Committee for Risk Assessment

RAC

Annex 1

Background document

to the 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

The background document is a compilation of information considered relevant by the dossier submitter or by RAC for the proposed classification. It includes the proposal of the dossier submitter and the conclusion of RAC. It is based on the official CLH report submitted to public consultation. RAC has not changed the text of this CLH report but inserted text which is specifically marked as 'RAC evaluation'. Only the RAC text reflects the view of RAC.

Adopted

13 June 2019

CLH report

Proposal for Harmonised Classification and Labelling

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

International Chemical Identification:

5-Chloro-2-methoxy-4-methyl-3-pyridyl)(4,5,6-trimethoxy-*o*-tolyl)methanone (Pyriofenone)

EC Number:

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

Index Number:

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United Kingdom

Version number: 2 **Date: February 2018**

ANNEX 1 - BACKGROUND DOCUMENT TO RAC OPINION ON (5-CHLORO-2-METHOXY-4-METHYL-3-PYRIDYL)(4,5,6-TRIMETHOXY-*O*-TOLYL)METHANONE; PYRIOFENONE

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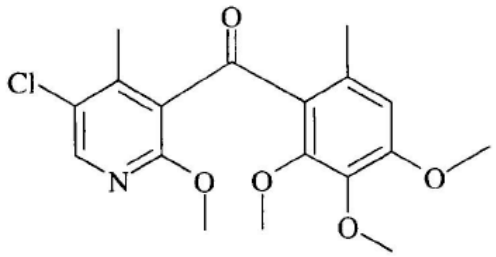
1 IDENTITY OF THE SUBSTANCE

1.1 Name and other identifiers of the substance

Table 1: Substance identity and information related to molecular and structural formula of the substance

Name(s) in the IUPAC nomenclature or other international chemical name(s)	*IUPAC:5-Chloro-2-methoxy-4-methyl-3-pyridyl)(4,5,6-trimethoxy-o-tolyl)methanone* *CA: Methanone, (5-chloro-2-methoxy-4-methyl-3-pyridinyl)(2,3,4-trimethoxy-6-methylphenyl)*
Other names (usual name, trade name, abbreviation)	Pyriofenone, IKF-309
ISO common name (if available and appropriate)	*Pyriofenone*
EC number (if available and appropriate)	Not assigned
EC name (if available and appropriate)	
CAS number (if available)	688046-61-9
Other identity code (if available)	
Molecular formula	$C_{18}H_{20}NO_5Cl$
Structural formula	
SMILES notation (if available)	
Molecular weight or molecular weight range	365.8
Information on optical activity and typical ratio of (stereo) isomers (if applicable and appropriate)	
Description of the manufacturing process and identity of the source (for UVCB substances only)	
Degree of purity (%) (if relevant for the entry in Annex VI)	*Minimum purity: 96.5 %* *Maximum purity: 98.9 %*

1.2 Composition of the substance

Pyriofenone includes no isomers or additives. A number of confidential impurities are present; however none of these are relevant for the classification of the substance.

Table 2: Constituants (non-confidential information)

Constituent (Name and numerical identifier)	**Concentration range (% w/w minimum and maximum in multi-constituent substances)**	**Current CLH in Annex VI Table 3.1 (CLP)**	**Current self-classification and labelling (CLP)**
Pyriofenone (CAS: 688046-61-9)	96.5 – 98.9 %	Not listed	Aquatic chronic 2 (H411 – toxic to aquatic life with long lasting effects)

2 PROPOSED HARMONISED CLASSIFICATION AND LABELLING

2.1 Proposed harmonised classification and labelling according to the CLP criteria

Table 3:

	Index No	International Chemical Identification	EC No	CAS No	Classification		Labelling			Specific Conc. Limits, M-factors	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	-	-	-	-	-	-	-	-	-	-	-
Dossier submitters proposal	-	*5-Chloro-2-methoxy-4-methyl-3-pyridyl)(4,5,6-trimethoxy-o-tolyl)methanone; pyriofenone*	-	688046-61-9	Carc. 2 Aquatic Chronic 1	H351 H410	GSH08 GSH09 Wng	H351 H410	-	M = 1	-
Resulting Annex VI entry if agreed by RAC and COM	-	*5-Chloro-2-methoxy-4-methyl-3-pyridyl)(4,5,6-trimethoxy-o-tolyl)methanone; pyriofenone*	-	688046-61-9	Carc. 2 Aquatic Chronic 1	H351 H410	GSH08 GSH09 Wng	H351 H410	-	M =1	-

Table 4: Reason for not proposing harmonised classification and status under public consultation

Hazard class	Reason for no classification	Within the scope of public consultation
Explosives	Data conclusive but not sufficient for classification	Yes
Flammable gases (including chemically unstable gases)	Hazard class not applicable	No
Oxidising gases	Hazard class not applicable	No
Gases under pressure	Hazard class not applicable	No
Flammable liquids	Hazard class not applicable	No
Flammable solids	Data conclusive but not sufficient for classification	Yes
Self-reactive substances	Data conclusive but not sufficient for classification	Yes
Pyrophoric liquids	Hazard class not applicable	Yes
Pyrophoric solids	Data conclusive but not sufficient for classification	Yes
Self-heating substances	Data conclusive but not sufficient for classification	Yes
Substances which in contact with water emit flammable gases	Data conclusive but not sufficient for classification	Yes
Oxidising liquids	Hazard class not applicable	No
Oxidising solids	Data conclusive but not sufficient for classification	Yes
Organic peroxides	Data conclusive but not sufficient for classification	No
Corrosive to metals	Data conclusive but not sufficient for classification	Yes
Acute toxicity via oral route	Data conclusive but not sufficient for classification	Yes
Acute toxicity via dermal route	Data conclusive but not sufficient for classification	Yes
Acute toxicity via inhalation route	Data conclusive but not sufficient for classification	Yes
Skin corrosion/irritation	Data conclusive but not sufficient for classification	Yes
Serious eye damage/eye irritation	Data conclusive but not sufficient for classification	Yes
Respiratory sensitisation	Hazard class not assessed in this dossier	No
Skin sensitisation	Data conclusive but not sufficient for classification	Yes
Germ cell mutagenicity	Data conclusive but not sufficient for classification	Yes
Carcinogenicity	**Carc. 2; H351**	**Yes**
Reproductive toxicity	Data conclusive but not sufficient for classification	Yes
Specific target organ toxicity-single exposure	Data conclusive but not sufficient for classification	Yes
Specific target organ toxicity-repeated exposure	Data conclusive but not sufficient for classification	Yes
Aspiration hazard	Hazard class not assessed in this dossier	No
Hazardous to the aquatic	**Aquatic chronic 1; H410**	**Yes**

Hazard class	Reason for no classification	Within the scope of public consultation
environment		
Hazardous to the ozone layer	Hazard class not assessed in this dossier. Not applicable as pyriofenone is not listed in Annex I to Regulation (EC) No. 1005/2009 (recognising the Montréal Protocol) and no Ozone Depleting Potential (ODP) is reported.	No

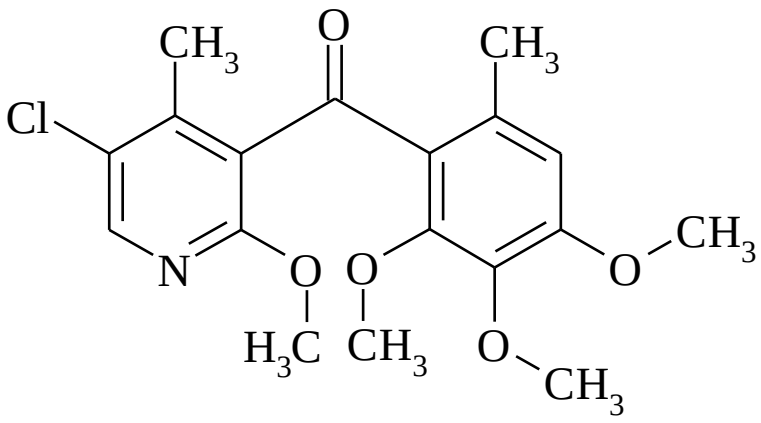
3 HISTORY OF THE PREVIOUS CLASSIFICATION AND LABELLING

Pyriofenone is a new pesticide active substance and has been reviewed in accordance with Directive 91/414/EEC with the UK as the Rapporteur Member State (RMS). There is no existing entry on Annex VI of CLP and there have been no previous classification and labelling discussions for this substance. In accordance with Article 36 (2) of the CLP Regulation, pyriofenone should now be considered for harmonised classification and labelling. Therefore, this CLH proposal considers all human health and environmental endpoints with the aim of achieving such.

At the time of submission the substance is not registered under REACH.

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 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.

4 JUSTIFICATION THAT ACTION IS NEEDED AT COMMUNITY LEVEL

Pyriofenone is a new active substance under Regulation 1107/2009.

There is no requirement for justification that action is needed at Community level.

5 IDENTIFIED USES

Pyriofenone is a fungicide, designed to treat mildew on crops such as wheat, rye, barley, spelt, oats, triticale and at larger concentrations, grapes.

6 DATA SOURCES

Pyriofenone is a new active substance under Regulation 1107/2009 and has not been placed on the market yet. The present evaluation exclusively relies on data submitted in the context of the application for approval as an active substance under Regulation 1107/2009 and any additional studies submitted directly by the applicant.

Draft assessment report (DAR): Volume 3 (B6) Toxicology (2012)

Draft assessment report (DAR): Volume 3 (B2) Physical Chemistry (2012)

Draft assessment report (DAR): Volume 3 (B8) Environmental fate (2012)

Draft assessment report (DAR): Volume 3 (B9) Ecoxtoxicology (2012)

Draft assessment report (DAR): Volume 4 Confidential information (2012)

7 PHYSICOCHEMICAL PROPERTIES

The physico-chemical properties of pyriofenone are summarised below. Reference should be made to the Draft Assessment Report – DAR – Volume 3, Annex B.2; Physical and Chemical properties – November 2012.

All studies were conducted to appropriate quality standards and were considered adequate during the peer review.

Table 5: Summary of physicochemical properties

Property	Value	Reference	Comment (e.g. measured or estimated)
Physical state at 20°C and 101,3 kPa	Crystalline powder	Turner, B., 2009a	Visual inspection Purity 99.19 % (w/w) DAR (B.2.1.7)
Melting/freezing point	Melting range: 93 – 95 °C	Turner, B., 2009a	EEC Method A1 (Metal block method) GLP Purity 99.19 %

Property	**Value**	**Reference**	**Comment (e.g. measured or estimated)**
			DAR (B.2.1.1)
Boiling point	Decomposes at temperatures > 100 °C	(Turner, 2009a)	EEC Method A2 (Siwoloboff method) GLP Purity 99.19 % DAR (B.2.1.2)
Relative density	1.33	(Turner, 2009a)	EEC Method A3 (Pycnometer) GLP Purity 99.19 % DAR (B.2.1.4)
Vapour pressure	1.9 x 10^{-6} Pa at 25 °C	(Turner, 2009b)	OECD 104/EEC Method A4 Vapour pressure balance GLP Purity 99.19 % DAR (B.2.1.5)
Surface tension	72.0 mN/m at 20 °C Not surface active	(Turner, 2009a)	EEC Method A5 90 % Saturated aqueous solution used with a surface tension torsion balance GLP Purity 99.19 % DAR (B.2.1.24)
Water solubility	1.56 mg/L at 20 °C and pH 6.6 Slightly soluble	(Turner, 2007)	OECD 105/EEC Method A6 (Column elution method) GLP Purity 99.19 % DAR (B.2.1.11) Not tested at pH range 4-10 as applicant asserts that pyriofenone is incapable of undergoing ionisation in this range, therefore water solubility would not be expected to be pH-dependent.
Partition coefficient n-octanol/water	$Log_{10}P_{ow}$ = 3.2 at 20 °C and pH 7.2-7.5 Potential for bioaccumulation	(Turner, 2009g)	OECD 107 GLP Purity 99.19 % DAR (B.2.1.13) Not tested at pH range 4-10 as applicant asserts that pyriofenone is incapable of undergoing ionisation in this range, therefore LogP would not be expected to be pH-dependent.
Flash point	Not tested or required as the melting point is above		DAR (B.2.1.21)

Property	Value	Reference	Comment (e.g. measured or estimated)
	40 °C		
Flammability	Not highly flammable. In a preliminary test, pyriofenone burned locally with a yellow flame which extinguished 2 seconds after removal of the heat source.	(Turner, 2009c)	EEC Method A10 – flammability GLP Purity 97.88 % DAR (B.2.1.20)
Explosive properties	Not explosive	(Turner, 2009c)	EEC Method A14 (Koenen test apparatus used) GLP Purity 97.88 % DAR (B.2.1.22)
Self-ignition temperature	Autoflammability 378 °C	(Turner, 2009c)	EEC Method A15 GLP Purity 97.88 % DAR (B.2.1.20)
Oxidising properties	Not oxidising. Burn rates 2:1, 1:1, 1:2 mixtures of pyriofenone: cellulose were significantly less than for barium nitrate/cellulose reference mixtures.	(Turner, 2009c)	EEC Method A17 GLP Purity 97.88 % DAR (B.2.1.23)
Dissociation constant	The applicant asserts that pyriofenone does not possess a dissociation constant in the pH range 4-10. Spectrophotometric evidence supplies indicates little variation in the pH range 1-12.9.	(Turner, 2009h)	OECD 112 GLP Purity 99.19 % DAR (B.2.1.18)

8 EVALUATION OF PHYSICAL HAZARDS

8.1 Explosives

Table 6: Summary table of studies on explosive properties

Method	Results	Remarks	Reference
EEC Method A14 (explosive properties)	Not explosive	GLP	(Turner, 2009c)

8.1.1 Short summary and overall relevance of the information provided on explosive properties

Pyriofenone was tested for explosive properties using EEC Method A14 and was found not to be explosive. Further, experience in handling and use indicates that it is not a pyrophoric solid and does not emit flammable gas on contact with water.

8.1.2 Comparison with the CLP criteria

Pyriofenone did not meet the criteria for classification as an explosive substance.

8.1.3 Conclusion on classification and labelling for explosive properties

Not classified – conclusive but not sufficient for classification

8.2 Self-heating substances

Table 7: Summary table of studies on self-heating substances

Method	Results	Remarks	Reference
EEC Method A15 (autoflammability)	Autoflammability: 378 °C	GLP	(Turner, 2009c)

8.2.1 Short summary and overall relevance of the provided information on self-heating substances

Pyriofenone was assessed for auto-flammability using EEC Method A15. The result showed no self-ignition up to the temperature of the melting point.

8.2.2 Comparison with the CLP criteria

Pyriofenone did not meet the criteria for classification as a self-heating substance.

8.2.3 Conclusion on classification and labelling for self-heating substances

Not classified – conclusive but not sufficient for classification

8.3 Oxidising solids

Table 8: Summary table of studies on oxidising solids

Method	Results	Remarks	Reference
EEC Method A17 (oxidising properties)	Not oxidising	GLP	(Turner, 2009c)

8.3.1 Short summary and overall relevance of the provided information on oxidising solids

Pyriofenone was tested for oxidising properties using a reference mixture of cellulose and barium nitrate. Burn rates for mixtures of pyriofenone: cellulose were found to be significantly less than the reference mixture. Therefore, pyriofenone is not oxidising.

8.3.2 Comparison with the CLP criteria

Pyriofenone does not meet the criteria for classification as an oxidising solid.

8.3.3 Conclusion on classification and labelling for oxidising solids

Not classified – conclusive but not sufficient for classification

8.4 Flammable solids

Table 9: Summary table of studies on flammable solids

Method	Results	Remarks	Reference
EEC Method A10 (flammability)	Not highly flammable	GLP	(Turner, 2009c)

8.4.1 Short summary and overall relevance of the provided information on flammable solids

In a standard study (EEC Method A10), a preliminary test showed that pyriofenone burned locally with a yellow flame which extinguished two seconds after removal of the heat source. Therefore, pyriofenone does not meet the classification criteria for classification as a flammable solid.

8.4.2 Comparison with the CLP criteria

Pyriofenone does not meet the criteria for classification as a flammable solid.

8.4.3 Conclusion on classification and labelling for flammable solids

Not classified – conclusive but not sufficient for classification

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.

Supplemental information - In depth analyses by RAC

Further clarification and comment on auto-flammability, or confirmatory data using EEC A.16.

The addendum to the DAR (2012), section B.2.3.1 supplied by the RMS (UK) to EFSA presented a reasoned argument to address concerns regarding data originally reported for auto-flammability, generated using method EEC A.15 rather than method EEC A.16, the more usual method for solids. Test EEC A.16 was not actually attempted. The test result for EEC A.15 / BS 4056 (378 °C), is for powdered material dispersed in air, which provides a reasonable simulation of how solid or molten pyriofenone might behave when subjected to further heat stress when dispersed. This test is less relevant to circumstances in which confined solid (or molten) pyriofenone is subject to continued heat stress. Pyriofenone may presumably be stored and transported in bulk as a solid in sealed containers. In a boiling point tests (B.2.1.1 / IIA.2.1), decomposition was noted in the melt at >100 °C and it is not clear that for the molten state self-heating leading to auto-flammability could be ruled out. The RMS noted that "*…on a balance of evidence basis, it is possible that the test result for EEC A.15 / BS 4056 alone might be a reliable*

result for auto-flammability".

The reason for the choice of method EEC A.15 was based on the practicalities of conducting the procedure by the traditional method for solids (EEC A.16). In the method 'Relative Self-Ignition Temperature for Solids', there is a requirement to place the test substance sample within a wire mesh cube and to then slowly heat the sample at a rate of 0.5 °C/minute to a temperature of 400 °C. If this procedure were to be conducted with the test substance, 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 test would probably not yield a positive result in the anticipated region of approximately 378 °C, as observed according to method EEC A.15.

Overall, the RMS concluded that the available data did not allow a definitive conclusion to be reached on the auto-flammability of pyriofenone for all relevant conditions. The RMS proposed that test EEC A.16 should be attempted but there is no evidence that this test was ever performed.

The DS concluded that pyriofenone did not meet the criteria for classification as a self-heating substance and appeared to accept the results of method EEC A.15.

RAC supports the DS in concluding no classification as a self-heating substance based on the available but limited data from method EEC A.15 (which concludes that auto-flammability below 378 °C for confined, molten pyriofenone is unlikely).

9 TOXICOKINETICS (ABSORPTION, METABOLISM, DISTRIBUTION AND ELIMINATION)

Table 10: Summary table of toxicokinetic studies

Method	Results	Reference
Metabolism in rats EU88/302/EEC GLP Fischer 4-9/sex/dose (depending on the investigation) ^{14}C-(phenyl)-pyriofenone and ^{14}C-(pyridyl)-pyriofenone Single dose: 5 mg/kg bw or 200 mg/kg bw Repeat dose: 5 mg/kg bw/day, 14 days	**Absorption:** 76 – 89 % following the low dose and 36-53 % following the high dose (saturation of absorption processes) **Distribution:** Widespread distribution in tissues after single and repeated dosing, declining with time. The concentration of pyriofenone in tissues was higher in males than females (generally 2-4 times higher). The pattern of distribution following repeated dosing was similar to that after a single dose but levels were 2-10 times higher suggestive of accumulation. The organs with the highest concentration of pyriofenone were the liver, kidney, whole blood and plasma. **Metabolism:** Metabolic pathway: After single and repeated dosing, demethylation of the methoxy groups at the 3- and 4-positions of the benzene ring to give two mono-hydroxy products and a di-hydroxy product. All three metabolites undergo glucuronide conjugation. In males – no individual metabolites accounted for > 3 % of the dose.	DAR: B.6.1.1 and B.6.1.2 (Anon, 2009)

Method	Results	Reference
	In females – one urinary metabolite was present at 9.5 % of the dose; this was the glucuronide of the di-hydroxy metabolite. There were no unidentified metabolites at > 5 % of the dose. Evidence of enterohepatic recirculation **Excretion:** ***Following single dosing:*** 87-97 % eliminated in urine and faeces within 48 h (mainly via the faeces) ***Following repeated dosing:*** > 90 % of the dose was eliminated within 24 h after the final dose (mainly via the faeces) **Toxicokinetics:** ***Following single dosing:*** The AUC_{120} and $T_{1/2}$ were lower in females than males $T_{1/2}$ was shorter for plasma than red blood cells ***Following repeated dosing:*** The T_{max} and AUC_{120} were increased in males but largely unchanged in females. $T_{1/2}$ was shorter for plasma than red blood cells Whole blood: Plasma ratio was increased – increased distribution of radioactivity into red blood cells.	

9.1 Short summary and overall relevance of the provided toxicokinetic information on the proposed classification(s)

The toxicokinetics of pyriofenone has been investigated in male and female Fischer rats (4-9/sex/group) using ^{14}C-(phenyl)-pyriofenone and ^{14}C-(pyridyl)-pyriofenone using single oral doses of 5 mg/kg bw and 200 mg/kg bw and after repeated dosing for 14-days with 5 mg/kg bw.

Absorption

Pyriofenone was found to be rapidly absorbed following low dose administration (5 mg/kg bw) with approximately 76-89 % absorbed. Following administration with the higher dose of pyriofenone (200 mg/kg bw), the absorption processes appeared to become saturated with approximately 50 % less being absorbed (36-53 %).

Distribution

Distribution of pyriofenone in the tissues of rats was widespread in both sexes. In general, the concentrations of radioactivity in the tissues of males was higher than in females (2-4 times) and the majority of tissues concentrations declined with time. The highest concentrations of pyriofenone were found in the liver, kidneys, whole blood and abdominal fat.

Following repeated dosing, there was some evidence of accumulation with higher tissues concentrations of radioactivity than after a single dose. In all dose groups, tissue levels were low at 120 hours post dosing.

Metabolism

Pyriofenone was found to be extensively metabolised and the pathway involved de-methylation of the two methoxy groups in the 3- and 4-positions of the benzene ring. This gave rise to the 3-hydroxy, 4-hydroxy and 3,4-dihydroxy analogues. These three metabolites could then undergo conjugation to glucuronide conjugates. In females, the 3,4 dihydroxy glucuronide was the major metabolite found in urine (9.5 % of the dose) but in males, not one metabolite was present at levels > 3 % of the dose.

Excretion

Following a single dose of pyriofenone, 87-97 % of the dose was eliminated in rats. This occurred mainly by the faeces. Only minor differences in excretion patterns occurred after repeated dosing.

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 size of the dose increase, indicating non-linear kinetics. These increases were greater in males than in females. After repeated dosing, the rate and extent of exposure was increased in the plasma and whole blood of males, but was essentially unchanged in the plasma of females (it was increased in the whole blood). The terminal half-life was longer after repeated dosing, indicating that changes in clearance and/or volume of distribution of radioactivity occurred during repeated dosing. Sequestation into red blood cells was evident after single and repeated dosing as the half-life was shorter for plasma than whole blood and also after a single dose, the whole blood: plasma ratio was higher in males than in females and increased with increasing dose. After repeated dosing, the whole blood: plasma ratio also increased, indicating an increased distribution of radioactivity into the red blood cells.

There were no findings from the toxicokinetic studies that might influence the proposed classification of pyriofenone. In general, pyriofenone was well-absorbed and fully eliminated. There was no evidence of tissue accumulation or the presence of any unidentified or toxic metabolites.

10 EVALUATION OF HEALTH HAZARDS

The human health hazards of pyriofenone are summarised below. Reference should be made to the Draft Assessment Report – DAR – Volume 3, Annex B.6; Mammalian Toxicology – November 2012.

Acute toxicity

10.1 Acute toxicity - oral route

Table 11: Summary table of animal studies on acute oral toxicity

Method, guideline, deviations if any	Species, strain, sex, no/group	Test substance	Dose levels, duration of exposure	Value LD_{50}	Reference
Acute oral toxicity study OECD 423 GLP	Rat, strain not specified, 6 females	IKF-309 technical Purity 97.88 %	2000 mg/kg bw in 1 % aq. methyl cellulose (1 % w/v)	> 2000 mg/kg bw No mortalities. No gross internal abnormalities. Abnormal body position in 2/6 females.	DAR: B.6.2.1 (Anon., 2008a)

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

Pyriofenone was tested for acute oral toxicity in one guideline study using rats (strain not specified). Six females received a single limit dose of 2000 mg/kg bw in by oral gavage in aqueous methyl cellulose (1 % w/v) (10 ml/kg bw). There were no mortalities and no gross abnormalities found at necropsy. Clinical signs were limited to the finding of abnormal body position in two rats. The LD_{50} was > 2000 mg/kg bw.

10.1.2 Comparison with the CLP criteria

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: $300 < LD_{50} \leq 2000$ mg/kg bw. The LD_{50} of pyriofenone for oral toxicity was found to be > 2000 mg/kg bw, therefore it does not require classification for acute toxicity by the oral route.

10.1.3 Conclusion on classification and labelling for acute oral toxicity

No classification – conclusive but not sufficient for classification

10.2 Acute toxicity - dermal route

Table 12: Summary table of animal studies on acute dermal toxicity

Method, guideline, deviations if any	Species, strain, sex, no/group	Test substance	Dose levels duration of exposure	Value LD_{50}	Reference
Acute dermal toxicity study OECD 402 (1987) GLP	Rats, strain not specified, 5males and 5 females	IKF-309 technical (Purity 97.88 %)	2000 mg/kg bw in 1 % aq. methyl cellulose (1 % w/v) Semi-occlusive 24 h topical application	> 2000 mg/kg bw No mortalities. No gross internal abnormalities. Slight erythema observed in all animals, resolved by day 7, scabbing in 1 female on Day 7 and 1 female from Day 4 – 14.	DAR: B.6.2.2 (Anon., 2008b)

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

One guideline study to assess acute toxicity via the dermal route is available. Male and female rats (strain not specified) received a single semi-occluded topical application of pyriofenone (2000 mg/kg bw) in aqueous methyl cellulose (1 % w/v) for 24 h. There were no mortalities in this study and no internal findings at necropsy. Clinical signs were limited to slight erythema in all animals and scabbing in one female on Day 7 and in another from Day 4 – 14. The LD_{50} was > 2000 mg/kg bw.

10.2.2 Comparison with the CLP criteria

In order to be classified with acute toxicity category 4 (dermal), the LD_{50} should be between $1000 < LD_{50} \leq 2000$ mg/kg bw. The results from the guideline study in rats showed that the LD_{50} for acute dermal toxicity was > 2000 mg/kg bw, therefore, classification for this endpoint is not required.

10.2.3 Conclusion on classification and labelling for acute dermal toxicity

No classification - conclusive but not sufficient for classification.

10.3 Acute toxicity - inhalation route

Table 13: Summary table of animal studies on acute inhalation toxicity

Method, guideline, deviations if any	Species, strain, sex, no/group	Test substance, , form and particle size (MMAD)	Dose levels, duration of exposure	Value LC_{50}	Reference
Acute inhalation toxicity study OECD 403 (1981) GLP	Rat, Sprague Dawley, 5 males and 5 females	IKF-309 technical (purity 97.88 %) Aerosol MMAD 3.9 µm, GSD 2.25	5.18 mg/l for 4 h (nose only) No mortalities No gross internal abnormalities No significant clinical findings	> 5.18 mg/L	DAR: B.6.2.3 (Anon., 2008)

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

There is one guideline acute inhalation toxicity study available, carried out in Sprague Dawley rats. Rats of both sex were exposed to an aerosol of pyriofenone for four hours (nose only) to an analysed concentration of 5.18 mg/l. 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 3 rats only 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.

10.3.2 Comparison with the CLP criteria

In order to be classified with acute toxicity category 4 (inhalation), the LC_{50} should lie between $1.0 < LC_{50} \leq 5.0$ mg/l (dusts and mists). As the LC_{50} for pyriofenone was > 5.18 mg/l there is no requirement to classify for acute inhalation toxicity.

10.3.3 Conclusion on classification and labelling for acute inhalation toxicity

No classification - conclusive but not sufficient for classification.

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)**.

