts in a similar manner to PB showing an induction of CYP2B1 protein and CAR along with translocation of CAR into the hepatocyte nuclear compartment. It did not investigate any other alternate systems but provides evidence for the CAR MoA in rat liver.

Study 11. Additional testing of Hepatic enzyme induction Study in Rats

This was a non-guideline, non GLP mechanistic study (Takeda, 2012) designed to investigate the induction and expression of a wide variety of isozymes from the cytochrome P450 family. The

liver samples originated from an earlier study (Study 4 described in section 2.1.2 above; 14-day feeding mechanistic hepatotoxicity study of IKF-309 in male rats; AN-2918; Anonymous, 2011a).

CYP3A2 protein (PXR marker) and gene expression levels of CYP1A1, CYP1A2, CYP2B1, CYP2B2, CYP3A2 and CYP3A23 were measured. mRNA levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were measured as a reference for quantitative real time RT-PCR since the GAPDH gene is considered to be a so called 'housekeeping' gene and constitutively expressed – it is therefore used for the normalisation of target gene expression data.

Male Fischer (F344/DuCrlCrlj) rats were used in a 14-day dietary study initiated when the animals were at 8 weeks of age. PB was used as the positive control. In addition to the 14-day treatment groups, a 14-day recovery group was also established to confirm the reversibility of the treatments.

Results:

1. CYP3A2 protein measured via western blot analysis. Following pyriofenone treatment, there were no significant increases in CYP3A2 protein (a marker for PXR activation) when compared to the corresponding control (maximum was just over 2-fold that for the controls at the 20000 ppm dose). However, CYP3A2 was strongly and significantly induced by PB (> 6-fold over that for the controls, statistically significant).

2. Pyriofenone effects on mRNA expression: Several mRNAs were significantly increased only in the top dose group when compared with the concurrent controls, the low dose group may in essence be regarded as a secondary control group. Pyriofenone had the following effects:

- 753-fold induction of CYP2B1 (marker for CAR),
- 28-fold induction of CYP2B2 (marker for CAR),
- 7-fold induction of CYP3A2 (marker for PXR),
- 18-fold induction of CYP3A23 (marker for PXR),
- 6-fold induction of CYP1A1 (marker for aryl hydrocarbon receptor (AhR)), and
- 2-fold induction of CYP1A2 (marker for AhR).
- There was no effect on GAPDH mRNA levels (reference marker).

In the 14-day recovery group, generally there were no significant changes between the 20000 ppm group and the corresponding control (except for CYP2B2 mRNA which was significantly decreased), thus illustrating the reversible nature of the gene induction following cessation of treatment with pyriofenone.

3. Phenobarbital effects on mRNA expression: Treatment with PB also showed CYP2B1, CYP2B2, CYP3A2 and CYP3A23 mRNAs were significantly increased to a very similar quantitative degree as pyriofenone when compared with the concurrent controls. In contrast, there was a down-regulation of CYP1A1 and CYP1A2 mRNAs.

There is discordance between the effects on CYP3A2 by pyriofenone and PB when comparing the amount of protein produced and the expression levels of the CYP3A2 mRNA. The western blot results indicate that PB has a greater effect on the expression of CYP3A2 while the mRNA results indicate pyriofenone has as great or an equal effect as PB.

CAR activators and PB generally increase levels of CYP1A1 and CYP1A2 by a small amount through what is regarded as a pathway independent of the aryl hydrocarbon receptor. Small increases in the expression of CYP1A isozymes are not unexpected.

To conclude, the results of this study generally indicate that pyriofenone acts in a similar manner to PB and specific markers of CAR activation are strongly induced by the substance.

Assessment and comparison with the classification criteria

3. *Carcinogenicity*

3.1 Introduction

Pyriofenone induced liver tumours in male rats with some indications of neoplasia in mice, thus there is a need to consider whether classification for carcinogenicity is appropriate. There is no information from studies in humans to inform on the carcinogenic potential of pyriofenone and so classification in category 1A may be excluded from further consideration.

The DS indicated that no link was observed between increased mortality and the incidence of liver tumours in rats. Although the authors in the original study report could not identify a common cause of health deterioration or death in the animals killed in extremis or found dead, they speculated that the increased mortality rate might be related to treatment. Mortality in the top dose animals affected males (34%) while females had a reduction in mortality from 30% in controls to 24% in the top dose animals. Tumours were only seen in males even though females received a higher dose of pyriofenone (197 vs 254 mg/kg bw/day respectively). In males, the differences in the mortality rate were visible in the top dose group relative to the other dose groups and controls from week 88 onwards but statistical significance was not attained until the last week of the study, i.e. week 101. In the male animals found dead before the end of the study, 3/17 (18%) were found to have liver adenoma and 1/17 (6%) had carcinoma of the liver. In those surviving to the end of the study, 3/33 (9%) had adenoma of the liver and 1/33 (3%) had liver carcinoma.

3.2 Rat Liver tumours

In males there was a numerical increase (6/50 (12%) vs 4/50 (8%) in control) in the incidence of hepatocellular adenomas at 197 mg/kg bw/day (5000 ppm) and hepatocellular carcinomas (2/50 (4%) vs 0/50 (0%) in control), which did not reach statistical significance (see table below). However, the incidences of hepatocellular adenoma and carcinoma in males at the top dose are above the upper limit of the most relevant historical control data (2003 – 2011) for this type of tumour in this strain of rat.

***Table**: Neoplastic findings in male F344 rats*

Dose (mg/kg bw/day) M/F	**0**	**7.25/9.13**	**36.4/46.5**	**197/254**	**HCD**
Males:					
Number examined	50	50	50	50	
Liver: Hepatocellular adenoma Hepatocellular carcinoma Combined total	4 (8%) 0 4 (8.0%)	1 (2%) 1 (2%) 2 (4.0%)	2 (4%) 1 (2%) 3 (6.0%)	6 (12%) 2 (4%) 8 (16%)	0-4% 0%
Females:					
Number examined	50	50	50	50	
Liver: Hepatocellular adenoma Hepatocellular carcinoma	0 0	0 0	0 0	1 (2%) 0	

HCD: 8 x studies (2003 – 2011); total animals = 400 males; total adenoma incidence = 5 (mean of 1.3%); total carcinoma incidence = 0 males.

3.2.1 The importance of the historical control data

The expanded historical control database

While there may appear to be an increased incidence of tumours at the top dose level only, this was not statistically significant when compared to the concurrent controls for either the hepatocellular adenomas or carcinomas. An expanded historical control database to include all the studies conducted at the performing laboratory for spontaneous hepatocellular adenoma and carcinoma in male F344 rats extending from 1978 to 2011 was supplied by the industry applicant. Taking this data into account, the adenoma incidence ranged from 0 – 12% (incidence of 12% was from a study conducted in 1991) with the carcinoma incidence ranging from 0 – 4% (incidence of 4% was from a study conducted in 1983).

Expanded HCD: 49 x studies (1978 – 2011), with the pyriofenone study excluded; total animals = 2528 males; total adenoma incidence = 70 animals (mean of 2.7%); total carcinoma incidence = 6 males (mean of 0.2%).

3.2.2 The Incidences of Liver Adenomas

The central question to ask is whether the incidence of tumours in the male rats is relevant or not. In the case of the mouse, it may be seen that the tumour profile is not relevant and therefore not substance related. In the case of the rat study, it is crucial to consider the validity of the concurrent controls and put them into perspective by looking more closely at the expanded HCD database that is available to RAC.

A look at the entire IET carcinogenicity study database using F344 rats presents 49 studies in total (not including the pyriofenone study) spanning the years 1978 – 2011 (33 years). A closer inspection of the HCD to investigate the variability of incidence of adenoma in male rats over time (figure below) indicates two clear and separate periods with respect to the spontaneous occurrence of F344 liver adenomas – period 1 (1978 – 1991) and period 2 (1991 – 2011). A second valid question is whether RAC should be merging the data for these two periods together.

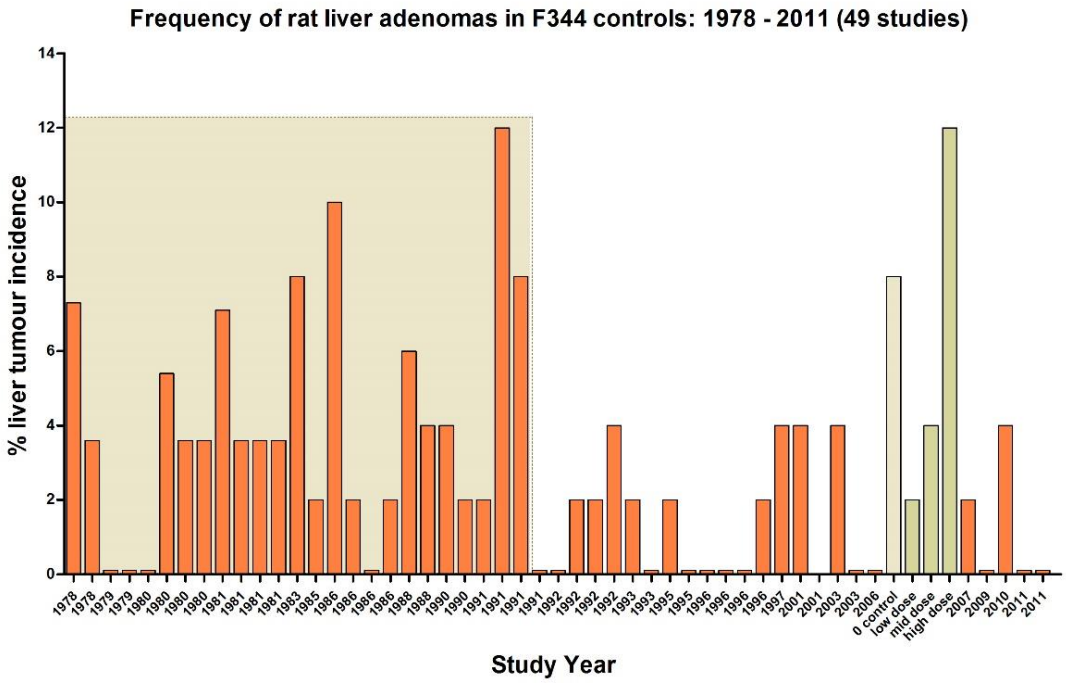


Figure: *Individual study incidences of spontaneous liver adenoma in male F344 rats from IET occurring from 1978 to 2011.*

According to CLP Guidance, the HCD should be contemporary to the study being evaluated (e.g. within a period of up to 5 years of the main study) and data older than this should be used with caution. This would appear to be prudent in the present case for two reasons: (1) hepatocellular adenomas occur at incidences > 4% only prior to 1991, and as seen above, the expanded historical control data may be clearly divided into two blocks with sporadic high adenoma incidences only recorded during the first period corresponding to 1978 – 1991. The second historical block (1991 – 2011) generally does not exceed 4% incidence except for the pyriofenone main study. This in itself also brings into question the extent to which the concurrent control group of the pyriofenone study is reliable or representative. (2) The extended HCD also illustrates just how rare is the incidence of hepatocellular carcinomas (6 out of 2528 males [0.2%; range 0 – 4%] from 49 studies spanning 1978 – 2011).

