Committee for Risk Assessment
RAC

Annex 1

Background document

to the Opinion proposing harmonised classification and labelling at EU level of

silthiofam (ISO); *N*-allyl-4,5-dimethyl-2-(trimethylsilyl)thiophene-3-carboxamide

EC Number: -
CAS Number: 175217-20-6

CLH-O-0000001412-86-245/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

30 November 2018

CLH report

Proposal for Harmonised Classification and Labelling

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

Substance Name: Silthiofam (ISO); N-allyl-4,5-dimethyl-2-(trimethylsilyl)thiophene-3-carboxamide

EC Number: **N/A**

CAS Number: **175217-20-6**

Index Number: **-**

Contact details for dossier submitter:

Pesticides Registration Division (PRD),
Department of Agriculture, Food & The Marine,
Backweston, Celbridge, Co. Kildare, W23 X3PH,
Ireland

Version number: **3.0**

Date: **Febuary 2018**

CONTENTS

Part A.

Part B.

Part A.

1 PROPOSAL FOR HARMONISED CLASSIFICATION AND LABELLING

1.1 Substance

Table 1: Substance identity

Substance name:	Silthiofam (ISO); N-allyl-4,5-dimethyl-2-(trimethylsilyl)thiophene-3-carboxamide
EC number:	N/A
CAS number:	175217-20-6
Annex VI Index number:	N/A
Degree of purity:	980 g/kg
Impurities:	The active substance as manufactured does not contain any impurities of toxicological/eco-toxicological relevance.

1.2 Harmonised classification and labelling proposal

Table 2: The current Annex VI entry and the proposed harmonised classification

	CLP Regulation
Current entry in Annex VI, CLP Regulation	Not classified
Current proposal for consideration by RAC	Repr. 2, H361d STOT RE 2, H373 Aquatic Chronic 2, H411
Resulting harmonised classification (future entry in Annex VI, CLP Regulation)	Repr. 2; H361d STOT RE 2; H373 Aquatic Chronic 2; H411

1.3 Proposed harmonised classification and labelling based on CLP Regulation criteria

Table 3: Proposed classification according to the CLP Regulation

CLP Annex I ref	Hazard class	Proposed classification	Proposed SCLs and/or M-factors	Current classification [1)]	Reason for no classification [2)]
2.1.	Explosives	None		None	Conclusive but not sufficient for classification
2.2.	Flammable gases	None		None	Substance is not a gas.
2.3.	Flammable aerosols	None		None	Substance is not an aerosol.
2.4.	Oxidising gases	None		None	Substance is not a gas.
2.5.	Gases under pressure	None		None	Substance is not a gas.
2.6.	Flammable liquids	None		None	Substance is not a liquid.
2.7.	Flammable solids	None		None	Conclusive but not sufficient for classification
2.8.	Self-reactive substances and mixtures	None		None	Data lacking.
2.9.	Pyrophoric liquids	None		None	Substance is not a liquid.
2.10.	Pyrophoric solids	None		None	Conclusive but not sufficient for classification.
2.11.	Self-heating substances and mixtures	None		None	Conclusive but not sufficient for classification
2.12.	Substances and mixtures which in contact with water emit flammable gases				Data lacking.
2.13.	Oxidising liquids	None		None	Substance is not a liquid.
2.14.	Oxidising solids	None		None	Conclusive but not sufficient for classification
2.15.	Organic peroxides	None		None	Conclusive but not sufficient for classification.
2.16.	Substance and mixtures corrosive to metals				Data lacking
3.1.	Acute toxicity - oral	None		None	Conclusive but not sufficient for classification
	Acute toxicity - dermal	None		None	Conclusive but not sufficient for classification
	Acute toxicity - inhalation	None		None	Conclusive but not sufficient for classification

ANNEX 1 - BACKGROUND DOCUMENT TO RAC OPINION ON SILTHIOFAM (ISO); *N*-ALLYL-4,5-DIMETHYL-2-(TRIMETHYLSILYL)THIOPHENE-3-CARBOXAMIDE

CLP Annex I ref	Hazard class	Proposed classification	Proposed SCLs and/or M-factors	Current classification [1)]	Reason for no classification [2)]
3.2.	Skin corrosion / irritation	None		None	Conclusive but not sufficient for classification
3.3.	Serious eye damage / eye irritation	None		None	Conclusive but not sufficient for classification
3.4.	Respiratory sensitisation				Data lacking
3.4.	Skin sensitisation	None		None	Conclusive but not sufficient for classification
3.5.	Germ cell mutagenicity	None		None	Conclusive but not sufficient for classification
3.6.	Carcinogenicity	None		None	Conclusive but not sufficient for classification
3.7.	**Reproductive toxicity**	**REPR 2, H361d**		**None**	
3.8.	Specific target organ toxicity –single exposure	None		None	Conclusive but not sufficient for classification
3.9.	**Specific target organ toxicity – repeated exposure**	**STOT RE 2, H373**		**None**	
3.10.	Aspiration hazard	None		None	Conclusive but not sufficient for classification
4.1.	**Hazardous to the aquatic environment**	**Aquatic Chronic 2, H411**	**None**	**None**	
5.1.	Hazardous to the ozone layer				Conclusive but not sufficient for classification

[1)] Including specific concentration limits (SCLs) and M-factors
[2)] Data lacking, inconclusive, or conclusive but not sufficient for classification

Labelling:

Signal word: Warning

Pictograms: GHS08; GHS09

Hazard statements:

Repr. 2; H361d: Suspected of damaging the unborn child.
STOT RE 2; H373: May cause damage to organs through prolonged or repeated exposure
Aquatic Chronic 2; H411: Toxic to aquatic life with long lasting effects.

Precautionary statements:

P391 Collect Spillage
P501 Dispose of contents/container to [in accordance with local/refional/international regulation]

Supplemental Hazard Statement:

EUH401 To avoid risks to human health and the environment, comply with the instructions for use.

2 BACKGROUND TO THE CLH PROPOSAL

2.1 History of the previous classification and labelling

Silthiofam was included into Annex I of Directive 91/414/EEC on 01 January 2004 (Commission Directive 2003/84/EC of 25 September 2003). Silthiofam has not been previously considered for inclusion in Annex VI of Regulation (EC) 1272/2008.

2.2 Short summary of the scientific justification for the CLH proposal

Health Effects CLH proposal

Developmental toxicity:

Silthiofam is currently not classified for reproductive toxicity (developmental). The significance of the findings in the rat developmental study have been re-evaluated in the context of current scientific knowledge and regulatory experience. A classification in Repr. 2 for developmental toxicity has been proposed based on significant adverse effects on development observed at the highest dose tested (1000 mg/kg) in the rat developmental toxicity study, which was also clearly toxic to the maternal animal. Reduced foetal weight, reduced ossification of centrum number 1 and sternebrea 1-4, increased 7th cervical rib and increased number of dead foetuses were considered related to treatment at this toxic dose. A rare finding, cleft palate, occurred in two litters; one foetus (1/15) in one litter and 8 (8/11) in a second. Even though 1/1 (0.3% per litter) is within the historical control data, the occurrence of a second litter with multiple malformed pups, including a high incidence of cleft palate contributes to an argument for a treatment-related adverse effect.

The effects on development reported above in the rat occurred in the presence of significant maternal toxicity. Some effects could be directly attributed to this, i.e., reduced foetal weight (possibly), reduced ossification and the increase in incidence of a commonly occurring skeletal anomaly, 7th cervical rib. The occurrence of cleft palate in two litters raises the possibility of a treatment-related effect which is difficult to dismiss. In addition, there was an increase in dead pups in rats at caesarian section which was practically unknown in the historical data provided. According to the criteria, classification into Repr. 2 H361d 'Suspected of damaging the unborn child' is forseen for such a situation.

Specific target organ toxicity (repeated exposure):

Silthiofam is currently not classified for specific target organ toxicity. The liver was identified as the main target organ following the short-term oral administration of silthiofam in the rat, mouse and dog. Elevated liver weights and serum enzyme markers for liver toxicity were observed in all three species. However, liver histopathology was observed only in rats and mice, and included hepatocellular hypertrophy, vacuolation, degeneration/necrosis of individual hepatocytes, bile duct hyperplasia/fibrosis, etc. No liver histopathological findings were observed in dogs, at a dose level that was significantly toxic. The dog and rat were identified as the most sensitive species; both of these species were more sensitive to the effects of silthiofam than the mouse (dog = rat > mouse).

Effects of treatment were seen in the dog at lower doses and within the numerical criteria for STOT RE 2. Silthiofam causes significant systemic toxicity in the dog on sub-chronic administration, including mortalities at doses of approx. 350/250 mg/kg bw after 24 doses

(female) and 12/18 doses (male), severe effects on food consumption and weight loss were seen. Some evidence of liver effects were also observed. Similarly, severe toxicity was seen at 125/75 mg/kg in the 90-day study with mortality (1), severe effects on body weight (loss) and some evidence of liver effects without histopathological correlation. The same pattern was seen in the one year study, at 80 mg/kg bw, with systemic toxicity, some evidence of liver toxicity but without histopathological correlation (outside the 1 year study extrapolated guidance value for STOT RE 2 of 25 mg/kg bw). Liver toxicity and systemic toxicity occurred in the rat and mouse but without mortality at significantly higher dose levels. However, the effects seen in the dog, which were within the cut-off criteria, are consistent with general systemic toxicity and also liver toxicity but were not seen in two other species tested to higher dose levels and classification is not proposed on the basis of systemic/liver toxicity.

However, in the rabbit range-finding developmental toxicity study (Report WI-95-239, 1997), increased mortaliy was observed. In this study 4/6 and 5/6 females died in the 100 and 150 mg/kg/day dose groups, respectively. Deaths occurred between days 13-16 and 15-22 for gravid females in these respective groups. All deaths (except for one intubation error in the 100 mg/kg group) were considered treatment-related. The two other females in the 100 mg/kg group aborted prior to scheduled euthanasia. These findings are within the criteria (mortality) and the numerical cut-of values (≤ 100 mg/kg bw) for studies of shorter duration (c.f. and classification as STOT RE 2 H373 'May cause damage to organs through prolonged or repeated exposure' is proposed on the this basis.

Long-term aquatic hazard:

Silthiofam classifies for ecotoxicological effects. It is non rapidly degradable. Based upon the results of the 28-day Early Life-Stage Toxicity Test with the Fathead Minnow (*Pimephales promelas*) and the 21 day semi-static life-cycle toxicity test with the cladoceran (*Daphnia magna*), both with a chronic NOEC ≤ 1 mg/L, Silthiofam classifies as Chronic Category 2 H411 'Toxic to aquatic organisms with long lasting effects' in accordance with CLP Regulation 1272/2008.

2.3 Current harmonised classification and labelling

No classification.

2.3.1 Current classification and labelling in Annex VI, Table 3.1 in the CLP Regulation

No entry.

2.3.2 Current classification and labelling in Annex VI, Table 3.2 in the CLP Regulation

No entry.

2.4 Current self-classification and labelling

Not applicable.

2.4.1 Current self-classification and labelling based on the CLP Regulation criteria

No classification.

RAC general comment
Silthiofam is a pesticide active substance approved as a selective fungicide under regulation (EC) No 1107/2009. There is no existing entry in Annex VI of the CLP regulation for silthiofam. Therefore, the proposal of the dossier submitter (DS) addressed all physical, human health and environmental endpoints.

3 JUSTIFICATION THAT ACTION IS NEEDED AT COMMUNITY LEVEL

Silthiofam is a pesticide active substance currently under review for approval to Regulation (EC) No 1107/2009 of the European Parliament and of the Council. The classification and labelling proposal includes mammalian and environmental toxicity endpoints and needs to be evaluated under the CLP Regulation.

Part B.

Scientific evaluation of the data

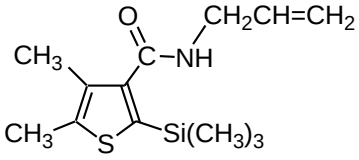
1 IDENTITY OF THE SUBSTANCE

1.1 Name and other identifiers of the substance

Table 4: Substance identity

EC number:	N/A
EC name:	N/A
CAS number (EC inventory):	N/A
CAS number:	175217-20-6
CAS name:	3-Thiophenecarboxamide, 4,5-dimethyl-N-2-propen-1-yl-2-(trimethylsilyl)
IUPAC name:	*N*-allyl-4,5-dimethyl-2-(trimethylsilyl)thiophene-3-carboxamide
CLP Annex VI Index number:	-
Molecular formula:	$C_{13}H_{21}NOSSi$
Molecular weight range:	267.47 g/mol

Structural formula:



1.2 Composition of the substance

Table 5: Constituents (non-confidential information)

Constituent	Typical concentration	Concentration range	Remarks
Silthiofam	≥ 980 g/kg		

Current Annex VI entry:

Table 6: Impurities (non-confidential information)

Impurity	Typical concentration	Concentration range	Remarks
Confidential data	A new specification based on the most recent 5-batch study from 2010 indicated 3 impurities of quantitative relevance. These impurities pose no concern from either a toxicological or environmental perspective in Silthiofam technical.		

Current Annex VI entry:

Table 7: Additives (non-confidential information)

Additive	Function	Typical concentration	Concentration range	Remarks
None	-	-	-	-

Current Annex VI entry:

1.2.1 Composition of test material

This information is confidential and in the case of Silthiofam not relevant for classification.

1.3 Physico-chemical properties

Table 8: Summary of physico - chemical properties

Property	Value	Reference	Comment (e.g. measured or estimated)
State of the substance at 20°C and 101,3 kPa	Fluffy crystalline powder, white in the case of the pure active substance (99.6%) and beige in the case of the T.G.A.I. (96.1%).	Herren, D. 1998. Report no. MSL-15542.	Observed.
Melting/freezing point	88°C or 361K (99.67% purity)	Krips, H. 2000. Notox Project 291353.	Measured.
Boiling point	321.9-340 °C (98.7% purity)	Bates, M. 1999. Report No. 64/93-D2141.	Measured.
Relative density	1.07 at 20.0°C (99.67% purity)	Krips, H. 2000. Notox Project 291375.	Measured.
Vapour pressure	$(8.1 \pm 0.7) \times 10^{-2}$ Pa at 20°C (99.67% purity)	Krips, H. 2000. Notox Project 291386.	Measured.
Surface tension	60.4 mN/m at 20 °C (90 % saturated solution) (97.3% purity)	Bates, M. 1996. Report no. 64/47-1014.	Measured.
Water solubility	39.9 µg/ml at 19.5 ±0.5°C at pH 8.7-9.1 (99.67% purity)	Brekelmans, M. 2000. Notox Project 291432.	Measured.
Partition coefficient n-octanol/water	log P_{OW} = 3.72 at 19.5 ± 0.5°C (pH 7.8) (99.67% purity)	Brekelmans, M. 2000. Notox Project no. 291443.	Measured.
Flash point	Not tested.		Silthiofam is not a liquid.
Flammability	Not flammable (97.3% purity)	Bates, M. 1996. Report no. 64/47-1014.	Measured.
Explosive properties	Non explosive (97.3% purity)	Bates, M. 1996. Report no. 64/47-1014.	Measured.
Self-ignition temperature	Not flammable (98.8% purity)	Edwards, D. 1998. Report no. 64/47-D2141	Measured.
Oxidising properties	Not oxidising	Edwards, D. 1998. Report no. 64/47-D2141	Justification.
Granulometry	Not tested.		
Stability in organic solvents and identity of relevant degradation products	Not tested.		

Dissociation constant	No dissociation (97.7% purity).	Bates, M. 1995. Report no. 64/46-1014.	Measured.
Viscosity	Not tested.		

2 MANUFACTURE AND USES

2.1 Manufacture

This information is confidential. Please refer to the Silthiofam RAR, Volume 4 - Annex C (Confidential Information).

2.2 Identified uses

Silthiofam is a selective seed applied fungicide with a limited systemic action and little or no translocation in the plant. Silthiofam is applied as a surface layer to the seed. It is a selective fungicide known to affect only one pathogen viz. *Gaeumannomyces graminis var tritici* (Take-All) and protect cereal crops including wheat, barley, triticale and spelt. The Take-all fungus is present in most soils where cereals are grown in the EU.

3 CLASSIFICATION FOR PHYSICO-CHEMICAL PROPERTIES

Table 9: Summary table for relevant physico-chemical studies

Method	Results	Remarks	Reference
EEC A14. -The BAM fall hammer test -The BAM friction test -The Koenen steel tube test	The test item was not explosive.	Carried out to GLP. No classification is required.	Bates, M. 1996. Report no. 64/47-1014.
Justification	Based on the structure of silthiofam the molecule does not have oxidising properties.	None.	Edwards, D. 1998. Report no. 64/47-D2141
EEC A10	Silthiofam does not support combustion.	Silthiofam is not flammable. No classification is required.	Bates, M. 1996. Report no. 64/47-1014.
EEC A10/A16.	In the initial evaluation, the test substance melted to a clear yellow liquid, white fumes were evolved but no flame was observed. In the train test the substance did not ignite. The sample was heated to 120°C which is ~30°C above the melting temperature. No significant thermal events were seen on a chart trace.	The experiment was conducted to GLP. Silthiofam is not auto-flammable. No classification is required.	Edwards, D. 1998. Report no. 64/47-D2141

3.1 Explosive properties

3.1.1 Summary and discussion of explosive properties for silthiofam

The BAM fall hammer test, the BAM friction test and the Koenen steel tube test were performed using a 6mm and a 2mm orifice. The test was conducted to GLP. The test item was not explosive.

3.1.2 Comparison with criteria

Not applicable, all standard studies investigating flammability, ignition and combustion properties were negative.

3.1.3 Conclusions on classification and labelling

Silthiofam is not explosive. No classification is required.

3.2 Oxidising properties

3.2.1 Summary and discussion of oxidising properties for silthiofam

Based on the structure of silthiofam the molecule does not have oxidising properties. The justification is acceptable.

3.2.2 Comparison with criteria

Not applicable.

3.2.3 Conclusions on classification and labelling

Silthiofam is not oxidising. No classification is required.

3.3 Flammability properties

3.3.1 Summary and discussion of flammability properties for silthiofam

Silthiofam does not support combustion.

3.3.2 Comparison with criteria

Not applicable, all standard studies investigating flammability, ignition and combustion properties were negative.

3.3.3 Conclusions on classification and labelling

Silthiofam is not flammable. No classification is required.

3.4 Auto-flammability properties

3.4.1 Summary and discussion of auto-flammability properties for silthiofam

In the initial evaluation, the test substance melted to a clear yellow liquid, white fumes were evolved but no flame was observed. In the train test the substance did not ignite. The sample was heated to 120°C which is ~30°C above the melting temperature. No significant thermal events were seen on a chart trace. The experiment was conducted to GLP.

3.4.2 Comparison with criteria

Not applicable.

3.4.3 Conclusions on classification and labelling

Silthiofam is not auto-flammable. No classification is required.

3.5 Overall conclusion on the classification for physical and chemical properties

Silthiofam is not explosive, oxidising, flammable or auto-flammable and does not classify from a physical and chemical point of view. Therefore, no classification is required.

RAC evaluation of physical hazards

Summary of the Dossier Submitter's proposal

The DS did not propose classification for physical hazards. The data on physico-chemical properties did not indicate any concerns and therefore silthiofam does not meet the criteria for classification.

Comments received during public consultation

No comments were received.

Assessment and comparison with the classification criteria

Tests conducted according to EEC A.10/A.16 showed that silthiofam is not flammable or auto-flammable. In addition, the structural formula of silthiofam does not contain any of the chemical groups characteristic of an oxidising solid. Test method EEC A.14 showed that silthiofam is not explosive. Therefore, RAC agrees with the DS that **classification is not required for physical hazards**.

4 HUMAN HEALTH HAZARD ASSESSMENT

4.1 Toxicokinetics (absorption, metabolism, distribution and elimination)

4.1.1 Non-human information

In the ADME study which included low-dose intravenous, low- and high-dose single oral, and low-dose repeat oral administration, silthiofam was rapidly and virtually completely absorbed by the rat at all dose levels (calculated absorption was 87.0% for the repeated oral dose, 91.6% for the single low oral dose, and 99.6% for the single high oral dose). The dose was readily eliminated with 87.3–93.7% of the dose being excreted within 48 hours after dosing. Urine was the major route of elimination accounting for 48.5–61.5% of the dose. There was no evidence of accumulation in the animal body. Less than 1.1% of the dose remained in the animal at 7 days after dosing. Levels of radioactivity in the tissues were low with fat containing the highest levels (0.11–0.61% of the dose). Except for fat, no single tissue of any individual animal contained greater than 0.25% of the dose. This pattern of absorption, distribution and elimination was not significantly changed either by a single high dose or by repeated administration of low doses. Similarly, the sex of the test animals did not affect the results. Metabolism of silthiofam by the rat was extensive with no unchanged parent observed in the urine or faeces. The major routes of metabolism were hydroxylation of the ring-attached

methyl groups and subsequent oxidation to the carboxylic acid, dihydroxylation of the allyl double bond, oxidative removal of the allyl group to form the primary amide, and hydrolysis of the amide to form the carboxylic acid. The metabolism of silthiofam was very similar regardless of the dose level, sex or dosing regimen (oral, intravenous or repeat oral).

4.1.2 Human information

No data.

4.1.3 Summary and discussion on toxicokinetics

The toxicokinetics of silthiofam were investigated *i.v.* and orally in single dose and repeat dose studies in rats. Following single and repeat administration, silthiofam was well absorbed and widely distributed. Silthiofam was extensively metabolised and excreted predominantly in the urine. There was no evidence of bioaccumulation.

4.2 Acute toxicity

Table 10: Summary table of relevant acute toxicity studies

Method	Results	Remarks	Reference
Acute oral toxicity (LD_{50}): Single dose of 5000 mg/kg Silthiofam 91.8% in corn oil -Rat (CD®(SD)BR)	>5000 mg/kg -No mortalities -Signs of non-specific toxicity in 3/10 animals	-	Blaszcak (1996). PL-95-241; DAR B6.2.1/01
Acute dermal toxicity (LD_{50}) Single dose of Silthiofam 91.8% in corn oil -Rat (CD®(SD)BR)	> 5000 mg/kg -No mortalities or abnormalities.	-	Blaszcak (1996). PL-95-242; DAR B6.2.2/01
Acute inhalation toxicity (LC50) Single dose of Silthiofam 97.4% as an aerosol: MMAD 3.6µm (GSD 2.0) 93% <10 µm 3.4% < 1µm Rat(CD®(SD)BR)	> 2.8 mg/L (max attainable conc.) -Transient (24 hours) non-specific signs of toxicity -No mortalities	-	Bechtel (1996). ML-96-158, MSL-14866; DAR B6.2.3/01

4.2.1 Non-human information

4.2.1.1 Acute toxicity: oral

Acute oral toxicity was tested in the rat using a single dose of 5000 mg/kg bw. There were no mortalities. Some 3/10 animals showed non-specific signs of toxicity including; ano-genitial staining, decreased activity, excessive salivation, red stains on snout/forepaws and decreased faecal volume. There were no abnormal findings at necropsy. The acute oral LD_{50} of silthiofam in the rat is greater than 5000 mg/kg.

4.2.1.2 Acute toxicity: inhalation

Acute inhalation toxicity was tested in the rat using a single dose of 2.8 mg/l, the maximum attainable concentration. No deaths occurred. Signs observed during exposure included laboured respiration and red ocular discharge. Signs observed in one or more rats immediately after exposure included red ocular discharge, urine and faeces stained hair, and test material on the nose. All rats were absent of clinical signs by day 1. No abnormalities were observed at the gross necropsy. The inhalation LC_{50} (4-hours) of silthiofam in the rat is greater than 2.8 mg/l

4.2.1.3 Acute toxicity: dermal

Acute dermal toxicity was tested in the SD rat using a single dose of 5000 mg/kg bw. No deaths or clinical signs occurred. No internal abnormalities were observed at the gross necropsy. The acute dermal LD_{50} of silthiofam in the rat is greater than 5000 mg/kg

4.2.1.4 Acute toxicity: other routes

No data.

4.2.2 Human information

No data.

4.2.3 Summary and discussion of acute toxicity

All studies were compliant with good laboratory practice guidelines and were carried out in accordance with current OECD guidelines. The acute toxicity potential of silthiofam following oral, percutaneous and inhalation exposure is low.

4.2.4 Comparison with criteria

The acute LD/LC_{50s} for silthiofam are greater than the cut-off values for classification for acute toxicity by oral, dermal and inhalation routes, i.e.,
Oral LD_{50}: >5000 mg/kg bw (Cat 4: 300 <ATE ≤ 2000).
Dermal LD_{50}: > 5000 mg/kg bw (Cat 4: 1000 < ATE ≤ 2000).
Inhalation LC_{50}: > 2.8 mg/l (Cat 4: 1.0 < ATE ≤ 5.0)

4.2.5 Conclusions on classification and labelling

Classification is not required.

RAC evaluation of acute toxicity

Summary of the Dossier Submitter's proposal

Oral route

No classification is proposed based on the absence of mortality at 5 000 mg/kg bw observed in an acute oral toxicity study in rats.

Dermal route

No deaths occurred in an acute rat dermal toxicity study. The acute dermal LD_{50} was greater than 5 000 mg/kg bw in the study. On this basis, no classification was proposed by the DS.

Inhalation route

The LC_{50} observed in rats exposed to a single dose of silthiofam (dust) was greater than 2.8 mg/L which was the highest technically attainable concentration. As no deaths occurred at this dose, no classification was proposed by the DS.

Comments received during public consultation

No specific comments were received.

Assessment and comparison with the classification criteria

Acute toxicity: oral

Silthiofam was tested in an OECD TG 401 study (GLP compliant) in the rats at 5 000 mg/kg bw. No deaths occurred and the LD_{50} was thus greater than 5 000 mg/kg bw in both sexes. In this study, signs of non-specific toxicity were observed in 3 out of 10 animals (e.g. decreased activity, excessive salivation, decreased faecal volume, red stains on the snout). Based on the criteria in the CLP regulation, RAC agrees with the DS that no classification is warranted.

Acute toxicity: dermal

The LD_{50} of silthiofam in rats was greater than 5 000 mg/kg bw in an OECD TG 402 study (GLP compliant) in both sexes. No classification is warranted based on the CLP criteria.

Acute toxicity: inhalation

In an OECD TG 403 study, rats were exposed nose-only to 2.8 mg/L/4h of silthiofam (dust). No mortality was observed in this group. The clinical signs from this study are described under the section on STOT SE (below). As 2.8 mg/L was the highest attainable concentration, there is no evidence that the LC_{50} in rats is below the 5 mg/L cut-off for classification for acute toxicity by inhalation. Therefore, RAC agrees with the DS that classification of silthiofam for acute toxicity following inhalation exposure is not warranted.

In conclusion, RAC agreed that **no classification is warranted for acute toxicity via the oral, dermal and inhalation routes**.

4.3 Specific target organ toxicity – single exposure (STOT SE)

4.3.1 Summary and discussion of Specific target organ toxicity – single exposure

There were no functional disturbances or morphological changes or severe toxicity impacting on health, observed in any of the acute animal studies.

4.3.2 Comparison with criteria

Not required.

4.3.3 Conclusions on classification and labelling

There was no toxicological basis to compare with guidance value ranges for STOT SE category 1 or 2 as set out in section 3.8.2.2.1 of the CLP guidance. Similarly, there was no evidence or indication of transient respiratory tract irritation or narcosis nor was there any human data relating to these effects. The criteria for STOT SE category 3 were not met. Classification for STOT SE is not supported.

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

Summary of the Dossier Submitter's proposal

The DS concluded that silthiofam was of low acute toxicity and that there is no basis for classification for STOT SE in category 1 or 2. Moreover, no evidence or indication of transient respiratory tract irritation or narcosis was observed in the available studies. Therefore, no classification was proposed by the DS.

Comments received during public consultation

No specific comments were received.

Assessment and comparison with the classification criteria

There was no relevant data on humans in the dossier. RAC agreed with the DS that that the data available from studies involving single exposure to silthiofam provided no basis for classification for STOT SE in category 1 or 2.

From the acute inhalation toxicity study, clinical signs suggestive of respiratory irritation such as laboured respiration and red ocular discharge and test material on the nose as well as hair stained with urine and faeces were observed in one or more rats during and immediately after exposure (exact number of animals not reported), at the highest attainable concentration tested (2.8 mg/L). These clinical signs were reversible by day 1 and no abnormalities were observed at necropsy. As the substance is a solid, the mechanical effect of solid particles may have contributed to the irritation observed. No lower concentrations were tested in the study. The substance was without irritant effect in the eyes or the skin of rabbits. No gross pathological findings in the lung were observed at necropsy. Therefore, RAC agrees with the DS' proposal not to classify silthiofam as STOT SE 3 for respiratory irritation.

Overall, RAC agrees with the DS that **classification for STOT SE is not warranted** for silthiofam.

4.4 Irritation

4.4.1 Skin irritation

Table 11: Summary table of relevant skin irritation studies

Method	Results	Remarks	Reference
Skin irritation Single semi-occlusive application (5 gms) of Silthiofam ,91.8% in corn oil NZW Rabbit	Non-irritant	-	Blaszcak (1996). PL-95-244; DAR B6.2.4/01

4.4.1.1 Non-human information

Silthiofam, purity: 91.8%, in corn oil was applied under semi-occlusive dressing to the clipped skin of 4 male and 2 female NZW rabbits. The exposure was for 4 hours to a dose of 5000 mg as. All six animals were free of dermal irritation for the duration of the study. Silthiofam was not irritant to the skin in this study.

4.4.1.2 Human information

No data.

4.4.1.3 Summary and discussion of skin irritation

Non irritant.

4.4.1.4 Comparison with criteria

Classification as a skin irritant according to the CLP criteria is based on evidence of reversible damage to the skin on application for up to 4 hours in experimental animals using criteria for erythema, oedema as defined in the Regulation. No evidence of an irritant effect was seen for silthiofam.

4.4.1.5 Conclusions on classification and labelling

Not required.

RAC evaluation of skin corrosion/irritation

Summary of the Dossier Submitter's proposal

In an OECD TG 404 study, silthiofam (ground and moistened with saline) was applied to the skin of 6 rabbits. Exposure was for 4 hours under a semi-occlusive dressing. As no irritation was observed, the DS proposed no classification for skin irritation/corrosion.

Comments received during public consultation

No specific comments were received.

Assessment and comparison with the classification criteria

In the absence of any signs of irritation in an OECD TG and GLP compliant study, RAC agrees with the proposal of the DS **not to classify silthiofam for skin irritation/corrosion.**

4.4.2 Eye irritation

Table 12: Summary table of relevant eye irritation studies

Method	Results	Remarks	Reference
Eye irritation Single application (5 gms) of Silthiofam 91.8% NZW Rabbit	Transient conjuntival irritation resolved by 48 hrs; not classifiable.	Non-irritant	Blaszcak (1996). PL-95-243; DAR B6.2.5/01

4.4.2.1 Non-human information

Silthiofam, purity: 91.8%, was instilled into one each of 3/sex NZW rabbits. The single dose administration was followed by a 3 day observation period. Slight to moderate conjunctival erythema were seen in all animals after one hour, which was resolved by 48 hours in all animals.

Table 13: Summary table of eye irritation scores

	Individual scores				
	1 hour	24 hours	48 hours	72 hours	mean (24,48,72)
Animal 1					
-conj. erythema.	2	2	0	0	0.7
Oedema	1	0	0	0	
Animal 2					
-conj. erythema.	2	1	0	0	0.3
Oedema	1	0	0	0	
Animal 3					
-conj. erythema.	1	0	0	0	0.0
Oedema	1	0	0	0	
Animal 4					
-conj. erythema.	2	1	0	0	0.3
Oedema	2	0	0	0	
Animal 5					
-conj. erythema.	2	1	0	0	0.3
Oedema	1	0	0	0	
Animal 6					
-conj. erythema.	1	0	0	0	0.0
Oedema	1	0	0	0	

There were no iridial or corneal effects.

4.4.2.2 Human information

No data.

4.4.2.3 Summary and discussion of eye irritation

Silthiofam can cause slight/moderate reversible effects to the eye.

4.4.2.4 Comparison with criteria

Classification as an eye irritant according to the CLP criteria is based on evidence of reversible damage to the eye in experimental animals using criteria for corneal opacity, iridial congestion and conjunctival inflammation as defined in the Regulation. Slight conjunctival irritation (24/48/72 mean of 0.3 – 0.7) was seen for silthiofam not meeting the classification criteria (≥ grade 2).

4.4.2.5 Conclusions on classification and labelling

Classification not required.

RAC evaluation of serious eye damage/irritation

Summary of the Dossier Submitter's proposal

In an OECD TG 405 study (GLP compliant), solid silthiofam was instilled in the eyes of six rabbits. Slight conjunctival irritation was observed after one hour which was resolved by 48 h (mean 24-72 h score: max. 0.7). On this basis, no classification was proposed by the DS.

Comments received during public consultation

No specific comments were received.

Assessment and comparison with the classification criteria

RAC agrees with the DS proposal **not to classify silthiofam for eye damage/irritation** based on the absence of irritation observed in an OECD TG 405 study (which was GLP compliant).

4.4.3 Respiratory tract irritation

No data.

4.4.3.1 Non-human information

No data.

4.4.3.2 Human information

No data.

4.4.3.3 Summary and discussion of respiratory tract irritation

Not relevant.

4.4.3.4 Comparison with criteria

4.4.3.5 Conclusions on classification and labelling

4.5 Corrosivity

No data. No evidence to support this hazard.

4.5.1 Non-human information

Not relevant.

4.5.2 Human information

Not relevant.

4.5.3 Summary and discussion of corrosivity

4.5.4 Comparison with criteria

4.5.5 Conclusions on classification and labelling

4.6 Sensitisation

4.6.1 Skin sensititsation

Table 14: Summary table of relevant skin sensitisation studies

Method	Results	Remarks	Reference
M and K (maximisation) Silthiofam 91.8% Intradermal induction: 5% w/v Topical induction: 100% w/vChallenge: 100%	Non-sensitising No reaction	-	Blaszcak (1996). PL-95-245; DAR B6.2.6/01

4.6.1.1 Non-human information

Silthiofam, (Lot Number: QPP-9509-6764-T, purity: 91.8%) was tested in a M&K sensitisation test. 5% (w/v) concentration was used for intradermal induction. Intradermal injections were made with Freund's Complete Adjuvant (FCA)/water emulsion (50%v/v), and a 5% w/v mixture of silthiofam in propylene glycol, and in FCA/water emulsion. The test substance was employed at a 100% concentration for topical induction and challenge having been moistened with 0.9% saline. Dose levels were based on the results of a range-finding test. The area to be treated was pre-treated with 10% sodium lauryl sulphate in petrolatum on the day before topical

induction to provoke a mild inflammatory reaction. Topical induction and challenge applications were made 7 and 21 days after the intradermal induction, respectively. Appropriately treated control animals were employed in the study design in order to differentiate between dermal responses produced by irritation and those produced by sensitisation.

All test and control animals were free of dermal responses 24 and 48 hours after administration of the challenge dose. Silthiofam did not exhibit a potential to produce dermal sensitisation in the guinea pig.

4.6.1.2 Human information

No data.

4.6.1.3 Summary and discussion of skin sensitisation

Silthiofam was negative in an appropriately conducted maximisation assay.

4.6.1.4 Comparison with criteria

Substances are classified as sensitisers on the basis of evidence in humans and/or animal studies. Silthiofam does not classify as there was no dermal response to challenge.

4.6.1.5 Conclusions on classification and labelling

No classification on the basis of the data submitted.

RAC evaluation of skin sensitisation

Summary of the Dossier Submitter's proposal

Silthiofam was tested in a guinea-pig maximisation test (GPMT), performed according to OECD 406 (GLP compliant). As no dermal response to challenge was observed in treated and control animals, no classification was proposed by the DS.

Comments received during public consultation

No comments were received.

Assessment and comparison with the classification criteria

RAC agrees with the DS that silthiofam **should not be classified as a skin sensitiser** based on the negative GPMT.

4.6.2 Respiratory sensitisation

4.6.2.1 Non-human information

No data.

4.6.2.2 Human information

No data.

4.6.2.3 Summary and discussion of respiratory sensitisation

Not relevant.

4.6.2.4 Comparison with criteria

4.6.2.5 Conclusions on classification and labelling

RAC evaluation of respiratory sensitisation

Summary of the Dossier Submitter's proposal

No classification was proposed by the DS due to lack of data.

Comments received during public consultation

No specific comments were received.

Assessment and comparison with the classification criteria

RAC agrees with the DS that silthiofam **should not be classified as respiratory sensitiser due to lack of data.**

4.7 Repeated dose toxicity

Table 15: Summary table of relevant repeated dose toxicity studies

Study	Dose levels	NOAEL	Key Findings	DAR Reference
28-day mouse (diet)	0, 10, 100, 1000 & 4000 ppm: 0, 1.4, 14.7, 147.7 and 578.0 (♂) and 0, 2.1, 25.8, 259.4 and 781.5 (♀)	1000 ppm (148/259 mg/kg) (M/F)	Liver toxicity at 4000 ppm included ↑ALT, ↑liver wts, gross lesions, and centrilobular vacuolation, degeneration and hypertrophy	Lemen and Ruecker (1996). ML-95-041, MSL-14538/15332; B6.3.1/01
28-day rat (diet)	0, 20, 200, 1000 & 8000 ppm: 0, 1.5, 14.5, 73.1 and 407.5	1000 ppm (73/77 mg/kg) (M/F)	Substantial liver toxicity at 8000 ppm as evidenced by ↑liver wts, clinical chemistry changes, and gross & microscopic findings (including hepatocellular necrosis, bile duct	Lemen and Thake (1996). ML-95-042, MSL-14537; 5

Study	Dose levels	NOAEL	Key Findings	DAR Reference
	(♂) and 0, 1.5, 16.1, 77.5 and 542.8 (♀)		hyperplasia, etc.). Other effects at 8000 ppm included ↓body weight, ↓food consumption, ↑reticulocytes, and possible effects on kidney and spleen.	B6.3.1/02
28-day dog (capsule)	0, 10, 50, 150 and 350/250 mg/kg bw (high-dose reduced from 350 to 250 after 2-3 weeks):	50/10 mg/kg (M/F)	High-dose (350/250) and perhaps 150 mg/kg/day were excessively toxic, as evidenced by mortality (high-dose only), clinical signs of toxicity and weight loss. Slight weight loss also seen in 50 mg/kg/day females. Increased liver weight and serum enzyme markers at 150 mg/kg/day but no histopath performed.	Lemen (1996). ML-95-204, MSL-14758; B6.3.1/03
60-day mouse (diet)	0, 50, 1000, 2500 & 5000 ppm: 0, 6.98, 140.32, 363.40 and 707.16 (♂) and 0, 10.50, 235.56, 519.31 and 1131.89 (♀).	1000 ppm (140/236 mg/kg/day) (M/F)	Liver toxicity at 2500 and/or 5000 included ↑ALT, ↑liver weights, and hepatocellular hypertrophy, vacuolation, degeneration and/or necrosis.	Lemen et al. (1997). ML-95-202, MSL-15251; B6.3.2/01
90-day rat with pilot repro phase (diet)	0, 25, 250, 2500 & 5000 ppm: 0, 1.45, 14.96, 145.73 and 290.04 (♂), and 0, 1.70, 17.55, 174.60 and 334.04 (♀)	250 ppm (15/18 mg/kg/day for general toxicity) 250 ppm (15/18mg/kg/day for development)	Excessive toxicity (including mortality) at 5000 ppm. Decreased weight gain at 2500 ppm. Liver toxicity at 2500 and/or 5000 ppm included ↑liver weights, clinical chemistry changes, hepatocellular vacuolation, bile duct hyperplasia/fibrosis, portal inflammation and/or pigment in Kupffer cells. Equivocal effects in kidney & spleen at 5000 ppm. Decreased pup weight ≥ 2500 ppm	Lemen and Ruecker (1996). ML-95-203, MSL-14816; B6.3.2/02
90-day dog (capsule)	0, 1,10, 50 & 125 mg/kg/day (high-dose females reduced to 75 mg/kg/day after 7 wks)	10 mg/kg/day	Excessive toxicity (including mortality) at 125/75 mg/kg/day. Liver toxicity at ≥50 mg/kg/day included ↑liver weights and serum enzymes but no histopath. Electrolyres phosphorus and K were significantly reduced.	Lemen and Ruecker (1997). ML-96-067, MSL-15197; B6.3.2/03
1-yr dog (capsule)	0, 1, 5, 20 & 80 mg/kg/day	5 mg/kg/day	Substantial ↓weight gain and some evidence of liver toxicity (↑liver weights and serum enzymes but no histopath, also altered electrolytes Phos and K) at 80 mg/k/day. Slight reduction in some serum electrolytes at ≥20 mg/kg/day was considered treatment-related.	Lemen and Thake (1998). ML-97-043, MSL-15574; B6..3.2/04
21-day rat dermal	0, 100, 300 & 1000 mg/kg/day	1000 mg/kg/daya	Slight ↑liver weight in 1000 mg/kg/day males was considered treatment related but was not associated with clinical chemistry or histopath findings. Increased spleen weights in 300 and 1000 mg/kg/day males not considered to be adverse due to lack of correlated findings.	Naylor and Thake (1998). ML-97-206, MSL-15527; B6.3.3/01
Rabbit	0,5, 15, 50, 100 or 150 mg/kg bw	50 mg/kg bw/day	Mortality, clinical signs and effects on body weight gain, food consumption and/or organ weights at ≥ 100 mg/kg bw	Holson, J.F. (1997b). WI-96-105 . B.6.6.2.2b

4.7.1 Non-human information

4.7.1.1 Repeated dose toxicity: oral

28-day/range-finding

Study 1

Four-week range-finding feeding study of silthiofam in CD-1 mice: Lemen, J.K. and Ruecker, F.A. (1996): Report MSL-14538 (MSL 15332 is an amended report of this study). OECD 407, EEC B.7. GLP compliant with the following exceptions: characterisation of the test substance and verification of the stability, homogeneity and concentration of the test substance in the diets were not performed as part of this study. The Quality Assurance Unit (QAU) did not inspect the study, and the report was not audited by the QAU.

Materials and methods:

Silthiofam, Lot number: NPD-9502-6403-T, purity: 96.6% was administered in the feed at concentrations of 0, 10, 100, 1000 and 4000 ppm for four weeks (5/sex/group). Calculated test substance consumption (mg/kg bw/day) for these dietary levels was, respectively: 0, 1.4, 14.7, 147.7 and 578.0 for males, and 0, 2.1, 25.8, 259.4 and 781.5 for females.

Results:

Table 16. Summary of findings

Endpoint	
Mortality	No effects observed
Clinical observations	No effects observed
Body weight	No effects observed
Food consumption	No effects observed
Haematology	No effects observed
Clinical chemistry	4000 ppm: ↑alanine aminotransferase (ALT) (♀/♂) (non-statistically significant)
Organ weights	4000 ppm: ↑relative liver weight(♀/♂): Stat sig trend ↑**absolute liver weight (♀/♂):
Gross pathology	4000 ppm: accentuated lobular pattern, liver; (2/5 males and 1/5 females)
Histopathology	4000 ppm: liver, centrilobular hepatocyte vacuolation (1 M, 3 F)and degeneration (2 males), mild centrilobular hypertrophy (1 M);

*** p≤ 0.01 dunnetts test*

Treatment-related findings of increased serum ALT, increased liver weight, macroscopic change and histopathological evidence indicate a toxic effect on the liver at the high dose level. Regenerative hyperplasia of the renal tubular epithelium was recorded in 1, 0, 1, 2 and 3 males at 0, 10, 100, 1000, and 4000 ppm, respectively. This was of minimal severity, not associated with other relevant changes and was not considered treatment-related.

Conclusion:

The liver was the target organ at the high dose 4000 ppm (578/781 mg/kg bw). The no observed effect level (NOAEL) was 1000 ppm in both sexes of mice based on effects on the liver (147.7 and 259.4 mg/kg bw/day in males and females, respectively).

Study 2.

Four-week range-finding feeding study of silthiofam in CD rats. Lemen, J.K. and Thake, D.C. (1996): Report SL-14537. Guidelines OECD 407, EEC B.7. GLP compliant with the following exceptions: characterisation of the test substance and verification of the stability, homogeneity and concentration of the test substance in the diets was not performed as part of this study. The study was not inspected by the QAU, and the report was not audited by the QAU.

Materials and methods:

Silthiofam, Lot number: NPD-9502-6403-T, purity: 96.6% was administered in the feed at concentrations of 0, 20, 200, 1000 and 8000 ppm for four weeks (5/sex/group). Calculated test substance consumption (mg/kg/day) for these dietary levels was, respectively: 0, 1.5, 14.5, 73.1 and 407.5 for males, and 0, 1.5, 16.1, 77.5 and 542.8 for females.

Table 17. Summary of relevant findings

Endpoint	Results
Mortality	No effects observed
Clinical observations	8000 ppm: discoloured urine; both sexes
Body weight	8000 ppm: ↓**weight (♀/♂) – body weight loss in ♂: ↓**weight gains (♀/♂)
Food consumption	8000 ppm: ↓mean intake (♂**♀*)
Haematology	8000 ppm: ↑reticulocyte counts increased(♀/♂) NS but BR
Clinical chemistry	8000 ppm: ↑↑AP (♂♀) ↑↑ALT (♂♀) ↑↑AST (♂♀) ↑CPK (♂♀) ↑↑γGT (♂♀) ↑direct bilirubin (♂♀) ↑↑total bilirubin (♂♀) ↑↑cholesterol (♂♀) ↑Calcium (♀) ↑↑globulin (♂♀) ↑↑blood urea nitrogen (♂♀) ↑APPT (**♂)
Urinalysis	8000 ppm: reddish-yellow colour; (♂♀) : ↑**volume, bilirubin and urobilinogen increased; females only
Organ weights	8000 ppm: abs (**♀) and rel liver weight increased; (**♂♀) ↑rel kidney weight (**♂♀)
Gross pathology	8000 ppm: accentuated lobular pattern and abnormal colour, liver; (♂♀) : abnormal colour, kidneys; (♂♀) : small/atrophy, spleen; males only (1 of 5 rats observed with finding)

Histopathology	8000 ppm: hepatocyte necrosis, karyomegaly and hypertrophy, multinucleated hepatocytes, increased apoptosis, periportal mononuclear infiltrate, bile duct hyperplasia and cholangiofibrosis, liver; one or both sexes : decreased cellularity of red pulp, lymphoid and marginal zone cell depletion, spleen; males only

*** p≤ 0.01 dunnetts test. NS = not significant; BR = biologically relevant.*

Conclusions:
Significantly lower body weights, failure to gain weight in males and reduced weight gain in females, in conjunction with reduced food intake were seen at the high dose. A significant treatment-related increase in reticulocyte count at the high dose indicated a possible targeting of the blood system. Activated partial thromboplastin time was also increased at this dose (statistically significantly in males and increased in females).

The main target organ was clearly identified as the liver. Absolute and relative weights were increased at the high dose level. There was evidence of toxic hepatocellular damage with increased serum levels of AST and ALP. Serum cholesterol was increased, albumen decreased and globulin levels increased. Together with an increase in ALP and γGT and serum bilirubin, these alterations provide evidence of cholestastic type of injury. These findings were correlated with microscopic evidence of bile duct hyperplasia and cholangiofibrosis. Increased AST, ALT, CPK and microscopic demonstration of hepatocyte necrosis, hypertrophy and apoptosis evidenced injury to the hepatocytes. All changes were confined to the top dose and were more evident in female animals.

Other potential target organs observed at the 8000 ppm dietary level included the kidney and spleen. The relative kidney weight was slightly increased and colour was abnormal. Blood urea nitrogen was increased in both sexes; urine volume was also increased in female rats. However, creatinine was unaffected and no microscopic changes were observed in this organ in both sexes. One male was observed grossly to have a small/atrophied spleen. 3 of 5 males each showed only one of the specific changes described in the table above.

The LOAEL was 8000 ppm/407 – 542 mg/kg bw/day, based on observation of clear liver toxicity.

Study 3.
Range-finding study of silthiofam administered orally to beagle dogs. Lemen, J.K. (1996). Report MSL-14758. No specific guidelines followed (range-finding study). GLP compliant with the following exceptions: characterisation and verification of the stability of the test substance was not performed as part of this study. The study was not inspected by the QAU, and the report was not audited by the QAU.

Materials and methods:
Silthiofam, Lot number: QPP-9509-6764-T, purity: 91.8% was administered *via* gelatin capsule (7 days/week) at dose levels of 0, 10, 50, 150 and 250/350 mg/kg bw/day for four weeks (2/sex/group). The high dose was reduced from 350 to 250 mg/kg bw/day after 2 (males) or 3 (females) weeks of administration because excessive toxicity was observed.

Results:

Table 18. Summary of relevant findings

Endpoint	Results
Mortality	250/350 mg/kg bw/day: 2 males and 1 female were sacrificed *in extremis*
Clinical observations	150 mg/kg bw/day: emesis, diarrhea (♂♀) 250/350 mg/kg bw/day: emesis, diarrhea, hypoactivity, pale integument, emaciation, dehydration and decreased defecation; one or both sexes
Body weight	≥150 mg/kg bwday: mean weight loss/weight gain reduced; (**♂*♀)
Food consumption	250/350 mg/kg bw/day: ↓mean intake reduced; (♂♀)
Haematology	≥150 mg/kg bw/day: ↓reticulocyte counts reduced; females only (↓NS/BS)
Clinical chemistry	50 mg/kg bw/day: ↑AP ♀ ≥150 mg/kg bw/day: ↑↑γGT, AP, APPT; one or both sexes
Organ weights	≥150 mg/kg bw/day: ↑ rel liver and kidney weight; one or both sexes (NS)
Gross pathology	250/350 mg/kg bw/day: emaciation, thickened stomach walls; one or both sexes
Histopathology	Not performed

**Dunnetts test p≤ 0.05* ** *Dunnetts test p≤ 0.01*
NS not statistically significant; BS biologically significant

The top dose level of 350/250 mg/kg bw/day and possibly also the 150 mg/kg bw/day dose levels were identified as excessively toxic. 2/2 males and 1/2 females died at the top dose. Clinical signs seen at these doses were emesis, diarrhoea and/or decreased defecation, poor appetite and lethargy. Weight loss was recorded for males and females at ≥ 150mg/kg bw/day.

Reticulocyte count was slightly reduced (not statistically significant) in females from ≥ 150 mg/kg bw/day. APTT times were increased in the 150 mg/kg bw/day animals but not in the remaining high dose female.

Increased ALP levels noted in males (single remaining male) at 150 mg/kg bw/day and females at ≥ 50 mg/kg bw/day were probably treatment-related. Slight increases in γGT were noted in the male at 150 mg/kg bw/day and in females at ≥ 150 mg/kg bw/day. A slight increase in absolute and relative liver weights (not statistically significant) was noted at 150 mg/kg bw/day in the male and at ≥ 150 mg/kg bw/day in females. Histopathology was not conducted. Thickening of the stomach wall was seen at necropsy in one female and the remaining male of the high dose groups.

Conclusion:
Administration of doses in excess of 150 mg/kg bw/day was found to be toxic to the dog, with mortalities occurring at the high dose and inappetance and weight loss at doses of ≥ 150 mg/kg bw/day. Increased organ weight and increased serum ALP and γGT provided some evidence

of adverse liver effects (cholestasis). Additional evidence of toxicity was seen in effects on blood chemistry and increased clotting times.

50 mg/kg bw/day was a NOAEL for male dogs while the NOAEL for females was 10 mg/kg bw/day based increased AP levels.

90-day oral

Study 4

Sixty-day feeding study of silthiofam in CD-1® mice. Lemen, J.K., Paul, P.M. and Ruecker, F.A. (1997). Report MSL-15251. OECD 408, FIFRA 82-1, EEC Directive 87/302, Part B; GLP compliant.

Material and methods:

Silthiofam, Lot number: QPP-9509-6764-T, purity: 91.8% was administered in the feed at concentrations of 0, 50, 1000, 2500 and 5000 ppm for 60 days (10/sex/group). Calculated test substance consumption (mg/kg bw/day) for these dietary levels was, respectively: 0, 6.98, 140.32, 363.40 and 707.16 for males, and 0, 10.50, 235.56, 519.31 and 1131.89 for females.

Results:

Table 19. Summary of relevant findings

Endpoint	Results
Mortality	No effects observed
Clinical observations	5000 ppm: discoloured urine; both sexes
Body weight	5000 ppm: mean weight gain reduced; (NS ♂♀)
Food consumption	No effects observed
Haematology	≥2500 ppm: platelet counts increased; both sexes 5000 ppm: haemaglobin, haematocrit, mean corpuscular volume and mean corpuscular haemaglobin decreased; (*/** in one or both sexes)
Clinical chemistry	5000 ppm: ↑ALT (**♂♀)
Organ weights	≥2500 ppm: ↑abs /rel liver weight (**♂♀) : spleen weight decreased; females only
Gross pathology	No effects observed
Histopathology	≥2500 ppm: centrilobular and/or mid-zonal region hypertrophy, vacuolation and degeneration and/or individual hepatocyte necrosis, liver; one or both sexes

* $p \leq 0.05$, ** $p \leq 0.01$ *Dunnetts test.*

There were no treatment-related mortalities and no treatment-related clinical signs other than a red-brown discolouration of the urine. Mean body weights were unaffected while there was a decrease in cumulative weight gains in mice of the high dose group (not statistically significant but presumably treatment-related). Food intake was not consistently affected.