10.4 Skin corrosion/irritation

Table 14: Summary table of animal studies on skin corrosion/irritation

Method, guideline, deviations if any	Species, strain, sex, no/group	Test substance,	Dose levels duration of exposure	Results -Observations and time point of onset -Mean scores/animal -Reversibility	Reference
Skin irritation study OECD 404 (2002) GLP	Rabbits, New Zealand White, 3 females	IKF-309 technical Purity 97.88 %	0.5 g, 4 h, semi-occlusive	No mortalities and no treatment-related signs. No dermal reactions in any animals	DAR: B.6.2.4 (Anon., 2008c)

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

In a single skin irritancy study, pyriofenone was applied to the clipped intact dorso-lumbar skin of three female New Zealand White rabbits for four hours, under a semi-occlusive dressing. There were no mortalities and no treatment-related clinical signs. No dermal reactions were observed in any rabbit, with a score of 0 across all timepoints.

10.4.2 Comparison with the CLP criteria

In a guideline skin irritation study with pyriofenone, there was no evidence of skin corrosion. In order to be classified with skin irritation, pyriofenone would be expected to cause erythema or oedema in at least two out of three rabbits with mean value of ≥ 2.3 - ≤ 4.0 from gradings at 24, 48 and 72 hours after patch removal. In all animals, at all timepoints, the gradings were 0 and therefore, pyriofenone does not meet the criteria for classification with skin irritation.

10.4.3 Conclusion on classification and labelling for skin corrosion/irritation

No classification - conclusive but not sufficient for classification.

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.**

10.5 Serious eye damage/eye irritation

Table 15: Summary table of animal studies on serious eye damage/eye irritation

Method, guideline, deviations if any	Species, strain, sex, no/group	Test substance,	Dose levels duration of exposure	Results - Observations and time point of onset - Mean scores/animal - Reversibility	Reference
Eye irritation study OECD 405 (2002) GLP	Rabbits, New Zealand White, 3 females	IKF-309 technical Purity 97.88 %	0.1 ml	No mortality or clinical signs Mean individual animal scores at 24,48 and 72 h: Corneal opacity: 0, 0, 0 Iritis; 0, 0, 0 Conjunctival redness: 0, 0.3, 0 Conjunctival erythema: 0, 0, 0 All effects were fully reversible by 48 h.	DAR: B.6.2.5 (Anon., 2008d)

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

There is one eye irritation study carried out with pyriofenone in rabbits. Three female New Zealand White rabbits each had pyriofenone (0.1 ml) instilled into the ocular sac of their right eye. The left eye remained untreated and acted as a control. Ocular reactions were assessed at 1, 24, 48 and 72 hours after treatment. There were no effects on the cornea or iris but slight irritation to the conjunctiva was observed. Redness of the conjunctiva was observed in 3/3 animals 1h 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.

10.5.2 Comparison with the CLP criteria

In a guideline eye irritation study in rabbits there were no evidence of any serious eye damage. There was some mild irritation to the conjunctiva of all rabbits (maximum grading of 1), however the mean score of this over 24 – 72 hours was less than 1 and was reversible within 48 h. According to the classification criteria, for a substance that has the potential to cause irreversible eye irritation, classification in category 2 is required when the substance produces:

At least in 2/3 tested animals, a positive response of:

- Corneal opacity ≥ 1 and/or
- Iritis ≥ 1, and/or
- Conjunctival redness ≥ 2 and/or
- Conjunctival oedema ≥ 2

Therefore pyriofenone does not meet the criteria for classification with eye irritation and no classification is required.

10.5.3 Conclusion on classification and labelling for serious eye damage/eye irritation

No classification - conclusive but not sufficient for classification.

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**.

Supplemental information - In depth analyses by RAC

***Table**: Eye irritation scores.*

Time	**Cornea**			**Iris**			**Conjunctiva**					
							Redness			**Chemosis**		
Animal number	56F	59F	60F	56F	59F	60F	56F	59F	60F	56F	59F	60F
after 1 hour	0	0	0	0	0	0	1	1	1	0	0	0
after 24 hours	0	0	0	0	0	0	1	0	1	0	0	0
after 48 hours	0	0	0	0	0	0	0	0	0	0	0	0
after 72 hours	0	0	0	0	0	0	0	0	0	0	0	0
mean scores 24-72h	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.33**	**0.00**	**0.33**	**0.00**	**0.00**	**0.00**

10.6 Respiratory sensitisation

No data are available on this endpoint.

10.7 Skin sensitisation

Table 16: Summary table of animal studies on skin sensitisation

Method, guideline, deviations if any	**Species, strain, sex, no/group**	**Test substance**	**Dose levels duration of exposure**	**Results**	**Reference**

<table>
<tr><th>Method, guideline, deviations if any</th><th>Species, strain, sex, no/group</th><th>Test substance</th><th>Dose levels duration of exposure</th><th>Results</th><th>Reference</th></tr>
<tr><td>Local Lymph Node Assay
OECD 429
GLP</td><td>Mice, CBA/JNCrlj, 5 females/group</td><td>IKF-309 technical
Purity 97.88 %
Positive control: α-hexylcinnamaldehyde</td><td>0, 5, 10 or 25 % (w/v)</td><td>No differences in stimulation index in any treatment groups:
<table>
<tr><th>Test material concentration (% w/v)</th><th>Lymph node weight (mg)</th><th>Test/control ratio (SI)</th></tr>
<tr><td>Vehicle control</td><td>4.6</td><td>1</td></tr>
<tr><td>Positive control</td><td>11.8</td><td>7.74</td></tr>
<tr><td>5</td><td>4.9</td><td>0.78</td></tr>
<tr><td>10</td><td>4.7</td><td>1.04</td></tr>
<tr><td>25</td><td>5</td><td>0.57</td></tr>
</table></td><td>DAR: B.6.2.6
(Anon., 2009a)</td></tr>
</table>

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

Pyriofenone was tested for skin sensitisation in a Local Lymph Node Assay (LLNA) using CBA mice. In this study, five females/group were given three consecutive daily topical applications of pyriofenone to the dorsal surface of both ears. The doses used were selected from a preliminary study and were 0, 5, 10 or 25 % w/v suspended in acetone/olive oil. The results showed there were no significant differences between the groups treated with pyriofenone and the vehicle control. The SI value was < 3 in all cases. Based on this study, pyriofenone is not a skin sensitiser.

10.7.2 Comparison with the CLP criteria

In a guideline LLNA, pyriofenone showed no evidence of skin sensisation in mice when tested up to a concentration of 25 % w/v. Therefore, it does not meet the criteria for classification for this endpoint.

10.7.3 Conclusion on classification and labelling for skin sensitisation

No classification - conclusive but not sufficient for classification.

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.

Additional key elements

The original study report had very little additional detail regarding the final dose selection. The preliminary dose-finding test was conducted at dose levels of 0, 10, 25 and 50% for a period of 3 days. Only a very brief description was provided in the original study report indicating that there was no toxicity or skin irritation (presumably at all of the tested doses, though the language used to describe this was not very clear). The poor solubility of the 50% test substance determined the dose selection for the main study. No further detail was provided

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**.

10.8 Germ cell mutagenicity

Table 17: Summary table of mutagenicity/genotoxicity tests in vitro

Method, guideline, deviations if any	**Test substance,**	**Relevant information about the study including rationale for dose selection (as applicable)**	**Observations**	**Reference**
Bacterial reverse mutation test OECD 471 GLP	IKF-309 technical Purity 97.88 %	*Test concentrations:* 0, 5, 15, 50, 150, 500, 1500 and 5000 µg/plate ± S9 Plate 1 – standard plate incorporation assay Plate 2 – pre-incubation stage included Strains tested: *S. typhimurium* TA1535, TA1537, TA98 and TA100 *E. coli* WP2*uvr*A	**Negative** No evidence of mutagenicity ±S9 No evidence of cytotoxicity Precipitation was observed in both plates at 1500 µg and above	DAR: B.6.4.1a (May, K. 2007
Mammalian chromosome aberration test in CHL cells OECD 473	IKF-309 technical Purity 97.88 %	Solubility in DMSO was assessed in a preliminary test (max solubility 2542 µg/ml). This was used as	**Positive** *Preliminary toxicity test* Cytotoxicity at concentrations ≥ 79.44 µg/ml (-S9) and ≥ 158.88 µg/ml (+S9)	DAR: B.6.4.1b (Pritchard, L. 2008)

<table>
<tr><th>Method, guideline, deviations if any</th><th>Test substance,</th><th>Relevant information about the study including rationale for dose selection (as applicable)</th><th>Observations</th><th>Reference</th></tr>
<tr>
<td>GLP

Study conducted in 2007</td>
<td></td>
<td>the maximum concentration in test 1 (initial test), however toxicity occurring ± S9 meant that the concentrations tested were reduced in test 1 (repeat test) and test 2.

Test concentrations:

Preliminary toxicity test: 18.86 – 2542 µg/ml

Test 1: 0, 60, 65 and 70 µg/ml (-S9)

0, 90, 110 and 120 µg/ml (+S9)

Test 2: 0, 20, 30 and 40 µg/ml (-S9)

0, 100, 110 and 130 µg/ml (+S9)

Test 1 – treatment for 3 h followed by 12 h recovery period (± S9)

Test 2 – treatment for 15 h (no recovery) (-S9), treatment for 3 h followed by a 12 h recovery period (+S9)

Positive controls: Mitomycin C or cyclophosphama</td>
<td>Test1 :

Metaphase analysis data (duplicate cultures)

No test-related effects on chromosome aberration frequency observed in the presence of S9. Results from the study without S9 are shown below:

<table>
<tr><td colspan="4">Without S9 (3 h exposure + 12 h recovery)</td></tr>
<tr><td></td><td colspan="2">Aberrant cells No. (% mean)</td><td>Relative mitotic index (%)</td></tr>
<tr><td>Concentration (µg/ml)</td><td>Excluding gaps</td><td>Including gaps</td><td></td></tr>
<tr><td>0</td><td>1,1 (1)</td><td>3,3 (3)</td><td>100</td></tr>
<tr><td>60</td><td>2,3 (2.5)</td><td>3, 5 (4)</td><td>65</td></tr>
<tr><td>65</td><td>4, 5 (4.5)</td><td>6,5 (5.5)</td><td>68</td></tr>
<tr><td>70</td><td>6,5 (5.5)**</td><td>6, 8 (7)</td><td>63</td></tr>
<tr><td>MTC (0.1 µg/ml)</td><td>24, 21 (22.5)***</td><td>30, 27 (28.5)***</td><td>47</td></tr>
</table>
HCD (excluding gaps): range 1.0 – 2.75, mean 1.9 (0.6 %) [Oct 2005-2007 studies]

Test 2:

Metaphase analysis data (duplicate cultures)

No test-related effects on chromosome aberration frequency observed in the presence of S9. Results from the study without S9 are shown below:

<table>
<tr><td colspan="4">Without S9 (15 h continuous exposure)</td></tr>
<tr><td></td><td colspan="2">Aberrant cells No. (% mean)</td><td>Relative mitotic index (%)</td></tr>
<tr><td>Concentration (µg/ml)</td><td>Excluding gaps</td><td>Including gaps</td><td></td></tr>
<tr><td>0</td><td>2, 2 (2.0)</td><td>4, 5 (4.5)</td><td>100</td></tr>
<tr><td>20</td><td>3, 3 (3.0)</td><td>3, 4 (3.5)</td><td>87</td></tr>
<tr><td>30</td><td>3, 3 (3.0)</td><td>4, 6 (5.0)</td><td>49</td></tr>
<tr><td>40</td><td>1, 3 (2.0)</td><td>10, 8 (9.0)</td><td>54</td></tr>
<tr><td>MTC (0.1 µg/ml)</td><td>32, 37 (34.5)***</td><td>37, 39 (38.0)***</td><td>96</td></tr>
</table>
HCD (including gaps): range 2.5 – 4.5, mean 3.8 (0.6 %) [Oct 2005-2007 studies]</td>
<td></td>
</tr>
</table>

Method, guideline, deviations if any	**Test substance,**	**Relevant information about the study including rationale for dose selection (as applicable)**	**Observations**	**Reference**
		ide		
In vitro mutagen test using mouse lymphoma L5178Y cells OECD 476 GLP	IKF-309 technical Purity 97.88 %	Solubility in DMSO was assessed in a preliminary test (max solubility 1271 µg/ml). This was used as the maximum concentration in a preliminary test to determine toxicity. The concentrations used in the main tests (test 1 and 2) were based on the results of the preliminary study. Test 1 – cell exposure for 3 h at concentrations of: 0, 19.86, 39.72, 79.44, 58.88, 317.75, 635.5 and 1271 µg/ml (± S9) Test 2 – cell exposure for 24 h at concentrations of: 0, 20, 40, 50, 60, 70 and 80 µg/ml (± S9) Positive controls: Methanesulphonate (MMS) (-S9) Benzo(a)pyrene (BaP) (+S9)	**Negative** No evidence of mutagenicity ±S9 with test substance but appropriate results with the positive controls were observed.	DAR: B.6.4.1c (Hynes, L. 2008)

Table 18: Summary table of mutagenicity/genotoxicity tests in mammalian somatic or germ cells in vivo

Method, guideline, deviations if any	**Test substance,**	**Relevant information about the study (as applicable)**	**Observations**	**Reference**
Mouse micronucleus test OECD 474 GLP	IKF-309 technical Purity 97.88 %	Mice, CD-1, (5/sex/dose) Oral gavage 0, 500, 1000 or 2000 mg/kg bw in aq. Methyl cellulose (1 % w/v) Positive control: Mitomycin C (12 mg/kg bw)	**Negative:** - No increase in the number of micronucleated polychromatic erythrocytes - No increase in the incidence of normochromatic erythrocytes - No significant decreases in the proportion of polychromatic erythrocytes - No bone marrow cell toxicity No mortalities or clinical signs.	DAR: B.6.4.2a (Anon., 2008e)
Unscheduled DNA synthesis (UDS) test OECD 486 GLP	IKF-309 technical Purity 97.88 %	Rats, Sprague Dawley, (3 males/dose) Oral gavage 0, 500, 1000 or 2000 mg/kg bw in aq. methyl cellulose (1 % w/v) Positive controls: Dimethylnitroamine (DMN) (10 mg/kg bw) or 2-acetylaminofluorene (50 mg/kg bw) Hepatocytes were isolated from livers at 2 and 16 h	**Negative:** - No induction of UDS in rat hepatocytes No mortalities or clinical signs.	DAR: B.6.4.2b (Anon., 2010)
Comet assay in rats OECD 489 GLP	IKF-309 technical Purity 97.88 %	Rats, Fischer (5 males/dose for pyriofenone and negative controls, 3 males/positive control) Oral gavage 0, 500, 1000 or 2000 mg/kg bw in aq. methyl cellulose (0.5 % w/v) Positive control: ethyl methanesulphonate (EMS) (200 mg/kg bw) The liver only was investigated.	**Negative:** - There was no induction of DNA damage in liver cells of rats.	Study report submitted to CA 2017 (Anon., 2017)
Comet assay in mice Non-	IKF-309 technical Purity 97.88	Mice, CD-1, (5/sex/dose) Oral gavage	**Negative:** - No induction of single strand DNA	DAR: B.6.4.2c (Anon., S.,

Method, guideline, deviations if any	**Test substance,**	**Relevant information about the study (as applicable)**	**Observations**	**Reference**
guideline Non-GLP	%	0, 500, 1000 or 2000 mg/kg bw/day suspended in corn oil Positive control: ethyl methanesulphonate (EMS) (200 mg/kg bw) The liver only was investigated.	damage in mouse liver	2011)

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

In vitro studies

The potential of pyriofenone to induce gene mutations in bacterial cells and gene mutation or chromosome damage in mammalian cells has been investigated in three well-conducted *in vitro* studies.

In bacteria, pyriofenone gave a clear negative result of mutagenicity in both the presence or absence of metabolic activation. Equally, when pyriofenone was assessed for its mutagenic potential in mouse lymphoma L5178Y cells, there were no increases in mean mutant frequency at the tk locuss in treated cells.

Reproducible, negative results were found when pyriofenone was tested for the potential to induce chromosome aberrations in Chinese Hamster Lung (CHL) cells in the presence of S9. 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, the number of aberrant cells both excluding gaps and including gaps increased in a concentration-dependent manner, reaching statistical significance at 70 µg/ml (excluding gaps). Given that mitotic index was reduced by only approximately 35%, this was not considered to have been influenced by the toxicity of the test substance to the cells. . In contrast, in a second test without S9, involving 15 h continuous exposure up to a concentration of 40 µg/ml, there was no increase in the number of aberrant cells (excluding gaps). The top dose produced a 50% redulction in mitotic index and is therefore considered to have been sufficiently high. An increase in aberration frequency including gaps was seen at the top dose, but this finding was not statitstically significant and, given the absence of an effect minus gaps, is considered of uncertain biological relevance. There is no explanation for the different results seen without S9 for the 3 h and 15 treatment periods, respectively; the positive result in the first test cannot be dismissed. Consequently, pyriofenone appears to have potential to induce chromosome damage in cultured mammalian cells, specifically in the absence of exogenous metabolic activation.

In vivo studies

Three guideline studies and one non-guideline study have been conducted to assess the potential of pyriofenone to induce chromosomal or DNA damage *in vivo*, in rodents.

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. The animals were sacrificed 24 or 48 hours after administration, with the bone marrow of the two femora being prepared from

each animal of each group at 24 hours and from animals of the vehicle and top dose group at 48 hours. For each animal, 2000 polychromatic erythrocytes were evaluated for the presence of micronuclei.

Administration of pyriofenone did not lead to any biologically relevant increase in the number of polychromatic erythrocytes that contained micronuclei and the rate of micronuclei was close to the concurrent negative control data. The positive-control, mitomycin C, led to the expected increase in the rate of polychromatic erythrocytes that contained micronuclei.

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 dimethylnitroamine (DMN) or 2-acetylaminofluorene (2-AAF) at 10 or 50 mg/kg bw, respectively. Hepatocytes were isolated from the livers at 2 and 16 hours post-administration of pyriofenone and were incubated in the presence of ^{3}H-thymidine before evaluation of UDS using autoradioagraphy.

In the pyriofenone-treated animals, there were no increases in net nuclear grains or percentage of cells in repair. Results were all comparable to the negative control data. The positive controls behaved appropriately. Therefore, under the conditions of this study, pyriofenone did not induce UDS in rat hepatocytes.

Two Comet assays are available. A recent, well-performed guideline test in rats and a non-guideline assay in mice. 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. A portion of the left lateral lobe was excised and evaluated for a change in mean % tail DNA. The results of this study were negative with no change in the range of mean % tail DNA any of the treatment groups. The positive control behaved accordingly. Therefore it was concluded that pyriofenone does not induce DNA damage in the liver cells of rats.

A similar study was 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. Pyriofenone does not induce DNA damage in the liver cells of mice under the conditions of this study.

10.8.2 Comparison with the CLP criteria

Pyriofenone has been tested for its potential genotoxic properties in a battery of *in vitro* assays and *in vivo* tests.

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 Chinese Hamster Lung cells there was evidence of an increase in cells containing chromosomal aberrations when incubated in the absence of metabolic activation. There was no evidence of mutagenic potential with pyriofenone in a mouse lymphoma cell mutation assay.

From the available studies it appears that pyriofenone has the ability to damage chromosomes *in vitro*. In accordance with the CLP regulation, positive results from *in vitro* studies alone are not sufficient to classify for germ cell mutagenicity. As there was no evidence of mutagenicity in 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.

10.8.3 Conclusion on classification and labelling for germ cell mutagenicity

No classification - conclusive but not sufficient for classification.

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 Mouse Lymphoma L5178Y Cells (thymidine kinase locus)	Hynes, 2008
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**.

10.9 Carcinogenicity

Table 19: Summary table of animal studies on carcinogenicity

↑↓ denote an increase or decrease in a parameter with respect to the control value
Statistical significance: * $p \leq 0.05$, ** $p \leq 0.01$
abs. = absolute
rel. = relative

Method, guideline, deviations if any, species, strain, sex, no/group	**Test substance, dose levels duration of exposure**	**Results**
OECD 451 GLP Rat, Fischer F344, 50/sex/dose Purity 97.88 % (w/w) Year of study: 2007 DAR: B.6.5.1b (Anon., 2010d)	0, 200, 1000 or 5000 ppm for 104 weeks Equivalent to: ♂ 7.25, 36.4 and 197 mg/kg bw/day ♀ 9.13, 46.5 and 254 mg/kg bw/day	Data includes all animals from the terminal kill and those found dead or sacrificed in extremis. **Non-neoplastic findings** **<u>5000 ppm (197/254 mg/kg bw/day):</u>** ***Observations:*** ↑ Cumulative mortality in males in weeks 101 (14 %)* and 104 (17 %)* ↓ Body weight in females week 104 (13 %)** ***Organ weights:*** ↑ Liver, males 30 %** (rel.) and females 13 %* (abs.) and 32 %** (rel.) ↑Kidneys, males 18 %** (rel.) and 39 %** (abs.) and females 36 %** (rel.) ↑Adrenals, males 46 %* (rel.) ↑Caecum, males 1.5 fold** (abs.) and 1.8 fold** (rel.) and females 2.1 fold** (abs.) and 2.5 fold** (rel.) ***Histopathology:*** **Liver** Necrosis: 8/50** males versus 0 in controls Fatty change: 23/50** males and 33/50** females versus 2/50 and 7/50 in controls (males and females respectively) Hypertrophy: 34/50** males and 37/50** females versus 0 in controls Focal congestion: 13/50** females versus 1/50 in controls **Kidneys** Coarse surface: 12/50* males versus 4/50 in controls

<table>
<tr><th>Method, guideline, deviations if any, species, strain, sex, no/group</th><th>Test substance, dose levels duration of exposure</th><th>Results</th></tr>
<tr><td></td><td></td><td>
Chronic nephropathy: 45/50** females versus 17/50 in controls

Large intestines
Caecum, distension: 15/50** males, 14/50** females versus 0/50 in controls
Black contents: 5/50 males* versus 0/50 in controls

Testis
Atrophy: 15/50 males versus 9/50 in controls

Skin
Loss of fur, 8/50* males and 35/50** females versus 2/50 and 12/50 in controls (males and females respectively)
Atrophy of hair follicles, 6/50* males versus 0 in controls
Perifolliculitis, 8/50* females versus 2/50 in controls

Lymph node (mesenteric)
Sinus dilation: 17/49** males versus 0 in controls

<u>1000 ppm (36.4/46.5 mg/kg bw/day):</u>
Histopathology:
Kidneys
Chronic nephropathy: 35/50** females versus 17/50 in controls

<u>200 ppm (7.25/9.13 mg/kg bw/day):</u>
No treatment-related findings

Neoplastic findings
Males:
<table>
<tr><td></td><td></td><td colspan="4">Dose (ppm)</td><td>HCD (2007 +/- 5 years)</td></tr>
<tr><td colspan="2">Number of animals affected (percentage)</td><td>0</td><td>200</td><td>1000</td><td>5000</td><td>Untreated F344 male rats</td></tr>
<tr><td rowspan="3">Liver:</td><td>Hepatocellular adenoma</td><td>4 (8 %)</td><td>1 (2 %)</td><td>2 (4 %)</td><td>6 (12 %)</td><td>0 – 4 %</td></tr>
<tr><td>Hepatocellular carcinoma</td><td>0</td><td>1 (2 %)</td><td>1 (2 %)</td><td>2 (4 %)</td><td>0 %</td></tr>
<tr><td>Combined total</td><td>4 (8 %)</td><td>2 (4 %)</td><td>3 (6 %)</td><td>8 (16 %)</td><td>-</td></tr>
</table>
HCD: Laboratory historical control data on hepatocellular tumour incidence in control male F344 rats 1978 – 2011 was provided by the Applicant. Hepatocellular adenoma
</td></tr>
</table>

Method, guideline, deviations if any, species, strain, sex, no/group	**Test substance, dose levels duration of exposure**	**Results**
		incidence ranged from 0 – 12 % and hepatocellular carcinoma ranged from 0 – 4% over the 33 year span. In the 5 years before or after the study year of 2007, the incidence of adenoma was 0 –4 % and there were no incidence of carcinoma in control animals. No statistical significance was observed. **There were no neoplastic findings in females.**
OECD 451 GLP Mouse, CD-1, 52/sex/dose Purity 97.88 % (w/w) Year of study: 2010 DAR: B6.5.2 (Anon., 2010e)	0, 600, 1800 or 5400 ppm (males) 0, 300, 1000 or 3000 ppm (females) for 78 weeks Equivalent to: ♂ 77.6, 237 and 716 mg/kg bw/day ♀ 49.4, 167 and 486 mg/kg bw/day	Data include all animals from the terminal kill and those found dead or sacrificed in extremis. **Non-neoplastic findings** **<u>5400 ppm (716 mg/kg bw/day) (males only):</u>** ***Observations:*** ↑ Incidence of perigenital staining 24/52 versus 7/52 in controls ***Histopathology:*** **Kidneys** Granular: 10/52** versus 1/52 in controls Cortical scarring: 28/52* versus 16/52 in controls Cortical tubular basophilia: 47/52* versus 37/52 in controls **Liver** Masses: 12/52* versus 4/52 in controls Hypertrophy: 12/52** versus 0 in controls Necrosis (individual hepatocytes): 7/52* versus 1/52 in controls **Prostate** Hyperplasia (acinar cells): 5/52* versus 0/51 in controls **<u>3000 ppm (486 mg/kg bw/day) (females only):</u>** ***Observations:*** ↓ Body weight gain week 76 (17 %)

<table>
<tr><th>Method, guideline, deviations if any, species, strain, sex, no/group</th><th>Test substance, dose levels duration of exposure</th><th>Results</th></tr>
<tr><td></td><td></td><td>
Histopathology:
Liver
Pigment in macrophages: 18/52* versus 8/52 in controls
<u>1800 ppm (237 mg/kg bw/day) (males only):</u>
Observations:
↑ Incidence of perigenital staining 13/52 versus 7/52 in controls
Histopathology:
Kidneys
Cortical tubular basophilia: 47/52* versus 37/52 in controls
Liver
Masses: 11/52* versus 4/52 in controls
Hypertrophy: 16/52** versus 0 in controls
Necrosis (individual hepatocytes): 8/52* versus 1/52 in controls
<u>1000 ppm (167 mg/kg bw/day) (females only):</u>
No treatment-related findings at this dose and below.
<u>600 ppm (77.6 mg/kg bw/day) (males only):</u>
Histopathology:
Liver
Masses: 10/52 versus 4/52 in controls
Hypertrophy: 13/52** versus 0 in controls
Neoplastic findings
Males:
<table>
<tr><td></td><td>Dose ppm (mg/kg bw/day)</td><td>0</td><td>600 (77.6)</td><td>1800 (237)</td><td>5400 (716)</td><td>HCD range (Sept 1997 - Jun 2007)</td></tr>
<tr><td rowspan="3">Liver</td><td>Hepatocellular adenoma</td><td>3 (5.8 %)</td><td>7 (13.5 %)</td><td>6 (11.5 %)</td><td>9 (17.3 %)</td><td>7.8 - 26 %</td></tr>
<tr><td>Hepatocellular carcinoma</td><td>1 (1.9 %)</td><td>2 (3.8 %)</td><td>3 (5.8 %)</td><td>3 (5.8 %)</td><td>0 - 8.0 %</td></tr>
<tr><td>Combined total</td><td>4 [7.7 %]</td><td>9 [17.3 %]</td><td>9 [17.3 %]</td><td>12* [23.1 %]</td><td>9.8 - 36 %</td></tr>
</table>
The historical control data provided was in the date range September 1997 to June 2007, whilst
</td></tr>
</table>

Method, guideline, deviations if any, species, strain, sex, no/group	**Test substance, dose levels duration of exposure**	**Results**
		the study itself was performed from October 2007 – January 2010. A detailed assessment of the data provided by the applicant indicates the percentages of adenoma and carcinoma showed no particular trends over the years. Many of the studies which resulted in percentages towards the higher end of the range fell in the years closest to the current study year. **There were no neoplastic findings in females.**

10.9.1 Carcinogenicity study in the rat

In a well-conducted and reliable study (Table 19), Fischer rats (50/sex/dose) were administered pyriofenone in the diet for 104 weeks at doses of 0, 200, 1000 or 5000 ppm [equivalent to 0, 7.25/9.13, 36.4/46.5, 197/254 mg/kg bw/day (males/females)].