A noted discrepancy was the high incidence of adenomas in the control group. In the rat two year study, the spontaneous rate of adenoma in the concurrent controls (8%) was double the incidence of adenomas observed in the most relevant time period of the available HCD (2003 – 2011). There was no explanation provided as to why this should have occurred. The low dose group (7.25 mg/kg bw/day; which some may consider a surrogate control group) had a low incidence of 2% (1/50 males). This result for the concurrent control group does pose a question of relevance and reliability for this group in this study because it is not representative of the expected spontaneous incidence of liver adenomas in male F344 rats. This may also explain why in any statistical comparison between a treated group and the controls in this case they are not significantly different. There was little discussion in the original study report; the authors simply concluded that pyriofenone showed no carcinogenic effect when in fact there is a small dose response (1, 2, 6 animals effected at the low, mid and top doses respectively). Spontaneous incidences of liver adenomas greater than or equal to 8% only occur prior to 1992. So what is the relevant HCD span RAC should normally consider?

There is a distinct change in the F344 male rat liver spontaneous adenoma tumour incidence profile circa 1991. This brings into question the wisdom of using such a broad range of time (33 years) to provide a meaningful HCD database in which to evaluate concurrent controls and incidence data with treatment. Consideration of the entire 33 year span would be more appropriate if the spontaneous background occurrence of liver adenomas was consistent. This is not the case here. Extending the HCD too far limits the ability of the HCD to detect true deviations from the spontaneous background if the rate of spontaneous occurrence is not in itself consistent; this effectively raises the noise floor. Other unknown factors must be at play to account for the lack of consistency. Caution must be exercised in going beyond the limits recommended in the CLP guidance.

3.2.3 The Incidences of Liver Carcinomas

In the main rat study, 4 males were affected across all dose groups. The incidence of hepatocellular carcinoma was weakly increased at all doses (0, 2%, 2% and 4% in the control, low, mid and high dose groups, respectively). What is important to note is just how rare this tumour is in the F344 rat. The HCD range specifies a 0 – 4% maximum incidence, however the range doesn't provide information on the representative or expected spontaneous rate of liver carcinomas in this strain of rat. The expected incidence is closer to zero. Out of a total of 49 carcinogenicity studies, only 4 studies showed any incidences of carcinoma, the remaining studies had a 0% incidence rate (see figure below). A total of 6 males had liver carcinomas (out of 2528 males); 2 in one study dating from 1981; 1 in another 1981 study; 2 in a 1983 study and 1 in 1993. In the pyriofenone study, there were 4 males in total affected with 0 in the concurrent controls. The top dose group had 2 males effected (4% incidence rate); consulting the HCD requires RAC to go back as far as 1983 to see a similar incidence.

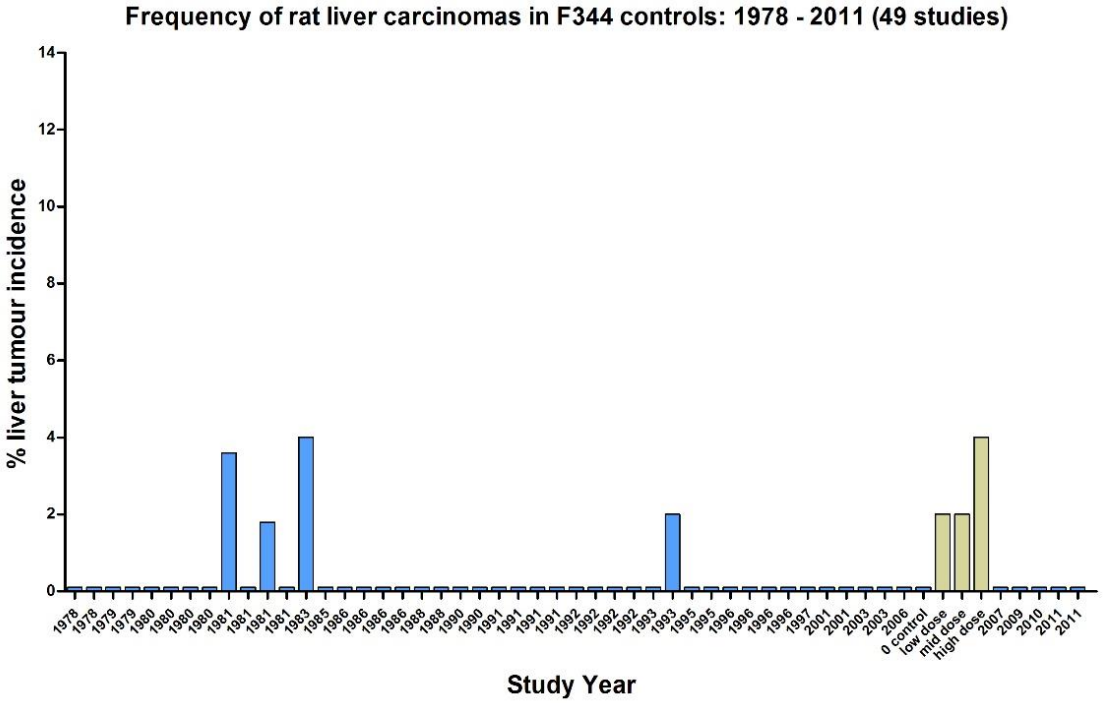


Figure: *Individual study incidences of spontaneous liver adenoma in male F344 rats from IET occurring from 1978 to 2011.*

Using the same approach of critically analysing the use of the HCD, the results of the carcinogenicity study on mice shows a clear lack of evidence for carcinogenicity by pyriofenone in this species (see figures in section 3.3 for adenomas and carcinomas). The incidences of tumours are well within the relevant HCD database and there is no clear signal signifying a carcinogenic response.

The evidence showing a small (not statistically significant), increase at the top dose for adenomas and carcinomas in male rats suggests that pyriofenone displays a low potency and weak carcinogenic effect. The detailed comparison with the HCD supports a weak but substance related effect. Perhaps hepatotoxicity and cytotoxicity and multiple modes of action are factors in the development of the hepatic tumours.

Several mechanistic studies were available and indicated that a CAR MoA was one plausible explanation for the increase in liver tumours. It must be noted, however, that the MoA studies in general used much higher doses of pyriofenone to show the CAR-mediated effects (10000 – 20000 ppm) relative to the highest dose used in the 2-year carcinogenicity study (5000 ppm). Only the CAR-knockout rat study used the same dietary concentration of 5000 ppm as was used in the main carcinogenicity study. Assuming the carcinogenic potency of pyriofenone is low then it may be expected that any investigation into MoA would be difficult to interpret. This is indeed the case and the MoA data was not very clear such that the relevance of these tumours to humans in the context of pyriofenone exposure remains unknown.

3.3 Mouse liver tumours

In males, there was an increase in hepatocellular adenoma incidence at all doses when compared to controls (6%, 13%, 11 and 17% in the control, low, mid and high dose groups respectively). There was also a small increased incidence of liver cell carcinoma at all doses when compared to controls (2%, 4%, 6%, 6% in the control, low, mid and high dose groups respectively) but statistical significance was only reached when combined incidences of adenoma and carcinoma

were compared to controls. It must be stated that the incidence rates of these tumours were well within the HCD provided for the performing laboratory (combined rate: 9.8 – 36%, see figures below). The HCD were relevant for this study and their analysis by the DS showed no concern for the range of values reported and did not identify any particular trend or change in tumour incidence over time. RAC supports the conclusion of the DS; pyriofenone does not appear to be carcinogenic in the mouse.

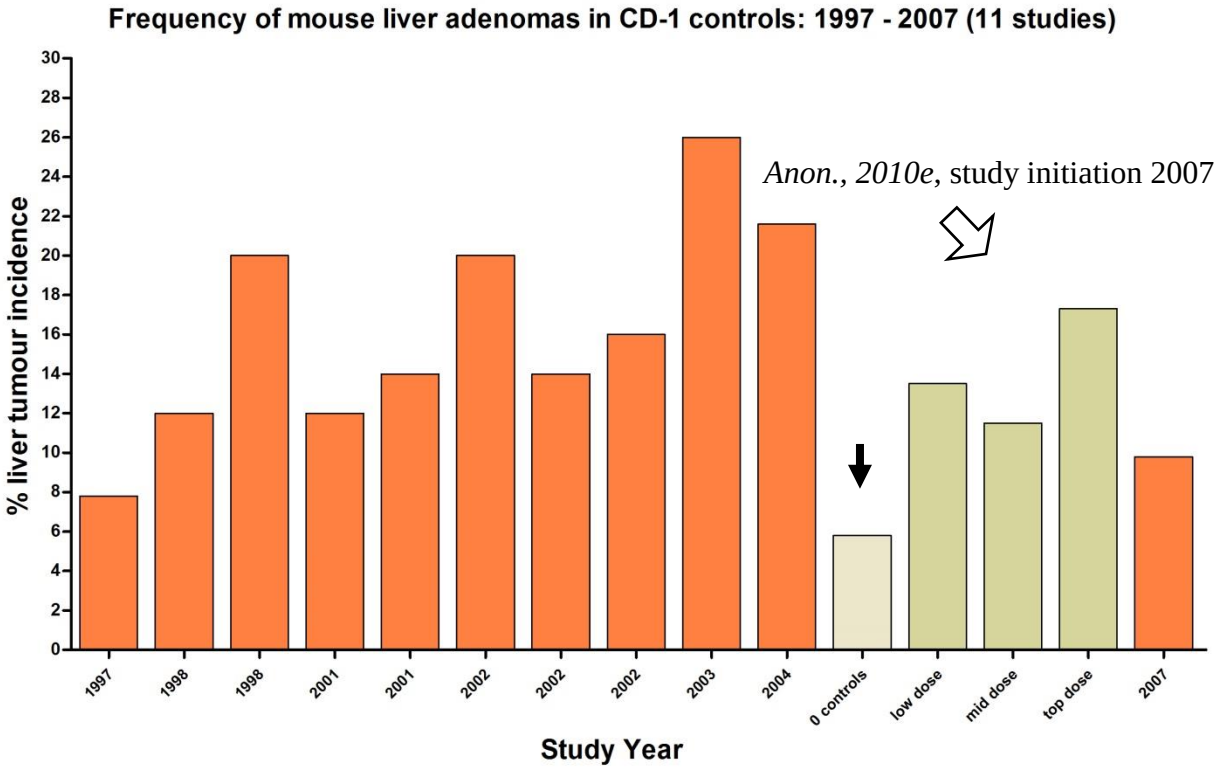


Figure: *Adenoma incidence rate in male CD-1 mice. Arrow head = concurrent control group*