There were a number of treatment-related (statistically significant) alterations in haematology parameters at the high dose as follows; decreased HGB, HCT, MCV in high dose animals and decreased MCH in high dose males. Platelet numbers were increased from 2500 ppm with a statistically significant trend also apparent at lower doses. Serum ALT was increased in high dose animals.

Absolute liver weight was increased at the 2500 ppm dose (♂) and the high dose (♂ and ♀). Relative liver was increased in animals at ≥2500 ppm. This was associated with histopathological evidence of liver damage at both dose levels in both sexes including the following; centrilobular/panlobular hepatocellular vacuolation (≥ 2500 ppm); centrilobular/midzonal or panlobular hepatocellular hypertrophy ≥ 2500); hepatocellular necrosis (high dose ♂ and ♀); hepatocellular degeneration (high dose ♂).

Conclusion:
There was clear evidence of a toxic effect on the liver in both males and females from 2500 ppm. Increased absolute and relative liver weights and increased serum levels of ALT were associated with histopathological evidence of liver damage at ≥ 2500 ppm in both sexes. There was some alteration of haematology parameters at the high dose which appeared treatment-related. The LOAEL was 5000 ppm in both sexes of mice based on effects on the liver (707 and 1132 mg/kg bw/day in males and females, respectively).

Study 5.
Three-month feeding study of MON 65500 in Sprague Dawley Rats with pilot reproduction phase. Lemen, J.K. and Ruecker, F.A. (1996). Report MSL-14816. OECD 408, FIFRA 82-1, EEC Directive 87/302, Part B. GLP compliant.

Material and methods:
Silthiofam, Lot number: QPP-9509-6764-T, purity: 91.8% was administered in the feed at concentrations of 0, 25, 250, 2500 and 5 000 ppm for 90 days (10/sex/group). Calculated test substance consumption (mg/kg bw/day) for these dietary levels was, respectively: 0, 1.45, 14.96, 145.73 and 290.04 for males, and 0, 1.70, 17.55, 174.60 and 334.04 for females. A satellite test system of 10 females/group was also included in this study; these rats were paired with the males from their corresponding groups for a period of up to 7 days after approximately 8 weeks of treatment. Females in the reproduction satellite were continued on treatment through post-natal day 4.

Results:

Table 20. Summary of relevant study findings

Endpoint	Results
Mortality	5000 ppm: 4/10 males died or were sacrificed *in extremis*
Clinical observations	≥2500 ppm: discoloured urine; one or both sexes
Body weight	≥2500 ppm: mean weight and/or weight gains reduced (**♂♀)
Food consumption	5000 ppm: mean intake reduced; (♂♀)
Haematology	5000 ppm: -red cell, lymphocyte and platelet counts increased; males only -mean corpuscular volume and mean corpuscular haemaglobindecreased; males only */** -haematocrit and reticulocyte counts decreased; females only -activated partial thromboplastin time and/or prothrombin time increased; one or both sexes
Clinical chemistry	5000 ppm: ↑AST; **♂ ↑ALT; **♂ ↑γglutamyl transferase: **♂, NS↑♀

	↑alkaline phosphatase: ♀** direct/total bilirubin; */** ♂ ↑cholesterol; **♂♀ ↓globulin; **♂*♀ ↑blood urea nitrogen; **♂*♀ ↓albumin; *♀ ↓glucose; **♂, NS↑♀
Urinalysis	No effects observed
Organ weights	≥2500 ppm: -↑abs * and rel** liver weight; ♂,♀ -↓ abs kidney weight; *♂ and rel **♂♀ 5000 ppm: -↓spleen weight;
Gross pathology	≥2500 ppm: -abnormal colour, liver; one or both sexes 5000 ppm: -abnormal colour, kidney, small seminal vesicles and prostate; males only
Histopathology	≥2500 ppm: -hepatocyte vacuolation, hyperplasia/fibrosis of bile duct, portal inflammation and/or pigment in Kupffer cells, liver; one or both sexes 5000 ppm: -brown pigment, tubule epithelium, kidney; males only
Ophthalmology	No effects observed
Reproduction	No effects observed on mating, fertility, pregnancy or offspring survival (to post-natal day 4)
Offspring body weight	≥2500 ppm: pup weights decreased (post-natal day 4) 5000 ppm: pup weights decreased (post-natal day 0)

**Dunnetts test p≤ 0.05 ** Dunnett's test p≤ 0.01*

Four of ten males of the high dose group died or were sacrificed *in extremis.* These deaths were considered treatment-related. The death of a single female from the low dose group was not considered related to treatment. Abnormal urine colour was recorded in high dose animals (9/10 ♂ and 10/10 ♀) and in 3/10 males at 2500 ppm. Mean body weights and weight gains were profoundly affected at the high dose (p≤0.01). Mean weights of males of the 5000 ppm group were 30% lower than controls while mean weight gains were 65% of controls by the end of the study. Mean weights of females of the 5000 ppm group were 16% lower than controls while mean weight gains were 54% of controls by the end of the study. Mean body weights and body weight gains were also lower at the 2500 ppm dose level but the difference was not statistically significant. Mean food intake was also reduced at the high dose level (statistically significant at all times in males and in females at intervals).

Table 21. Summary of treatment-related clinical pathology findings

Dose (ppm)►	**0**		**25**		**250**		**2500**		**5000**	
	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀
Haematology										
RBC	8.9	-	8.8	-	8.8	-	8.7	-	9.7**	-
WBC	8.0	5.7	8.4	5.1	8.0	5.5	8.2	6.0	9.95*	6.6+
MCV	52.7	56	53.6	54.7	53.6	55.5	51.9	54.6	47.6	53.4
MCH	17.3	-	17.6	-	17.6	-	17	-	15.8**	-
Haem	-	14	-	15**	-	14.2	-	13.9	-	13.5--
Haematocrit	-	42.4	-	45**	-	42.8	-	42	-	39.9*
Reticulocyte	-	186	-	163	-	155	-	148.7	-	154.2*
Platelets	811	822	813	789	808	754	854	838	1098**	908++
Clinical chemistry										
ALP	207	75	167	87.7	186	83.2	254	73.3	435**	72
ALT	37.5		35.3	37.9	37.2	27	53.8	22	165**	25
AST	77.4	-	77.9	87.1	82.4	69.9	78.2	61.8	133**	65
γGT	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	2.83**	0.4
Bilirubin –direct	0.07	-	0.07	-	0.07	-	0.07	-	0.15*	-
-total	0.1	-	0.1	-	0.1	-	0.09	-	0.2**	-
Cholesterol	57.9	87.4	59.5	81.4	66.3	90.4	71.4	86.1	126**	114**
Globulin	2.46	2.35	2.35	2.24	2.5	2.01	2.7	2.4	3.2**	2.75*
Albumin	4.83259	5.5	4.75	5.9	4.8	6.0	4.7	5.5	3.9**	5.4
Glucose	19.4	220	264	194	245	217	239	200	148**	171
BUN		19.8	18.8	20.1	19.8	18.7	21.5	23.4*	26.9**	23.5*
Organ weights										
(% of control)										
Liver -absolute	-	-	97	96	100	93	109	108	84*	115*
-relative	-	-	97	99	102	96	116**	117**	126**	136**
Kidney -absolute	-	-	102	94	102	98	105	105	80**	99
-relative	-	-	101	97	104	102	111	114*	119**	117**

+ Linearly related to dose with positive trend (p<0.05).
++ Linearly related to dose with positive trend (p<0.01).
-- Linearly related to dose with negative trend (p<0.01).
*Dunnetts test: * (p≤0.05) ** (p≤0.01)*

Alterations in haematological parameters in the high dose males (and/or females) were considered related to treatment, although increases in RBC, lymphocyte counts (and platelets) have not generally been associated with chemical toxicity. Serum enzyme markers of hepatocellular damage and cholestasis were significantly elevated as were bilirubin and globulin in males at the high dose. Glucose and albumin levels were decreased in males also. Cholesterol and globulin levels were increased in high dose females.

Relative liver weights were significantly increased in rats from 2500 ppm. Lesions were identified at microscopic examination at both dose levels as follows; centrilobular hepatocellular vacuolation and bile duct hyperplasia/fibrosis in males at ≥ 2500 ppm and in females also at 5000 ppm; pigment deposition in the Kuppfer cells in males at 5000 ppm and portal inflammation in males from ≥ 2500 ppm. There was some evidence of an effect on the kidneys with increased relative weight in females from ≥ 2500 ppm and in males at 5000 ppm (and also increased BUN). Deposition of brown pigment was seen the tubular epithelial cells in males of the high dose group.

Reproduction Phase

Reproductive parameters were not affected by treatment. Maternal body weights were not significantly affected while there was a reduction in body weight gain at the high dose level (75% of control on gestation days 0-7 and 54% of control on lactation days 0-4. Weight gain may also have been affected in the 2500-ppm group (29% of control during days 0-4 of lactation).

Mean male pup weights were significantly reduced on days 0 and 4 postpartum and combined pup weights were reduced at day 4. Mean pup weights were also significantly lower than controls on day 4 in the 2500-ppm group ($p<0.05$).

Table 22. Summary of reproduction phase data.

OBSERVATIONS	**VALUES**				**123**
Dosage (mg/kg bw/day)	**0(control)**	**25**	**250**	**2500**	**5000**
Dams (N)	10	9	10	10	9
Females achieving pregnancy (%)	100	97	113	100	111
Dam mean body weight gain: GD 7-14 GD 14-21 LD 0-4	30 ±5 83 ± 18 16 ± 10	34 ± 6 81 ± 16 15 ± 12	241±5 852±2 25±8	30 ±8 86 ±9 5 ±26	28 ±8 78 ±25 9 ±7
Live pups/dam at termination: mean N (%)	12.6	13.7(109)	14(111)	14.7(114)	13.8(110)
Dead foetuses/dam: mean n (%)	0.87	0	0.11 (13)	0	0.12 (14)
Foetus weight (mean): day 0 : day 4	6.9 11.3	6.3(91) 9.8 (87)	6.56 (95) 10.6 (93)	6.2 (90)↓ 8.9 (79)↓	6.0 (87)↓ 9.3* (82)

Conclusions:
The liver was the main target organ. Effects were observed at the 2500 and 5000 ppm dietary levels in one/both sexes and included increases in organ weight, enzymes (ALP, AST, ALT and γGT), bilirubin and cholesterol, and/or microscopic changes that involved hepatocytes, Kupffer cells and the biliary system. Increased platelet counts and clotting times were observed primarily in males at a dietary level of 5000 ppm and may have resulted from the liver changes. Other potential target organs observed at the 5000 ppm dietary level included the kidney and spleen. With respect to the kidneys, they were of abnormal colour and organ weight and/or blood urea nitrogen was increased in one or both sexes. Non-specific microscopic changes consisting of brown pigment in tubule epithelial cells were observed in males.

No effects were observed on reproduction. Pup weights were decreased at the 2500 and 5000 ppm dietary levels, statistically significant at the high dose. There were possible marginal effects on maternal body weight gains at the higher dose. Three or fewer males at the 5000 ppm dietary level were observed grossly and/or microscopically with a small/atrophied prostate and/or seminal vesicles; these effects appeared to have no functional consequence with respect to reproduction.

The LOAEL was 2500 ppm in both sexes of rats based on effects on the liver (146 and 175 mg/kg bw/day in males and females, respectively). The LOAEL for developmental toxicity was 2500 ppm based on reduced mean foetal weight at birth and on day 4 of lactation. Fertility was not affected in this pilot study.

Study 6.
Three-month study of MON 65500 administered by capsule to beagle dogs. Lemen, J.K. and Ruecker, F.A. (1997). Report MSL-15197. OECD 409, FIFRA 82-1, EEC Directive 87/302, Part B. GLP compliant.

Materials and methods:
Silthiofam, Lot number: SRL-9601-6895-T, purity: 97.4% was administered *via* gelatin capsule (7 days/week) at dose levels of 0, 1, 10, 50 and 125 mg/kg bw/day for 90 days (5/sex/group). The high dose was reduced from 125 to 75 mg/kg bw/day after 7 weeks of study (females only) because excessive toxicity was observed.
Results:

Table 23. Summary of relevant findings

Dose level (mg/kg bw/day))►	**0**		**1**		**10**		**50**		**75/125**	
Endpoint ▼	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀
Mortality	0	0	0	0	0	0	0	0	0	1#
Clinical observations	-	-	-	-	-	-	↑	↑	↑↑	↑↑
Body weight gain	0.53 ± 0.7	0.04 ± 0.3	0.3 ± 0.3	0.06 ± 0.6	0.72 ± 0.4	-0.03 ± 0.4	0.57 ±0.46	0.5 ± 0.6	-0.45 ±0.4*	-0.48 ±0.46
Food consumption	-	-	-	-	-	-	-	-	-	↓*
Haematology	-	-	-	-	-	-	-	-	-	-
Clinical chemistry										
ALP	53	65.6	64.6	59.6	71.4	78	82	152**	197**	155*
γGT	-	-	-	-	-	-	-	-	↑*	↑*
AST	-	-	-	-	-	-	-	-	-	↓
ALT	-	-	-	-	-	-	-	-	↓	↓
CPK	-	-	-	-	-	-	-	-	-	↓*
Albumen	-	-	-	-	-	-	-	↑*	↑	↑**
K	-	-	-			-	-	-	↓	-
Phos.	-	-	-			-	-	-	↓*	↓*
Na	-	-	-			↑*	-	↑**	-	↑*
Organ weights										
abs. liver wt.	100	100	110	98	110	96	115	109	121*	112
rel. liver wt.	100	100	105	99	107	98	112	115	129**	119*

 # sacrificed in extremis
*Dunnets test: * (≤ p0.05), ** (p≤0.01)*

A single female of the high dose group was sacrificed *in extremis* on day 50, with the following clinical signs of toxicity; emesis, weight loss, hypothermia, hypoactivity , pale mucose and decreased defaecation. There were no other mortalities. Emesis was noted in both sexes at the high dose level and sporadically at 50-ppm. A statistically significant decrease in mean body weight gain was recorded at the high dose at all intervals in males and in females up to day 50 (time of reduction of the 125-ppm dose to 75-ppm). There was a treatment and dose-related increased in absolute and relative liver weight in both sexes.

Haematology parameters were not affected. A number of alterations in clinical chemistry parameters were considered related to treatment as follows; increased serum ALP (alkaline phosphatase) in both sexes at interim and terminal sampling; increased γGT in both sexes at the high dose; increased albumen in females at ≥ 50 mg/kg bw/day and a slight increase in males at the high dose. Levels of AST and ALT were apparently decreased at the high dose in males and females. Reduced levels of these enzymes are not normally associated with toxicity and do not appear (from the data) to be related to treatment. Increased serum albumen may be related to liver toxicity although the direction of change is not usually associated with liver toxicity.

A slight but statistically significant increase in APTT (activated partial prothrombin time) was noted at ≥ 50 mg/kg bw/day in males at the interim sampling time. An increase (not statistically significant) was also noted at termination in both sexes. Serum phosphorus levels were reduced in both sexes at the high dose and potassium levels in were slightly reduced in males only. Serum sodium levels were elevated from ≥ 10 mg/kg bw/day in females. The relationship between electrolyte changes and treatment is unclear. There is no evidence of an adverse effect on the kidney and these alterations may not be of biological relevance.

Conclusion:
The liver was identified as the target organ in this study; effects were observed at doses of ≥ 50 mg/kg bw/day in both sexes and included an increased organ weight and/or enzymes (peripherally). Increased clotting times observed primarily in males at dose levels of ≥ 50 mg/kg bw/day may have resulted from the liver toxicity.

The no observed effect level (NOAEL) was 10 mg/kg bw/day in male and female dogs, based on effects on the liver.

Study 7.
One-year study of MON 65500 administered by capsule to beagle dogs. Lemen, J.K. and Thake, D.C. (1998). Report MSL-15574. OECD 452, FIFRA 83-1, EEC Directive 87/302, Part B. GLP compliant.

Materials and methods:
Silthiofam, Lot number: SRL-9601-6895-T, purity: 97.4% was administered *via* gelatin capsule (7 days/week) at dose levels of 0, 1, 5, 20 and 80 mg/kg bw/day for 1 year (5/sex/group).

Results:

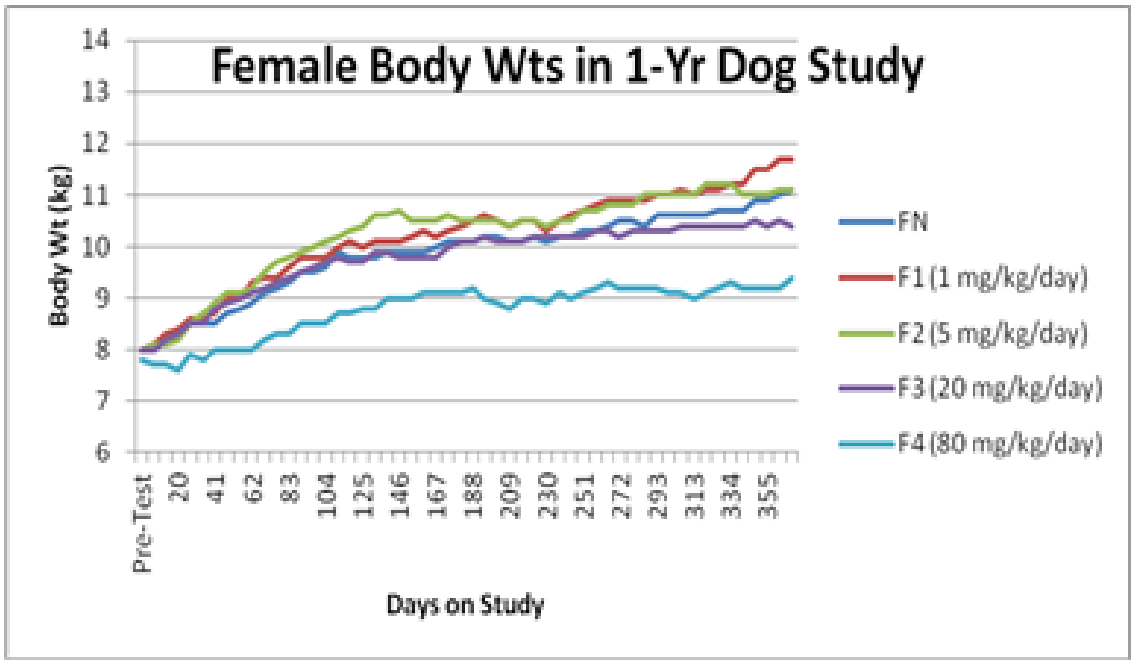
Table 24. Summary of possibly treatment-related findings

	Results									
Dose (mg/kg bw/day)►	**0**		**1**		**5**		**20**		**80**	
Endpoint ▼	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀
Clinical observations (emesis)	-	-	-	-	-	-	-	-	↑	↑
Body weight (gain)	-	3.07 ±1.0	-	3.65 ±1.4	-	3.16 ±1.3	-	2.41 ±0.4	-	↓1.61 ±1.1
Haematology -platelets	-	-	-	-	-	-	-	-	(↑*)	(↑*)
Clinical chemistry										
ALP Terminal sacrifice *Historical control* *41 U/L (8-234) 95% interval 10-128[4]* *21-170[2]*	49.6	64.4	44	85.8	58.2	72	64.0	88.6	157	232.8**[5]
γGT 9 months 12 months *HC: 0-6 U/L[1]*	5.0 5.2	6.2 5.8	7.2 5.0	5.2 4.6	5.0 4.0	6.0 5.8	5.4 4.2	6.6 5.8	6.2 6.2	8.8** 8.8↑
Phos. 9 months 12 months *HC[2]: 3-6.2 mg/dl*	4.3 4.2	3.8 3.2	4.0 4.1	3.6 3.0	3.9 3.8	3.78 2.7	3.7** 3.26**	3.5 3.0	4.0 3.12**	3.0↓ 2.56↓
K 9 months 12 months HC: 4.4mmol/L[1], 3.6-5.3mmol/l[2] 4.2 ± 0.2 mmol/L[3]	4.5 4.4	4.4 4.2	4.8 4.5	4.5 4.3	4.4 4.3	4.6 4.1	4.1 4.1*	4.4 4.4	4.1 4.0**	4.1 3.8*
Cl. 12 months *HC:* *107 mmol/L[1]* *117 (92-150) mmol/L[4]* *110 ±3.1mmol/L[3]*	118.2	112.6	114.6**	110.4	115.6**	111.0	112.2	111.6	107.2**	110.6

Organ weights Liver wt. (rel.)	-	-	-	-	-	-	-	-	↑*	↑**

(*) Trend towards increase with statistically significance at some time points.

A biologically significant reduction in body weight gain was observed in females of the high dose group, particularly at the early time points. At the end of the study, weight gains in this group were approximately 52% of control values. Food intakes were not consistently affected.



There was a trend towards increased platelet counts was observed in both males and females throughout the study, which was significant at some sampling times.

A profile of clinical chemistry alterations similar to that recorded in the three-month study was recorded in this study. A statistically significant increase in alkaline phosphatase (statistically significant in females only) and γGT levels was seen in both sexes at the high dose (statistically significant in females). NOAEL was considered to be 5 mg/kg bw per day taking into account the dose-dependent decreased bwg of females (↓20%), weakly increased ALP (+27%) (however observed at all timepoints, and more so at 90d), decreased Pi (↓21%) and K (↓8%) at 20 mg/kg bw/d. The reduction in serum potassium and phosphorus seen in the three-month study was more clearly identified in this study with a statistically significant reduction in males from ≥ 20 mg/kg bw/day and in females at 80 mg/kg bw/day.

Conclusion:
The liver was identified as the target organ in this study; effects were observed at 80 mg/kg bw/day only, in both sexes, and included increased organ weight and enzymes. Slight reductions in serum potassium and phosphorous were observed in one (males) or both sexes at dose levels of 20 and 80 mg/kg bw/day, respectively. Serum chloride was also reduced in males at a dose level of 80 mg/kg bw/day. The reduction in these elements peripherally was considered a treatment related effect of unknown toxicological importance. There was an absence of obvious physiological, gross or microscopic correlates in the animals from these dose levels.5 mg/kg bw/day was a no observed adverse effect level (NOAEL) in this study.

This was based on treatment-related reductions in serum potassium and phosphorous at 20 mg/kg bw/day in males, only with effects on liver weight, marker enzymes and reductions in serum potassium and phosphorous seen in both sexes at 80 mg/kg bw/day.

4.7.1.2 Repeated dose toxicity: inhalation

Silthiofam is not volatile (vapour pressure $<10^{-2}$ Pa); therefore inhalation (28 or 90-day) studies have not been conducted with the test substance. The particle size distribution of unmilled technical silthiofam indictates that the potential for this substance to penetrate beyond the nasopharyngeal region of the respiratory tract is low. This limits the potential for the generation of harmful effects, either on the respiratory tract, or as a result of particulate clearance mechanisms.

4.7.1.3 Repeated dose toxicity: dermal

Range-finding and 21-day dermal study of MON 65500 in rats. Naylor, M.W. and Thake, D.C. (1998). Report MSL-15527. OECD 410, FIFRA 82-2, EEC Directive 92/69, Part B9. GLP compliant with the following exceptions:

1) On day 16 of the 21-day dermal study no trained personnel were available to administer the test substance. An individual who had previously assisted in the dermal administration procedures applied the test substance on this day. Two male rats died shortly after the start of the exposure period on day 16 from asphyxiation; the result of the occlusive wrapping having being applied too tightly around the torso. These events did not affect the interpretation of the study. Trained personnel administered the test substance on all other days of the study.

2) The reagent for the determination of reticulocyte counts was employed in the study after the manufacturer's stated expiration date. The reference control values were, however, within the manufacturer's stated accepted range. This deviation was not considered to have adversely affected the reticulocyte count results, or the conclusions of the study.

Materials and methods:
Silthiofam, Lot number: SRL-9601-6895-T, purity: 97.7% was administered dermally using occluded procedures. The test was applied each morning 5 days/week for 3 weeks to a sterile gauze wrap, moistened with 0.9% saline and applied to an area of skin approximately 25-35 cm2 on the back of each rat. An occlusive wrapping was applied. The application period each day was 6 hours. The dose levels were 0, 100, 300 and 1000 mg/kg bw/day (8/sex/group).

Results:

Table 25. Summary of relevant findings.

Endpoint	Results
Mortality	300 mg/kg bw/day: 2 males died on day 16; the deaths were incidental to treatment (asphyxiation by the occlusive dressing).
Clinical observations	No effects observed
Body weight	No effects observed
Food consumption	No effects observed
Haematology	Slight decrease in platelets (91-93% of controls) @ ≥ 300 mg/kg bw/day (♀)
Clinical chemistry	No effects observed
Organ weights	300 and 1000 mg/kg bw/day: reduced spleen weights (absolute and relative to body weight); males only. Slight increase in relative liver weight @1000 mg/kg bw/day (♂).
Gross pathology	No effects observed
Histopathology	No effects observed

Two mid-dose males died apparently of asphyxiation. There were no treatment-related clinical signs, effects on body weight or food consumption. A slight, but statistically significant decrease in platelet count (91 and 93% of controls) was observed in the mid and high dose females (1086, 1082, 1004, and 990 103/mm3 at 0, 100, 300 and 1000 mg/kg/day, respectively. The values were within the historical control range: 667-1348 103/mm3 and were considered unrelated to treatment.

Moderate reductions in spleen weight (82-84% of control values) were observed in males only, from the 300 and 1000 mg/kg bw/day dose groups. This effect was not considered to be toxicologically significant, as there was an absence of corroborative haematological, gross or microscopic changes to the organ in either sex. There was a statistically significant positive trend towards increased relative liver weight in males. The increase at the high dose was significantly different from controls ($p<0.05$).

Table 26. Summary of relevant organ weight changes

Affected Organ	0		100		300		1000	
	♂	♀	♂	♀	♂	♀	♂	♀
Liver weight								
-Absolute	**9.42**	6.42	**9.96**	6.61	**9.89**	6.28	**10.09**	6.51
-Relative	**3.08**	3.22	**3.24**	3.29	**3.25**	3.21	**3.35***	3.22
Spleen weight								
-Absolute	**0.69**	0.43	**0.64**	0.44	**0.57***	0.43	**0.56***	0.42
-Relative	**0.22**	0.21	**0.21**	0.22	**0.18****	0.22	**0.187****	0.20

Some treatment-related alterations in relative liver and spleen weight were observed in male rats following the short-term dermal administration of silthiofam. However, in the context of the preceding evidence of liver toxicity in three species, it is considered likely by the reviewer

that the increase in liver weight seen in this study is, in fact, treatment-related. As there is no histopathological change in the liver and no clinical chemical change, this effect may be adaptive rather than toxic. The LOAEL for short-term dermal toxicity is thus considered to be 1000 mg/kg/day.

4.7.1.4 Repeated dose toxicity: other routes

No data on other routes.

4.7.1.5 Human information

No data.

4.7.1.6 Other relevant information

No other data.

4.7.1.7 Summary and discussion of repeated dose toxicity

The sub-chronic toxicity of silthiofam was investigated in the rat, mouse and dog via the oral route and 21-day via the dermal route. Studies were of 28-day (mouse, rat and dog) and 60/90 day (mouse, rat and dog), and one year (dog) duration. The liver was identified as the main target organ following the short-term oral administration of silthiofam in the rat, mouse and dog. Elevated liver weights and serum enzyme markers for liver toxicity were observed in all three species. However, liver histopathology was observed only in rats and mice, and included hepatocellular hypertrophy, vacuolation, degeneration/necrosis of individual hepatocytes, bile duct hyperplasia/fibrosis, etc. No liver histopathological findings were observed in dogs, at a dose level that was significantly toxic. Also considered relevant to the discussion of sub-chronic toxicity was the findings of the range-finding developmental study in the rabbit (*Holson, JF,* (1997a).

The dog and rat were identified as the most sensitive species; both of these species were more sensitive to the effects of silthiofam than the mouse (dog = rat > mouse).

4.8 Specific target organ toxicity (CLP Regulation) – repeated exposure (STOT RE)

4.8.1 Summary and discussion of repeated dose toxicity findings relevant for STOT RE according to CLP Regulation

The liver was identified as the main target organ following the short-term oral administration of silthiofam in the rat, mouse and dog. Elevated liver weights and serum enzyme markers for liver toxicity were observed in all three species. However, liver histopathology was observed only in rats and mice, and included hepatocellular hypertrophy, vacuolation, degeneration/necrosis of individual hepatocytes, bile duct hyperplasia/fibrosis, etc. No liver histopathological findings were observed in dogs, at a dose level that was significantly toxic.
The dog and rat were identified as the most sensitive species; both of these species were more sensitive to the effects of silthiofam than the mouse (dog = rat > mouse). All findings in rats and mice occurred at doses in excess of the guidance values for classification.

In addition, the occurrence of mortalities in the rabbit range-finding study (DRAR B.6.6.2.2a: Holson, J.F., 1997a) was also considered relevant to the STOT RE evaluation. The 100 and 150 mg/kg bw/day dose levels employed in this study exceeded the maternal MTD based on mortality, clinical signs and effects on body weight gain, food consumption and/or organ weights. No maternal toxicity was observed at dose levels of <50 mg/kg bw/day.

Dog:
Effects of treatment were seen in the dog at lower doses and within the numerical criteria for STOT RE 2:

28 day dog: One female died at day 24 and two males on day 12 and 18 at the top dose 250 mg/kg bw (reduced from 350 mg/kg at weeks 3/2 respectively). Reduced food consumption and statistically significant body weight loss occurred at this dose. The liver was targeted with increased absolute (male/female; 15/10%) and relative (male/female; 15%/26%) weights greater than respective controls. Serum ALP was greatly increased (>100% greater than controls; not statistically significant). γGT was also slightly increased (ns). There was no histologically investigation, so it is not possible to differentiate between adaptive *vs* toxic liver effects, other than increased ALP.

90-day dog: The top dose of 125 mg/kg was reduced to 75 mg/kg after 7 weeks due to excess toxicity. A single female was sacrificed *in extremis* on day 50. Statistically significant body weight loss occurred at this dose, also increased absolute (m/f 21%/12%) and relative (m/f 29%/19%) liver weights. ALP was significantly increased ($p<0.01$) in high dose animals and females at 50 mg/kg bw ($p<0.05$). γGT was significantly increased ($p< 0.05$) in high dose dogs also. There was no histopathological evidence of liver damage.

1 year dog: At the high dose of 80 mg/kg bw, there were clinical signs of toxicity, significantly reduced body weight gain (52% of controls at termination), increased ALP and γGT (stat sig), reduced phosphate and potassium and increased relative liver weights (stat sig). There was no gross pathology or histomorphologic alterations which could be attributed to treatment. Reduced electrolytes were also considered relevant at 20 mg/kg bw/day in the 1-yr dog study

Rabbit developmental studies:
During the course of the EFSA Peer evaluation (February, 2016), it was identified that the results of the dose-range finding rabbit developmental toxicity study (DRAR B.6.6.2.2a: Holson, J.F., 1997a) may trigger STOT RE. This position was agreed at the EFSA PRAPr Meeting 414, 22th Feb 2016).

In this study, 6/dose pregnant rabbits were dosed by gavage with 0, 5, 15, 50, 100 or 150 mg/kg bw/day from days 7 to 19 of pregnancy. Four of six and 5/6 females died in the 100 and 150 mg/kg/day dose groups, respectively. Deaths occurred between days 13-16 and 15-22 for gravid females in these respective groups. All deaths except for one intubation error in the 100 mg/kg group were considered treatment-related. The two other females in the 100 mg/kg group aborted prior to scheduled euthanasia. Clinical signs in decedents and/or survivors from the affected groups were hypoactivity/lethargy, faecal changes, dried or wet staining of body surfaces. Mean body weight losses occurred from 100 mg/kg during GD 7-19 (specifically GD10-13 ($p\leq0.01$@ 150 mg/kg) and 13-19). A trend in body weigh loss continued post treatment. Food consumption was reduced over the treatment period (specifically GD 10-13 and 13-19) with a trend for reduced food consumption continuing post treatment. One rabbit in the 100 mg/kg and two in the 150 mg/kg groups had red fluid contents in the urinary bladder,

considered treatment-related. This was apparently also observed at 150 mg/kg in non-pregnant rabbits in a 5 day study (not reported). The single surviving dam in the 150 mg/kg dose group had an elevated liver and reduced spleen weight relative to controls.

These findings at 100 mg/kg bw are well above the cut-off criteria for Cat 1, for studies of shorter duration (i.e. 28-day: ≤ 30 mg/kg bw/d). However, it was agreed that the pregnant rabbit is a particularly sensitive species (a clear steep dose response was observed because no toxic effects or lethality were observed at 60 mg/kg bw per day in the main study). Lethality was observed below the trigger values for classification in STOT RE category 2 (28-day: ≤ 300 mg/kg bw/d), and a proposal for STOT RE 2 is therefore considered appropriate.

Table 27: Summary of short-term NOAELs/LOAELs

Type of study	Species	NOAEL ppm (mg/kg bw/day): males - females	LOAEL ppm (mg/kg bw/day): Males - females
Oral route, 28-days	Mouse	1000 (147.7 - 259.4)	4000 (578-782)
Oral route, 28-days	Rat	1000 (73.1 - 77.5)	8000 (407-542)
Oral route, 28-days	Dog	50 - 10	150 -50 mg/kg bw
Oral route, 60-days	Mouse	1000 (140.3 - 235.5)	2500 (363-256)
Oral route, 90-days	Rat	250 (14.9 - 17.5)	2500 (146-175)
Oral route, 90-days	Dog	10 mg/kg	50 mg/kg
Oral route, 1-year	Dog	5 mg/kg	20 mg/kg
Percutaneous route, 21-days	Rat	300 mg/kg	1000 mg/kg
Range-finding developmental tox	Rabbit	50 mg/kg bw	100 mg/kg bw

4.8.2 Comparison with criteria of repeated dose toxicity findings relevant for STOT RE

STOT RE 1: Classification in this category is based on "…*observations from appropriate studies in experimental animals in which <u>significant and/or severe toxic effects</u>, of relevance to human health, were produced at generally low exposure concentrations".*

The equivalent guidance values for oral 28-day and 90-day studies:
Rat: 28-day: ≤ 30 mg/kg bw/d; 90-day: ≤ 10 mg/kg bw/d.

All treatment-related findings occurred in the dog studies at dose levels in excess of the guidance values for STOT RE 1. The findings of mortalities in the rabbit developmental toxicity study do not satisfy the criteria for placement into STOT RE 1 because they were observed only at > 60 mg/kg bw, which is greater than the cut-off criteria for STOT RE 1 for studies of shorter duration as indicated above.

STOT RE 2: Classification in this category is based on "…*the basis of observations from appropriate studies in experimental animals in which <u>significant toxic effects</u>, of relevance to human health, were produced at generally moderate exposure concentrations".*

Equivalent guidance values for 28-day and 90-day studies:
Oral, rat: 28-day: ≤ 300 mg/kg bw/d and 90-day: ≤ 100 mg/kg bw/d.

Silthiofam causes significant systemic toxicity in the dog on sub-chronic administration, including mortalities at doses of approx. 250 mg/kg bw after 24 doses (female) and 12/18 doses (male), severe effects on food consumption and weight loss were seen at this dose. Some evidence of liver effects were seen. Similarly, severe toxicity was seen at 125/75 mg/kg in the 90-day study with mortality (1), severe effects on body weight (loss) and some evidence of liver effects without histopathological correlation. The same pattern was seen in the one year study, at 80 mg/kg bw, with systemic toxicity, some evidence of liver toxicity but without histopathological correlation (outside the 1 year study extrapolated guidance value for Cat 2 of 25 mg/kg bw). Liver toxicity and systemic toxicity occurred in the rat and mouse but without mortality at significantly higher dose levels.

The effects seen in the dog are consistent with severe general systemic toxicity rather than a specific target organ effect and were not seen in two other species tested to higher dose levels, therefore classification is not proposed on the basis of these findings.

It is noted that annex 1, Part a, para 3.9.2.7.3 of the Regulation identifies morbidity/death within the evidence for classification with STOT RE: ('*morbidity or death resulting from repeated or long-term exposure. Morbidity or death may result from repeated exposure, even to relatively low doses/concentrations, due to bioaccumulation of the substance or its metabolites, and/or due to the overwhelming of the de-toxification process by repeated exposure to the substance or its metabolites).*

It is proposed that the pregnant rabbit is a particularly sensitive species (a clear steep dose response was observed as no toxic effects were observed at 60 mg/kg bw per day in the main study (DRAR B.6.6.2b; Holson, J.F.(1997b)). The increased mortaliy observed in the rabbit range-finding study is within the criteria for significant toxic effects (mortality). Because lethality (3/6 animals substance related at 100 mg/kg bw/d) was observed below the trigger value for classification with category 2 (28-day: ≤ 300 mg/kg bw/d); a proposal for STOT RE 2 is considered appropriate.

Conclusions on classification and labelling of repeated dose toxicity findings relevant for classification as STOT RE

The effects seen in the dog, which were within the cut-off criteria, are consistent with general systemic toxicity and also liver toxicity but were not seen in two other species tested to higher dose levels and classification is not proposed on the basis of systemic/ liver toxicity. However, the increased mortaliy observed in the rabbit range-finding study is within the criteria (mortality) and the numerical cut-of values for studies of shorter duration and classification as STOT RE 2 is proposed on this basis.

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

Summary of the Dossier Submitter's proposal

The evaluation of STOT RE was based on nine repeated-dose toxicity studies. The studies consisted of three oral studies in dogs (28-day range-finding, 90-day and one year), two oral

studies in mice (28-day and 60-day), two oral studies in rats (28-day, 90-day with a pilot reproductive toxicity phase) and one rat 21-day dermal toxicity study. In addition, a rabbit range-finding developmental toxicity study was considered relevant for this endpoint.

The liver was identified as the main target organ in dogs, rats and mice. Elevated liver weight and serum enzyme markers for liver toxicity were observed in all three species. However, liver histopathology was observed only in rats and mice. No liver histopathological findings were observed in dogs up to significantly toxic doses.
Liver findings in rats and mice occurred at doses in excess of the guidance values for classification as STOT RE 2. As the liver effects observed in dogs occurred without histopathological correlates and were consistent with severe general systemic toxicity rather than on a specific target organ, no classification was proposed by the DS for the liver.

***Table**: Selected findings in repeated-dose toxicity studies in dogs exposed to silthiofam*

	28-day (0, 10, 50, 150, 350/250 mg/kg bw/d) *(STOT RE 2 ≤ 300 mg/kg bw/d)*	**90-day** (0, 1, 10, 50, 75*/125 mg/kg bw/d) *(STOT RE 2 ≤ 100 mg/kg bw/d)*	**1-year** (0, 1, 5, 20, 80 mg/kg bw/d) *(STOT RE 2 ≤ 25 mg/kg bw/d)*
Mortality	350/250 mg/kg bw/d: 1/2 (f) (d24) + 2/2 (m) (d12, d18)	75/125 mg/kg bw/d: 1/5 (f)(d50)	-
Body weight (bw) and food consumption (fc)	At 350/250 mg/kg bw/d: ↓ fc, bw loss	75/125 mg/kg bw/d: ↓ fc, bw loss	At 20 mg/kg bw/d: ↓ bw gain (f) At 80 mg/kg bw/d: bw loss
Biochemistry	≥ 150 mg/kg bw/d: ↑ APPT (m), ↑ γGT, ↑ ALP (m/f) 50 mg/kg bw/d: ↑ ALP (f)	75/125 mg/kg bw/d: ↑ ALP (m/f), ↑ γGT (f), ↑ APPT (m/f) At 50 mg/kg: ↑ APPT (m/f) ↑ ALP (f)	80 mg/kg bw/d: ↑ ALP, γGT, potassium, phosphorus 20 mg/kg bw/d: ↑ potassium, phosphorous; ↑ ALP (f)
Liver relative weight	≥ 150 mg/kg bw/day: ↑ weight in m/f (15%/26%)	≥ 75/125 mg/kg bw/d: ↑ weight in m/f (29%/19%)	80 mg/kg bw/d: ↑ weight (m/f) ≤ 20 mg/kg bw/d: no effects
Liver necropsy	Not investigated	No effects	No effects

APPT: activated partial prothrombin time; ALP: alkaline phosphatase; γGT : gamma glutamyltransferase; *top dose was reduced to 75 mg/kg bw/d in females after 7 weeks; m: males, f: females

However, the DS proposed to classify silthiofam as STOT RE 2 for lethality observed in the rabbit range-finding developmental toxicity study. In this range-finding study, rabbits were dosed with 0, 5, 15, 50, 100 and 150 mg/kg, from days 7 to 19 of pregnancy. Four of six and 5/6 females died in the 100 and 150 mg/kg/day dose groups, respectively. Deaths occurred between gestation days 13-16 and 15-22 in these respective groups. All deaths except for one intubation error in the 100 mg/kg group were considered treatment-related. A clear steep dose response relationship was observed as no toxic effects were observed at the top dose of 60 mg/kg bw/d in the main study. Mortality occurring at ≥ 100 mg/kg bw/d fall within the guidance values for classification in category 2 (28-day study ≤ 300 mg/kg bw/d).

Comments received during public consultation

One Member State (MS) supported the classification as STOT RE 2 based on mortality observed in dogs and rabbits at relevant dose levels and in rats at higher dose levels. Moreover, the MS

further asked for the potential relevance of the mechanism of action (MoA) in fungi (e.g. inhibition of ATP export from the mitochondrial matrix to the cytosol) with regard to humans because this MoA may add support to the relevance of mortality seen in animals to humans. In addition, according to the MS, effects observed in the liver also fulfilled the criteria for STOT RE 2 (no further explanation provided). The DS agreed that mortality in dogs may also be relevant for STOT RE. No further information on the relevance of the MoA in fungi to humans was included by the DS.

A second MS supported the DS's proposal to classify silthiofam for STOT RE based on the mortality observed in pregnant rabbits. Nevertheless, the MS proposed to use the actual time of death. At 100 mg/kg bw/d, the death of dams, observed within 7-9 days, were within the range of the guidance values for STOT RE 1 classification (for 7 days, Cat. 1 ≤ 130 mg/kg bw/d), and the death observed at day 10 was only just above the extrapolated guidance value. Due to the severity of the effect and as higher sensitivity of pregnant rabbits compared to pregnant humans has not been shown, the MS considered that STOT RE 1 could also be considered for this severe effect. This was agreed by the DS in their response to comments.

Assessment and comparison with the classification criteria

Based on the available repeated-dose toxicity studies, the main effects of concern were liver toxicity and lethality.

Liver toxicity

There were several studies available in rats and mice on the repeated-dose toxicity of silthiofam. In rats, liver toxicity was noted in the oral 28-day, 90-day and 2-year studies and in the dermal 21-day toxicity study above the guidance value level for classification as STOT RE 2. In the two-generation reproduction dietary study (1998), effects on liver consisted of increased organ weight and incidence of microscopic changes (hepatocyte vacuolation, bile duct hyperplasia). These effects occurred at 226 mg/kg bw/d in F0 males and at 273 mg/kg bw/d in F0 females which are also above the guidance values.

In mice, liver toxicity was observed in the feeding 28-day, 60-day or 18-month studies at doses above the guidance values.

In dogs, levels in ALP and γGT were consistently increased in both sexes after 28-day, 90-day or 1-year exposure at relevant dose levels for classification as STOT RE 2, indicating possible bile duct effects/cholestasis. Moreover, the increase in activated partial thromboplastin time and/or prothrombin time in the 28-day and 90-day studies might also support liver disease. An increase in absolute and relative liver weight was noticed at relevant dose levels only in the 28-day and 90-day toxicity studies. No histopathological findings were found in the 90-day and one-year studies (necropsy was not performed in the 28-day study). Overall, the increase in weight and the changes in enzyme activity are not considered sufficient for classification. This is supported by the absence of histopathological liver findings even at the highest dose level, at which marked general toxicity was shown in the one-year study. Thus, RAC agrees with the DS's proposal for no classification for liver.

Lethality

In rats, treatment-related mortality in males was observed in the 90-day repeated-dose toxicity study above the relevant guidance value doses. No effect on survival was reported in the 28-day range-finding study or in the carcinogenicity study in rat. In the rat developmental toxicity study,

a single death was considered treatment-related but occurred at a dose above the guidance values for classification (e.g. 1 000 mg/kg bw/d).

In mice, no deaths occurred in the 4-week range-finding study and in the 60-day feeding toxicity study. In the mouse 18-month study, a statistically significant decrease in survival was observed in females only at 2 and 20 mg/kg bw/d. Nevertheless, as the effect was not dose-related and did not occur at higher dose levels, this finding was not considered treatment-related.

In rabbits, in the range-finding developmental toxicity study, 4/6 and 5/6 females died in the 100 and 150 mg/kg bw/d dose group, respectively. The deaths were all treatment-related, except one intubation error at 100 mg/kg bw/d and 150 mg/kg bw/d occurred between gestation days 13-16 and 15-22 in the respective groups. Clinical observations reported at 100 and 150 mg/kg in the decedents included hypoactivity/lethargy, decreased defecation, discoloured faeces and/or staining of body surfaces/cage bedding. Moreover, body weight losses and reduced food consumption was observed during treatment and post-treatment periods. Gross pathology performed on the decedents revealed in three dams red fluid in the contents of the urinary bladder. Red-fluid in the urinary bladder was considered treatment related based on similar observations made in non-pregnant rabbits at 150 mg/kg or greater in the five-day repeated dose toxicity study. Other findings noted in decedents included dark red contents in the stomach of two females, of the caecum in one female and/or trachea in one female. One female had also dark red lungs and a pale heart. The cause of death was not reported. As stated in the guidance on the application of CLP criteria, for exposure durations shorter than 9 days (GD7 to GD 13-16), the effect should be compared to a guidance value of 100 mg/kg bw/d ≤ STOT RE 2 ≤ 1 000 mg/kg bw/d. At 150 mg/kg bw/d effects were also within the criteria for classification as STOT RE 2 (70-100 ≤ STOT RE 2 ≤ 700-1 000 mg/kg bw/d for GD7 to GD15-22 with last day of treatment at GD 19). Although the effects were observed at doses just above the guidance value for STOT RE 1 at 100 mg/kg bw/d, lethality occurring at a higher dose level (150 mg/kg bw/d) was consistent with STOT RE 2.RAC agrees with the DS that a very steep dose-response curve exists for mortality as no effects were observed in the main study at up to 60 mg/kg bw/d in rabbits. Although no target organs were identified, no findings were observed to suggest that the pregnant rabbit would not be a relevant species for investigating toxicity in humans. Indeed, the substance is not irritating/corrosive and clinical signs and gross-necropsy did not reveal severe toxicity in the gastro-intestinal tract of rabbits. Thus, mortality did not appeared to be the result of a non-relevant higher sensitivity of this species.

In dogs, in the 28-day range-finding study, 2/2 males (days 12, 18) and 1/2 females (day 24) were sacrificed at 350 mg/kg bw/d. The dose was reduced to 250 mg/kg bw/d following the two first weeks of exposure in males and following 3 weeks of exposure in females. At this dose level, clinical signs were observed (emesis, diarrhoea, hypoactivity, pale integument, emaciation, dehydration and decreased defecation). Liver was identified as the target organ in this study. Taking into account Haber's rule, the deaths were observed at relevant dose levels for classification as STOT RE 2 (≤ 375-750 mg/kg bw/d for 12-24 days). In the 90-day study, one out of 5 females was sacrificed (day 50) at 125 mg/kg bw/d. The high dose was then reduced to 75 mg/kg bw/d in females because of excessive toxicity. Clinical signs noted in the female sacrificed *in extremis* were emesis, weight loss, hypothermia, hypoactivity, pale mucosa and decreased defecation. No other findings were reported. The cause of death was not reported. Using Haber's rule, this is in line with the guidance values for STOT RE 2 classification (18 ≤ STOT RE 2 ≤ 180 mg/kg bw/d for 50 days). No death occurred in males in the 90-day study. In the one-year dog study, no deaths were reported up to 80 mg/kg bw/d. Overall, mortality was observed in both sexes in the 28-day range-finding study and in females in the 90-day study, at doses below the guidance values for STOT RE 2. As observed in rabbits, a very steep dose-

response existed for this effect and there are uncertainties as to whether Haber's rule could be applied here (no mortality in longer term studies: no mortality in males in the 90-day study and no mortality observed in the one-year dog study). Nevertheless, data on dogs could be used as supporting evidence for classification. Indeed, mortality observed in dogs suggested that this effect is not rabbit-specific due to a higher sensitivity of this species.

Moreover, silthiofam is a selective fungicide acting through the inhibition of the exportation of ATP from the mitochondrial matrix to the cytosol in fungi leading to cell death due to the disruption of energy-dependent processes. *In vitro*, in rats and human hepatocytes, decreased intracellular ATP production was indeed observed (confidential report, 2013). Reduction of ATP concentration in cells might affect all tissues. Thus, although clinical symptoms and findings at necropsy reported in the dossier in dogs and rabbits prior to death did not allow the identification of a target organ, mortality observed may be still of relevance to humans. Nevertheless, RAC noted that additional MOA data would be needed to confirm the relevance of the MOA in fungi to mammals.

Overall, RAC supports the DS proposal to **classify silthiofam as STOT RE 2** based on lethality observed in rabbits and supported by mortality observed in dogs. As no specific target organ was identified, no organ will be specified for the STOT RE 2 classification.

4.9 Germ cell mutagenicity (Mutagenicity)

Table 28: Summary table of relevant *in vitro* and *in vivo* mutagenicity studies

Method	Results	Remarks	Reference
In vitro studies			
Bacterial gene mutation (Ames) TS: purity 96.6% 0, 0.015, 0.05, 0.15, 0.5 and 1.5 mg/plate *Salmonella typhimurium* TA98, TA100, TA102, TA1535 and TA1537	Negative	-	Stegeman and Kier (1995). ML-95-057, MSL-14335; RAR B6.4.1/01
Mammalian gene mutation TS: 97.4% 0, 62.5, 125, 150, 250 and 500 µg/ml CHO/HGPRT	Negative	-	Stegeman *et al.* (1996). ML-96-144, MSL-14936; RAR B6.4.1/02
In vitro chromosomal aberration TS purity: 96.6% Test 1: 0, 50.5, 101, 20, 401, 601 and 801 µg/ml ± S9 Test 2: 0, 3.18, 6.35, 12.7, 25.3, 50.5 and 101 µg/ml. Human lymphocytes	Negative	-	Murli (1995). HL-95-159; RAR B6.4.1/03
In vivo studies			
In vivo micronucleus TS: 97.4% 0, 500, 1000 and 2000 mg/kg b.w Mouse (bone marrow)	Negative	-	Stegeman et al (1996). ML-96-143; MSL-14933; RAR B6.4.2/01
In vivo UDS 0, 500, 1000 and 2000 mg/kg b.w Rat (liver)	Negative	-	Bakke (2002). SR-2001-127; May 2002 Addendum, RAR B6.4.2.2

4.9.1 Non-human information

4.9.1.1 *In vitro* data

All studies negative.

4.9.1.2 *In vivo* data

All studies negative.

4.9.2 Human information

No data.

4.9.3 Other relevant information

Not relevant.

4.9.4 Summary and discussion of mutagenicity

No evidence of genotoxicity was noted in a battery of in vitro and in vivo studies which were previously evaluated during the EU review and which are summarised in Table B.6.4-1.

4.9.5 Comparison with criteria

Not required.

4.9.6 Conclusions on classification and labelling

No classification for germ cell mutatgenicity.

RAC evaluation of germ cell mutagenicity

Summary of the Dossier Submitter's proposal

In a battery of *in vitro* genotoxicity studies performed under GLP and OECD TG, silthiofam did not cause gene mutations or chromosome aberrations. *In vivo*, negative results were obtained in a micronucleus assay in mice (up to 2 000 mg/kg bw) and in an unscheduled DNA synthesis test (UDS). On this basis, no classification was proposed by the DS for germ cell mutagenicity.

Comments received during public consultation

One MS commented that there was no information on proof of exposure to bone marrow in the *in vivo* micronucleus test. According to the MS, the negative results of the study should not be taken into account in the event that no such information was available. The DS responded that silthiofam was highly absorbed by oral route and was widely distributed. Therefore, the DS believed that the target tissue had been exposed to silthiofam.

Assessment and comparison with the classification criteria

Silthiofam was negative in the three available *in vitro* assays (bacterial mutation assays, a mammalian gene mutation assay, a mammalian cytogenicity test) and in two *in vivo* assays (mouse micronucleus and UDS). RAC agrees with the comment that no proof of exposure was reported in the dossier and therefore the negative results obtained in the micronucleus test are difficult to interpret. Nevertheless, on the basis of the negative *in vitro* test and negative *in vivo* results, RAC agrees with the conclusion of the DS that silthiofam **did not meet the criteria for classification for germ cell mutagenicity**.