The main target organs for non-neoplastic effects were the liver, kidneys and large intestines, with the majority of effects occurring in animals of the top dose group [5000 ppm (197/254 mg/kg bw/day). There were no treatment-related findings at the low dose of 200 ppm (7.25/9.13 mg/kg bw/day).

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).

Kidney weights were also increased in animals of the top dose group [males 18 % (relative) and 39 % (absolute) and females 36 % (relative)]. The surface of the kidneys were found to be coarse in a number of males and chronic nephropathy was more prevalent in females of the top and mid dose groups than in controls.

Caecum weight was increased in males and females (approximately 2-fold when compared to control animals) and distension was observed as well as black contents in some males.

Other findings included a slight increase in the number of males of the top dose group with testicular atrophy, loss of fur in both males and females and sinus dilation of the mesenteric lymph node in males.

The only significant neoplastic findings were observed in the liver of male rats. There was a dose-related 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 given that hepatocellular adenoma was also seen in 8 % of control males. The incidence of hepatocellular carcinoma was increased marginally at all doses (0, 2%, 2% and 4 % in the control, low, mid and high dose groups respectively). These small increases in the frequencies of adenoma and carcinoma were not found to be statistically significant.

Increased mortality was seen among top dose male rats during the last 3 weeks of this study when compared to all the other dose groups. In week 101, cumulative mortality was 14 %; in the final week 104, cumulative mortality was 17 %. Strictly, this top dose was therefore above the MTD recommended for a carcinogenicity study. However, on the basis of the individual animal data, there was no link seen between increased

mortality and the incidence of liver tumours. In the 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.

Historical control data

Historical control data (HCD) were provided from the laboratory where the carcinogenicity study in rats was carried out. This included the incidences of hepatocellular adenoma and carcinoma in control male F344 rats in studies carried out from 1978 – 2011. The incidence ranges of adenoma and carcinoma during this period were 0 – 12 % and 0 – 4 %, respectively. The findings in the concurrent study are within these ranges, however according to CLP, HCD should be contemporary to the study being evaluated (e.g. within a period of up to 5 years of the study) and data older than this should be used with caution and acknowledgement of its lower relevance and reliability. Further, closer analysis of the HCD showed that the majority of the higher incidences of adenoma and carcinoma occurred between the years 1980 and 1986, which indicates that tumour incidences in control animals may have changed with time. Taking this into account, and utilising only the studies within a 5 year time period of the concurrent study, the incidence of adenoma ranged from 0 – 4 % and carcinoma incidence was 0. Thus the finding of adenoma (12 %) at the top dose of 5000 ppm was above the HCD data range. It is noted that the control incidence of 8 % in this study was also above the HCD. The findings of carcinoma in the low, mid and top dose group (2, 2 and 4 %) were also all above the HCD range.

The Applicant provided further examples of HCD for spontaneous hepatocellular adenoma and carcinoma in male F344 rats taken from national databases. 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). These HCD indicate that spontaneous incidences of adenoma and carcinoma have been shown to occur in males of this strain of rat, however, given the relatively low incidences in this study, the Dossier Submitter believes that more weight should be given to the laboratory control data.

Overall, pyriofenone appears to have produced a weak carcinogenic response in the liver of male rats. No response was seen in female rats. Among males, there were dose-related increases in adenoma, carcinoma and adenoma/carcinoma combined, but the response rates were small and, in the case of the adenomas, the incidence in the control group was greater than the HCD provided, giving less weight to the increase at the top dose. The incidence of carcinoma in the low and mid dose groups was limited to only one animal per dose group, but the incidence of 4 % in the top dose group was above the contemporary laboratory HCD data provided. Overall, the slight dose-response seen in males cannot be dismissed entirely as a chance finding.

10.9.2 Carcinogenicity study in the mouse

In a well-conducted and reliable study, CD-1 mice (52/sex/group) were administered pyriofenone in the diet for 78 weeks (Table 19) at doses of 0, 600, 1800 or 5400 ppm in males and 0, 300, 1000 or 3000 ppm in females [equivalent to 0, 77.6/49.4, 237/167 or 716/486 mg/kg bw/day (males/females)].

Non-neoplastic findings in this study were generally limited to the liver and the kidneys. There were no treatment-related findings at doses of 1000 ppm and below in females.

In males livers were found to have an increase in masses (10/52, 11/52 and 12/52 in the low, mid and high groups respectively versus 4/52 in controls). Hypertrophy was also observed (13/52, 16/52 and 12/52 in the low, mid and high groups respectively versus 0 in controls) and necrosis of individual hepatocytes was seen in an increased number of males (5400 ppm: 7/52 and at 1800 ppm: 8/52 versus 1/52 in controls). The only finding related to the liver in females was an increase in animals with macrophage pigmentation (18/52 versus 8/52 in controls) following a dose of 3000 ppm.

In top dosed males, kidneys were observed to be granular in an increased number of animals and corticol scarring was also seen. There was an increase in cortical tubular basophilia in males of the mid and top dose groups.

There was no increased mortality among mice at the highest dose level. No significant tumour findings were evident in females. 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 600 ppm, yet only 17 % at 5400 ppm, an almost 10-fold increase in dose. There was also a small increased 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 historical control data (HCD) provided for this laboratory (combined rate: 9.8 – 36 %). The HCD appear to be relevant for this study; they derived from comparable studies carried out between 1997 and 2007. Analysis of the historical data showed no concern about the range of values or any particular trends in percentages of tumours found over the years.

To conclude, small increases in liver adenoma, carcinoma and adenoma/carcinoma combined were observed in male mice only. However, the relationship to dose was also weak and the findings in all treatment groups were within the HCD provided. Therefore, pyriofenone does not appear to be carcinogenic in the mouse.

10.9.3 Mechanistic studies relevant to findings in the liver

A consideration of potential modes of action that could attribute for the weak carcinogenic effect seen in the rat study is provided in Section 10.9.4. In wanting to understand the biological events occurring in the rat liver, the Applicant undertook a series of studies that focused on the possibility of pyriofenone being an activator of the constitutive androstane receptor (CAR). Several non-guideline, non-GLP mechanistic studies were conducted in order to investigate this mode of action. These included an assessment of cytochrome P450 (CYP P450) gene expression and replicative DNA synthesis in isolated rat and human hepatocytes and enzyme induction and cell proliferation in rats and mice. Also available is a mechanistic study comparing CAR-knock-out rats to wild-type rats. The main findings are summarised in Tables 20-23.

Table 20: *In vitro* studies in rat hepatocytes

<table>
<tr><th>Type of study/data</th><th>Relevant information about the study (as applicable)</th><th>Observations</th></tr>
<tr><td>Expression of CYP genes in rat hepatocytes (PCR measurement of mRNA)

Non-guideline
Non-GLP

Taken from a study report submitted directly to the CA:

(Shikama, H., 2013a)</td><td>F344 rat hepatocytes (freshly isolated and pooled from male rats)

Pyriofenone: 1.25, 2.5 and 5 ppm

Positive control: PB 3, 30 and 300 ppm</td><td>Altered gene expression of CYP2B is a marker for activation of CAR whereas altered expression of CYP1A is a marker for activation of the aryl hydrocarbon receptor (AhR)
<table>
<tr><td>Concentration (ppm)</td><td>Rat</td><td></td></tr>
<tr><td></td><td>CYP2B1</td><td>CYP1A2</td></tr>
<tr><td></td><td colspan="2">Relative quantity of CYP mRNA</td></tr>
<tr><td>Pyriofenone</td><td colspan="2"></td></tr>
<tr><td>Vehicle control (DMSO 0.005 %)</td><td>1</td><td>1</td></tr>
<tr><td>1.25</td><td>1</td><td>2</td></tr>
<tr><td>2.5</td><td>3</td><td>3.5</td></tr>
<tr><td>5</td><td>11</td><td>4.5</td></tr>
<tr><td>PB</td><td colspan="2"></td></tr>
<tr><td>Vehicle control (dH_20)</td><td>1</td><td>1</td></tr>
<tr><td>3</td><td>8</td><td>0.5</td></tr>
<tr><td>30</td><td>9</td><td>0.5</td></tr>
</table></td></tr>
</table>

<table>
<tr><th>Type of study/data</th><th>Relevant information about the study (as applicable)</th><th>Observations</th></tr>
<tr><td></td><td></td><td>
<table>
<tr><td></td><td></td><td></td></tr>
<tr><td>300</td><td>14</td><td>1</td></tr>
</table>

Little study information was given, it was unclear as to how many replicates were performed. No statistical analysis was performed.

There was a concentration-dependent increase of both CYP2B1 and CYP1A2 following exposure to pyriofenone indicating activation of both CAR and AhR.</td></tr>
<tr><td>Effect of pyriofenone on DNA replication in rat hepatocytes (measured by BrdU incorportation)

Non-guideline

Non-GLP

Taken from a study report submitted directly to the CA:

(Shikama, H., 2013b)</td><td>F344 rat hepatocytes (freshly isolated from male rats, number and age unknown)

Pyriofenone: 5 and 10 ppm

PB 30 and 300 ppm

Positive control: epidermal growth factor (EGF) 25 ng/ml</td><td>Table showing BrdU incorporation into rat hepatocytes (average of three replicates from a single study):
<table>
<tr><td></td><td></td><td>EGF (ng/mL)</td><td colspan="2">PB (ppm)</td><td></td><td colspan="2">Pyriofenone (ppm)</td></tr>
<tr><td></td><td>control</td><td>25</td><td>300</td><td>30</td><td>control</td><td>5</td><td>10</td></tr>
<tr><td>% of Control</td><td>100</td><td>132.4</td><td>125.8</td><td>112.9</td><td>100</td><td>99</td><td>107.2</td></tr>
</table>

No statistics were performed on the results of this study.

Increased DNA replication occurred with EGF and PB (30 and 300 ppm)

A “slight” increase in DNA replication occurred with pyriofenone 10 ppm – however no clear conclusion can be reached.</td></tr>
</table>

In vitro studies in rat hepatocytes

A study to investigate the effects of pyriofenone on the expression of the CYP genes CYP2B6 and CYP1A2 in rat hepatocytes was conducted in isolated male F344 rat hepatocytes (Shikama, H., 2013a). A preliminary cytotoxicity test was first carried out to determine appropriate test concentrations. PB caused no cytotoxicity at the highest dose tested (300 ppm); whereas pyriofenone caused no cytotoxicity at concentrations of 12.5 ppm and below. The test concentrations used in the main study were PB: 3, 30 and 300 ppm and pyriofenone: 1.25, 2.5 and 5 ppm. Cells were pooled from an unspecified number of rats and exposed to pyriofenone or PB for 24 h. RNA was extracted and gene expression was measured using PCR.

The results of the study showed that pyriofenone increased levels of CYP2B1 expression genes concentration-dependently from 2.5 ppm and also increased CYP1A2 concentration-dependently from 1.25 ppm. Treatment with the known CAR-activator PB resulted in a concentration-dependent increase of CYP2B1 levels only from 3 ppm. However, the study consisted of only one experiment with only one replicate, and so the results can offer only a preliminary view of the effects of pyriofenone on the expression of CYP2B1 and 1A2 and it was not possible to perform any statistical analysis.

A second study assessed the effect 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) (Shikama, H., 2013b). Test concentrations used were PB: 30 and 300 ppm, pyriofenone: 5 and 10 ppm and epidermal growth factor (EGF): 25 ng/mL. Both PB and the positive control, EGF were clearly shown to cause DNA replication. At 10 ppm, pyriofenone showed, what was described by the study authors as, a “slight” increase in DNA replication. This increase of 7 % above the control value compared to the increases of about 25 – 30 % seen with the positive control substances appears to be very small. The result of this study is inconclusive with respect to the potential of pyriofenone to induce

increased DNA replication in rat liver. In the absence of any statistical analysis the Dossier Submitter believes the results can only show that pyriofenone *may* induce increased DNA replication in rat liver.

Table 21: *In vivo* studies in rats

↑↓ denote an increase or decrease in a parameter with respect to the control value
Statistical significance: * $p \leq 0.05$, ** $p \leq 0.01$
abs. = absolute
rel. = relative

Type of study/data	Relevant information about the study (as applicable)	Observations
Hepatocyte proliferation in rats (Cell proliferation measurement using immunohistochemical staining of histopathology slides for BrdU of duodenum and liver sections. The duodenum sections were used to validate the immunostaining method. Nuclei were stained using haematoxylin and eosin). Non-guideline Non-GLP DAR: B.6.8.3b (Anon., 2009b)	Fischer Rats (males/5/dose) Pyriofenone: 0, 200 and 20000 ppm (dietary administration) Equivalent to 0, 15.7/14.4 and 849/1109 mg/kg bw/day (3 day group/7 day group) The difference in average intake in the top dose, 7-day treated animals is thought to be due to palatability of the test diet. Positive control: Chloroform 1000 mg/kg bw/day Treatment time: 3 or 7 days (2 days for positive control)	**After 3 days:** **<u>Pyriofenone (20000 ppm/849 mg/kg bw/day):</u>** ↑Mean RDS (replicative DNA Synthesis) index 2.98 versus 1.23 in control (no statistical significance) **<u>Pyriofenone (200 ppm/15.7 mg/kg bw/day):</u>** No increase in liver weight and no increase in mean RDS index compared to controls. **After 7 days:** **<u>Pyriofenone (20000 ppm/1109 mg/kg bw/day):</u>** ↑Mean RDS index 3.91** versus 1.42 in controls ↑ Liver weight 16 % (abs.) and 27 % (rel.)** No adverse liver pathology **<u>Pyriofenone (200 ppm/14.4 mg/kg bw/day):</u>** No increase in liver weight and no increase in mean RDS index compared to controls. **Chloroform (1000 mg/kg bw/day):** ↑Mean RDS index 14.3** versus 1.42 in controls
Hepatic enzyme induction in rats Non-guideline Non-GLP DAR: B.6.8.3a (Anon., 2011a)	Fischer, F344 rats (males/5/dose) Pyriofenone: 0, 200 and 20000 ppm Equivalent to 0, 14.3 and 1300/1289 mg/kg bw/day (Group 1/2) Positive control: Phenobarbital sodium (PB) 500 ppm Group 1: 14 days treatment Group 2: 14 days treatment + 14 days recovery	**Pyriofenone** **<u>20000 ppm (1300/1289 mg/kg bw/day):</u>** ↑Liver weight (rel.) 43 % ↑PROD 20 fold**, ↑ CYP2B1 content, 8 fold** ↑ECOD 1.5 fold**, ↑ CYP1A2 content, 1.6 fold Effects were all reversible within 14 days. **<u>200 ppm (14.3 mg/kg bw/day):</u>** No effects observed.

Type of study/data	Relevant information about the study (as applicable)	Observations
	Livers were excised and homogenised. The microsomal suspension was then used to determine microsomal protein content, total CYP 450 content and ECOD and PROD activities Ethoxycoumarin O-dealkylase (ECOD) is a marker for CYP1A2 activity and penoxyresorufin O-dealylase (PROD) is a marker for CYP2B1 activity	**PB** **500 ppm:** ↑Liver weight (rel.) 35 % ↑ Total cytochrome P450 2.6 fold ↑ PROD 71 fold**, ↑ CYP2B1 content, 40 fold** ↑ ECOD 4.7 fold**, no effect on CYP1A2 levels
In vivo mechanism analysis study in CAR-knock-out (KO) rats Non-guideline Non-GLP Taken from a study report submitted directly to the CA: (Anon.., 2017a)	CAR-KO rats (5 males/dose) Wild type (WT) rats (Sprague-Dawley) (5 males/dose) Pyriofenone (purity 97.88 %) Dietary administration: 0 or 5000 ppm for 7 days (Equivalent to 357.2 mg/kg bw/day in CAR-KO rats and 363.6 mg/kg bw/day in WT rats) Livers were removed, weighed and then the left lateral lobe was used for measurement of CYP2B1 gene expression, measurement of CYP2B1 enzyme activity and hepatocyte proliferation. Ki-67 is a protein marker of cellular proliferation, present during all active phases of the cell cycle	**5000 ppm (357.2/363.6 mg/kg bw/day):** Food consumption: ↓ Food consumption: WT 16 % (days 1-3 only) KO 12 % (days 1-3 only) Clinical chemistry: ↑ Total cholesterol 20 % (WT only) ↓ Triglyceride 50 % (WT only) Findings in the liver: ↑ Weight WT: 16 % (abs. and rel.) KO: 20 % and 19 % (abs. and rel. respectively) Hypertrophy (minimal): WT: 1/5 (versus 0 in controls) KO: 1/5 (versus 0 in controls) CYP2B1 gene expression: WT: ↑ 130-fold (Normalised mRNA amount was 129.0 versus 0.99 in controls)* KO: ↑ 120-fold (Normalised mRNA amount was 14.4 versus 0.12 in controls)** CYP2B1 activity: WT: ↑ 8-fold** KO: No increase. Hepatocyte proliferation: Ki-67 positive ratio: there was no evidence of hepatocyte

Type of study/data	Relevant information about the study (as applicable)	Observations
		proliferation in WT or KO rats.

In vivo studies in rats

An *in vivo* study, 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) (100 mg/kg bw) *(i.p.*). At the higher concentration of pyriofenone, after three days, there was no increase in liver weight but there was a very slight increase in hepatocellular proliferative activity. However, this was not statistically significant (mean RDS was increased to 2.98 versus 1.23 in controls). After seven days of treatment, liver weight was statistically significantly increased (absolute liver weight 16 % and relative liver weight 27 % of controls) and there was a statistically significant increase in replicating DNA following treatment with 20000 ppm (1109 mg/kg bw/day) pyriofenone (mean RDS was increased to 3.91 versus 1.42 in controls). Chloroform also increased cell proliferation in this study.

The results of this study showed that pyriofenone can cause an increase in replicative proliferation in Fischer rats at high doses.

Pyriofenone was also tested for its effects on hepatic enzyme induction in male F344 rats.

In this study, the rats were divided into two groups; group one received pyriofenone in the diet at either 0, 200 or 20000 ppm for 14 days and group two 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. Liver microsomes taken from treated rats were then used to determine the microsomal protein content, the total P450 content and ethoxycoumarin O-dealkylase (ECOD), a marker for CYP1A2 and penoxyresorufin O-dealylase (PROD), a marker for CYP2B1 activities.

The results showed that following 14 days of treatment with pyriofenone (20000 ppm) or PB (500 ppm), liver weight was increased (pyriofenone 43 % and PB 35 % with respect to controls). Phase I liver enzymes were induced following treatment with PB, with total CYP450 content, increasing 2.6-fold that of controls and PROD and ECOD activities increasing 71 and 4.7-fold of controls (respectively). Pyriofenone also increased PROD and ECOD activities, somewhat less markedly than PB at 20 and 1.5 fold of controls (respectively). The results from group two indicated that this effect was reversible. Treatment with pyriofenone caused an increase in CYP2B1 content (8-fold) and a small increase in CYP1A2 (1.6-fold). PB induced CYP2B1 (40-fold) but CYP1A content was not affected. Induction of other P450 isoforms were not assessed in this study.

Therefore, pyriofenone has been shown to cause enzyme induction *in vivo* in Fischer rats.

An *in vivo* mechanistic study was carried out comparing 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) for 7 days. At the end of the study period body weight, food consumption, clinical chemistry, liver weight, CYP2B1 gene expression, CYP2B1 activity and hepatocyte proliferation were all measured and statistically analysed.

No deaths occurred during the dosing period and there were no abnormal clinical signs. Body weight in the 5000 ppm groups remained comparable to the 0 ppm groups. There was a reduction in food consumption in both WT and KO animals treated with 5000 ppm on days 1-3 (12/16 % KO/WT compared to untreated groups) which was resolved during days 3-8. Some changes to blood chemistry were noted in WT animals. These were an increase in total cholesterol (20 % compared to controls) and a decrease in triglycerides (50 % compared to controls). Minimal hypertrophy of the liver was noted in 1/5 WT and 1/5 KO animals only.

Measurement of the Ki-67 positive ratio in hepatocytes, a cellular marker for proliferation (Scholzen, T. and Gerdes, J., 2000), did not show any increase in either WT or KO rats. In fact, the ratio actually decreased in both treated groups compared to controls. However, the observation associated with increased cell proliferation of increased liver weight was noted in both WT and KO treated animals. 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).

CYP2B1 gene expression was increased in both WT and KO groups. In WT rats the increase was 130-fold of the controls and in the KO rats expression increased 120-fold of the controls. Pyriofenone did not increase in CYP2B1 activity in the CAR-KO rats, whilst in WT rats CYP 2B1 activity was found to increase by 8-fold of the controls.

Overall, the observation of increased CYP2B1 activity in WT but not KO rats is consistent with CAR activation. However, the observation of increased CYP2B1 gene expression in both WT and KO animals suggests that there may be other mechanisms of action by which pyriofenone effects the liver.

Table 22: *In vivo* studies in mice

↑↓ denote an increase or decrease in a parameter with respect to the control value
Statistical significance: * $p \leq 0.05$, ** $p \leq 0.01$
abs. = absolute
rel. = relative

Type of study/data	Relevant information about the study (as applicable)	Observations
Hepatic enzyme induction in mice and assessment of hepatocyte proliferation Non-guideline Non-GLP DAR: B.6.8.4 (Anon., 2010f)	CD-1 mice (males, 12/dose) Dietary administration for 4 weeks Pyriofenone: 0, 5000 or 10000 ppm Equivalent to 0, 854 or 1714 mg/kg bw/day Liver sections from all animals were stained for proliferating cell nuclear antigen (PCNA) to demonstrate presence of proliferating cells. Enzyme assays were performed on pooled livers of the 3 dose groups to measure microsomal protein concentration, EROD activity, testosterone hydroxylase and dehydrogenase activities and lauric acid hydroxylase activities.	No clinical signs of toxicity, no reductions in body weight and no macroscopic findings at necropsy. **<u>10000 ppm/1714 mg/kg bw/day</u>** ↑ Liver weight 14 % (rel.)** ↑ Cytochrome P450 1.5 fold ↑EROD activity 1.4 fold **<u>5000 ppm/854 mg/kg bw/day</u>** ↑ Liver weight 12 % (rel.)** ↑ Cytochrome P450 1.3 fold ↑EROD activity 1.37 fold

In vivo studies in mice

Hepatic enzyme induction and cell proliferation were studied *in vivo* in male CD-1 mice. Groups of 5 mice received pyriofenone in the diet at doses of 0, 5000 or 10000 ppm for 4 weeks. Liver sections were taken from all animals and stained for proliferating cell nuclear antigen (PCNA) to demonstrate presence of proliferating cells. Enzyme assays were performed on pooled livers of each dose group and the following parameters measured: i) microsomal protein content, ii) cytochrome P450 concentration, iii) 7-ethoxyresorufin O-deethylase activity (EROD) (a marker for CYP1A1 and 1A2), iv) testosterone hydroxylase and dehydrogenase activities (a monitor for CYP2A, 2B, 2C and 3A) and v) lauric acid hydroxylase activities (a monitor for CYP2E and 4A). The results showed an increase in relative liver weight following both doses of pyriofenone (12 % and 14 % at 5000 and 10000 ppm respectively) and a small increase in cytochrome P450 in both dose groups, indicating Phase I enzyme induction. There was no evidence of induction of CYP2A, CYP 2B, CYP2C, CYP 2E, CYP 3A or CYP4A. A small increase in EROD activity was observed (approximately 1.4 fold) following treatment with 10000 and 5000 ppm pyriofenone. There was no evidence of any increase in the rate of cell proliferation in the liver. The results of this study indicate that pyriofenone has little effect of on hepatic phase I enzyme induction or cell proliferation in mice.

Table 23: *In vitro* studies in human hepatocytes

<table>
<tr><th>Type of study/data</th><th>Relevant information about the study (as applicable)</th><th>Observations</th></tr>
<tr><td>Expression of CYP genes in human hepatocytes (PCR measurement of mRNA)

Taken from a study report submitted directly to the CA:
(Shikama, H., 2013a)</td><td>Human hepatocytes (Cryopreserved cells from males, number and health status unknown)
Pyriofenone: 1.25, 2.5 and 5 ppm

Positive control: PB 3, 30 and 300 ppm</td><td>Altered gene expression of CYP2B is a marker for activation of CAR whereas altered expression of CYP1A is a marker for activation of the aryl hydrocarbon receptor (AhR)
<table>
<tr><td>Concentration (ppm)</td><td colspan="2">Human</td></tr>
<tr><td></td><td>CYP2B6</td><td>CYP1A2</td></tr>
<tr><td></td><td colspan="2">Relative quantity of CYP mRNA</td></tr>
<tr><td>Pyriofenone</td><td colspan="2"></td></tr>
<tr><td>Vehicle control (DMSO 0.005 %)</td><td>1</td><td>1</td></tr>
<tr><td>1.25</td><td>Not tested</td><td>Not tested</td></tr>
<tr><td>2.5</td><td>1</td><td>0.5</td></tr>
<tr><td>5</td><td>7</td><td>1</td></tr>
<tr><td>PB</td><td colspan="2"></td></tr>
<tr><td>Vehicle control (dH_20)</td><td>1</td><td>1</td></tr>
<tr><td>3</td><td>Not tested</td><td>Not tested</td></tr>
<tr><td>30</td><td>5</td><td>0.5</td></tr>
<tr><td>300</td><td>14</td><td>0.5</td></tr>
</table>
Little study information was given, it was unclear as to how many replicates were performed. No statistical analysis was performed.
An increase in CYP2B6 was seen with 5 ppm pyriofenone and with increasing concentrations of phenobarbital indicative of CAR activation.
There was no increase in CYP1A2 activity in the presence of pyriofenone or phenobarbital, suggesting no activation of AhR.</td></tr>
<tr><td>Effect of pyriofenone on DNA replication in</td><td>Human hepatocytes (sex, number and health status unknown)
Pyriofenone: 0.6, 2.5</td><td>Table showing BrdU incorporation into human hepatocytes(average of four replicates from a single study):
<table>
<tr><td></td><td>EGF (ng/mL)</td><td>PB (ppm)</td><td></td><td>Pyriofenone (ppm)</td></tr>
</table></td></tr>
</table>

<table>
<tr><th>Type of study/data</th><th>Relevant information about the study (as applicable)</th><th>Observations</th></tr>
<tr><td>human hepatocytes (measured by BrdU incorporation)

Taken from a study report submitted directly to the CA:
(Shikama, H., 2013b)</td><td>and 10 ppm
PB 30 and 300 ppm
Positive control: epidermal growth factor (EGF) 25 ng/ml</td><td>
<table>
<tr><td></td><td>control</td><td>25</td><td>300</td><td>30</td><td>control</td><td>10</td><td>2.5</td><td>0.6</td></tr>
<tr><td>% of Control</td><td>100</td><td>148.1</td><td>95.1</td><td>92.2</td><td>100</td><td>99.9</td><td>96.6</td><td>97</td></tr>
</table>
No statistical analysis was performed.
DNA replication was evident following exposure to the positive control EGF
No DNA replication was observed with PB or pyriofenone.
</td></tr>
</table>

In vitro studies in human hepatocytes

Pyriofenone was tested in human hepatocytes for its ability to cause changes in expression of CYP genes and to assess cell proliferation.

In the first study, pyriofenone was tested for its ability to cause changes in expression of the genes CYP2B6 and CYP1A2 using cryopreserved human hepatocytes (males, number of donors, age and health status unknown) (Shikama, H., 2013a). The test concentrations used in the main study were PB: 30 and 300 ppm and pyriofenone: 2.5 and 5 ppm and cells were exposed for a period of 24 h. RNA was extracted and gene expression was measured using PCR.