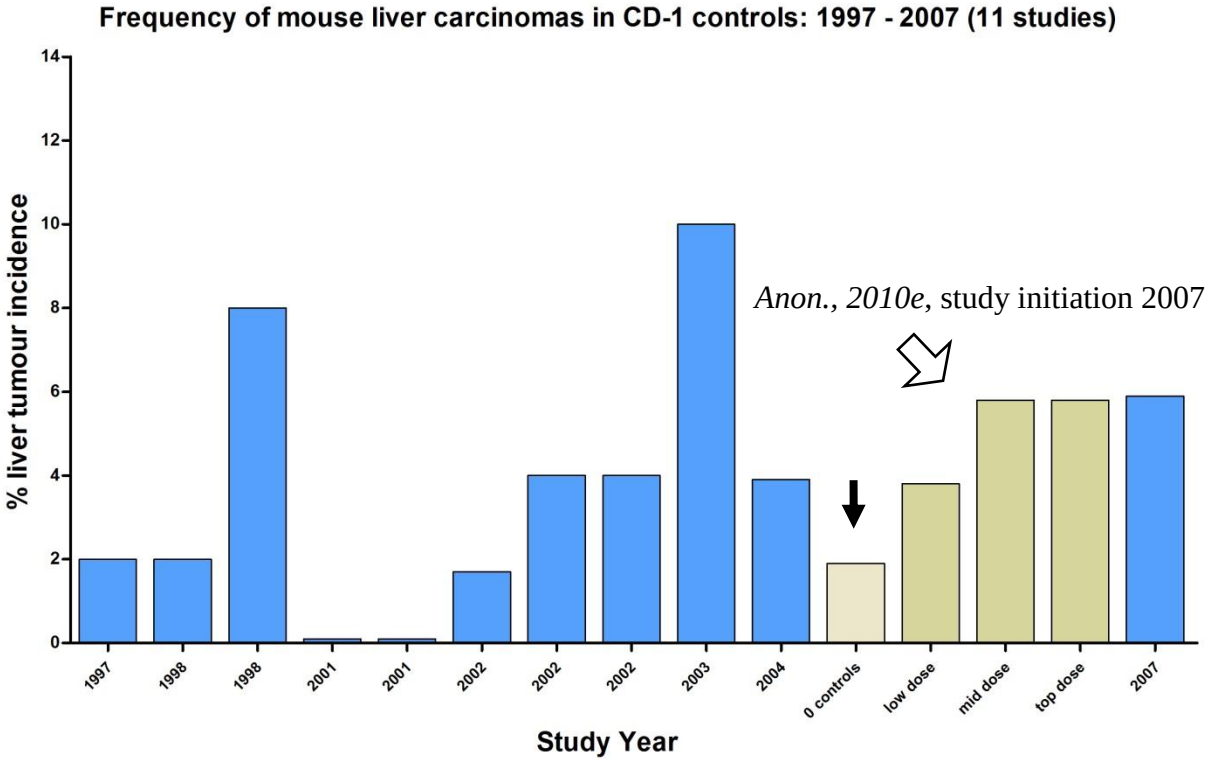


Figure: *Carcinoma incidence rate in male CD-1 mice. Arrow head = concurrent control group*

3.4 Rat liver tumours: assessment of the MoA

The tumour profile observed in the pyriofenone carcinogenicity bioassay was typical of a non-genotoxic mechanism (single species, single sex and single organ involvement without decreased latency). The potency of pyriofenone is low, borderline responses are seen: in male rats it appears to be a weak carcinogen; in female rats there was no clear carcinogenic response.

Several mechanistic explanations can be considered for this weak carcinogenic response in rats. Increased liver weight and hepatocellular hypertrophy are not specific surrogate markers for CAR activation because the induction of other CYP P450 isoforms or peroxisome proliferation can also produce these findings. Hepatocellular cytotoxicity and subsequent regenerative proliferation, such as that caused by chloroform, is another mechanism by which carcinogenesis can occur. This mechanism is typically characterised by sustained diffuse necrosis and cellular proliferation. A limited investigation into these and other modes of action was undertaken and they may be summarised as follows:

- genotoxicity → the overall conclusion is negative for genotoxicity though there were some indications pyriofenone may have the ability to damage chromosomes *in vitro* → **conclusion**: *an unlikely MoA for rat liver tumours.*
- cytotoxicity → the liver was the target organ, there was an increase in mortality in male rats in the top dose group in the rat carcinogenicity study and also an increase in single cell necrosis. Other findings in the liver included increases in relative liver weight, fatty changes and hypertrophy. Some mechanistic findings showed an increase in DNA replicative synthesis but there was no definitive data for cytotoxicity and the increases were not as large as with the chloroform positive control in one mechanistic study (study 3, section 2.1.2). The *in vitro* studies on isolated rat and human hepatocytes are considered unreliable but the use of very low concentrations of pyriofenone suggests some cytotoxicity. A clear link between exposure to pyriofenone, toxicity and the formation of pre-neoplastic lesions in the liver is lacking.

Cytotoxicity was not actively investigated in an effort to discount this MoA → **conclusion**: *plausible, but not definitive.*

- PPARα receptor activation → there were no studies performed to investigate β-oxidation or markers of PPARα activation in rat liver. From the repeated dose studies in rats and mice, there was no evidence of peroxisome proliferation (a key marker of PPARα receptor activators) following histopathological examinations. In mice, there was no change in expression of genes coding for CYP4A upon treatment with pyriofenone → **conclusion**: *an unlikely MoA for rat liver tumours in this case but not sufficiently investigated.*
- CAR/PXR receptor activation → *in vitro* and *in vivo* mechanistic studies generally indicate that pyriofenone induces changes in rats consistent with this MoA but the evidence is mixed as in the case of the CAR KO vs WT study (study 5, section 2.1.2), e.g. CYP2B1 gene expression was increased in KO rats as was liver weight. This indicates uncertainty with regards to CAR activation being the exclusive MoA in operation. Additional MoA studies showed increased CAR staining in hepatocytes and western blot results confirmed CAR translocation into the nucleus as a result of pyriofenone and PB exposure (study 10). In additional investigations in rat livers which assessed CYP3A2 and CYP3A23 transcription (associated with CAR/PXR activation), pyriofenone had a similar effect as PB in increasing mRNA levels but the effect was not as great as with the CAR marker CYP2B1. There was no reliable human *in vitro* hepatocyte data to investigate this MoA → **conclusion**: *plausible, but not sufficiently investigated.*
- AhR receptor activation → results indicate that pyriofenone has the potential to induce AhR markers: *in vivo* studies in rats with pyriofenone showed induction of ECOD activity (a marker of CYP1A activity) but this was small relative to the increase in PROD activity (study 4). There was an increase in CYP1A2 in contrast to PB in the *in vitro* rat hepatocytes (study 1) and small increases were also noted in CYP1A1 and CYP1A2 mRNA in mechanistic study 11. It is not clear if pyriofenone has the potential to activate the AhR or through what is regarded as a pathway independent of this nuclear receptor. Possible crosstalk between receptors and their gene/regulatory DNA sequences is recognised so the evaluation of positive increases in markers for AhR must be taken under caution → **conclusion**: *plausible but unlikely as a primary mechanism*.
- Porphyria → no evidence.
- Endocrine mediated proliferation → no evidence from other studies.
- Immunosuppression → no data

From the data available, the most plausible modes of action that could account for the weak carcinogenic response to pyriofenone in male rats would appear to be non-genotoxic, involving either cytotoxicity or CAR activation. Evidence to show that this carcinogenic response was driven by the hepatotoxicity of pyriofenone is limited.

3.4.1 The proposed Mode of Action for pyriofenone induced liver tumours

A pathway of changes in the liver stemming from CAR activation has been well characterised in recent years as a potential mechanism of action for some rodent liver carcinogens. This MoA involves activation of CAR which results in changes in expression of a wide range of genes, including genes involved in phase I and phase II xenobiotic metabolism, induction of phase III transporters and regulation of genes associated with various physiological processes such as cell

proliferation, apoptosis and metabolism, eventually leading to liver tumour formation (see figure below).

Most of the mechanistic data presented in the CLH report suggests the CAR MoA as a plausible cause of the liver events in the rat. However, the data from pyriofenone is quite mixed and in many cases contradictory. There were also no reliable data from human hepatocytes to support the proposed MoA in male F344 rats and the *in vivo* CAR KO study has numerous uncertainties associated with it such that data is lacking to conclude on a qualitative difference in the established CAR activation MoA for hepatocarcinogenesis between rodents and humans in this case.

3.4.2 Mechanistic study findings related to key and associative events of CAR-activation

Key event 1 – CAR activation

In a recently performed mechanistic study comparing the effects of pyriofenone in CAR knock-out (KO) rats and wild-type (WT) rats CYP2B enzyme activity was increased only in the group of WT rats (by 8-fold of controls). However, liver weight was found to increase in both groups and an increase in CYP2B gene expression occurred to a similar extent in both WT and KO rats. There was no evidence of hepatocyte proliferation in either WT or KO rats with the dose and timeframe used. There is some evidence to indicate that CAR activation can occur in male rats following exposure to pyriofenone but there are major uncertainties as to whether it is the sole mechanism involved.