4.10 Carcinogenicity

Table 29: Summary table of relevant carcinogenicity studies

Method	Results	Remarks	Reference
18-month mouse dietary 0, 10, 100, 1000 & 4000 ppm Exposure M: 0, 1.4, 13.7, 141 & 564 mg/kg bw/day F: 0, 2.03, 20.7, 203 and 855 mg/kg bw/day n = 50 (terminal sacrifice) n = 10 (interim sacrifice)	Evidence of liver toxicity at 4000 ppm included ↑ALT & AST, ↑liver weights, hepatocellular hypertrophy, cystic degeneration, karyomegaly, mixed cell foci, pigment deposition in Kuppfer cells, cytoplasmic vacuolation and individual cell necrosis. Cholelith (stones/calculi) were observed in the gall bladders of several high-dose animals but no microscopic evidence of biliary obstruction was noted. There was a slight but statistically significant increase in the incidence of hepatocellular adenomas in high-dose females that was considered to be treatment related and secondary to liver toxicity.	-	Dudek (1998). ML-96-126, MSL-15585; RAR B.6.5/01
2-yr rat dietary 0, 10, 100, 1000 & 3000 ppm Exposure M: 0, 0.5, 5.02, 50.52 & 150 mg/kg bw/day F: 0, 0.65, 6.42, 65 and 195 mg/kg bw/day n = 50 (terminal sacrifice) n = 10 (interim sacrifice) 1000/100 ppm (6.46.4 mg/kg/day, males)	Evidence of liver toxicity at 3000 ppm included ↑ γGGT, ↑ liver weights, hepatocellular hypertrophy, vacuolation, eosinophilic foci and cystic degeneration. Hepatocellular vacuolation was also noted in 1000 ppm females. Hepatocellular pallor was noted at 1000 & 3000 ppm but was considered indicative of an increase in cytoplasmic smooth endoplasmic reticulum resulting from enzyme induction and thus an adaptive rather than a toxic response. A small increase in hepatocellular tumours was noted in high-dose males and considered related to treatment. A slight increase in thyroid follicular tumours was also noted in high-dose males and considered treatment-related.	-	Stout and Thake (1998). ML-96-035, MSL-15713; DAR B6.5/02

4.10.1 Non-human information

4.10.1.1 Carcinogenicity: oral

Study 1:
Oncogenicity study of MON 65500 administered in feed to CD-1 mice for 18 months. Dudek, B.R. (1998). Report MSL-15585. OECD Guideline 451, FIFRA 83-2, EEC OJ L 133. GLP compliant.

Materials and methods:
Silthiofam, Lot number: SRL-9601-6895-T, purity: 97.4% was administered in the feed at concentrations of 0, 10, 100, 1000 and 4000 ppm for 18 months (50/sex/main group). An interim sacrifice was performed at 12 months (10/sex/group). Calculated test substance

consumption (mg/kg bw/day) for these dietary levels was: 0, 1.4, 13.7, 141 and 564 for males, and 0, 2.03, 20.7, 203 and 855 for females respectively. Test material stability for the duration of the study was confirmed.

Results:

Interim 12 month sacrifice.

An interim sacrifice of 10 mice/sex/dose group was performed at 12 months. Organs weighted were brain, kidneys, liver, spleen, and testes. Blood samples were collected for haematology and clinical chemistry. Tissues from control and high dose animals were examined microscopically. Histopathogy of the liver indicated changes consistent with hepatotoxicity in the high dose groups (Table 32).

Haematology parameters were unaffected; there were no treatment-related changes and no dose response. ALT was raised slightly in males and females. Liver weights were slightly raised (table 30).

Table 30: Summary of treatment-related findings at interim sacrifice (12 months)

Dose levels (mg/kg)←	**♂**	**♀**	**♂**	**♀**	**♂**	**♀**	**♂**	**♀**	**♂**	**♀**
	0	**0**	**1.4**	**2.0**	**13.7**	**20.7**	**141**	**203**	**564**	**855**
Endpoint										
Body weight (%)	100	100	109	101	99	95	106	88	100	91
ALT (U/L)	55	52	31	36	30	41	25	36	83	62
AST (U/L)	161	165	103	104	100	108	94	121	129	123
Liver –absolute (%)	100	100	101	94	97	108	94	88	110	108
Liver –relative (%)	100	100	91	93	97	114	86	101	107	**115****

**Significantly elevated at 12 months ($p \leq 0.01$)

Several treatment-related pathological lesions were found in high dose animals only. These included hepatocellular hypertrophy, pigment deposition, vacuolisation, necrosis of individual hepatocytes and karyomegaly (Table 32). Cell proliferation results (PCNA analysis) from the livers of high dose females were non-significant.

Terminal sacrifice.

The mean survival was 74% for males and 71% for females at termination. There was a statistically significant reduction in survival in the female 10 and 100 ppm groups. The causes of death were commonly occurring findings. Overall, there was no dose-related increase across all groups and the finding was not considered treatment-related. There were no apparently treatment-related clinical signs throughout the study.

Haematology parameters were not affected in a treatment-related manner. Serum ALT and AST were significantly elevated in males at the high dose and were elevated (but not statistically significantly in females), indicating some toxicity to the liver at this dose level (Table 31). Other clinical chemistry parameters were not affected by treatment.

There was a treatment-related increase in absolute and relative liver weight in both males and females at the high dose level. This was associated with pathological findings at microscopic examination in the centrilobular and midzonal regions at interim and terminal sacrifices. The findings at interim considered related to treatment are as follows; hepatocellular hypertrophy, vacuolation, pigment deposition, necrosis of individual hepatocytes and/or karyomegaly. The pigment was described as yellow/brown foamy pigment and was observed in the cytoplasm of the Kuppfer cells and was thought to be either lipofuscin or haemosiderin.

Table 31: Summary of treatment-related findings at terminal sacrifice.

Dose levels (mg/kg)←	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀
	0	**0**	**1.4**	**2.0**	**13.7**	**20.7**	**141**	**203**	**564**	**855**
Endpoint										
BODY WEIGHT (%)	100	100	99	97	101	95	99	93	98[1]	94
ALT (U/L) AST (U/L)	56 115	62 213	56 127	80 218	42 124	49 127	46 164	38 116	**189**** **200****	88 266
Liver –absolute (%) Liver –relative (%)	100 100	100 100	100 100	98 102	99 96	101 105	100 100	100 107	128**[2] 128**[2]	111*[2] 117**[3]

[1] Statistically significant ($p \leq 0.01$ or $p \leq 0.05$) at months 3, 6, 12, 14 and 15.
[2] Significantly elevated at 18 months ($p \leq 0.01$)
[3] Significantly elevated at 12 and 18 months ($p \leq 0.01$)

Additional to the liver changes observed in the interim sacrifice animals, degeneration, cystic and mixed cell foci were also increased in males at the high dose. The two incidences of hypertrophy seen in males of the 1000 ppm group were within the historical control range (mean 10%; range 0-78%).

Cholelith (stone/calculus) was present in the gall bladders of 4 males of the high dose group at interim sacrifice. Cholelith occurred in a total of 15 males ($p<0.01$) of the high dose group (including all deaths) by 18 months. The single occurrence in the female high dose group was considered probably related to treatment. These were not accompanied by evidence of inflammatory change, degeneration or proliferative change. There was no microscopic evidence of biliary obstruction.

Conclusion:
The liver was identified as the primary target organ in this study. The gall bladder was also identified as a target primarily in males of the high dose group. Effects on the liver were observed at the 564/855 mg/kg bw/day dose (male/female 4000 ppm dietary level), and included increases in organ weight, enzymes (peripherally), and/or microscopic changes in the centrilobular and/or midzonal regions. Such changes were indicative of hepatotoxicity and also included hepatocellular hypertrophy, cystic degeneration, karyomegaly, mixed cell foci, pigment deposition in Kupffer cells, cytoplasmic vacuolation, karyomegaly and/or individual hepatocyte necrosis.

An increase in the incidence of in the incidence of hepatic adenoma was observed in females, at a dietary level of 4000 ppm. This finding was considered to be biologically significant and treatment related; the observed incidence (5/50 mice; 10%) was above that in the concurrent control group (1/50; 2%), and outside of the laboratory (0-3%) and Charles-River (1 – 8%) historical control ranges.

The toxicological significance of this finding was considered low as it was considered that the data from the study supported a threshold effect related to the underlying hepatotoxic effect (most notably, individual hepatocyte necrosis). The increase was observed in one sex and organ and did not affect survival, i.e., was not contributory to unscheduled deaths (all adenomas were observed in females that survived to study termination) and did not progress to malignancy. No increase was seen at non-hepatotoxic doses. Mechanistic studies evaluating the human relevance of the observed tumours is are presented in section 4.10.3.

The low observed adverse effect level (LOAEL) for long-term (chronic) toxicity was 4000 ppm in both sexes of mice based on effects on the liver and gall bladder (564 and 855 mg/kg bw/day in males and females, respectively).

The low observed adverse effect level (LOAEL) for carcinogenicity was 4000 ppm (855 mg/kg bw/day) in females. silthiofam induced an increase in hepatocellular adenoma in females at this high dose level which was also hepatotoxic.

Table 32: Summary of selected histopathological findings.

	Interim sacrifice									
Dose levels (mg/kg)	**0** ♂	**0** ♀	**1.4** ♂	**2.0** ♀	**13.7** ♂	**20.7** ♀	**141** ♂	**203** ♀	**564** ♂	**855** ♀
NUMBER OF ANIMALS	10	10		10	10	10	10	10	10	10
LIVER										
HEPATOCELLULAR HYPERTROPHY	0	0	-	0	0	0	1	0	**7****	**3**
Pigment deposition	1	3	-	4	1	2	0	2	**9****	**8**
Necrosis, individual cells	0	0	-	0	0	0	0	0	**7****	**5**
Karyomegaly	2	2	-	0	3	2	4	0	**8****	2
Vacuolisation	-	0	-	0	-	0	-	1	-	**5**
Cholelith	0	-	-	-	0	-	0	-	**4**	-
	Terminal sacrifice									
NUMBER OF ANIMALS	38	41	41	30	34	39	38	28	34	39
LIVER										
HEPATOCELLULAR HYPERTROPHY	0	0	0	0	0	0	2	0	**25****	**12****
Pigment deposition	32	16	-	24	14	27	15	22	**51****	**42**
Cystic degeneration	0	1	-	0	0	0	0	0	**16****	1
Mixed cell focus	0	0	-	0	0	0	0	0	**7***	2
Basophilic focus	0	0	-	0	0	0	2	0	0	2
Eosinophilic focus	0	0	-	0	0	0	0	0	1	1
Hepatocellular adenoma	4	1	-	0	3	1	8	0	4	5
Hepatocellular adenoma, multifocal	2	0	-	0	1	0	1	0	2	0
Hepatocellular carcinoma	6	0	-	0	2	0	2	0	1	0
Necrosis, individual cells	3	5	-	1	3	0	3	3	**40****	**19****
Karyomegaly	11	6	-	3	10	8	11	5	**36****	**15**
Vacuolisation	-	0	-	0	-	0	-	2	-	**6**
Cholelith	0	0	-	0	0	0	0	0	**7***	1

Bold = considered biologically relevant.

Study 2.

Combined chronic toxicity/oncogenicity study of MON 65500 administered in feed to CD™rats for 24 months. Stout, L.D. and Thake, D.C. (1998). Report MSL-15713. Guidelines; OECD 453, FIFRA 83-5, EEC Directive 87/302/EEC, Part B. GLP compliant.

Materials and methods:

Silthiofam, Lot number: SRL-9601-6895-T, purity: 97.4% was administered in the feed at concentrations of 0, 10, 100, 1000 and 3000 ppm for approximately 24 months (60/sex/group). An interim sacrifice was performed at 12 months (10/sex/group). Calculated test substance

consumption (mg/kg bw/day) for these dietary levels was, respectively: 0, 0.5, 5.02, 50.52 and 150 for males, and 0, 0.65, 6.42, 65 and 195 for females. Due to decreased survival rates in most groups (< 50%) towards the end of the study period, the study was terminated at about 23.5 months.

Results:
The test substance was found to be homogenously distributed and stable throughout the study. Dietary concentrations were analysed and found to be acceptable. There was no statistically significant effect on survival in male or females. Percentage survival was low at termination for the 0, 10, 100, 1000 and 3000 ppm levels and were 34%, 40%, 34%, 28%, and 50% for males and 32%, 32%, 38%, 36% and 30% for females.

The occurrence of unusual urine colour in high dose males was considered treatment-related. There were no other clinical signs of toxicity.

Mean body weight and body weight gains of males were not significantly affected by treatment. Mean body weights and body weight gains of females were increased ($p<0.01$ or $P<0.05$) at 10 and 100 ppm throughout the study and decreased at the high dose, 3000 ppm (statistically significant at various time points ($p<0.01$ or $p<0.05$)).

Table 33. Summary of treatment-related findings at terminal sacrifice

		Cumulative weight gain (% control)			
Sex	**Month**	**0.5 / 0.65**	**5.0 / 6.4**	**51 / 65**	**150 / 195**
Male	3	105	100	97	96
	12	101	93	97	98
Female	3	110	113	105	94
	12	110	108	102	90

Slight increases and decreases in food intake in males over the study period were not considered related to treatment due to the small magnitude and/or lack of dose response. Intermittent alterations in food intake in females of the 10 – 1000 ppm groups were not considered related to treatment. The statistically significant decrease in food consumption in females of the high dose group was considered treatment-related.

A number of haematology parameters were altered intermittently throughout the study but none were altered in a biologically significant way. Haemoglobin and haematocrit were decreased in males at month 3, but not thereafter. There was a trend towards increased platelet count in males at months 3 and 6 only. This increase may be related to treatment, given the increased platelet counts recorded in males of the 90-day study at 5000 ppm. The effect was not seen at the later time intervals in the present study however, and is unlikely to be biologically significant. Similarly, the decrease in haemoglobin levels (3 months only) and decreased platelet (3, 6 and 12 months) and lymphocyte counts (3 months only) seen in females were not clearly dose-or time-related (table 34).

γGT was consistently elevated in high dose males from month 6 onward (144-205% of control), indicating possible bile duct effects/cholestasis. The increase was not statistically significant and it was not considered toxicologically significant. A slight ($p<0.05$) increase in serum alkaline phosphatase (ALP) was noted in the high dose males at the 3 month period only. These findings were without histological correlate in the bile ducts of the males. There was a possibly treatment-related increase in bile duct hyperplasia and fibrosis in the high dose females

($p<0.01$). The study author concluded that this increase was within the historical control range and considered incidental to treatment (HC data were not included).

Statistically significant alterations were seen in a number of other clinical chemistry parameters (ALT, AST, CPK, BUN, creatinine, total protein, albumin, calcium, sodium, chloride, and potassium). These were not considered related to treatment due to the lack or dose or time relatedness, small magnitude of the change or the change was opposite in direction to that normally associated with toxicity (AST, ALT). Blood clotting potential was not affected by treatment.

Table 34: Summary of treatment-related findings at terminal sacrifice.

		Results									
Endpoint		**0 / 0**		**0.5 / 0.65**		**5.0 / 6.4**		**51 / 65**		**150 / 195**	
		♂	♀	♂	♀	♂	♀	♂	♀	♂	♀
Survival		34	32	40	32	34	38	28	36	50	30
CLINICAL OBSERVATIONS		-	-	-	-	-	-	-	-	↑	-
Body weight gain (%)		100	100	101	110	93	108	97	102	98	90*/**
Food consumption		-	-	-	-	-	-	-	-	-	↓*
Haemaglobin (%)		100	100	109	96	107	94	95	91	99	92
Haemocrit (%)		100	100	109	97	108	96	95	92	99	92
Platelet count (%)		100	100	90	102	97	107	94	103	93	94
Lymphocytes (%)		100	100	85	122	98	111	101	106	117	105
γGT		100	100	107	71	86	64	121	21	193	86
ALT		100	100	129	98	145	88	147	128	123	75
AST		100	100	113	105	229	99	147	129	107	85
CPK		100	100	99	92	125	89	149	71	93	54
		100						90			
Liver; interim	-abs.	100	100	119	104	103	107	109	113	127**	104
	-rel.	100	100	115	104	102	111	108	114	125**	104
Liver; term.	-abs	100	100	103	102	111	111	111	116	120**	107
	-rel.	100	100	102	100	110	110	109	114	119**	107

* P ≤ 0.05; ** P ≤ 0.01

A statistically significant increase in liver weights both absolute and relative to body weight were recorded for high dose males. Relative liver was significantly elevated in high females also at this dose. Alterations in other organ weights were not clearly dose or treatment related.

Table 35. Selected histopathological findings.

Dose levels (ppm)	**0 / 0**		**0.5 / 0.65**		**5.0 / 6.4**		**51 / 65**		**150 / 195**	
	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀
NUMBER OF ANIMALS	**10/50** [a]	**10/50**	**10/50**	**10/50**	**10/50**	**10/50**	**10/50**	**10/50**	**10/50**	**10/50**
LIVER										
HEPATOCELLULAR HYPERTROPHY	0/0b	-	0/0	-	0/0	-	0/0	-	0/2	-
Cystic degeneration	0/11	-	1/20	-	3/22	-	2/20	-	1/30**	-
Eosinophilic foci	0/7	1/5	1/12	1/4	0/7	0/10	2/9	1/10	4/29**	4/23**
Centrilobular pallor	0/0	0/0	0/0	0/0	4/4	4/4	6*/11*	6*/12**	9**/22**	10**/29**
Vacuolisation	1/3	0/0	1/1	2/2	1/2	1/4	1/5	8**/11**	9**/24**	8**/24**
Bile duct hyperplasia/fibrosis	-	2/19	-	4/27	-	3/27	-	3/27	-	5/33*

* P ≤ 0.05; ** P ≤ 0.01; - not found/relevent
a – no. of animals at interim/ no. of animals at termination.
b – no. of findings at interim/no. of findings at termination

There were no effects on several organ systems (except liver) from the 12-month interim sacrifice including pituitary, thyroid, kidney, testes etc. Results confirm that the liver is the target organ in both males and females with effects observed at the two highest doses. Treatment-related microscopic findings were observed in the liver at terminal sacrifice. Hepatocellular vacuolation (females) and pallor (centrilobular and/or midzonal) (both sexes) were seen at ≥ 1000 ppm. Centrilobular pallor was also observed at 100 ppm in 4 males and 4 females at both sacrifices. The hepatocellular pallor was further described as a tinctoral change, without changes in nuclear morphology. This change was considered to be consistent with an increase in cytoplasmic smooth endoplasmic reticulum, which is a morphological correlate to enzyme induction. It was also noted that there were no degenerative or progressive changes present in hepatocytes in which pallor was observed at termination. This change was therefore interpreted as an adaptive response rather than a toxic change. Cystic degeneration was seen in males at the high dose at termination.

There was an increase in incidence of combined[1] hepatocellular adenomas and carcinomas, and of hepatocellular adenomas and carcinomas in males of the top dose group (Table 36). These increases were outside of concurrent controls. While the study pathologist stated that these lesions could not be unequivocally associated with treatment, the converse is also true. There is an apparently dose-related increased incidence of adenoma, carcinoma and tumours combined, which supports an association with treatment. The incidence of adenoma and carcinoma exceeds the Charles River historical data (1995 – 2001). A treatment-relationship can also be supported by the observation that hepatotoxicity was more pronounced in males in the 90-day study (RAR B.6.3.2.2) and the finding that hepatocellular tumour induction is exclusive to males only in the present study.

Table 36. Liver tumours in male rats

	Incidences of hepatic adenoma and carcinoma in males					
Dose (mg/kg)	**0**	**0.5**	**5.0**	**51**	**150**	**HCD**
Adenoma	4/50 (8%)	3/50 (6%)	2/50 (4%)	5/50 (10%)	7/50 (14%)	1.4 – 8%
Carcinoma	0/50 (0%)	2/50 (4%)	2/50 (4%)	3/50 (6%)	4/50 (8%)	0.8 – 6.7%
Tumours combined	4/50 (8%)	5/50 (10%)	4/50 (8%)	8/50 (16%)	11/50 (22%)	

Historical control data:
adenoma - mean 2.4% (37/1531 (no. affected/no. observed)).
Carcinoma – mean 2.1% (32/1531).
Combined adenoma/carcinoma – mean 4.5% (69/1531).

There were small increased incidences of combined thyroid follicular cell adenomas and carcinomas, and of follicular cell hyperplasias (table 37). Some lesions were associated with grossly observed thyroid enlargement and mass/nodules. It was the opinion of the pathologist that these lesions may represent a progressive morphological continuum. However, this does not negate an association with treatment. The incidence of carcinoma in this study is at the upper limit of the historical control background incidence (4% incidence at 150 mg/kg *vs* 1%

[1] The pathology report (Appendix 3) refers to an increase in combined adenoma and carcinoma and multifocal adenoma. Examination of the individual data identifies 4 multifocal and 3 focal adenoma..

and a range of 0.9 – 3.9% in the historical controls). The incidence of adenoma (10%) does not exceed the historical control data range but is once again in the upper boundry (1.7% – 12%).

Table 37. Thyroid tumours in male rats

	Incidences of thyroid follicular epithelium adenoma and carcinoma in males					
Dose (mg/kg)	**0**	**0.5**	**5.0**	**51**	**150**	**HCD**
Adenoma	3/50 (6%)	0/50	0/50	1/50 (2%)	5/50 (10%)	1.7 – 12%
Carcinoma	0/50	0/50	1/50 (2%)	1/50 (2%)	2/60 (4%)	0.9 – 3.9%
Tumours combined	3/50 (6%)	0/50	1/50 (2%)	2/50 (4%)	7/50 (14)	

Historical control data:
adenoma – mean 2.7% (41/1531 (no. affected/no. observed)).
Carcinoma – mean 1.0% (15/1531).
Combined – mean 3.7% (56/1531).

Conclusions:
The liver was the target organ identified in this study. Effects were observed at the 51 and 150 mg/kg bw/day dose levels in males and 65 and 195 mg/kg bw/day in females. Effects included increases in organ weight, increased serum γGT (males) and/or microscopic changes. Microscopic change included hepatocellular vacuolization and hypertrophy, eosinophilic foci and/or cystic degeneration. There were treatment-related increases in incidences of hepatocellular tumours and of thyroid tumours in mid- and high- dose males.

The overall LOAEL was based on non-neoplastic effects and set at 65 mg/kg bw/day based on centrilobular vacuolation, increased eosinophilic foci, and increased liver in females.

The LOAEL for carcinogenicity was set at 51 mg/kg bw/day in male rats based on increased incidences of hepatic adenomas at 51 and 150 mg/kg bw/day. However, these tumours are not considered to be relevant to humans. Please see data from the mechanistic studies below.

Study 3:

Human Relevance Assessment of Rodent Tumours.
The following assessment is based largely on the human relevance framework (HRF) developed by the International Programme on Chemical Safety (IPCS, 2007; Boobis et al., 2006). This framework is generally considered to be a useful approach to aid in the weight-of-evidence evaluation of the mode of action by which chemicals induce tumours in laboratory animals and the relevance of such data to human health risk assessment. The mechanistic studies referred to here are described later under section 4.10.3

Rat Liver Tumours
The human relevance framework (HRF) applies a stepwise approach:

Step 1: Evaluate if the weight of evidence is sufficient to establish the mode of action (MOA) in animals;

Step 2: Evaluate if the human relevance of the proposed MOA can be reasonably excluded on the basis of qualitative differences in key events between animals and humans;

Step 3: Evaluate if the human relevance of the proposed MOA can be reasonably excluded on the basis of quantitative differences in key events between animals and humans.

Step 1. Evaluate if the weight of evidence is sufficient to establish the mode of action (MOA) in animals

Postulated MOA

Silthiofam activates the CAR and PXR nuclear receptors in male rats. This results in the altered expression of a number of genes as well as an increase in hepatic cell proliferation that eventually leads to a slight increase in hepatocellular tumours.

Key events

1. Activation of CAR and PXR nuclear receptors
2. Increased hepatocellular proliferation

Strong evidence of CAR and, to a lesser extent, PXR activation by silthiofam was observed in the 14-day mode of action study in male rats. Dietary administration of 3000 ppm silthiofam (which was also the high-dose level in the chronic rat study) resulted in substantial induction of hepatic CYP2B1, CYP2B2 and (to a lesser extent) CYP3A1, as assessed by enzyme activity, mRNA expression and Western blot analysis. Although there is not total specificity, CYP2B1 and CYP2B2 are generally considered markers for CAR activation while CYP3A1 is considered to be a marker for PXR activation. A substantial increase in replicative DNA synthesis (cell proliferation) was also observed in the livers at 7 and 14 days. However, there was little to no other evidence of hepatotoxicity, as indicated by unchanged or minimally decreased serum enzyme markers (ALT, AST, ALP) and only slight histopathology (minimal to slight hypertrophy, decreased glycogen-induced vacuolation, and slightly increased lipid accumulation). Significant induction of CYP2B1, CYP2B2, CYP3A and cell proliferation was also observed in an in vitro study with rat hepatocytes.

Cytochrome P450 enzymes and hepatocellular proliferation were not evaluated in any of the longer-term studies with silthiofam. However, a number of findings in the liver, including increased liver weights, hepatocellular hypertrophy, and hepatocellular pallor (a tinctoral change consistent with an increase in cytoplasmic smooth endoplasmic reticulum) were observed in these studies and are consistent with P450 enzyme induction.

Dose-Response

Only a single dose level (3000 ppm) was utilized in the in vivo mode of action study so in vivo dose-response relationships cannot be evaluated. However, the dose level used in the in vivo mode of action study was the same as the high-dose level in the chronic rat study. Thus, activation of the CAR and PXR nuclear receptors and increased hepatocellular cell proliferation have been demonstrated at the same dose level that produced a slightly increased incidence of liver tumours. Dose response was evaluated in the in vitro study with rat hepatocytes, in which the level of cell proliferation appeared to be consistent with the level of CAR and PXR induction.

Temporal Association

Only limited data are available to evaluate the temporal relationships for the key events. Activation of CAR and PXR and stimulation of cell proliferation were observed within 7 to 14 days of administration of 3000 ppm silthiofam to male rats. Only minimal evidence of hepatotoxicity was noted at 3000 ppm in this study but slightly greater evidence of hepatotoxicity was noted at ≥2500 ppm in the 90-day rat study. A slightly increased incidence of liver tumours, along with indirect evidence of increased enzyme induction and other signs of liver toxicity, was noted in males at 3000 ppm in the chronic rat study. These results suggest that short-term exposure to silthiofam activates CAR and PXR, leading to a number of hepatic changes, including enzyme induction, hepatocellular hypertrophy and increased hepatocellular proliferation. It is not known how long the cell proliferation is sustained. However, an early, short-term burst of cell proliferation is sufficient to cause liver tumours for a number of chemicals, including phenobarbital, which has been shown to act via a similar CAR/PXR-mediated mode of action as is being proposed for silthiofam. Thus, the existing temporal data are supportive of the proposed MOA.

Strength, consistency and specificity

Silthiofam has been shown to activate CAR and PXR and stimulate hepatocellular proliferation in rats both in vivo and in vitro. However, the proliferative response was species-specific as silthiofam activated CAR and PXR but did not stimulate hepatocyte proliferation in isolated human hepatocytes. The *in vitro* results with silthiofam were also very similar to those for phenobarbital, which has been shown to cause rodent liver tumours *via* a similar CAR/PXR-mediated mode of action as is proposed for silthiofam.

Biological plausibility and coherence

The proposed CAR/PXR-mediated MOA for silthiofam is biologically plausible. Activation of the CAR and/or PXR nuclear receptors is known to lead to changes in expression of a number of genes, including many that are involved in metabolism, apoptosis, cell proliferation, etc. It has been well-established that increased hepatocellular proliferation, even if not sustained for an extended period of time, can lead to an increased incidence of tumours in rodents.

The proposed CAR/PXR-mediated mode of action is also consistent with the general pattern of hepatotoxic effects seen in the short-term and long-term studies with silthiofam, and probably also accounts for the increased levels of hepatic T4-UDPGT, an enzyme known to be regulated by CAR and PXR.

Finally, the proposed CAR/PXR-mediated MOA for silthiofam is consistent with the growing body of literature indicating a similar MOA for phenobarbital and a few other chemicals that have been shown to induce liver tumours in rats and/or mice. In fact, the results from silthiofam were very similar to those from phenobarbital, when tested in vitro in both rat and human hepatocytes.

Other modes of action

Silthiofam is not genotoxic so a genotoxic mode of action for the rat liver tumours can be ruled out.

The slightly increased incidence of liver tumours in the high-dose (3000 ppm) male rats in the chronic rat study occurred in the absence of substantial liver toxicity. In addition, a substantial increase in hepatocellular proliferation in the absence of significant hepatotoxicity was noted at 3000 ppm in the 14 day *in vivo* mode of action study. Therefore, cytotoxicity can probably be ruled out as the mode of action for the rat liver tumours.

No evidence of activation of PPARα (as measured by enzyme activity, gene expression and Western blot) was noted in the 14-day *in vivo* mode of action study and no evidence of peroxisomal proliferation was reported in any of the other studies with silthiofam. Therefore, peroxisomal proliferation can also be ruled out as a mode of action for the liver tumours.

Other known non-genotoxic modes of action for liver tumours include hormonal perturbation, immunosuppression, porphyria and activation of the AhR receptor.

CYP1A (a marker for AhR) was not included in the in vivo mode of action study. However, silthiofam does not appear to have the chemical structure that would likely activate this receptor. No evidence for any of the other potential MOAs has been observed in any of the studies with silthiofam. Thus, none of these potential MOAs are considered likely to be involved. (Note from evaluator: CYP1A was not tested).

Uncertainties, inconsistencies and data gaps

There are no inconsistencies in the data base regarding the proposed carcinogenic MOA for silthiofam. However, there are a few data gaps and uncertainties, including the following:

1. Lack of *in vivo* dose-response data for CAR/PXR activation and hepatocellular proliferation. It is likely that CAR/PXR activation would be observed at much lower dose levels than 3000 ppm. But, while dose-response data for this endpoint may have been of use for correlating with non-neoplastic findings, it would likely not be useful in evaluating the mode of action for the marginal increase in liver tumours that occurred only at the high-dose level. Dose-response data for hepatocellular proliferation may have been of more value in confirming the proposed MOA but lack of this data is not considered to be critical, especially since a correlation between activation of CAR/PXR and the level of cell proliferation was observed in the in vitro rat mode of action study.

2. No studies with CAR/PXR knockout animals. The available data provide convincing evidence that silthiofam activates CAR and PXR and, especially when evaluated along with literature data on phenobarbital and other chemicals, strongly suggest that activation of these nuclear receptors is responsible for the increase in cell proliferation. However, these studies do not allow a determination as to the relative contributions of CAR *vs*. PXR or provide proof that another mode of action was not also involved. Answering both of these questions would have required use of both CAR and PXR single knockout and CAR/PXR double knockout rats. However, a double knockout rat was not available. In addition, understanding the relative contributions of CAR *vs*. PXR to the proposed silthiofam MOA is considered to be of limited value.

Overall Assessment of postulated MOA

The available data provide convincing evidence that silthiofam activates the CAR and PXR nuclear receptors in rats, and strongly suggest that this leads to increased hepatocellular proliferation, which is a well-established key event in the development of liver tumours. Due to the lack of availability of a double-knockout CAR/PXR rat, the possibility that another MOA may also be involved cannot be definitively excluded. However, the *in vitro* results from silthiofam were very similar to those for phenobarbital, for which a similar MOA has been established. Therefore, it is considered to be highly likely that a CAR/PXR-mediated mode of action is responsible for the slightly increased incidence of liver tumours in high-dose male rats.

Step 2. Evaluate if the human relevance of the proposed MOA can be reasonably excluded on the basis of qualitative differences in key events between animals and humans.

The *in vivo* and *in vitro* mode of action studies demonstrated that silthiofam activated the CAR and PXR nuclear receptors and caused hepatocellular proliferation in rats and isolated rat hepatocytes. Silthiofam also activated CAR and PXR in isolated human hepatocytes. However, the response was weaker than in rats (see Table 38). More importantly, there was no proliferative response. Therefore, although there may still be some minor uncertainties regarding the initial key event leading to the proliferative response in rat, the absence of a proliferative response in human hepatocytes, even at cytotoxic doses of silthiofam, makes this a moot point and indicates that the occurrence of liver tumours in humans would be extremely unlikely.

Table 38. Comparison of results from *in vitro* studies with rat and human hepatocytes (max increase relative to control)

	Silthiofam		**Phenobarbital**	
	Rat	**Human**	**Rat**	**Human**
PROD	2X	-	4X	2X
BROD	2X	3X	5X	8X
BQ	5X	2X	4X	5X
CYP2B mRNA	13X	5X	36X	9X
CYP3A mRNA	97X	4X	34X	9X
S-phase	2X	-	2X	-

The *in vitro* responses with silthiofam were generally similar to those with phenobarbital, which has been shown to cause liver tumours in rats and mice via a CAR/PXR-mediated mode of action [2],[3]. Phenobarbital also causes cell proliferation in rat hepatocytes but not in human hepatocytes and, based on a long history of safe use, is generally considered not to be carcinogenic to humans.

Therefore, the slightly increased incidence of liver tumours observed in high-dose male rats (which was not definitively attributed to silthiofam treatment) is not considered to be relevant to humans.

Step 3. Evaluate if the human relevance of the proposed MOA can be reasonably excluded on the basis of quantitative differences in key events between animals and humans.

[2] Holsapple *et al.* (2006). Mode of action in relevance of rodent liver tumors to human cancer risk. Toxicol Sci. 89(1):51-56

[3] Elcombe *et al.* (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. Crit Rev Toxicol. 44(1): 64-82.

This step is not necessary since the human relevance of the proposed CAR/PXR MOA can be reasonably excluded on the basis of qualitative differences in Step 2.

Rat Thyroid Follicular Tumours
The slightly higher incidence of thyroid follicular tumours in high-dose male rats in the chronic rat study was not statistically significant and was considered to be only possibly related to treatment. In addition, only limited MOA data are available. Therefore, a formal assessment using the IPCS HRF has not been conducted. However, induction of hepatic T4-UDPGT was observed after 14 days of dosing with 3000 ppm silthiofam. Although thyroid hormone and TSH levels were not evaluated, induction of hepatic T4-UDPGT is a well-known mode of action leading to disruption of thyroid hormone homeostasis and ultimately the development of thyroid follicular tumours in rats. UDPGT is known to be regulated by CAR and PXR, and increases in rat thyroid follicular tumours are often observed in conjunction with liver tumours. Therefore, if treatment related, it is likely that the slightly increased incidence of thyroid follicular tumours in male rats was a result of a disruption in thyroid hormone homeostasis arising from induction of hepatic T4-UDPGT caused, at least in part, by CAR/PXR activation. The rat is known to be particularly sensitive to thyroid follicular tumours arising from disruption of thyroid hormone homeostasis, and this mode of action is generally believed to be of little to no relevance to humans. Therefore, the possibly treatment related increase in the incidence of thyroid follicular tumours in high-dose males rats in the chronic rat study with silthiofam is not considered to be relevant to humans.

Mouse Liver Tumours
A slight but statistically significant increase in benign liver tumours was noted in the high-dose (4000 ppm) female mice. However, these animals also exhibited a high incidence of individual hepatocellular necrosis. The liver tumours did not progress to malignancy and did not affect survival. No increase in tumours was seen at non-hepatotoxic dose levels. Therefore, the slight increase in liver tumours in high-dose female mice was considered to be secondary to a regenerative response to hepatotoxicity. Although no mode of action data is available in mice, it is possible that CAR and/or PXR activation may also have played a role. Thus, the slightly increased incidence of benign liver tumours in high-dose female mice in the chronic toxcity study is not considered to be relevant to humans.

4.10.1.2 Carcinogenicity: inhalation

No data.

4.10.1.3 Carcinogenicity: dermal

No data.

4.10.2 Human information

No data

4.10.3 Other relevant information

Supplementary studies on the active substance
The liver was the primary target organ following repeated oral administration of silthiofam to rats, mice and dogs. Increased liver weights were observed in all three species but liver

histopathology was observed only in rats and mice. The histopathological findings in the long-term rodent studies included an increase in liver tumours in male rats and a small but statistically significant ($p \leq 0.05$) increase in liver tumours in female mice. The incidences of the liver tumours in both male rats and female mice were above the upper limits of the available historical control data and thus the increases in both species were considered related to silthiofam administration. The increased incidence of liver tumours in high-dose (855 mg/kg bw/day) female mice was accompanied by significant hepatotoxicity, including a high incidence of individual cell necrosis. Therefore, these tumours were considered likely to be a result of a secondary regenerative response to cytotoxicity at high dose levels and thus of low toxicological significance. Hepatotoxicity was also noted in high-dose (150 mg/kg bw/day) male rats, and may be considered to account for the neoplastic response seen at this dose level. Therefore, three mechanistic studies have been conducted in order to investigate the potential modes of action for the hepatotoxic and potentially carcinogenic effects of silthiofam in male rats.

The results of these studies indicate that silthiofam activated the CAR and PXR nuclear receptors and caused an increase in hepatocellular proliferation in rodents both *in vivo* and *in vitro*. The increased hepatocellular proliferation was likely responsible for the increased incidence of liver tumours. Silthiofam also activated CAR and PXR in human hepatocytes. However, the response in human hepatocytes was less than that in rats and, most importantly, did not lead to cell proliferation. The results with silthiofam were very similar to those for phenobarbital, which has been shown to produce liver tumours in rodents *via* a predominantly CAR mediated mechanism and perhaps also include a PXR mediated mode of action, but does not cause tumours in humans. The newly available SD rat CAR/PXR double knockout model illustrated that the presence of functional CAR/PXR is required for increased CYP2B2, CYP3A1 mRNA levels and certain enzyme expression levels and the initial proliferative burst observed in rats following either PB or silthiofam treatment (Chatham, 2015a).

Accordingly, the apparently small increase in liver tumours in high-dose male rats is not considered to be relevant for human risk assessment.

The mechanistic studies conducted with silthiofam (table 39) are listed below.

Table 39. Mechanistic studies conducted with silthiofam.

Study	Reference
1. *In vivo* mode of action in CD rats	Haines C (2013). CXR-2012-0359 B.6.8.2.1
2. *In vitro* mode of action in rat hepatocytes	Elcombe B (2013). CXR-2012-0616 B.6.8.2.2
3. *In vitro* mode of action in human hepatocytes	Elcombe B (2013). CXR-2013-0132 B.6.8.2.3
4. Cytochrome P450 enzyme and DNA-synthesis induction in cultured male rat CARKO/PXRKO hepatocytes	Chatham L (2015a). CXR-2015-0230 B.6.8.2.4
5. *In vitro* mode of action in female human hepatocytes: Cytochrome P450 enzyme and DNA-synthesis induction in cultured human female hepatocytes	Chatham L (2015). CXR-2015-0229 B.6.8.2.5

4.10.3.1 Study 1: Mechanistic 7 & 14 day dietary study in rats

Elucidation of mode of action: A 7 & 14 day dietary study with silthiofam in male Sprague-Dawley CD rats. Haines, C. (2013). Report: CXR-2012-0359, MSL0024815. Non-guideline and non-GLP mechanistic study. However, study was conducted according to established SOPs and good laboratory practices.

Materials and methods:
Silthiofam, Lot number: GLP-1008-20873-T, purity: 99.7% was administered in the feed at concentrations of 0 and 3000 ppm for 7 days and 14 days (10 animals per group, 40 in total). The dietary concentration (3000 ppm) of silthiofam used in this study was the same as the highest concentration tested in the chronic rat study (DRAR B.6.5.2). Blood was collected from all animals at sacrifice and evaluated for serum enzyme markers of liver damage. The livers from all animals were weighed and evaluated for hepatocellular proliferation using BrdU immunohistochemistry. Histopathological and biochemical assessments were conducted on the livers from the 14-day exposure animals. The hepatic biochemical parameters evaluated included enzyme activity, gene expression and protein levels for several cytochrome P450s (CYP2B, CYP3A and CYP4A). Acyl CoA oxidase and UDPGT-glucuronosyl transferase (UDPGT) enzyme activities and ACOX gene expression were also evaluated. Calculated test substance consumption (mg/kg bw/day) for these dietary levels was 215 and 248 mg/kg body weight/day for the 7-day and 14-day exposure groups, respectively.

Results:
The test diets were found to be homogeneous and within 6% of the intended concentration. All animals survived until scheduled termination. No mortality or clinical signs of toxicity were noted in any of the animals.

Silthiofam-treated animals exhibited slightly decreased food consumption and body weight and/or weight gain, after 7 and 14 days of exposure (table 6.8.2.1-1, DRAR). Animals sacrificed after 14 days administration of silthiofam at 3000 ppm showed a 24 % decrease in bodyweight gain after week 1, and a 16 % decrease after week 2.

Increased liver weights (7-18%), small changes in liver histopathology, and substantial increases in hepatocellular proliferation (about 7-9 fold) were also evident. A minimal to slight centrilobular hepatocellular hypertrophy was noted in rats treated with silthiofam at 3000 ppm for 14 days, often associated with a decreased hepatocellular glycogen induced vacuolation and slightly increased lipid accumulation (fatty change). These alterations are considered to be related to the treatment. There were no toxicologically relevant changes in serum chemistry or serum enzymes (table 40).

PROD (marker of CYP2B activity, CAR), BROD (marker of CYP2B and CYP3A, CAR/PXR) and BQ (marker of CYP3A, PXR) activities were increased approximately 13×, 14× and 3× fold, respectively. A slight (1.5-fold) but statistically significant increase was observed for UDPGT. Decreased activity was noted for 12-OH LAH (marker of CYP4A1 activity, PPARα) and PCO activity (marker of PPARα), (table 41).

Hepatic CYP2B1, CYP2B2 and CYP3A1 mRNA levels were increased about 1200-fold, 62-fold, and 3-fold, respectively. No significant changes were noted in ACOX or CYP4A1 mRNA levels.

The Western blot results quite clearly illustrate that hepatic microsomal cyp2B and cyp3A proteins were markedly induced by silthiofam. The cyp2B1 protein is under constitutive control by CAR so there is always a certain baseline level of protein present (as seen in the controls for example). The inducibility of CAR by silthiofam is confirmed by the large increase in expression of cyp2B1 protein. In addition, the increased expression of cyp3A protein indicates the involvement of PXR in addition to CAR. CYP4A protein appeared to be decreased, which suggests that exposure to silthiofam does not lead to activation of the peroxisome proliferator-activated receptor alpha (PPARα). There is no data about the involvement of the aryl hydrocarbon receptor (AHR) in liver effects mediated by silthiofam exposure (figure 1).

Conclusions:
Administration of diets containing 3000 ppm silthiofam to rats for 14 days resulted in slightly decreased food consumption and body weights, slightly increased liver weights, minor changes in liver histopathology, and substantial increases in hepatocellular proliferation. Marked increases were noted in PROD and BROD enzyme activities, as well as CYP2B mRNA and protein expression, and are indicative of strong CAR activation. Increased BQ enzyme activity and CYP3A mRNA and protein expression were also observed, indicating that PXR was also activated. UDPGT activity (which is regulated by CAR, PXR and several other transcription factors) was also slightly increased, which may account for the possibly slightly increased incidence of thyroid tumours that was also observed in the chronic rat study. Acyl CoA oxidase and CYP4A were either unchanged or slightly decreased, as reflected by enzyme activities, mRNA expression and protein expression, indicating a lack of activation of PPARα.

These results strongly suggest that the mode of action for the hepatic effects of silthiofam, including the slightly increased incidences of liver tumours, is mainly *via* activation of CAR with small contributions through activation of PXR.

Table 40: Body weight, food consumption, liver weights, blood chemistry and hepatocellular proliferation in rats after 7 or 14 days of silthiofam administration

Parameter	7-Day Exposure Group		14-Day Exposure Group	
	Control	Silthiofam	Control	Silthiofam
Terminal Body Wt (g)	349	348 (100)[a]	406	381 (94)**
Weight Gain - Week 1(g)	42.8	37.8 (88)	45.1	34.1 (76)**
Weight Gain - Week 2 (g)	NA	NA	37.3	31.4 (84)
Food Consumption (g/kg/day)	84.6	72.5 (86)***	84.1	80.8 (96)
Food Consumption (g/rat/day)	29.6	25.2 (85)***	32.4	29.5 (91)**
Liver weight (g)	14.2	16.7 (117)**	16.6	17.8 (107)
Relative Liver Weight (%)	4.07	4.79 (118)***	4.10	4.67 (114)***
ALT (U/L)	72.0	61.4 (85)	69.6	61.4 (88)*
AST (U/L)	136	139 (102)	106	107 (101)
ALP (U/L)	153	145 (95)	172	147 (85)*
BrdU Labelling Index (%)	0.08	0.50 (657)***	0.04	0.35 (892)***

[a] Mean (% Control)
NA - not applicable
* p≤0.05; **p≤0.01; ***p≤0.001

Table 41. Hepatic enzyme activities and gene expression changes relative to controls in rats after 14 days of silthiofam administration

Parameter	Control	Silthiofam	Gene Ex. (fold change)
PROD (pmol/min/mg)	3.65	47.0 (1287)***	CYP2B1 (×1208 ***)
BROD (pmol/min/mg)	16.6	235.3 (1420)***	CYP2B2 (×62 ***)
BQ (nmol/min/mg)	0.73	2.09 (288)***	CYP3A1 (×3.3 ***)
12-OH LAH (nmol/min/mg)	4.51	2.29 (51)**	CYP4A1 (×1.6)
PCO (nmol/min/mg)	12.0	8.72 (73)***	ACOX (×1.2)
T4-UDPGT (pmol/min/mg)	1.42	2.08 (146)***	

[a] Mean (% Control)
**p≤0.01; ***p≤0.001

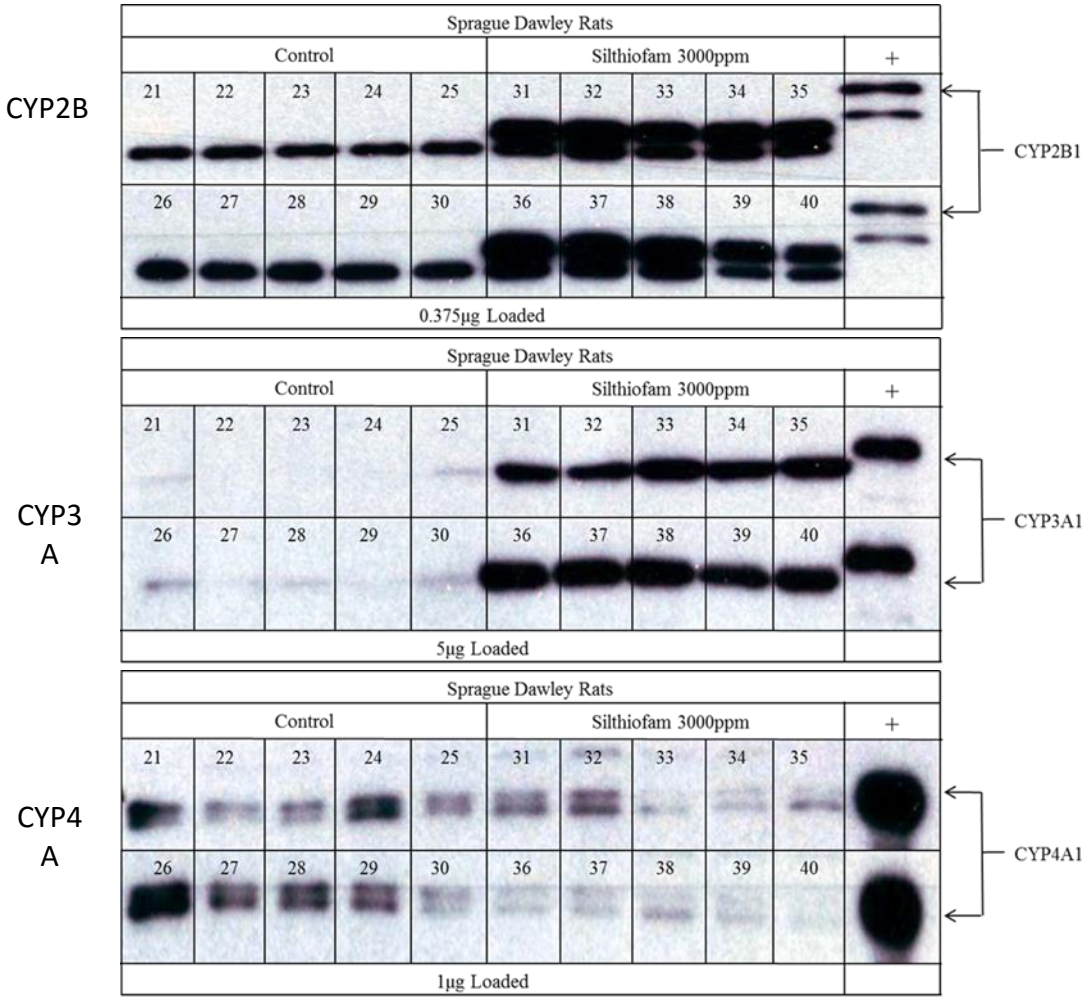


Figure 1: Hepatic protein expression of CYP2B, CYP3A and CYP4A in rats after 14 days of silthiofam administration

4.10.3.2 Study 2: *In vitro* mode of action in rat hepatocytes

Silthiofam - Cytochrome P450 enzyme and DNA-synthesis induction in cultured Sprague-Dawley CD rat hepatocytes. Elcombe, B. (2013). Report CXR-2012-0616, MSL0025452. Non guideline no-GLP mechanistic study.GLP. However, the study was conducted according to established SOPs and good research practices.

Materials and methods:
Silthiofam, Lot number: GLP-1008-20873-T, purity: 99.7% was used where male rat hepatocytes were exposed for 96 hours to 1 to 500 µM silthiofam, 10 or 1000 µM phenobarbital, or 25 ng/ml EGF (cell proliferation assay only).

Male Sprague-Dawley CD rats (Crl:CD(SD)), 7 weeks (approximately 250 g) on arrival, were terminally anaesthetised using Euthatal and hepatocytes isolated by *in situ* perfusion. Viabilities of the hepatocyte preparations, determined by trypan blue exclusion, were in excess of 80%.

A preliminary experiment was initially performed to select suitable silthiofam concentrations for the mode of action (MOA) study. For this preliminary study, cytotoxicity was measured as the change in cellular adenosine-5'-triphosphate (ATP) released from viable hepatocytes after 96 hours in culture. The hepatocytes were exposed to the test item at 9 concentrations (0.03, 0.1, 0.3, 1, 3, 10, 30, 100 and 500 µM silthiofam) and included vehicle control (0.1% v/v dimethyl sulfoxide). Dose levels of 30, 100, 300 and 500µM silthiofam were chosen to progress into the main study.

In the main study, rat hepatocytes were cultured with medium containing either phenobarbital sodium salt at 2 concentrations (10 and 1000 µM), silthiofam at one of 4 concentrations (30, 100, 300 and 500 µM, with an expanded range for investigating PROD dose responses at 1, 3, 10, 30, 100 and 300 µM), EGF (25 ng/mL) or vehicle (0.1% v/v DMSO) alone. The total duration of exposure to the test substances was 96 h, with daily medium changes. There were 3 replicates for each concentration in 25 cm^2 flasks for PROD, BROD and BQ analyses, 5 replicates for each concentration in 6-well plates for replicative DNA synthesis (proliferation) analyses and 6 replicates for each concentration in 96-well plates for ATP measurements. Rat hepatocytes were evaluated for the following:

- Cytotoxicity was measured as the change in intracellular adenosine-5'-triphosphate (ATP) concentration.
- Levels of rat cyp2B activity were determined spectrofluorometrically by the formation of resorufin from pentoxyresorufin (PROD) and benzyloxyresorufin (BROD).
- Levels of rat cyp3A activity were determined spectrofluorometrically by the formation of 7-hydroxyquinoline from benzyloxyquinoline (BQ).
- The number of hepatocytes undergoing replicative DNA synthesis (S-phase of the cell cycle) was determined immunocytochemically following the incorporation of 5-bromo-2'-deoxyuridine (BrdU, 10 µM) into hepatocyte nuclei over the last 72 h of culture. EGF (25 ng/mL, n=5) was used as a positive control.
- RNA was extracted from cultured hepatocytes and cDNA synthesised from all available RNA samples. TaqMan analysis was performed using primers specific for CYP2B1, CYP2B2 and CYP3A1. Rat β-actin was used as the internal standard. Data was analysed by generation of CT and delta CT values for all genes.

Results:

Cytotoxicity

Substantially decreased intracellular ATP levels were noted at 500 µM silthiofam in the preliminary experiment (data not shown) and at both 300 and 500 µM silthiofam in the main experiment (figure 2). The morphological effects at 500 µM were excessive and replicative DNA synthesis could not be measured. Cytotoxicity was also observed at 300 µM and, to a lesser extent, at 100 µM silthiofam in the PROD expanded range experiment (table 42 and table 43).