The results showed that pyriofenone, at the higher concentration, increased CYP2B6 gene expression. PB also caused increased expression of CYP2B6. There was no effect on CYP1A2 levels following exposure to either substance. There was no statistical analysis of the results. Consistent with a CAR mode of action, pyriofenone caused an increase in CYP2B6 expression.

A second study assessed the effect of pyriofenone on DNA replication in human hepatocytes by measurement of incorporation of BrdU (Shikama, H., 2013b). Test concentrations used were PB; 30 and 300 ppm, pyriofenone: 0.6, 2.5 and 10 ppm and epidermal growth factor (EGF): 25 ng/mL. 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 under S-phase DNA synthesis. This finding was consistent with the hypothesis that a CAR mode of action is not relevant to humans.

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

The carcinogenic potential of pyriofenone has been investigated in rats and mice. In a carcinogenicity study in rats, a marginal increase in the number of hepatic adenoma and carcinoma was observed in males treated with pyriofenone (5000 ppm). The increases observed were above that of the concurrent control and above the contemporary historical control data provided. No such increase was observed in females or in mice.

The increase in liver tumours in rats were associated with an increase in mortality towards the end of the study. Non-neoplastic findings in the liver included an increase in relative organ weight, fatty changes and hypertrophy in both males and females and an increase in the number of males with necrosis of the liver and increase in the number of females with focal congestion. The increase in mortality and hepatic cell death at the highest dose might be indicative of excessive toxicity, although body weight was largely unaffected in

males. If this were the case, it could be argued that the tumours observed were a non-specific consequence of the hepatoxicity of pyriofenone under extreme dosing conditions, making them of less relevance to humans. However, no excess toxicity was observed at 200 or 1000 ppm where tumours were also seen. There are other potential modes of action that should be considered, as discussed below.

10.9.4.1 Potential Modes Of Action (MOA)

Alongside the possibility of the tumour findings in male rats being related to the toxicity of pyriofenone, there are various possible mechanistic explanations that can be considered for this weak carcinogenic response in rats (summarised in the Table 24).

Table 24: Potential modes of action (MOA) relating to tumour formation in the liver of rats

Mode of Action	Data relating to pyriofenone	Conclusion
Genotoxicity	Assessed in a battery of standard *in vitro* and *in vivo* mutagenicity studies (Section 10.8). Although induction of chromosomal damage was observed in an *in vitro* test, the weight of evidence from a micronucleus test in mice, an unscheduled DNA synthesis test in rats (targeting the liver) and two Comet assays investigating the liver of rats and mice, suggests that this is not the mode of action of pyriofenone.	Unlikely
Cytotoxicity	There was an increase in mortality in male rats of the top dose group in the rat carcinogenicity study and also an increase in single cell necrosis. Therefore, there is a possibility that cytotoxicity could be a potential mode of action of pyriofenone. However, no significant liver toxicity was observed in the low or mid dose groups of the rat carcinogenicity study at which there were single incidences of liver adenoma and carcinoma. Other findings in the liver included increases in relative liver weight, fatty changes and hypertrophy.	Plausible, but not definitive
Peroxisome proliferator-activated receptor alpha (PPARα) activation	Investigated by substance-mediated induction of CYP4A mRNA levels or enzymatic activity. There were no studies conducted to investigate the induction of CYP 4A1 gene transcription in rodent hepatocytes. In mice, there was no change in expression of genes coding for CYP4A indicating activation of the PPAR α receptor does not occur. There were no equivalent studies in rats. 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.	Unlikely
Constitutive androgen receptor (CAR)/Pregnane X receptor (PXR) activation	*In vitro* and *in vivo* mechanistic studies indicate that pyriofenone induces changes in rats and humans consistent with this mode of action (see details in Table 27).	Plausible, but not definitive

	A recent study designed to definitively address the CAR-dependence of pyriofenone showed that whilst CYP2B1 enzyme activity was increased in WT rats, it was not increased in CAR-KO rats following administration of pyriofenone in the diet. However, CYP2B1 gene expression was increased in KO rats as was liver weight. However, the study did not demonstrate the ability of pyriofenone to cause proliferation in either WT or KO rats. Therefore, the results of this study leaves uncertainties as to whether the effects of pyriofenone are entirely due to CAR activation. See Section 10.9.4.2 for further details. There were no studies carried out in rats to assess CYP3A transcription (associated with CAR/PXR activation), although in mice, there was no evidence of an increase in CYP3A activity (there was no substance-related increases in liver tumours in mice).	
Aryl hydrocarbon receptor (AhR) activation	In rat hepatocytes, pyriofenone was shown to induce expression of CYP1A genes in a concentration-dependent manner. *In vivo* studies in rats with pyriofenone showed induction of ECOD activity (a marker of CYP1A activity) and an increase in CYP1A2 content. Sodium phenobarbital also increased ECOD activity, however there were no associated changes to CYP1A content. These results indicate that pyriofenone has potential to activate the AhR, however the magnitude of the increase in ECOD was much lower than the increase in PROD activity (associated with the CAR MOA). There was a small increase in EROD activity in mice (a marker of CYP1A activity). No such increase in expression was observed for CYP1A2. A change in expression of CYP1A2 might be under some influence of CAR, however, it is widely recognised as a marker of the aryl hydrocarbon receptor (AhR). This receptor is involved in various signalling pathways and dysregulation of these cellular processes may also provoke a carcinogenic response. It can occur in rats and humans. The absence of an induction of this enzyme therefore suggests this MoA is not of relevance to pyriofenone.	Plausible, but unlikely

Porphyria	There were some modest changes to red blood cell parameters in many of the repeated dose studies, although in most cases the changes were less than 5 % when compared to control values. Wainstok de Calmanovici et al (1984) indicated that the precursors of haem synthesis, aminolevulinic acid (ALA) and porphobilinogen (PBG) would be elevated in the development of porphyria. This was not the case in any of the studies available. In the 2-generation study in rats, brown deposition was described in the Glisson's capsule of the liver and necrosis of the liver was observed in mice in the carcinogenicity study.	Unlikely
Endocrine Activity	There were no adverse effects observed in the ovaries or testes of rats, mice or dogs in any of the repeated dose or reproduction studies. In one 2-generation reproduction study in rats, there was an increase in the incidence of follicular cell hypertrophy of the thyroid of parental female rats dosed with 5000 ppm (307 - 677 mg/kg bw/day) pyriofenone. This finding alone is not considered to warrant an endocrine effect following treatment with pyriofenone. There were no hormonal disturbances observed.	Unlikely
Immunosuppression	In the short term and chronic studies provided, there was no evidence of changes to the immune system or immune cells.	Unlikely

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. From the data available, summarised in the table above, 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 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 is lacking. The Applicant has therefore considered CAR activation to be the most likely explanation for the tumour response seen.

10.9.4.2 Activation of the Constitutive Androstane Receptor (CAR)

A pathway of changes in the liver stemming from activation of the constitutive androstane receptor (CAR) has been well characterised in recent years as a potential mechanism of action for some rodent liver carcinogens. Whilst there is strong evidence for this pathway being relevant to rats and mice, data relating to the rodent liver carcinogen phenobarbital (PB) suggest it to be qualitatively not plausible for humans (Elcombe et al 2014).

The 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. Many of the substances that activate the CAR receptor also activate the pregnane X receptor (PXR), producing a combined response pattern of gene expression and functional changes. The key and associative events involved in this process are illustrated below (Figure 1):

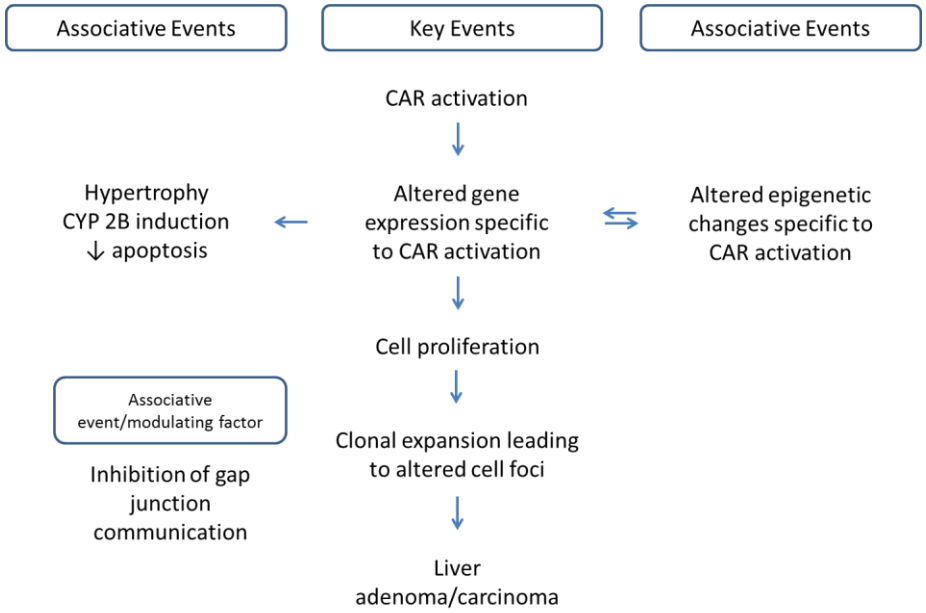


Figure 1. Key and associative events involved in CAR-mediated liver toxicity and tumour formation

A number of the effects produced in the rodent liver by these substances have also been seen in humans or model systems that are representative of the human liver. These include activation of CAR and induction of the appropriate forms of cytochrome P450 (e.g. CYP2B) and liver hypertrophy. However, evidence from humans who have been exposed to phenobarbital for long periods do not seem to be at increased risk of developing liver cancer. Similarly, in experimental systems, the substances concerned have been shown to induce cell proliferation in rodent liver cells, but not in human cells. Therefore, if the CAR activation can be proven to be key to the slightly increased liver tumour incidence in male rats following treatment with pyriofenone, then this would show that no classification for carcinogenicity would be justified in the absence of relevance to humans.

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

The results obtained from the mechanistic studies carried out *in vitro* in rats and humans and *in vivo* in rats and mice have been critically assessed in relation to the key and associative events involved in the CAR-mediated formation of tumours in rodents. Reference is made to the model CAR-activating substance PB. The evidence is summarised in Table 25.

10.9.4.3.1 Key event 1 – CAR activation

Induction of the key enzymes CYP2B and CYP3A occur largely via activation of CAR and the pregnane X receptor (PXR) (respectively). It is known that many of the molecules that activate CAR, also activate PXR, producing a combined response pattern of gene expression and functional changes (Moore JT *et al*. 2000). Therefore, induction of CYP2B (and CYP3A) enzymes are indicative of CAR activation.

The activation of CAR is the molecular initiating event in this pathway. It is possible, using genetically engineered CAR "knock-out" rats (or mice) to show the CAR-dependency of the increase in liver weight, induction of CYP2B enzymes and cellular proliferation seen in wild type animals.

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 at the dose and timeframe used.

Therefore, there is some evidence to indicate that CAR activation can occur in male rats following exposure to pyriofenone but it is not definitive.

10.9.4.3.2 Key event 2 – Altered gene expression specific to CAR activation

Altered gene expression specific to CAR activation is considered to be the second Key event in the mechanism of action for phenobarbital-induced rodent liver formation.

In rat hepatocytes, a concentration-dependent increase of CYP2B1 and CYP1A2 gene expression was found following exposure of cells to pyriofenone (concentrations of 2.5 ppm and above). In a similar experiment using human hepatocytes, there was a similar increase in expression of CYP2B6 (at 5 ppm). However, in an *in vivo* study comparing CAR-KO and WT rats, expression of CYP 2B1 genes was observed in both treated groups, indicating strictly the lack of CAR-dependency for this finding.

Associated events

Induction of CYB 2B isoforms is considered an associative event to key event 2. In a study using CAR-null rats, it was found that the CAR receptor was essential for induction of CYP 2B1 activity following treatment with pyriofenone (5000 ppm/~360 mg/kg bw/day). The results of this study showed no increase in CYP2B1 activity in KO rats, whilst a small (8-fold) increase in activity was observed in WT rats.

CYP450 enzyme induction was assessed in a second *in vivo* study in rats and also in mice. Following treatment with pyriofenone (20000 ppm/~1300 mg/kg bw/day) in rats for 14 days, there was an increase in PROD activity (a marker of CYP 2B activity) and also an increase in transcription of CYP 2B1 (8-fold when compared to controls). The magnitude of this effect was much lower than that seen with PB. BROD activity (a marker of both CYP2B and CYP3A) was not measured nor was transcription of CYP3A enzymes. In a similar study in mice, there was no such increase in CYP2B1 content. However, this could be considered consistent with the findings in the main carcinogenicity study in mice, where there were no treatment-related increases in liver tumours.

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 (and also 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 causes changes to gene expression and liver weight associated with CAR activation, it has also been shown to cause these changes in the absence of the CAR receptor. Enzyme induction of CYP 2B1 caused by pyriofenone appeared to be dependent on CAR.

10.9.4.3.3 Key event 3 – Cell proliferation

Increased cell proliferation is considered the third Key event in the CAR mode of action leading to the formation of tumours in rodents. It is known that in humans, following CAR activation by PB, altered gene expression and enzyme induction of CYP2B6 occurs, however there is no increase in hepatic cell proliferation. In contrast to rats and mice, there is no decrease in apoptosis or increase in the incidence of liver tumours.

As an indicator of cell proliferation, increased DNA replication was measured in an *in vitro* mechanistic study using rat hepatocytes. The results of the study showed a clear, dose-dependent increase in uptake of BrdU by the cells after exposure to PB. Following treatment with pyriofenone (10 ppm) there was a small increase in BrdU uptake. However, the magnitude of this response was considerably less than seen with PB. In a similar experiment using human hepatocytes, a clear negative result was obtained indicating no DNA replication following exposure of the cells to a maximum concentration of 10 ppm pyriofenone.

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.

Therefore, at high doses only, pyriofenone does have potential to cause hepatocellular proliferation in rats but in a study to determine a definitive CAR mode of action, no proliferation was demonstrated in either WT or CAR-KO rats. Consequently, it is uncertain whether pyriofenone causes cell proliferation by activation of CAR.

10.9.4.3.4 Key event 4 – Clonal expansion leading to altered cell foci

Altered liver foci are precursor lesions for subsequent tumour formation. In the standard and mechanistic studies provided by the applicant, the only evidence of focal congestion was in the livers of top-dosed female rats of the 2-year carcinogenicity study. It is possible that pyriofenone can induce these changes in male rats, but a study optimised to show this was not conducted.

10.9.4.3.5 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. The number of rats with tumours was above the concurrent control and were above the contemporary HCD, therefore the possibility of a weak carcinogenic response cannot be excluded.

Table 25: Summary of evidence in rodents and humans associated with the key and associative events involved in the CAR-mediated formation of tumours in rats

Key and Associative events	Evidence in rats and mice	Evidence in humans
Activation of CAR	Some evidence In an *in vivo* mechanistic study using CAR-KO rats, CYP2B1 enzyme activity was increased in WT but not KO out rats. However, changes in gene expression and liver weight were noted in both KO and WT rats and there was no evidence of hepatocellular proliferation at the doses used.	No data
Altered gene expression	Strong positive In an *in vitro* study using male Fischer rat hepatocytes, there was a concentration-dependent increase in gene expression of CYP2B1 after exposure to pyriofenone. An increase in CYP2B1 gene expression was observed in both WT and KO rats indicating that the effect is not CAR-dependent.	Positive evidence In an *in vitro* study using human male hepatocytes there was an increase in gene expression of CYP2B6 only.

Hypertrophy	Strong positive Observed in male and female rats and in male mice *in vivo*.	No data
Increased hepatocellular proliferation	Some evidence *In vitro*, slight increases in DNA replication in rat hepatocytes were indicative of increased proliferation. In rats, there was a small increase in hepatocyte proliferation after 7 days of exposure to pyriofenone (1109 mg/kg bw/day). The magnitude of this was lower than with the positive control, chloroform and was not seen at all at a dose of ~15 mg/kg bw/day). Hepatocellular proliferation was not observed at a cellular level in either WT or KO rats (administered with the lower dose of ~ 360 mg/kg bw/day), however liver weight was increased in both groups of animals.	Negative In an *in vitro* study using human male hepatocytes there was no evidence of DNA replication.
Altered hepatic foci	Some evidence Only observed in female rats in the *in vivo* carcinogenicity study. However, absence of an effect in rats has not been established.	No data
Liver tumours	Positive evidence A slight increase in the number of adenoma and carcinoma in male rats only, observed following two years of treatment with pyriofenone. No such increase was observed in females or in mice.	No data

Following a critical assessment of all mechanistic data provided, it appears that the CAR mode of action is a plausible explanation for the increase in liver tumours observed in male rats treated with pyriofenone. However, some 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.
- *In vitro* studies in rat hepatocytes, investigating the effect of pyriofenone on DNA replication - the magnitude of the BrdU incorporation into the cells following exposure to pyriofenone (10 ppm) was very small. The experiment was performed in isolation and there was no statistical analysis of the data performed.
- *In vivo* studies in rats to investigate enzyme induction and hepatocyte proliferation - the top concentration tested was four times higher than that causing liver tumours *in vivo*. Whilst use of a

more representative dose did lead to enzyme induction in WT rats, it did not lead to increased cell proliferation in either WT or CAR-KO rats.

- In the *in vitro* studies in human hepatocytes investigating expression of CYP genes and the effect of pyriofenone on DNA replication cells used were pooled cryopreserved cells from males. The number of donors, age and health status remain unknown. Therefore, the results should be used with caution before reaching conclusions about the relevance to the human population as a whole.
- Inhibition of apoptosis and other associative events in the CAR-mediated tumour model have not been investigated, however there is some debate as to the importance of inhibition of apoptosis as a determinant of tumour promotion in rodents, therefore, the lack of studies on this do not represent a major knowledge gap.
- No data was provided on the ability of pyriofenone to cause an increase in gene transcription and enzyme activity consistent with activity of the PXR - many of the substances that activate the CAR receptor also activate the PXR receptor, producing a combined response pattern of gene expression and functional changes. Whilst this doesn't form a data gap in itself, it would have been more informative and added more weight to the evidence.

Given these study limitations and data gaps, the weight of evidence supporting the view that pyriofenone induced liver cancer in male rats via a non-genotoxic pathway solely involving CAR-activation is not entirely persuasive.

10.9.4.4 Conclusion

In a carcinogenicity study in rats, a small increase in the incidence of liver tumours was observed in males treated with pyriofenone (5000 ppm in the diet). The increases observed were above the concurrent control values; and above the contemporary laboratory control incidence of 0. There was no carcinogenic effect seen in female rats or in male or female mice.

The most likely mechanism of action underpinning this activity is considered to involve CAR-activation. However, based on the evidence currently available there remains some key areas of uncertainty. As such, in the absence of a definitive conclusion about the MoA, the relevance of the rat liver tumour findings for humans cannot be discounted completely.

10.9.5 Comparison with the CLP criteria

An increase in tumours has been observed in the livers of male rats following treatment with pyriofenone.

As there is no evidence to suggest that pyriofenone causes carcinogenicity in humans, classification with Category 1A is not considered appropriate.

In order to be classified in Category 1B, the evidence provided must be considered sufficient to presume the substance has carcinogenic potential in humans. As the liver tumours observed occurred in just one species (rats), in one sex (males), in one tissue (the liver) and the increased incidences were slight, only just over the concurrent control level yet within the literature historical control levels for this species (laboratory control data was not provided) the data is not considered sufficiently convincing for this category.

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 categories 1A or 1B.

Consequently, pyriofenone could either be classified in category 2 for carcinogenicity, based on limited evidence of carcinogenicity in rats, or be not classified for this endpoint, based on the tumour findings being explained as no relevance to humans.

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. However, for pyriofenone this mode of action has not been definitively established. A cytotoxic mode of action is also plausible but, again, definitive evidence is lacking. There appears to be no other plausible mechanisms of action.

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

(a) The evidence is limited to a single experiment

(b) There are unresolved questions about the interpretation of the study results

It is possible, on the basis of the total weight of evidence, that the overall likelihood that pyriofenone poses a carcinogenic hazard to humans is low. However, as discussed above, there is currently insufficient evidence to underpin the proposed species-specific mechanism of action. This provides sufficient uncertainty to support a category 2 classification for this endpoint.

10.9.6 Conclusion on classification and labelling for carcinogenicity

Category 2 carcinogen; H351 – Suspected of causing cancer

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.

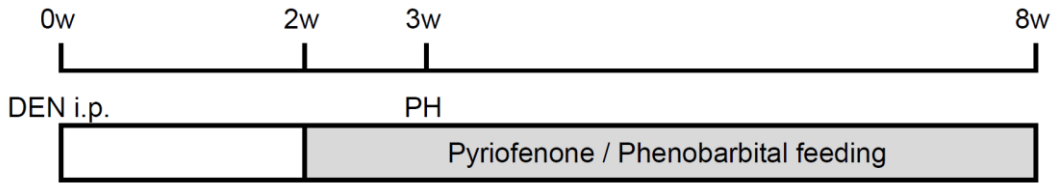


Figure: *Design of the dosing schedule for the medium term carcinogenesis bioassay.*

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	4 (8%)	1 (2%)	2 (4%)	6 (12%)	0-4%
Hepatocellular carcinoma	0	1 (2%)	1 (2%)	2 (4%)	0%
Combined total	4 (8.0%)	2 (4.0%)	3 (6.0%)	8 (16%)	
Females:					
Number examined	50	50	50	50	
Liver: Hepatocellular adenoma	0	0	0	1 (2%)	
Hepatocellular carcinoma	0	0	0	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.

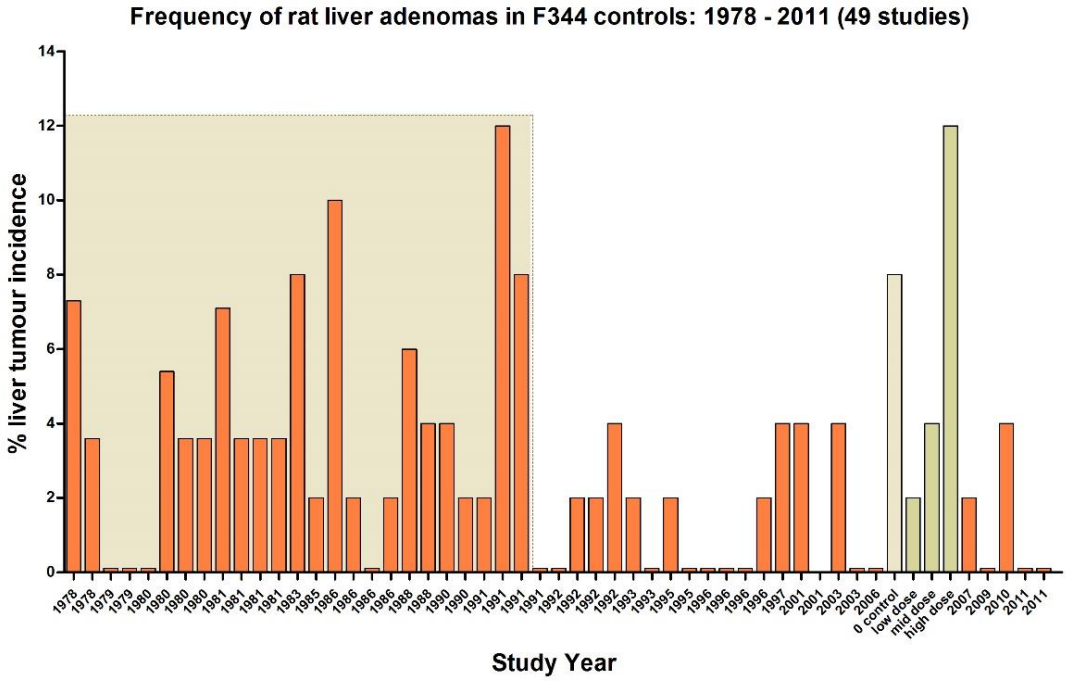


Figure: *Individual study incidences of spontaneous liver adenoma in male F344 rats from IET occurring from 1978 to 2011.*

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.

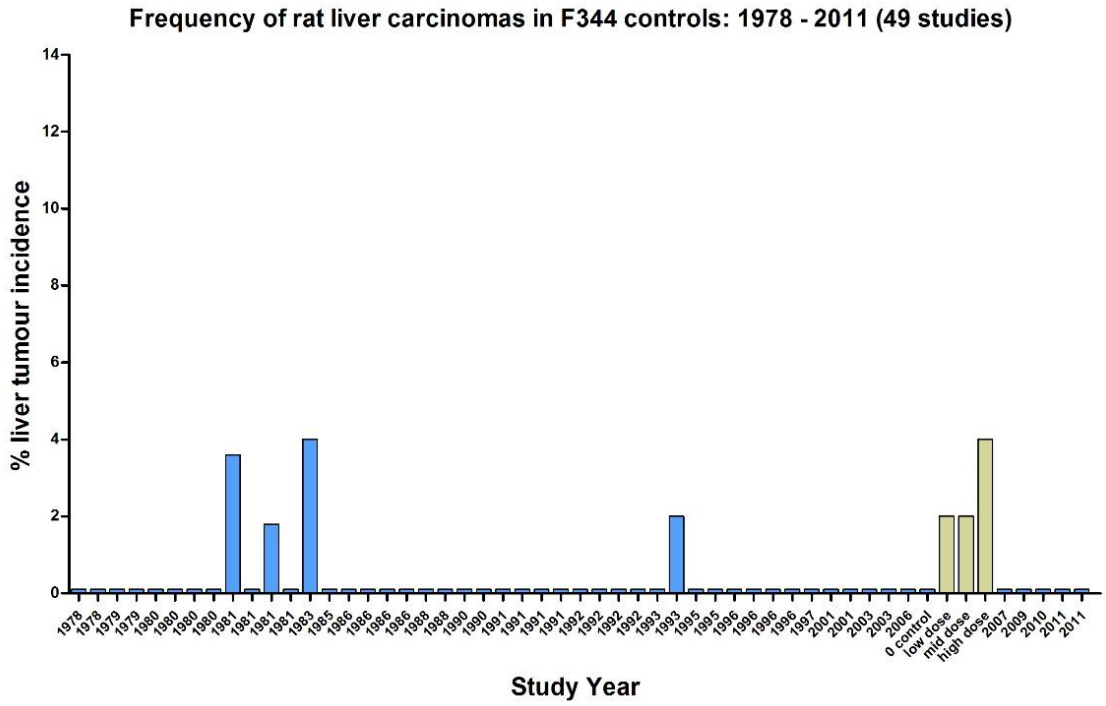


Figure: *Individual study incidences of spontaneous liver adenoma in male F344 rats from IET occurring from 1978 to 2011.*

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.