Key event 2 – Altered gene expression specific to CAR activation

There is no data for altered gene expression specific to CAR activation that may be considered key to the promotion of events responsible for tumourigenesis. There is data to show associative events indicative of CAR activation and altered gene expression such as large selective increases in CYP2B1 mRNA and protein in addition to increased PROD enzyme activity. Many of the mechanistic studies have focused on showing how pyriofenone acts in a similar manner to phenobarbital and mostly this is true. Enzyme activity studies were limited (only PROD, ECOD and EROD [in mice] were measured) to investigating CYP1 and CYP2 activity.

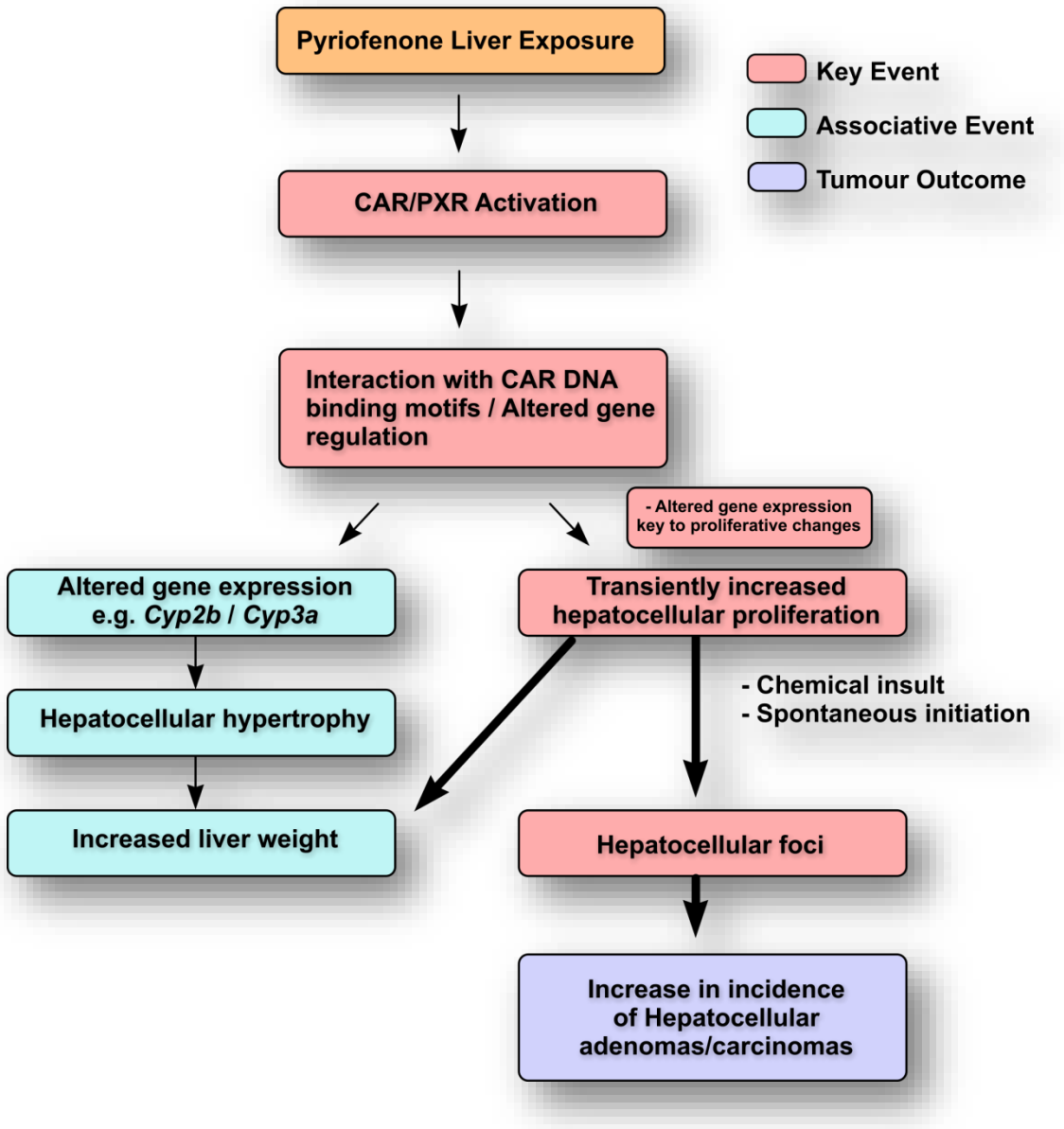


Figure: *Mode of Action hypothesis for pyriofenone-induced liver tumour formation in male rats.*

Liver enlargement, due to both hypertrophy and hyperplasia is also considered an associative event in the MoA for CAR activation leading to tumour formation. Centrilobular hypertrophy and liver weight increases were observed in all of the standard repeated dose rat studies (as well as in the mouse and dog studies) from doses of 226 mg/kg bw/day and above. Liver weight was also increased in the CAR-KO mechanistic study, occurring in CAR-KO rats as well as the WT rats.

Therefore, whilst pyriofenone can cause changes to gene expression and liver weight associated with CAR activation, the problem is it has also been shown to cause these changes in the absence of the CAR receptor.

Key event 3 – Cell proliferation

In *in vivo* mechanistic studies in rats, a statistically significant increase in liver cell proliferation was observed following treatment with 20000 ppm (1109 mg/kg bw/day) pyriofenone administered via the diet for 7 days [mean replicative DNA synthesis (RDS) index 3.91 versus

1.42 in controls] but at a dose of 5000 ppm (~360 mg/kg bw/day) for 7 days no cell proliferation was noted in either WT or CAR-KO rats. There was no evidence of cell proliferation in the livers of mice.

Increased DNA replication was measured in an *in vitro* mechanistic study using rat hepatocytes and no increase in a similar experiment using human hepatocytes. However, RAC recognises severe limitations in these *in vitro* hepatocyte studies and cannot place much weight on their results and consequently considers them unreliable for hazard assessment.

Therefore, it has been shown that only at high doses pyriofenone has the potential to cause hepatocellular proliferation in rats. In a study to determine a definitive CAR mode of action, no proliferation was demonstrated in either WT or CAR-KO rats at the tumourigenic dose (5000 ppm, identical to that in the rat long-term study).

Key event 4 – Clonal expansion leading to altered cell foci

Altered liver foci are precursor lesions for subsequent tumour formation. There was no evidence in the standard studies that pyriofenone induced these changes in male rats. The only mechanistic evidence is that from Nagaike (2012), where pyriofenone was shown to act as a tumour promoter in male rats in a similar way to phenobarbital, showing a clear increase in GST-P positive liver foci but only after the animals were treated with DEN. Pyriofenone treatment without DEN initiation did not promote the development of GST-P positive foci.

Therefore, there is no clear data to support the development of altered liver foci in male rats from treatment with pyriofenone.

Key event 5 – Liver adenoma/carcinoma

In the 2-year carcinogenicity study in rats, a small, dose related increase in the incidence of liver adenoma and carcinoma was observed in males, but not females, indicating positive support for this key event.

3.5 Conclusions

3.5.1. Human Relevance

There was no reliable experimental data that demonstrated pyriofenone did not produce the key event of cell proliferation in human liver cells. Results from an *in vitro* study with human hepatocytes indicated there was no proliferative potential but due to lack of data regarding the study details, number of samples, number of replicates, number of donors, cytotoxicity, weak EGF response etc., RAC cannot consider this sufficient for hazard assessment purposes.

3.5.2 Conclusion on Carcinogenicity

Following a critical assessment of all the mechanistic data provided, the CAR mode of action appears to be a plausible explanation for the increase in liver tumours observed in male rats treated with pyriofenone. However, a number of uncertainties remain:

- In a study intended to provide definitive evidence of the CAR dependence in the mechanism of action of pyriofenone, the key event of altered gene expression occurred in both wild-type and knock-out rats, indicating a lack of CAR-dependence.
- In the same study, liver weight was found to be increased in both CAR-KO and WT rats – this indicates pyriofenone might cause induction of P450 enzymes and cell proliferation independently of CAR activation*. It could also indicate problems with the CAR-knockout rat model employed.*

- There was no increased cell proliferation in either WT or CAR-KO rats (study 5). Another study (study 3) showed cell proliferation. Thus, there are contradictions in the data that differ from what would be expected from a CAR MoA.
- *In vitro* studies in rat and human hepatocytes investigating the effect of pyriofenone on DNA replication were considered unreliable and insufficient for hazard assessment. In both cases, the EGF positive control response for the DNA replicative synthesis investigations was weak; this raises questions about the quality and reliability of these *in vitro* hepatocyte studies.
- The results in several of the mechanistic studies were contradictory or non-supportive of the CAR mediated MoA.
- Inhibition of apoptosis and other associative events in the CAR-mediated tumour model have not been investigated.
- There was no reliable experimental data that demonstrated pyriofenone did not produce the key event of cell proliferation in human liver cells.
- The data for alternate modes of action in the rat are limited and generally confined to a few candidates for enzyme activity (PROD and ECOD), mRNA transcription (CYP1A1, CYP1A2, CYP2B1, CYP2B2, CYP3A2, CYP3A23) and protein detection (CYP2B1, Ki-67, CAR).
- Altered liver foci were not observed.
- The doses required to illustrate a CAR MoA were much greater than the highest tumorigenic dose used in the rat carcinogenicity study.

3.5.3. Classification into Category 1A

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

3*.5.4 Classification into Category 1B*

In order to be classified in Category 1B, the evidence provided must be considered sufficient to presume the substance has carcinogenic potential in humans.

The substance was not found to be genotoxic. The liver tumours observed occurred in just one species (rats), in one sex (males), in one tissue (the liver) and the increased incidences were small (12% for adenoma; 4% for carcinoma), greater than the concurrent control level (8% for adenoma; 0% for carcinoma) and outside the most relevant laboratory HCD for this species (0 – 4% for adenoma; 0% for carcinoma). The strength of evidence relating to a carcinogenic effect following exposure to pyriofenone is considered limited and not sufficiently convincing to place the substance in category 1B.

3.5.5. Classification into Category 2

Pyriofenone could be classified in category 2 for carcinogenicity, based on limited evidence of carcinogenicity in rats.

Pyriofenone is non-genotoxic. A clear non-genotoxic mechanistic basis to account for the increased tumour incidence in male rats is lacking. The possibility is that this could have occurred via a CAR-mediated mode of action, which is generally agreed to be of limited relevance to humans with regard to the formation of liver tumours. However, for pyriofenone this mode of action has not been definitively established. A cytotoxic mode of action is also plausible but definitive evidence is also lacking.