Table 42. Biochemical and mRNA results in rat hepatocytes from the Main Experiment

	DMSO	Phenobarbital		Silthiofam				EGF 25 ng/mL
		10 µM	1000 µM	30 µM	100 µM	300 µM	500 µM	
ATP (units released)	93259 (100)[a]	88876 (95.3)	97343 (104)	95613 (103)	84549 (91)	9127*** (10)	9104*** (10)	-
S-phase labelling index (%)	9.27 (100)	14.90*** (161)	16.56*** (179)	14.04*** (151)	16.20*** (175)	11.72** (126)	-	46.32*** (499)
PROD (pmol/min/mg)	0.124 (100)	0.122 (99)	0.377** (305)	0.241 (194)	0.109 (88)	0.208* (168)	0.172** (139)	-
BROD (pmol/min/mg)	0.420 (100)	0.621** (148)	2.077*** (495)	0.878*** (209)	0.921*** (219)	0.582* (139)	0.365 (87)	-
BQ (nmol/min/mg)	0.034 (100)	0.037 (108)	0.125*** (367)	0.068*** (199)	0.170*** (498)	0.063** (187)	0.021*** (61)	-
CYP2B1 mRNA (fold change)[b]	1	4	36*	10***	13*	3	3***	-
CYP2B2 mRNA (fold change)	1	2*	25	8*	11**	6	3	-
CYP3A1 mRNA (fold change)	1	1	34*	8***	94***	97**	86***	-

[a] Values in parenthesis represent % of DMSO vehicle control value

[b] Fold change for gene expression (mRNA) results were calculated by the formula $2^{-\Delta\Delta Ct}$, where ΔΔCt=ΔCt values from the target *minus* ΔCt value for the first control sample, and ΔCt=Ct values from the gene of interest minus Ct values for the internal control gene (β-actin)

* p≤0.05; **p≤0.01; ***p≤0.001

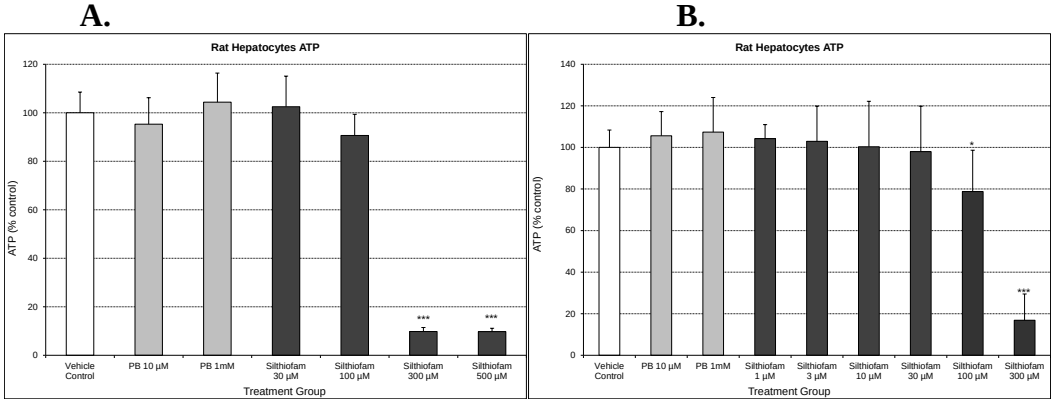
Table 43. Biochemical and mRNA results in rat hepatocytes from the expanded PROD experiment

	DMSO	Phenobarbital		Silthiofam					
		10 µM	1000 uM	1 µM	3 µM	10 µM	30 µM	100 µM	300 uM
ATP (units released)	89248 (100)	94226 (106)	95811 (107)	93055 (104)	91860 (103)	89537 (100)	87445 (98)	70314* (79)	15054*** (17)

PROD (pmol/min/mg)	0.220 (100)	0.400* (181)	0.858*** (389)	0.143 (65)	0.200 (91)	0.226 (102)	0.425** (193)	0.373* (169)	0.330* (150)

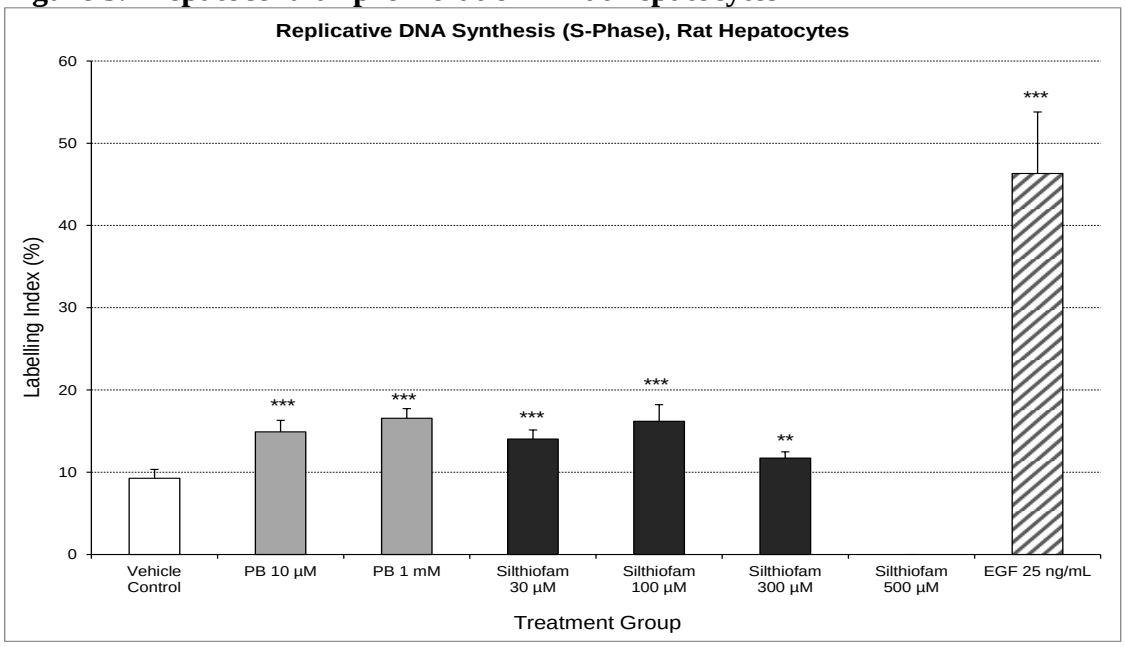
[a] Values in parenthesis represent % of DMSO vehicle control value
* p≤0.05; **p≤0.01; ***p≤0.001

Figure 2. Adenosine 5'-Triphosphate (ATP) release, (A) Main & (B) PROD expanded range experiments in rat hepatocytes



Hepatocellular Proliferation:
Statistically significant increases in hepatocellular proliferation (~1.5 to 1.8-fold relative to vehicle control) were observed with 10 and 1000 µM phenobarbital and with 30 and 100 µM silthiofam (table 42 and figure 3). A slightly lower (~1.3 fold) increase was noted at 300 µM silthiofam, probably due to the cytotoxicity noted at this concentration. Treatment with EGF resulted in a 5-fold increase in replicative DNA synthesis, demonstrating the suitability of this system for assessing cell proliferation.

Figure 3. Hepatocellular proliferation in rat hepatocytes

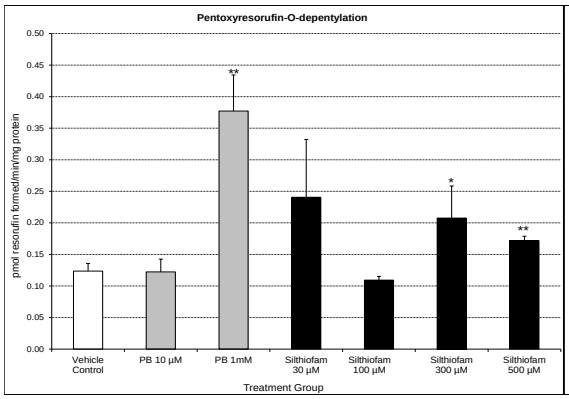


Cytochrome P450 Enzyme Activity:
Statistically significant increases in PROD (up to 1.9-fold relative to vehicle control), BROD (up to 2.2-fold) and BQ (up to 5-fold) were noted at 30, 100 and/or 300 µM silthiofam (*tables*

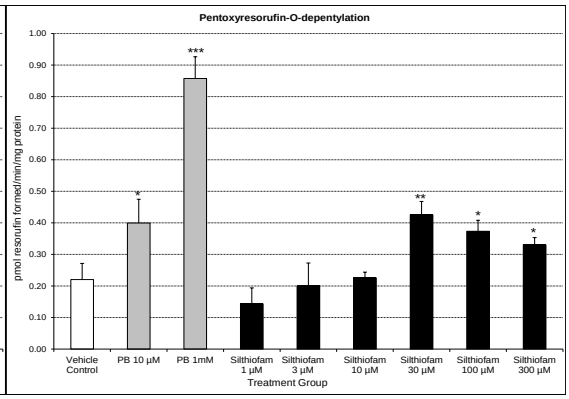
42 and 43). Statistically significant increases in PROD (up to 3.9-fold), BROD (up to 4.9-fold) and BQ (up to 3.7-fold) were also seen at 1000 µM phenobarbital. BROD was also significantly increased (~1.5-fold) at 10 µM phenobarbital (figure 4).

Figure 4. Pentoxyresorufin-O-dependentylation (PROD) activity, (A) Main & (B) PROD expanded range experiments in rat hepatocytes; (C) Benzyloxyresorufin-O-debenzylation (BROD) & (D) Benzyloxyquinoline-O-debenzylation (BQ) activity in rat hepatocytes.

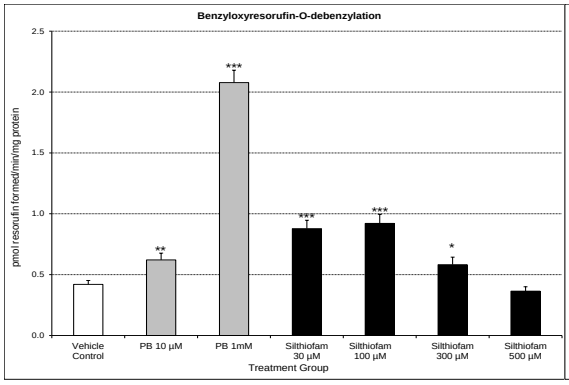
A



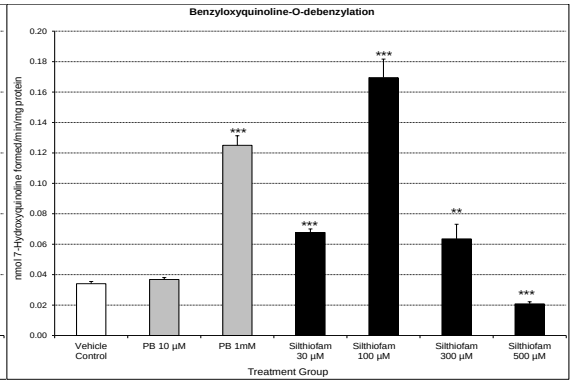
B



C.



D



Gene Expression:
Statistically significant increases in CYP2B1, CYP2B2 and CYP3A1 mRNA expression (up to 13-, 11- and 97-fold, respectively, relative to vehicle control) were noted in rat hepatocytes treated with 30, 100 and/or 300 µM silthiofam. Statistically significant increases in CYP2B1, CYP2B2 and CYP3A1 mRNA expression (up to 36-, 25- and 34-fold, respectively) were also noted with 10 and/or 1000 µM phenobarbital (figure 5).

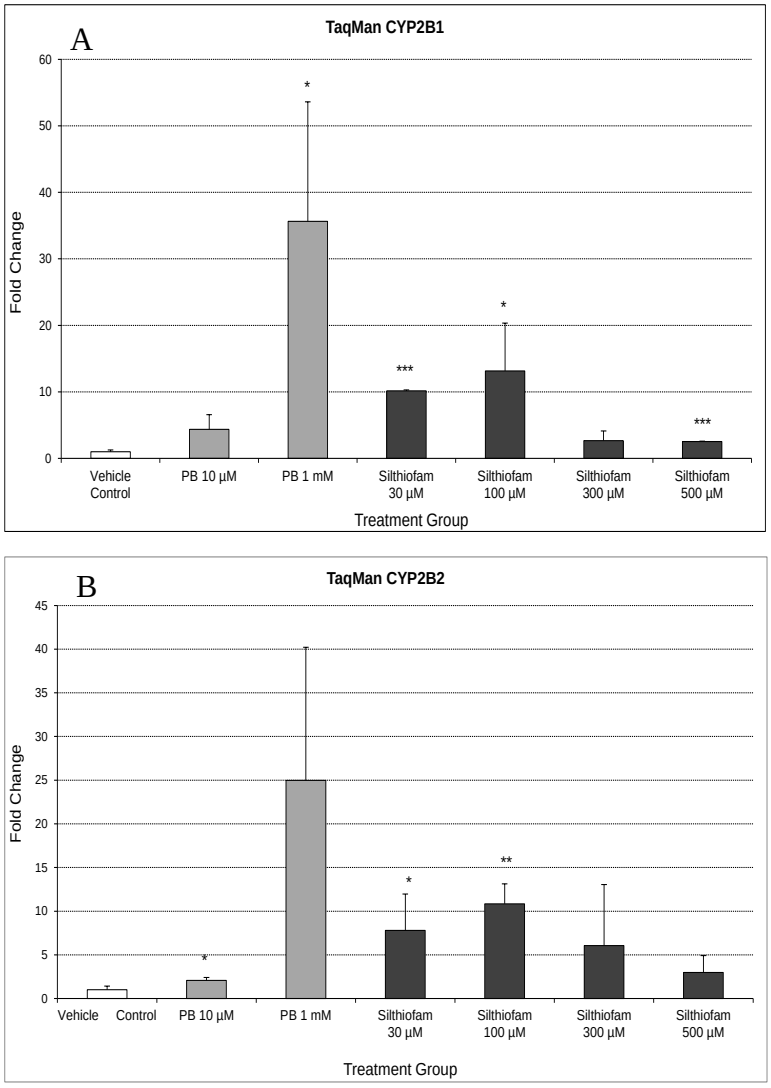
Conclusions
The ATP cytotoxicity observed following treatment with 300 and 500 µM demonstrated that silthiofam had been tested to a suitably high concentration.

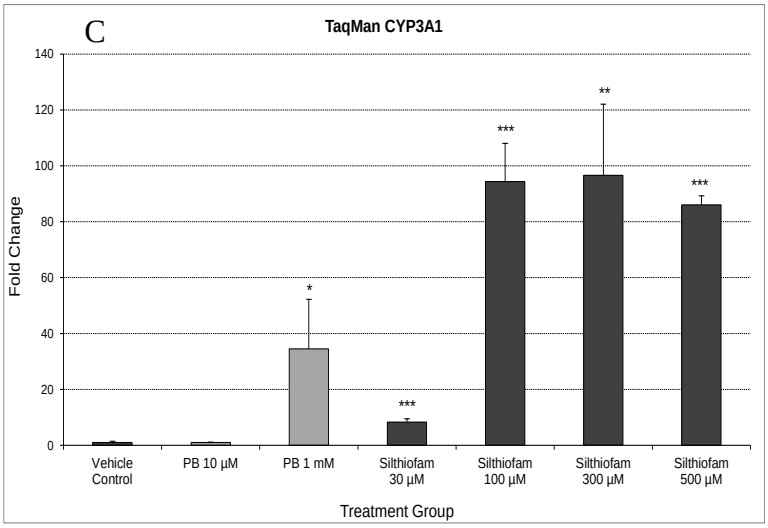
Exposure of rat hepatocytes to silthiofam or phenobarb resulted in similar increases in hepatocellular proliferation.

Both silthiofam and phenobarbital were inducers of PROD, BROD and BQ – treatment with silthiofam resulting in increases in PROD, BROD and BQ of up to 1.9-, 2.2- and 5-fold control,

respectively, and treatment with PB resulting in increases in PROD, BROD and BQ of up to 3.9-, 4.9- and 3.7-fold control, respectively. In the rat, PROD and BROD are primarily CYP2B-mediated reactions, while BQ is primarily a CYP3A-mediated reaction. However, there is not total specificity (e.g. BROD is also mediated partly by CYP3A and BQ may also be mediated partly by CYP2B), induction of CYP2B is linked primarily to CAR activation while induction of CYP3A is linked primarily to PXR activation.

Figure 5: (A) CYP2B1, (B) CYP2B2 and (C) CYP3A1 mRNA expression in rat hepatocytes.





These data indicate that silthiofam is a more potent CYP3A-inducer than CYP2B-inducer. This was confirmed by the levels of mRNA expression seen following exposure to silthiofam. Silthiofam is an activator of both CAR and PXR in isolated rat hepatocytes with PXR activation being predominant.

4.10.3.3 Study 3: *In vitro* mode of action in human hepatocytes

Silthiofam - Cytochrome P450 enzyme and DNA-synthesis induction in cultured human hepatocytes. Elcombe, B. (2013). Report CXR-2013-0132, MSL0025453. Non guideline, non-GLP study mechanistic study. However, study was conducted according to established SOPs and good research practices.

Materials and methods:
Silthiofam, Lot number: GLP-1008-20873-T, purity: 99.7% was used where human hepatocytes were exposed for 96 hours to 1 to 300 µM silthiofam, 10, 100 or 1000 µM phenobarbital, or 25 ng/ml EGF (cell proliferation assay only).

Primary male human hepatocytes, plateable cryopreserved, were sourced from Invitrogen, 7 Kingsland Grange, Warrington, Cheshire, WA1 4SR. Viabilities of the hepatocyte preparation, determined by trypan blue exclusion, were in excess of 70%. Hepatocytes from one donor were used.

A preliminary experiment was initially performed to select suitable silthiofam concentrations for the mode of action (MOA) study. For this preliminary study, cytotoxicity was measured as the change in cellular adenosine-5'-triphosphate (ATP) released from viable hepatocytes after 96 hours in culture. The hepatocytes were exposed to the test item at 9 concentrations (0.03, 0.1, 0.3, 1, 3, 10, 30, 100 and 300 µM silthiofam) and included vehicle control (0.1% v/v dimethyl sulfoxide). Dose levels of 1, 3, 10, 30, 100 and 300µM silthiofam were chosen to progress into the main study.

In the main study, human hepatocytes were cultured with medium containing either phenobarbital sodium salt at 3 concentrations (10, 100 and 1000 µM), silthiofam at one of 6

concentrations (1, 3, 10, 30, 100 and 300 µM), EGF (25 ng/mL) or vehicle (0.1% v/v DMSO) alone. The total duration of exposure to the test substances was 96 h, with daily medium changes. There were 3 replicates for each concentration in 25 cm2 flasks for PROD, BROD and BQ analyses, 5 replicates for each concentration in 6-well plates for replicative DNA synthesis (proliferation) analyses and 6 replicates for each concentration in 96-well plates for ATP measurements. Human hepatocytes were evaluated for the following:

- Cytotoxicity was measured as the change in intracellular adenosine-5'-triphosphate (ATP) concentration.
- Levels of human cyp2B activity were determined spectrofluorometrically by the formation of resorufin from pentoxyresorufin (PROD) and benzyloxyresorufin (BROD). In contrast to the rat, BROD is a better marker for CYP2B induction in humans than PROD.
- Levels of human cyp3A activity were determined spectrofluorometrically by the formation of 7-hydroxyquinoline from benzyloxyquinoline (BQ).
- The number of hepatocytes undergoing replicative DNA synthesis (S-phase of the cell cycle) was determined immunocytochemically following the incorporation of 5-bromo-2'-deoxyuridine (BrdU, 10 µM) into hepatocyte nuclei over the last 72 h of culture. EGF (25 ng/mL, n=5) was used as a positive control.
- RNA was extracted from cultured hepatocytes and cDNA synthesised from all available RNA samples. TaqMan analysis was performed using primers specific for CYP2B6 and CYP3A4. Human β-actin was used as the internal standard. Data was analysed by generation of CT and delta CT values for all genes.

Results:

Cytotoxicity:

Substantially decreased intracellular ATP levels were noted at 100 and 300 µM silthiofam in the preliminary experiment (data not shown) and in the main experiment with levels being reduced to 78.4% and 22.0% of control, respectively (figure 6). The morphological effects at 300 µM were excessive and replicative DNA synthesis and PROD activity could not be measured (table 44).

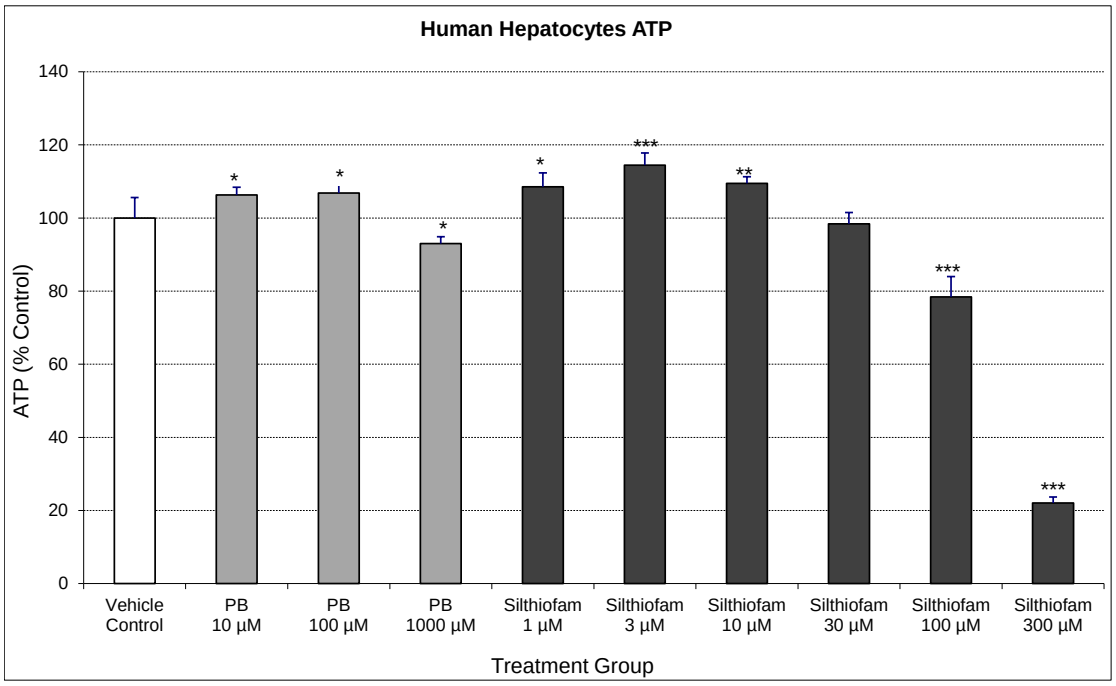
Table 44. Biochemical and mRNA results in human hepatocytes

	DMSO	Phenobarbital			Silthiofam						EGF 25 ng/mL
		10 µM	100 µM	1000 uM	1 µM	3 µM	10 µM	30 µM	100 µM	300 uM	
ATP (units released) (%)	103437 (100) [a]	109946* (106)	110508* (107)	96177* (93)	112242* (109)	118380*** (114)	113241** (109)	101809 (98)	81132*** (78)	22802*** (22)	-
S-phase labelling index (%)	0.38 (100)	0.37 (96)	0.35 (92)	0.35 (92)	0.40 (104)	0.30 (79)	0.40 (103)	0.37 (97)	0.34 (88)	[b]--	4.12*** (1073)
PROD (pmol/min/mg)	0.188 (100)	0.163 (87)	0.184 (98)	0.331** (176)	0.168 (89)	0.148 (79)	0.184 (98)	0.190 (101)	0.118* (63)	[b]--	-

BROD (pmol/min/mg)	0.524 (100)	0.634 (121)	1.352*** (258)	4.186*** (798)	1.003*** (191)	1.251** (239)	1.474*** (281)	0.922*** (176)	0.633 (121)	0.212** (40)	-
BQ (nmol/min/mg)	0.146 (100)	0.153 (105)	0.349*** (238)	0.790** (540)	0.174 (119)	0.195* (133)	0.253*** (173)	0.268*** (183)	0.045*** (31)	0.009*** (6)	-
CYP2B6 mRNA (fold change)[c]	1	1.6	4.6	8.6**	1.7	2.8	4.8*	4.2***	3.4***	1	-
CYP3A4 mRNA (fold change)	1	1.7	5.3***	9.4**	1.1	1.9**	2.8**	4.3***	2.9***	0.4*	-

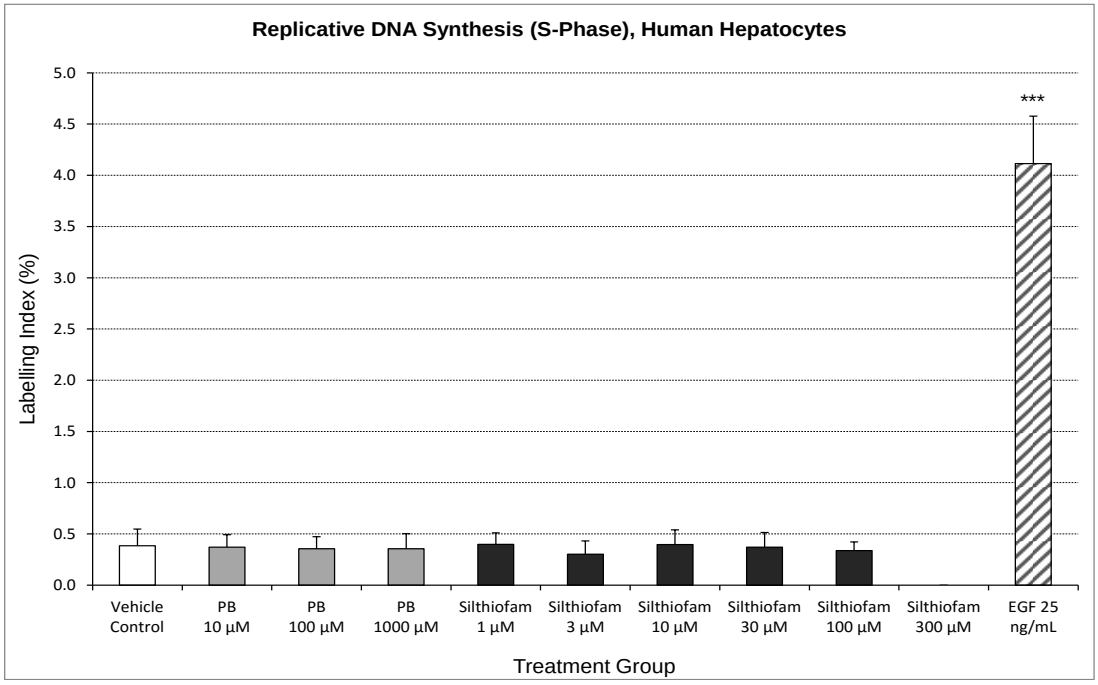
[a] Values in parenthesis represent % of DMSO vehicle control value
[b] Analysis unable to be performed due to excessive cytotoxicity
[c] Fold change for gene expression (mRNA) results were calculated by the formula $2^{-\Delta\Delta Ct}$, where ΔΔCt=ΔCt values from the target *minus* ΔCt value for the first control sample, and ΔCt=Ct values from the gene of interest minus Ct values for the internal control gene (β-actin)
* p≤0.05; **p≤0.01; ***p≤0.001

Figure 6: Silthiofam cytotoxicity: Adenosine 5'-Triphosphate (ATP) release in human hepatocytes



Hepatocellular Proliferation:
No statistically significant increases in cell proliferation were observed (table 46 and figure 7) following treatment with either phenobarbital (10 to 1000 µM) or silthiofam (1 to 100 µM). Treatment with EGF resulted in an 11-fold increase in replicative DNA synthesis, demonstrating the suitability of this system for assessing cell proliferation. Note the Y-axis (figure 8) is about 10-fold less than that used for the rat hepatocyte data. This illustrates the low proliferative potential of human hepatocytes relative to rat hepatocytes when treated with PB, silthiofam and EGF.

Figure 7. Hepatocellular proliferation in human hepatocytes. Note the Y-axis is about 10-fold less than that used for the rat hepatocyte data.



Cytochrome P450 Enzyme Activity:
No increases in PROD activity were noted at any concentration of silthiofam (figure 9). Treatment with 100 µM silthiofam resulted in a statistically significant decrease in PROD activity to 62.7% of the control value, whilst treatment with 300 µM silthiofam resulted in cytotoxicity precluding PROD measurement. The response to PB treatment was similar in magnitude to that seen in rat hepatocytes. PROD activity is not increased with silthiofam treatment.

Statistically significant increases in BROD (up to 2.8-fold over the control value) activity were noted with increasing silthiofam concentrations up to 10 µM. Higher concentrations attenuated the response. 300 µM silthiofam decreased BROD activity to 40% of the control value indicating toxicity at this dose level. The response to PB treatment at the highest dose was about 2-fold greater to that seen in rat hepatocytes. Treatment with 100 and 1000 µM PB resulted in a dose-dependent, statistically significant increase in BROD activity to a maximum 8.0-fold over control values.

A weak BQ response (a marker for CYP3A) and increase in enzyme activity is also noted (up to 1.8-fold) with silthiofam concentrations of 1, 3, 10 and 30 µM. Treatment with 100 and 1000 µM PB resulted in dose-dependent, statistically significant increases in BQ activity to a maximum 5.4-fold control value. Rat hepatocytes however show a much greater response (up to 5-fold over controls) that even exceeds the response to the highest dose of PB.

Gene Expression:
Statistically significant increases in mRNA expression of CYP2B6 and CYP3A4 (up to 4.8-fold and 4.3-fold, respectively) were noted in human hepatocytes treated with silthiofam (figure 10). Larger increases in mRNA expression of CYP2B6 and CYP3A4 (up to 8.6-fold and 9.4-fold, respectively) were observed after treatment with 100 or 1000 µM phenobarbital.

The highest dose of 300 μM silthiofam had no effect on CYP2B6 expression but did decrease CYP3A4 mRNA expression to 35% of the control value.

The magnitude of the responses (relative to controls) to silthiofam and phenobarbital is much less in human hepatocytes when compared to the responses observed for rat hepatocytes. Rat hepatocytes in particular, have a very marked CYP3A1 response to silthiofam but have lower BQ specific activities than those found in human hepatocytes.

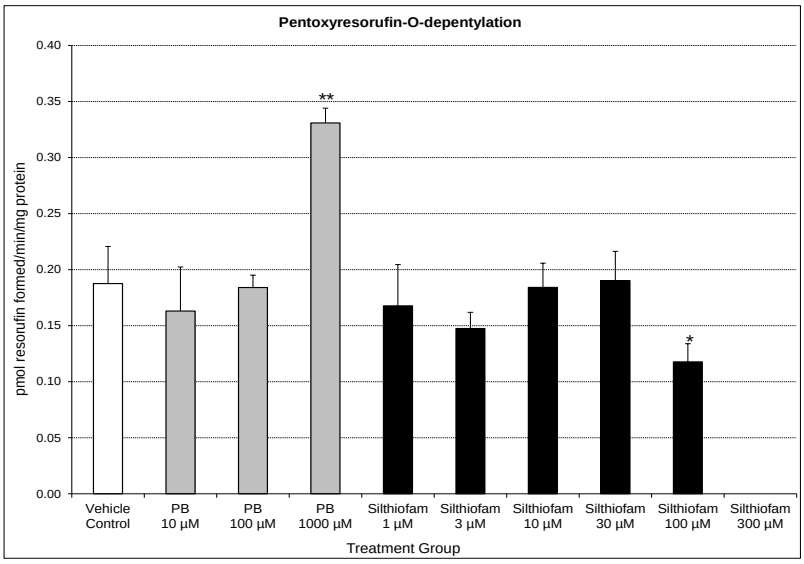
Conclusions:
The ATP cytotoxicity observed following treatment with 100 and 300 μM demonstrated that silthiofam had been tested to a suitably high concentration. As a result of significant cytotoxicity observed following treatment with 300 μM silthiofam, replicative DNA synthesis and PROD activity could not be measured at this concentration.

PB is an inducer of CYP2B and CYP3A. At the mRNA expression level, PB induced increases in CYP2B6 and CYP3A4 of up to 8.6- and 9.4-fold control, respectively. Treatment of cultured human hepatocytes with silthiofam resulted in weak induction of CYP2B and CYP3A at both the protein enzyme activity level and the gene expression level.
There was no evidence of PB- or silthiofam-stimulated DNA synthesis in cultured human hepatocytes. Exposure to 25ng/mL EGF resulted in a statistically significant increase in replicative DNA synthesis to 11-fold control values indicating that human hepatocytes could proliferate following exposure to proliferative stimuli.

In conclusion, silthiofam is a weak activator of CAR and PXR (as shown by the effects on CYP2B and CYP3A respectively) with no compound-stimulated DNA synthesis in human hepatocytes.

Figure 8. (A) PROD activity, (B) BROD and (C) BQ activity in human hepatocytes.

A.



B.

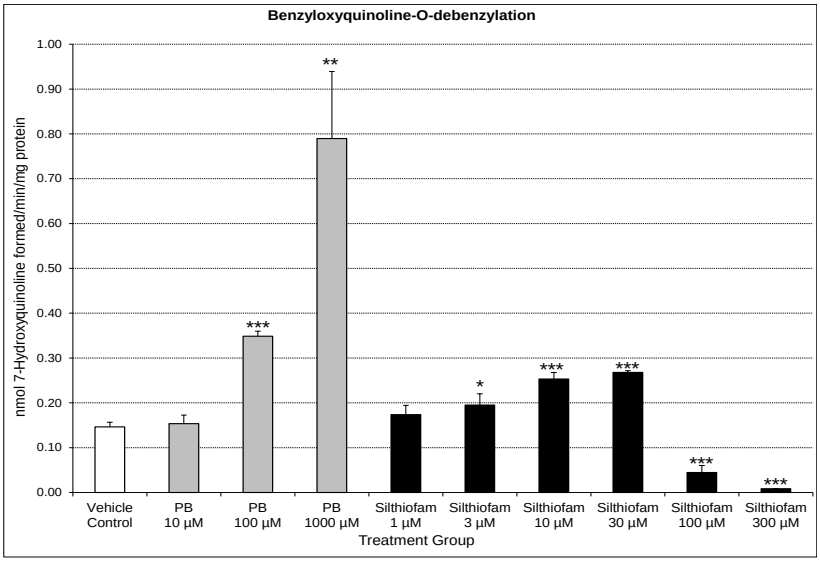
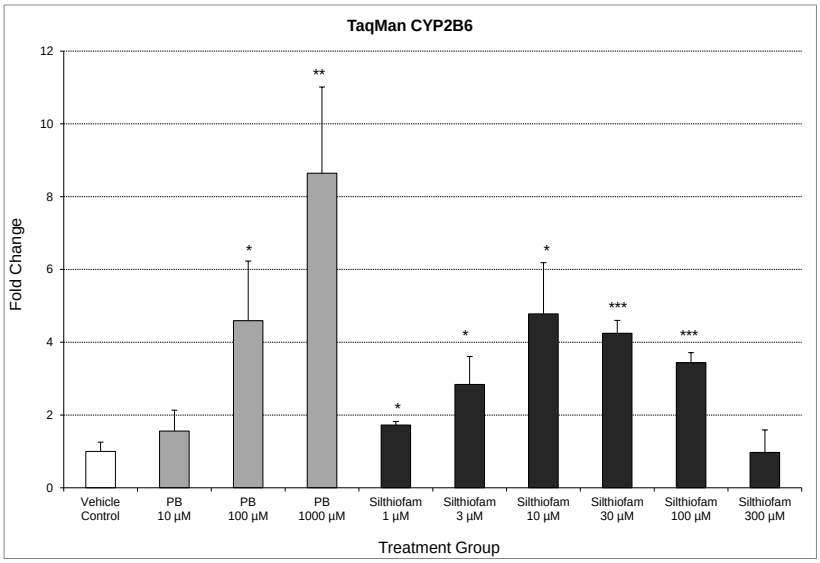
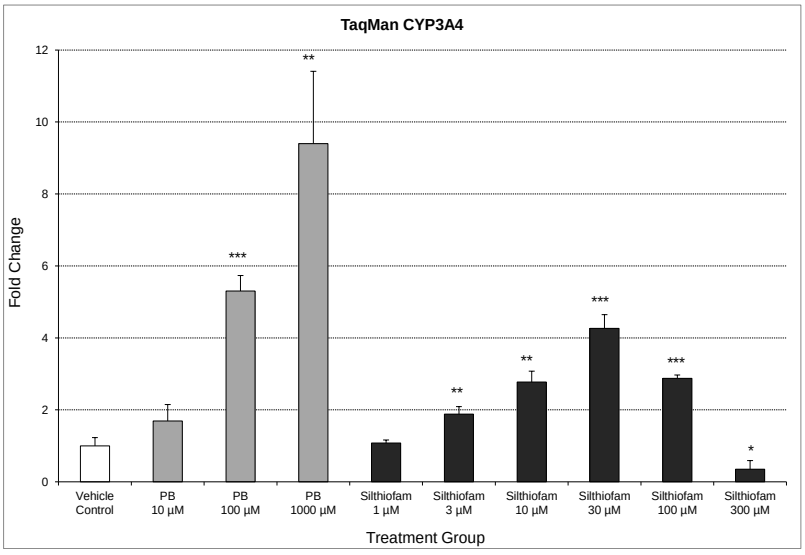


Figure 9. (A) CYP2B6 and, (B) CYP3A4 mRNA expression in human hepatocytes.

A.



B.



4.10.3.4 Study 4: Enzyme induction in cultured male rat CARKO/PXRKO hepatocytes

Reference: Chatham, L. (2015a): Silthiofam - Cytochrome P450 enzyme an DNA-synthesis induction in cultured male rat CARKO/PXRKO hepatocytes.
Study / Report: CXR-2015-0230, MSL0027211
Guidelines: Non guideline, mechanistic study.
GLP: No. However, study was conducted according to established SOPs and good research practices.

Executive summary:
Silthiofam has previously been shown to activate the Constitutive Androstane Receptor (CAR) and Pregnane X Receptor (PXR) nuclear receptors and stimulate hepatocellular proliferation in male Sprague-Dawley rats both *in vivo* and *in vitro*. The objective of this study was to confirm that activation of CAR and/or PXR was a key event in the proliferative response by investigating the ability of silthiofam to induce cell proliferation in cultured hepatocytes from male CAR/PXR double-knockout rats. CAR and PXR activation were assessed by cytochrome P450 (CYP) 2B and CYP3A enzyme activity, respectively, along with mRNA expression. Phenobarbital sodium salt (PB) and epidermal growth factor (EGF) were included as controls.

Exposure to silthiofam resulted in a slight increase in gene expression for CYP2B1 but no increase in CYP2B2 or CYP3A gene expression or PROD, BROD or BQ enzyme activity. Most importantly, there was no increase in cell proliferation. This is in contrast to the significant increase in cell proliferation previously reported following silthiofam treatment in wild-type rat hepatocytes. Increased cell proliferation was observed following treatment with EGF but not with phenobarbital. Thus, the results from this study provide strong evidence that the mode of action for silthiofam-induced proliferation in rat hepatocytes is CAR and/or PXR-mediated.

Materials and methods:
Silthiofam, Lot number: GLP-1502-23805-T, purity: 100% and Sodium Phenobarbital, Lot number: 080M1276V, purity: 100% were added to medium used to culture isolated hepatocytes for 96 hours. Hepatocytes were isolated by *in situ* perfusion according to the method of *Mitchell et al. (1984)*[4]. Hepatocytes from one animal were used for the range-finding study while hepatocytes from two animals were pooled for the main study. Cell viability was acceptable (>80%). No control rats (wild-type) were used, only double knock out animals. CARKO/PXRKO rats do not produce functional CAR or PXR. Isolated hepatocytes were cultured with medium containing the appropriate test or control substance for 96 hours, with medium changed daily. Hepatocytes were exposed to either 0.1% (v/v) DMSO, sodium phenobarbital (100 or 1000 µM) or one of seven concentrations of silthiofam (1, 3, 10, 30, 100, 300 and 500 µM). Epidermal growth factor (EGF; 25 ng/mL) was included as a positive control for the replicative DNA synthesis assay. There were 3 replicates for each concentration in 25 cm^2 flasks for PROD, BROD and BQ analyses, 5 replicates for each concentration in 6-well plates for replicative DNA synthesis (proliferation) analyses and 6 replicates for each concentration in 96-well plates for ATP measurements.

[4] *Mitchell et al. (1984).* Factors influencing peroxisome proliferation in cultured rat hepatocytes. Arch. Toxicol. 55:239-246

Several assay systems were employed in this study. (i) Cell viability was assessed using a bioluminescence assay to determine the amount of adenosine 5' triphosphate (ATP) released from the hepatocytes. (ii) cell proliferation was assessed by exposing the hepatocytes to bromodeoxyuridine (BrdU, 10 µM) and measuring the BrdU incorporation into nuclear DNA over the last 72 hours of culture. The results were reported as a labelling index representing the percentage of hepatocytes that were replicating and thus incorporated BrdU into their DNA. Approximately 1500 hepatocytes were counted per replicate for each treatment. (iii) CYP2B and CYP3A activities were assessed by measuring cytochrome P450-dependent O-dealkylation of 7 pentoxyresorufin, 7 benzyl-oxyresorufin and 7 benzyloxyquinoline (PROD, BROD and BQ, respectively). (iv) Gene expression was assessed by measuring CYP2B1, CYP2B2 and CYP3A mRNA levels using quantitative RT-PCR (TaqMan®). Rat β-actin was used as the internal standard.

Results:
(i) Cell viability - substantial cytotoxicity (almost 80% reduction in ATP relative to control) was observed at both 300 and 500 µM silthiofam (table 45 and figure 10). There was no cytotoxicity associated with lower levels of silthiofam or with the two tested concentrations of sodium phenobarbital.

(ii) Hepatocellular Proliferation: - Cell proliferation rates (replicative DNA synthesis) were comparable in the vehicle control, phenobarbital and silthiofam-treated groups (the 300 µM silthiofam group could not be evaluated due to excessive toxicity) see figure 11. Treatment with EGF resulted in a 4-fold increase in replicative DNA synthesis, demonstrating the suitability of this system for assessing cell proliferation.

No increase in cell proliferation was observed following treatment of CARKO/PXRKO rat hepatocytes with silthiofam or phenobarbital (figure 11). This is in contrast to the significant increases in cell proliferation previously reported for both compounds in wild-type rat hepatocytes.

(iii) Cytochrome P450 Enzyme Activity: - PROD, BROD and BQ activities in all silthiofam-treated groups were comparable to or lower than control values (figure 12). PROD, BROD and BQ activities were comparable to control in the 100 µM phenobarbital group but were slightly increased relative to control in the 1000 µM phenobarbital group.

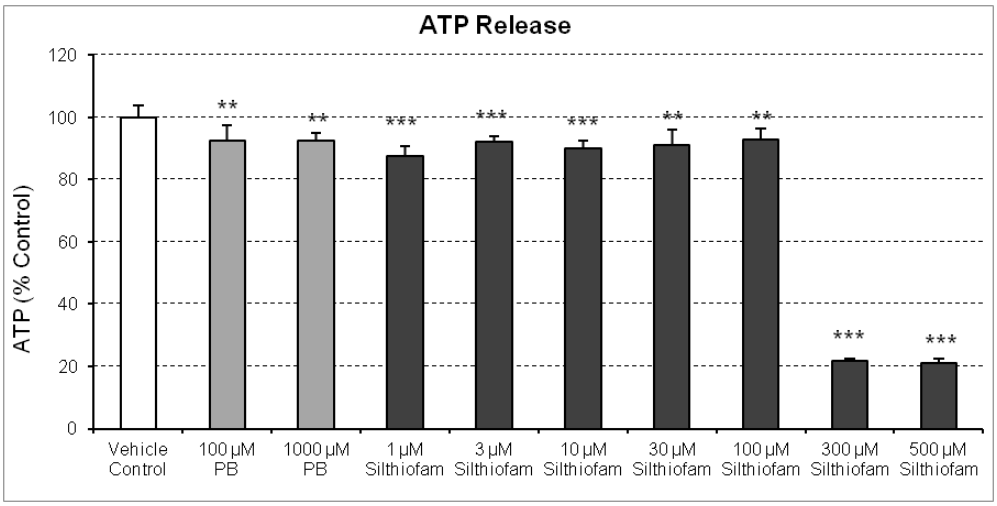
(iv) Gene Expression: - CYP2B2 and CYP3A1 mRNA expression were either comparable to vehicle control or substantially decreased following treatment with silthiofam (figure 13). However, statistically significant increases in mRNA expression (up to 5-fold relative to vehicle control) were observed for CYP2B1. No meaningful changes in mRNA expression were observed following treatment with phenobarbital.

Table 45: Biochemical and mRNA results in rat CARKO/PXRKO hepatocytes.

	DMSO	Phenobarb		Silthiofam							EGF
		100 µM	1000 µM	1 µM	3 µM	10 µM	30 µM	100 µM	300 µM	500 µM	
ATP (units released)	327138 (100)[a]	302337** (92.4)	302379** (92.4)	286293*** (87.5)	301665*** (92.2)	294675*** (90.1)	297909** (91.1)	303649** (92.8)	71488*** (21.9)	68744*** (21.0)	-
S-phase labelling index (%)	11.54 (100)	11.32 (98.1)	11.06 (95.8)	-	-	11.73 (101.7)	12.14 (105.2)	10.63 (92.1)	[b]	[b]	50.15*** (434.6)
PROD (pmol/min/mg)	0.313 (100)	0.297 (94.9)	0.538* (172.1)	-	-	0.320 (102.4)	0.270 (86.3)	0.210* (67.3)	0.201* (64.3)	[b]	-
BROD (pmol/min/mg)	0.958 (100)	1.030 (107.5)	2.424** (252.9)	-	-	0.953 (99.4)	0.861 (89.9)	0.670 (69.9)	0.782 (81.6)	[b]	-
BQ (nmol/min/mg)	0.072 (100)	0.072 (99.4)	0.116** (160.5)	-	-	0.075 (104.5)	0.074 (103.1)	0.049* (68.5)	0.022*** (30.9)	[b]	-
CYP2B1 mRNA (fold change) [c]	1.00	0.38**	0.63	-	-	0.96	1.07	2.54***	5.32***	[b]	-
CYP2B2 mRNA (fold change) [c]	1.00	2.09	0.58	-	-	1.04	0.61*	0.09***	0.04***	[b]	-
CYP3A1 mRNA (fold change) [c]	1.00	2.25	1.10	-	-	1.26	0.85	0.30***	0.08***	[b]	-

[a] Values in parenthesis represent % of DMSO vehicle control value
[b] Analysis not possible due to excessive cytotoxicity
[c.] Fold change for gene expression (mRNA) results were calculated by the formula $2^{-\Delta\Delta Ct}$, where ΔΔCt=ΔCt values from the target *minus* ΔCt value for the first control sample, and ΔCt=Ct values from the gene of interest minus Ct values for the internal control gene (β-actin).
* p≤0.05; **p≤0.01; ***p≤0.001

Figure 10: Adenosine 5'-Triphosphate (ATP) release from cultured rat CARKO/PXRKO hepatocytes.



* p≤0.05; **p≤0.01; ***p≤0.001

Figure 11: Hepatocellular proliferation in rat CARKO/PXRKO hepatocytes.

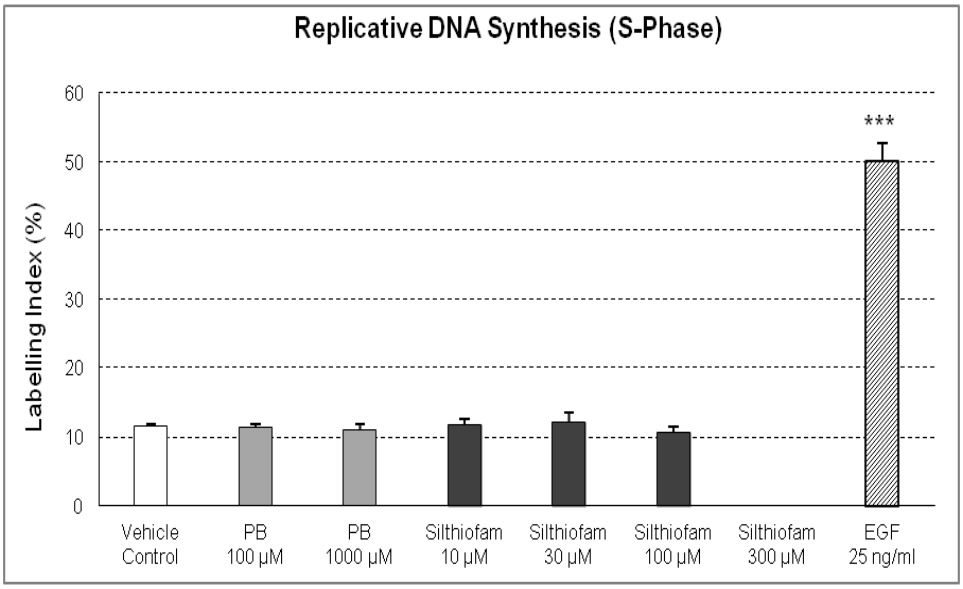
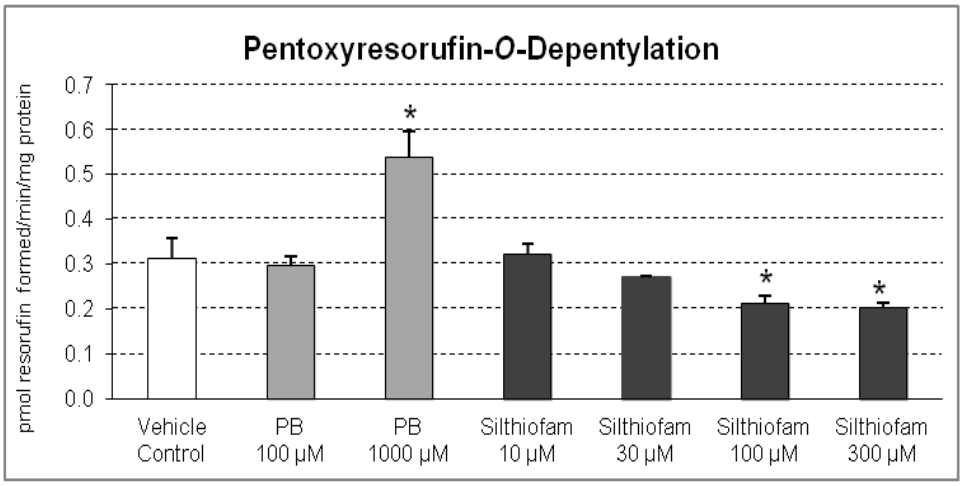
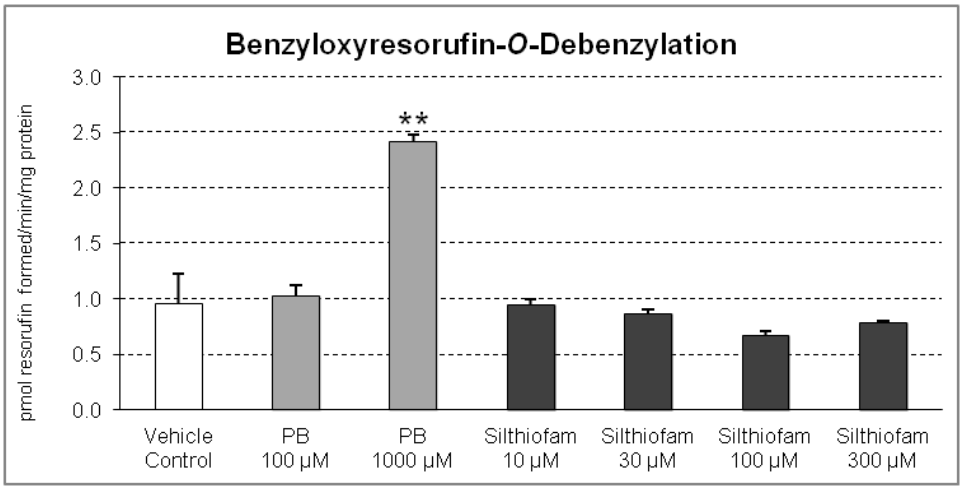
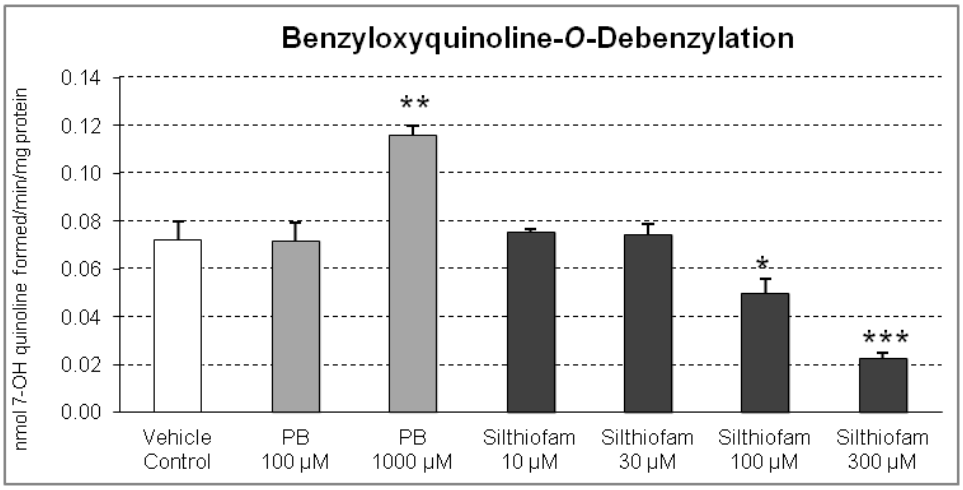


Figure 12: Hepatocellular enzyme activity in rat CARKO/PXRKO hepatocytes.