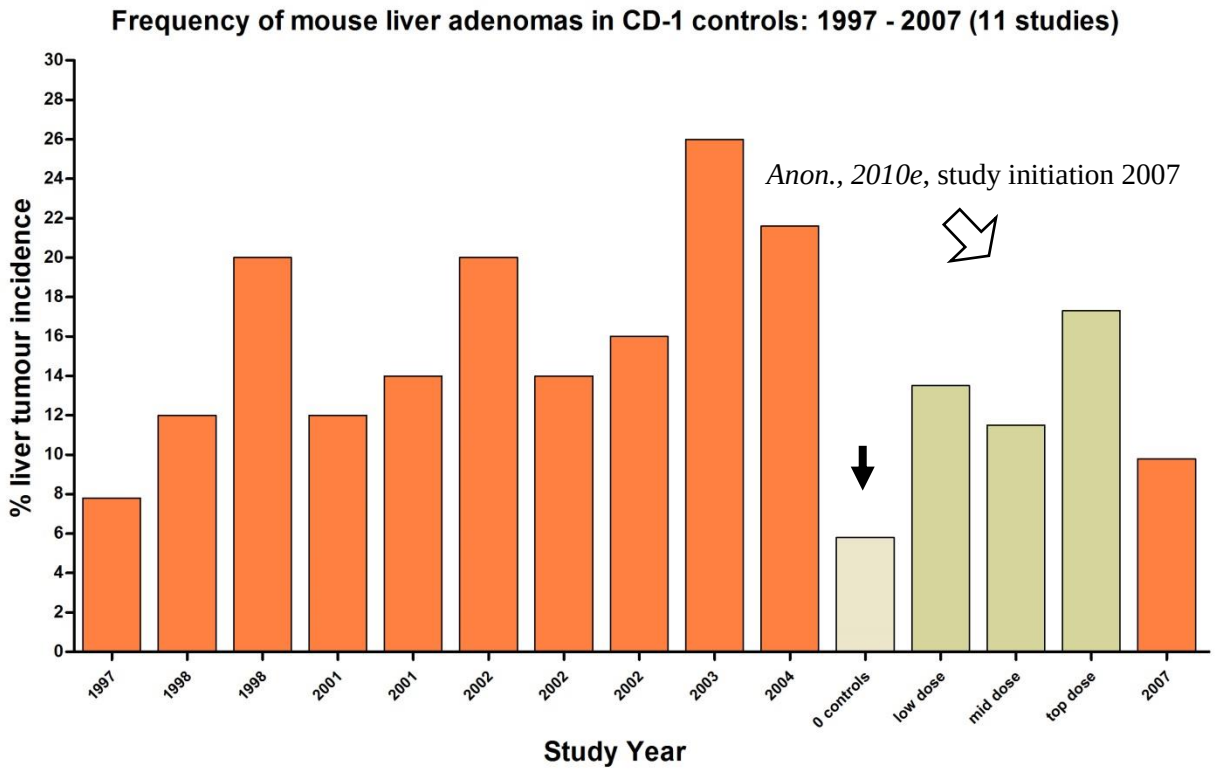


Figure: *Adenoma incidence rate in male CD-1 mice. Arrow head = concurrent control group*

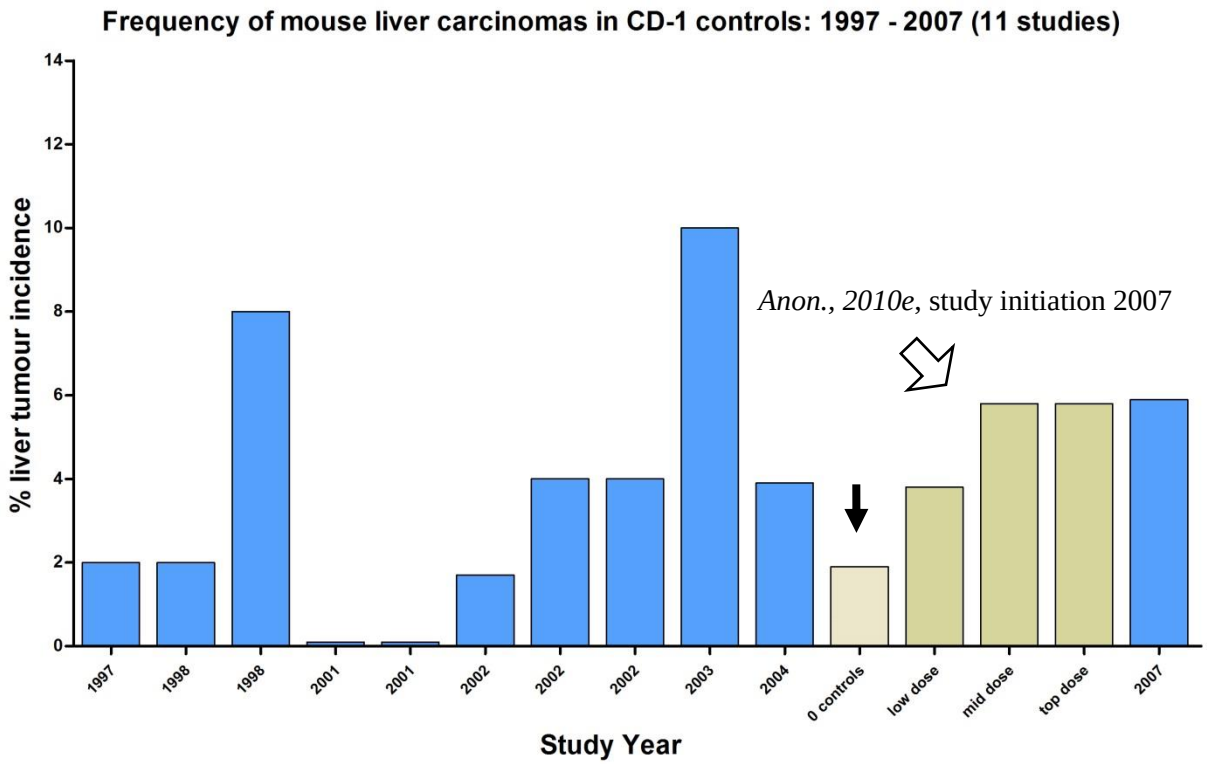


Figure: *Carcinoma incidence rate in male CD-1 mice. Arrow head = concurrent control group*

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.

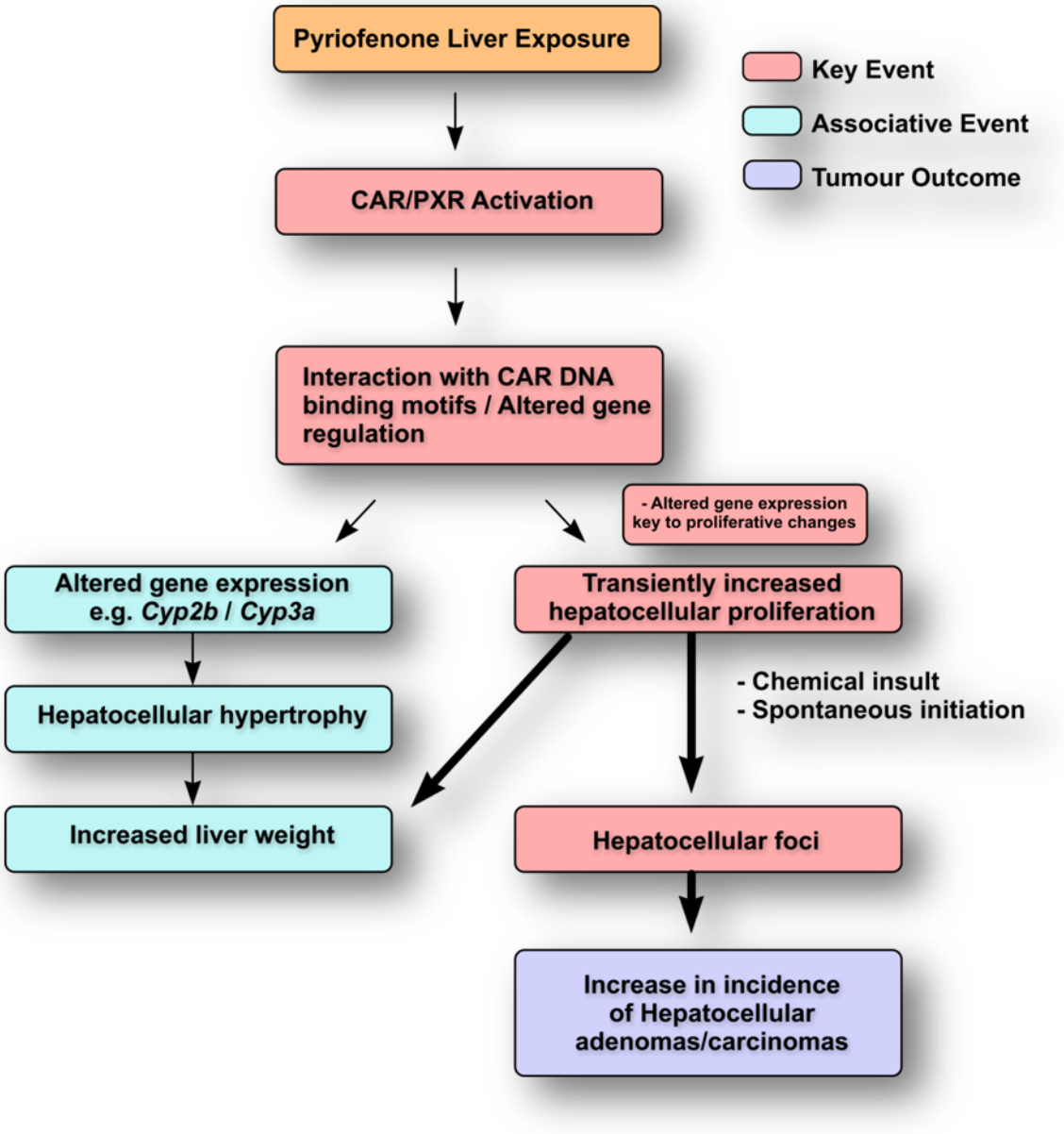


Figure: *Mode of Action hypothesis for pyriofenone-induced liver tumour formation in male rats.*

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).**

10.10 Reproductive toxicity

10.10.1 Adverse effects on sexual function and fertility

Table 26: Summary table of animal studies on adverse effects on sexual function and fertility

↑↓ denote an increase or decrease in a parameter with respect to the control value
Statistical significance: * $p \leq 0.05$, ** $p \leq 0.01$
abs. = absolute
rel. = relative

Method, guideline, deviations if any, species, strain, sex, no/group	**Test substance, dose levels duration of exposure**	**Results**
Multi-generation toxicity study in rats (Dose-range finding study) OECD 416 GLP Rats, Wistar 8/sex/group DAR: B.6.6.1a (Anon., 2009d)	IKF-309 technical Purity 97.88 % 0, 300, 3000, 10000 or 20000 ppm Equivalent to: ♂ 17.9, 185, 591 and 1159 mg/kg bw/day ♀ 31.9, 328, 1004 and 1828 mg/kg bw/day	***General toxicity:*** ***Parents*** **20000 ppm (1159/1828 mg/kg bw/day):** ↓Bodyweight gain: 21 %* in males at week 10, 10 % in females during weeks 0-3 (pre-mating) and 24 % during weeks 14-20 (gestation) ↓ Food consumption: 13 %** during weeks 0-3, 17 % during weeks 14-20 and 20 %* during weeks 14-21 (lactation) in females ***Organ Weights:*** ↑ Liver weight: 36 %** (abs.) and 50 %** (rel.) in males and 80 %** (abs.) and 88 %** (rel.) in females ↑ Kidneys weight: 27 %** (abs.) and 39 %** (rel.) in males and 11 %** (rel.) in females ↑ Caecum weight: 2.4 fold** (abs.) and 1.6 fold** (rel.) in males and 1.9 fold* (abs. and rel.) in females ↓ Ovaries weights: 38 %** (abs.) and 33 %** (rel.) ***Histopathology:*** **Liver** Diffuse hepatocyte hypertrophy:8/8*** males and 8/8*** females Enlargement: 8/8*** males and 8/8*** females (versus 0 in controls) Dark colour: 8/8*** males and 5/8 females (versus 0 in controls) **Kidney** Increased hyaline droplet deposition in proximal tubule cells: 5/8* males ***Clinical Chemistry:*** ↑ γ-Glutamyl transpeptidase 4-fold** in males, 14-fold** in females ↓ Creatinine 18 %** in males, 24 %** in females ↓ Glucose 16 %** in males, 17 %** in females ↑ Total Cholesterol 77 %** in females ↓ Total bilirubin 63 %** in females Haematology: ↓ Haemoglobin 12 %** in females, (5 %** in males) ↓ Haematocrit 11 %** in females, (4 %* in males) ↑ Platelet count 40 %** in males, 55 %** in females

Method, guideline, deviations if any, species, strain, sex, no/group	**Test substance, dose levels duration of exposure**	**Results**
		<u>10000 ppm (591/1004 mg/kg bw/day):</u> ***Organ Weights:*** ↑ Liver weight: 27 %** (abs.) and 30 %** (rel.) in males and 62 %** (abs. and rel.) in females ↑ Kidneys weight: 26 %** (abs.) and 29 %** (rel.) in males ↑ Caecum weight: 2.2 fold** (abs. and rel.) in males and females ↓ Ovaries weights: 24 %** (abs.) and 23 %** (rel.) ***Histopathology:*** **Liver** Diffuse hepatocyte hypertrophy:8/8*** males and 8/8*** females (versus 0 in controls) Enlargement: 6/8** males and 8/8*** females (versus 0 in controls) Dark colour: 8/8*** males and 4/8* females (versus 0 in controls) **Kidney** Increased hyaline droplet deposition in proximal tubule cells: 5/8* males (versus 0 in controls) ***Clinical Chemistry:*** ↑ γ-Glutamyl transpeptidase 2-fold** in males, 5-fold** in females ↓ Creatinine 15 %** in males, 18 %** in females ↓ Glucose 15 %** in females ↑ Total Cholesterol 48 %** in females ↓ Total bilirubin 43 %** in females ***Haematology:*** ↓ Haemoglobin 10 %** in females ↓ Haematocrit 10 %** in females ↑ Platelet count 28 %** in males, 25 %* in females **<u>3000 ppm (185/328 mg/kg bw/day);</u>** ***Organ Weights:*** ↑ Liver weight: 13 %* (abs.) and 12 %** (rel.) in males and 24 %* (abs.) and 22 % (rel.) in females ↑ Kidneys weight: 17 %** (abs.) and 15 %** (rel.) in males ↑ Caecum weight: 1.7-fold (abs. and rel.) in males and 1.6-fold* (abs. and rel.) in

Method, guideline, deviations if any, species, strain, sex, no/group	**Test substance, dose levels duration of exposure**	**Results**
		females ***Clinical Chemistry:*** ↑ Total Cholesterol 35 %* in females ↓ Total bilirubin 43 %** in females **300 ppm (17.9/31.9 mg/kg bw/day):** No treatment-related findings ***F_1 generation:*** **20000 ppm (1159/1828 mg/kg bw/day):** ↓Bodyweight [25-26 days of age]: 36 %** in males and 35 %** in females ***Organ Weights:*** ↓ Brain weight: 10 %** (abs.) and 41 %** (rel.) in males and 10 %** (abs.) and 38** % (rel.) in females ↓ Spleen weight: 54 %** (abs.) and 30 %** (rel.) in males and 60 %** (abs.) and 37 %** (rel.) in females ↓ Thymus weight: 44 %** (abs.) and 14 % (rel.) in males and 47 %** (abs.) and 19 % (rel.) in females ↓ Uterus weight: 16 % (abs.) and 27 %* (rel.) **10000 ppm (591/1004 mg/kg bw/day):** ↓Bodyweight [25-26 days of age]: 21 %** in males and 12 %** in females ***Organ Weights:*** ↓ Brain weight: 22 %** (rel.) in males ↓ Spleen weight: 35 %** (abs.) and 18 %* (rel.) in males and 23 %** (abs.) (rel.) in females ↓ Thymus weight: 25 %** (abs.) in males and 424 %** (abs.) and 13 % (rel.) in females **≤ 3000 ppm (185/328 mg/kg bw/day):** No treatment-related findings ***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 F_1 generation.

<table>
<tr><th>Method, guideline, deviations if any, species, strain, sex, no/group</th><th>Test substance, dose levels duration of exposure</th><th>Results</th></tr>
<tr><td></td><td></td><td><u>Parents</u>
<u>20000 ppm (1159/1828 mg/kg bw/day):</u>
1/8 females was non-pregnant
1/8 females lost the entire litter by lactation Day 4

<u>≤ 10000 ppm (591/1004 mg/kg bw/day:</u>
No treatment-related findings

<u>F₁ generation:</u>
No effects observed at any doses

NOAELs were not set for this study.</td></tr>
<tr><td>Two-generation reproduction toxicity study in rats
OECD 416
GLP
Rats, Wistar
(24/sex/dose)

DAR: B.6.6.1b

(Anon., 2009c)</td><td>IKF-309 technical
Purity 97.88 %
0, 150, 1000 or 5000 ppm</td><td>There were no effects on reproductive indices or on the development of offspring in this study.

Mean intake of test material:

<table>
<tr><td></td><td>Parental Males</td><td colspan="3">Parental Females</td></tr>
<tr><td>Concentration (ppm)</td><td>Pre-mating (weeks 0-10)</td><td>Pre-mating (weeks 0-10)</td><td>Gestation (Days 0-20)</td><td>Lactation (Days 1-14)</td></tr>
<tr><td>150</td><td>9.61</td><td>11.9</td><td>9.3</td><td>21.2</td></tr>
<tr><td>1000</td><td>64.1</td><td>79.2</td><td>62</td><td>138.1</td></tr>
<tr><td>5000</td><td>334</td><td>395</td><td>307</td><td>677</td></tr>
<tr><td></td><td>F1 males</td><td colspan="3">F1 Females</td></tr>
<tr><td>150</td><td>11.4</td><td>13</td><td>9.6</td><td>20.6</td></tr>
<tr><td>1000</td><td>76.8</td><td>84.4</td><td>61.6</td><td>130</td></tr>
<tr><td>5000</td><td>393</td><td>434</td><td>321</td><td>709</td></tr>
</table>

<u>General toxicity:</u>
<u>Parents:</u>
<u>5000 ppm (334/307-677 mg/kg bw/day):</u>
Organ Weights:
↑ Liver weight: 27 %** (abs.) and 29 %** (rel.) in males and 42 %** (abs.) and 38 %** (rel.) in females</td></tr>
</table>

Method, guideline, deviations if any, species, strain, sex, no/group	**Test substance, dose levels duration of exposure**	**Results**
		↑ Kidneys weight: 22 %** (abs.) and 24 %** (rel.) in males and 13 %** (abs.) and 10 %** (rel.) in females ↑ Thyroids weight: 21 %** (abs.) and 23 %** (rel.) in males ↑ Caecum weight: 1.8 fold** (abs. and rel.) in males and 1.9 fold** (abs. and rel.) in females ***Histopathology:*** **Liver** Diffuse hepatocyte hypertrophy:13/23** males and 20/23** females (versus 0 in controls) Brown deposition in Glisson's capsule: 13/23** males (versus 0 in controls) Dark colour: 14/23** males and 20/23** females (versus 0 in controls) **Kidney** Increased hyaline droplet deposition in proximal tubule cells: 11/23** males (versus 0 in controls) **Thyroid** Follicular cell hypertrophy: 14/23** females (versus 3/21 in controls) ***Haematology:*** ↓ Haemoglobin 10 %** in females ↓ Haematocrit 9 %* in females ↑ Platelet count 11 % in females ↓ Lymphocyte count 20 %* in females ↓ Basophil count 67 %** in females **1000 ppm (64.1/62-138.1 mg/kg bw/day):** ***Organ Weights:*** ↑ Liver weight: 11 %** (abs.) and 9 %** (rel.) in females **150 ppm (9.6/9.3-21.2 mg/kg bw/day):** No treatment-related findings. ***F_1 generation:*** **5000 ppm (393/321-709 mg/kg bw/day):** ***Organ Weights:*** ↑ Liver weight: 31 %** (abs.) and 27 %** (rel.) in males and 56 %** (abs.) and 48 %** (rel.) in females ↑ Kidneys weight: 24 %** (abs.) and 21 %** (rel.) in males and 18 %** (abs.) and 10

Method, guideline, deviations if any, species, strain, sex, no/group	**Test substance, dose levels duration of exposure**	**Results**
		%** (rel.) in females ↑ Caecum weight: 1.9 fold** (abs. and rel.) in males and 1.5 fold** (abs. and rel.) in females ***Histopathology:*** **Liver** Diffuse hepatocyte hypertrophy:22/24** males and 22/24** females (versus 0 in controls) Brown deposition in Glisson's capsule: 8/24** males (versus 0 in controls) **Kidney** Increased hyaline droplet deposition in proximal tubule cells: 11/24** males (versus 0 in controls) **Thyroid** Follicular cell hypertrophy: 19/24** males (versus 5/22 in controls) and 16/23** females (versus 5/22 in controls) **Caecum** Distension: 18/24** males and 6/23* females (versus 0 in controls) ***Haematology:*** ↑ Platelet count 18 %** in females NOAEL general toxicity (parental):150 ppm (9.6/9.3-21.2 mg/kg bw/day) NOAEL reproductive toxicity: > 5000 ppm (> 334/307-677 mg/kg bw/day) NOAEL general toxicity (F_1 generation): 1000 ppm (76.8/61.6-130 mg/kg bw/day)

10.10.2 Short summary and overall relevance of the provided information on adverse effects on sexual function and fertility

Pyriofenone was tested in a preliminary dose range-finding study and a definitive two-generation study in rats in order to assess its effects on sexual function and fertility.

Preliminary Study

In the preliminary study, male and female Wistar rats (8/sex/group) were administered pyriofenone in their diet at concentrations of 0, 300, 10000 or 20000 ppm for three weeks prior to mating, throughout gestation and lactation until weaning of the F_1 offspring (doses were equivalent to 17.9/31.9, 185/328, 591/1004 and 1159/1828 mg/kg bw/day males/females).

No 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 with reduced food consumption during weeks 0-3, 14-20 and during the lactation period, days 14-21.

In the parental generation, the main organs affected in this study were the liver, kidneys and caecum. From a dose of 3000 ppm (185/328 mg/kg bw/day) the weights of these organs were increased, dose-dependently. In males and females of the top dose group, absolute and relative liver weights were increased by 36 and 50 % in males and 80 and 88 % in females compared to controls (absolute and relative weights respectively). Histopathology revealed diffuse hepatocyte hypertrophy and enlargement in all males and females of the 10000 ppm and 20000 ppm dose groups.

All males of the top two dose groups had darkened livers as did 5/8 females of the 20000 ppm group and 4/8 females of the 10000 ppm dose group. Kidney weight was increased in males of the top dose group by 27 and 36 %, compared to controls, (absolute and relative respectively) with increased hyaline droplet deposition in proximal tubule cells (5/8 males) whilst females were shown to be slightly less sensitive with a 11 % relative increase in weight when treated with 20000 ppm pyriofenone only. The large intestine was affected, with the weight of the caecum increasing approximately 2-fold in both males and females of the top two dose groups.

Females treated with 20000 ppm and 10000 ppm were also found to have a decrease in ovaries weight (20000 ppm: 38 % absolute and 33 % relative; 10000 ppm: 24 % absolute and 23 % relative). In the absence of any associated histopathology or effects to sexual function, this decrease in weight was likely to be associated with the decrease in body weight gain.

Changes in clinical chemistry were consistent with the toxicity occurring in the liver and kidney. In females of the top dose and those dosed with 10000 ppm, there were decreases to haemoglobin and haematocrit levels. Both males and females dosed with 20000 and 10000 ppm had elevated platelet counts.

In the F_1 generation, body weight was reduced in males and females treated with doses ≥ 10000 ppm (20000 ppm: 36 % in males and 35 % in females, 10000 ppm: 21 % in males and 12 % in females). Organs affected following treatment with pyriofenone were the brain, spleen, thymus and uterus with dose-dependent reductions in weight observed from a dose of 10000 ppm. There was no associated histopathology with the organ weight changes and in the absence of such, they are considered to be a consequence of generalised toxicity and the reduction in body weight.

There were no treatment-related effects to oestrous cycle, mating index, fertility index, gestation index, the duration of gestation or the number of implantations. There was also no effect on the development of the offspring. At the top dose of 20000 ppm, one female was non-pregnant and one female lost her entire litter by lactation Day 4. The cause was likely due to ill-health; however no firm conclusions can be drawn from this study due to the low numbers of animals used and the decreased pre-mating period.

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 [approximately 10-20, 70-130, 320 – 700 mg/kg bw/day (exact doses in mg/kg bw/day can be found in Table 26, depending on sex and study period)]. There were no treatment-related mortalities, clinical findings or effects on body weight or body weight gain in P or F_1 parental animals.

In P and F_1 parental animals the main organs affected following treatment with pyriofenone were the liver, kidneys, thyroids and caecum.

In the P generation, liver weight was increased from a dose of 1000 ppm (64.1/62-138.1 mg/kg bw/day) in males and females. At the top dose of 5000 ppm (334/307-677 mg/kg bw/day) the increased liver weight [27 % and 29 % in males and 42 % and 38 % in females (absolute and relative respectively) compared to controls] was accompanied by diffuse hepatocyte hypertrophy (13/23 males and 20/23 females versus 0 in controls), brown deposition in the Glisson's capsule in males (13/23 males versus 0 in controls) and dark colouring (14/23 males and 20/23 females).

Kidney weight (absolute and relative) was increased in males and females of the top dose by up to 24 % and in males, this was accompanied by increased hyaline droplet deposition in proximal tubule cells (11/23 males versus 0 in controls).

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), indicating that whilst the increase in thyroid weight might be due to stress and general toxicity, the hypertrophy was more likely to be due to the effects of the ingested substance.

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 of the top dose and the female platelet count was increased (11 % compared to controls). Changes to white blood cell parameters were noted, with a decrease in lymphocyte count and a decrease in the basophil count in females of the top dose.

Males and females of the top dose of the F_1 parental generation also had increased liver weights [31 % and 27 % in males and 56 % and 48 % in females (absolute and relative respectively)] with accompanying diffuse hepatocyte hypertrophy (22/24 males and females, versus 0 in controls) and brown deposition in the Glisson's capsule in males only (8/24 males versus 0 in controls).

Kidney weight was also increased in both sexes [24 % and 21 % in males and 18 % and 10 % in females (absolute and relative)]. In males, increased hyaline droplet deposition in proximal tubule cells was also noted (11/24 males versus 0 in controls).

The caecum weight was increased in both males and females (1.5 – 1.9 fold) and distension was noted (18/24 males and 6/23 females, versus 0 in controls).

Follicular cell hypertrophy of the thyroid was also noted in both males and females (19/24 males and 16/23 females versus 5/22 in controls) and haematology revealed an 18 % increase in platelet count in females compared to controls.

There were no treatment-related changes to sexual function or fertility in males or females in both the P and F_1 generations. There were no developmental adverse effects to F_1 or F_2 offspring.

The results indicate that pyriofenone does not cause adverse effects to sexual function or fertility in rats.

10.10.3 Comparison with the CLP criteria

Pyriofenone has been well-tested in a guideline-compliant two-generation study in Wistar rats. Further information has also been 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. At the top dose of 20000 ppm in the preliminary study, one female failed to become pregnant and another lost all of her litter by lactation Day 4, however these effects occurred only at a dose that also resulted in marked parental toxicity (decreased food consumption and body weight gain and significant changes to liver, kidney and caecum weight with associated histopathogical adverse effects).

Pyriofenone is not known as a human reproductive toxicant; therefore classification in Category 1A is not necessary.

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 in Category 1B is not appropriate.

Classification in Category 2 is reserved for substances where there is some evidence from human or experimental animals of an adverse effect on sexual function and fertility. Such effects should be observed in the absence of other toxic effects. 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 no classification for this endpoint is required.

10.10.4 Adverse effects on development

Table 27: Summary table of animal studies on adverse effects on development

↑↓ denote an increase or decrease in a parameter with respect to the control value
Statistical significance: * $p \leq 0.05$, ** $p \leq 0.01$
abs. = absolute
rel. = relative

<table>
<tr><th>Method, guideline, deviations if any, species, strain, sex, no/group</th><th>Test substance, dose levels duration of exposure</th><th>Results</th></tr>
<tr><td>Teratogenicity study in rats
(Dose-range finding study)
OECD 414
GLP
Rats, Wistar
(8 females/ group)

DAR: B.6.6.2a

(Anon., 2009f)</td><td>IKF-309 technical
Purity 97.88 %
0, 30, 100, 300 or 1000 mg/kg bw/day
In aq. Carboxymethyl cellulose (1 %)</td><td><u>Maternal effects</u>
<u>1000 mg/kg bw/day):</u>
↑ Caecum weight: 23%* (rel.)