In accordance with the criteria provided in Annex I of the CLP Regulation, "limited evidence" of carcinogenicity in animals is provided for pyriofenone:

1. The evidence is limited to a single experiment.
2. There are unresolved questions about the interpretation of the study results.

There is currently insufficient evidence to support the proposed species-specific mode of action. The incidence of liver adenomas is clearly raised but the concurrent controls are also very high therefore, RAC places less weight on these findings. However, the increase in the liver carcinoma incidence is of concern and appears to be clearly substance related and above the HCD though it is considered a weak effect. This provides sufficient uncertainty for RAC to agree with the DS and support a category 2 classification for carcinogenicity.

3.5.6 No Classification

No classification may be considered for pyriofenone only if the tumour findings can be shown to have no relevance to humans. However, RAC considers insufficient evidence was presented to indicate no concern for human health. The sole MoA for liver tumours in rats being secondary to hepatocellular proliferation induced by activation of the CAR nuclear receptor has not been adequately investigated in human hepatocytes. There were also insufficient robust data to conclude on other alternative modes of actions.

3.5.7 Conclusion

RAC considers the increased incidence in liver carcinomas to be biologically relevant with less weighting placed on the increased incidence of liver adenomas. The mechanistic evidence was inconclusive and gave mixed results. The concern for human health remained.

Consequently, RAC agrees with the DS' proposal to classify pyriofenone as **Carc. 2, H351 (Suspected human carcinogen).**

RAC evaluation of reproductive toxicity

Summary of the Dossier Submitter's proposal

Pyriofenone was evaluated in a preliminary dose range-finding study (Anonymous, 2009d) and a definitive two-generation study (Anonymous, 2009c) in rats in order to assess its effects on sexual function and fertility. The effects of pyriofenone on development following exposure during pregnancy have been well tested in preliminary (dose range-finding) teratogenicity studies in rats (Anonymous, 2009f) and rabbits (Anonymous, 2009e) and similarly in the definitive pre-natal developmental toxicity studies, also in rats (Anonymous, 2009g) and rabbits (Anonymous, 2009h). The main studies were guideline (OECD TG 416 and 414) and GLP compliant.

1. Reproductive toxicity

1.1 Preliminary Study

In the preliminary dose range-finding study, male and female Wistar rats (8/sex/group) were administered pyriofenone in their diet at concentrations of 0, 300, 3000, 10000 or 20000 ppm for three weeks prior to mating, throughout gestation and lactation until weaning of the F1 offspring.

***Table**: Mean dose received (mg/kg bw/day)*

Dietary concentration of pyriofenone (ppm)	0	300	3000	10000	20000
Males	0	17.9	185	591	1159
Females	0	31.9	328	1004	1828

1.1.1 General toxicity

No parental mortalities occurred during the study and there were no clinical signs. Body weight gain was reduced in males of the top dose group throughout the ten week study (21% lower than controls) and in females of the top dose group during the premating period (weeks 0-3, 17% lower than controls) and throughout the gestation period (weeks 14-20, 24% lower than controls). The reduction in weight gain was accompanied by reduced food consumption during weeks 0-3, 14-20 and during the lactation period, days 14-21.

There was clear parental toxicity at the top dose as indicated by numerous significant weight increases recorded for various organs including liver, kidney and caecum and significant reductions in ovarian weight. Clinical chemistry and haematological parameters were also adversely affected (table 26, CLH report). Significant weight reductions in various organs (brain, spleen, thymus and uterus) were also noted at doses of 10000 ppm and above in the F1 offspring. There was no associated histopathology with the organ weight changes and in their absence, they are considered to be a consequence of generalised toxicity and the reduction in body weight.

1.1.2 Reproductive effects

There were no treatment related effects on the oestrous cycle, mating index, fertility index, gestation index, duration of gestation or number of implantations in the parent generation or the F1 generation. At the top dose of 20000 ppm, one female was non-pregnant and one female lost her entire litter by lactation day 4.

1.2 Two-generation study

In the main study, male and female Wistar rats (24/sex/dose) received pyriofenone in their diet at concentrations of 0, 150, 1000 or 5000 ppm for two generations (10 weeks pre-mating, mating and through to weaning).

***Table**: Mean dose received (mg/kg bw/day)*

Concentration (ppm)	P-males	P-females		
	Pre-mating Weeks 0-10	Pre-mating Weeks 0-10	Gestation Days 0-20	Lactation Days 1-14
150	9.61	11.9	9.3	21.2
1000	64.1	79.2	62.0	138.1
5000	334	395	307	677
	F1-males	**F1-females**		
150	11.41	13.0	9.6	20.6
1000	76.8	84.4	61.6	130.0
5000	393	434	321	709

1.2.1 General toxicity

There were no treatment related mortalities, clinical findings or effects on body weight or body weight gain in P or F1 parental animals.

There were clear effects at the top dose as indicated by numerous significant weight increases recorded for various organs including liver, kidney, thyroid and caecum. The thyroid weight was increased in males of the top dose [21% (abs.) and 23% (rel.) compared to controls] but an increase in the incidence of follicular cell hypertrophy was observed in females only (14/23 versus 3/21 in controls). Caecum weight was increased in both males and females of the top dose by just under 2-fold (absolute and relative). Similarly to the preliminary study, haemoglobin and haematocrit levels were decreased in females given the top dose and the female platelet count was increased (11% compared to controls). Similar general effects were noted in the F1 generation.

1.2.2 Reproductive effects

There were no treatment related changes to sexual function or fertility in males or females in both the P and F1 generations. There were no developmental adverse effects to F1 or F2 offspring.

According to the DS, from the animal data available, there was no clear evidence to suggest that pyriofenone should be presumed to be a human reproductive toxicant, therefore, classification for fertility was not proposed.

2. Developmental toxicity

Developmental toxicity was investigated in the rat and the rabbit in GLP and guideline compliant studies with preliminary range-finding studies in both species performed prior to the main studies.

2.1 Rat studies

2.1.1 Preliminary Study

A preliminary developmental toxicity study (Anonymous, 2009f), was conducted in the Wistar Hannover (BrlHan:WIST@Jcl[GALAS]) rat at doses of 0, 30, 100, 300 or 1000 mg/kg bw/day. There were no treatment related deaths, nor any effects on body weight or clinical signs. At the top dose of 1000 mg/kg bw/day there was an increase in relative caecum weight in dams (23% greater than controls, significant). There were increases in liver weight (< 10%) exhibiting a dose related trend but this was not statistically significant at any dose level. There were no treatment related findings on pregnancy or the caesarean section parameters and no foetal abnormalities were observed in any dose group.

2.1.2 Main Study

In the main developmental toxicity study (Anonymous, 2009e), mated female Wistar Hannover (BrlHan:WIST@Jcl[GALAS]) rats (24/group) were orally (gavage) administered dose levels of 0, 30, 300 and 1000 mg/kg bw/day on day 6 to day 19 of gestation.

No animals were found dead or killed in extremis during the study period. No treatment related clinical signs or effects on body weight, adjusted body weight or body weight gain were observed even on gestation days 6-12. There were no treatment related gross findings in the females at necropsy.

Dams dosed with 1000 mg/kg bw/day were found to have increased liver weight [16% and 14% greater than controls (absolute and relative respectively)] and also increased caecum weight [1.3 fold greater than controls (absolute and relative)]. There were no treatment related findings affecting pregnancy.

At doses ≥ 300 mg/kg bw/day there were statistically significant and dose-dependent increases in the number of foetuses with skeletal variations (see table Summary of the main external, visceral and skeletal findings, in the section "Assessment and comparison with the criteria"). These consisted of an increase in incidence of supernumerary ribs and discontinuous rib cartilage. A dose response relationship was seen in the total foetal response for variations, the foetal response for supernumerary ribs and the foetal and litter responses for discontinuous rib cartilage, all suggesting a treatment related response. The responses noted at the top dose were just outside the HCD.

In summary, following administration of pyriofenone to rats, the developmental effects included an increase in skeletal variations; an increased incidence of supernumerary ribs and discontinuous rib cartilage were noted in top-dose animals. This increase was above the concurrent control and above the laboratory HCD.

The DS concluded there were no permanent adverse findings on foetal development in rats and did not propose classification for development.

2.2 Rabbit studies

2.2.1 Preliminary Study

A preliminary developmental toxicity study (Anonymous, 2009g), was conducted in artificially inseminated (pooled sperm) Japanese White (Kbl:JW) female rabbits (8/group). Pyriofenone at concentrations of 0, 30, 100, 300, 1000 mg/kg bw/day was orally (gavage) administered on day 6 to day 27 of gestation. At doses of 300 mg/kg bw/day and above, administered over GD 6-28, body weight gain was affected with a much reduced body weight gain to only 10% of that in the controls (i.e. an overall 90% reduction in bw gain relative to controls) in dams treated with 1000 mg/kg bw/day and body weight loss occurring in dams treated with 300 mg/kg bw/day. At the top dose, liver weight was also increased, +27% (absolute) and +35% (relative) in comparison to controls.

There were no maternal deaths during this study. In the high dose group, 2 animals aborted on GD24 and 2 animals had a premature delivery on GD28 prior to scheduled necropsy on this day. At doses ≥ 100 mg/kg bw/day the percentage of resorptions and foetal deaths was higher than controls or the low dose group (see table below). There was no dose response relationship to this effect; it was remarkably stable at around 20-24% in the affected groups:

***Table**: Summary of the percentage of resorptions and foetal deaths*

Dose: (mg/kg bw/day)	**0**	**30**	**100**	**300**	**1000**
% incidence:	7.4	6.8	21.4	24.1	20.1

Ten females were observed to have ceased eating as the study progressed, one at 30 mg/kg bw/day, three at 300 mg/kg bw/day and 6 at 1000 mg/kg bw/day. The 4 rabbits that aborted/delivered prematurely were amongst the 6 in the high dose group that had ceased eating from around GD 15 onwards. There were no treatment related increases in the number of variations or malformations in foetuses of this study.

2.2.2. Main Study

In the main teratogenicity study (Anonymous, 2009h), artificially inseminated female Japanese White rabbits (25/group) were orally gavaged with pyriofenone at concentrations of 0, 30, 100 or 300 mg/kg bw/day on days 6 - 27 of gestation. There was no significant difference in the mean body weight or body weight gain in the treated groups compared to the control group.