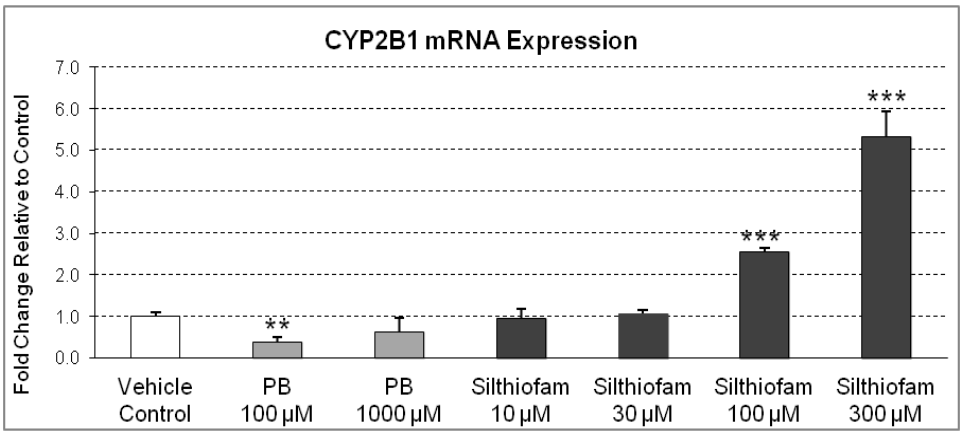


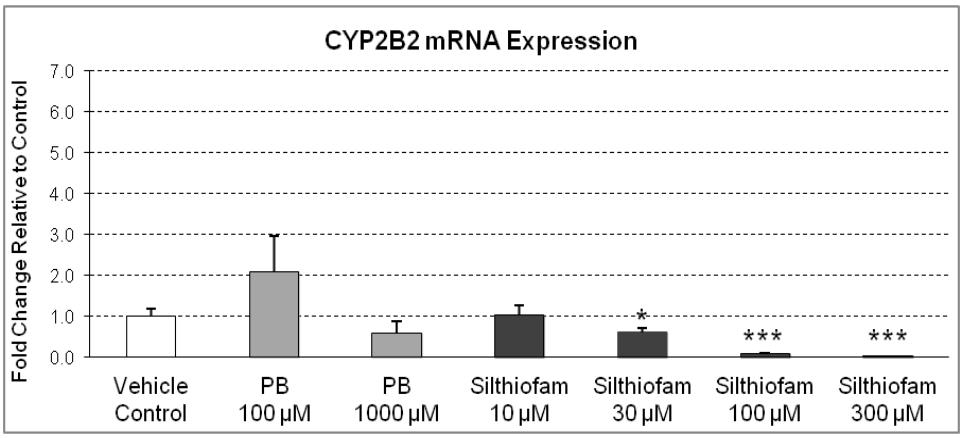


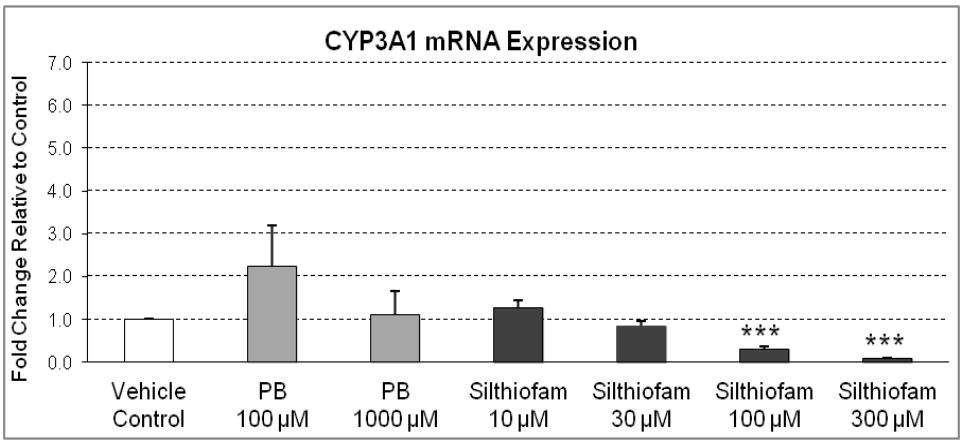


** p≤0.05; **p≤0.01; ***p≤0.001

Figure 13: CYP2B1, CYP2B2 and CYP3A1 mRNA expression in rat CARKO/PXRKO hepatocytes.







* p≤0.05; **p≤0.01; ***p≤0.001

Conclusions:
The presence of functional CAR and/or PXR appears essential for the initial hepatic proliferative response from both silthiofam and phenobarbital. No increase in cell proliferation was observed following treatment of CARKO/PXRKO rat hepatocytes with either of these substances.

Silthiofam failed to induce CYP2B2 or CYP3A1 mRNA expression levels as shown by TaqMan analysis in male CARKO/PXRKO rat hepatocytes. However, a small dose-dependent increase in CYP2B1 mRNA expression levels was observed after treatment with silthiofam (100, 300µM levels only), which did not result in an increase in enzyme activity as determined by PROD or BROD activity measurements. This observation could be due to the known level of redundancy between various nuclear receptors. Cross-talk between different nuclear receptors in the absence of active CAR and PXR and due to exposure to silthiofam, may cause a small increase in CYP2B1 mRNA levels that is not sufficient to result in detectable increases in PROD or BROD enzyme activity. However, this remains unsubstantiated and untested.

PB treatment failed to induce CYP2B1, CYP2B2 or CYP3A1 mRNA expression levels in male CARKO/PXRKO rat hepatocytes. This is in contrast to the significantly increased expression of these genes following PB treatment in wild-type rat hepatocytes and confirms the close association between these genes and the CAR and PXR nuclear receptors.

These results strongly suggest that the mode of action for the hepatic effects of silthiofam is predominantly *via* activation of CAR and PXR. Treatment of male CARKO/PXRKO rat hepatocytes with silthiofam did not stimulate cell proliferation and neither were there increases in CAR/PXR target gene expression and cytochrome P450 activity.

4.10.3.5 Study 5: Enzyme induction in cultured human female hepatocytes

Reference: Chatham, L. (2015b): Silthiofam - Cytochrome P450 enzyme and DNA-synthesis induction in cultured human female hepatocytes.
Study / Report: CXR-2015-0229, MSL0027258
Guidelines: Non guideline, mechanistic study.
GLP: No. However, study was conducted according to established SOPs and good research practices.

Executive summary:
Silthiofam has previously been shown to weakly activate the Constitutive Androstane Receptor (CAR) and Pregnane X Receptor (PXR) nuclear receptors in isolated <u>male</u> human hepatocytes (from one donor) but to have no effect on cell proliferation. The objective of this study was to evaluate the potential for silthiofam to activate CAR and PXR and stimulate cell proliferation in isolated female human hepatocytes (one donor).

Exposure of the female human hepatocytes to silthiofam resulted in weak induction of CYP2B6 and CYP3A4 as indicated by slightly increased enzyme activity and mRNA expression. Slightly greater increases in enzyme activity and gene expression were noted with phenobarbital. However, there was no evidence of an increase in replicative DNA synthesis (cell proliferation) with either silthiofam or phenobarbital, which was in contrast to a substantial increase in cell proliferation noted with EGF.

Materials and methods:
Silthiofam, Lot number: GLP-1502-23805-T, purity: 100% and Sodium Phenobarbital (PB), Lot number: 080M1276V, purity: 100% were added to medium used to culture isolated hepatocytes for 96 hours. Hepatocytes from one donor were used (68 year old female). Cell viability was acceptable (>70%). DMSO (0.1% v/v), phenobarbital (100, 1000µM), silthiofam (1, 3, 10, 30, 100µM) and EGF (25 ng/ml) were tested. There were 3 replicates for each concentration in 25 cm^2 flasks for PROD, BROD and BQ analyses, 5 replicates for each concentration in 6-well plates for replicative DNA synthesis (proliferation) analyses and 6 replicates for each concentration in 96-well plates for ATP (cytotoxicity) measurements.

Several assay systems were employed in this study. (i) Cell viability was assessed using a bioluminescence assay to determine the amount of adenosine 5' triphosphate (ATP) released from the hepatocytes. (ii) cell proliferation was assessed by exposing the hepatocytes to bromodeoxyuridine (BrdU, 10 µM) and measuring the BrdU incorporation into nuclear DNA over the last 72 hours of culture. The results were reported as a labelling index representing the percentage of hepatocytes that were replicating and thus incorporated BrdU into their DNA. Approximately 1500 hepatocytes were counted per replicate for each treatment. (iii) CYP2B and CYP3A activities were assessed by measuring cytochrome P450-dependent O-dealkylation of 7 pentoxyresorufin, 7 benzyl-oxyresorufin and 7 benzyloxyquinoline (PROD, BROD and BQ, respectively). In contrast to the rat, BROD is a better marker for CYP2B induction in humans than PROD. (iv) Gene expression was assessed by measuring CYP2B6 and CYP3A4 mRNA levels using quantitative RT-PCR (TaqMan). Human β-actin was used as the internal standard.

In a first experiment, hepatocytes were exposed to silthiofam at concentrations of 10, 30, 100 and 300 µM. However, a second experiment was performed due to cytotoxicity at the higher silthiofam concentrations (>30 µM), additional hepatocytes were thus subsequently exposed to silthiofam at 1, 3 or 10 µM, EGF (25 ng/mL) and DMSO (0.1% v/v).

Results:
(i) Cell viability - substantial cytotoxicity (almost 80% reduction in ATP relative to control) was observed from 100µM and greater silthiofam (table 46 and figure 14). There was little to no cytotoxicity associated with lower levels of silthiofam (1, 3, 10 and 30 µM) or with the two tested concentrations of sodium phenobarbital.

(ii) Hepatocellular Proliferation: - No statistically significant increases in S-phase labelling index (cell proliferation) were observed following treatment with either silthiofam (1 to 30 µM) or phenobarbital (100 or 1000 µM). Analyses of hepatocytes exposed to 100 and 300 µM silthiofam were excluded due to excessive cytotoxicity. Treatment with EGF resulted in a 4- to 7-fold increase in replicative DNA synthesis, demonstrating the suitability of this system for assessing cell proliferation, see figure 15.

In summary, no increase in cell proliferation was observed following treatment of human female hepatocytes with silthiofam or phenobarbital (figure 15).

(iii) Cytochrome P450 Enzyme Activity: - Treatment with silthiofam did not result in any meaningful changes in PROD activity. Slight (statistically significant) increases in BROD and BQ activities (1.4-fold and 1.1-fold, respectively) were noted after treatment with 1 µM

silthiofam **but** decreased BROD and BQ activity were noted at 3 to 100 µM silthiofam. Increased PROD, BROD and BQ activities (up to 1.9-fold) were noted with both 100 and 1000 µM phenobarbital (16).

(iv) Gene Expression: - Statistically significant increases (up to 2.5-fold) in mRNA expression of CYP2B6 were noted in human hepatocytes treated with 1 to 30 µM silthiofam. A statistically significant increase (1.9-fold) in mRNA expression of CYP3A4 was also noted at 3 µM silthiofam but not at other concentrations. Larger increases in mRNA expression of CYP2B6 and CYP3A4 (up to 3.7-fold and 6.2-fold, respectively) were observed after treatment with phenobarbital (figure 17).

Conclusions:
Exposure of female human hepatocytes to silthiofam resulted in a low level induction of CYP2B and CYP3A, as indicated by increased mRNA expression. Enzyme activity levels were variable with decreases in BROD and BQ activities with silthiofam treatment in contrast to PB treatment.

PB was observed to be an inducer of human CYP2B and CYP3A. At the mRNA expression level, PB induced increases in CYP2B6 and CYP3A4 of up to 3.7- and 6.2-fold control, respectively. At the protein enzyme activity level, treatment with PB resulted in increases in PROD, BROD and BQ activity of up to 1.5-, 1.9- and 1.9-fold of control, respectively.

In agreement with the *in vitro* human male hepatocyte study, no hepatocyte proliferation (BrdU) was seen in response to PB or silthiofam at any concentration. Positive control with EGF responded successfully.

Silthiofam is a weak activator of human CAR and PXR (as shown by the effects on CYP2B and CYP3A mRNA levels, respectively), there is evidence for inhibition of CYP2B and CYP3A protein activities (BROD and BQ) with no effect on DNA-synthesis in female human hepatocytes.

Table 46: Biochemical and mRNA results in human female hepatocytes.

Treatment	ATP (units released)	S-phase (label index (%))	PROD (pmol/min/mg)	BROD (pmol/min/mg)	BQ (nmol/min/mg)	CYP2B6 (fold change)[c]	CYP3A4 (fold change)
Vehicle control 1 (0.1% v/v DMSO)	315830 (100)[a]	0.17 (100)	0.139 (100)	1.914 (100)	0.150 (100)	1.00	1.00
PB 100 µM	308849 (98)	0.18 (106)	0.205* (148)	3.634** (190)	0.216 *** (144)	1.85*	1.83
PB 1000 µM	272945*** (86)	0.21 (123)	0.215* (155)	3.100 ** (162)	0.285*** (190)	3.72***	6.18***
Silthiofam 10 µM	249916 *** (79)	0.19 (112)	0.135 (97)	0.769** (40)	0.039*** (26)	2.13**	1.57
Silthiofam 30 µM	211482*** (67)	0.19 (112)	0.132 (95)	0.744** (39)	0.024*** (16)	2.13**	1.47
Silthiofam 100 µM	138421*** (44)	b	0.121 (87)	0.555** (29)	0.017*** (11)	b	b
Silthiofam 300 µM	21836*** (7)	b	b	b	b	b	b
EGF (25 ng/mL)	-	1.14*** (673)	-	-	-		
Vehicle control 2 (0.1% v/v DMSO)	259193 (100)	0.21 (100)	0.488 (100)	2.941 (100)	0.257 (100)	1.00	1.00
Silthiofam 1 µM	273584 (106)	0.17 (81)	0.554 (114)	3.998** (136)	0.280* (109)	1.59*	0.58
Silthiofam 3 µM	287122* (111)	0.15 (73)	0.334* (68)	2.261* (77)	0.219* (85)	2.48***	1.87*
Silthiofam 10 µM	233388* (90)	-	-	-	-	-	-
EGF (25 ng/mL)	-	0.86*** (411)	-	-	-	-	-

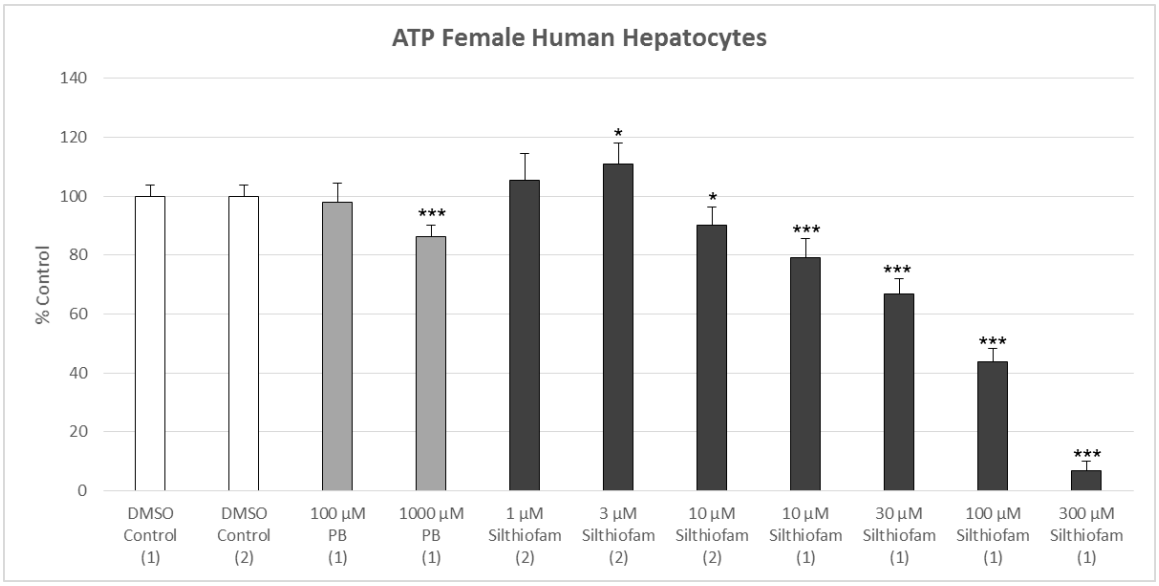
[a] Values in parenthesis represent % of DMSO vehicle control value

[b] Analysis unable to be performed due to excessive cytotoxicity

[c] Fold change for gene expression (mRNA) results were calculated by the formula $2^{-\Delta\Delta Ct}$, where ΔΔCt=ΔCt values from the target *minus* ΔCt value for the first control sample, and ΔCt=Ct values from the gene of interest minus Ct values for the internal control gene (β-actin)

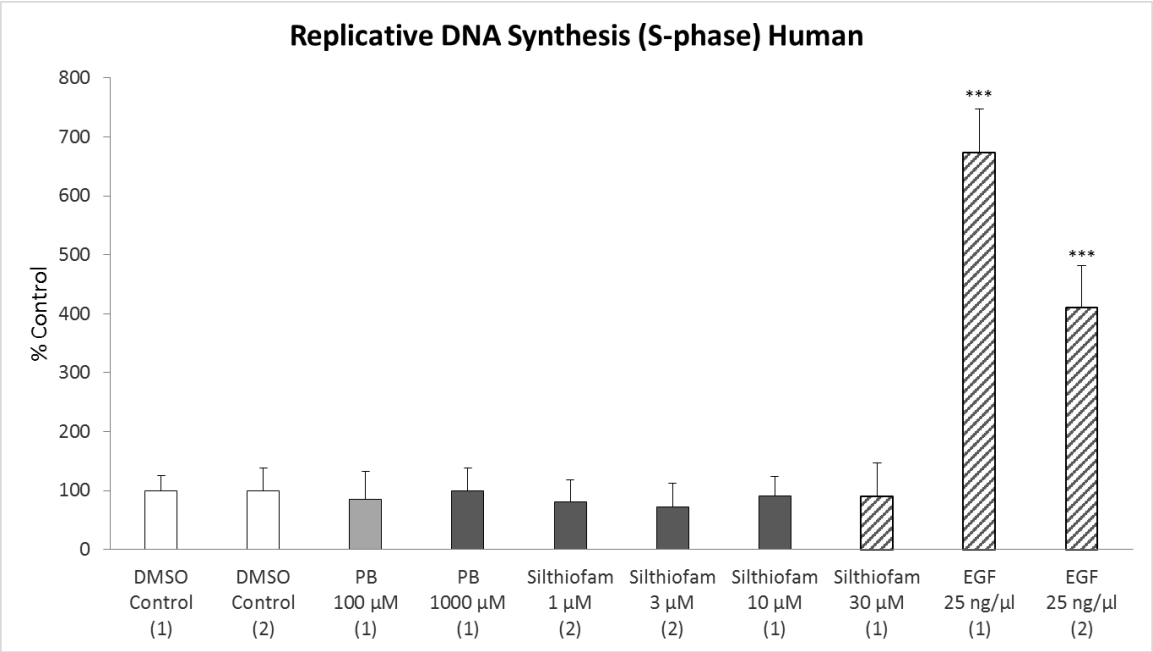
* p≤0.05; **p≤0.01; ***p≤0.001

Figure 14: Adenosine 5'-Triphosphate (ATP) release from cultured human female hepatocytes.



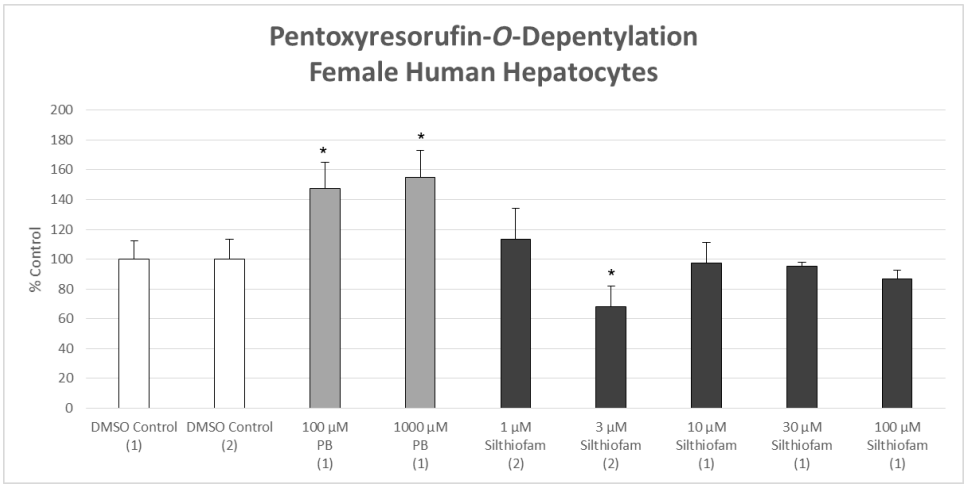
Numbers in parenthesis refer to 1st and 2nd experiments
* p≤0.05; **p≤0.01; ***p≤0.001

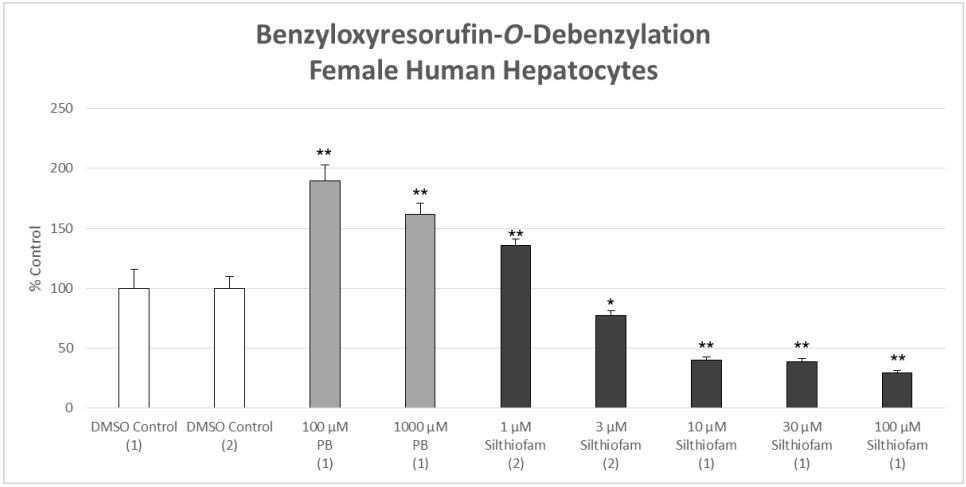
Figure 15: Hepatocellular proliferation in human female hepatocytes.

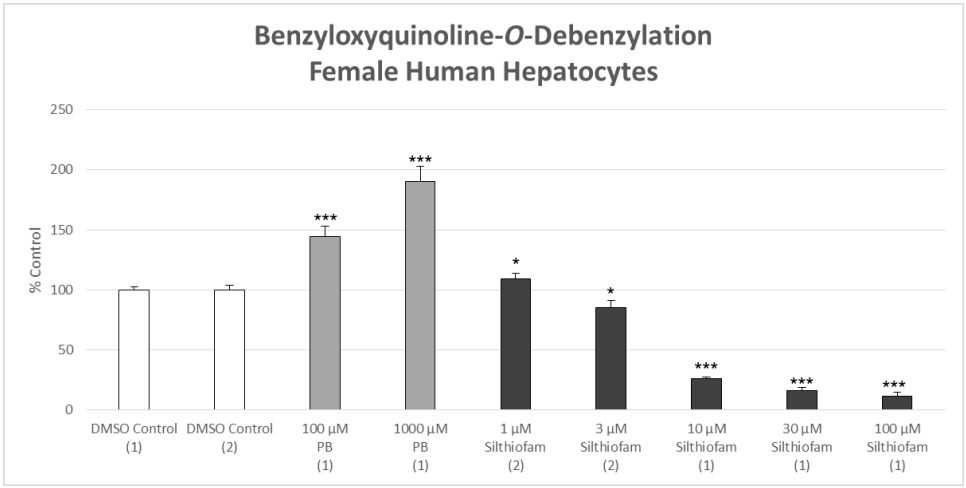


Numbers in parenthesis refer to 1st and 2nd experiments
* p≤0.05; **p≤0.01; ***p≤0.001

Figure 16: Hepatocellular enzyme activity (PROD, BROD & BQ) in human female hepatocytes.

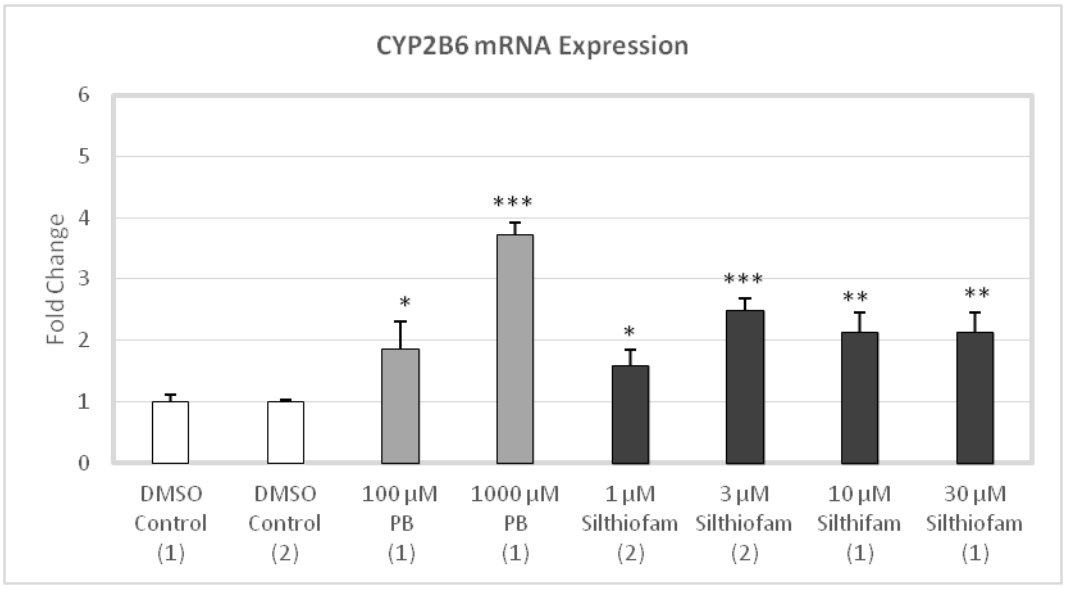


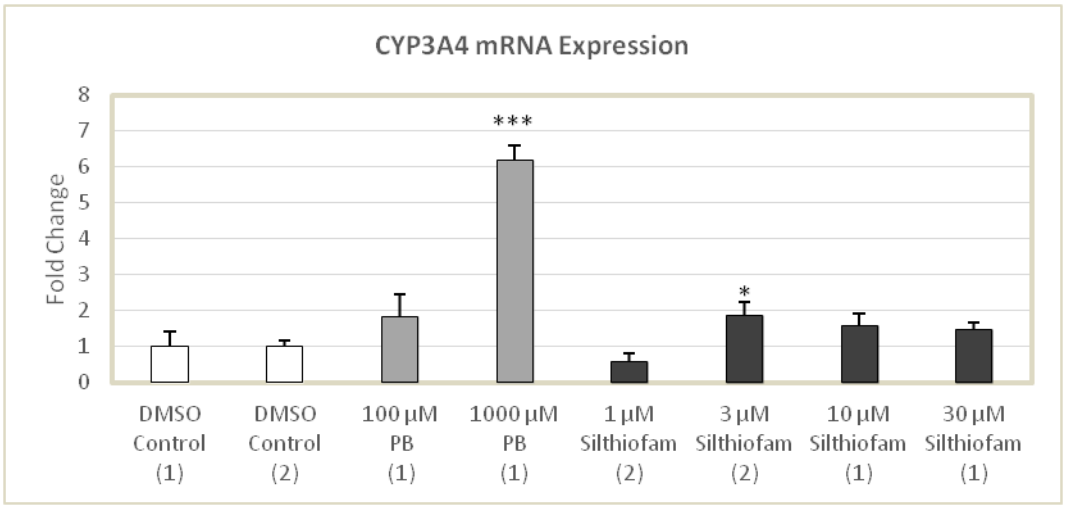




Numbers in parenthesis refer to 1st and 2nd experiments
* p≤0.05; **p≤0.01; ***p≤0.001

Figure 17: CYP2B6 and CYP3A4 mRNA expression in human female hepatocytes.





Numbers in parenthesis refer to 1st and 2nd experiments
* p≤0.05; **p≤0.01; ***p≤0.001

4.10.3.6 Overall Summary of Mechanistic Studies

The mechanistic data supports a CAR/PXR mode of action for tumour development in rodents. The *in-vivo* mode of action rat study established that cytochrome P450 enzyme protein levels and activities and mRNA transcripts were consistent with activated CAR. Indicators of PPARα activity were decreased. CYP1 and the involvement of the aryl hydrocarbon receptor (AHR) were not investigated.

The *in-vitro* rat hepatocyte study confirmed CAR/PXR activation and up-regulation of their respective target genes and proteins. The PXR response was greater than the CAR response.

Statistically significant increases in hepatocellular proliferation were observed with 30 and 100 µM silthiofam.

The *in-vitro* rat CARKO/PXRKO double knockout hepatocyte study confirmed that the presence of functional CAR and/or PXR appears essential for the initial hepatic proliferative response from both silthiofam and phenobarbital

The *in-vitro* human male hepatocyte study confirmed a weaker CAR/PXR activation relative to rat hepatocytes. CAR and PXR responses were roughly similar. There was no evidence of increased hepatocellular proliferation with silthiofam exposure. Similarly, the *in-vitro* human female hepatocyte study confirmed a weaker CAR/PXR activation relative to rat hepatocytes. There was a small induction of CYP2B and CYP3A but increases in protein activity were either unconvincing or decreased with silthiofam treatment. In agreement with the results for human male hepatocytes, there was no evidence of increased hepatocellular proliferation with silthiofam exposure.

There was no activation of PPARα or evidence of significant hepatocellular toxicity in rats.

The *in vitro* results with silthiofam were very similar to those from phenobarbital, which induces rodent hepatocarcinogenicity *via* CAR activation but is generally not considered to pose a carcinogenic risk to humans.

The data are considered 'limited' but supportive of a CAR/PXR mediated effect on rodent liver following exposure to silthiofam.

4.10.3.7 Summary and discussion of carcinogenicity

The results from the long-term toxicity (rat) and carcinogenicity (mouse) studies previously evaluated during the EU were used to assess the carcinogenicity potential of silthiofam. Additional important mechanistic studies have recently been conducted to evaluate the mode of action for the findings in the chronic rat study.

The liver was identified as the primary target organ following the long-term administration of silthiofam in the rat and mouse. In both species, hepatotoxicity was characterised by elevated organ weight and serum enzymes, and microscopic changes that included hepatocellular hypertrophy, vacuolation, and cystic degeneration. Individual hepatocyte necrosis was noted only in mice at the highest dose level (males: 564 and females: 855 mg/kg/day) that was also much higher than the high-dose (males: 150 and females: 195 mg/kg/day) utilized in the chronic rat study. Gallstones were also observed in several high-dose mice but, although considered to be treatment related, were not accompanied by any microscopic evidence of biliary obstruction or inflammatory, degenerative or proliferative changes.

Weak evidence of carcinogenicity was noted in both species. A slight but statistically significant increase in the incidence of hepatocellular adenomas was observed in high-dose (855 mg/kg/day) female mice (Table 47). The original study summaries were incorrect in expressing tumour incidences as a proportion of 60 animals, only 50 animals maximum were assigned to the full term of the study, 10 animals were used for the interim sacrifice at 12 months. The correct incidences are presented in this re-evaluation. Variable incidences of liver tumours also occurred in male mice, but against a very high background level, observed in both in the controls and historical control data from

CR labs (Table 48). CR HCD was not exceeded for males. The tumours in female mice occurred in animals that also exhibited severe liver toxicity (e.g., chronic inflammation and individual cell necrosis). These tumours did not progress to malignancy and did not affect survival. No increase in tumours was seen at non-hepatotoxic dose levels. The mouse liver tumours may were originally considered to be a result of a secondary, regenerative response to cytotoxicity.

A slight increase in the incidence of hepatocellular tumours was also observed in mid- and high-dose (150 mg/kg/day) male rats (Table 49). Although not statistically significant, the incidence of hepatocellular carcinomas exceeded the upper limits of the testing laboratory's historical control data from 7 studies conducted within 10 years of this study (although the incidence was within the lab's historical range when a longer period was utilised). In addition, the slightly increased incidence of liver tumours in males but not females in this study is consistent with the observation that hepatotoxicity was more pronounced in males than females in the 90-day rat study. Increased incidence of liver tumours in high-dose male rats was considered to be treatment related.

There was also a slight, increase in the incidence of thyroid follicular tumours in high-dose male rats (Table 49). The incidences of these tumours were within the published historical control ranges for the Charles River CD rats that were utilised in this study. However, a relationship with treatment is indicated by the increased incidence at the high dose and supported by increased hepatic UDPGT.

Overall, the NOAEL for long-term toxicity and carcinogenicity following the oral administration of silthiofam is considered to be 6.4 mg/kg/day based on centrilobular vacuolation in the livers of females.

Table 47: Incidence of Liver Tumours in Females from 18-Month Mouse Study with Silthiofam

Dose Level ppm (mg/kg bw)	**0 (0)**	**10 (2.03)**	**100 (20.7)**	**1000 (203)**	**4000 (855)**	**Lab HCD**	**CR CD-1 HCD**
Adenoma (%)	1/50 (2)	0/50	1/50 (2)	0/50	**5/50* (10)**	0 – 3%	1 – 8%
Carcinoma (%)	0/50	0/50	0/50	0/50	1/50 (2)	0 – 3%	1 – 4%

Table 48: Incidence of Liver Tumours in Males from 18-Month Mouse Study with Silthiofam

Dose Level ppm (mg/kg bw)	**0 (0)**	**10 (1.4)**	**100 (13.7)**	**1000 (141)**	**4000 (564)**	**Lab HCD**	**CR CD-1 HCD**
Adenoma (%)	6/50 (12)	nd	4/50 (8)	9/50 (18)	6/50 (12)	nd	3 – 28%
Carcinoma (%)	6/50 (12)	nd	2/50 (4)	2/50 (4)	1/50 (2)	nd	2 – 16%

Table 49: Incidence of Liver & Thyroid Tumours in Males from 2-Year Rat Study with Silthiofam

Dose Level (ppm) (mg/kg bw)	**0 (0)**	**10 (0.50)**	**100 (5.0)**	**1000 (51)**	**3000 (150)**	**(SD) BR rat HCD**
	Liver tumours					

Adenoma (%)	4/50 (8)	3/50 (6)	2/50 (4)	5/50 (10)*	7/50 (14)*	0 – 8%
Carcinoma (%)	0/50	2/50 (4)	2/50 (4)	3/50 (6)	4/50 (8)*	0 – 6.7%
Adenoma and/or carcinoma	4/50 (8)	5/50 (10)	4/50 (8)	8/50 (16)*	10/50 (20)*	0.9 – 10%
	Thyroid follicular tumours					
Adenoma (%)	3/50 (6)	0/50	0/50	1/50 (2)	5/50 (10)	1.7 – 12%
Carcinoma (%)	0/50	0/50	1/50 (2)	1/50 (2)	2/50 (4)	0.9 – 3.9%
Adenoma and/or carcinoma	3/50	0/50	1/50	2/50	7/50	0.0 – 14%

*Incidence in high dose is greater than the published rat HCD for Charles River (23 studies; 1995 - 2001). nd: no data. No evidence of a tumourigenic effect in female rats. Incidences of thyroid follicular tumours is within historical control data as published by Charles River Labs (23 studies, 1995 – 2001).

4.10.4 Comparison with criteria

CLP: Regulation (EC) No. 1272/2008
The criteria for classification as a carcinogen under the CLP Regulation are as follows;

Category 1: Known or presumed human carcinogens. A substance is classified in Category 1 for carcinogenicity on the basis of epidemiological and/or animal data. A substance may be further distinguished as:

a. Category 1A, known to have carcinogenic potential for humans, classification is largely based on human evidence, or
b. Category 1B, presumed to have carcinogenic potential for humans, classification is largely based on animal evidence.

Differentiation between classification in Category 1A and 1B is based on strength of evidence together with additional considerations of all relevant information.

Category 2: Suspected human carcinogen.

"Substances are classified as a category 2 Carcinogen when evidence is obtained from human and/or animal studies, but which is not sufficiently convincing to place the substance in Category 1A or 1B, based on strength of evidence together with additional considerations. Such evidence may be derived either from limited evidence of carcinogenicity in human studies or from limited evidence of carcinogenicity in animal studies."

Relevant data
There is weak evidence of a carcinogenic effect in mice and rats (liver). In addition, rats show evidence of an increased incidence in thyroid follicular tumours.

In the female CD-1 mouse at the high dose (855 mg/kg bw/day), there was an increased incidence of liver adenomas (10%) that exceeded concurrent controls and the historical control incidence data for both the testing laboratory (0-3%) and an assortment of Charles River laboratories from 1987 to 1999 (1–8%). The liver tumours did not progress to malignancy and did not affect survival. There is no mode of action data available for mice treated with silthiofam.

Male CD rats had an increase in incidence of combined hepatocellular adenomas and carcinomas (20%), and of hepatocellular adenomas alone (14%) and carcinomas alone (8%) in the top dose group (3,000ppm equiv to 150 mg/kg bw/day). At the mid dose (1,000ppm equiv to 51 mg/kg bw/day) males also showed an increase in hepatocellular adenomas (10%) that exceeded both concurrent controls and historical control data. Liver toxicity was evident at the high and mid doses with non-neoplastic histopathological changes that included increases in the incidences of cystic degeneration and vacuolisation.

Follicular cell tumours of the thyroid gland were also slightly increased in high dose males but did not exceed historical control data incidences. Induction of hepatic T_4-UDPGT was observed after 14 days of dosing with 3000 ppm silthiofam and is supportive of disruption of thyroid homeostasis secondary to increased clearance of thyroid hormones by increased hepatic UDPGT activity. Thyroid hormone and TSH levels were not evaluated.

Silthiofam is not genotoxic.

There was evidence of pre-neoplastic changes in rat liver at the highest dose, a statistically significant increase in eosinophilic foci was observed in both male and female rats at 3000 ppm silthiofam.

Some mechanistic data was presented; a CAR/PXR mode of action for tumour development in rodents can be supported. An *in-vivo* mode of action rat study established cytochrome P450 enzyme protein levels and activities and mRNA transcripts consistent with activated CAR. Indicators of PPARα activity were decreased. CYP1 and the involvement of the aryl hydrocarbon receptor (AHR) were not investigated.

An *in-vitro* rat hepatocyte study confirmed CAR/PXR activation and up-regulation of their respective target genes and proteins. The PXR response was greater than the CAR response. Statistically significant increases in hepatocellular proliferation were observed with 30 and 100 µM silthiofam. The newly available SD rat CAR/PXR double knockout model illustrated that the presence of functional CAR/PXR is required for increased CYP2B2, CYP3A1 mRNA levels and PROD, BROD and BQ enzyme activity levels and the initial hepatocyte proliferative burst observed following either PB or silthiofam treatment.

Two *in-vitro* human hepatocyte studies (one using isolated male hepatocytes and one using female hepatocytes) confirmed a weaker CAR/PXR activation relative to rat hepatocytes. CAR and PXR responses were roughly similar. There was no evidence of increased hepatocellular proliferation with silthiofam exposure.

No activation of PPARα or evidence of significant hepatocellular toxicity was noted in rats.

In vitro results with silthiofam were very similar to those from phenobarbital, which induces rodent hepatocarcinogenicity via CAR activation but is generally not considered to pose a carcinogenic risk to humans.

The data are considered 'limited' but supportive of a CAR/PXR mediated effect on rodent liver following exposure to silthiofam.

Classification in Category 1A

Because there is no epidemiological evidence regarding the carcinogenicity of silthiofam to humans, a classification in Category 1A is not appropriate.

Classification in Category 1B

Since an increase in tumour incidence has been observed in two species (mouse and rat), with an additional tumour type also observed in rats, an argument for classification in category 1B can be made. However, on consideration of the available data, there are a number of factors that indicate that classification in category 2 might be more appropriate in this case:

- the available genotoxicity data on silthiofam do not support a genotoxic mode of action for tumour induction;
- mechanistic data suggests that silthiofam is carcinogenic in liver by a secondary mechanism involving the nuclear receptors CAR/PXR;
- the additional tumour type (thyroid follicular adenoma) in rats was not statistically significant and does not exceed the historical control incidence for the particular strain of rat at the highest dose of silthiofam. In addition, the increased hepatic UDPGT activity observed from the rat *in-vivo* mechanistic study suggests this tumour is not relevant to humans.
- the hepatic tumours in mice at very high doses (855 mg/kg bw/day) did not progress to malignancy, did not show a dose response and did not affect survival. In addition, liver cytotoxicity (high incidence of individual hepatocellular necrosis), suggests the tumours could be secondary to a regenerative hyperplasia arising from cytotoxicity. Also the rat data strongly indicates a CAR/PXR mechanism may also play a role;

Classification in Category 2

On the basis of the 2 long-term rodent toxicity studies alone a good argument can be made for classification in Category 2 for silthiofam. The increase in thyroid follicular tumours in the male rat is within historical control data, is of low concern and considered not to be relevant to humans. The rat liver tumours show a partial dose response and there is little doubt of a treatment related effect. However, the mechanistic data suggests a role for activated CAR/PXR nuclear receptors with downstream effects considered to be rodent specific.

No Classification

Increased tumour incidences have been seen in two species so a simple argument for Category 2 classification can be made. However, on consideration of all the available data, there are a number of factors that indicate classification in Category 2 may not be appropriate. Silthiofam has no structural relationship with other known carcinogens. There is a complete lack of genotoxicity seen with silthiofam in *in vitro* and *in vivo* studies. The mechanistic evidence provides support for a CAR-mediated mechanism to explain the liver responses, tumours and enzyme induction profiles in silthiofam treated animals. Some data gaps remain however, transgenic mouse models such as the humanised CAR/PXR model were not employed to investigate the mouse liver tumours. However, a new study investigating the use of rat CAR/PXR knockouts provided strong evidence that the mode of action for silthiofam-induced proliferation in rat hepatocytes is CAR and/or PXR-mediated. Overall, data has been presented showing silthiofam has very similar responses to those seen with phenobarbital so the involvement of the CAR/PXR system in rodents in this case is strengthened.

In view of these considerations, the available evidence is deemed to support a mechanism of action that is not relevant for human health (activation of CAR, induction of CYP isozymes, leading to increased hepatocellular proliferation with subsequent induction of proliferative lesions and tumours in the liver). On the weight of the available evidence, classification for carcinogenicity is not proposed by the Dossier Submitter.

4.10.5 Conclusions on classification and labelling

No classification

RAC evaluation of carcinogenicity

Summary of the Dossier Submitter's proposal

The carcinogenic potential of silthiofam was investigated in a 2-year rat study and in an 18-month mouse study. In addition, several mechanistic studies were available to investigate the potential tumour MoA.

In the carcinogenicity rat study, a treatment-related increase in the incidence of liver adenoma and carcinoma was observed at the mid and high dose male rats. The increases in adenoma and carcinoma were not statistically significant but were above concurrent and historical control data (HCD) (See the table below). Moreover, an increase in thyroid follicular adenoma and/or carcinoma was observed in male rats (not statistically significant) at the highest dose above the concurrent controls and at the upper range of the HCD.

In the mouse carcinogenicity study, a slight statistically significant positive trend (Peto analysis) in liver adenoma was observed in females at the highest dose (855 mg/kg bw/day; 5/50 animals, 10 %). The increase was not statistically significant by pairwise comparison. The incidence was above concurrent controls (1/50 animals, 2 %), historical control data ranges from the testing laboratory (maximum 3 %) and HCD ranges from Charles River laboratories for the same strain of mice (maximum 8 %). The tumours occurred in presence of severe liver toxicity (cell necrosis, chronic inflammation).

Mechanistic data were provided in the dossier to investigate a potential CAR/PXR-mediated MoA for liver tumours. Five mechanistic studies were conducted with silthiofam and the results were summarised by the DS as follows:

(1) 14 day *in vivo* rat study (silthiofam):
- Substantial induction of hepatic CYP2B1, CYP2B2 and (to a lesser extent) CYP3A1 (based on enzyme activity, mRNA expression and Western blot data);
- substantial increase in replicative DNA synthesis (cell proliferation) in the liver at 7 and 14 days;
- No evidence of activation of PPARα (as measured by enzyme activity, gene expression and Western blots);
- Increased induction of hepatic T4-UDPGT activity after 14 days of dosing.

(2) Rat wild-type hepatocyte *in vitro* study (phenobarbital and silthiofam tested):
- Silthiofam acted in a phenobarbital-like manner;

- Increased induction of CYP2B1, CYP2B2 and CYP3A1 (revealed by enzyme activity, mRNA expression);
- Increase in replicative DNA synthesis (cell proliferation);
- Cytotoxicity at silthiofam concentrations > 100 µM.

(3-4) Human hepatocyte *in vitro* studies (phenobarbital and silthiofam tested, one study with one male donor and one study with one female donor):

- Silthiofam acted in a phenobarbital-like manner;
- No increase in PROD activity (CYP2 marker), weak response in BROD and BQ activity (CYP2/ CYP3 and selective CYP3 markers respectively);
- Weak induction of CYP2 and CYP3 mRNA expression;
- No increase in replicative DNA synthesis (cell proliferation);
- Cytotoxicity at silthiofam concentrations > 100 µM.

(4) Rat CARKO/PXRKO hepatocyte *in vitro* study (phenobarbital and silthiofam tested):

- No increase in PROD, BROD or BQ enzyme activity relative to controls;
- Weak effect on CYP2B1 but no effect in CYP2B2 or CYP3A gene expression;
- No increase in cell proliferation;
- Cytotoxicity at silthiofam concentrations > 100 µM.

According to the DS the results were consistent with the proposed CAR/PXR-mediated effect on rodent liver which is not relevant for human health: activation of CAR/PXR, induction of CYP isoenzymes, increased hepatocellular proliferation leading to tumours in the liver. Some uncertainties were noted by the DS as some MoA were not investigated (involvement of AhR) and as no *in vivo* studies with CAR/PXR knock out animals were performed.

Overall, no classification was proposed by the DS based on a weight-of-evidence analysis which took into account the following:

- Silthiofam was not genotoxic;
- Mechanistic data were supportive of a CAR/PXR-mediated effect on rodent liver not relevant for human health supporting no classification for liver tumours observed in rats;
- Thyroid follicular tumours observed in rats were not statistically significant and did not exceed the HCD. In addition, the increase in hepatic UDPGT activity observed in the *in vivo* mechanistic study suggested that these tumours were also not relevant to humans;
- Finally, hepatic tumours in mice at a very high dose did not progress to malignancy and did not affect survival. In addition, liver toxicity observed in mice suggested that tumours could be secondary to a regenerative hyperplasia from cytotoxicity. The rat mechanistic data indicated that a CAR/PXR nuclear receptor may also play a role.

Comments received during public consultation

One MS disagreed with the DS's proposal and supported a Carc. 2 classification. Although the MS acknowledged that some evidence suggested a CAR/PXR mediated MoA, they considered that other potential MoA were not sufficiently excluded. The MS was of the opinion that hepatotoxicity and cytotoxicity of silthiofam might be the main potential MoA in the rat and mouse studies.

Assessment and comparison with the classification criteria

Rat

In the combined chronic toxicity and carcinogenicity study in rats, an increase in hepatocellular adenoma and carcinoma was observed in males. The increases were not statistically significant and were inside the relevant laboratory historical control range. Survival, mean body weight and body weight gain were not affected in the study in males. A treatment-related increase in absolute and relative liver weight was observed at the top dose in males (↑ 19 % in relative weight). Eosinophilic foci were increased in the liver at the high dose in both males and females. Centrilobular pallor was increased in both male and female rats at the mid and high dose levels. An increase in the incidence of cystic degeneration was also observed at the top dose in males. No degenerative changes were noted (See table below).

In this study, an increase in thyroid follicular cell tumours was also observed in males. The increase was not statistically significant but was observed at the upper range of HCD for this strain of rats and slightly above laboratory historical control data.

***Table**: Selected non-neoplastic findings at terminal sacrifice (carcinogenicity rat study, 1998)*

	n = 50									
	Males					**Females**				
Dose (mg/kg bw/d)	**0**	**0.5**	**5**	**51**	**150**	**0**	**0.65**	**6.4**	**65**	**195**
Rel. liver wt (% bw)	100	102	110	109	119**	100	100	110	114	107
Hypertrophy	0	0	0	0	2	-	-	-	-	-
Eosinophilic Foci	7	12	7	9	29**	5	4	10	10	23**
Centrilobular pallor	0	0	4	11*	22**	0	0	4	12**	29**
Cystic degeneration	11	20	22	20	30**	-	-	-	-	-
vacuolation	3	1	2	5	24**	0	4	1	11**	24**

*p≤0.05; **p≤0.01; -: not found; wt: weight

***Table:** Incidence of liver and thyroid tumours in male rats (carcinogenicity study, 1998)*

Tumour type	**Tumour incidence (%) Dose (mg/kg bw/d)**						
	0	**0.5**	**5**	**51**	**150**	**HCD***	**HCD****
Liver							
Adenoma	8	6	4	10	14	0-8	0-18
Carcinoma	0	4	4	6	8	0-6.7	
Adenoma and/or carcinoma	8	10	8	16	20	0.9-10	
Thyroid follicular tumours							
Adenoma	6	0	0	2	10	1.7-12	0-8
Carcinoma	0	0	2	2	4	0.9-3.9	0-2
Adenoma and/or carcinoma	6	0	2	4	14	0-14	

*Charles river (SD)BR rat HCD (23 studies, 1995-2001), ** Historical control range from the laboratory

Mode of action of liver tumours

The human relevance framework has been used by the DS to assess the human relevance of the rodent tumours. Five mechanistic studies were performed in the dossier to investigate a potential CAR-mediated MoA in rodents. The postulated MoA was that the activation of CAR and PXR nuclear receptors in male rats results in the altered expression of a number of genes as well as an increase in hepatic cell proliferation leading to hepatocellular tumours.

Is the WOE provided sufficient to establish the MoA in animals in the case of silthiofam?

Two key events have been considered by the DS: the activation of CAR/PXR nuclear receptors and hepatocellular proliferation.

- Activation of CAR and PXR nuclear receptors

CAR activation has been investigated in an *in vivo* 14-day MoA study in male rats (2013). A single dose was tested which was equivalent to the top dose used in the carcinogenicity study.

BROD (a marker for CYP2B and CYP3A, CAR/PXR), PROD (a marker for CYP2B, CAR) and BQ (a marker for CYP3A, PXR) enzyme activities were increased 13×, 14× and 3×, respectively. Altered gene expression was also noted, as hepatic CYP2B1, CYP2B2 and CYP3A mRNA levels were increased about 1 200-fold, 62-fold and 3-fold, respectively.

The liver induction profile of silthiofam was thus consistent with CAR/PXR activation. Although no comparison with a positive control was performed in the study, CYP2B induction was higher than CYP3A activity as observed with CAR/PXR activators.

Associated events to CAR/PXR activation were also noted in the study. Indeed, increased liver weights and minimal to slight hepatocellular hypertrophy were observed in the 14-day rat study. Cytochrome P450 enzymes were not evaluated in longer term studies. However, liver weight was increased in both males and females in the carcinogenicity study and hepatocellular pallor was consistent with P450 enzyme induction. Nevertheless, RAC noted the absence of liver hypertrophy in both the 90-day and carcinogenicity studies (except in 2 out of 50 animals).

- Increased hepatocellular proliferation.

Hepatocellular proliferation as shown by BrDU labelling of hepatocytes was statistically significantly increased (about 7-fold after 7-day exposure and 9-fold after 14-day exposure) in the *in vivo* 14-day rat study (2013).

Although hepatocellular proliferation was not investigated in longer term studies, an increase in a pre-neoplastic lesion (altered foci) was observed in both males and females at the top dose in the rat carcinogenicity study, which was consistent with hepatocellular proliferation. As liver tumours were only increased in males, the cause of this sex difference is unknown.

It is unknown how long the cell proliferation was sustained but RAC agrees with the statement in the dossier that an early, short term burst of cell proliferation could be sufficient to cause liver tumours.

- Exclusion of alternative MOA

The *in vitro* rat CARKO/PXRKO double knockout study (Chatham, 2015a) showed that the presence of functional CAR and/or PXR appeared essential for the initial hepatic proliferative response from both silthiofam and phenobarbital. Indeed, in contrast with the results observed in the *in vitro* study performed with wild-type rat hepatocytes, no cell proliferation was observed at non-cytotoxic concentrations either with silthiofam or phenobarbital. PROD, BROD and BQ activities were in all silthiofam-treated groups comparable or lower than control. Gene expression of CYP2B2 and CYP3A1 mRNA were either comparable to control or decreased. Nevertheless, a statistically significant dose-related 5-fold increase in mRNA expression was observed for CYP 2B1 (no change was observed with phenobarbital).

Silthiofam was not genotoxic.