There were no other treatment-related findings or effects on pregnancy or fetal abnormalities.

NOAELs were not set for this study.</td></tr>
<tr><td>Teratogenicity study in rats
OECD 414
GLP
Rats, Wistar
(24 females/ group)

DAR: B.6.6.2b

(Anon., 2009e)</td><td>IKF-309 technical
Purity 97.88 %
0, 30, 300 or 1000 mg/kg bw/day
In aq. Carboxymethyl cellulose (1 %)</td><td><u>Maternal effects</u>
<u>1000 mg/kg bw/day):</u>
↑ Liver weight: 16%** (abs.) and 14 %** (rel.)
↑ Caecum weight: 1.3 fold** (abs. and rel.)

There were no treatment-related effects on pregnancy.
<u>Foetal effects</u>
There were no treatment-related increases in malformations observed in this study. However, a number of variations were observed as shown in the table below:
<table>
<tr><td>Dose mg/kg bw/day</td><td></td><td>0</td><td>30</td><td>300</td><td>1000</td><td>HCD range</td></tr>
<tr><td></td><td></td><td colspan="5"></td></tr>
<tr><td>No. foetuses [litters]</td><td></td><td>284 [24]</td><td>290 [24]</td><td>284 [24]</td><td>301 [24]</td><td></td></tr>
<tr><td>No. of litters with variations</td><td></td><td>24</td><td>23</td><td>23</td><td>23</td><td></td></tr>
<tr><td>No. of foetuses with variations:</td><td>Visceral</td><td>30</td><td>29</td><td>35</td><td>18*</td><td></td></tr>
</table>
</td></tr>
</table>

<table>
<tr><th>Method, guideline, deviations if any, species, strain, sex, no/group</th><th>Test substance, dose levels duration of exposure</th><th>Results</th></tr>
<tr><td></td><td></td><td>
<table>
<tr><td></td><td></td><td>Skeletal</td><td>84</td><td>83</td><td>105*</td><td>121**</td><td></td></tr>
<tr><td colspan="8"></td></tr>
<tr><td rowspan="2">Discontinuous rib cartilage</td><td>Foetuses</td><td>38 (25.5 %)</td><td>45 (54.2 %)</td><td>47 (31.8 %)</td><td>62 (39.5 %)*</td><td>29.7 - 44.8 %</td></tr>
<tr><td>Litters</td><td>15 (62.5 %)</td><td>17 (70.8 %)</td><td>20 (83.3 %)</td><td>23 (95.8 %)*</td><td></td></tr>
<tr><td rowspan="2">Supernumeracy ribs:</td><td>Foetuses</td><td>64 (43.0 %)</td><td>51 (34.0 %)</td><td>85 (57.4 %)*</td><td>98 (62.4 **)</td><td>35.7 - 61.0 %</td></tr>
<tr><td>Litters</td><td>21 (87.5 %)</td><td>18 (75.0 %)</td><td>23 (95.8 %)</td><td>22 (91.7 %)</td><td></td></tr>
<tr><td rowspan="2">Full supernumerary ribs:</td><td>Foetuses</td><td>5 (3.4 %)</td><td>3 (2.0 %)</td><td>10 (6.8 %)</td><td>14 (8.9 %)#</td><td>5.6 - 6.8 %</td></tr>
<tr><td>Litters</td><td>3 (12.5 %)</td><td>3 (12.5 %)</td><td>7 (29.2 %)</td><td>7 (29.2 %)</td><td></td></tr>
</table>
statistically significant (Fischers test p = 0.03)

NOAEL general toxicity: 300 mg/kg bw/day
NOAEL developmental toxicity: 30 mg/kg bw/day
NOAEL teratogenicity: 1000 mg/kg bw/day</td></tr>
<tr><td>Teratogenicity study in rabbits
(Dose-range finding study)
OECD 414
GLP
Rabbits, Japanese White
(8 females/ group)

DAR: B.6.6.3a

(Anon., 2009g)</td><td>IKF-309 technical
Purity 97.88 %
0, 30, 100, 300 or 1000 mg/kg bw/day

In aq. Carboxymethyl cellulose (1 %)</td><td><u>Maternal effects:</u>
<u>1000 mg/kg bw/day):</u>
General toxicity
↓ Body weight gain: 10 %
↑ Liver weight: 27% (abs.) and 35 % (rel.)
Pregnancy
No. of abortions/premature deliveries: 4/8 (versus 0 in controls)
Resorptions and foetal deaths: 20.1 % (versus 7.4 % in controls)

<u>300 mg/kg bw/day):</u>
General toxicity
Body weight loss: -130 g (versus gain of 280 g in control group)
Pregnancy
Resorptions and foetal deaths: 24.1 % (versus 7.4 % in controls)

<u>100 mg/kg bw/day):</u>
Pregnancy
Resorptions and foetal deaths: 21.4 % (versus 7.4 % in controls)</td></tr>
</table>

Method, guideline, deviations if any, species, strain, sex, no/group	**Test substance, dose levels duration of exposure**	**Results**
		Foetal effects: There were no toxicologically relevant findings at any doses. NOAELs were not set for this study.
Teratogenicity study in rabbits OECD 414 GLP Rabbits, Japanese White (25 females/ group) DAR: B.6.6.3b (Anon., 2009h)	IKF-309 technical Purity 97.88 % 0, 30, 100 or 300 mg/kg bw/day In aq. Carboxymethyl cellulose (1 %)	There were no effects on body weight or body weight gain in any treated groups. **Maternal effects:** **300 mg/kg bw/day:** ***Pregnancy*** No. of abortions/premature deliveries: 2/25 (versus 0 in controls) **≤100 mg/kg bw/day:** No treatment-related findings **Foetal effects:** There were no toxicologically relevant findings at any doses (including the frequency of resorptions and foetal deaths). NOAEL general toxicity: 100 mg/kg bw/day NOAEL developmental toxicity:300 mg/kg bw/day NOAEL teratogenicity: 300 mg/kg bw/day

10.10.5 Short summary and overall relevance of the provided information on adverse effects on development

The effects of pyriofenone on development following exposure during pregnancy have been well tested in a preliminary (dose range-finding) teratogenicity study, in rats and rabbits and a definitive teratogenicity study, also in rats and rabbits.

Studies in rats

In a preliminary teratogenicity study, female Wistar rats received pyriofenone in aqueous sodium carboxymethylcellulose (1 %) by oral gavage (8/group). Doses used were 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). There were no other treatment-related findings, effects on pregnancy or foetal abnormalities in any dose group.

In the main, guideline-compliant, teratogenicity study, pyriofenone was administered to time-mated female Wistar rats, (24/group), by oral gavage at 0, 30, 300 or 1000 mg/kg bw/day. 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. 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. These were an increase in incidence of supernumaracy of ribs and discontinuous rib cartilage. At 300 mg/kg bw/day the number of foetuses affected was 105/284 and at 1000 mg/kg bw/day the number was 121/301 (versus 84/284 in controls). At 1000 mg/kg bw/day the percentage of foetuses with discontinuous rib cartilage was statistically significantly increased to 39.5 % (versus 25.5 % in controls). Whilst this value is above the concurrent control value for this variation, it was within the laboratory historical control data (HCD) provided: range 29.7 – 44.8 %. At the same dose there was a statistically significant increase in the foetuses with supernumaracy ribs (62.4 % versus 43 % in controls). At 300 mg/kg bw/day the percentage of foetuses affected was also increased (57.4 %), however, the value was within the range of the given HCD (35.7 – 61.0 %). At 1000 mg/kg bw/day the percentage of foetuses affected with supernumaracy ribs was above the concurrent control and also above the HCD. Given that the increase occurred in a dose-dependent manner and is above the HCD, it could be considered treatment-related.

Following administration of pyriofenone to rats, an increase in skeletal variations, including an increased incidence of supernumaracy ribs noted in top-dose animals. This increase was above the concurrent control and above the laboratory historical control data.

Studies in rabbits

A preliminary teratogenicity study was carried out in Japanese White rabbits in order to determine dose levels for the main experiment. In this study, artificially inseminated females (8/group) were given an oral dose of pyriofenone (by gavage) at 0, 30, 100, 300 or 1000 mg/kg bw/day suspended in aqueous sodium carboxymethyl cellulose (1 %). There were no maternal deaths during this study. At doses of 300 mg/kg bw/day and above, body weight gain was affected with reduced body weight gain of 10 % in dams treated with 1000 mg/kg bw/day and body weight loss occurring in dams treated with 300mg/kg bw/day. It was noted that six animals in the top dose group had ceased eating during the study and three animals dosed with 300 mg/kg bw/day. Of the six animals in the top dose group that had ceased eating, 4 aborted or delivered prematurely. At the top dose, liver weight was also increased, 27 % (absolute) and 35 % (relative) in comparison to controls. This was accompanied by a marginal increase in the incidence of fatty change (8/8 animals versus 6/8 in controls), however the extent of the fatty change was found to be more severe in the livers of animals dosed with 1000 mg/kg bw/day. From a dose of 100 mg/kg bw/day and above, the percentage of resorptions and foetal deaths appeared to be higher than controls, however, with such small animal numbers no statistics were performed and no definitive conclusions can be drawn as to whether this was treatment-related. There were no treatment-related increases in the number of variations or malformations in foetuses of this study.

In the main teratogenicity study, artificially inseminated female Japanese White rabbits (25/group) were orally gavaged with pyriofenone suspended in aqueous sodium carboxymethylcellulose (1 %) at concentrations of 0, 30, 100 or 300 mg/kg bw/day (as determined from the preliminary study) on days 6 - 27 of gestation. Two females from the top dose group were sacrificed on gestation day 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 are considered to have occurred by chance and not related to treatment with the test substance. 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.

Pyriofenone did not appear to be a developmental toxicant in rabbits.

10.10.6 Comparison with the CLP criteria

Developmental toxicity has been assessed in both rats and in rabbits.

Classification in Category 1A for effects on development is required when a substance is a known human reproductive toxicant. Pyriofenone is not a known human reproductive toxicant, therefore, classification in this category is not required.

In order to be classified in Category 1B, there must be clear evidence from animal studies of an adverse effect on development occurring in the absence of other toxic effects. As there was no clear evidence to suggest pyriofenone should be a presumed human reproductive toxicant, classification in this category is not appropriate.

Classification in Category 2 is required if a substance is considered a suspected human reproductive toxicant. In a guideline-compliant study in rats, there was an increased incidence in the number of skeletal variations (supernumeracy ribs) in animals of the top dose group that was marginally above the laboratory control data. This finding is considered a variation and not a malformation as the growth of supernumeracy 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. Therefore, they cannot represent a permanent structural change. This finding was not observed in the dose-range finding study carried out in advance of the main study. There were no adverse findings on foetal development in rabbits. Overall, it can be concluded that pyriofenone is not a developmental toxicant. Classification for this endpoint is not required.

10.10.7 Adverse effects on or via lactation

10.10.8 Short summary and overall relevance of the provided information on effects on or via lactation

There were no adverse effects on or *via* lactation observed in the two-generation studies in rats or the teratogenicity studies in rats and rabbits.

10.10.9 Comparison with the CLP criteria

There was no evidence to suggest that pyriofenone had an adverse effect on lactation or via lactation, therefore classification with this endpoint is not appropriate.

10.10.10 Conclusion on classification and labelling for reproductive toxicity

Not classified. Data conclusive but not sufficient for classification.

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 No of pups: Found dead:	21 168 1	20 160 0	23 176 0	23 181 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	22	24	22	24
	No of pups:	255	251	217	257
	Found dead:	5	0	1	0
Lactation days 1-4:	No of litters examined	22	24	22	24
	No of pups:	250	251	216	257
	Found dead:	0	0	0	1
	Lost:	2	1	0	1
Lactation days 5-21:	No of litters examined	22	24	22	23
	No of pups:	171	181	156	180
	Found dead:	0	0	0	0
	Lost:	1	0	0	0
Male pups weights (g): (litter mean & SD)	Lactation day 0	6.0 (0.5)	6.1 (0.5)	6.0 (0.6)	6.0 (0.5)
	Lactation day 21	55.0 (4.0)	55.3 (4.8)	54.6 (4.9)	53.1 (3.3)
Female pups weights (g): (litter mean & SD)	Lactation day 0	5.7 (0.5)	5.8 (0.6)	5.7 (0.5)	5.9 (0.7)
	Lactation day 21	52.7 (3.8)	53.2 (4.4)	52.8 (3.9)	51.8 (3.2)
Gross findings:	No of litter examined	22	24	22	23
	No of pups:	170	181	156	180
	No of pups without abnormalities:	164	167	141	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

* $p \leq 0.05$. 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**.

10.11 Specific target organ toxicity-single exposure

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

Pyriofenone has been well investigated in a number of acute studies by the oral, dermal and inhalation routes using the limit dose of 2000 mg/kg. In these studies there were few clinical signs and no macroscopic abnormalities. Therefore, there is no evidence of any effects that might be concluded to be due to specific target organ toxicity.

10.11.2 Comparison with the CLP criteria

Pyriofenone did not cause specific organ toxicity following a single exposure in rats by the oral, inhalation or dermal routes. There are no reports of specific target organ toxicity following a single exposure in humans either. Therefore, pyriofenone should not be classified for this endpoint.

10.11.3 Conclusion on classification and labelling for STOT SE

Not classified. Data conclusive but not sufficient for classification.

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.

10.12 Specific target organ toxicity-repeated exposure

The specific target organ toxicity of pyriofenone upon repeated exposure has been investigated in 28-day and 90-day studies in rats, mice and dogs, in one-year studies in rats and dogs. Additional information is provided by chronic/carcinogenicity studies in rats and mice, which are reported in section 10.9 (Table 19) and from a two-generation reproduction study, reported in section 10.10 (Table 26).

Table 28: Summary table of animal studies on STOT RE

↑↓ denote an increase or decrease in a parameter with respect to the control value
Statistical significance: * $p \leq 0.05$, ** $p \leq 0.01$
abs. = absolute
rel. = relative

Method, guideline, deviations if any, species, strain, sex, no/group	**Test substance, dose levels and guidance values for classification**	**Results**
	Oral studies	
28-Day oral dietary study OECD 407 GLP Rats, Fischer 6/sex/dose DAR: B.6.3.1a (Anon., 2010g)	IKF-309 technical Purity 98.04 % 0, 300, 3000, 10000 or 20000 ppm Equivalent to: ♂ 24.2, 251, 823 and 1657 mg/kg bw/day ♀ 26.1, 261, 841 and 1660 mg/kg bw/day	**<u>20000 ppm (1657/1660 mg/kg bw/day):</u>** ***Organ weights:*** ↑ Liver 38 %** (abs), 45 %** (rel.) in males and 59 %** (abs.and rel.) in females ↑Kidneys 22 %** (abs.), 28 %** (rel.) in males and 22 %** (abs.), 21 %** (rel.) in females ↑Thyroids 38 %** (rel.) in males ***Histopathology:*** **Liver** Diffuse hepatocyte hypertrophy: 6/6 males** and 6/6 females** (versus 0 in controls) Dark in colour: 6/6 males** and 6/6 females** (versus 0 in controls) **Kidney**

	STOT-RE 1: ≤ 30 mg/kg bw/day STOT-RE 2: > 30, ≤ 300 mg/kg bw/day	Increased hyaline droplet deposition in proximal tubule cells: 6/6 males** (versus 0 in controls) Increased calcification, corticomedullary junction: 4/6 females * (versus 0 in controls) **Caecum** Distended with contents: 6/6 males** and 6/6 females** (versus 0 in controls) ***Clinical chemistry:*** ↓ Alkaline phosphatase 29 %** in males and 31 %** in females ↑ γ-Glutamyl transpeptidase 20 %** in males, 40 %** in females ↓ Creatinine 19 %** in males, 17 %* in females ↑ Total Cholesterol 27 %** in males, 29%** in females ↓ Total bilirubin 33 %** in males and 75 %** in females ***Haematology:*** ↑ Platelet count 16.7 %** in males, 15 %** in females ↑ Lymphocyte count 17 %* in males **<u>10000 ppm (823/841 mg/kg bw/day):</u>** ***Organ weights:*** ↑ Liver 22 %** (abs), 28 %** (rel.) in males and 36 %** (abs.and rel.) in females ↑ Kidneys 16 %** (abs.), 18 %** (rel.) in males and 15 %** (abs.and rel.) in females ***Histopathology:*** **Liver** Diffuse hepatocyte hypertrophy: 6/6 males** and 6/6 females** (versus 0 in controls) Dark in colour: 6/6 males** (versus 0 in controls) **Kidney** Increased hyaline droplet deposition in proximal tubule cells: 6/6 males** (versus 0 in controls) **Caecum** Distended with contents: 6/6 males** and 6/6 females** (versus 0 in controls) ***Clinical chemistry:*** ↓ Alkaline phosphatase 19 %** in males and 13 % in females ↑ γ-Glutamyl transpeptidase 10 %* in males, 20 %* in females ↓ Creatinine 13 %* in males, 11 % in females ↑ Total Cholesterol 25 %** in males, 11%** in females ↓ Total bilirubin 33 %** in males and 75 %** in females

		<u>3000 ppm (251/261 mg/kg bw/day):</u> ***Organ weights:*** ↑ Liver 10 %** (rel.) in males ***Histopathology:*** **Kidney** Increased hyaline droplet deposition in proximal tubule cells: 6/6 males** (versus 0 in controls) **Caecum** Distended with contents: 6/6 males** and 6/6 females** (versus 0 in controls) ***Clinical chemistry:*** ↓ Alkaline phosphatase 10 %** in males ↓ Total bilirubin 50 %** in females **<u>300 ppm (24.2/26.1 mg/kg bw/day):</u>** No treatment-related findings NOAEL: 300 ppm (24.2/26.1 mg/kg bw/day)
90-Day oral dietary study OECD 408 GLP Rats, Fischer 10/sex/dose DAR: B.6.3.1b (Anon., 2010h)	IKF-309 technical Purity 98.04 % 0, 300, 1000, 2500 or 5000 ppm Equivalent to: ♂ 17.9, 60.5, 150 and 305 mg/kg bw/day ♀ 20.6, 69.0, 171 and 350 mg/kg bw/day STOT-RE 1: ≤ 10 mg/kg bw/day STOT-RE 2: >10, ≤ 100 mg/kg bw/day	**<u>5000 ppm (305/350 mg/kg bw/day):</u>** ***Organ weights:*** ↑ Liver 21 %** (abs), 20 %** (rel.) in males and 13 %** (abs), 16 %** (rel.) in females ↑ Kidneys 15 %** (abs.), 16 %** (rel.) in males and 10 %** (rel.) in females ↑ Caecum 2.6 fold** (abs. and rel.) in males and 2 fold** (abs. and rel.) in females ***Histopathology:*** **Liver** Diffuse hepatocyte hypertrophy: 9/10** males and 6/10** females (versus 0 in controls) **Kidney** Increased hyaline droplet deposition in proximal tubule cells: 9/10**males (versus 0 in controls) Tubular basophilic change: 7/10** males **Caecum** Distended with contents: 10/10** males and 5/10* females (versus 0 in controls) ***Clinical chemistry:*** ↓ Aspartate aminotransferase 20 %** in males ↓ Alanine aminotransferase 19 %** in males

		↑ Blood urea nitrogen 20 %** in males ↓ Creatinine 11 % in males, 26 %** in females ↑ Total cholesterol 24 %** in males ↓ Total bilirubin 50 %** in males and none detected** in females ***Haematology:*** ↑ Lymphocyte count 24 %** in males **2500 ppm (150/171 mg/kg bw/day):** ***Organ weights:*** ↑ Liver 14 %** (abs), 10 %** (rel.) in males ↑ Caecum 1.3fold (abs. and rel.) in males and 1.4 fold** (abs. and rel.) in females ***Clinical chemistry:*** ↓ Aspartate aminotransferase 14 %** in males ↓ Alanine aminotransferase 20 %** in males ↓ Creatinine 11 % in males ↓ Total bilirubin 25 %** in males and 50 %** in females **1000 ppm (60.5/69 mg/kg bw/day):** ***Clinical chemistry:*** ↓ Alanine aminotransferase 10 %** in males ↓ Total bilirubin 25 % in males and 25 %** in females **300 ppm (17.9/20.6 mg/kg bw/day):** No treatment-related findings NOAEL: 300 ppm (17.9/20.6 mg/kg bw/day)
13-Weeks oral dietary study OECD 408 GLP Mice, CD-1 10/sex/dose DAR: B.6.3.2a (Anon., 2009h)	IKF-309 technical Purity 97.88 % 0, 300, 1000, 3000 or 7000 ppm Equivalent to: ♂ 53, 176, 515 and 1318 mg/kg bw/day ♀ 61, 214, 695 and 1504 mg/kg bw/day STOT-RE 1: ≤ 10 mg/kg bw/day	**7000 ppm (1318/1504 mg/kg bw/day):** ***Organ weights:*** ↑ Liver 12 %** (rel.) in males and 14 %** (abs.), 18 %** (rel.) in females ***Histopathology:*** **Liver** Periportal hepatocytes hypertrophy: 3/10 males and 7/10 females (versus 0 in controls) ***Clinical chemistry:*** ↓ Aspartate aminotransferase 38 %* in males **3000 ppm (515/695 mg/kg bw/day):** ***Organ weights:*** ↑ Liver 9.5 %* (rel.) in males and 8.5 %* in females

	STOT-RE 2: >10, ≤ 100 mg/kg bw/day	**<u>1000 ppm (176/214 mg/kg bw/day):</u>** ***Organ weights:*** ↑ Liver 11.5 %* (rel.) in females **<u>300 ppm (53/61 mg/kg bw/day):</u>** No treatment-related findings NOAEL: 300 ppm (53/61 mg/kg bw/day)
90-Day oral dietary study OECD 409 GLP Dogs, Beagle 4/sex/dose DAR: B.6.3.3b (Anon., 2010k)	IKF-309 technical Purity 97.88 % ♂ 0, 500, 3000 or 25000 ppm ♀ 0, 500, 3000 or 15000 ppm Equivalent to: ♂ 15.0, 90.3 and 776 mg/kg bw/day ♀ 15.3, 89.9 and 475 mg/kg bw/day Based on a 90 day study in rats: STOT-RE 1: ≤ 10 mg/kg bw/day STOT-RE 2: > 10, ≤ 100 mg/kg bw/day	**<u>25000 ppm (776 mg/kg bw/day) (males only):</u>** ***Observations:*** ↓ Body weight gain 35 % ***Organ weights:*** ↑ Liver 32 %** (abs), 40 %** (rel.) ↓ Testis 19 % (abs), 15 % (rel.) ↓ Prostate 27 % (abs) 24 % (rel.) ***Histopathology:*** **Liver** Centrilobular hypertrophy: 3/4 males (versus 0 in controls) ***Clinical chemistry:*** ↑Alkaline phosphatase 5.8 fold ↑ Triglyceride 84 %** **<u>15000 ppm (475 mg/kg bw/day) (females only):</u>** ***Organ weights:*** ↑ Liver 23 % (abs), 26 % (rel.) ↓ Spleen 28 % (rel.) ↓ Ovaries 53 % (abs), 52 % (rel.) ***Histopathology:*** **Liver** Centrilobular hypertrophy: 3/4 females (versus 0 in controls) ***Clinical chemistry:*** ↑Alkaline phosphatase 4.9 fold ***Haematology:*** ↑ Platelet count 42 %* in females **<u>3000 ppm (90.3/89.9 mg/kg bw/day):</u>** ***Clinical chemistry:***

		↑Alkaline phosphatase 2.2 fold in females **<u>500 ppm (15.0/15.3 mg/kg bw/day):</u>** No treatment-related findings NOAEL: 500 ppm (15.0/15.3 mg/kg bw/day)
1-Year oral dietary study OECD 452 GLP Rats, Fischer 20/sex/dose DAR: B.6.5.1a (Anon., 2010i)	IKF-309 technical Purity 97.88 % 0, 200, 1000 or 5000 ppm Equivalent to: ♂ 8.5, 42.9 and 226 mg/kg bw/day ♀ 10.6, 53.5 and 275 mg/kg bw/day STOT-RE 1: ≤ 2.5 mg/kg bw/day STOT-RE 2: > 2.5, ≤ 25 mg/kg bw/day	**<u>5000 ppm (226/275 mg/kg bw/day):</u>** ***Observations:*** ↑ Soiled fur 12/20** females (versus 0 in controls) ***Organ weights:*** ↑ Liver 16 %** (abs), 20 %** (rel.) in males and 29 %** (abs), 27 %** (rel.) in females ↑ Kidneys 15 %** (abs.), 20 %** (rel.) in males and 12 %** (abs) and 21 %** (rel.) in females ↑ Caecum 2.1 fold** (abs. and rel.) in males and 1.9 fold** (abs. and rel.) in females ↑ Adrenals 11 %** (abs.), 20 %** (rel.) in males and 13 %** (rel.) in females ***Histopathology:*** **Liver** Centrilobular hypertrophy: 18/20** males (versus 0 in controls) **Kidney** Tubular basophilic change: 10/20** males (versus 1/20 in controls) Increased deposition of brown pigment in tubular cells: 20/20** females (versus 0/20 in controls) **Bone marrow (sternum)** Increased haematopoiesis: 9/20** males (versus 0 in controls) **Bone marrow (femur)** Increased haematopoiesis: 9/20** males (versus 0 in controls) **Caecum** Distended large intestine: 5/20* males and 10/20** females ***Clinical chemistry:*** ↓Alkaline phosphatase 27 %** in males and 33 %** in females ↓Aspartate aminotransferase 34 %** in males and 40 %** in females ↓Alanine aminotransferase 45 %** in males and 33 %** in females ↓Creatine 14 %** in males and 27 %** in females ↓Bilirubin 83 %** in females **<u>1000 ppm (42.9/53.5 mg/kg bw/day):</u>** No toxicologically significant findings

		NOAEL: 1000 ppm (42.9/53.5 mg/kg bw/day)
1-Year oral dietary study OECD 452 GLP Dogs, Beagle 4/sex/dose DAR: B.6.3.3c (Anon., 2010l)	IKF-309 technical Purity 97.88 % ♂ 0, 500, 3000 or 25000 ppm ♀ 0, 500, 3000 or 15000 ppm Equivalent to: ♂ 13.7, 83.5 and 701 mg/kg bw/day ♀ 14.1, 86.2 and 448 mg/kg bw/day Extrapolating from 90 day study cut-off values in rats: STOT-RE 1: ≤ 2.5 mg/kg bw/day STOT-RE 2: > 2.5, ≤ 25 mg/kg bw/day	**<u>25000 ppm (701 mg/kg bw/day) (males only):</u>** ***Observations:*** ↓ Body weight 23 % ↓ Body weight gain 71 % Vomiting feed: 3/4 males versus 1/4 in controls Loose stool: 4/4 males versus 2/2 in controls ***Organ weights:*** ↑ Liver 43 %** (abs), 86 %** (rel.) ↑ Kidney 29 %** (rel.) ***Histopathology:*** **Liver** Centrilobular hypertrophy: 3/4 males (versus 0 in controls) Dark colour: 4/4* males (versus 0 in controls) Enlargement: 4/4* males (versus 0 in controls) **Gallbladder** Intraluminal black granules: 3/4 males (versus 1/4 in controls) ***Clinical chemistry:*** ↑Alkaline phosphatase 10 fold** ↑ γ-Glutamyl transpeptidase 2.3 fold* **<u>15000 ppm (448 mg/kg bw/day) (females only):</u>** ***Observations:*** ↓ Body weight 10 % ↓ Body weight gain 40 % Vomiting feed: 2/4 females versus 0 in controls Loose stool: 4/4 females versus 1/4 in controls ***Histopathology:*** **Liver** Dark colour: 3/4 females (versus 0 in controls) Enlargement: 4/4 females (versus 0 in controls) **Gallbladder** Intraluminal black granules: 2/4 males (versus 1/4 in controls) ***Clinical chemistry:*** ↑Alkaline phosphatase 8 fold** **<u>3000 ppm (83.5/86.2mg/kg bw/day):</u>** ***Observations:*** ↓ Body weight gain 49 % in males and 30 % in females

		Loose stool: 3/4 females versus 1/4 in controls ***Organ weights:*** ↑ Liver 29 % (rel.) in males ↑ Kidney 29 %** (rel.) in males ***Clinical chemistry:*** ↑Alkaline phosphatase 2.5 fold in males and 2.2 fold in females **500 ppm (13.7/14.1mg/kg bw/day):** ***Observations:*** ↓ Body weight gain 44 % in males NOAEL not determined
Dermal studies		
28-Day dermal study OECD GLP Rats, Sprague Dawley 10/sex/dose DAR: B.6.3.4a (Anon., 2010m)	IKF-309 technical Purity 97.88 % 0, 100, 300 or 1000 mg/kg bw/day STOT-RE 1: ≤ 60 mg/kg bw/day STOT-RE 2: > 60, ≤ 600 mg/kg bw/day	**1000 mg/kg bw/day:** No treatment-related findings. NOAEL > 1000 mg/kg bw/day

Further details about these studies and the results are described below. In addition consideration of the information relevant for classification is available in Table 29.