Two females from the top dose group were sacrificed on GD 18 as they showed signs of abortion. Gross pathology of these females revealed that one had white spots on the liver and the other had a coarse-surfaced and enlarged spleen. These incidences of abortion were considered to have occurred by chance and were not related to treatment with the test substance. No treatment related gross abnormalities were seen in the remaining animals.

One female in the control group and two females in the top dose group failed to become pregnant, all other females successfully conceived.

There were no treatment related gross abnormalities in the remaining animals. There were no treatment related increases in variations or malformations in foetuses of this study.

The DS concluded there were no adverse findings on foetal development in rabbits and did not propose classification for development.

Comments received during public consultation

No comments were received during the public consultation.

Assessment and comparison with the classification criteria

3. Assessment of reproductive and developmental studies.

3.1 Fertility

3.1.1 Parental effects

In the definitive multi-generation study, there were haematological changes, gross findings (dark coloured livers and distension of the large intestine), and increased organ weights and histopathological findings in the liver, kidneys and thyroid.

There were no treatment related mortalities, clinical findings or effects on body weight or body weight gain in P or F1 parental animals. Sperm counts, motility and morphology in P and F1 males were similar to control values. There were no treatment related effects on the P or F1 oestrous cycles. Mean oestrous cycle lengths were between 4.0 to 4.2 days in all groups in the P and F1 generations. There were no treatment related effects on the mating index, fertility index, gestation index, viability index, the duration of gestation, the number of implantations, the number of pups delivered and the pup sex ratio in the P and F1 generations (see table below).

***Table**: Summary of reproductive and pup data*

Parameter		**Concentration (ppm)**			
		0	150	1000	5000
P generation females and pups					
No females pregnant		22	20	23	23
Mean implantation sites/female parent		13.2 (1.4)	13.4 (1.9)	12.6 (2.0)	13.2 (1.9)
Mean pups/litter		12.8 (1.8)	12.7 (2.2)	11.3 (2.8)	11.8 (2.4)
Duration of gestation (days)		22.3 (0.5)	22.1 (0.2)	22.1 (0.5)	22.1 (0.3)
Lactation day 0:	No of litters examined No of pups: Found dead:	22 265 4	20 253 5	23 260 3	23 272 1
Lactation days 1-4:	No of litters examined No of pups: Found dead: Lost:	22 261 5 10	20 248 0 1	23 257 0 2	23 271 0 3
Lactation days 5-21:	No of litters examined	21	20	23	23

	No of pups: Found dead:	168 1	160 0	176 0	181 0
Male pups weights (g): (litter mean & SD)	Lactation day 0 Lactation day 21	5.9 (0.6) 54.9 (4.4)	5.7 (0.4) 54.4 (3.1)	6.0 (0.5) 54.3 (3.7)	5.8 (0.6) *51.7 (4.4)
Female pups weights (g): (litter mean & SD)	Lactation day 0 Lactation day 21	5.6 (0.6) 52.9 (4.2)	5.4 (0.3) 53.1 (2.7)	5.6 (0.5) 51.8 (3.4)	5.5 (0.6) *49.6 (4.3)
Gross findings:	No of litter examined No of pups: No of pups without abnormalities:	21 120 114	20 112 97	22 128 121	23 133 125
Sex ratio		0.536	*0.455	0.465	0.533
F1 generation females and pups					
No females pregnant		22	24	22	24
Mean implantation sites/female parent		12.2 (1.6)	11.1 (3.0)	10.9 (2.9)	11.3 (3.3)
Mean pups/litter		11.6 (1.5)	10.5 (2.8)	9.9 (3.6)	10.7 (3.2)
Duration of gestation (days)		22.1 (0.3)	22.1 (0.3)	22.0 (0.4)	22.3 (0.4)
Lactation day 0:	No of litters examined No of pups: Found dead:	22 255 5	24 251 0	22 217 1	24 257 0
Lactation days 1-4:	No of litters examined No of pups: Found dead: Lost:	22 250 0 2	24 251 0 1	22 216 0 0	24 257 1 1
Lactation days 5-21:	No of litters examined No of pups: Found dead: Lost:	22 171 0 1	24 181 0 0	22 156 0 0	23 180 0 0
Male pups weights (g): (litter mean & SD)	Lactation day 0 Lactation day 21	6.0 (0.5) 55.0 (4.0)	6.1 (0.5) 55.3 (4.8)	6.0 (0.6) 54.6 (4.9)	6.0 (0.5) 53.1 (3.3)
Female pups weights (g): (litter mean & SD)	Lactation day 0 Lactation day 21	5.7 (0.5) 52.7 (3.8)	5.8 (0.6) 53.2 (4.4)	5.7 (0.5) 52.8 (3.9)	5.9 (0.7) 51.8 (3.2)
Gross findings:	No of litter examined No of pups: No of pups without abnormalities:	22 170 164	24 181 167	22 156 141	23 180 176
Sex ratio		0.486	0.458	0.493	0.463

a) Sex ratio = total number of male pups/total number pups delivered. b) Lost (probably due to maternal cannibalism). c) * $p \leq 0.05$.

3.1.2 Offspring effects

Sexual development in F1 males (preputial separation) and F1 females (vaginal opening) were similar to the control groups as was body weight at time of pubertal maturation (see the table below). Time to attainment of puberty was unaffected by pyriofenone.

***Table**: Pubertal development data for F1 juveniles*

Genera-tion	Dietary level (ppm)	Completion of preputial separation in males		Completion of vaginal opening in females	
		Days of age	Body weight (g)	Days of age	Body weight (g)
F1	0	42.3	182	31.9	101
		2.4	19	2.4	11
		24	24	24	24
	150	41.9	182	31.6	100
		2.1	10	2.4	13
		24	24	24	24
	1000	42.4	182	31.2	97
		1.7	15	2.9	15
		24	24	24	24
	5000	42.0	181	31.6	100
		2.0	16	2.4	14
		24	24	24	24

Values represent mean, S.D., and no. examined.
There were no statistically significant differences noted.

3.1.3 Conclusion

Pyriofenone was tested in a guideline-compliant two-generation study in Wistar rats. Further information was provided by a preliminary dose range-finding study, also in Wistar rats. A specific effect on fertility, reproduction and pregnancy outcome was not demonstrated by these studies.

The RAC agrees with the assessment of the DS, that there were no adverse effects on reproductive performance, mating behaviour or conception. Furthermore, there was no evidence to suggest that pyriofenone had an adverse effect on lactation or via lactation.

3.2 Development

3.2.1 Rat

3.2.1.1 The preliminary study

No females were found dead or killed in extremis during the study period. There were no treatment related effects on pregnancy or the caesarean section parameters and no foetal abnormalities were observed.

3.2.1.2 The main rat teratogenicity study

No animals were found dead or killed in extremis during the study period. No treatment related clinical signs or effects on body weight, adjusted body or body weight gain were observed.

Mean gravid uterine weight, the number of corpora lutea and implants, and percentage pre-implantation losses in all treated groups were comparable to those in the control group. There was no statistically significant difference in the mean number of live foetuses, resorptions or foetal deaths in the treated groups (see table below).

***Table**: Pregnancy and caesarean section data*

Dose (mg/kg bw/day)	0	30	300	1000
Number of pregnant females	24	24	24	24
Number of females with live foetuses	24	24	24	24
Number of corpora lutea (mean ± s.d.)	13.5 (1.1)	14.4 (2.4)	13.8 (1.3)	14.1 (1.6)
Number of implants (mean ± s.d.)	12.8 (1.6)	13.0 (1.9)	12.8 (1.7)	13.3 (1.4)
Pre-implantation losses (%)	5.4	8.9	6.7	5.3
Number of live foetuses (mean ± s.d.)	11.8 (1.7)	12.1 (1.7)	11.8 (1.9)	12.5 (1.8)
Resorptions and foetal deaths (%)	7.5	6.4	7.8	5.9
Foetal weight male (mg. mean ± s.d.)	3606 (213)	3627 (193)	3560 (201)	3517 (202)
Foetal weight female (mg. mean ± s.d.)	3432 (173)	3468 (221)	3401 (235)	3336 (226)
Placental weight (mg. mean ± s.d.)	431 (40)	441 (43)	435 (34)	419 (30)
Sex ratio	0.496	0.459	0.461	0.495

* p ≤ 0.05. b) Sex ratio = total number of male pups/total number pups delivered.

No treatment related foetal malformations or visceral variations were evident in any dose group. From a dose of 300 mg/kg bw/day there was a statistically significant and dose-dependent increase in the number of foetuses with skeletal variations (see table below). The main skeletal variations were supernumerary ribs and discontinuous rib cartilage. Supernumerary ribs consist of two different structures: full supernumerary ribs which have a cartilaginous segment at the distal end and rudimentary supernumerary ribs which are small rounded ossification sites without cartilage.

***Table**: Summary of the main external, visceral and skeletal findings*

Dose (mg/kg bw/day)	0	30	300	1000
Overall summary				
No foetuses [litters]	284 (24)	290 (24)	284 (24)	301 (24)
No foetuses (external examination)	284	290	284	301
No foetuses (visceral examination)	135	140	136	144
No foetuses (skeletal examination)	149	150	148	157
Number of litters with malformations	1	2	1	2
Number of foetuses with malformations: External Visceral Skeletal	 0 1 0	 1 2 0	 0 0 1	 2 0 1
Number of litters with variations	24	23	23	23
Number of foetuses with variations: Visceral Skeletal	 30 84	 29 83	 35 **105***	 **18*** **121****
Incidence of main skeletal findings				
Discontinuous rib cartilage: Foetuses Litters	 38 (25.5%) 15 (62.5%)	 45 (30.0%) 17 (70.8%)	 47 (31.8%) 20 (83.3%)	 **62 (39.5%)*** 23 (95.8%)*
Supernumerary ribs; Foetuses Litters	 64 (43.0%) 21 (87.5%)	 51 (34.0%) 18 (75.0%)	 **85 (57.4%)*** 23 (95.8%)	 **98 (62.4%)**** 22 (91.7%)
Full supernumerary ribs: Foetuses Litters:	 5 (3.4%) 3 (12.5%)	 3 (2.0%) 3 (12.5%)	 10 (6.8%) 7 (29.2%)	 **14 (8.9%) #** 7 (29.2%)

* p ≤ 0.05; ** p ≤ 0.01. # Statistically significant (Fishers test p = 0.03).