In the carcinogenicity study, liver tumours were observed in the absence of significant liver toxicity such as necrosis, fibrosis or inflammation. Cystic degeneration in liver was observed in males. This non neoplastic lesion is derived from altered ito cells and was not considered as evidence of pre-neoplastic lesions by RAC. Indeed, the finding was also statistically significantly increased in male mice but did not lead to tumours. Clinical chemistry changes such as γGT and ALP were elevated in the study indicating possible bile duct effects but the increase was not statistically significant. In the 90-day rat repeated-dose toxicity study, liver toxicity was observed but necrosis was not found. In this study, histopathological findings consisted of hepatocyte vacuolation, hypertrophy/fibrosis of the bile duct, portal inflammation and/or pigment in Küpffer cells. In the 14-day *in vivo* rat study, hepatocellular cell proliferation was observed in the absence of significant hepatotoxicity. Although excessive cytotoxicity was observed in the *in vitro* studies at ≥ 100 µM in males and females, there was no toxicokinetic data in the dossier to compare this concentration to *in vivo* dose levels. Therefore, cytotoxicity may not be the main MoA for rat liver tumours.

No evidence of activation of PPARα was noted in the 14-day study. Therefore, peroxisomal proliferation can be ruled out.

Nevertheless, CYP1A1 was not tested in the study, thus AhR activation cannot be ruled out.

There is no data in the dossier suggesting that other MoA such as Porphyria, statins/altered cholesterol synthesis, estrogenic activity and immunosuppression would be likely for silthiofam.

Overall, RAC agrees that the proposed MoA could be plausible in male rats. Nevertheless, the following uncertainties remained:
- Absence of dose-response data for CAR/PXR activation (only a single dose tested);
- No decrease in apoptosis as a consequence of alterations in gene expression was noted;
- No hypertrophy was observed in rats in the carcinogenicity study; this finding would also have been expected;
- No *in vivo* studies using CAR/PXR knock out animals were performed to confirm the *in vitro* results;
- No exclusion of AhR activation;
- Sex differences in tumour induction have not been investigated. Indeed, no mechanistic data in female rats (*in vitro* and *in vivo*) have been provided.

Evaluate if the human relevance of the proposed MoA can be reasonably excluded on the basis of qualitative/quantitative differences in key events between animals and humans

The two *in vitro* studies in human hepatocytes used cells from one male and one female donor. Silthiofam did not cause hepatocellular proliferation. Although to a lesser extent than in the *in vitro* rat study, silthiofam activated PROD, BROD (2-fold) and BQ in human hepatocytes. In these studies, treatment with EGF resulted in an increase in replicative DNA synthesis, demonstrating the suitability of the system for assessing cell proliferation.

***Table** : Comparative in vitro studies in human and rat wild-type hepatocytes*

	Human hepatocytes				**Male rat hepatocytes**	
	Male donor		**Female donor**			
Concentrations tested (µM)	Silthiofam (1-30 µM)	Phenobarbital (10-1 000 µM)	Silthiofam (1-30 µM)	Phenobarbital (100-1 000µM)	Silthiofam 30-100 µM	Phenobarbital 10-1 000 µM
Cell proliferation (by BrdU incorp.)	-	-	-	-	2×	2×
PROD activity (Cyp2b)	-	2×	-	1.5×	2×	4×
BROD activity (Cyp2b/Cyp3a)	3×	8×	-	1.6×	2×	5×
BQ activity (Cyp 3a)	2×	5×	-	1.9×	5×	4×
CYP2B mRNA	5×	9×	2.5×	3.7×	13×	36×
CYP3A mRNA	4×	9×	1.9×	6.8×	97×	34×

In grey cells, results obtained with silthiofam

These studies showed that the increase in cell proliferation observed in rat was not observed in human donors. One limitation was the use of only one male and one female donor in the *in vitro* experiments. Moreover, in the dossier there is no information on the male donor and only little information on the female donor (a 68-year old female). Overall, these studies showed that there were quantitative differences in the activation of CAR by silthiofam in rats and humans. Indeed, activation of human CAR is lower than rat CAR, and cell proliferation was not detected in human hepatocytes while this was observed in the rat. These two differences are consistent with the lack of relevance of the CAR activation mechanism in humans.

Mode of action of thyroid tumours

These tumours were not considered relevant by the DS based on the increase in hepatic UDGPT observed in the *in vivo* 14-day mode of action rat study. In the absence of data on thyroid hormone levels (TSH, T4, T3) and further mechanistic data to support this hypothesis, thyroid tumours need to be considered relevant to humans.

Mouse

In the mouse carcinogenicity study (50 mice/sex/group), an increase in the incidence of hepatocellular adenoma was observed in females at the top dose (855 mg/kg bw/d) which was above concurrent controls and historical control data from the laboratory or CD-1 mice from Charles River laboratories. The two hepatocellular carcinoma observed at the top dose was inside the historical control data from the laboratory. In this study, no treatment-related effects on survival, clinical signs or body weight was observed. Liver toxicity included a treatment-related increase in absolute and relative liver weight in males and females and

elevated serum AST and ALT in males only at the top dose. Liver hypertrophy was significantly increased in both males and females at the top dose. Additionally, at the top dose, liver foci (mixed cell focus) were significantly increased in males. Although not statistically significant, foci were also observed in females (mixed cells, basophilic and eosinophilic focus). Necrosis (individual cells) was observed in both males and females and was associated with karyomegaly, and cystic degeneration in males.

Table: *Selected neoplastic and non-neoplastic liver findings in mice at terminal sacrifice (mouse carcinogenicity study, 1998)*

	Males					**Females**				
Dose (mg/kg bw/d)	**0**	**1.4**	**13.7**	**141**	**564**	**0**	**2.03**	**20.7**	**203**	**855**
No. of animals	38	41	41	30	34	39	38	28	34	39
Rel. liver wt (% bw)	100	100	96	100	128**	100	102	105	107	117**
Hypertrophy	0	0	0	2	25**	0	0	0	0	12**
Focus - Mixed cell - Basophilic - Eosinophilic	 0 0 0	 - - -	 0 0 0	 0 2 0	 7* 0 0	 0 0 0	 0 0 0	 0 0 0	 0 0 0	 2 2 1
Necrosis	3	-	3	3	40**	5	1	0	3	19**
Cystic degeneration	0	0	0	0	16**	1	0	0	0	1
Karyomegaly	11	-	10	11	36**	6	3	8	5	15
Hepato-cellular adenoma	6	-	4	9	6	2	0	2	0	**10**
Hepato-cellular carcinoma	6	-	2	2	1	0	0	0	0	2

**: statistically significant; In grey cells, bold text: statistically significant according to the DS, not stated in the study summaries available in the DAR and RAR for silthiofam.

The table shows that the increase in the incidence of adenoma correlated with increased body weight, preneoplastic lesions (foci) and liver hypertrophy. Nevertheless, although these non-neoplastic lesion were observed in male rats (with higher incidences than in females), no neoplastic findings were observed.

Tumours were observed in presence of hepatotoxicity, as necrosis was observed in both males and females at the top dose. The DS considered that tumours observed in female mice were mainly secondary to cytotoxicity. RAC further noted that in the 90-day study, centrilobular hypertrophy, vacuolation and degeneration and/or individual hepatocyte necrosis were already observed in males and females at 707/1 132 mg/kg bw/d (males/females, respectively). However, although higher cytotoxicity was observed in males compare to females, no tumours were observed in males and therefore cytotoxicity may not explain the observed tumours. The DS considered that the rat mechanistic data supported the argument that CAR/PXR mechanism may play a role. Nevertheless, this potential MoA is not supported by RAC, as no mechanistic data were available in mice.

Comparison with criteria

Liver tumours have been observed in male rats and female mice. Additionally an increase in thyroid tumours has been observed in male rats.

The increases in the incidences of liver adenoma and carcinoma observed at the top dose in males were not statistically significant and were within the HCD range provided by the laboratory. Although liver toxicity was observed in the study, there was no clear evidence of a link between liver non-neoplastic findings and tumour induction as similar findings were observed in both males and females without tumour induction in females. Based on the mechanistic data available in the dossier, a CAR/PXR mediated effects are plausible although uncertainties have been noted by RAC. Overall, RAC considers this finding of increased top-dose liver male tumours to be insufficient evidence to support classification.

Thyroid follicular adenoma and/or carcinoma in male rats were not statistically significant. Although the tumours were slightly outside the range of laboratory HCD, the incidences did not exceed the HCD incidence for this strain of rat (Charles River HCD). Overall, RAC agrees with the DS that the evidence is insufficient to support classification.

With regard to the mouse liver tumours, no mechanistic data were provided and therefore human relevance cannot be excluded. There is no clear evidence that tumours could be secondary to cytotoxicity. Indeed, tumours where not seen in males having higher liver cytotoxicity. The increases in mouse liver tumours were only observed in females, at the top dose only and did not progress to malignancy. Moreover, silthiofam was not genotoxic. Overall, no classification is proposed based on this type of tumour.

In conclusion, RAC agrees with the DS's proposal **not to classify silthiofam for carcinogenicity.**

4.11 Toxicity for reproduction

Table 50: Summary table of relevant reproductive toxicity studies.

Study	Strain, route	Dose levels	NOAEL	LOAEL	Targets / main effects
Rat 2-gen, Lemen & Ruecker (1998), OECD 416 (1981), GLP.	CD, oral dietary feed.	0, 40, 400, 4000 ppm	Parental, systemic: 400ppm (in both sexes based on effects on the liver and/or adrenal gland (25 and 30 mg/kg bw/day in males and females, respectively [average test substance intake across both generations]) Reproductive: 400 ppm in both sexes (25 and 30 mg/kg bw/day in males and females, respectively).	Parental systemic: 4000ppm (256 and 293 mg/kg bw/day in males and females respectively over 2 generations). Reproductive: 4000 ppm in both sexes.	*Parental*: liver: ↑organ weight, bile duct hyperplasia and hepatocyte vacuolation; adrenal gland: vacuolation of the cortex at the high dose. *Off-spring*: reduced mean pup weights from LD 4-21 at the high dose.

Study	Strain, route	Dose levels	NOAEL	LOAEL	Targets / main effects
Rat developmental, Holson (1996), OECD 414, GLP. All surviving females euthanized on GD20.	Sprague-Dawley Crl:CD BR, oral gavage, a.i. in corn oil.	0, 50, 500 and 1000 mg/kg bw/day, GD6 – GD15			Parental:
Rabbit dose range finding developmental study, Holson (1997), OECD All surviving females euthanized on GD29.	NZW, oral gavage, a.i. in CMC	0, 5, 15, 50, 100 and 150 mg/kg bw/day, GD7 – GD19	Range finding study.	Range-finding study.	Parental: 4/6 and 5/6 females died in the 100 and 150 mg/kg bw/day dose groups respectively. Deaths occurred between GD 13-16 and 15-22 for the gravid females in these respective groups. 2 females from the 100 mg/kg/day dose group aborted on GD25 and GD29. 1 death at the 100mg/kg/day group attributed to intubation error. Clinical signs related to treatment were only seen at the 100 and 150 mg/kg/day doses. No evidence of developmental toxicity was observed at 5-50 mg/kg/day.
Rabbit developmental, Holson (1997), OECD					

4.11.1 Effects on fertility

4.11.1.1 Rat two-generation reproduction dietary study

Two-generation reproduction study of MON 65500 administered in the diet to CD®rats. Lemen, J.K. and Ruecker, F.A. (1998). Report MSL-15554. Guidelines: OECD 416, FIFRA 83-4, EEC Annex 5 Guidelines (Two-Generation Reproduction Study). GLP compliant.

Materials and methods:
Silthiofam, Lot number: SRL-9601-6895-T, purity: 97.4% was administered in the feed at concentrations of 0, 40, 400 and 4000 ppm (30/sex/group). Two generations (F_0 and F_{1A}) were evaluated; one litter was produced per generation. Calculated test substance consumption (mg/kg bw/day) for these dietary levels was, respectively: 0, 2.29, 23.52 and 226.19 for males, and 0, 2.83, 28.37 and 273.39 for females (F_0 generation). Corresponding values for the F_{1A} generation were as follows: 0, 2.65, 26.98 and 286.76 (males), and 0, 3.13, 30.81and 311.83 (females).

Results:
The homogeneity and stability of the dietary preparation was within acceptable limits. The average analytical values of the dietary concentrations were within 95 – 98% of target.

Parental effects

Body weigh/food consumption: Mean body weights were significantly reduced ($p<0.05/p\leq0.01$) in both sexes at the high dose throughout the growth phase. Mean body weight was slightly but significantly reduced in pregnant and lactating females at the high dose throughout gestation and lactation. Body weight gains were not affected (possibly a slight increase), except that body weight loss exhibited between lactation days 14 and 21 by females of the 4000 ppm group was significantly ($p<0.01$) less than controls of both generations. Food consumption was reduced during the growth phase of both sexes and slightly during lactation in both generations. Food consumption was not affected during gestation.

Table 51. Mean body weights of dams

	Female mean body weight gms(%)							
Dose level ppm (numbers)	**Go**	**G7**	**G14**	**G21**	**L0**	**L7**	**L14**	**L21**
0 F0 (19) F1A	325 310	358 334	386 360	474 433	360 342	369 354	380 367	349 356
40 F0 (22) F1A	319 (98) 308(99)	356(99) 336(100)	384(99) 358(100)	478(101) 438(101)	356(99) 336(98)	370(100) 350(99)	390(102) 366(100)	355(102)330(98)
400 F0 (25) F1A	324(100) 311(100)	360(100) 349(101)	394(102) 364(101)	491(104) 435(100)	361(100) 332(97)	378(102) 351(99)	391(103) 366(100)	352(101) 339(101)
4000 F0 (27) F1A	↓*303(93) ↓*280(90)	↓**334(93) ↓*304(91)	↓368(95) ↓*329(91)	↓456(96) ↓410(95)	↓**335(93) ↓**300(88)	↓*347(94) ↓**310(88)	↓**357(94) ↓**329(90)	353(102) 322(96)

*Dunnetts test: * significantly different from control $p \leq 0.05$, ** $p\leq0.01$*

Table 52. Mean body weight change dams.

	Female mean body weight gms(%)					
Dose level ppm (numbers)	**Go-G7**	**G7-14**	**G14-21**	**L0-7**	**L7-14**	**L14-21**
0 F0 (19) F1A	33 24	28 25	88 74	9 8	12 10	-33 -27
40 F0 (22) F1A	36 (111) 27(113)	28(101) 22(88)	94(106) 79(107)	18(150) 13(154)	20(170) 13(138)	-35 -34
400 F0 (25) F1A	34(105) 28(116)	↑*35(135) 24(97)	97(110) 71(96)	17(187) 19(224)	13(110) 10(105)	-39 23
4000 F0 (27) F1A	30(92) 23(96)	↑*34(125) 25(100)	87(100) 81(111)	12.5(140) 10(120)	9(74) ↑18(191)	↓**-3 ↓**-7

*Body weigh loss exhibited LD 14-21 by dams in the 4000 ppm group was significantly less than controls: Dunnetts test: ** $p\leq0.01$*

Clinical signs: A number of mortalities were not considered treatment-related; 2 females from the 40 and 400 ppm groups and one male from the 4000 ppm group. Orange-red urine colour was noted in males of the F_0 animals and in both sexes of the F_{1A} high dose groups. There were no other apparently treatment-related clinical signs in adults or pups of either generation.

Pathology: Terminal body weights were significantly lower in both males and females, F_0 and $F_{1A,}$ ($p\leq0.01$). The liver was identified as a target organ in parental rats of both generations; effects were observed at the 4000 ppm dietary level only, in one or both sexes. Effects on the liver consisted of

increased organ weight and statistically significantly increased incidence ($p<0.01$) of microscopic changes that included the hepatocyte vacuolation and bile duct hyperplasia.

Absolute ovarian weights were increased from 400 ppm in the F_O generation but were not altered in the F_{1A} generation. The apparent increase was within the historical control range (0.1156 – 0.1766 g). Relative ovary weight was statistically significantly increased from 400 ppm in the F_0 and at 4000 ppm in the F_{1A}. This may have been related to the decrease in body weight at this dose level. A statistically significant increase in ovarian cysts occurred at 4000 ppm in the F_0 generation (1,1,1,4). This was not observed in the F_{1A} females (0,0,2,0). These cysts were considered to be follicular cysts which arise from secondary follicles which fail to ovulate or become atretic. This finding was considered incidental to treatment as it did not occur in both generations and had no correlation with reproductive function (5/6 females with cysts successfully mated, beaome pregnant and weaned normal litters).

There was an increased incidence ($p<0.05/p<0.01$) of vacuolation of the adrenal gland cortex at the high dose.

Some effects seen in the kidney may have been treatment-related. Statistically significant increases in relative kidney weight were seen at 4000 ppm in the F_0 animals and from 40 ppm in the F_{1A}. The increases seen at 40 and 400 ppm were less than 10%. Grossly, this organ was of abnormal colour in parental males of both generations. This finding was without concurrent microscopic changes.

Table 53. Summary of relevant microscopic lesions

Dietary level			**0**	**40**	**400**	**4000**
Organ/lesion	**Generation**	**Sex**				
Adrenal						
Vacuolation, cortex	Fo	M	5/30 (17)	5/30 (17)	7/30 (23)	15/39 (50)**
	F1A	M	5/30 (17)	3/30 (10)	4/30 (13)	12/30 (40)**
Liver						
Hyperplasia, bile ducts	Fo	M	0/30	0/30	0/30	13/30 (43)**
	F1A	M	0/30	0/30	1/30 (3)	29/30 (97)**
	F1A	F	0/30	0/30	0/30	13/30 (43)**
	Fo	M	2/30 (7)	1/30 (3)	3/30 (10)	17/30 (57)**
Hepatocellular vacuolation	F1A	M	1/30 (3)	0/30	0/30	28/30 (93)**
Ovaries						
Cysts	Fo	F	1/30	1/30	1/30	4/30 (13)*
	F1A	F	0/30	0/30	2/30	0/30

*Dunnetts test: * significantly different from control $p \leq 0.05$, ** $p \leq 0.01$*

Reproductive: There were no treatment-related effects on mating or fertility indices. An apparent increase in male fertility and pregnancy rates at 400 and 4000 ppm was related to the lower values in the control groups.

Offspring effects

There were no treatment-related effects on litter size, viability or survival in either generation. Mean pup weights were similar at birth, but were significantly lower than controls ($p<0.01$, $p<0.05$) from PN day 4 to PN day 21 at the high dose level. This observation was associated with maternal toxicity at this dose. The (statistically) significant lesser body weight losses in dams of this group from days 14 to 21 of lactation when compared to controls possibly indicates a diversion of nutrients from lactation to maintenance of body mass and thus causing poor thriving of pups. This group had significantly lower mean body weight (but not body weight gain) throughout gestation (Table 51/52).

Reduced mean pup weigh may also coincide with the beginning of ingestion of the chow containing the test material.

Table 54. Summary of relevant reproductive and offspring effects

	Gen	0 ppm	40 ppm	400 ppm	4000 ppm
Females on study (F_1 and F_0)	**F_0** **F_{1A}**	30 30	29 30	30 30	29 30
No. dams pregnant	**F_0** **F_{1A}**	67 63	83 77	83 67	97 83
Insemination index %	**F_0** **F_{1A}**	97 100	93 97	90 93	100 100
Fertility index %	**F_0** **F_{1A}**	69 63	89 79	93 71	97 83
Mean litter size	**F_0** **F_{1A}**	13.7 13.1	15 13.1	14.8 12.6	14 13.2
Duration of pregnancy	**F_0** **F_{1A}**	22.8 22.6	22.8 22.6	22.8 22.5	22.7 22.4
No of pups (total)	**F_0** **F_{1A}**	274(20) 250(19)	365(24) 302(23)	370(25) 252(20)	384(28) 332(24)
No of pups dead	**F_0** **F_{1A}**	7 1	5 5	12 2	10 0
Mean implantation sites per dam	**F_0** **F_{1A}**	Not counted.			
Live birth index	**F_1** **F_2**	97% 99.6	98.6 98.3	96.7 99.2	97.3 100
% males	**F_0** **F_{1A}**	44% 48%	55% 50%	47% 44%	47% 47%
Viability index (PN 4)	**F_0** **F_{1A}**	94 99..8	99 96.9	96.3 98	97.8 99
Mean pup weight during lactation	**F_0** 0 4 7 14 21 **F_{1A}** **0** 4 7 14 28	 7 12.2 18.9 38.7 64 6.7 11.2 17.9 36.3 60.7	 6.8 11.4 18.7 39.7 66 6.8 11.2 17.9 36.4 61.6	 6.8 11.7 19.3 39.8 65.5 6.6 11.1 17.8 35.8 60.9	 6.7 10.9* 17.6** 33.9** 54.1** 6.4 10.1* 15.7* 30** 49.6**
Survival (%) (PND4-21)	**F_0:** **F_{1A}:**	100 100	99 100	100 99	100 100

*Dunnetts test: * significantly different from control $p \leq 0.05$, ** $p \leq 0.01$*

Conclusion:

The primary target organ identified in parental rats from both generations in the reproduction study was the liver; hepatotoxic effects were restricted to the 4000 ppm dietary level and included increases in organ weight, and microscopic changes that included bile duct hyperplasia and hepatocyte vacuolation. The adrenal gland was also identified as a target organ in male rats from both generations

at the 4000 ppm dietary level; vacuolation of the cortex was observed in this organ microscopically. Mean body weights of high dose dams were significantly reduced throughout mating and gestation. This group gained weight normally during gestation and lactation but did not lose body weight from days 14-21 of lactation in contrast to controls and lower dose groups. This probably reflects the reduced mean body weight of these dams throughout treatment.

Pup weights were reduced in both generations at the high dose; the effects were mild, occurred at, or after postnatal day 4 and were considered to be secondary to maternal toxicity and also possibly to the ingestion of treated feed. No effects were observed on reproduction in this study.

The low observed effect level (LOAEL) for systemic and reproductive toxicity was 4000 ppm (256 and 293 mg/kg bw for males and females respectively, averaged over 2 generations) in both sexes based on reduced mean body weight and effects on the liver and/or adrenal gland and reduced mean pup weights from LD 4-21. The no observed effect level (NOAEL) for systemic and reproductive toxicity was 400 ppm (25 and 30 mg/kg bw for males and females respectively, averaged over 2 generations).

Human information:

No data.

4.11.2 Developmental toxicity

4.11.2.1 Study 1: Rat developmental toxicity study

A developmental toxicity study of MON 65500 in rats. Holson, J.F.(1996). Report: WIL 50240 OECD 414, FIFRA 83-3, EEC Part B. Methods for the Determination of Toxicity: Teratogenicity Test (OJ L133, May 30, 1988). GLP compliant.

Materials and Methods:
Silthiofam, Lot number: QPP-9509-6764-T, purity: 91.8% was administered by gavage in corn oil at dose levels of 0, 50, 500 and 1000 mg/kg b.w./day (25 mated rats/group) once daily, from gestation days 6 through 15. All dams were examined twice daily for moribundity and mortality. Clinical observations were recorded daily from GD0 to GD20. Maternal body weights were recorded on days 0, 6, 12, 16, 18 and 20 and mean body weight changes calculated. Gravid uterine weight and net body weight (body weight minus weight of uterus and contents) and net body weight gain were recorded. Liver, kidney and pleen weights were recorded for each dam. The number and location of implantation sits were recorded at necropsy. Foetuses were examined for external, soft-tissue (visceral) and skeletal dysmorphology following laparohysterectomy on gestation day 20.

Results:
Maternal observations

Clinical observations: A single mortality in the high dose group at day 17 of gestation was considered treatment-related. Treatment-related observations included; severe body staining, rocking, lurching and swaying movement, unkempt appearance, dark faeces and lack of faeces. Lethargy, rubbing face on cage and walking on tiptoe were also seen. Other indications of toxicity included severe weight loss and swollen liver. Other animals of the high dose also exhibited some of the clinical signs of toxicity during treatment.

Body weight gain was slightly lower in the high dose animals during days 6-12 of dosing. Thereafter, a significant reduction in body weight gain was recorded for this group with a 37% reduction in weight gain over the treatment period. Mean gravid uterine weight, net body weight and net body weight gain were reduced in the high dose group relative to controls ($p<0.01$). Food consumption followed a similar pattern in this group with a statistically significant reduction in food consumption over the treatment-period.

Table 55. Summary of maternal effects

	Dose levels (mg/kg bw/day) No. of animals			
Parameter	**0 25**	**50 24**	**500 24**	**1000 23**
Mean body wt. during gestation D0 D6 D9 D12 D16 D18 D20	247±10.4 283±13 288±13 303±17 328±16 358±19 392±20	247± 280±13 285±15 299±16 329±17 359±20 393±21	 250±11 284±15 291±15 307±17 331±20 359±20 392±23	 244±11 275±15 277±21 297±20 ↓**304±35 ↓**315±34 ↓**335±43
Mean body wt. change during gestation D0-6 D6-16 D16-20 D0-20	 36±8 46±8 63±8.6 145±16	 33±7 49±8.5 64±6.8 145±16	 34±9 47±6 61±11 142±18	 31±7.5 ↓19±14** ↓29±32** ↓27±23** ↓92±38**
Gravid uterine weight	80.9±10	82.2±10	78.6±14	↓59.5±14**
Net body wt.	311±16	310±16	313±18	↓275±33**
Net body wt. change	64±12	63±11	63±11	↓32±27**
Organ weights Liver (mean±SD) Kidney Spleen	 15.7(1.2) 2.12(0.2) 0.64(0.09)	 16.3(1.2) 2.14(0.15) 0.68(0.09)	 ↑17.97(1.4)* * 2.2(0.2) 0.66(0.1)	 ↑21.8(2.9)** ↑2.28(0.16)** ↑ 0.49(0.13)**

Dunnett's test: * $p\leq0.05$, ** $p\leq0.01$

Necropsy: The 1000 mg/kg bw/day female which died on day 17 was found to have a swollen liver, red vaginal contents and 16 early resorptions *in utero*. Two other high dose animals had white patches on the liver. Mean liver and kidney weights in the 1000 mg/kg bw/day group were increased by 39% and 8%, respectively. Splenic weight was reduced by 23% in this group. These weight changes were considered treatment-related. There were no abnormal findings at other dose levels.

Foetal observations:

Mean foetal weight was statistically significantly reduced in the 1000 mg/kg bw/day group ($p\leq0.01$). Four dead foetuses from two litters were found at 1000 mg/kg bw/day (3 foetuses in 1 litter and 1 foetus in 1 litter)per litter). This finding was likely to be treatment-related even though 3 incidences were within one litter (the WIL historical control incidence was 1 in 127 data sets.). It was reported that no malformations were apparent in all 4 dead fetuses. Other reproductive and foetal parameters were not affected.

Table 56. Summary of mean caesarian data

OBSERVATIONS	**VALUES**			
Dosage (mg/kg bw/day)	**0(control)**	**50**	**500**	**1000**
Dams (N)	25	25	25	25
Females achieving pregnancy (N)	25	24(96)	24(96)	23(96)
Live pups/dam at termination (%)	94(±5.3)	96.9(±4.1)	96.8(±4.8)	94.5(±7.3)
Dead foetuses/dam (%)	0	0	0	**1.2 (±4.2)#**

Sex ratio (m/f) at termination (%)	47.6/52.4	53.9/46.1	50.9/49.1	53.3/46.17
Litter weight at termination				
Foetus weight at termination (mean)	3.5 (±0.2)	3.5(±0.2)	3.6(±0.22)	**2.6(±0.5)****
Implantation sites (mean)	15.8(±1.9)	15.6(±2.2)	14.8(±2.5)	15.1(±1.8)
Corpora lutea (mean)	17.9 (±2.8)	17.8(±2.8)	16.5(±2.1)	16.8(±2.5)
Pre-implantation loss (mean±SD)	10.5(10.9)	10.9(14.3)	10.4(12.7)	9.1(9.7)
Early resorptions (mean)	5.6(±5.3)	3.1(±4.1)	3.2(±4.9)	3.1(±4.7)
Late resorptions (mean)	0	0	0	1.2(±5.7)
Post-implantation loss (mean)	5.6(±5.3)	3.1(±4.13)	3.2(±4.9)	5.5(±7.36)

4 dead fetuses (3/1, 1/1). WIL historical control data: 0.0a. There was a single incidence of a dead foetus at laparohysterectomy in the HC data base which cannot compute due to rounding.

Table 57. Relevant findings (Foetuses/litter and mean percent of litter)

Observation	0	50	500	1000
Malformations: - *foetus/litter (prop % per litter)*				
External				
Umbilical herniation of the intestine[2]	0	0	0	**1/1(0.3±1.39)**
Cleft palate	0	0	0	***9/2 (4.7±20.84)**
Mandibular micrognathia[1]	0	0	0	**1/1 (0.3±1.30)**
Microphthalia and/or anophthalmia	0	0	0	1/1 (0.5±2.61)
Microglossia[1]	0	0	0	1/1 (0.3±1.30)
Vertebral agenesis	1/1(0.3±1.43)	0	0	0
Visceral:				
Situs inversus	1/1(0.2±1.00)	1/1(0.2±1.20)	0	0
Hydrocephaly	0	2/1 (0.5±2.55)	0	0
Retroesophagal aortic arch	0	1/1 (0.2±1.07)	0	0
Historical control data: foetus/litters (proportional % per litter)				
Umbilical herniation of the intestine	5/5 (0.0 – 0.4%)			
Cleft palate	7/7 (00.0-0.3 %)			
Mandibular micrognathia	4/4 (0.0 – 0.4 %)			
Microphthalia and/or anophthalmia	24/22 (0.0 – 1.3 %)			
Vertebral agenesis	3/3 (0.0 – 0.5 %)			
Situs inversus	12/12 (0.0 – 0.7 %)			
Hydrocephaly	12/12 (0.0 – 0.3 %)			
Retroesophagal aortic arch	1/1 (0.0 – 0.3%)			
Skeletal variations				
F Foetus/litter (proportional % of litter)				
-Cervical centrum				
-#1 ossified	79/24 (21%±15)	66/20(18±18)	68/17(19±21)	**↓36/12(10.1±16)****
-Unossified 1,2,3,4 sternebrae	0/0	1/1(0.2±1.2)	0/0	**↑7/5(2±4)**
-7th cervical ribs	1/1 (0.2±1.18%)	0/0	1/1(0.3±1.7%	**↑18/8 (5.1±9)**
Historical control data*: *foetus/litters (% per litter)				
-Cervical centrum #1 ossified	1610/497 (0.0 – 27.6%)			
-Unossified 1,2,3,4 sternebrae	105/97 (0.0 – 3.5%)			
-7th cervical ribs	174/143 (0.0 – 5.6%)			
Malformations (% litters (%per litter))				
-external	4.0	0.0	0.0	↑17.4 (5.2)[1]
-soft tissue	4.0	8.3	4.2	0.0 (0.0)
-skeletal	0.0	4.2	0.0	↑4.3 (0.3)
Total	8.0	8.3	4.2	↑17.4 (5.5)
Variations (% per litter)				
-external	0.0	0.0	0.0	0.0
-soft tissue	0.2 (±1)	0.0	0.0	1.1(±5.2)
-skeletal	25.2(±14.8)	24.3(±19.2)	23.3(±20.5)	↑35.6(±25.6)
Total	25.4(±14.89)	24.3(±19.2)	23.3(±20.5)	↑35.6(±25.6)

[1] Mandibular micrognathia with microglossia (1/1:litter 43125)
*Cleft palate (9/2:litter 43109 (1) and litter 43188 (8))
Microphthalmia/anophthalmia (1/1: litter 43188)
[2]Umbilical herniation of intestine (1foetus/1litter: litter 43106)

Historical control data: WIL Laboratories CR COBS CD rats 1982 – 1994

127 studies (approximately 2843 litters/40497 foetuses)).

External malformations:
One control foetus and 11 high dose foetuses had external malformations. 8/9 foetuses with cleft palate were from a single litter. Note: One of these foetuses also had microphthalmia, one had a major blood vessel variation (no brachiocephalic trunk) and all had unossified entire sternum. The high incidence (cluster) of cleft palate in this litter was considered by the study author to be related to developmental delay, as demonstrated by the severely reduced mean body weight in this group. However, current interpretation of the occurrence of severe and specific malformations, which are rare in the relevant historical data (such as cleft palate in CRL(CD)SD rats) as a result of maternal toxicity), does not support a causative effect (Hood, R., and Rogers, J, 2012). The single high dose litter in which all fetuses were malformed or resorbed may not to be attributable to treatment and may reflect a total failure of foetal development in this dam. The remaining single occurrence of cleft palate occurred at the same dose level. The incidence per litter is 0.3%, which is within the historical control range (0.0 to 0.3% per litter) if the cluster litter is excluded. If the cluster of cleft palates in a single litter is counted however, then there are two treatment-related occurrences with a proportional per litter % of 4.6). Single incidences of umbilical herniation of the intestine and microglossia combined with micrognathia were observed in separate litters at 1000 mg/kg bw/day. These may occur within the HC data, but are both rare.

Soft tissue malformations:
Hydrocephaly (2/1) at 500 mg/kg bw/day; retroesophageal aortic arch (1/1) at 50 mg/kg bw/day; *situs inversus* (1/1) at 50 mg/kg bw/day and (1/1) in the controls. Major blood vessel variations/anomalies were noted in one control (right carotid and subclavian arteries arise from the aorta without the brachiocephalic trunk) and the same finding in 1/1 litters at 1000 mg/kg bw/day. A single foetus at 1000 mg/kg bw/day had an accessory spleen. These were not considered treatment-related.

Skeletal anomalies:
The following were noted in 1/1 foetuses at 50 and 1000 mg/kg bw/day : absent or malpositioned thoracic arches and centra and absent rib. There was a significant reduction in ossification of the cervical centrum from 21.7% in the control group to 10.6% in the 1000 mg/kg bw/day group ($p \leq 0.01$). This was considerable lower than concurrent controls; at the lower end of the historical control data presented (9.9-27% per litter) but likely to be related to the reduced foetal weights at this dose. There was also a reduction in ossification of sternebrae 1, 2, 3, and 4 in the high dose litters (2.0% compared to 0.0% per litter in controls) which may be related to treatment. This was at the upper margin of the historical control range (0.0 to 3.5% per litter) and consistent with reduced foetal weights. It is noted that all fetuses with cleft palate in litter 43188 also had entire sternum unossified giving rise to an overall increase in incidence for the group. An increased incidence of 7th cervical rib (pinpoint to intermediate, generally unilateral) observed at the high dose (mean % litter 5.1% (control 0.2%)) was considered treatment-related (historical control range: 0.0-5.6% per litter with 1/127 data sets having an incidence greater than 5.1%). This finding is described as a variation. It can be considered an anomaly in rats where for conformational reasons it does not appear to cause problems post-natally; an extra cervical rib can have serious adverse effects when it occurs in humans *(Thoracic Outlet Syndrome TOS), (Paumgarten, 2011. An Introduction to problems of postnatal anomalies regarding their integration into new terminology and classification (2011). In: 7th Workshop on the Terminology in Develomopental Toxicology, Federal Institute of Risk Assessment – Berlin, Germany; May 4-6, 2011)).*

Conclusion

The 1000 mg/kg bw/day dose level employed in this study caused considerable maternal toxicity as demonstrated by; mortality (1); clinical signs; significant effects on body weight/weight gain, food consumption and organ weights. Some evidence of maternal toxicity was also observed at the 500 mg/kg bw/day dose level by an increase in liver weight.

Developmental toxicity was observed at the 1000 mg/kg bw/day dose level. A number of observations were considered treatment-related. There was a clear reduction in foetal weight (74% of control value). A reduction of ossification of the cervical centrum and also of the 1, 2, 3 and 4 sternebrae was observed and was probably related to general developmental retardation. An increase in 7th cervical rib was seen which could be interpreted as significant to human health. An increase in the number of dead fetuses, a rare finding in the rat, was also observed at the 1000 mg/kg bw/day dose level. An increased incidence of a single malformation (cleft palate) was within the historical control data values; if a second litter with multiple occurrences is disregarded. All of the developmental effects observed in this study occurred at a dose level which caused severe maternal toxicity. Endpoints which may be related to maternal toxicity were reduced ossification and foetal weights. The relationship to treatment of specific rare events such as cleft palate and dead fetuses is more difficult to elucidate; even at very high doses such as 1000 mg/kg bw. The increased incidence of 7th cervical rib, a relatively frequently observed finding in the HC data, is likely to be treatment-related and not without some relevance to man.

4.11.2.2 Study 2a: Rabbit dose-range finding developmental toxicity study

A dose-range finding developmental toxicity study of MON 65500 in rabbits. *Anon.* (1997a), Report WI-95-239. Guidelines OECD 414, FIFRA 83-3, EEC Part B. Methods for the Determination of Toxicity: Teratogenicity Test (OJ L133, May 30, 1988). GLP compliant with the following exception: assays to verify the concentration, stability and homogeneity of the test substance in the carrier were not performed.

Materials and Methods:
Silthiopham, Lot number: QPP-9509-6764-T, purity: 91.8% was administered by gavage in 1% carboxymethylcellulose/0.1% polysorbate 80 at dose levels of 0, 5, 15, 50, 100 and 150 mg/kg bw/day (6 artificially inseminated rabbits/group) daily, from gestation days 7 through 19. Foetuses were examined for external dysmorphology following laparohysterectomy on gestation day 29.

Results:

Maternal observations:
Four of six and 5/6 females died in the 100 and 150 mg/kg/day dose groups, respectively. Deaths occurred between days 13-16 and 15-22 for gravid females in these respective groups. All deaths except for one intubation error in the 100 mg/kg group were considered treatment-related. The two other females in the 100 mg/kg group aborted prior to scheduled euthanasia. Clinical signs in decedents and/or survivors from the affected groups were hypoactivity/lethargy, faecal changes, dried or wet staining of body surfaces. Mean body weight losses occurred from 100 mg/kg during GD 7-19 (specifically 10-13 ($p \leq 0.01$@ 150 mg/kg) and 13-19). A trend in body weight loss continued post treatment. Food consumption was reduced over the treatment period (specifically GD 10-13 and 13-19) with a trend for reduced food consumption continuing post treatment. One rabbit in the 100 mg/kg and two in the 150 mg/kg groups had red fluid contents in the urinary bladder that were considered treatment-related. This was apparently also observed at 150 mg/kg in non-pregnant rabbits in a 5 day study (not reported). The single surviving dam in the 150 mg/kg dose group had an elevated liver and reduced spleen weight relative to controls.

Table 58. Summary of relevant data

Endpoint	Results
Mortality	100 mg/kg bw/day: 4 females died; gestation days 13-16 150 mg/kg bw/day: 5 females died; gestation days 15-22
Clinical observations	≥100 mg/kg bw/day: hypoactivity/lethargy, decreased defecation, discoloured faeces and/or staining of body surfaces/cage bedding
Body weight	≥100 mg/kg bw/day: body weight losses during treatment and post-treatment periods
Food consumption	≥100 mg/kg bw/day: food consumption reduced during treatment and post-treatment periods
Organ weights	150 mg/kg bw/day: increased liver weight and decreased spleen weight in the single surviving female
Gross pathology	≥100 mg/kg bw/day: red fluid contents in urinary bladder, dark red contents in stomach, caecum and/or trachea; decedents only
Number of gravid rabbits	5/6, 5/6, 6/6, 4/6, 5/6 and 5/6 rabbits in the 0, 5, 15, 50, 100 and 150 mg/kg bw/day groups, respectively
Laparohysterectomy/Foetal examination	No effects observed

Uterine parameters were not affected up to 60 mg/kg bw and gravid uterine weights were unaffected. There were no surviving 100 mg/kg dams and only one litter from the 150 mg/kg bw group. No malformations or variations were observed in any foetuses examined.

Conclusion
The 100 and 150 mg/kg bw/day dose levels employed in this study exceeded the maternal MTD based on mortality, clinical signs, effects on body weight gain, food consumption and/or organ weights. No maternal toxicity was observed at dose levels of <50 mg/kg bw/day.

A sharp dose-response with respect to lethality was identified between 50 and 100 mg/kg bw/day. The evaluation of developmental endpoints was limited at ≥ 100 mg/kg bw/day due to the low number of surviving dams. This data was used to select dose levels of 5, 20 and 60 mg/kg bw/day for the main developmental toxicity study.

4.11.2.3 Study 2b: Rabbit developmental toxicity study

Reference: *Anon.* (1997b): A developmental toxicity study of MON 65500 in rabbits.
Report: WI-96-105
Guidelines: OECD 414, FIFRA 83-3, EEC Part B. Methods for the Determination of Toxicity: Teratogenicity Test (OJ L133, May 30, 1988)
GLP: Yes.

Materials and Methods
Silthiofam, Lot number: QPP-9509-6764-T, purity: 91.8% was administered by gavage in 1% carboxymethylcellulose/0.1% polysorbate 80 at dose levels of 0, 5, 20 and 60 mg/kg bw/day (20 artificially inseminated rabbits/group) daily, from gestation days 7 through 19. Dose levels were chosen on the basis of the results of the preceeding range-finding study in which a steep dose response was apparent for maternal toxicity between the dose levels of 50 and 100 mg/kg bw/day. The high dose (60 mg/kg) was chosen to exceed the NOAEL for maternal toxicity and to avoid excessive mortality; which would preclude assessment of developmental toxicity. Maternal toxicity was evaluated through clinical observations, body weights, and food consumption. Liver, kidney and spleen were weighted at sacrifice. Foetuses were examined on gestation day 29 for external, soft-tissue (visceral) and skeletal dysmorphology following laparohysterectomy.

Results:

Maternal observations:
There were no treatment-related clinical findings. A single mid-dose dam aborted. Periodic statistically significant fluctuations in maternal weight parameters occurred but were not considered treatment-related. Some alterations in food consumption before and during treatment were inconsistent and not considered treatment-related. No abnormalities noted at necropsy were considered treatment-related and liver, kidney and spleen weights were unaltered. There was no effect on corpora lutea, implantation sites or post-implantation loss.

Table 59. Summary of maternal findings

Parameter (means)	**Mean gravid uterine weight and body weight change (gms)**			
	0	**5**	**20**	**60**
Initial body weight	3237± 140	3250 ±198	3241± 179	3185± 1553
Terminal body weight	4143± 250	4089± 320	4122± 271	3979 ±255
Gravid uterine weight	326 ±147	347± 151	405 ±146	357 ±157
Net body wt	3817± 247	3741± 334	3717± 323	3622 ±259
Net body weight change	580± 213	491 ±236	476 ±262	437± 227
Liver weight	135± 20	127± 23	128 ±21	125 ±21
Kidney weight	21 3±	19± 2.6	18± 2	19 ±1.8
Spleen weight	2.12 ±0.4	1.8 ±0.45	2 ±0.35	1.75± 0.4

Foetal observations:

Reproductive parameters were unaffected by treatment (post-implantation loss, number of viable foetuses, foetal body weights, sex ratios, numbers of corpora lutea and implantation sites). A number of external, soft tissue and skeletal malformations were identified; (foetuses (litters)) (5(4), 2(2), 4(3) and 3(2) at 0, 5, 20 and 50 mg/kg bw/day, respectively. These occurred across the groups and no treatment-related trends in type or numbers of malformations were identifiable. Foetal developmental variations occurred similarly in the control and treated groups and no relationship to treatment was apparent.

Table 60. Summary of developmental/foetal effects.

OBSERVATIONS	**VALUES**			
Dosage (mg/kg bw/day)	**0(control)**	**5**	**20**	**60**
Dams (N)	20	20	20	20
Females achieving pregnancy (N)	12	18	14	13
Live pups/dam at termination (%)	84(±29)	89(±25)	90(±17)	98(±4)
Dead foetuses/dam (%)	0	0	0	0
Male pups termination (%)	57	41	43.7	53
Litter weight at termination				
Foetus weight at termination (mean)	49.6 (±4)	51(±5)	49(±4)	48(±6)
Implantation sites (mean)	5.6(±2)	5.3(±2.3)	6.8(±2.5)	5.8(±3)
Corpora lutea (mean)	9.4 (±3)	9.3(±4)	10.2(±2.9)	10(±4)
Pre-implantation loss (mean±SD)	38.5(±23)	40.7(±24.3)	32.8(±17.3)	42.9(±22)
Early resorptions (mean)	16(±29)	10(±25)	9.1(±15)	1.7(±4)

OBSERVATIONS	**VALUES**			
Dosage (mg/kg bw/day)	**0(control)**	**5**	**20**	**60**
Late resorptions (mean)	0	0.6 (±2.3)	0.9 (±3)	0
Post-implantation loss (mean)	16(±29)	11(±25)	10(±17)	1.7(±4)
Malformations: foetuses(litters)				
-external	2(2)	1(1)	0	1(1)
-soft tissue	2(1)	0	1(1)	1(1)
-skeletal	2(2)	1(1)	4(3)	2(2)
Total	5(4)	2(2)	4(3)	3(2)
Variations (% per litter)				
-external	0.0	0.0	0.0	0.0
-soft tissue	31 (±18)	25(20)	19(18)	27.6(±30)
-skeletal	67(±24)	64(±34)	70.3(±34)	68(±23)
Total	76(±23)	71(±32)	72(±34)	79(±24)

Conclusion:
There was no maternal or developmental toxicity apparent in this study. The high dose of 60 mg/kg /day was an NOAEL for both maternal and developmental toxicity.

4.11.3 Other relevant information

None.

4.11.4 Summary and discussion of reproductive toxicity

Reproduction/fertility
The liver was the primary target organ identified in parental rats from both generations in the reproduction study; hepatotoxic effects were restricted to the 4000 ppm dietary level and included increases in organ weight, microscopic changes including bile duct hyperplasia and hepatocyte vacuolation. The adrenal gland was also identified as a target organ in male rats from both generations at the 4000 ppm dietary level; vacuolation of the cortex was observed in this organ microscopically. Mean body weights of high dose dams were significantly reduced throughout mating and gestation. This group gained weight normally during gestation and lactation but did not lose body weight from days 14-21 of lactation in contrast to controls and lower dose groups. This probably reflects the reduced mean body weight of these dams throughout treatment.

Pup weights were reduced in both generations at the high dose. The effects were mild, occurred at or after postnatal day 4, and were considered to be secondary to maternal toxicity. They may also have been related to the ingestion of treated feed. No effects were observed on reproduction in this study.

The low-observed-effect-level (LOAEL) for systemic and reproductive toxicity was 4000 ppm in both sexes. This was based on reduced mean body weight and effects on the liver and/or adrenal gland (226 and 311 mg/kg bw/day in males and females, respectively (average test substance intake across both generations)).

The low-observed effect level for reproductive toxicity (LOAEL) was 4000 ppm in both sexes based on reduced mean pup weights from LD 4-21.

Developmental toxicity
Developmental toxicity was investigated in the rat and in the rabbit. In the rat study, the 1000 mg/kg bw/day dose level employed in this study caused considerable maternal toxicity as demonstrated by

mortality, clinical signs and significant effects on body weight/weight gain, food consumption and organ weights. Some evidence of maternal toxicity was also observed at the 500 mg/kg bw/day dose level by an increase in liver weight.

Developmental toxicity was observed at the 1000 mg/kg bw/day dose level. A number of observations were considered treatment-related. There was a clear reduction in foetal weight (74% of control value). A reduction of ossification of the cervical centrum and also of the 1,2,3 and 4 sternebrae was observed and was probably related to general developmental retardation. An increase in 7th cervical rib was seen which could be interpreted as significant to human health. An increase in the number of dead foetuses, a rare finding in the rat, was also observed at the 1000 mg/kg bw/day dose level. Cleft palate, a rare finding, occurred in two litters; one foetus (1/15) in one litter and 8 (8/11) in a second. An increased incidence of a single malformation (cleft palate) was within the historical control data values. However, the occurrence of a second litter with multiple malformed pups, including a high incidence of cleft palate, may contribute to an argument for a treatment-related adverse effect. All of the developmental effects observed in this study occurred at a dose level which caused severe maternal toxicity.

A range-finding study was conducted in the rabbit in which the higher doses employed (100 and 150 mg/kg bw/day) exceeded the maternal MTD based on mortality, clinical signs and effects on body weight gain, food consumption and/or organ weights. No maternal toxicity was observed at dose levels of ≤50 mg/kg bw/day.

A sharp dose-response with respect to maternal lethality was identified between 50 and 100 mg/kg bw/day. There were no adverse effects on developmental parameters up to 50 mg/kg bw. The evaluation of developmental endpoints was limited at ≥ 100 mg/kg bw/day due to the low number of surviving dams (1 at 100 mg/kg, 0 at 150 mg/kg bw). This data was used to select dose levels of 5, 20 and 60 mg/kg bw/day for the main developmental toxicity study. There were no effects on maternal animals up the top dose of 60 mg/kg bw. Foetal and developmental endpoints were not affected in this dose range. Therefore the test substance goes from no effect to fully lethal within the range of 60 – 100 mg/kg bw, making the assessment of specific developmental toxicity difficult within this range. The NOAEL for both maternal and developmental toxicity was 60 mg/kg bw.

4.11.5 Comparison with criteria

According to the criteria for Regulation (EC) 1272/2008, classification may be required where a substance causes an adverse effect on sexual function/fertility in adult males or females or/and an adverse effect on normal development in the offspring.

Sexual function/fertility:

On the basis of the data presented above for silthiofam there is no evidence for an adverse effect on parameters of sexual function or fertility as defined by the criteria. The highest dose tested (4000 ppm/287-312 mg/kg bw) was significantly toxic to the parental animals but without effect on reproductive parameters. At this dose, mean pup weights were reduced compared to controls only from lactation day 4. This effect is more likely to reflect maternal toxicity and possibly also ingestion of treated feed. Classification for sexual function/fertility is therefore not proposed.

Development:

Some significant adverse effects on development were seen at the highest dose tested (1000 mg/kg) in the rat, which was also clearly toxic to the maternal animal and above the MTD. The effects observed were not considered sufficient to propose a Repr. 1 classification. Reduced foetal weight, reduced ossification of centrum number 1 and sternebrea 1-4, increased 7th cervical rib and increased number of dead fetuses were considered related to treatment at this high dose. A rare finding, cleft palate, occurred in two litters; one foetus (1/15) in one litter and 8 (8/11) in a second. The incidence of 1/1 (0.3% per litter) is within the historical control data; however, the occurrence of a second litter with multiple malformed pups including a high incidence of cleft palate, may contribute to an argument for a treatment-related adverse effect.

Criteria for Repr. 2 reproductive toxicant (development):

Substances are classified in Category 2 for reproductive toxicity when there is some evidence from humans or experimental animals, possibly supplemented with other information, of an adverse effect on sexual function and fertility, or on development, and where the evidence is not sufficiently convincing to place the substance in Category 1. If deficiencies in the study make the quality of evidence less convincing, Category 2 could be the more appropriate classification.

Such effects shall have been observed in the absence of other toxic effects, or if occurring together with other toxic effects the adverse effect on reproduction is considered not to be a secondary non-specific consequence of the other toxic effects.

The effects on development in the rat reported above occurred in the presence of significant maternal toxicity. Some effects could be directly attributed to this, i.e., reduced foetal weight (possibly), reduced ossification and the increase in incidence of a commonly occurring skeletal anomaly - 7th cervical rib. The occurrence of cleft palate in two litters raises the possibility of a treatment-related effect which is difficult to dismiss. In addition, there was an increase in dead pups in rats at caesarean section which was practically unknown in the historical data provided.

According to the criteria, classification with Repr. 2 is foreseen for such a situation.

4.11.6 Conclusions on classification and labelling

No classification is considered warranted for sexual function and fertility.

Classification for development [Repr. 2; H361d] is proposed on the basis of adverse effects on rat development; reduced foetal weight, increased 7th cervical rib, cleft palate, and increase in numbers of dead pups at doses causing maternal toxicity. A high dam mortality rate with a steep dose response made assessment of developmental toxicity difficult in the rabbit. It is not possible to conclude whether the effects on development in the rat are related to maternal toxicity or not; therefore classification with Repr. 2 should be considered.

RAC evaluation of reproductive toxicity

Summary of the Dossier Submitter's proposal

Fertility

The DS based its evaluation on a 2-generation reproductive toxicity study in rats (GLP-compliant, OECD TG 416) from 1998. In this study, no effects on parameters relevant for sexual function and fertility were observed. Therefore, no classification was proposed by the DS.

Developmental toxicity

Two developmental toxicity studies were considered by the DS, one in rats and one in rabbits.

In the range-finding developmental toxicity study in rabbits, dose levels exceeded the maternal maximum tolerated dose at the two highest dose levels (lethality). No maternal toxicity was observed at the next lower doses (≤ 50 mg/kg bw/d). In this study no malformations or variations were observed. Nevertheless, the evaluation of developmental toxicity was limited at 100 and 150 mg/kg bw/d due to the low number of litters (only one litter from the 150 mg/kg bw/d group).

In the main developmental toxicity rabbit study, no maternal toxicity was observed up to the top dose of 60 mg/kg. No developmental effects were observed at this dose range. Nevertheless, assessment of specific developmental toxicity was difficult in rabbits as the test substance went from no maternal effect at 60 mg/kg bw/d to fully lethal at 100 mg/kg bw/d.

In the rat developmental study, significant adverse effects on development were seen at the top dose level of 1 000 mg/kg bw/d:

- Statistically significantly reduced foetal weight (↓ 25 %);
- Skeletal variations: reduced ossification of centrum number 1 and sternebrae 1-4; increased 7th cervical rib;
- Increased incidences of dead foetuses (very rare in historical control data);
- External malformations: cleft palate observed in two litters: 1/15 foetuses and 8/22 foetuses. One foetus per litter was within historical control data. Nevertheless, the occurrence of a second litter with cleft palate raised the possibility of a treatment-related effect.