10.12.1 Oral studies in rats

Pyriofenone has been tested in rats by the oral route in a 28 day, a 90-day and a 1-year study. Also available are data from a 2-year carcinogenicity study and a 2-generation reproduction study.

Twenty-eight day study in rats

The doses relevant for classification in this 28-day study were 300 and 3000 ppm (~25 and 255 mg/kg bw/day) (STOT RE 1: C ≤ 30 mg/kg bw/day and STOT RE 2: 30 < C ≤ 300 mg/kg bw/day).

Fischer rats (6/sex/group) were fed dietary concentrations of 0, 300, 3000, 10000 or 20000 ppm of pyriofenone (equivalent to 0, 24.2, 251, 823 and 1657 mg/kg bw/day in males and 0, 26.1, 261, 841 and 1660 mg/kg bw/day in females) for 28 days.

There were no unscheduled deaths in any group of either sex nor were there any effects to body weight or food consumption. Clinical signs were limited to one female of the 10000 ppm group and two females of the 20000 ppm group which were found to have soiled fur in the external genital region. There were no

treatment-related adverse findings at concentrations relevant for classification with STOT-RE 1. At 3000 ppm (251/261 mg/kg bw/day) findings were limited to an increase in relative liver weight in males only (10 % greater than controls), with no associated pathology, an increase in hyaline droplet deposition in proximal tubule cells of the kidney in all males (versus none in controls) and distention of the caecum in all males and females (versus none in control groups). There were some changes in clinical chemistry at this dose associated with liver and kidney toxicity. These were a decrease in alkaline phosphatase (10 % lower than controls) in males and a decrease in bilirubin (50 % lower than controls) in females.

At doses greater than 3000 ppm effects to the liver and kidneys described at lower doses became more marked.

Ninety day study in rats

The doses relevant for classification with STOT-RE 2 in this 90-day study were 300 and 1000 ppm (~20 and 65 mg/kg bw/day). There were no doses relevant for classification with STOT-RE 1 (STOT-RE 1: $C \leq 10$ mg/kg bw/day and STOT RE 2: $10 < C \leq 100$ mg/kg bw/day).

Fischer rats (10/sex/dose) were administered pyriofenone in the diet at doses of 0, 300, 1000, 2500 or 5000 ppm for 90 days (equivalent to 17.9, 60.5, 150 and 305 mg/kg bw/day in males and 20.6, 69.0, 171 and 350 mg/kg bw/day in females).

At 1000 ppm there were small disturbances to the clinical chemistry, in particular a decrease in levels of alanine aminotransferase in males (10 % lower than controls) and a decrease in total bilirubin in both males and females (25 % lower than controls). The main effects observed at doses ≥ 2500 ppm were increases in absolute and relative weight of the liver, kidney and caecum and associated histopathology. These changes became more apparent with increasing dose. In the liver, diffuse hypertrophy was observed in most males and just over half of the females of the top dose. In the kidney, increased hyaline droplet deposition in proximal cells and tubular basophilic changes were noted in most males. Similar to the 28-day study, the caecum was found to be distended with contents and this occurred in all males of the top dose group and 5/10 females. Clinical chemistry showed various changes to the parameters associated with liver and kidney toxicity.

One year study in rats

The doses relevant for classification with STOT-RE 2 in this 1-year study were 200 and 1000 ppm (~45 mg/kg bw/day) (derived by adjusting the standard guidance values for rats, using Haber's rule). There were no doses relevant for classification with STOT-RE 1 (STOT-RE 1: $C \leq 2.5$ mg/kg bw/day and STOT RE 2: $2.5 < C \leq 25$ mg/kg bw/day).

Fischer rats (20/sex/dose) were fed concentrations of pyriofenone in their diet for 52 consecutive weeks. The concentrations used were 0, 200, 1000 or 5000 ppm (equivalent to 8.51, 42.9 and 226 mg/kg bw/day in males and 10.6, 53.5 and 275 mg/kg bw/day in females).

At doses ≤ 1000 ppm there were no overt toxicologically significant findings. At 5000 ppm the main organs affected were the liver, kidneys and caecum. Organ weights were increased in both males and females and centrilobular hypertrophy was evident in most males. In the kidney, tubular basophilic change was observed in half of the males and increased deposition of brown pigment in tubular cells was observed in all females. Distention of the caecum was observed in 5/20 males and 10/20 females. Also observed were increased incidences of haematopoiesis in the bone marrow of males. Changes in clinical chemistry were consistent with liver and kidney toxicity.

Two year carcinogenicity study in rats

Only data from animals dosed with 200 ppm (~ 8 mg/kg bw/day) were relevant for classification with STOT-RE 2 (doses were derived by adjustment of the standard guidance values for rats, using Haber's rule). There

were no doses relevant for classification with STOT-RE 1 (STOT-RE 1: C ≤ 1.25 mg/kg bw/day and STOT RE 2: 1.25 < C ≤ 12.5 mg/kg bw/day).

Fischer rats (50/sex/dose) were administered pyriofenone in the diet for 104 weeks (Table 21) at doses of 0, 200, 1000 or 5000 ppm [equivalent to 0, 7.25/9.13, 36.4/46.5, 197/254 mg/kg bw/day (males/females)]. There were no toxicologically relevant findings at 200 ppm, the only dose relevant for classification. In brief, there were statistically significant increases in mortality at the top dose (5000 ppm, 197/254 mg/kg bw/day males/females) in the last three weeks of the study. The target organs were the liver (increased incidences of liver spots, fatty changes, hypertrophy in males and females, necrosis in males only and focal congestion in females only), kidneys (increased incidences of coarse surface in males and chronic nephropathy in females only) and large intestines (increased incidences of distension of the caecum in males and females and mucosal haemorrhage in males).

Two-generation study in rats

The overall length of this study is approximately 90 days and so taking into consideration the relevant guidelines for a 90-day study in rats (STOT-RE 1: C ≤ 10 mg/kg bw/day and STOT RE 2: 10 < C ≤ 100 mg/kg bw/day), only toxicity observed at doses of 1000 ppm and below are is relevant for classification purposes.

Wistar rats (24/sex/dose) received pyriofenone in their diet for a 10 week period prior to mating and then for the duration of the gestation and lactation periods. Concentrations used were 0, 150, 1000 or 5000 ppm [(approximately 10-20, 70-130, 320 – 700 mg/kg bw/day (exact doses in mg/kg bw/day can be found in Table 28, depending on generation, sex and study period)]. The main target organs were liver, kidney and caecum with organ weights increased in males and females of the top dose group and increased incidences of hepatocyte hypertrophy, brown deposition in the Glisson's capsule, darkened liver colour and hyaline droplet deposition in proximal tubule cells of the kidney. At doses relevant for classification with STOT-RE 2 there were small increases in absolute and relative liver weight in parental females of 11 % and 9 % (respectively) compared to the controls, however there were no adverse findings from histopathological examinations pertaining to the liver. There were no adverse findings from the histopathological examination associated with the increase in liver weight.

10.12.2 Oral studies in mice

Pyriofenone has been tested in mice by the oral route in a 90-day study. Data from an 18-month carcinogenicity study is also available.

Ninety day study in mice

The dose relevant for classification with STOT-RE 2 in this 90-day study was 300 ppm ~55 mg/kg bw/day). There were no doses relevant for classification with STOT-RE 1 (STOT-RE 1: C ≤ 10 mg/kg bw/day and STOT RE 2: 10 < C ≤ 100 mg/kg bw/day).

CD-1 mice (10/sex/dose) were fed pyriofenone in the diet at concentrations of 0, 300, 1000, 3000 or 7000 ppm for 13 weeks (equivalent to 53, 176, 515 and 1318 mg/kg bw/day in males and 61, 214, 695 and 1504 mg/kg bw/day in females).

There were no mortalities, clinical signs of significant effects on body weights or food consumption. At 300 ppm there were no toxicologically significant findings. At doses > 300 ppm the liver was the target organ. Liver weight was increased in both males and females and periportal hepatocyte hypertrophy was apparent in both sexes also. Clinical chemistry revealed decreased aspartate aminotransferase in males only. There were some haem effects in males from a dose of 300 ppm in females from a dose of 1000 ppm. However, there

was a lot of inter-individual variation and the majority of these effects occurred at doses greater than the guidance values for classification.

Eighteen month carcinogenicity study in mice

All doses used in this study were above the extrapolated guideline cut-off values (as taken from a 90 day rat study), STOT-RE 1: C ≤ 1.6 mg/kg bw/day and STOT RE 2: 1.6 < C ≤ 16 mg/kg bw/day.

CD-1 mice (52/sex/dose) received an oral dose of pyriofenone in their diet at concentrations of 0, 600, 1800 or 5400 ppm for males (equivalent to 0, 77.6, 237 and 716 mg/kg bw/day) or 0, 300, 1000 or 3000 ppm for females (equivalent to 0, 49.4, 167 and 486 mg/kg bw/day) for 78 weeks (Table 21). The main non-neoplastic findings in this study included an increased incidence of abnormal yellow staining (perigential region) in males, reduced bodyweight gain in females and liver and kidney findings. In males, the main findings in the liver were increased organ weight and masses were found. Also observed was centrilobular hepatocyte hypertrophy and necrosis of individual hepatocytes. In the kidney, increased basophilic and eosinophilic foci with granular kidneys and cortical tubular basophilia was observed. In females, pigment in the liver macrophages was observed and granular kidneys and chronic progressive nephropathy.

10.12.3 Oral studies in dogs

Pyriofenone has been tested in dogs by the oral route in a 28-day, a 90-day and a 1-year study.

Twenty-eight day study in dogs (DAR: B.6.3.3a, Anon., 2010j)

This study was carried out according to OECD guidelines but with a significant deviation of only one dog being used per dose group. Due to this significant deviation, this study was not included in Table 28. The objective of the study was to clarify the toxic characteristics of pyriofenone in dogs and to select dose levels for a 90-day study.

Beagle dogs (1/sex/dose) were administered pyriofenone in their diet at concentrations of 0, 500, 1500, 5000 or 15000 ppm (equivalent to 14.9, 37.9, 144 and 390 mg/kg bw/day in males and 15.9, 47.5, 143 and 485 mg/kg bw/day in females). There was no mortality or treatment-related clinical signs observed and no significant changes in behaviour observations. As this study was intended to be a sighting study it is hard to ascertain the true effects of pyriofenone in dogs, however there were indications of increased liver, kidney, adrenals, thymus, spleen and ovary weight in the top-dosed female. There were no treatment-related gross findings in the male or female.

Ninety day study in dogs

Using the guideline cut-off values for a 90-day rat study, the doses relevant for classification in this 90-day dog study are 500 ppm and 3000 ppm only (15 mg/kg bw/day and 90 mg/kg bw/day). There were no doses relevant for classification with STOT-RE 1 (STOT-RE 1: C ≤ 10 mg/kg bw/day and STOT RE 2: 10 < C ≤ 100 mg/kg bw/day).

Beagle dogs (4/sex/dose) received pyriofenone in their diet at concentrations of 0, 500, 3000, 15000 (females only) or 25000 ppm (males only) for 90 days (equivalent to 15.0, 90.3 and 776 mg/kg bw/day in males and 15.3, 89.9 and 475 mg/kg bw/day in females). There was no mortality or treatment-related clinical signs observed and no significant changes to behaviour. Body weight and food consumption did not show any treatment-related differences, however body weight gain was reduced in males of the top dose group.

Animals dosed with 500 ppm had no treatment-related adverse effects. Those dosed with 3000 ppm had small changes in clinical chemistry only [increased alkaline phosphatase levels in females only (2.2 fold higher than controls]. At doses higher than 3000 ppm, the liver was the target organ, with increased absolute

and relative weight in males and females and increased incidences of centrilobular hypertrophy in males and females. In addition, the mean relative spleen weight was statistically significantly reduced in females of the top dose. Males of the top dose were observed to have decreased testes (19 % absolute and 15 % relative in comparison to controls) and prostate (27 % absolute and 24 % relative in comparison to controls). These findings occurred at a very high dose in the presence of severe effects to body weight gain and the liver. There were no such findings at lower doses or in other studies; therefore they are unlikely to be a specific effect caused by pyriofenone. In females of the top dose, ovary weight was decreased (53 % absolute and 52 % relative, in comparison with controls). Again, these effects occurred in the presence of severe toxicity (increased liver weight and associated histopathology). The study report also described the presence of four sexually immature females, which may have had an impact on the reproductive organ weights.

One year study in dogs

Using the guideline cut-off values for a 90-day rat study and extrapolating to one year as a guide, the dose relevant for classification with STOT-RE 2 in this 1-year dog study was 500 ppm only (~14 mg/kg bw/day). There were no doses relevant for classification with STOT-RE 1 (STOT-RE 1: C ≤ 2.5 mg/kg bw/day and STOT RE 2: 2.5 < C ≤ 25 mg/kg bw/day).

Beagle dogs (4/sex/dose) were administered pyriofenone in their diet at concentrations of 0, 500, 3000, 15000 (females only) or 25000 (males only) ppm for 12-months (equivalent to 13.7, 83.5 and 701 mg/kg bw/day in males and 14.1, 86.2 and 448 mg/kg bw/day in females). There were no unscheduled deaths and at the doses relevant for classification, there were no overt toxicological signs. In animals dosed with 3000 ppm and above, bodyweight gain was affected in a dose-dependent manner. Emesis of feed was noted in males and females and also loose stools. The liver was the primary target organ, with increased weight and centrilobular hypertrophy in males of the top dose and dark colour and enlargement in both sexes at the top dose. Clinical chemistry revealed a dose-dependent increase in alkaline phosphatase in males and females at doses ≥ 3000 ppm. There were small but statistically significant effects to haem parameters in males and females of the top dose. These were not observed at doses of 3000 ppm and less.

10.12.4 Dermal studies in rats

Pyriofenone has been tested for its ability to induce systemic toxicity in rats following dermal administration in a short-term dermal 28-day study.

Twenty-eight day study in rats

In this study, the doses relevant for classification with STOT-RE 2 were 100 and 300 mg/kg bw/day. There were no doses relevant for classification with STOT-RE 1 (STOT-RE 1: C ≤ 60 mg/kg bw/day and STOT RE 2: 60 < C ≤ 600 mg/kg bw/day).

Pyriofenone was applied to the clipped dorsal skin of Sprague Dawley rats (10/sex/dose), under a semi-occlusive dressing daily for 6 hours at doses of 0, 100, 300 or 1000 mg/kg bw/day for four weeks. There were no unscheduled deaths, no treatment-related clinical signs, no treatment-related skin irritation or effects on body weight or food consumption. There were no treatment-related findings at any dose other than an increase in partial thromoplastin time in females of the top dose.

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

The repeated-dose oral toxicity of pyriofenone has been 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.

Following oral administration, the main target organs were the liver in rats, mice and dogs, the kidney in rats and mice and the caecum in rats.

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). These findings were observed from doses of 150 mg/kg bw/day in rats (90-day study), 176 mg/kg bw/day in mice (90-day study) and 83.5 mg/kg bw/day in dogs (1-year study). Clear liver toxicity (including liver foci and hepatocellular necrosis) was reported in the carcinogenicity studies in rats and mice from 197 mg/kg bw/day in mice and 486 mg/kg bw/day in rats.

The main effects to the kidney included increased absolute and relative weight and increased hyaline droplet deposition in proximal tubule cells. These occurred in rats from a dose of 226 mg/kg bw/day (1-year study) and in dogs from a dose of 701 mg/kg bw/day (1-year study). In the chronic/carcinogenicity studies, increased incidences of chronic nephropathy were observed in rats from doses of 36 mg/kg bw/day and cortical tubular basophilia was observed in mice from doses of 237 mg/kg bw/day.

Effects to the large intestine were observed in rats at doses of 150 mg/kg bw/day and higher. The effects to 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 that were observed included effects on haem in rats and mice, although these changes tended to be very small (less than 5 %) and in some cases there was a lot of inter-individual variation and always at doses higher than the recommended cut-off values for classification. In rats prolongation of activated partial thromboplastin time was observed in most studies, again, always at doses far higher than those relevant for classification.

The majority of the adverse effects observed occurred at doses much higher than the guidance cut-off 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. Table 29 shows the studies with effects at doses relevant for classification.

Table 29: Adverse effects occurring at doses relevant for classification with STOT RE 2 in rats, mice and dogs following oral administration of pyriofenone

Study	**(Adjusted) guidance value for STOT RE 2** **mg/kg bw/day**	**Effects at doses below guidance cut-off values**
28-Day rat study	300	**<u>251/261 mg/kg bw/day</u>** ↑ Liver 10 %** (rel.) in males Increased hyaline droplet deposition in proximal tubule cells of the kidney: 6/6 males** (versus 0 in controls) Distended caecum with contents: 6/6 males** and 6/6 females** (versus 0 in controls) ↓ Alkaline phosphatase 10 %** in males ↓ Total bilirubin 50 %** in females
28-Day dog study	300	No clear data trends due to only one dog per dose group
90-Day rat study	100	**<u>60.5/69 mg/kg bw/day</u>** ↓ Alanine aminotransferase 10 %** in males ↓ Total bilirubin 25 % in males and 25 %** in females

2-Generation rat study	100	**(64.1/62-138.1 mg/kg bw/day):** ↑ Liver weight: 11 %** (abs.) and 9 %** (rel.) in females
90-Day mouse study	100	No treatment-related findings at doses ≤ 100 mg/kg bw/day
90-Day dog study	100	**90.3/89.9 mg/kg bw/day** ↑Alkaline phosphatase 2.2 fold in females
1-Year rat study	25	No treatment-related findings at doses ≤ 25 mg/kg bw/day
1-Year dog study	25	**13.7/14.1mg/kg bw/day** ↓ Body weight gain 44 % in males (not observed in females)
18-Month mouse carcinogenicity study	16	No doses used that are relevant for classification
2-Year rat carcinogenicity study	12.5	No treatment-related findings at doses ≤ 12.5 mg/kg bw/day

10.12.6 Comparison with the CLP criteria

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/d (for a classification in category 2) obtained in a 90-day rat study.

'Significant' toxicity is taken to mean changes that clearly indicate functional disturbance or morphological changes that are toxicologically relevant. 'Severe' toxicity is considered to be more profound or serious and indicates changes that are of a considerably adverse nature with a significant impact on health.

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 Table 29.

In the majority of the studies, there were no doses used that were relevant for STOT-RE 1. In the one study that did use a dose relevant for classification in category 1, no toxicologically adverse effects were observed (28-day oral study in rats). All treatment-related findings occurred at doses relevant for classification in Category 2 only. Therefore, classification in Category 1 for specific target organ toxicity following repeated dosing is not required.

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. Changes to the proximal cells of the kidney were observed in males of the 28-day study, however in the absence of any such changes occurring at doses relevant for classification in the 90-day study, the UKCA concludes that this change in rats does not warrant classification. 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 and were more indicative of adaptive changes occurring in the liver and kidney due to exposure to pyriofenone. Therefore, whilst they are considered treatment-related, they are not considered adverse and do not support classification.

Therefore, it is concluded that there is no evidence of significant or severe toxicity at doses below the guidance values for classification for specific target organ toxicity following repeated oral or dermal administration of pyriofenone. No classification for this endpoint is warranted.

10.12.7 Conclusion on classification and labelling for STOT RE

Not classified. Data conclusive but not sufficient for classification.

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**.

10.13 Aspiration hazard

There are no data available for this endpoint.

10.13.1 Conclusion on classification and labelling for aspiration hazard

Not classified. Data lacking.

11 EVALUATION OF ENVIRONMENTAL HAZARDS

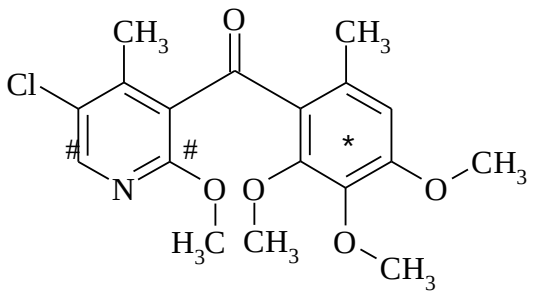
Pyriofenone (often referred to in test reports as IFK-309) is a fungicide intended for use against mildew in cereals and grapes. Available environmental fate and hazard studies have been considered under Regulation (EC) No 1107/2009 and summarised in the Draft Assessment Report (DAR) 2012.

The key information pertinent to determining a classification is presented below.

The water solubility of pyriofenone in pure water has been experimentally determined (OECD 105, column elution method, GLP) to be 1.56 mg/l at 20 °C and pH 6.6 (Turner, 2007). Based on available data is unclear if solubility is pH dependant.

A dissociation constant is not available and it is unclear if a pKa value for the substance would lie within and environmentally relevant pH range.

All radiolabelled studies used ^{14}C-pyriofene with a purity of ≥ 97% as shown in Figure 2.



*Position of the ^{14}C-(phenyl) uniform radiolabel
position of the ^{14}C-(pyridy) radiolabelled in the 2 and 6 positions of the pyridyl ring

Figure 2: Structure of pyriofenone indicating positions of the ^{14}C labels

A summary of reliable valid information on the aquatic fate of pyriofenone is presented in Table 30 below. Available soil data have not been presented as suitable aquatic data are available.

11.1 Rapid degradability of organic substances

Table 30: Summary of relevant information on rapid degradability

Method	Results	Remarks	Reference
Aquatic hydrolysis OECD 111, GLP	Hydrolytically stable at pH 4, 7 and 9 at 50 °C DT_{50} considered >16 days at environmentally relevant pH and temperature	Valid	Juozenaite, 2009
Ready biodegradation OECD Guideline 310, GLP	0.6% mineralisation day 28 Not readily biodegradable	Valid	Dickinson, 2009
Aquatic photolysis SETAC and EPA Guidelines (Pesticides Assessment Guidelines,	DT_{50} = 33 to 54 days spring sunlight at 35°N	Valid	Kane, 2009

Method	Results	Remarks	Reference
Subdivision N, Series 161-2, GLP			
Freshwater aerobic mineralisation in surface water (simulation biodegradation), OECD Guideline 309, GLP	DT_{50} = 15.9 days at 12 °C based on geometric mean of test systems and primary degradation Maximum 16.8% AR mineralisation as CO_2 by day 100	Valid	Crowe, 2009

11.1.1 Ready biodegradability

Study 1 – Dickinson (2009)

A GLP ready biodegradation study following OECD Test Guideline 310 (CO_2 Evolution) is available using pyriofenone at 10 mgC/l dissolved in acetone (acetone was also included in control and reference samples). A reference substance was included and considered valid. A toxicity control was also included indicating the test item was not inhibitory to the microbial inoculum. The study included 5 replicates although 2 gave anomalous results and were removed from calculations as it was suggested the test vessels may have leaked. During review under Regulation 1107/2009, this was not considered to have affected study results.

By day 28 a mean of 0.6% minerlaisation was observed. On this basis, pyriofenone is not considered readily biodegradable.

11.1.2 BOD_5/COD

No data.

11.1.3 Hydrolysis

Study 1 – Juozenaite (2009)

A preliminary hydrolysis test at 50 °C over 5 days at pH 4, 5, 7 and 9 is available conducted according to OECD Test Guideline 111 with radiolabelled pyriofenone at a concentration approximately half the water solubility. Radioactivity recoveries were 95.1-100%. No hydrolysis of pyriofenone was observed and the test substance was found to be stable in aqueous solution under sterile conditions. Thus, the main test at 25 °C was not performed.

Overall, pyriofenone is considered hydrolytically stable at an environmentally relevant pH and temperature with a half-life greater than 16 days.

11.1.4 Other convincing scientific evidence

11.1.5 Field investigations and monitoring data (if relevant for C&L)

No data.

11.1.6 Inherent and enhanced ready biodegradability tests

No data.

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

Study 1 – Crowe (2009)

A freshwater aquatic biodegradation simulation study is available following OECD Guideline 309 and GLP. The study used ^{14}C-phenyl and ^{14}C-pyridyl labelled pyriofenone and two natural aquatic systems: Calwich Abbey Lake, England and Swiss Lake, England. Table 31 presents the characteristics of each aquatic system.

Table 31: Physiochemical parameters of the pyriofenone water/sediment systems

Sediment Parameter	Calwich Abbey Lake	Swiss Lake
Geographic Location	Calwich Abbey Lake, Calwich, Ashbourne, Derbyshire, England	Swiss Lake, Chatsworth, Derbyshire, England
Texture Class	Silt Loam	Sand
% Sand	7	98
% Silt	82	2
% Clay	11	0
pH (1:5 soil:water ratio)	7.7	6.0
% Organic Carbon (%)	4.1	0.6
CEC (meq/100 g)	14.2	1.9
Water Parameter	**Calwich Abbey Lake**	**Swiss Lake**
Temperature (°C)	20 °C	20 °C
pH	8.26	5.8
Hardness mg equivalent $CaCO_3$/l (ppm)	250	19
Organic Carbon (mg/l)	4.0	10.8
Total Suspended Solids (ppm)	6	50

Test systems were prepared with filtered water at a ratio of 4:1 water:sediment (w:w). The test item was applied to the water layer at a rate of 0.033 mg/l. The study ran for 100 days in the dark at 20 ºC.

For analysis, water and sediment layers were separated by centrifugation with subsequent decanting of the water layer. The radioactivity was quantified by Liquid Scintillation Counting (LSC) and then analysed by HPLC with UV and radiochemical detection. Overall recoveries were 86.5 to 99.8% Applied Radioactivity (AR).

Pyriofenone was observed to dissipate to sediment. This dissipation was slower in the Swiss Lake system which is considered a function of higher proportion of sand and less organic carbon.

Low levels of mineralisation observed in the Calwich Abbey system (both radiolabels) and in the pyridyl radiolabel in the Swiss Lake system with a maximum of 2.4% AR by day 60 in the Calwich Abbey phenyl radiolabel system. Increased CO_2 levels were observed in the Swiss Lake system using the phenyl radiolabel with 16.8% AR observed on day 100.

Whole systems DT_{50} values (representing primary degradation) at 20 ºC were calculated using SFO kinetics as follows:

- Calwich Abbey: 4.5 to 5.5 days
- Swiss Lake: 13.8 to 14.5 days
- Combined geometric mean: 8.4 days

For the purpose of classification these values have been converted to 12 ºC, in line with ECHA guidance and Member State Committee testing protocols, to reflect a more environmentally relevant temperature.

- Calwich Abbey: 8.5 to 10.4 days
- Swiss Lake: 26.4 to 27.5 days
- Combined geometric mean: 15.9 days

The following degradants were observed during the study:

Calwich Abbey:

- 3HDPM: water max. 3.1% AR and sediment max. 5.3% AR
- 2MDPM: water max. 0.3% AR and sediment max. 2.6% AR
- 4MDPM: water max. 1.2% AR and sediment max. 0.6% AR
- 3HDHP: sediment only at max. 2.4% AR

Swiss Lake:

- 3HDPM: water max. 1.8% AR and sediment max. 4.0% AR
- 2MDPM: water max. 4.0% AR and sediment max. 6.3% AR
- 4MDPM: sediment only at max. 4.4% AR
- 3HDHP: sediment only at max. 5.3% AR
- 4HDPM: sediment only at max. 1.4% AR PTBA: sediment only at max. 3.5% AR

The proposed degradation pathway is show in Figure 3.