Based on the IET historical control (table below) for supernumerary ribs (35.7 - 61.0%), the foetal incidence of 57.4% at 300 mg/kg bw/day and the incidence of 62.4% at 1000 mg/kg bw/day are just within or just exceed the upper limit boundary of the HCD for the IET laboratory. The foetal incidences of supernumerary ribs and discontinuous rib cartilage exhibit a dose-response relationship and so are likely to be a treatment related effect.

***Table**: IET Historical control data for skeletal variations in Wistar rats*

Observations	**Study year-IET studies**						
	2002	**2004**	**2007**	**2007**	**2008**	**2009**	**2009**
No of foetuses examined	143	143	145	143	146	141	142
Supernumerary ribs	51 (35.7%)	69 (48.3%)	55 (37.9%)	54 (37.8%)	89 (61.0%)	61 (43.3%)	74 (52.1%)
Full supernumerary ribs	-	-	-	-	10 (6.8%)	8 (5.7%)	8 (5.6%)
Discontinuous cartilage	-	-	43 (29.7%)	64 (44.8%)	44 (30.1%)	47 (33.3)	55 (38.7)

The DAR reported a company position paper stating that both rudimentary supernumerary and full supernumerary ribs were all located at the thoracic-lumbar boundary as 14th thoracic or lumber ribs. There was some detail added about the fate of these skeletal variations in the 2012 DAR on pyriofenone. The rudimentary ribs, it was stated, have the same post-natal fates in experimental animals and humans, i.e. they disappear and probably become part of the lateral transverse processes. "*Therefore they cannot represent 'a permanent structural change', which is the agreed definition of a 'malformation' or 'alterations from baseline or normal conditions that diminish an organism's ability to survive, reproduce or adapt to the environment' which is the definition of an 'adverse effect'*." Furthermore, the position paper quoted above also stated that the growth of the full supernumerary ribs is similar to the other thoracic ribs but unlike extra cervical ribs, extra complete lumbar ribs in humans are not associated with congenital abnormalities.

The incidence of discontinuous rib cartilage in foetuses and litters in this study was significantly increased at 1000 mg/kg bw/day. This finding was observed at the distal portion of costal cartilages of the 10-12th ribs. The foetal incidence of 39.5% at the top dose lay within the HCD (range 29.7 - 44.8%), and showed a statistically significant but weak dose response relationship. In addition, the litter incidence showed a significant increase in this effect at 1000 mg/kg bw/day along with a dose response relationship, which supports the conclusion that this effect was treatment related. The toxicological significance of discontinuous rib cartilage was considered somewhat unclear, since there are no relevant clinical reports in humans and there is no evidence that discontinuous rib cartilage results in a functional disorder. However, this finding is a common variation in the rats of this strain as seen from the range of HCD (29.7 - 44.8%).

3.2.1.3 Conclusion

Pyriofenone was tested in a guideline-compliant developmental study in Wistar rats. There was an increased incidence in the number of skeletal variations (supernumerary ribs and discontinuous rib cartilage) in animals of the top dose group that was marginally above the laboratory control data in the case of the supernumerary ribs. RAC agrees with the DS that this finding is considered a variation and not a malformation as the growth of supernumerary ribs is similar to the other thoracic ribs but unlike extra cervical ribs, extra complete lumbar ribs in humans are not associated with congenital abnormalities.

3.2.2 Rabbit

3.2.2.1 The preliminary study

There were no treatment related increases in the number of variations or malformations in foetuses of this study. From a dose of 100 mg/kg bw/day and above, the percentage of resorptions and foetal deaths was higher than controls. For the 100 mg/kg bw/day and 300 mg/kg bw/day dose groups this was mainly due to complete implantation loss in a single doe in each dose group. In the top dose group, there were only data for 4 animals and their litters and in 2 of these litters, the total number of resorptions and foetal deaths was high. With such small numbers, it was not possible to draw any conclusions about this being a treatment effect. In the main study, which dosed to a maximum level of 300 mg/kg bw/day there was no evidence of such an effect with treatment, the high dose group had a slightly lower incidence than the concurrent control group.

There were no maternal deaths during this study. It was noted that six animals in the top dose group (1000 mg/kg bw/day) had ceased eating during the study. As a consequence, maternal body weight gain over GD 6-28 was considerably affected in rabbits at the mid and high dose as compared to controls (90% reduction at 1000 mg/kg bw/day and even body weight loss at 300 mg/kg bw/day). Of the six animals in the top dose group that had ceased eating, 4 aborted or delivered prematurely (2 abortions on GD24, 2 premature deliveries on GD28). Necropsy was conducted on all animals but failed to find any explanation for these 4 litter losses. It may simply be the disruption to the diet on cessation of eating that made these 4 dams susceptible to abortion/premature delivery. The study authors concluded on the basis of these effects to use a dose of 300 mg/kg bw/day for the highest dose in the definitive developmental study in rabbits.

3.2.2.2 The main rabbit teratogenicity study

In contrast to the preliminary study there was no discernible effect on the % of resorptions and foetal deaths, in fact there was a small decrease at the top dose relative to concurrent controls (yellow highlight in table below).

***Table**: Rabbit pregnancy and caesarean section data*

Dose (mg/kg bw/day)	**0**	**30**	**100**	**300**
Number of pregnant females	24/25	25/25	25/25	23/25
[c]Number of non-pregnant females	1	-	-	2
Number of abortions/premature delivery	0	0	0	2
[d]Number of animals with no grossly observable conceptus	0	1	2	0
Number of females examined	24	24	23	21
Number of females with live foetuses	24	23	23	21
Number of corpora lutea (mean ± s.d.)	10.7 ± 2.0	10.2 ± 1.8	10.7 ± 1.7	10.1 ± 2.2
Number of implants (mean ± s.d.)	8.7 ± 2.5	7.6 ± 2.7	8.4 ± 2.5	8.4 ± 3.2
Pre-implantation losses (%)	18.7	26.4	21.5	19.1
Number of live foetuses (mean ± s.d.)	7.7 ± 2.3	6.7 ± 3.0	7.4 ± 2.2	7.8 ± 3.2
Resorptions and foetal deaths (%)	10.8	16.5	10.9	8.8
Foetal weight male (g ± s.d.)	36.5 ± 5.5	36.2 ± 4.9	36.8 ± 4.9	**40.1 ± 4.2***
Foetal weight female (g. mean ± s.d.)	35.5±5.8	37.3 ± 6.0	37.7 ± 4.0	36.4 ± 4.2
Placental weight (mg. mean ± s.d.)	5114 ± 683	5224 ± 1076	5317 ± 828	5734 ± 1060
Sex ratio	0.459	**0.575***	0.524	0.518

* $p \leq 0.05$; Sex ratio = total number of male pups/total number pups delivered. [C] No stained implantation sites or grossly observable conceptus. [D] Stained implantation sites but not grossly observable conceptus

One female in the control group and two females in the top dose group failed to become pregnant, all other females successfully conceived. There was a significant increase in the male foetal weight at 300 mg/kg bw/day.

Two females from the top dose group were sacrificed on GD 18 as they showed signs of abortion. There was no evidence to suggest this was a treatment related effect. There was no cessation of food intake but it was noted in the 300 mg/kg bw/day group that the mean food consumption during gestation days 12-15 only was significantly lower (-18%) than in the control group. All other time points were similar to controls.

There were no treatment related increases in variations or malformations in the foetuses of this study (see table below). The supernumerary ribs have been grouped together, and unlike in the rat, do not show an increase or dose response with treatment.

***Table**: Summary of the main rabbit external, visceral and skeletal findings*

Dose (mg/kg bw/day)	**0**	**30**	**100**	**300**
Overall summary				
Number of foetuses [litters]	185 [24]	160 [23]	170 [23]	164 [21]
Number of litters with malformations	3	4	6	2
Number of foetuses with malformations: External Visceral Skeletal	 0 2 1	 3 2	 2 4 5	 0 1 1
Number of litters with variations	22	17	20	16
Number of foetuses with variations: External Visceral Skeletal	 0 27 47	 0 24 26	 0 19 58	 0 16 31
Incidence of main findings				
Thymic remnant in the neck: Foetuses Litters	 10/185 [7/24]	 7/160 [3/23]	 6/170 [3/23]	 ****0/164** ****[0/21]**
Supernumerary ribs: Foetuses (%) Litters	 37/185 (20) [18/24]	 20/160 (12.5) [11/23]	 44/170 (25.9) [18/23]	 28/164 (17) [14/21]

* p ≤ 0.05; ** p ≤ 0.01

3.2.2.3 Conclusion

Pyriofenone was tested in a guideline-compliant developmental study in rabbits. RAC agrees with the assessment of the DS, there were no treatment related increases in variations or malformations in foetuses from this study.

3.3 Comparison with the criteria

3.3.1 Consideration of Category 1A classification

According to the CLP criteria, classification in Category 1A is largely based on evidence from human data, which were not present in the CLH report. Therefore, classification as Repr. 1A is not warranted.

3.3.2 Consideration of Category 1B or 2 classification

According to the CLP criteria, classification in Category 1B is quite stringent and must be based on robust and strong evidence from animal studies. Such data shall provide clear evidence of an adverse effect on sexual function and fertility or on development. As there was no clear evidence

to suggest pyriofenone had any such effects in the animal studies, classification in this category is not warranted.

3.3.2.1 Sexual function or fertility

On the basis that there is no evidence that pyriofenone causes any adverse effects to sexual function or fertility, it should not be classified in this category. Therefore, in agreement with the DS, RAC considers that no classification for this endpoint is warranted.

3.3.2.2 Developmental toxicity

There are no effects in rabbits. In rats, there was an increased incidence of skeletal variations (supernumerary ribs and discontinuous rib cartilage), in the absence of maternal toxicity, and with the foetal incidences for full and rudimentary supernumerary ribs being just below/at (mid dose, 300 mg/kg bw/d) or just above (high dose, 1000 mg/kg bw/d) the upper limit of the HCD. Given that both types of variations are common variations in rats, that incidences are slightly outside the HCD range only at a very high dose (supernumerary ribs only), and that supernumerary ribs (in particular rudimentary) are thought to resolve postnatally, it seems the toxicological significance of these findings is limited. It is further noted by RAC that in the ECETOC Guidance on Evaluation of Reproductive Toxicity Data, "discontinuous (incomplete) ribs" and "supernumerary (additional, extra thoracolumbar) ribs" are designated a low to moderate level of concern.