This top dose was clearly toxic to dams (one death, clinical signs, significant decrease in net body weight and food consumption, liver and kidney toxicity). The DS considered that some of the above-mentioned findings may have been secondary to maternal toxicity (delayed ossification, reduced weight, and skeletal variations) but that the cleft palate and dead foetuses occurring in two litters may have been treatment-related. Thus, the DS proposed to classify silthiofam as Repr. 2; H361d.

Comments received during public consultation

Fertility

One MS commented that the control in the 2-generation study should be considered invalid as fertility was lower than 70 % in this group. The DS still considered the study valid as no effects on treatment groups were observed.

Developmental toxicity

One MS supported Repr. 2, H361d but asked for individual data to better understand whether dams where foetal mortality and cleft palate occurred were particularly affected by treatment. The MS also asked if historical control data for the cluster of litters with cleft palate were available. The DS responded that markedly reduced weight and ossification were observed in the litters having cleft palate and marked maternal toxicity above the maximum tolerated dose (MTD) was observed in the dams. The DS reviewed the published papers provided during public consultation by industry supporting the argument that cleft palate could be associated with foetal weight retardation but highlighted that marked toxicity is not always associated with cleft palate caused by a non-specific mechanism. HCD on a cluster of malformations were not available but the DS pointed out that on a litter basis, 2 litters were outside the range of historical control data supplied. The DS also noted that the severity of the effects may warrant classification but that a strong argument can also be made for non-classification.

One MS requested more information on the malformations occurring in the rabbit study before taking a position on classification. The DS provided tabulated data for the rabbit developmental toxicity study but did not considered this study to be relevant for classification.

One MS supported the classification as Repr. 2 but further noted that classification for effects on or via lactation need to be considered based on the clear decreases in pup weight from GD 4 onwards in the 2-generation study from both generations. Moreover, the MS noted that based on the physico-chemical properties of the substance (e.g. log P_{ow}), transfer to milk may be possible.

Two comments from industry disagreed with the proposal to classify silthiofam as a reproductive toxicant and provided additional references on the link between maternal toxicity and cleft palate. The marked toxicity observed in dams of which the litters exhibited cleft palate and dead foetuses explained the observed effects. One of the industry comments also noted that although the incidences of dead foetuses were increased (not statistically significant), no concurrent decrease in viable foetuses and post-implantation losses were noted. The DS responded that based on severe and rare malformations in conjunction with pup deaths and the increased incidence of 7th cervical ribs, classification is warranted. Nevertheless, the DS acknowledged that maternal and foetal toxicity is relevant and important to the discussion of the proposal for classification in category 2.

Additional key elements

Individual data have been provided by industry during public consultation. Selected findings are reported in the table and figure below.

Table: Description of the malformations in the rat prenatal developmental toxicity study

Dose (mg/kg bw/d)	Litter	Description (litter number)	Maternal toxicity
0	43166	1 foetus with situs inversus	-
	43201	1 foetus with vertebral agenesis	-
50	43164	1 foetus with vertebral anomaly with or without associated rib anomaly	-
	43185	1 foetus with retroesophageal aortic arch	-
500	43165	2 foetuses with hydrocephaly	-
1 000	43106	1 foetus with umbilical herniation of intestine, present in HCD	-
	43125	1 foetus with mandibular micrognathia with microglossia 1 foetus with vertebral anomaly with or without associated rib anomaly	↓ net bw (↓ 10 % compare to mean observed in controls), Hair loss
	43188	8/11 foetus with cleft palate and evidence of delayed ossification (e.g. complete absence of ossification of the entire sternum). In one of these 8 foetuses with cleft palate, micropthalmia was also evident. In this litter other foetuses had late resorptions 3/11.	↓ net bw (↓ 13 % compare to mean observed in controls), ↓ net bw gain (66 % of control mean) ↓ food consumption (27 %, GD0-20 compare to mean of controls)
	43149	1 foetus with cleft palate	↓ net bw (↓ 26 % compare to mean observed in controls), ↓ food consumption (↓ 27 %, GD0-20 compare to mean of controls) Brown staining of neck, abdominal, forelimb, hind limb, inguinal, urogenital and anogenital area, red/brown material around nose and mouth, lacrimation, decreased defecation, dark faeces, rocking, swaying.
	43196	one dead foetus without apparent malformations, 0 in historical controls	↓ net bw (↓ 13 % compare to mean observed in controls) ↓ food consumption (↓ 27 %, GD0-20 compare to mean of controls) Hair loss
	43162	3 dead foetuses, without apparent malformations, 0 in historical controls	↓ net bw (↓ 30 % compare to mean of the control group), ↓ food consumption (↓ 27 %, GD0-20 compare to mean of controls) Brown staining of forelimb, anogenital and urogenital area, drooping eye lids, red material around eyes, dark faeces, rocking, swaying. Brown matting, liver white area at macroscopic examination

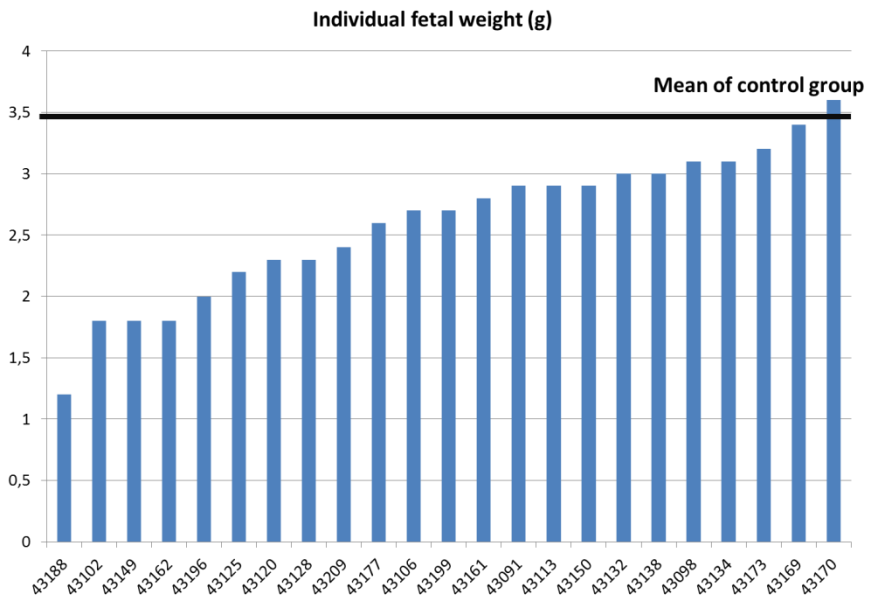


Figure: Individual foetal weight in the 1 000 mg/kg bw/d dose group

Assessment and comparison with the classification criteria

Fertility

Effects in the multigenerational study, available in rats, which could be possibly linked to fertility, were changes in absolute ovary weight in F0 rats which were within historical control ranges and in relative weight in both F0 and F1 generations. Moreover, in F0 an increased in ovarian cysts was observed at the top dose level (but not in F1). As the effects did not correlate with fertility effects, these changes are not considered to be of sufficient concern for classification. No effects were observed in the reproductive organs in other repeated-dose toxicity studies in any species (rats, dogs or mice).

Small prostate and seminal vesicles were found at the top dose in the rat 90-day repeated-dose toxicity study but they occurred in presence of marked general toxicity and were not observed in other studies.

In conclusion, RAC agrees with the DS's proposal of no classification for fertility.

Developmental toxicity

In the developmental prenatal toxicity study performed in rabbits, no effects relevant for classification were observed. Nevertheless, due to severe toxicity (mortality), developmental toxicity may not have been identified.

In the rat developmental toxicity study, three main findings were highlighted by the DS: severe malformations (cleft palate, dead foetuses) and increased incidence of the 7th cervical rib (variations).

Cleft palate

The increase in malformations was primarily due to cleft palates (entire length). This severe malformation was observed in 8 out of 11 foetuses in one litter and in one foetus in a second litter. The incidence was above the HCD on both a foetus and litter basis (max: 7 foetuses in 7 litters; 0-0.3 % per litter). Eight out of the 9 foetuses were clustered to a

single litter. Moreover, in this litter, all foetuses were malformed or late resorptions. This may reflect a total failure of foetal developmental in this dam. According to the study authors, these malformations were related to developmental delay as demonstrated by the severely reduced foetal body weight in this litter (46 % of the dose group mean and 33 % of the control group mean). Moreover, cleft palates were associated with reduced ossification (absence of ossification of the entire sternum).

Dead foetuses

Dead foetuses were observed in two litters at 1 000 mg/kg bw/d. No dead foetuses were reported either in the control and above the historical control data provided (0-0.3 % per litter). In these litters a low mean weight of the foetuses was noted (↓ 43-49 % of mean of controls).

7th cervical ribs

An increase in the 7th cervical ribs (pinpoint to intermediate) was observed at 1 000 mg/kg bw/d. This type of variations was observed in 13 foetuses in 7 litters (maximum 4 per litter, 5.1 % per litter) which was inside the highest value observed in the HCD (9 foetuses in 6 litters, 5.6 % per litter).

Maternal toxicity

At 1 000 mg/kg bw/d, considerable maternal toxicity was observed, exceeding the MTD, including mortality (one death), clinical signs, and significant effects on body weight and body weight gain, food consumption and organ weight changes (e.g. liver). Nevertheless, in some dams with comparable marked general toxicity (litter 43102), no malformations, dead foetuses or 7th cervical ribs were observed suggesting that maternal toxicity might not completely explain the occurrence of malformation at this dose level.

Comparison with criteria

RAC agrees that reduced foetal weight, reduced ossification and increased incidence of variations (7th cervical ribs) may be explained by the marked maternal toxicity observed at 1 000 mg/kg bw/d. There is some concern from potential developmental effects such as cleft palate and dead foetuses observed at the high dose level in rats. Nevertheless, cleft palates were mainly clustered in one dam that had total failure in foetal development. The occurrence of one cleft palate in the other litter is insufficient for classification. With regard to dead foetuses, although rarely occurring, this finding may have been secondary to the high maternal toxicity observed in the dams.

Overall, RAC considers that **no classification is warranted for developmental toxicity**.

Effects on or via lactation

In the 2-generation rat study, a reduction in mean pup weight (over LD4-21) was observed in both generations at the top dose that may indicate an effect on or *via* lactation (> 10 %, see table 54 of CLH report). At this dose, reduced body weight was observed but body weight loss was statistically significantly less than controls in both generations between lactation days 14-21. No data are available in the concentration of silthiofam and its metabolites in the milk. ADME data showed a wide distribution of silthiofam including fat suggesting that transfer to milk may be possible. Nevertheless, the reduced mean pup

weight may also coincide with the beginning of ingestion of the chow containing the test material. Therefore, **no classification is proposed for lactation**.

4.12 Other effects

4.12.1 Non-human information

4.12.1.1 Neurotoxicity

No specific acute or subchronic neurotoxicity studies are available for Silthiofam. Some clinical signs indicative of potential neurotoxicity were observed in the rat developmental study such as lethargy and gait changes (rocking, lurching, swaying during walking), but only against a background of marked general toxicity. There is no firm evidence of specific neurotoxicity.

4.12.1.2 Immunotoxicity

No evidence for a specific effect on the immune system.

4.12.1.3 Specific investigations: other studies

No data.

4.12.1.4 Human information

No data.

4.12.2 Summary and discussion

4.12.3 Comparison with criteria

Not relevant.

4.12.4 Conclusions on classification and labelling

Not required.

5 ENVIRONMENTAL HAZARD ASSESSMENT

5.1 Degradation

5.1.1 Stability

Note: Company designation MON 65500 = the active substance Silthiofam.

Table 61: Summary of Degradation

Method	Results	Remarks	Reference
Hydrolysis			
(^{14}C)-MON 65500: Hydrolytic Stability *EEC Method C.7 and EPA Pesticide Assessment Guidelines, Subdivision N, paragraph 161-1 (October 1982).*	Silthiofam is relatively stable hydrolytically at alkaline pH but is unstable in acidic conditions. The hydrolysis half-lives for silthiofam at 25°C ranged from 45 hours (pH 4) to 448 days (pH 7). The hydrolysis half-life at 25°C for pH 9 was 314 days. Using Arrhenius plots, the half-lives at 20°C were estimated to be 73 hours for pH 4 and 77 years for pH 9. Therefore silthiofam is rapidly hydrolysed at pH 4 and 20°C but can be considered to be stable at pH 9 and 20°C. The principal hydrolysis product of silthiofam was CP 240659, resulting from acid-catalysed desilylation of the parent molecule. This metabolite was also shown to form hydrolytically in acidic soils. There was no evidence of any significant degradation of CP 240659 during the hydrolysis study with Silthiofam.	The study has been evaluated for the previous inclusion into the Annex I of the Directive 91/414/EEC. Performed in accordance with GLP (excluding sterilisation procedure and sterility measurements which were respectively performed by Isotron PLC, Bradford, West Yorkshire, and Grange Laboratories, Wetherby, West Yorkshire, which laboratories are not included in the UK GLP Compliance Programme)	Lewis, C.J. (1996b): (^{14}C)-MON 65500: Hydrolytic Stability. Corning Hazleton (Europe), Report no.: 64/55-1015. Not published. RAR B8.2.1.1
Photochemical degradation in water			
(^{14}C)-MON 65500: Photodegradation in Sterile, Aqueous Solution. *SETAC Procedures for Assessing the Environmental Fate and Ecotoxicology of Pesticides, Section 10 (1995); and* *EPA Pesticide Assessment Guidelines, Subdivision N, paragraph 161-2 (1982)*	A review of the aqueous photolysis study concluded that photolysis was a significant route of degradation for silthiofam. In the study [^{14}C]silthiofam was rapidly photodegraded in pH 7 aqueous solution under artificial sunlight, with a DT_{50} value of 16 days. There were no major degradation products and each individually accounted for less than 10% of applied radioactivity. CP 240659 was identified as accounting for up to 2% of applied radioactivity. Considering the relatively short DT_{50} value of 16 days for the irradiated samples it could be	Study performed according to GLP criteria.	Lewis, C.J. (1997): (^{14}C)-MON 65500: Photodegradation in Sterile, Aqueous Solution. Covance, Report no.: 64/66-1015. Not published. RAR B8.2.1.2

Method	Results	Remarks	Reference
	concluded that if silthiofam was exposed to sunlight under field conditions then photochemical degradation could be expected to be an important factor in its degradation.		
Photochemical degradation in soil			
Silthiofam is used as a seed treatment at drilling and, therefore, the amount of silthiofam that would be exposed to sunlight is expected to be very low. In the 2000 EU evaluation of silthiofam it was concluded that a silthiofam soil photolysis study is not required since the seeds are mixed with the soil the percentage of the seed-treated dose left at the soil surface is expected to be minimal.			

Hydrolytic degradation

(^{14}C)-Silthiofam: Hydrolytic Stability; Lewis, C.J. (1996b)

The rate of degradation of Silthiofam was studied in buffered, sterile, aqueous solutions at pH 4, 5, 7 and 9, using (^{14}C)-Silthiofam (specific activity 32.1 mCi/mmol, radiochemical purity >97%) labelled at the 2-position of the thiophene ring. In a preliminary study, units were incubated at 50°C (pH 4, 7 and 9). In the definitive study, units were incubated at 25°C (pH 4, 5, 7 and 9) and an additional incubation was carried out at 38°C (pH 9).

Silthiofam is relatively stable hydrolytically at alkaline pH but is unstable in acidic conditions. The hydrolysis half-lives for silthiofam at 25°C ranged from 45 hours (pH 4) to 448 days (pH 7). The hydrolysis half-life at 25°C for pH 9 was 314 days. Using Arrhenius plots, the half-lives at 20°C were estimated to be 73 hours for pH 4 and 77 years for pH 9. Therefore silthiofam is rapidly hydrolysed at pH 4 and 20°C but can be considered to be stable at pH 9 and 20°C. The principal hydrolysis product of silthiofam was CP 240659, resulting from acid-catalysed desilylation of the parent molecule. This metabolite was also shown to form hydrolytically in acidic soils. There was no evidence of any significant degradation of CP 240659 during the hydrolysis study with Silthiofam.

Photochemical degradation in water

(^{14}C)-Silthiofam: Photodegradation in Sterile, Aqueous Solution; Lewis, C.J. (1997)

The photodegradation of (^{14}C)-Silthiofam in aqueous buffered solution was investigated at 25 ± 1°C under sterile conditions. The study was conducted at pH 7, the pH at which (^{14}C)-Silthiofam was found to be most hydrolytically stable.

(^{14}C)-Silthiofam was rapidly photodegraded n pH 7 aqueous solution under artificial sunlight, with a DT_{50} value of 16 days. There were no major degradation products and each individually accounted for less than 10% of applied radioactivity. CP 240659 was identified as accounting for up to 2% of applied radioactivity. Comparison of the relatively short DT_{50} value of 16 days for the irradiated samples with the long DT_{50} value of 1490 days for the dark control samples indicates that if Silthiofam was exposed to sunlight under field conditions then photochemical degradation could be expected to be an important factor in its degradation.

5.1.2 Summary and discussion of abiotic degradation

Silthiofam is relatively stable hydrolytically at alkaline pH but is unstable in acidic conditions. The hydrolysis half-lives for silthiofam at 25°C ranged from 45 hours (pH 4) to 448 days (pH 7). The hydrolysis half-life at 25°C for pH 9 was 314 days. Using Arrhenius plots, the half-lives at 20°C were estimated to be 73 hours for pH 4 and 77 years for pH 9. Therefore silthiofam is rapidly hydrolysed at pH 4 and 20°C but can be considered to be stable at pH 9 and 20°C. The principal hydrolysis product of silthiofam was CP 240659, resulting from acid-catalysed desilylation of the parent molecule. This metabolite was also shown to form hydrolytically in acidic soils. There was no evidence of any significant degradation of CP 240659 during the hydrolysis study with silthiofam.

Photolysis was a significant route of degradation for silthiofam. In the study [^{14}C]silthiofam was rapidly photodegraded in pH 7 aqueous solution under artificial sunlight, with a DT_{50} value of 16 days. The degradation products each individually accounted for less than 10% of applied radioactivity. CP 240659 was identified as accounting for up to 2% of applied radioactivity. Considering the relatively short DT_{50} value of 16 days for the irradiated samples it could be concluded that if silthiofam was exposed to sunlight under field conditions then photochemical degradation could be expected to be an important factor in its degradation.

5.1.3 Biodegradation

Table 62: Summary of relevant information on biodegradation of Silthiofam

Method	Results	Remarks	Reference
Ready biodegradability of Silthiofam			
SILTHIOFAM: Assessment of ready biodegradability by measurement of carbon dioxide evolution, *EC Directive 92/69/EEC Part C4-C; and OECD Guideline 301B (1992).*	Carbon dioxide evolution from silthiofam reached a maximum of 2% at the applied concentration over a period of 28 days. Although the outcome of the carbon dioxide evolution test was negative, it does not necessarily indicate that Silthiofam is non-biodegradable under more lenient conditions. Silthiofam is not considered to be "readily biodegradable".	This study was performed according to GLP criteria.	Bealing, D.J. (1996): MON 65500: Assessment of ready biodegradability by measurement of carbon dioxide evolution. Corning Hazleton (Europe), Report no.: 64/59-1018. Not published. RAR B8.2.2.1
Water/Sediment			
(^{14}C)-SILTHIOFAM: Degradation and Retention in Water-Sediment Systems. *BBA Guidelines Part IV, Section 5-1 (1990), SETAC Procedures for Assessing the Environmental Fate and Ecotoxicology of Pesticides, Section 8.2 (1995) and EPA*	The fate of silthiofam in two different water/sediment systems was evaluated. Silthiofam degradation resulted mainly in the formation of bound residues and numerous low levels degradation products. The degradation products were each less than 10% of the applied radioactivity in all cases. In the total system of one water/sediment system, one component accounted for 6% of the radioactivity and another for 5% of the radioactivity at the end of the study (100 days). These compounds were <5% of AR in surface	This study was performed according to GLP criteria.	Lewis, C.J. (1997): (^{14}C)-MON 65500: Degradation and Retention in Water-Sediment Systems. Covance Laboratories, Report no.: CHE 64/67-1015. Not published.

Method	Results	Remarks	Reference
Pesticide Assessment Guidelines, Subdivision N, Paragraph 162-4 (1982)	water and sediment respectively and were not identified in the study. The test article disappeared quickly from the water phase in both systems (DT_{50} values of 5 and 52 days for the pond and run-off systems respectively). Degradation in the systems as a whole was somewhat slower due to uptake of Silthiofam by the sediments, with DT_{50} values of 269 and 147 days for the pond and run-off systems respectively. By the end of the study (100 days) levels of Silthiofam detected in the total system accounted for 74% and 58% of applied radioactivity for the pond and run-off systems respectively.		RAR B8.2.2.2
Silthiofam: Route and Rate of Degradation of [^{14}C]Silthiofam in Aerobic Aquatic Sediment Systems *OECD Guidelines for the Testing of Chemicals, Guideline 308: Aerobic and Anaerobic Transformation in Aquatic Sediment Systems; April 2002.*	The route and rate of degradation of [^{14}C]silthiofam in two aquatic systems (river and pond) under aerobic conditions were investigated at 20 °C in the dark. [^{14}C]silthiofam dissipated from the water phases the river and pond aquatic systems relatively fast and continued steadily and by the end of incubation after 118 days ca. 23.1% and 17.6% of the applied test item was detectable in the river and pond water phase, respectively. The test item dissipation from water was mainly due to the adsorption to the sediment layers. Maximum values of test item found in the sediment extract accounted for 39.7% (day 28) for river and 46.2% (day 28) of applied for the pond. Thereafter, [^{14}C]silthiofam continued to slowly decrease to levels of 29.2% (river) and 32.5% (pond) of applied radioactivity by the end of the study. Consequently, degradation of [^{14}C]silthiofam in the total systems was significantly slower than in the water phase alone. By the end of the study (118 days) levels of [^{14}C]silthiofam detected in the total system accounted for 52.2% and 50.2% of applied radioactivity for the river and pond systems, respectively. In the water phase and in the sediment extracts several minor degradation products were detectable, but most of them did not exceed 5% of applied radioactivity. In the total system, only two significant degradation products (designated as metabolites M1 and M2) were detected in both systems. Metabolite M1 was identified as Silthiofam (MON 65500) Amide accounting for 5.8% and 4.8% of applied in the river and pond entire system. The second metabolite M2 was identified as Silthiofam (MON 65500) Allyl Acid, accounting for 6.6% and 6.2% of applied at	This study was performed according to GLP criteria.	Irmer A. (2013); Silthiofam: Route and Rate of Degradation of [^{14}C]Silthiofam in Aerobic Aquatic Sediment Systems. Harlan Laboratories Ltd. Report No. MSL0025181. Not published. RAR B8.2.2.2

Method	Results	Remarks	Reference
	the end of the study. The formation of radioactive carbon dioxide and other volatile products was insignificant.		
Determination of persistence and modelling endpoints for silthiofam and its metabolites from water/sediment studies according to FOCUS kinetics guidance. *FOCUS (2006) Guidance Document on Estimating the Persistance and Degradation Kinetics from Environmental Fate Studies on Pesticides in EU registration, Report of the FOCUS Work Group on Degradation Kinetics, EC Document Sanco/10058/2005, version 2.0., June 2006; FOCUS (2011): Generic Guidance for Estimating Persistence and Degradation Kinetics from Environmental Fate Studies on Pesticides in EU Registration, version 1.0*	For FOCUS surface water modelling the silthiofam bias-corrected geometric mean DT_{50} values of 152.3 days for total system, 78.2 days for water column, and 193.6 days for sediment phase are recommended. For the metabolites M1 and M2, no decline phase was established so a worst case default value of 1000 days is recommended.	Study acceptable.	Carnall, J. (2014b); Determination of persistence and modelling endpoints for silthiofam and its metabolites from water/sediment studies according to FOCUS kinetics guidance. Cambridge Environmental Assessments ADAS UK Ltd. Cambridge UK. (Report # CEA.1150). Report No. MSL0025594. Not published. RAR B8.2.2.2
Biodegration in Soil			
Aerobic Degradation [^{14}C]-SILTHIOFAM: Soil Metabolism and Degradation. *SETAC – Procedures for Assessing the Environmental Fate and Ecotoxicity of Pesticides, March 1995.*	Silthiofam (MON 65500) was degraded readily in the sandy loam test soil at 20ºC under aerobic conditions. Degradation resulted in the formation of bound residues, carbon dioxide and minor degradation products. None of the degradation products exceeded 10% of the applied radioactivity at any time during the study.	Study previously evaluated for the previous inclusion into the Annex I of the Directive 91/414/EEC. This study was performed according to GLP criteria.	Lewis, C.J. (1996): [^{14}C]-MON 65500: Soil Metabolism and Degradation. Corning Hazleton (Europe), Report no.: 64/49-1015. RAR B8.1.1.1.1
Aerobic Degradation (^{14}C)-SILTHIOFAM: Aerobic Route of Soil Degradation. *SETAC – Procedures for Assessing the Environmental Fate and Ecotoxicity of Pesticides, March 1995.*	Following application at twice the recommended field rate Silthiofam (MON 65500) was degraded steadily in the sandy loam test soil (PT 102) at 20ºC under aerobic conditions, resulting in the formation of bound residues, carbon dioxide and minor degradation products. None of the degradation products exceeded 10% of the applied radioactivity at any time during the study. Comparison of this study with the previous study indicates that an increase in the SILTHIOFAM application rate from 40 g a.s./ha to 80 g a.s./ha has no	Study previously evaluated for the previous inclusion into the Annex I of the Directive 91/414/EEC. This study was performed according to GLP criteria	Goodyear, A. (1999): (^{14}C)-MON 65500: Aerobic Route of Soil Degradation. Covance, Report no.: CLE 64/87-D2142. RAR B8.1.1.1.1

Method	Results	Remarks	Reference
	significant effect on the aerobic route of degradation in soil PT 102 at 20°C		
Aerobic Degradation Degradation of silthiofam metabolites (M2, M5 and M6) in three soils incubated under aerobic conditions. OECD Guideline 307 and SETAC (Europe), March 1995, Part 1.	Silthiofam rapidly degraded in three agricultural soils. Mineralization was high during the study with $^{14}CO_2$ representing maximum values of 41.3, 24.6 and 39.7% of the applied radioactivity in Soils I, II and III, respectively, at the end of the incubation on Day 123. Besides the test item, up to eleven degradates were detected in the soil extracts. M1 reached a maximum of 10.2% of the applied radioactivity in one soil, while metabolites M2 and M6 accounted for maximum levels of 9.5 and 6.6% of the applied radioactivity. Metabolite M5 was found to be associated with metabolite fraction M4, which reached a maximum level of 11.2% of the applied radioactivity, but was found by further chromatographic analyses to consist of up to fifteen subfractions. All other unknown degradates remained below 4% of the applied radioactivity. The mean half-lives for M2 and M6 were calculated to be 42 days and 26 days, respectively.	Study considered acceptable. This study was performed according to GLP criteria.	Völkel, W (2008a); Degradation of MON 65500 metabolites (M2, M5 and M6) in three soils incubated under aerobic conditions. Report No. MSL0020216. RCC Ltd, Environmental Chemistry & Pharmanalytics, Itingen / Switzerland (Report # 850903). RAR B8.1.1.1.1
Aerobic Degradation Characterization and Identification of Unknown Metabolites from the Aerobic Soil Incubation of Silthiofam. No relevant guidance.	The major soil metabolites observed in aerobic soil metabolism studies of silthiofam have been identified, and pathways for metabolism of silthiofam in soil have been elucidated. Silthiofam is metabolized in soil by either oxidative removal of the allyl group to form the primary amide metabolite M1 (MON 65500 Amide), or oxidation of the ring-attached 5-methyl group to a carboxylic acid forming metabolite M2 (MON 65500 Allyl Acid). Further oxidative metabolism generates metabolite M6 (MON 65500 Acid Amide) by oxidation of the ring-attached 5-methyl group of metabolite M1, or oxidative removal of the allyl group from metabolite M2 to form the primary amide. All three of the identified soil metabolites of silthiofam retained the trimethylsilyl group.	Study considered acceptable. This study was performed according to GLP criteria.	Miller, M.J. and Whitehead, T.L. (2009); Characterization and Identification of Unknown Metabolites from the Aerobic Soil Incubation of Silthiofam. Monsanto Company Missouri, USA. Report No. MSL0020621. RAR B8.1.1.1.1
Aerobic Degradation Silthiofam: Route and rate of degradation of 14C-silthiofam in four soils incubated under aerobic conditions.	An aerobic soil degradation study of silthiofam was conducted on four soils using a [^{14}C]silthiofam dose rate of 0.27 mg a.i./kg dry soil incubated in the dark at 20 ºC for up to 120 days. [^{14}C]Silthiofam degraded rapidly in all four soils.	Study considered acceptable. This study was performed according to GLP criteria.	Fahrbach M. (2013a); Silthiofam: Route and rate of degradation of 14C-silthiofam in four soils incubated under

Method	Results	Remarks	Reference
U.S. EPA Fate, Transport and Transformation Test Guidelines OPPTS 835.4100, Aerobic Soil Metabolism (adopted October 2008); *OECD Guideline 307 for Testing of Chemicals: Aerobic and Anaerobic Transformation in Soil (adopted April 2002);* *SETAC-EUROPE Procedures for Assessing the Environmental Fate and Ecotoxicity of Pesticides: Section 1.1 (Laboratory aerobic soil degradation studies) (March 1995).*	Besides the parent compound, several degradation products were detected. Three predominant degradation products, designated M1, M2 and M6, were formed. Metabolite M1 was identified as MON 65500 Amide and reached a maximum mean amount of 6.6% of applied on day 7 in soil I. Metabolite M2 was identified as MON 65500 Allyl Acid and reached a maximum mean amount of 12.0% of applied in soil IV on day 28. Metabolite M6 was identified as MON 65500 Amide Acid and reached a maximum mean amount of 13.1% of applied on day 90 in soil III. The formation of radioactive carbon dioxide was significant. The amount of non-extractable radioactivity was also significant during the entire incubation period. Organic matter fractionation on day 120 indicated that the majority of the non-extractable radioactivity was bound to the immobile humic acids and humin. Lower amounts of radioactivity were detected in the more mobile fulvic acid fraction.		aerobic conditions. MSL0024763. Harlan Laboratories Ltd., Itingen, Switzerland (Report # Harlan D57133). RAR B8.1.1.1.1
<u>Anaerobic Degradation</u> (^{14}C)-silthiofam: Anaerobic Soil Degradation Study. *SETAC – Procedures for Assessing the Environmental Fate and Ecotoxicity of Pesticides, March 1995.*	Following application at twice the recommended field rate Silthiofam (MON 65500) was degraded slowly in a flooded UK sandy loam soil (PT 102) at 20°C under anaerobic conditions. Degradation resulted in the formation of unextracted soil residues, carbon dioxide and minor unidentified degradation products. None of the degradation products exceeded 10% of applied radioactivity at any time during the study.	Study previously evaluated for the previous inclusion into the Annex I of the Directive 91/414/EEC. This study was performed according to GLP criteria.	Goodyear, A. (1999): (^{14}C)-MON 65500: Anaerobic Soil Degradation Study. Covance, Report no.: 64/83-D2142. RAR B8.1.1.1.1
<u>Anaerobic Degradation</u> Silthiofam: Route and rate of degradation of ^{14}C-silthiofam in one soil incubated under anaerobic conditions. *OECD Guideline 307 for Testing of Chemicals: Aerobic and Anaerobic Transformation in Soil (adopted April 2002);* *SETAC-EUROPE Procedures for Assessing the Environmental Fate and Ecotoxicity of Pesticides.*	The route of degradation of [^{14}C]silthiofam was investigated in one soil incubated at 20 °C for an initial period of 28 days under aerobic conditions followed by incubation for 120 days under flooded anaerobic conditions. The levels of [^{14}C]silthiofam in soil extracts decreased to 63.4% of the dose within the 28-day aerobic incubation period. After flooding on day 28 [^{14}C]silthiofam was degraded relatively slower with its levels in the total soil/water system declining from the initial level of 63.4% at the time of flooding to 46.3% of the applied radioactivity at the last sampling day. Besides the parent compound, numerous low levels degradation products were detected. Only one predominant degradation product (> 5% of applied), designated as M1, was formed. Metabolite M1 was identified as	Study considered acceptable. This study was performed according to GLP criteria.	Fahrbach M. (2013b); Silthiofam: Route and rate of degradation of 14C-silthiofam in one soil incubated under anaerobic conditions.

Method	Results	Remarks	Reference
	MON 65500 Amide. Metabolite M1 reached a maximum concentration of 8.2% in the total soil/water system after 7 days of anaerobic incubation and subsequently declined to 7.1% after 120 days of incubation. Up to 33 minor degradation products were detected, but none of them exceeded 3.0% of the applied radioacitivity. The formation of radioactive carbon dioxide was significant reaching a maximum of 18.0% of the applied radioactivity at the end of the anaerobic incubation period.		

5.1.3.1 Biodegradation estimation

5.1.3.2 Screening tests

Ready biodegradability of Silthiofam

The ready biodegradability of Silthiofam was determined by measurement of carbon dioxide evolution in a CO_2 Evolution assay (Modified Sturm Test) which corresponds to the OECD 301B test setup. Silthiofam (MON 65500) was suspended in a buffered mineral salts medium at a concentration of 25.7 mg/l, nominally equivalent to 15 mg organic carbon per litre. The medium was inoculated with micro-organisms derived from a sample of activated sludge not previously exposed to the test substance. The test vessels were incubated in the dark at 20.3-22.3°C for 28 days, during which time the vessels were continually sparged with carbon dioxide-free air (50-100 ml/min). Measured pH values were in the range 7.45-7.76. Exhaust air was passed through a series of dedicated carbon dioxide scrubbers containing barium hydroxide solution. The extent of biodegradation was determined by expressing the corrected cumulative recovered yield as a percentage of the theoretical, calculated from the carbon content of the test substance. Two vessels containing a reference substance (sodium benzoate) were run under the same conditions as vessels containing Silthiofam (MON 65500), in order to check the procedure and activity of the inoculum. An additional vessel containing Silthiofam (MON 65500) and the reference substance served as a toxicity control to assess whether the test substance was inhibitory at the concentration at which it was applied.

Carbon dioxide evolution from Silthiofam reached a maximum of 2% at the applied concentration over a period of 28 days. Silthiofam cannot therefore be classified as readily biodegradable. Although the outcome of the carbon dioxide evolution test was negative, it does not necessarily indicate that Silthiofam is non-biodegradable under more lenient conditions. The method used in the study was not devised as a simulation of real aquatic environments but as a stringent fail-safe screen to classify substances that would degrade rapidly and completely in natural water bodies.

5.1.3.3 Simulation tests

Water/Sediment Studies

Study 1: (^{14}C)-SILTHIOFAM: Degradation and Retention in Water-Sediment Systems; Lewis, C.J. (1997b)

The degradation of (^{14}C)-silthiofam was studied over a 100 day period in two water-sediment systems (Mill stream pond and Iron hatch run-off) containing sediments of different biomass, textural class, organic carbon, nitrogen and phosphorus contents. The results of the study indicated that silthiofam degradation resulted mainly in the formation of bound residues and numerous low levels degradation products. The degradation products were each less than 10% of the applied radioactivity in all cases. In the total system of one water/sediment system, one component accounted for 6% of the radioactivity and another for 5% of the radioactivity at the end of the study (100 days). These compounds were <5% of AR in surface water and sediment respectively and were not identified in the study. The physicochemical properties of the sediment and the data detailing the degradation of silthiofam are presented below.

Table 63: Sediment characteristics in the water/sediment study

	Mill Stream pond	**Iron Hatch run-off**
pH (water)	7.8	8.4
pH (1M KCl)	7.4	8.1
Organic Carbon (%)	7.2	0.3
Textural Class (UK)	Clay loam	Sand
Clay (<2 µm; %) Silt (2-63 µm; %) Sand (63-2000 µm; %)	24.59 43.48 27.04	0.51 0.71 98.99
CEC (mEq/kg dry sediment)	42.4	2.6
Total nitrogen (mg/kg)	6781.8	392.0
Total phosphorous (mg/kg)	2010.7	194.9

Table 64: Percentages of applied radioactivity present as silthiofam in two water-sediment systems

		Mill Stream Pond		**Iron Hatch run-off**	
Time (days)	**Replicate**	**Water**	**Sediment**	**Water**	**Sediment**
0	**A**	**92.92**	**4.57**	**95.48**	**1.22**
0	**B**	**94.69**	**3.4**	**94.54**	**1.47**
0.25	**A**	**89.74**	**8.69**	**94.63**	**6.32**
0.25	**B**	**85.75**	**10.78**	**91.78**	**5.91**
1	**A**	**69.22**	**27.14**	**81.6**	**14.17**
1	**B**	**74.43**	**19.07**	**81.49**	**13.24**
2	**A**	**66.39**	**29.06**	**82.07**	**14.16**
2	**B**	**66.04**	**28.85**	**83.67**	**12.56**
7	**A**	**37.17**	**55.12**	**72.53**	**20.74**
7	**B**	**45.59**	**43.54**	**71.14**	**22.3**
14	**A**	**34.55**	**55.75**	**61.59**	**29.4**
14	**B**	**37.46**	**52.95**	**61.29**	**28.74**
30	**A**	**26.85**	**63.33**	**53.02**	**34.01**
30	**B**	**21.94**	**64.29**	**59.75**	**32.99**
59	**A**	**19.47**	**66.42**	**47.36**	**34.43**
59	**B**	**16.93**	**62.94**	**45.01**	**33.4**
100	**A**	**14.5**	**59.03**	**30.9**	**26.32**
100	**B**	**16.08**	**57.85**	**30.6**	**27.77**

Study 2: Silthiofam: Route and Rate of Degradation of [^{14}C] Silthiofam in Aerobic Aquatic Sediment Systems: Irmer A. (2013)

The route and rate of degradation of [^{14}C]silthiofam was investigated in two representative aquatic sediment systems (river and pond) incubated under aerobic conditions at 20 °C in the dark. Two natural water/sediment systems, a river (i.e. the river Rhine Mumpf, AG, Switzerland) and a pond (Fröschweiher, Möhlin, AG, Switzerland), were freshly sampled by Harlan Laboratories Ltd. The sampling locations are not subject to nearby run-offs from farmland or effluents from water treatment plants. The water was sampled at a depth of about 30 cm and the sediment was sampled from the top 10 cm layer. The sampling sites were located 1 metre from firm land.

Mass Balance
The material balance was determined as the sum of radiocarbon in soil extracts, volatile traps and residual soil radiocarbon. The total mean recovery for all samples was on average 99.7±7.7% and 99.1±5.1% of the applied radioactivity for the river and pond systems, respectively

Bound Extractable Residues
The proportion of the total radioactivity in the sediment increased constantly with time, from an initial level (time 0) of 6.3% (river) and 2.2% (pond) of the applied radioactivity to maximum levels of 46.9% (river on day 118) and 56.2% (pond systems on day 28). In the pond sediment afterwards the radioactivity decreased slightly to 49% at the end of incubation. The radioactivity extracted from the sediment (total of cold extraction and Soxhlet) reached maximum levels of 41.4% on day 56 for river and 49.8% of applied on day 28 for the pond systems. The levels of extractables then decreased steadily to 37.5% (river) and 36.8% (pond) on day 118. Soxhlet extraction was performed on the sediment of samples from day 56 onwards. It released mean maximum amounts of 2.6% and 5.4% of the applied radioactivity from river and pond aquatic system on day 90 and 118, respectively. The amount of non-extractable radioactivity in the sediment represented <1.5% of dose at time 0 but continuously increased with time to levels of 9.4% and 12.2% of applied at day 118 in the river and pond sediments, respectively. Acidic harsh extraction under reflux followed by organic matter fractionation were conducted on the non-extracted residues from sediment collected at the last sampling interval (day 118). Reflux extraction released mean amounts of 1.5 and 2.9% of the applied radioactivity for river and pond aquatic systems, respectively. Organic matter fractionation of the remaining non-extractable radioactivity of day 118 sediment samples showed that the majority of the non-extractable radioactivity was bound to the insoluble humin fraction accounting for 5.4% and 5.7% of the applied radioactivity for river and pond systems, respectively. The corresponding values for the fulvic acids were 1.9% and 2.3% and for the humic acids were 0.7% and 1.2% of applied.

Volatilization
A minor level of mineralization of the radioactive residue to $^{14}CO_2$ was observed in both systems. Small amounts of carbon dioxide were observed from day 14 (river) or day 28 (pond) onwards and they represented cumulative maximum values of 4.1% (river) and 2.8% (pond) of applied radioactivity on day 118. Additionally formation of organic volatiles other than 14CO2 was not significant, accounting for maximum of 2.6% (river) on day 56 and 1.9% (pond) on day 118 in the two systems.

Transformation of the Parent Compound
In the total systems, silthiofam decreased steadily with time from an initial level of 102.4% and 100.8% of applied radioactivity to 52.2% and 50.2% at day 118 in the total river and pond systems, respectively. The parent substance dissipated from the water phase mainly due to adsorption the

sediment layer and to a lesser extent due the metabolic degradation. Initial levels of 98.4% and 100.8% of parent substance declined steadily in the course of the study and on day 118, [14C]silthiofam accounted for 23.1% and 17.6% of the applied radioactivity in the water phases of the river and pond system, respectively. The levels of parent substance found in the sediment extracts on day 0 accounted for only 4.0% in the river system. In the pond system no significant level of the parent was found in the sediment extracts on day 0. However, levels of silthiofam in the sediment extracts increased to maximum values of 39.7% and 46.2% on day 28, for river and pond sediments, respectively. Thereafter, [14C]silthiofam concentration decreased in the sediment extracts to levels of 29.2% (river) and 32.5% (pond) of applied radioactivity on day 118.

There were only two significant degradation products of silthiofam (designated as metabolites M1 and M2) at maximum concentrations of 5.8 and 6.6% of the applied radioactivity in the river system, respectively. In the pond total system, metabolites M1 and M2 increased to a maximum of 4.8% and 6.2% of applied, respectively. Metabolites M1 and M2 were identified as MON 65500 Amide and MON 65500 Allyl Acid, respectively. In addition, the HPLC chromatogram of only one of the replicate sample of the day 118 concentrated sediment extracts from river system showed the presence of 5.3% of CP 240659 (MON 65500 desilated). However, the TLC chromatogram (no concentration step needed) of the same sample did not show any significant presence of CP 240659. In previous environmental fate studies, degradation of silthiofam to metabolite CP 240659 has been shown to occur via acid-catalyzed hydrolysis and not as a result of biological degradation. Thus, detection of CP 240659 in the HPLC chromatogram of the concentrated sediment extract of the replicate B of the day 118 sample is almost certainly due to the catalytic desilylation of the parent molecule formed during concentration step needed for the HPLC analysis. Similarly in the replicate A of day 118 water sample from the pond system, formation of metabolite CP 240659 was also detected. Again since the other replicate (replicate B of day 118) showed no significant formation of CP 240659, it could be concluded that the detection of CP 240659 is an artifact of sample work-up and not a genuine metabolite in both water-sediment systems.

Conclusion

The route and rate of degradation of [^{14}C]silthiofam in two aquatic systems (river and pond) under aerobic conditions were investigated at 20 °C in the dark. [^{14}C]silthiofam dissipated from the water phases the river and pond aquatic systems relatively fast and continued steadily and by the end of incubation after 118 days ca. 23.1% and 17.6% of the applied test item was detectable in the river and pond water phase, respectively. The test item dissipation from water was mainly due to the adsorption to the sediment layers. Maximum values of test item found in the sediment extract accounted for 39.7% (day 28) for river and 46.2% (day 28) of applied for the pond. Thereafter, [^{14}C]silthiofam continued to slowly decrease to levels of 29.2% (river) and 32.5% (pond) of applied radioactivity by the end of the study. Consequently, degradation of [^{14}C]silthiofam in the total systems was significantly slower than in the water phase alone. By the end of the study (118 days) levels of [^{14}C]silthiofam detected in the total system accounted for 52.2% and 50.2% of applied radioactivity for the river and pond systems, respectively.

In the water phase and in the sediment extracts several minor degradation products were detectable, but most of them did not exceed 5% of applied radioactivity. In the total system, only two significant degradation products (designated as metabolites M1 and M2) were detected in both systems. Metabolite M1 was identified as MON 65500 Amide accounting for 5.8% and 4.8% of applied in the river and pond entire system. The second metabolite M2 was identified as MON 65500 Allyl Acid, accounting for 6.6% and 6.2% of applied at the end of the study. The formation of radioactive carbon dioxide and other volatile products was insignificant.

Study 3: Determination of persistence and modelling endpoints for silthiofam and its metabolites from water/sediment studies according to FOCUS kinetics guidance; Carnall, J. (2014b)

This study analyzed the degradation data from the four water-sediment systems presented in Studies 1 and 2 under FOCUS kinetics guidance.

For FOCUS surface water modelling the silthiofam bias-corrected geometric mean DT_{50} values of 152.3 days for total system, 78.2 days for water column, and 193.6 days for sediment phase are recommended. For the metabolites M1 and M2, no decline phase was established so a worst case default value of 1000 days is recommended by the RMS evaluator.

Table 65: Persistence and modeling endpoints of silthiofam in water-sediment systems

Study	**System**	**Persistence endpoints at Level P-I**			**Modelling endpoints at Level P-I**	
		Model	**DT_{50}[a] (days)**	**DT_{90}[a] (days)**	**Model**	**SFO DT_{50}[a] (days)**
Silthiofam (total system)						
Lewis, (1997b)	Mill stream pond	SFO	263	872	SFO	263
	Iron hatch run-off	SFO	154	512	SFO	154
Irmer, (2013)	River Rhine	SFO	106.2	352.9	SFO	106.2
	Fröschweiher Pond	SFO	125.1	415.7	SFO	125.1
Minimum			106.2	352.9	Minimum	106.2
Maximum			263	872	Maximum	263
Bias-corrected Geometric mean						152.3
Silthiofam (water phase)						
Lewis, (1997b)	Mill stream pond	FOMC	4.71	335	DFOP	55.9[b]
	Iron hatch run-off	DFOP	49.1	240	DFOP	82.5[b]
Irmer, (2013)	River Rhine	FOMC	19.4	481.0	HS	86.8[b]
	Fröschweiher Pond	FOMC	12.2	628.4	DFOP	93.4[b]
Minimum			4.71	240	Minimum	55.9
Maximum			49.1	628.4	Maximum	93.4
Bias-corrected Geometric mean						78.2
Silthiofam (sediment phase)						
Lewis, (1997b)	Mill stream pond	-[c]	-[c]	-[c]	-[c]	-[c]
	Iron hatch run-off	-[c]	-[c]	-[c]	-[c]	-[c]
Irmer, (2013)	River Rhine	SFO	184.3	612.3	SFO	184.3
	Fröschweiher Pond	SFO	203.8	676.9	SFO	203.8
Minimum			184.3	612.3	Minimum	184.3
Maximum			203.8	676.9	Maximum	203.8
Bias-corrected Geometric mean						193.6

[a] DT_{50} = $DegT_{50}$ for total system but DT_{50} for water and sediment phase
[b] Calculated from slower k-rate
[c] no reliable fit achieved

Biodegradation in Soil

Aerobic Degradation
[^{14}C]-silthiofam: Soil Metabolism and Degradation; Lewis, C.J. (1996)

Silthiofam was applied to the surface of a sandy loam soil (PT 102) in a laboratory study. Test article was applied at a rate of approximately 0.04 ppm, equivalent to 40 g a.s./ha ; the recommended field rate.

Conclusion
Silthiofam was degraded readily in the sandy loam test soil at 20ºC under aerobic conditions. Degradation resulted in the formation of bound residues, carbon dioxide and minor degradation products. None of the degradation products exceeded 10% of the applied radioactivity at any time during the study.

An additional study was carried out on the aerobic degradation of silthiofam, in the same soil type, in order to assess the effect of an application rate equivalent to 80 g a.s./ha - twice the recommended field rate.

(^{14}C)-silthiofam: Aerobic Route of Soil Degradation; Goodyear, A. (1999)

Silthiofam was applied to the surface of a sandy loam soil (PT 102) in a laboratory study at a rate of approximately 0.08 ppm, equivalent to 80g a.s./ha , which is twice the recommended field rate. It should be noted that the microbial biomass of the sample of soil PT 102 used in the present study (557.6 µg C per g soil, pre-study) was significantly higher than that of the sample of soil PT 102 used in the previous study mentioned above (384 µg C per g soil, pre-study).

Conclusion
Following application at twice the recommended field rate Silthiofam (MON 65500) was degraded steadily in the sandy loam test soil (PT 102) at 20ºC under aerobic conditions, resulting in the formation of bound residues, carbon dioxide and minor degradation products. None of the degradation products exceeded 10% of the applied radioactivity at any time during the study. Comparison of this study with the previous study indicates that an increase in the silthiofam application rate from 40 g a.s./ha to 80 g a.s./ha has no significant effect on the aerobic route of degradation in soil PT 102 at 20°C.

Degradation of Silthiofam (MON 65500) metabolites (M2, M5 and M6) in three soils incubated under aerobic conditions; Völkel, W (2008a)

The degradation of silthiofam was investigated in three soils incubated under aerobic conditions at 20 °C and 40% maximum water holding capacity in the dark for a period of 123 days. Several metabolites were observed and their structures were determined in the study by Miller, M.J. and Whitehead, T.L. (2009).

Conclusion
Silthiofam rapidly degraded in three agricultural soils. Mineralization was high during the study with $^{14}CO_2$ representing maximum values of 41.3, 24.6 and 39.7% of the applied radioactivity in Soils I, II and III, respectively, at the end of the incubation on Day 123.

Besides the test item, up to eleven degradates were detected in the soil extracts. M1 reached a maximum of 10.2% of the applied radioactivity in one soil, while metabolites M2 and M6 accounted for maximum levels of 9.5 and 6.6% of the applied radioactivity. Metabolite M5 was found to be associated with metabolite fraction M4, which reached a maximum level of 11.2% of the applied radioactivity, but was found by further chromatographic analyses to consist of up to fifteen subfractions. All other unknown degradates remained below 4% of the applied radioactivity. The mean half-lives for M2 and M6 were calculated to be 42 days and 26 days, respectively.

Characterization and Identification of Unknown Metabolites from the Aerobic Soil Incubation of Silthiofam; Miller, M.J. and Whitehead, T.L. (2009)

This study was undertaken in order to establish the chemical structure of the metabolites M1, M2 and M6 identified in the previous study undertaken by (Völkel, W 2008a).

The major soil metabolites observed in aerobic soil metabolism studies of silthiofam have been identified, and pathways for metabolism of silthiofam in soil have been elucidated. Silthiofam is metabolized in soil by either oxidative removal of the allyl group to form the primary amide metabolite M1 (MON 65500 Amide), or oxidation of the ring-attached 5-methyl group to a carboxylic acid forming metabolite M2 (MON 65500 Allyl Acid). Further oxidative metabolism generates metabolite M6 (MON 65500 Acid Amide) by oxidation of the ring-attached 5-methyl group of metabolite M1, or oxidative removal of the allyl group from metabolite M2 to form the primary amide. All three of the identified soil metabolites of silthiofam retained the trimethylsilyl group.

Silthiofam: Route and rate of degradation of ^{14}C-silthiofam in four soils incubated under aerobic conditions; Fahrbach M. (2013a)

An aerobic soil degradation study of silthiofam was conducted on four soils using a [^{14}C]silthiofam dose rate of 0.27 mg a.i./kg dry soil incubated in the dark at 20 ºC for up to 120 days.

[^{14}C]Silthiofam degraded rapidly in all four soils. Besides the parent compound, several degradation products were detected. Three predominant degradation products, designated M1, M2 and M6, were formed. Metabolite M1 was identified as MON 65500 Amide and reached a maximum mean amount of 6.6% of applied on day 7 in soil I. Metabolite M2 was identified as MON 65500 Allyl Acid and reached a maximum mean amount of 12.0% of applied in soil IV on day 28. Metabolite M6 was identified as MON 65500 Amide Acid and reached a maximum mean amount of 13.1% of applied on day 90 in soil III. The formation of radioactive carbon dioxide was significant. The amount of non-extractable radioactivity was also significant during the entire incubation period.

Organic matter fractionation on day 120 indicated that the majority of the non-extractable radioactivity was bound to the immobile humic acids and humin. Lower amounts of radioactivity were detected in the more mobile fulvic acid fraction.

Anaerobic degradation

(^{14}C)-silthiofam: Anaerobic Soil Degradation Study; Goodyear, A. (1999b)

The anaerobic route of degradation of (^{14}C)-silthiofam was studied in a UK sandy loam test soil at 20°C for 118 days, following application of test article at a rate of approximately 0.08 ppm, equivalent to 80 g a.s./ha . The soil type (PT 102) was the same as that utilized in the aerobic route of degradation studies.