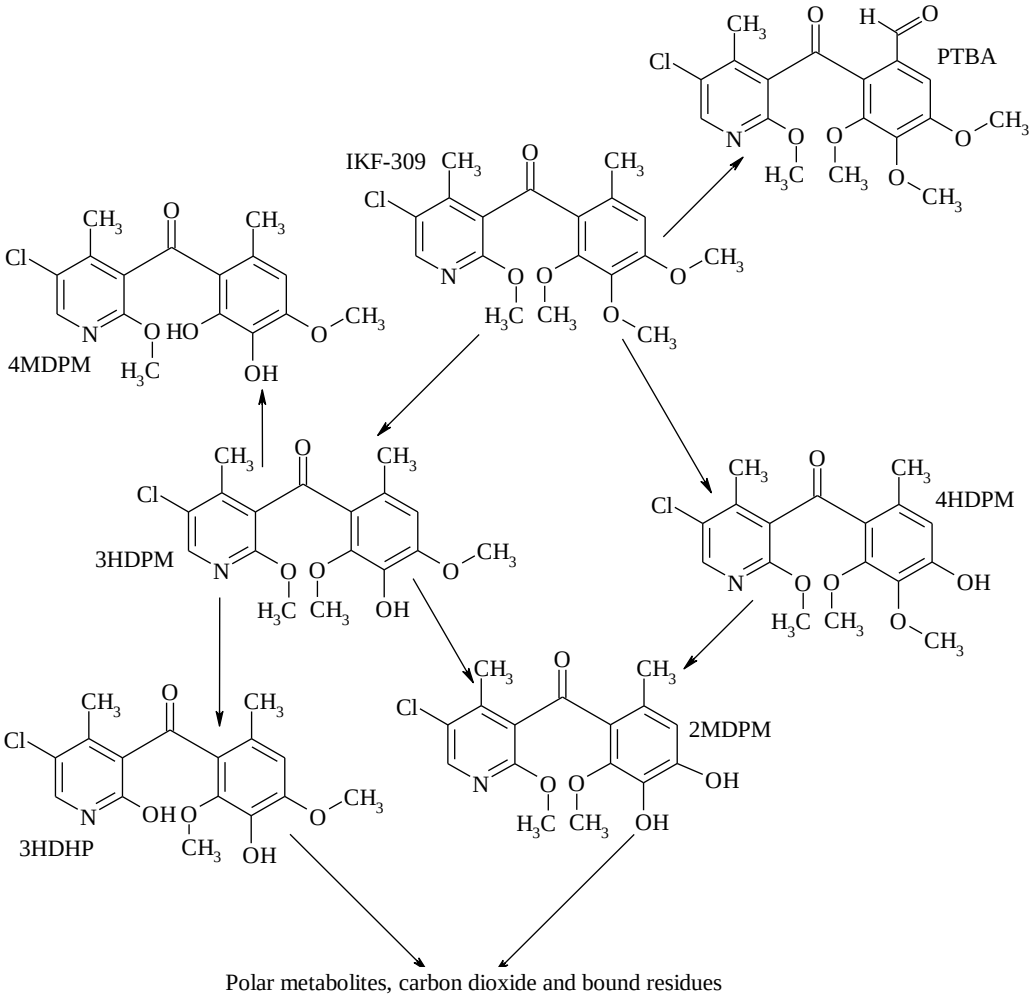


Figure 3 – Proposed degradation pathway for pyriofenone in aquatic sediment systems

11.1.7.1 Photochemical degradation

A GLP aquatic photolysis study is available following SETAC and EPA Guidelines (Pesticides Assessment Guidelines, Subdivision N, Series 161-2) using pyriofenone. Following a preliminary test, the main test used sterile natural river water and purified water to which the radiolabelled test item was added at approximately 0.7 mg/l. Samples were irradiated at 25 ± 2 ºC, with stirring for up to 7 days using a xenon arc light source (wavelengths <290 nm removed). Samples were analysed by Liquid Scintillation Counting (LSC) and High Performance Liquid Chromatography (HPLC) with radiodetection. Recoveries for both natural and purified media test systems were 92.6 to 102.7%. Volatile radioactivity reached a maximum of 9.6% CO_2 in both natural and purified systems

In both the natural and purified irradiated media, the test item decreased over the 7 days to 40.5% in natural water and 54.8% in purified water. In non-irradiated media 98.6-98.8% test item was present at study termination. Up to 13 degradants were observed between the two media. None were present at greater than 6.8% Applied Radioactivity (AR).

The study calculated DT_{50} for pyriofenone in natural water was 159 hours of continuous irradiation equivalent to 33 days spring sunlight at latitude 35ºN.

The study calculated DT_{50} for pyriofenone in purified water was 261 hours of continuous irradiation equivalent to 54 days spring sunlight at latitude 35ºN.

Using GCSOLAR and the calculated quantum yield, the study also calculated theoretical lifetimes at the water surface at latitude 40ºN as follows:
- Spring = 29 days
- Summer = 22.7 days
- Autumn = 52.1 days
- Winter = 98 days

Summary

Pyriofenone is not readily biodegradable and is hydrolytically stable. Under experimental conditions, limited photodegradation was observed in pure water. In a water/sediment simulation study pyriofenone underwent primary degradation with low levels of ultimate degradation. Overall, pyriofenone is not considered to be rapidly degradable for the purpose of classification.

11.2 Environmental transformation of metals or inorganic metals compounds

Not relevant.

11.3 Environmental fate and other relevant information

Following OECD 106 and GLP, pyriofenone has experimentally determined log K_{foc} values in the range 705 to 2720 ml/g for sandy clay loam to loamy sand soils (Kane, 2008) indicating it will be of low mobility in soil.

The calculated Henry's Law Constant of 1.9 x 10^{-4} Pa.m^3.mol (Turner, 2009b) indicating it is unlikely to partition from air.

11.4 Bioaccumulation

Table 31: Summary of relevant information on bioaccumulation

Method	Results	Remarks	Reference
Partition coefficient, OECD 107, purity	logPow 3.2 at pH 7.2-7.5, 20 ºC)	Unclear if pH dependant	Turner, 2009g

Method	Results	Remarks	Reference
99.19%, GLP			
Experimental aquatic BCF test in fish to OECD Guideline 305, GLP, purity 97.88%,	Steady state whole fish BCF_{ss}: 142 to 160 l/kg (not lipid normalised) Kinetic whole fish BCF_k: 137 to 176 l/kg (not lipid normalised or growth corrected) Depuration half-life DT_{50}: 0.6 to 0.93 days	Flow through, 28 days exposure, 6 days depuration Valid	Anon., 2009

11.4.1 Estimated bioaccumulation

No data.

11.4.2 Measured partition coefficient and bioaccumulation test data

Study 1 – Turner (2009g)

The octanol:water partition coefficient of pyriofenone was determined following OECD Test Guideline 107 (shake flask method). The study was conducted using pure water at pH 7.2-7.5 and 20 °C. In the absence of measurements at higher and lower pHs, it is unclear if a pH dependence may occur. The $logP_{OW}$ was 3.2.

Study 2 – Anon. (2009)

The study followed GLP and OECD Guideline 305. It used pyriofenone (97.88%), a flow-through system with carp (*Cyprinus carpio*) and two exposure concentrations; 0.01 and 0.001 mg/l with the aid of the solvent dimethylformamide (DMF). A solvent control was included. 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.

Kinetic Bioconentration Factors (BCF_k) were determined as 137 to 176 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 BCFs have not been lipid normalised, the mean lipid content at the end of the depuration is only slightly above 5% and lipid normalised would not result in a BCF_{ss} above 500 l/kg.

During the study the degradants 3HDPM and 4HDPM were detected in fish indicating pyriofenone parent underwent metabolism as neither degradant were detected in test water. The maximum concentrations were as follows:

4HDPM: 0.3145 mg/kg (high concentration group) and 0.04108 mg/kg (low concentration group)

3HDPM: 0.0363 mg/kg (high concentration group) and <0.01 mg/kg (low concentration group)

During the depuration phase, pyriofenone, 4HDPM and 3HDPM were rapidly depurated from the fish with the following half-lives:

- Pyriofenone: 0.6 to 0.93 days
- 4HDPM: 1.32 to 1.73 days
- 3HDPM: 5.65 days

ANNEX 1 - BACKGROUND DOCUMENT TO RAC OPINION ON (5-CHLORO-2-METHOXY-4-METHYL-3-PYRIDYL)(4,5,6-TRIMETHOXY-*O*-TOLYL)METHANONE; PYRIOFENONE

Summary

Pyriofenone has a logPow below the CLP threshold of 4 and experimental BCFs below the CLP threshold of 500. It is therefore considered to have a low potential for bioaccumulation.

11.5 Acute aquatic hazard

A summary of available valid information on the aquatic toxicity of pyriofenone is presented in Table 32. Where available, a summary of valid information for degradants is also included in Annex I, Table A1. Based on the limited available data, degradants are not considered more toxic than the parent substance and not considered further for classification.

Table 32: Summary of relevant information on acute aquatic toxicity

Method	Species	Test material	Results	Remarks	Reference
Acute toxicity to fish, OECD 203, GLP	Rainbow Trout (*Oncorhynchus mykiss*)	Pyriofenone (97.88%)	96-h LC_{50} >1.44 mg a.s./l (mm)	Valid	Anon., 2007
Acute toxicity to fish, OECD 203, GLP	Common Carop (*Cyprinus carpio*)	Pyriofenone (97.88%)	96-h LC_{50} >1.41 mg a.s./l (mm)	Test fish were longer length than test guideline recommendation	Anon., 2008
Daphnia sp Acute Immobilisation OECD 202, GLP	*Daphnia magna*	Pyriofenone (97.88%)	48-h EC_{50} >1.55 mg a.s./l (mm)	Valid	Burke, Manson and Scholey, 2008b
Freshwater Algal Growth Inhibition OECD 201, GLP	*Pseudokirchneriella subcapitata*	Pyriofenone (97.88%)	72-h E_rC_{50} 1.77 mg a.s./l (mm)	Valid	Burke, Manson and Scholey, 2008d

Notes:
mm refers to mean measured concentrations

11.5.1 Acute (short-term) toxicity to fish

Two valid semi-static, acute toxicity to fish studies using pyriofenone following GLP and OECD Test Guideline 203 are discussed below.

Study 1 - Anon., (2007)
Using Rainbow Trout (*Oncorhynchus mykiss*) a nominal exposure range of 0.02, 0.2 and 2 mg a.s./l was employed with the aid of acetone as a solvent (a solvent control was included). Study conditions were acceptable and validity criteria were met. Analytical concentrations by HPLC-UV were 72 to 79% of nominal. No mortality was observed. The study 96-h LC_{50} was >1.44 mg a.s./l based on mean measured concentrations.

Study 2 – Anon., (2008)
Using Common Carp (*Cyprinus carpio*) a single test concentration of 2 mg a.s./l nominal was employed prepared with the aid of acetone as solvent (a solvent control was included). Study conditions were acceptable and validity criteria were met. However, it is noted that at the start of the study, the test fish were 5.5 to 6.6 cm which is longer than the guideline recommendation of 3 cm ± 1 cm any may have influenced sensitivity. Analytical concentrations by HPLC UV were 70 to 72% of nominal. No mortality was observed. The study 96-h LC_{50} was >1.41 mg a.s./l based on mean measured concentrations.

11.5.2 Acute (short-term) toxicity to aquatic invertebrates

Study 1 – Burke, Manson and Scholey (2008b)

A semi-static acute toxicity to *Daphnia magna* study is available following GLP and OECD Test Guideline 202. Study conditions were acceptable and validity criteria were met. The exposure range was nominally 0.002, 0.02, 0.2 and 2 mg a.s./l prepared with the aid of acetone as a solvent (a solvent control was included). Analytical measurement by HPLC-UV was 78 to 91% of nominal with mean measured concentrations 0.00164, 0.0181, 0.181 and 1.55 mg a.s./l. There was no effect on immobilisation at any concentration. Based on mean measured concentrations, the 48-h EC_{50} was >1.55 mg a.s./l.

11.5.3 Acute (short-term) toxicity to algae or other aquatic plants

Study 1 – Burke, Manson and Scholey (2008b)

A 72-hour static algal growth inhibition test using the freshwater algae *Pseudokirchneriella subcapitata* is available following GLP and OECD Test Guideline 201 (2006). Study conditions were acceptable and validity criteria were met. The nominal exposure range was 0.023, 0.052, 0.114, 0.250, 0.550, 1.21 and 2.66 mg a.s./l. Exposure concentrations were prepared with the aid of acetone as a solvent and a solvent control was included. Analytical measurement by HPLC-UV was 75 to 124% of nominal with mean measured concentrations 0.0274, 0.0591, 0.141, 0.249, 0.575, 1.00 and 1.98 mg a.s./l.

In three highest treatments, suspended particulate test material was observed.

The 72-h E_rC_{50} was calculated to be 1.77 mg a.s./l based on mean measured concentrations. The 72-h NOE_rC was determined to be 0.249 mg a.s./l based on mean measured concentrations.

11.5.4 Acute (short-term) toxicity to other aquatic organisms

No data.

11.6 Long-term aquatic hazard

Table 33: Summary of relevant information on chronic aquatic toxicity

Method	Species	Test material	Results	Remarks	Reference
Fish Early-Life Stage toxicity, OECD 210, GLP	Fathead Minnow (*Pimephales promelas*)	Pyriofenone (97.88%)	28-d NOEC 1.27 mg a.s./l (mm)	Valid	Anon. , 2008a
Daphnia magna Reproduction OECD 211, GLP	*Daphnia magna*	Pyriofenone (97.88%)	21-d NOEC 0.0899 mg a.s./l (mm)	Valid	Burke, Manson and Scholey, 2008c
Freshwater Algal Growth Inhibition OECD 201, GLP	*Pseudokirchneriella subcapitata*	Pyriofenone (97.88%)	72-h NOE_rC 0.249 mg a.s./l (mm)	Valid	Burke, Manson and Scholey, 2008d

Notes:
mm refers to mean measured concentrations
n refers to nominal concentrations

11.6.1 Chronic toxicity to fish

Study 1 – Anon. (2008a)

A semi-static chronic toxicity to fish study using pyriofenone following GLP and OECD Test Guideline 210 is available. The study used Fathead Minnow (*Pimephales promelas*) and the following endpoints: hatching success, survival and growth (length and dry weight). General observations were also recorded.

Study conditions were acceptable and validity criteria were met. It is noted that although test eggs were >24 hours past fertilisation they were considered acceptable as they were in the 'tail bud' stage of development. The nominal exposure range was 0.019, 0.061, 0.195, 0.625 and 2 mg a.s./l. Exposure solutions were prepared with the aid of a solvent (DMF at 0.1 ml/l) and a solvent control was included.

Analytical verification was by HPLC-UV with measured values 63.5 to 73.3% of nominal with mean measured concentrations 0.0126, 0.0416, 0.143, 0.435 and 1.27 mg a.s./l.

No statistically significant effects were observed for any parameter.

The 28-d NOEC for all parameters was considered to be 1.27 mg a.s./l based on the highest treatment and mean measured concentrations.

11.6.2 Chronic toxicity to aquatic invertebrates

Study 1 – Burke, Manson and Scholey (2008c)

A semi-static chronic toxicity to *Daphnia magna* study is available following GLP and OECD Test Guideline 211. The nominal exposure range was 0.0519, 0.104, 0.208, 0.415, 0.830 and 1.66 mg a.s./l. Exposure solutions were prepared with the aid of a solvent (DMF 0.1 ml/l) and a solvent control was included Analytical measurement by HPLC-UV and time-weight mean measured concentrations were calculated to be 0.0442, 0.0899, 0.188, 0.359, 0.670 and 1.16 mg a.s./l. Study conditions were acceptable and the study is considered valid.

The 21-day NOEC for reproduction was 0.0899 mg a.s./l based on time-weight mean measured concentrations.

The 21-day NOEC for weight was 0.670 mg a.s./l and the 21-day for length was 0.359 mg a.s./l, both based on time-weight mean measured concentrations.

11.6.3 Chronic toxicity to algae or other aquatic plants

A toxicity to algae study is available using pyriofenone. Study details are presented in section 11.5.3 above with the chronic endpoint detailed below.

Study 1 – Burke, Manson and Scholey (2008b)

The 72-h NOE_rC was determined to be 0.249 mg a.s./l based on mean measured concentrations.

11.6.4 Chronic toxicity to other aquatic organisms

Study 1 – Burke and Scholey (2009)

A GLP, 28-day toxicity to *Chironomus riparius* study using pyriofenone is available following OECD Test Guideline 219. The study employed a static water-sediment system and spiked overlying water (nominally 0.2, 0.4, 0.8, 1.6 and 3.2 mg a.s./l).

Significant losses were observed in the aqueous phase as the test item partitioned to the sediment phase and potentially underwent primary degradation. By day 28, concentrations in the water phase were 5.03 to 9.38% of the applied nominal dose.

The study reported a 28-day NOEC of 1.6 mg a.s./l for emergence and a 28-day NOEC of 3.2 mg a.s./l for development. Both NOECs are based on nominal applied water phase concentrations.

The above information is presented for completeness although the nominal endpoints are not considered for hazard classification due to difficulties interpreting data from water-sediment test systems.

11.7 Comparison with the CLP criteria

11.7.1 Acute aquatic hazard

Pyriofenone acute toxicity data are available for fish, invertebrates, algae and aquatic plants.

All acute ecotoxicity endpoints are >1 mg/l. Algae are the most acutely sensitive trophic level with an E_rC_{50} of 1.77 mg a.s./l.

Based on available data, degradation products are not considered more acutely toxic than the parent substance (see Annex I) and are not considered further for classification.

Based on these data, pyriofenone does not require an Aquatic Acute classification.

11.7.2 Long-term aquatic hazard (including bioaccumulation potential and degradation)

Pyriofenone is not readily biodegradable and is hydrolytically stable.

Under experimental conditions, photodegradation was observed in pure water. The study calculated a DT_{50} of 54 spring days sunshine at 35°N. However, information on photochemical degradation in the aquatic environment is difficult to use for classification purposes because the degree of degradation is dependent on local conditions.

In a water/sediment simulation study pyriofenone underwent primary degradation with low levels of ultimate degradation (maximum of 16.8% AR as CO_2 after 100 days). Whole system DT_{50} values at 12 °C based on primary degradation were 8.5 to 27.5 days with a geometric mean of 15.9 days for the two systems.

Multiple aquatic degradants were observed although none at >10% AR.

Overall, pyriofenone is not considered to be rapidly degradable for the purpose of classification.

Pyriofenone has a logPow below the CLP threshold of 4 and experimental BCFs below the CLP threshold of 500, therefore it is considered to have a low potential for bioaccumulation.

Chronic toxicity data for fish, invertebrates and algae are available. The fish 28-day NOEC is >1 mg/l and the algal 72-hour NOE_rC is 0.249 mg/l. However, invertebrates (*Daphnia magna*) are the most chronically sensitive trophic level with a 21-day NOEC of 0.0899 mg/l.

Based on available data, degradation products are not considered more chronically toxic than the parent substance (see Annex I) and are not considered further for classification.

Given the chronic ecotoxicity data and that pyriofenone is not rapidly degradable, it should be classified for the environment as Aquatic Chronic 1. Based on the *Daphnia magna* endpoint, a chronic M-factor of 1 is appropriate (0.01 mg/l < NOEC ≤ 0.1 mg/l).

11.8 CONCLUSION ON CLASSIFICATION AND LABELLING FOR ENVIRONMENTAL HAZARDS

No Aquatic Acute classification.

Aquatic Chronic 1, Chronic M-Factor: 1

Data conclusive and sufficient for classification

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
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.**

12 EVALUATION OF ADDITIONAL HAZARDS

12.1 Hazardous to the ozone layer

Not assessed in this dossier.

13 ADDITIONAL LABELLING

Not applicable.

14 REFERENCES

Physchem. references:

Study Author	Year	Reference
Turner, B.	2009a	IKF-309 (PAI): Physico-chemical properties Huntingdon Life Sciences Ltd Laboratory no. ISK0399

		Ishihara Sangyo Kaisha Ltd. GLP, unpublished
Turner, B.	2009b	IKF-309 (PAI): Vapour Pressure and Calculation of Volatility (Henry's Law Constant) Huntingdon Life Sciences Ltd Laboratory no. ISK0396 Ishihara Sangyo Kaisha Ltd. GLP, unpublished
Turner, B.	2009c	IKF-309 (TGAI): Physico-chemical properties Huntingdon Life Sciences Ltd Laboratory no. ISK0392 Ishihara Sangyo Kaisha Ltd. GLP, unpublished
Turner, B.	2007	IKF-309 PAI: Water solubility Huntingdon Life Sciences Ltd Laboratory no. ISK0290 Ishihara Sangyo Kaisha Ltd. GLP, unpublished
Turner, B.	2009g	IKF-309 (PAI): Partition coefficient Huntingdon Life Sciences Ltd Laboratory no. ISK0397 Ishihara Sangyo Kaisha Ltd. GLP, unpublished
Turner, B.	2009h	IKF-309 (PAI): Dissociation constant Huntingdon Life Sciences Ltd Laboratory no. ISK0398 Ishihara Sangyo Kaisha Ltd. GLP, unpublished

Toxicology and human health references:

Study Author	**Year**	**Reference**
May, K.	2007	IKF-309 technical: Bacterial reverse mutation test Huntingdon Life Sciences Ltd Laboratory no. ISK0311/073744 Ishihara Sangyo Kaisha Ltd. GLP, unpublished
Pritchard, L.	2008	IKF-309 technical: *In vitro* mammalian chromosome aberration test in CHL cells Huntingdon Life Sciences Ltd

		Laboratory no. ISK0322 Ishihara Sangyo Kaisha Ltd. GLP, unpublished
Hynes, L.	2008	IKF-309 technical: *In vitro* mutation test using mouse lymphoma L5178Y cells Huntingdon Life Sciences Ltd Laboratory no. ISK0310 Ishihara Sangyo Kaisha Ltd. GLP, unpublished
Haseman, J. K.	1998	Spontaneous neoplasm incidences in Fischer 344 rats and B6C3F1 mice in two-year carcinogenicity studies: A National Toxicology Program Update Toxicologic Pathology, 1998 vol. 26, no. 3, pages 428 – 441 published
Lang, P.	1990	Spontaneous neoplastic lesions in the CDF® (F344)/CrlBR rat. Published Report, Febraury 1990, Charles River
Shikama, H.	2013a	Effects of IKF-309 on Expression CYP genes in cultured hepatocytes Applied Science Group, Bioscience Research Laboratory, Central Research Institute Report ref. AS0081-01 Ishihara Sangyo Kaisha Ltd Non-GLP, unpublished
Shikama, H.	2013b	Effects of IKF-309 on DNA Replication in cultured hepatocytes Applied Science Group, Bioscience Research Laboratory, Central Research Institute Report ref. AS0082-01 Ishihara Sangyo Kaisha Ltd Non-GLP, unpublished
Scholzen, T. and Gerdes, J.	2000	The Ki-67 protein: From the known and the unknown Journal of Cellular Physiology 2000, 182: 311-322
Moore, JT et al.	2000	Orphan Nuclear Receptors Constitutive Androstane Receptor and Pregnane X Receptor Share Xenobiotic and Steroid Ligands J. Biol. Chem. 2000 275: 15122
Elcombe, C.R.	2014	Mode of action and human relevance analysis for nuclear receptor-mediated liver toxicity: A case study with phenobarbital as a model constitutive androstane receptor (CAR) activator Critical Reviews in Toxicology, Volume 44 – Issue 1 pages 64-82
Wainstok de Calmanovici, R, *et al*	1984	Mechanism of hexachlorobenzene-induced porphyria in rats. Effect of phenobarbitone pretreatment Biochemical Journal, Volume 218 (3) pages 753-763

<u>Environmental fate and ecotoxicology references:</u>

Author	**Year**	**Reference**
Turner, B.	2007	IKF-309 PAI: Water solubility. Huntingdon Life Sciences Ltd. Laboratory no. ISK0290. Unpublished.
Turner, B.	2009b	IKF-309 PAI: Vapour Pressure and Calculation of Volatility (Henry's Law Constant). Huntingdon Life Sciences Ltd. Laboratory no. ISK0396. Unpublished.
Turner, B.	2009g	IKF-309 (PAI): Partition coefficient Huntingdon Life Sciences Ltd Laboratory no. ISK0397 Ishihara Sangyo Kaisha Ltd. GLP, unpublished
Kane, T.	2008	Adsorption/desorption in five soils. Huntingdon Life Sciences Ltd. Laboratory no. ISK0283. Unpublished.
Kane, T.	2009	IKF-309: Photodegradation in water and determination of the quantum yield Huntingdon Life Sciences Ltd Laboratory no. ISK0285 Ishihara Sangyo Kaisha Ltd. GLP, unpublished
Juozenaite, A.	2009	IKF-309: Hydrolysis under simulated processing conditions Huntingdon Life Sciences Ltd Laboratory no. ISK0332 Ishihara Sangyo Kaisha Ltd. GLP, unpublished
Dickinson, R. A.	2009	IKF-309 technical – assessment of ready biodegradability: Sealed-vessel carbon dioxide evolution test Huntingdon Life Sciences Ltd Laboratory no. ISK0387 Ishihara Sangyo Kaisha Ltd. GLP, unpublished
Crowe, A.	2009	IKF-309: Aerobic transformation in aquatic sediment systems Huntingdon Life Sciences Ltd Laboratory no. ISK0288 Ishihara Sangyo Kaisha Ltd. GLP, unpublished

Burke, J., Manson, P.S. and Scholey, A.	2008b	IKF-309 technical: Acute toxicity to *Daphnia magna* Covance Laboratories Ltd Laboratory no. 2244/025-D2149 Ishihara Sangyo Kaisha Ltd GLP, unpublished
Burke, J., Manson, P.S. and Scholey, A.	2008c	IKF-309 technical: Chronic effects to *Daphnia magna* Covance Laboratories Ltd Laboratory no. 2244/034-D2149 Ishihara Sangyo Kaisha Ltd. GLP, unpublished
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Wilby, J	2010	2MDPM: Algal growth inhibition assay. Huntingdon Life Sciences Ltd. Report no. JSM0071. Unpublished.

15 ANNEXES

ANNEX I – Aquatic toxicity data for pyriofenone degradants.

Ecotoxicity studies using pyriofenone degradants are described in the DAR (2012). The majority of studies were considered unreliable and endpoints not valid due to study deficiencies including lack of analytical verification and GLP status (refer to DAR, 2012). However, the studies are a useful indicator that the degradants 2MDPM and 3HDPM are unlikely to be more ecotox than the parent pyriofenone.

The remaining valid ecotoxicity data is presented below.

Table A1: Summary of relevant information on aquatic toxicity for pyriofenone degradants

Degradant / Guideline / GLP	Species	Exposure		Results		Notes / observations	Reference
		Design	Duration	Endpoint	Toxicity (mg/l)		
2MDPM Freshwater Algal Growth Inhibition OECD 201, GLP, pyriofenone (98.17%)	*Pseudokirchneriella subcapitata*	Static	72 hours	E_rC_{50} NOE_rC	>0.418 (mm) 0.418 (mm)	Single treatment of nominal 2mg/l 0 h concentrations were 67-100% nominal 72 h concentrations were < LoD of 0.05 mg/l No microscopic abnormalities were observed.	Wilby, 2010

Notes:
mm refers to mean measured concentrations

ANNEX II – Confidential reference list (separate document).