On the basis that there is no evidence that pyriofenone causes any permanent or severe adverse effects to development, it should not be classified for developmental toxicity. Therefore, in agreement with the DS, RAC considers that no classification for this endpoint is warranted.

Overall, RAC concludes that **no classification is warranted for effects on fertility, development or for effects on or via lactation**.

ENVIRONMENTAL HAZARD EVALUATION

RAC evaluation of aquatic hazards (acute and chronic)

Summary of the Dossier Submitter's proposal

Overall, the DS considered that pyriofenone is not rapidly degradable, has a low potential for bioaccumulation, did not propose a classification for acute aquatic hazard and proposed a classification as Aquatic Chronic 1 with an M factor of 1, based on the lowest NOEC value for aquatic invertebrates *(Daphnia magna)* of 0.0899 mg/L.

Degradation

The results of a hydrolysis study following OECD TG 111 showed that pyriofenone is hydrolytically stable at environmentally relevant pH and temperature (pH 4, 7 and 9 at 50°C) under sterile conditions with a half-life greater than 16 days (Juozenaite, 2009).

In a ready biodegradation study following OECD TG 310, a mean mineralisation of pyriofenone of 0.6% was observed by day 28 (Dickinson, 2009).

An aquatic photolysis study following SETAC and EPA guidelines showed that photochemical degradation with DT_{50} of 33 to 54 days under spring sunlight at 35°N (Kane, 2009) observed in

pure water under experimental conditions. However, the DS concluded that the information on photochemical degradation is not useful for classification purposes, as the degree of degradation is dependent on local conditions.

A freshwater aerobic mineralisation in surface water study following OECD TG 309 and GLP was conducted in two natural aquatic systems, Calwich Abbey Lake and Swiss Lake, England, for 100 days in the dark at 20°C (Crowe, 2009). Based on the results of the water/sediment simulation study, the DS considered that pyriofenone underwent primary degradation with low levels of ultimate degradation (max of 16.8% AR as CO2 after 100 days). Whole system DT_{50} values at 12°C based on primary degradation were 8.5 to 27.5 days (geometric mean 15.9 days) for the two systems. Multiple aquatic degradants were observed although none at > 10% AR.

Overall, due to the results summarized above, the DS concluded that pyriofenone can be considered as a not rapidly degradable substance in the environment, according to the CLP criteria.

Aquatic Bioaccumulation

A study was conducted following OECD TG 107 at 20°C and pH 7.2-7.5 (Turner, 2009g). The determined Log Pow was 3.2. However, in the absence of measurements at higher and lower pH, it was unclear if pH dependence may occur. In spite of this, the BCF has been determined following GLP and OECD TG 305 with carp *(Cyprinus carpio)* (Anonymous, 2009). The exposure period ran for 28 days followed by a 6 day depuration period. The steady-state fish residue concentration was reached on day 14 with steady-state bioconcentration factors (BCF_{ss}) determined as 142 to 160 L/kg. The mean lipid content was 4.9% at the start of the exposure period and 5.2% at the end of the depuration phase. While the above steady-state BCF have not been lipid normalised, the mean lipid content at the end of the depuration was only slightly above 5% and, if lipid normalised, it would not result in a BCFss above 500 L/kg.

Overall, due to the results summarized above, the DS concluded that pyriofenone can be considered as not bioaccumulative in the aquatic environment.

Aquatic Toxicity

The aquatic toxicity test results from available acute and chronic studies for all trophic levels of pyriofenone are summarised in the following table and sections. Only the valid acute and chronic studies on pyriofenone which are relevant for hazard classification purposes are included in the following table and relevant endpoints from these studies are discussed in further detail below.

The majority of the studies for pyriofenone degradants (2MDPM, 3HDPM, 4MDPM) were considered by the DS as unreliable and the endpoints not validated. However, the studies were a useful indicator that the degradants are unlikely to be more ecotoxic than the parent substance (pyriofenone). Therefore, the DS concluded that the degradants are not considered more toxic than the parent substance and not considered further for classification purposes.

Test organism / guideline, test method	Short-term result (endpoint)	Long-term result (endpoint)	Test material/ remarks	Reference
Fish				
Rainbow Trout *(Oncorhynchus mykiss)* / Acute toxicity to fish, OECD TG 203, GLP	96-h LC_{50} ≥ 1.44 mg/L (mean measured)		Pyriofenone (97.88%) / Valid	Anonymous, 2007

Test organism / guideline, test method	Short-term result (endpoint)	Long-term result (endpoint)	Test material/ remarks	Reference
Common Carp *(Cyprinus carpio)* / Acute toxicity to fish, OECD TG 203, GLP	96-h LC_{50} ≥ 1.41 mg/L (mean measured)		Pyriofenone (97.88%) / Test fish were longer length than test guideline recommendation	Anonymous, 2008
Fathead Minnow *(Pimephales promelas)* / Fish Early-Life Stage toxicity, OECD TG 210, GLP		28-d NOEC = 1.27 mg/L (mean measured)	Pyriofenone (97.88%) / Valid	Anonymous, 2008a
Aquatic invertebrates				
Water flea (*Daphnia magna*) / Acute Immobilisation OECD TG 202, GLP	48-h EC_{50} ≥ 1.55 mg/L (mean measured)		Pyriofenone (97.88%) / Valid	Burke *et al.*, 2008b
Water flea (*Daphnia magna*) / Reproduction OECD TG 211, GLP		**21-d NOEC = 0.0899 mg/L** (mean measured)	Pyriofenone (97.88%) / Valid	Burke *et al.*, 2008c
Algae				
Algae *(Pseudokirchneriella subcapitata)* / Freshwater Algal Growth Inhibition OECD TG 201, GLP	72-h E_rC_{50} = 1.77 mg/L (mean measured)	72-h NOE_rC = 0.249 mg/L (mean measured)	Pyriofenone (97.88%) / Valid	Burke *et al.*, 2008d

Reliable acute and chronic aquatic toxicity data are available for the three trophic levels fish, aquatic invertebrates and algae.

As all acute endpoints for classification purposes were above 1 mg/L and the degradation products were not considered more acutely toxic than the parent substance, the DS concluded that pyriofenone does not require classification as Aquatic Acute 1, based on the lowest E_rC_{50} of 1.77 mg/L for algae *(Pseudokirchneriella subcapitata)*.

The lowest reliable chronic endpoint for classification purposes was the NOEC for invertebrates *(Daphnia magna)* of 0.0899 mg/L. This is in the range >0.01 to ≤0.1 and, therefore, the DS considered that pyriofenone should be classified as Aquatic Chronic 1 (H410) with an M-factor of 1 (as a not rapidly degradable substance).

Comments received during public consultation

Four MSCA submitted comments on the environmental part of the DS' proposal. All of them agreed with the proposed classification by the DS although one MSCA indicated that study results with *Pimephales promelas* from the ELS toxicity test are not complete and favoured the use of the 28-day NOEC for wet weight of fish of 0.435 mg/L, which is below than given NOEC of 1.27 mg/L for mortality, hatch and length of fish. However, as invertebrates are the most chronically sensitive trophic level with a NOEC of 0.0899 mg/L for *Daphnia magna*, this doesn't change proposed classification. In reply, the DS noted that NOEC for fish of 1.27 mg/L was agreed in the DAR and EFSA conclusion.

Assessment and comparison with the classification criteria

Degradation

Pyriofenone is hydrolytically stable at environmentally relevant pH and temperature (pH 4, 7 and 9 at 50°C) under sterile conditions with a half-life above16 days.

Pyriofenone was not demonstrated to be readily biodegradable in a 28-day test for ready biodegradability (0.6% by day 28).

Pyriofenone underwent primary degradation with low levels of ultimate degradation (max of 16.8% AR as CO_2 after 100 days) in a surface water simulation test. Whole system DT_{50} values at 12°C based on primary degradation, were 8.5 to 27.5 days (geometric mean 15.9 days) for the two systems. Multiple aquatic degradants were observed but none of them at levels above 10% AR.

Although photochemical degradation was observed in pure water with DT_{50} of 54 days (spring days sunshine at 35°N), this information is not useful for classification purposes.

Consequently, RAC confirms that pyriofenone is considered to be not rapidly degradable for the purpose of classification.

Aquatic Bioaccumulation

The determined Log Pow 3.2 (at pH 7.2-7.5, 20°C) is less than the CLP trigger of ≥ 4. However, due the absence of measurements at higher and lower pHs, it is unclear if pH dependence may occur. The determined steady-state BCF with carp *(Cyprinus carpio)* was 142 to 160 L/kg, which is substantially less than the CLP BCF trigger value of 500.

The mean lipid content was 4.9% at the start of the exposure period and 5.2% at the end of the depuration phase. While the above steady state BCF has not been lipid normalised, the mean lipid content at the end of the depuration was only slightly above 5% and lipid normalisation would not result in a BCF above 500 L/kg.

Consequently, RAC confirms that pyriofenone is considered as not bioaccumulative in the aquatic environment.

Aquatic Toxicity

RAC notes that there are reliable acute and chronic aquatic toxicity data for fish, aquatic invertebrates and algae. RAC agrees that the parent substance (pyriofenone) is more toxic than the degradation products, based on the available data provided by the DS in the CLH report.

RAC confirms that all reliable acute endpoint values for aquatic acute classification purpose of pyriofenone show $L(E)C_{50}$ values above 1 mg/L for all species and **pyriofenone does not warrants classification for acute aquatic toxicity**, based on the CLP criteria.

RAC confirms that the lowest reliable chronic endpoint value for aquatic chronic classification purposes of pyriofenone is a 21-d NOEC for invertebrates *(Daphnia magna)* of 0.0899 mg/L, based on mean measured concentrations. As this value is in the range of > 0.01 to ≤ 0.1 mg/L, pyriofenone warrants classified as Aquatic Chronic 1 (H410) with an M-factor of 1 (as a not rapidly degradable substance).

Pyriofenone is considered as not rapidly degradable and does not fulfil the CLP criteria for bioaccumulation. Based on the available and reliable information, RAC is of the opinion that **Pyriofenone warrants classification as Aquatic Chronic 1** based on a NOEC of 0.0899 mg/L for *Daphnia magna*. As this chronic toxicity value falls within the 0.01 < NOEC ≤ 0.1 mg/L range, the **chronic M-factor is 1.**

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).