Following application at twice the recommended field rate Silthiofam (MON 65500) was degraded slowly in a flooded UK sandy loam soil (PT 102) at 20°C under anaerobic conditions. Degradation resulted in the formation of unextracted soil residues, carbon dioxide and minor unidentified degradation products. None of the degradation products exceeded 10% of applied radioactivity at any time during the study

Silthiofam: Route and rate of degradation of ^{14}C-silthiofam in one soil incubated under anaerobic conditions; Fahrbach M. (2013b)

The route of degradation of [^{14}C]silthiofam was investigated in one soil incubated at 20 °C for an initial period of 28 days under aerobic conditions followed by incubation for 120 days under flooded anaerobic conditions.

The levels of [^{14}C]silthiofam in soil extracts decreased to 63.4% of the dose within the 28-day aerobic incubation period. After flooding on day 28 [^{14}C]silthiofam was degraded relatively slower with its levels in the total soil/water system declining from the initial level of 63.4% at the time of flooding to 46.3% of the applied radioactivity at the last sampling day. Besides the parent compound, numerous low levels degradation products were detected. Only one predominant degradation product (> 5% of applied), designated as M1, was formed. Metabolite M1 was identified as MON 65500 Amide. Metabolite M1 reached a maximum concentration of 8.2% in the total soil/water system after 7 days of anaerobic incubation and subsequently declined to 7.1% after 120 days of incubation.

Up to 33 minor degradation products were detected, but none of them exceeded 3.0% of the applied radioacitivity. The formation of radioactive carbon dioxide was significant reaching a maximum of 18.0% of the applied radioactivity at the end of the anaerobic incubation period.

5.1.4 Summary and discussion of degradation

Biodegradability: silthiofam is not considered as “readily biodegradable”.

Water/sediment systems

The fate of silthiofam in two new water/sediment systems was investigated to complement the findings of the two systems presented during the 2000 EU evaluation. In the new water/sediment study silthiofam dissipated reasonably rapidly from water in both the river and pond water/sediment systems, consistent with what was observed in the earlier study. Dissipation from water phase proceeded mainly through binding to the sediment and via degradation to form numerous low levels degradation products. Degradation of [^{14}C]silthiofam in the total system as a whole was somewhat slower than water phase due to its adsorption by the sediments. Additionally, the two minor degradation products (> 5% of applied), designated as M1 (identified as MON 6550 Amide) and M2 (identified as MON 65500 Allyl Acid) were definitively identified in this study.

In water/sediment systems: FOCUS surface water modelling, for Silthiofam, bias-corrected geometric mean DT_{50} values of 152.3 days for total system, 78.2 days for water column, and 193.6 days for sediment phase are recommended. For the metabolites M1 and M2, no decline phase was established so a worst case default value of 1000 days is recommended by the RMS evaluator.

Aerobic degradation

Aerobic metabolism studies in soil under laboratory conditions indicated that silthiofam degradation resulted mainly in the formation of bound residues, carbon dioxide and numerous low levels degradation products. The degradation products were each less than 10% of the applied radioactivity,

except in one soil (acidic sandy loam), where the metabolite CP 240659 was present at levels up to 21% of the applied radioactivity. Laboratory aerobic soil studies also demonstrated that from 10 to 26% of the applied silthiofam is mineralized to carbon dioxide. In the primary route and rate of degradation conducted on 4 soils (Study 1, Lewis, 1996a), four unknown metabolites were observed in the range 5-10% of applied dose. In a second study (Study 2, Goodyear, 1999a), conducted on a single soil at a higher rate (80g/ha), two unknown metabolites exceeded 5% of applied. These low level metabolites were not identified but these studies were assessed as "acceptable" based on the evaluation criteria and guidance in force at that time. Although these low level metabolites were not identified as a concern in the 2000 EU review of silthiofam, individual Member States subsequently requested identification/additional information on these minor metabolites.

Following the 2000 EU review, efforts were thus made to further characterize minor metabolites designated as M1, M2, and M6 in the previous aerobic soil degradation studies in response to these inquiries and in order to comply with the more recent guidance documents to identify the metabolites present at levels >5% of applied radioactivity (AR). In a study by Völkel, 2008 (Study 3) [^{14}C] silthiofam was incubated in soil under aerobic conditions in order to generate soil degradation products for the purpose of isolation and identification of low levels silthiofam soil metabolites. The identification of silthiofam soil metabolites was then accomplished using soil extracts from the study by Völkel (2008) and the results were reported in a subsequent study report authored by Miller and Whitehead (2009). Three additional major soil metabolites observed in the aerobic soil metabolism studies of silthiofam were identified unequivocally by a combination of liquid chromatography/electrospray (LC/ESI) mass spectrometry, gas chromatography/electron ionization (GC/EI) mass spectrometry and reprivatisation (methyl ester formation) where appropriate. Additionally after Annex I inclusion, a new aerobic soil study (Fahrbach, 2013a) was conducted in order to address new technical guidance for study design and to comply with the requirements of the new OECD guideline no. 307 (OECD, 2002) for aerobic and anaerobic transformation in soil.

The results of the new studies are consistent with the conclusions of the first EU review of silthiofam (2000) and confirm that silthiofam is degraded in soil over time to carbon dioxide and several low level metabolites. Based on the identities of the minor metabolites, it is proposed that silthiofam is metabolized in soil by either oxidative removal of the allyl group to form the primary amide metabolite M1 (MON 65500 Amide), or oxidation of the ring-attached 5-methyl group to a carboxylic acid forming metabolite M2 (MON 65500 Allyl Acid). Further oxidative metabolism generates metabolite M6 (MON 65500 Acid Amide) by oxidation of the ring-attached 5-methyl group of metabolite M1, or oxidative removal of the allyl group from metabolite M2 to form the primary amide. All three of the identified soil metabolites of silthiofam retained the trimethylsilyl group. It was also proposed that only in a very acidic soil desilylation of silthiofam via chemical hydrolysis may occur to form CP 240659 as a potential soil metabolite.

Anaerobic degradation

During the 2000 EU evaluation, laboratory studies on anaerobic degradation of silthiofam in soil showed that its degradation was negligible under anoxic anaerobic conditions as dictated by the old SETAC anaerobic soil test guideline. In this laboratory anaerobic study, silthiofam dissipated rapidly from the surface water phase, however, its degradation in the total system (consisting of the surface water and soil phases together) was much slower. Up to five unidentified degradation products were observed in the surface water phase but individually none of these products exceeded 1.5% of applied radioactivity at any sampling interval. One unidentified component (in addition to parent compound) was detected for the soil compartment, accounting for 1.7% of applied radioactivity by the end of the study. Carbon dioxide evolution was minimal, with the level of $^{14}CO_2$ at study termination accounting for 0.2% of applied radioactivity.

A new laboratory anaerobic degradation study with silthiofam was conducted according to the requirements of the new OECD Guideline 307 for anaerobic transformation in soil. The results of the new study show that silthiofam also degrades under anaerobic conditions although at slower rate than the aerobic conditions. Only one predominant degradation product (> 5% of applied), designated as M1, was formed. Metabolite M1 was identified as MON 65500 Amide and reached a maximum concentration of 8.2% in the total soil/water system after 7 days of anaerobic incubation and subsequently declined to 7.1% after 120 days of incubation. Up to 33 other minor degradation products were also detected, but none of them exceeded 3.0% of the applied radioactivity. The metabolites resulting from the degradation of silthiofam in soil are the same under both aerobic and anaerobic conditions. Therefore, the metabolic pathway for degradation of silthiofam in soil under anaerobic conditions is the same as in the aerobic conditions. The results from the new study by Fahrbach 2013b) are in contrast to the results obtained from the previous anaerobic degradation of silthiofam (Goodyear, 1999b) conducted under the old SETAC anaerobic soil test guideline. The Goodyear (1999b) study examined the degradation of silthiofam when applied directly to sandy loam topsoil that had been flooded and converted to anaerobic conditions during a pre-incubation period of 32 days. Under the anoxic conditions established for the study, silthiofam did not degrade significantly. Prolonged and fully anaerobic conditions are expected to be rare throughout the surface soil zone where silthiofam is expected to be present.

5.2 Environmental distribution

5.2.1 Adsorption/Desorption

The adsorption/desorption behaviour of the active substance silthiofam was studies in two UK soils (sandy silt loam and clay loam soil types) and two US soils (sand and sandy loam soil types) by means of the Freundlich adsorption/desorption isotherm. The adsorption coefficient K_F for silthiofam ranged from 0.98 to 6.41 mL/g. When these values were normalized to the organic carbon content of the soils the corresponding K_{Foc} values ranged between 173 and 328 mL/g. The results indicate that silthiofam is moderately mobile in soil. The data shows a linear pH dependency between the coefficient of adsorption to soil organic carbon (K_{FOC}) and soil pH.

At the time of the first EU silthiofam review, the only relevant soil metabolite of silthiofam detected in the various soil metabolism studies was identified as CP 240659. Therefore, for the first Annex I inclusion, the adsorption/desorption behaviour of CP 240659 was investigated in three soil types. This is listed as Study 2 below. The results of the adsorption isotherms showed that adsorption was independent of the concentration of MON 65500 Amide (M1). The 1/n values ranging from 0.97 to 1.06 showed near linearity for the adsorption process. There was a correlation between the adsorption constant K_F and the pH measured in the soils. The desorption process was also found to be independent of the test substance concentration. The calculated $K_{des,Foc}$ values were in the same range as those observed in the adsorption, reflecting a similar sorption behaviour as found for the adsorption process. There was no correlation between the desorption constant $K_{des,\ Foc}$ and the pH of any of the soils. The K_{Foc} values ranged from 60 to 94, while the $K_{des,\ Foc}$ values ranged from 56 to 112.

In the renewal dossier, new adsorption-desorption studies have been conducted for other relevant silthiofam soil degradation products - M1 (identified as MON 6550 Amide), M2 (MON 65500 Allyl Acid), and metabolite M6 (MON 65500 Amide Acid). The adsorption/desorption behaviour of silthiofam metabolites M2 and M6 was investigated by Völkel, W. (2008d) on three different soil types. The result obtained show that both compounds have a high potential for mobility in soil. The

K_{Foc} values, derived by Allan, J. (2013 a-b), place metabolites M2 and M6 as very highly mobile to mobile according to the FAO classification.

Table 66: Summary of adsorption and desorption results for Silthiofam

Method	Results	Remarks	Reference
Adsorption and desorption in soil			
Study 1. Adsorption and desorption in soil of the Active Substance (Silthiofam) (^{14}C)-silthiofam: Adsorption/Desorption in four soils. *OECD Guideline 106 (1981); US EPA Pesticide Assessment Guidelines, Subdivision N, Section 163-1 (1982).*	The results of the study indicate that soil organic carbon content is an important factor influencing adsorption of Silthiofam. The K_{oc} values for the adsorption phase range from 173 to 328, with the lowest value being found for the soil which has the highest organic carbon content. The K_{oc} values for the first desorption phase range from 207 to 863, with the values for the second desorption phase covering the range 240 to 2903. In all cases the desorption constants are higher than the corresponding adsorption constants, indicating partial irreversible adsorption of Silthiofam to the soils used. The adsorption K_{oc} values for Silthiofam in the four soil types used in the study cover the range 173 to 328 and therefore Silthiofam can be classified as moderately mobile in these soils since the observed K_{oc} values are in the range 75 to 500 (Hollis, 1991).	The study has been evaluated for the previous inclusion into the Annex I of the Directive 91/414/EEC. This study was performed according to GLP criteria (except the soil characterisation work carried out by Land Research Associates (Derby, UK), Landis International Inc (Valdosta, Georgia, USA) and the Macaulay Research Institute (Aberdeen, Grampian, UK).	Lewis, C.J., (1995): (^{14}C)-MON 65500: Adsorption/Desorption in four soils. Corning Hazleton (Europe), Report no.: 64/42-1015. Not published. RAR B.8.1.2.1
Study 2. Adsorption and desorption in soil of the CP 240659, a metabolite of Silthiofam (MON65500). (^{14}C)-CP 240659 (MON 65500 Metabolite): Adsorption/Desorption in three soils. *OECD Guideline 106 (1981).*	The adsorption K_{oc} values for CP 240659 in the three soil types used in the study cover the range 77 to 135 and therefore CP 240659 can be classified as moderately mobile (tending towards mobile) in these soils since the observed K_{oc} values are in the range 75 to 500 (Hollis, 1991).	The study has been evaluated for the previous inclusion into the Annex I of the Directive 91/414/EEC. This study was performed according to GLP criteria.	Lewis, C.J., (1997a): (^{14}C)-CP 240659 (MON 65500 Metabolite): Adsorption/Desorption in three soils. Covance Laboratories, Report no.: 64/72-1015. Not published. RAR B.8.1.2.2
Study 3. Adsorption and desorption in soil of Amide (M1), a metabolite of Silthiofam (MON65500) MON 65500 Amide (M1) metabolite,	The results of the adsorption isotherms showed that adsorption was independent of the concentration of MON 65500 Amide (M1). The 1/n values ranging from 0.97 to 1.06 showed near linearity for the adsorption process. There was a correlation	This study was performed according to GLP criteria. Study considered acceptable.	Völkel, W. (2008c); MON 65500 Amide (M1) metabolite, Adsorption/desorption on soil. RCC Ltd. Environmental Chemistry & Pharmanalytics,

Adsorption/desorption on soil. *OECD Guideline 106 (Adopted January 21, 2000) Adsorption/desorption using a batch equilibrium method.*	between the adsorption constant K_F and the pH measured in the soils. The desorption process was also found to be independent of the test substance concentration. The calculated $K_{des,Foc}$ values were in the same range as those observed in the adsorption, reflecting a similar sorption behaviour as found for the adsorption process. There was no correlation between the desorption constant $K_{des,\ Foc}$ and the pH of any of the soils. The K_{Foc} values ranged from 60 to 94, while the $K_{des,\ Foc}$ values ranged from 56 to 112.		Itingen / Switzerland. Report No. MSL20934. Not published. RAR B.8.1.2.2
Study 4. Adsorption and desorption in soil of M2 and M6, metabolites of Silthiofam (MON65500) Adsorption/desorption of MON 65500 metabolites (M2 and M6) on soils. *OECD Guideline 106 (Adopted January 21, 2000) Adsorption/desorption using a batch equilibrium method.*	The adsorption/desorption characteristics of two silthiofam soil depredates, MON 65500 Allyl Acid (M2) and MON 65500 Amide Acid (M6) were studied using the batch equilibrium method. These were isolated from the samples generated in a study of the degradation of silthiofam reported in Study 3 (see above). The adsorption/desorption behaviour of silthiofam metabolites M2 and M6 was investigated on three different soil types. The result obtained show that both compounds have a high potential for mobility in soil.	This study was performed according to GLP criteria. Study considered acceptable.	Völkel, W. (2008d); Adsorption/desorption of MON 65500 metabolites (M2 and M6) on soils. RCC Ltd. Environmental Chemistry & Pharmanalytics, Itingen / Switzerland. Report No. MSL0020217. Not published. RAR B.8.1.2.2
Study 5. Adsorption and desorption in soil of M2, a metabolite of Silthiofam (MON65500). Determination of Adsorption – Desorption of Silthiofam Metabolite M2 Using the Batch Equilibrium Method. *OECD Guideline 106 (Adopted January 21, 2000) Adsorption/desorption using a batch equilibrium method;*	The adsorption/desorption isotherm of the test substance, metabolite M2, or MON 6550 Allyl Acid (MON 65533), a soil metabolite of silthiofam, was determined in three soils varying in properties and in geographical source. K_F values ranged from 0.13 in the Quilen loam to 0.67 in the Chelmorton silt loam. The organic carbon normalized Freundlich adsorption isotherm coefficients K_{Foc} ranged from 4.0 in the Quilen loam to 16 in the Chelmorton silt loam. The values for 1/n ranged from 0.86 to 0.89 across all the test soils. Coefficients of determination (r^2) for the isotherms ranged from 0.9649 to 0.9990 for	This study was performed according to GLP criteria. Study considered acceptable.	Allan, J. (2013a); Determination of Adsorption – Desorption of Silthiofam Metabolite M2 Using the Batch Equilibrium Method ABC Laboratories Report No. MSL0025058. Not published. RAR B.8.1.2.2

U. S. EPA OPPTS Guidelines 835.1230.	the adsorption phase, indicating the Freundlich equation adequately predicts the adsorption of the test substance over the concentration range studied. The K_{Foc} values place Metabolite M2 as very highly mobile to mobile according to the FAO classification.		
Study 6. Adsorption and desorption in soil of M6, a metabolite of Silthiofam (MON65500). Determination of Adsorption – Desorption of Silthiofam Metabolite M6 Using the Batch Equilibrium Method. *OECD Guideline 106 (Adopted January 21, 2000) Adsorption/desorption using a batch equilibrium method;* *U. S. EPA OPPTS Guidelines 835.1230.*	The adsorption/desorption isotherm of the test substance, metabolite M6, or MON 6550 Amid Acid (MON 65534), a soil metabolite of silthiofam, was determined in three soils. K_F values ranged from 0.11 in the Quilen loam to 0.64 in the Chelmorton silt loam. The organic carbon normalized Freundlich adsorption isotherm coefficients K_{Foc} ranged from 3.2 in the Quilen loam to 29 in the Ingleby sand. The values for 1/n ranged from 0.91 to 1.2 across all the test soils. Coefficients of determination (r^2) for the isotherms ranged from 0.9246 to 0.9978 for the adsorption phase, indicating the Freundlich equation adequately predicts the adsorption of the test substance over the concentration range studied. The K_{Foc} values place Metabolite M6 as very highly mobile to mobile according to the FAO classification.	This study was performed according to GLP criteria. Study considered acceptable.	Allan, J. (2013b); Determination of Adsorption – Desorption of Silthiofam Metabolite M6 Using the Batch Equilibrium Method. ABC Laboratories Report No. MSL0025059. Not published. RAR B.8.1.2.2
Column Leaching Studies			
(^{14}C)-silthiofam: Leaching Characteristics of Aged Residues Applied as a Seed Treatment in Two Soils. *OECD Draft Document (1999): Leaching in Soil Columns;* *SETAC-Europe, Procedures for Assessing the Environmental Fate and Ecotoxicity of Pesticides*	The test article, Silthiofam, showed low mobility in the sandy loam soil (Itingen) and the sandy clay loam soil (Foster) under the conditions of the study. It was not detected in the leachates from either soil. Neither was it detected below segment 2 in the extracts of soil segments taken after the leaching had finished. The levels of Silthiofam in the top segment (no. 1) were 10.5% and 20.8% of applied radioactivity for soils Itingen and Foster respectively.	The study has been evaluated for the previous inclusion into the Annex I of the Directive 91/414/EEC. Performed according to GLP criteria.	Mamouni, A, (1999) (^{14}C)-MON 65500: Leaching Characteristics of Aged Residues Applied as a Seed Treatment in Two Soils. RCC Ltd (Switzerland), Report no.: RCC Project 729538. Not published. RAR B8.1.3.1.
Field Leaching Study			
Field Study of the Potential of silthiofam to Leach and Accumulate.	Under worst case conditions for leaching (porous soil profile, wet winters succeeded by dry summers) and following three	The study has been evaluated for the previous inclusion into	Fogg, P.; Brown, C.D.; Carter, A.D. (1998): Field Study of the Potential of MON

Guideline: Not specified (in-house method used).	consecutive years of application at a rate of 40 g a.s./ha, silthiofam may have a potential to leach and accumulate in soil water at the depth of 1 meter. The year mean concentrations were <0.1 µg/l after a single application, a maximum of 0.124 µg/l after two consecutive years of treatment and a maximum of 0.41 µg/l after three consecutive years of treatment. The mean concentrations over the whole period of the study (three-year mean concentrations) were <0.1 µg/l for the strip that received one application, 0.027 µg/l for the strip that received two applications and 0.106 µg/l for the strip that received three applications. When the applications ceased, silthiofam concentrations one year after treatment were <0.1 µg/l at 1 m depth. The results of the field leaching study showed that there was a considerable decrease in concentrations of silthiofam between 50 and 100 cm depth. Therefore, a further significant decrease in leaching concentrations can be expected before leaching water reaches the groundwater body. The concentration in water at 10 m depth is likely to be less than 0.1 µg/l. It should also be noted there is great potential for dilution within the groundwater body itself. Consequently, it can be concluded that silthiofam used in accordance with good agricultural practice poses low risk to groundwater contamination.	the Annex I of the Directive 91/414/EEC. Performed according to GLP criteria.	65500 to Leach and Accumulate. Soil Survey and Land Research Centre, Cranfield University (UK), Report no.: 94/FLD/08/UK (SSLRC Project Number: 82/3158). Not published. RAR B8.1.3.3.

Adsorption and Desorption Summary

Table 67: Summary for the Active substance

Reference	Soil, origin	Soil type	OC [%]	pH (in H_2O)	Adsorption Coeficient		
					K_F	K_{Foc}	1/n
Lewis (1995)	LA95-154, USA	Sand	0.3	5.5	0.98	328	0.92
	PT 070, UK	Sandy silt loam	2.1	6.4	4.93	235	0.95
	SK9560682, UK	Clay loam	3.7	7.6	6.41	173	0.92
	Elder, USA	Sandy loam	1	6.3	2.65	265	0.81

	pH dependance: Yes

Table 68: Summary for CP240659

Study	Soil, origin	Soil Type	OC [%]	pH (in H_2O)	Adsorption Coeficient		
					K_d/K_F	K_{oc}/K_{Foc}	1/n
Lewis (1997a)	PT 183 (UK)	Sandy loam	0.8	7.4	0.61	77	0.83
	PT 195 (UK)	Sandy clay loam	1.6	6.5	1.51	94	0.78
	SK 566696 (UK)	Loamy sand	0.5	4.4	0.67	135	0.88
							pH dependance: Yes

Table 69: Summary for M1

Reference	Soil, origin	Soil type	OC [%]	pH (in H_2O)	Adsorption Coeficient		
					K_F	K_{Foc}	1/n
Völkel (2008c)	Speyer 2.2 (Germany)	Loamy sand	2.36	5.6 (KCl)	2.15	94	0.97
	Attenschwiller (France)	Silt loam	1.1	7.4 (KCl)	0.66	60	1.02
	Hesingue (France)	Silty clay	2.73	5.4 (KCl)	1.87	68	1.06
	Mechtildshausen (Germany)	Loam	1.46	7.1 (KCl)	1.2	82	1
							pH dependance: Yes

Table 70: Summary for M2

Reference	Soil, origin	Soil type	OC [%]	pH (in H_2O)	Adsorption Coeficient		
					K_F	K_{Foc}	1/n
Völkel (2008d)	Speyer 2.2 (Germany)	Sandy Loam	2.3	5.6 (KCl)	0.3 (K_d)	12 (K_{oc})	-
	Speyer 2.3 (Germany)	Loam	1.3	7.4 (KCl)	0.2 (K_d)	16 (K_{oc})	-
	Speyer 2.4 (Germany)	Silty Clay Loam	2.7	5.0 (KCl)	0.4 (K_d)	14 (K_{oc})	-
Allan (2013a)	Chelmorton (UK)	Silt loam	4.6	5.7	0.67	16	0.87
	Quilen (France)	Loam	3.3	6.9	0.13	4	0.89
	Ingleby, (UK)	Sand	0.9	5.4	0.16	13	0.86
							pH dependance: No

Table 71: Summary for M6

Reference	Soil, origin	Soil type	OC [%]	pH (in H_2O)	Adsorption Coeficient		
					K_F	K_{Foc}	1/n
Völkel (2008d)	Speyer 2.2 (Germany)	Sandy Loam	2.3	5.6 (KCl)	0.2 (K_d)	9 (K_{oc})	-
	Speyer 2.3 (Germany)	Loam	1.3	7.4 (KCl)	0.1 (K_d)	8 (K_{oc})	-
	Speyer 2.4 (Germany)	Silty Clay Loam	2.7	5.0 (KCl)	0.3 (K_d)	11 (K_{oc})	-
Allan (2013b)	Chelmorton (UK)	Silt loam	4.6	5.7	0.64	15	0.91
	Quilen (France)	Loam	3.3	6.9	0.11	3.2	0.91
	Ingleby, (UK)	Sand	0.90	5.4	0.36	29	1.2
							pH dependance: No

5.2.2 Volatilisation

Not relevant for this dossier.

5.2.3 Distribution modelling

From the adsorption/desorption study it can be concluded that Silthiofam is moderately mobile in soil. The identified metabolites, M1, M2 and M6 are very highly mobile to mobile according to the FAO classification.

5.3 Aquatic Bioaccumulation

Table 72: Summary of relevant information on aquatic bioaccumulation

Method	Results	Remarks	Reference
(^{14}C)-silthiofam: Flow-through fish bioconcentration test *OECD 305E and US EPA Chapter 165-4 Subdivision N. There were no deviations from the guidelines.*	The mean bioconcentration factor for Silthiofam (MON 65500) in whole fish was determined to be 98 mg/L.	Performed according to GLP criteria.	Yeomans P. (1998). (^{14}C)-MON 65500: Flow-through fish bioconcentration test, Covance Laboratories, unpublished report No.: 64/68. DAR B9.2.3.1

5.3.1 Aquatic bioaccumulation

The log K_{ow} for Silthiofam is 3.72. Although no prolonged or repeated exposure of the aquatic environment is expected, a fish bioconcentration study was carried out because the log $P_{ow} \geq 3.0$. The bioconcentration factor (BCF) for fish was determined experimentally to be 98 mg/L.

5.3.1.1 Bioaccumulation estimation

Since, the log Kow of Silthiofam (3.72) is lower than the threshold values (CLP Regulation ≥4), the potential risk for bioaccumulation in tissues of aquatic organisms is low.

5.3.1.2 Measured bioaccumulation data

Study: (^{14}C)-silthiofam: Flow-through fish bioconcentration test; Yeomans P. (1998)

Rainbow trout (*Oncorhynchus mykiss*) were exposed to (^{14}C)-MON65500 (Silthiofam) diluted with unlabelled MON 65500 (Silthiofam) at nominal concentrations of 0.14 and 0.7 mg a.s./l. There was one negative control (dilution water with solvent at 0.1 ml/l). The test system was flow-through (medium exchange rate of 8 volumes/24 h/tank), exposure duration was 28 days, after which test fish were transferred to clean tanks containing dilution water only for a depuration period of 56 days. One hundred fish were allocated to each test group (divided into two replicates/dose level), and eighty to

the control group. Trout mean fork length was 7.6 cm and mean fresh weight 6.0 g (measured for 10 control fish at start of test).

Findings

There was no mortality in the controls or in the group exposed to (^{14}C)-MON 65500 (Silthiofam) at 0.14 mg/l. At 0.7 mg/l (^{14}C)-MON 65500 (Silthiofam), one fish died on Day 28 and two further mortalities (not treatment-related) occurred during the depuration phase. Residues observed in edible and non-edible tissues consisted of the parent molecule (37 to 50%) and numerous unknown components. Only one unknown component exceeded 10% of radioactivity, ranging from 11 to 19% of total radioactivity. Depuration of the parent molecule and its degradates was rapid, with calculated half-lives < 6 days for all sample types. At the end of the 56 day depuration period, 12% of total radioactivity remained in whole fish tissues.

The average BCF for the study was 98. Therefore, if fish were exposed to silthiofam once per season at low levels, they will not take up the compound in high amounts and will subsequently release any residues rapidly.

Depuration of residues of Silthiofam from whole fish tissues was faster in those fish exposed to the higher concentration of Silthiofam (0.7 a.i. mg/l). This is due to the fact that the rate of depuration did not follow first order kinetics (single phase exponential loss) but second order kinetics (two phase exponential loss) thus, resulting in a faster depuration rate of 1.17 days in the 0.7 a.i. mg/l treatment group compared to 4.72 days in the 0.14 a.i. mg/l

Table 73: Kinetic bioconcentration factors for Silthiofam in Rainbow trout (*Oncorhynchus mykiss*)

	0.14 mg a.s./l			**0.7 mg a.s./l**		
	Edible	**Non-edible**	**Whole fish**	**Edible**	**Non-edible**	**Whole fish**
k_1 (days^{-1})	8.468	34.470	16.780	19.316	107.282	48.529
k_2 (days^{-1})	0.129	0.186	0.147	0.423	0.781	0.592
DT_{50} (days)	5.37	3.73	4.72	1.64	0.89	1.17
DT_{95} (days)	23.20	16.10	20.41	7.08	3.84	5.06
BCF_k	66	185	**114**	46	137	**82**

k_1 - uptake rate constant; k_2 - depuration rate constant; BCF_k - kinetic bioconcentration factor

Conclusion

The mean bioconcentration factor for Silthiofam in whole fish was determined to be 98 mg/L.

5.3.2 Summary and discussion of aquatic bioaccumulation

Although no prolonged or repeated exposure of the aquatic environment is expected, a fish bioconcentration study was carried. Silthiofam has a log P_{ow} 3.72 and the bioconcentration factor (BCF) for fish was determined experimentally to be 98 mg/L. The BCF is below the classification criteria of 500 mg/L and is therefore not considered for classification purposes.

5.4 Aquatic toxicity

Table 74: Summary of relevant information on aquatic toxicity

Method	Results	Remarks	Reference
Fish			
Silthiofam: A 96-hour static acute toxicity test with the rainbow trout (*Oncorhynchus mykiss*) *OECD 203 and FIFRA Chapter 72-1 Subdivision E.*	LC_{50} (mg a.s./l) = 14 (95% CI: 13 - 16) LOEC(mg a.s./l) = 5.3 NOEC(mg a.s./l) = 3.2 Silthiofam is only slightly toxic to rainbow trout up to the water solubility limit.	Performed according to GLP criteria.	Graves W.C. and Swigert J.P. (1996). MON 65500: A 96-hour static acute toxicity test with the rainbow trout (*Oncorhynchus mykiss*) Generated by: Wildlife International Ltd Submitted by: Monsanto Company Report No.: WL-95-276 GLP, Unpublished. RAR B9.2.1
Silthiofam: A 96-hour static acute toxicity test with the Bluegill (*Lepomis macrochirus*) *OECD 203 and FIFRA Chapter 72-1 Subdivision E.*	LC_{50} (mg a.s./l) = 11 (95% CI: > 8.4) LOEC(mg a.s./l) = 6.0 NOEC(mg a.s./l) = 3.7 Silthiofam is slightly toxic to bluegill sunfish up to the water solubility limit.	Performed according to GLP criteria.	Graves W.C. and Swigert J.P. (1996). MON 65500: A 96-hour static acute toxicity test with the Bluegill (*Lepomis macrochirus*). Generated by: Wildlife International Ltd Submitted by: Monsanto Company Report No.: WL-95-280 GLP, Unpublished. RAR B9.2.1
MON 65513: A 96-hour static acute toxicity test with the rainbow trout (*Oncorhynchus mykiss*). *OECD 203, EEC method C1 and FIFRA Chapter 72-2 Subdivision E.*	LC_{50} (mg a.s./l) = 71 (95% CI: 60 – 98) LOEC(mg a.s./l) = 35 NOEC(mg a.s./l) = 21 MON 65513 is slightly toxic to Rainbow Trout up to the water solubility limit.	Performed according to GLP criteria.	Palmer S.J. and Krueger H.O. (1998). MON 65513: A 96-hour static acute toxicity test with the rainbow trout (*Oncorhynchus mykiss*). Generated by: Wildlife International Ltd.

			Submitted by: Monsanto report No.: WL-98-092 GLP, Unpublished RAR B9.2.1
MON 65513: A 96-hour static acute toxicity test with the Bluegill (*Lepomis macrochirus*). *OECD 203, EEC method C1 and FIFRA Chapter 72-1 Subdivision E.*	LC_{50} (mg a.s./l) = 86 (95% CI: 72 – 119) LOEC(mg a.s./l) = 72 NOEC(mg a.s./l) = 44 MON 65513 is slightly toxic to Bluegill sunfish up to the limit of water solubility.	Performed according to GLP criteria.	Palmer S.J. and Krueger H.O. (1998). MON 65513: A 96-hour static acute toxicity test with the Bluegill (*Lepomis macrochirus*). Generated by: Wildlife International Ltd. Submitted by: Monsanto report No.: WL-98-091 GLP, Unpublished RAR B9.2.1
MON 65500 Allyl Acid (MON 65533): A 96-Hour Static Acute Toxicity Test with the Rainbow Trout (*Oncorhynchus mykiss*). *EEC-C1; OECD 203; OPPTS 850.1075; ASTM Standard E729-96*.	The 96-hour LC_{50} for rainbow trout, *Oncorhynchus mykiss*, exposed to MON 65533 (metabolite M2) under static test conditions was >113 mg MON 65533/L, the highest concentration tested. The no-mortality concentration and the no-observed-effect concentration (NOEC) were both 113 mg MON 65533/L.	Performed according to GLP criteria.	Schneider, S.Z., Kendall, T. Z., Krueger, H.O. (2010). MON 65500 Allyl Acid (MON 65533): A 96-Hour Static Acute Toxicity Test with the Rainbow Trout (*Oncorhynchus mykiss*). Monsanto report no. WL-2010-233 RAR B9.2.1
MON 65500 Amide Acid (MON 65534): A 96-Hour Static Acute Toxicity Test with the Rainbow Trout (*Oncorhynchus mykiss*). *EEC-C1; OECD 203; OPPTS 850.1075; ASTM Standard E729-96*	The 96-hour LC_{50} for rainbow trout, *Oncorhynchus mykiss*, exposed to MON 65534 (metabolite M6) under static test conditions was >110 mg MON 65534/L, the highest concentration tested. The no-mortality concentration and the no-observed-effect concentration (NOEC) were both 110 mg MON 65534/L.	Performed according to GLP criteria.	Schneider, S.Z., Kendall, T.Z., Krueger, H.O. (2010). MON 65500 Amide Acid (MON 65534): A 96-Hour Static Acute Toxicity Test with the Rainbow Trout (*Oncorhynchus mykiss*). Monsanto report no. WL-2010-234. RAR B9.2.1
MON 65561: A 96-Hour Static Acute Toxicity Test with the	The 96-hour LC_{50} for rainbow trout, *Oncorhynchus mykiss*,	Performed according to GLP criteria.	Minderhout T, Kendall TZ,

Rainbow Trout (*Oncorhynchus mykiss*). *OECD 203, OPPTS 850.1075, EEC C.1.*	exposed to MON 65561 under static test conditions was greater than 24.7 mg/L, the highest concentration tested. The no-mortality concentration was 10.1 mg/L and the no-observed-effect concentration (NOEC) was 4.7 mg/L.		Krueger HO. (2008). MON 65561: A 96-Hour Static Acute Toxicity Test with the Rainbow Trout (*Oncorhynchus mykiss*). Monsanto report no. WL-2007-116. RAR B9.2.1
Silthiofam: An Early Life-Stage Toxicity Test with the Fathead Minnow (*Pimephales promelas*). *ASTM Standard E-1241-05; FIFRA Subdivision E, Section 72-4a; OECD 210; OPPTS 850.1400*	Fathead minnows (*Pimephales promelas*) were exposed to silthiofam at mean measured concentrations ranging from 0.12 to 1.8 mg/L under flow-through conditions for 33 days (a 5-day hatching period plus a 28-day post-hatch growth period). There were no significant treatment-related effects on hatching success or survival at concentrations ≤1.8 mg/L. Growth, measured as total length, wet and dry weight, was the most sensitive biological endpoint measured in this study. Fathead minnows exposed to silthiofam at concentrations ≥1.8 mg/L had statistically significant reductions in total length, wet weight and dry weight in comparison to the pooled controls. Consequently, the NOEC, based on growth, was 0.89 mg/L. The LOEC was 1.8 mg/L and the MATC was calculated to be 1.3 mg/L.	Performed according to GLP criteria.	Minderhout T, VanEvera SM, Gallagher SP, Martin KH. (2014). MON 65500: An Early Life-Stage Toxicity Test with the Fathead Minnow (*Pimephales promelas*) Monsanto report no. WL-2013-0381.
Aquatic Invertebrates			
Silthiofam: A 48-hour static toxicity test with the cladoceran (*Daphnia magna*). *OECD 202, FIFRA Chapter 72-2 Subdivision E.*	EC_{50} (mg a.s./l) = 14.0 (95% CI: 12 – 16) LOEC(mg a.s./l) = 7.8 NOEC(mg a.s./l) = 4.9 Up to the limit of water solubility, Silthiofam is only slightly toxic to the waterflea, *Daphnia magna*.	Performed according to GLP criteria.	Graves W.C. and Swigert J. P. (1996). MON 65500: A 48-hour static toxicity test with the cladoceran (*Daphnia magna*) Generated by: Wildlife International Ltd Submitted by: Monsanto Company Report No.: WL-95-275

			GLP, Unpublished. RAR B9.2.4
MON 65513: A 48-hour static acute toxicity test with the cladoceran (*Daphnia magna*). *OECD 202, EEC method C2, FIFRA Chapter 72-2 Subdivision E.*	EC_{50} (mg a.s./l) = 78.0 (95% CI: 59 – 119) LOEC(mg a.s./l) = 59.0 NOEC(mg a.s./l) = 30.0 Up to the limit of water solubility, MON 65513 is only slightly toxic to the waterflea *Daphnia magna*.	Performed according to GLP criteria.	Palmer S.J. and Krueger H.O. (1998). MON 65513: A 48-hour static acute toxicity test with the cladoceran (*Daphnia magna*), Generated by: Wildlife International Ltd., unpublished Submitted by: Monsanto Company Report No.: WL-98-090. GLP, Unpublished. RAR B9.2.4
Silthiofam: A semi-static life-cycle toxicity test with the cladoceran (*Daphnia magna*). *OECD 211; ASTM Standard E1193-87*	LOEC(mg a.s./l) survival = 3.7 LOEC(mg a.s./l) reproduction = 3.7 LOEC(mg a.s./l) growth = 0.96 NOEC(mg a.s./l) survival= 1.8 NOEC(mg a.s./l) reproduction = 1.8 NOEC(mg a.s./l) growth = 0.47 Silthiofam has a moderate chronic toxicity to *Daphnia magna* with a NOEC of 0.47 mg a.i./l.	Performed according to GLP criteria.	Drottar, K.R., Kendall, T.Z. and Kreuger, H.O. (2000). MON 65500: A semi-static life-cycle toxicity test with the cladoceran (*Daphnia magna*) Generated by: Wildlife International, Ltd. Submitted by: Monsanto Company Report No.: WL-2000-93 GLP, Unpublished. RAR B9.2.5
MON 65500 Allyl Acid (MON 65533): A 48-Hour Static Acute Toxicity Test with the Cladoceran (*Daphnia magna*). *ASTM Standard E729-96; EEC-C2; OECD 202; OPPTS 850.1010.*	The 48-hour EC_{50} for *Daphnia magna* exposed to MON 65533 under static test conditions was >110 mg MON 65533/L, the highest concentration tested. The no-immobility and no-observed-effect concentrations (NOEC) were both 110 mg MON 65533/L.	Performed according to GLP criteria.	Schneider, S.Z., Kendall, T.Z., Krueger, H.O. (2010). MON 65500 Allyl Acid (MON 65533): A 48-Hour Static Acute Toxicity Test with the Cladoceran (*Daphnia magna*). Monsanto report no. WL-2010-235.

			RAR B9.2.4
MON 65500 Amide Acid (MON 65534): A 48-Hour Static Acute Toxicity Test with the Cladoceran (*Daphnia magna*). *ASTM Standard E729-96; EEC-C2; OECD 202; OPPTS 850.1010*	The 48-hour EC_{50} for *Daphnia magna* exposed to MON 65534 under static test conditions was >109 mg MON 65534/L, the highest concentration tested. The no-immobility and no-observed-effect concentrations (NOEC) were both 109 mg MON 65534/L	Performed according to GLP criteria.	Schneider, S.Z., Kendall, T.Z., Krueger, H.O. (2010). MON 65500 Amide Acid (MON 65534): A 48-Hour Static Acute Toxicity Test with the Cladoceran (*Daphnia magna*). Monsanto report no. WL-2010-236. RAR B9.2.4
MON 65561: A 48-Hour Static Acute Toxicity Test with the Cladoceran (*Daphnia magna*). *OECD 202, OPPTS 850.1010, ASTM Standard E-729-96*	The 48-hour EC_{50} for *Daphnia magna* exposed to MON 65561 under static test conditions was greater than 26.8 mg/L, the highest concentration tested and the apparent solubility limit of the test substance in the test system. The no-mortality or immobility concentration was 12.3 mg/L and the no-observed effect concentration (NOEC) was 6.4 mg/L.	Performed according to GLP criteria.	Minderhout T, Kendall TZ, Krueger HO. (2008). MON 65561: A 48-Hour Static Acute Toxicity Test with the Cladoceran (*Daphnia magna*). Monsanto report no. WL-2007-117. RAR B9.2.4
Algae			
Silthiofam: A five-day toxicity test with the freshwater alga (*Selenastrum capricornutum*). *OECD 201, EEC method C3 and FIFRA Chapter 123-2.*	There were no statistically significant reductions in cell density, area under the growth curve or growth rate of *Selenastrum capricornutum* exposed to Silthiofam (MON 65500) at concentrations of 2.3 mg a.s./l. Day 3 EC_{50} values for biomass and growth rate were: EbC_{50} (0-72 h) = 8.6 (confidence limits: 2.9 and 11) and ErC_{50} (0-72 h) = 13 (confidence limits: 13 and 13), respectively. NOEbC = 4.6 mg a.s./L NOErC = 2.3 mg a.s./L Therefore, Silthiofam is slightly to moderately toxic to the green algae, *Selenastrum capricornutum.*	Performed according to GLP criteria.	Drottar K.R. and Krueger H.O. (1998). MON 65500: A five-day toxicity test with the freshwater alga (*Selenastrum capricornutum*), Wildlife International Ltd., unpublished report No.: WL-97-167. RAR B9.2.6
MON 65513: A 72 h toxicity test with the freshwater alga (*Selenastrum capricornutum*). *OECD 201 and EEC method C3.*	There were no statistically significant reductions in cell density, area under the growth curve or growth rate of *Selenastrum capricornutum*	Performed according to GLP criteria.	Palmer S.J. and Krueger H.O. (1998). MON 65513: A 72 h toxicity test with

	exposed to silthiofam at concentrations of 15 mg a.s./l. Day 3 EC_{50} values for biomass and growth rate were: EbC_{50} (0-72 h) = 21 (confidence limits: 18 and 23) and ErC_{50}(0-72 h) = 28 (confidence limits: 24 and 31), respectively. MON 65513 is slightly toxic to the green algae *Selenastrum capricornutum*.		the freshwater alga (*Selenastrum capricornutum*), Generated by: Wildlife International Ltd., unpublished Submitted by: Monsanto Company Report No.: WL-98-089. GLP, Unpublished. RAR B9.2.6
MON 65500 Allyl acid (MON 65533): A 72-hour toxicity test with the freshwater alga (*Pseudokirchneriella subcapitata*). *OECD 201 (2006) and EU Directive 92/69/EEC, Method C.3.*	Based on mean measured concentrations, the 72-hour EC_{50} values for MON 65533 toxicity to *Pseudokirchneriella subcapitata* under static test conditions were determined to be: EC_{50} (cell density): >112 mg MON 65533/L (95% confidence interval: n/a) E_rC_{50} (growth rate): >112 mg MON 65533/L (95% confidence interval: n/a) The 72-hour NOAEC for both cell density and growth rate was 112 mg MON 65533/L.	Performed according to GLP criteria.	Porch, J.R.; Kendall, T.Z.; Krueger, H.O. (2010). MON 65500 Allyl acid (MON 65533): A 72-hour toxicity test with the freshwater alga (*Pseudokirchneriella subcapitata*). Monsanto study no. WL-2010-237. RAR B9.2.6
MON 65500 amide acid (MON 65534): A 72-hour toxicity test with the freshwater alga (*Pseudokirchneriella subcapitata*). *OECD 201 (2006) and EU Directive 92/69/EEC, Method C.3.*	Based on mean measured concentrations, the 72-hour EC_{50} values for MON 65534 toxicity to *Pseudokirchneriella subcapitata* under static test conditions were determined to be: EC_{50} (cell density): >110 mg MON 65534/L (95% confidence interval: n/a) E_rC_{50} (growth rate): >110 mg MON 65534/L (95% confidence interval: n/a) The 72-hour NOAEC for cell density and growth rate were 54 and 110 mg MON 65534/L, respectively.	Performed according to GLP criteria.	Porch, J.R.; Kendall, T.Z.; Krueger, H.O. (2010). MON 65500 amide acid (MON 65534): A 72-hour toxicity test with the freshwater alga (*Pseudokirchneriella subcapitata*). Monsanto study no. WL-2010-238. RAR B9.2.6
MON 65561: A 72-hour Toxicity Test with the Freshwater Alga (*Pseudokirchneriella subcapitata*).	The 72-hour EC_{50}, based on cell density, was 19 mg/L with the 95% confidence intervals of 15 to 23 mg/L.	Performed according to GLP criteria.	Minderhout, T.; Kendall, T.Z.; Krueger, H.O. (2008). MON 65561: A 72-hour

OECD Guideline 201, EU Directive 92/69/EEC, Method C.3.	The E_bC_{50}, based on area under the growth curve, was 19 mg/L, with the 95% confidence intervals of 16 to 23 mg/L. The E_rC_{50}, based on growth rate, was the least sensitive and was determined to be greater than 23 mg/L, the highest concentration tested. Dunnett's test indicated that cell density, area under the growth curve and growth rate were significantly reduced (p<0.05) in the 5.8, 11 and 23 mg/L treatment levels. Consequently, the 72-hour NOEC in this study was determined to be 3.2 mg/L.		Toxicity Test with the Freshwater Alga (*Pseudokirchneriella subcapitata*). Monsanto study no. WL-2007-118. RAR B9.2.6

5.4.1 Fish

5.4.1.1 Short-term toxicity to fish

Silthiofam: A 96-hour static acute toxicity test with the rainbow trout (Oncorhynchus mykiss); Graves W.C. and Swigert J.P. (1996)

The acute toxicity of Silthiofam (MON 65500) to rainbow trout *(Oncorhynchus mykiss)* was investigated by Graves W.C. and Swigert J.P. in accordance with the *OECD 203 Fish, Acute Toxicity Test* and *FIFRA Subdivision E § 72-1*.

Rainbow trout were exposed to Silthiofam (MON 65500) at nominal concentrations of 3.2, 5.4, 9.0, 15 and 25 mg a.s./l (measured concentrations: 3.2, 5.3, 8.4, 13 and 16 mg a.s./l) under static conditions for a period of 96h. One negative control (well water) and one solvent control were included. Twenty fish were allocated to each group, divided into two replicates/dose level. Animals were fasted during acclimation and test periods. Mortality, signs of toxicity or abnormal behaviour were monitored at approximately 2.5, 24, 48, 72 and 96 h.

No mortalities occurred in the 5.3 and 8.4 mg a.i./l treatment groups, however, sublethal toxicological effects did occur and these included lethargy, dark coloration, loss of equilibrium and lying on the bottom of the test chamber with only gill movements.

Table 75: Toxicity of silthiofam to Rainbow trout (*Oncorhynchus mykiss*)

Test Substance	TG a.s.
Test Object	Rainbow Trout
Exposure	96h, static
LC_{50} mg a.s./l	14 (95% CI: 13 - 16)

Lowest Observed Effect Concentration (LOEC) mg a.s./l	5.3
Highest Tested conc. without toxic effect (NOEC) mg a.s./l	3.2

The study concluded Silthiofam (MON 65500) is only slightly toxic to rainbow trout up to the water solubility limit.

Silthiofam: A 96-hour static acute toxicity test with the Bluegill (Lepomis macrochirus); Graves W.C. and Swigert J.P. (1996)

The acute toxicity of Silthiofam (MON 65500) to Bluegill (*Lepomis macrochirus*) was investigated by Graves W.C. and Swigert J.P. in accordance with in accordance with the *OECD 203 Fish, Acute Toxicity Test* and *FIFRA Subdivision E § 72-1*.

Bluegill (*Lepomis macrochirus*) fish were exposed to Silthiofam (MON 65500) at measured concentrations of 2.4, 3.7, 6.0, 8.4 and 12 mg a.s./l under static conditions for a period of 96h. There was one negative control (well water) and one solvent control. Twenty fish were allocated to each group, divided into two replicates/dose level. Animals were fasted during acclimation and test periods. Mortality, signs of toxicity or abnormal behaviour were monitored at approximately 1, 24, 48, 72 and 96 h. Water temperature, pH, dissolved oxygen, hardness, alkalinity and specific conductance were monitored as required during the test. Water samples for determination of silthiofam concentrations were collected from each replicate test chamber at 0, 48 and 96 h.

Although no mortalities were observed in the 6.0 and 8.4 mg a.i./l treatment groups throughout the test, sublethal signs of toxicity such as loss of equilibrium, erratic swimming and lethargy were observed.

Table 76: Acute toxicity to Bluegill sunfish (*Lepomis macrochirus)*

Test Substance	TG a.s.
Test Object	Bluegill sunfish
Exposure	96h, Static
LC_{50} mg a.s./l	11 (95% CI: > 8.4)
Lowest observed effect (LOEC) mg a.s./l	6.0
Highest test conc. without toxic effect (NOEC) mg a.s./l	3.7

The study concluded Silthiofam (MON 65500) is slightly toxic to bluegill sunfish up to the water solubility limit.

MON 65513: A 96-hour static acute toxicity test with the rainbow trout (Oncorhynchus mykiss); Palmer S.J. and Krueger H.O. (1998)

The acute toxicity of the Silthiofam metabolite CP 240659 (referred to as MON 65513) to rainbow trout (*Oncorhynchus mykiss*) was investigated by Palmer S.J. and Krueger H.O. in accordance with *OECD 203 Fish, Acute Toxicity Test, EEC Method C1* and *FIFRA Subdivision E § 72-2*.

Rainbow trout (*Oncorhynchus mykiss*) were exposed to MON 65513 at nominal concentrations of 13, 22, 36, 60 and 100 mg a.s./l (mean measured concentrations: 13, 21, 35, 60 and 98 mg a.s./l) under static conditions for a period of 96h, with one negative control (well water). Twenty fish were allocated to each group, divided into two replicates/dose level. Animals were fasted during acclimation and test periods. Mortality, signs of toxicity or abnormal behaviour were monitored at approximately 3, 24, 48, 72 and 96 h. Water temperature, pH, dissolved oxygen, hardness, alkalinity and specific conductance were monitored as required during the test. Water samples for determination of silthiofam concentrations were collected from each replicate test chamber at 0, 48 and 96 h.

No mortalities occurred in the 35 and 60 mg a.i./l treatment groups, however, sublethal signs of toxicity were observed such as surfacing, lethargy, erratic swimming and / or lying on the bottom.

Table 77: Toxicity of MON 65513 to Rainbow trout (*Oncorhynchus mykiss*)

Test Substance	TG pm*
Test Object	Rainbow Trout
Test Exposure	96h, Static
LC_{50} mg a.s./l	71 (95% CI: 60 – 98)
Lowest observed effect concentration (LOEC) mg a.s./l	35
Highest tested conc. without toxic effect (NOEC) mg a.s./l	21

pm*=pure metabolite

The study concluded the Silthiofam metabolite (CP 246059) MON 65513 is slightly toxic to Rainbow Trout up to the water solubility limit.

MON 65513: A 96-hour static acute toxicity test with the Bluegill (Lepomis macrochirus); Palmer S.J. and Krueger H.O. (1998)

The acute toxicity of the Silthiofam metabolite CP 240659 (referred to as MON 65513) to Bluegill (*Lepomis macrochirus*) was investigated by Palmer S.J. and Krueger H.O. in accordance with *OECD 203 Fish, Acute Toxicity Test, EEC Method C1* and *FIFRA Subdivision E § 72-1*.

Bluegill sunfish (*Lepomis macrochirus*) were exposed to MON 65513 at nominal concentrations of 16, 26, 43, 72 and 120 mg a.s./l (measured concentrations: 15, 26, 44, 72 and 119 mg a.s./l), under static conditions for a period of 96h, with one negative control (well water). Twenty fish were allocated to each group, divided into two replicates/dose level. Animals were fasted during acclimation and test period. Mortality, signs of toxicity or abnormal behaviour were monitored at approximately 2, 24, 48, 72 and 96 h. Water temperature, pH, dissolved oxygen, hardness, alkalinity and specific conductance were monitored as required during the test. Water samples for determination of MON 65513 concentrations were collected from each replicate test chamber at 0, 48 and 96 h.

After two hours of exposure, all fish in the 72 mg a.i./l treatment group appeared lethargic, however, all fish appeared normal within in 24 hours. Also, in the 72 mg a.i./l treatment group, fish were found lying on the bottom of the aquaria during the 48, 72 and 96 hours observations. After two hours, all fish were observed to be lying on the bottom in the 119 mg a.i./l treatment group.

Table 78: Toxicity of MON 65513 to Bluegill sunfish (*Lepomis macrochirus*)

Test Substance	TG pm*
Test Object	Bluegill Sunfish
Test Exposure	96h, Static
LC_{50} mg a.s./l	86 (95% CI: 72 – 119)
Lowest observed effect concentration (LOEC) mg a.s./l	72
Highest tested conc. without toxic effect (NOEC) mg a.s./l	44

pm* = pure metabolite

The study concluded the Silthiofam metabolite (CP 246059) MON 65513 is slightly toxic to Bluegill sunfish up to the limit of water solubility.

MON 65500 Allyl Acid (MON 65533): A 96-Hour Static Acute Toxicity Test with the Rainbow Trout (Oncorhynchus mykiss); Schneider, S.Z., Kendall, T. Z., Krueger, H.O. (2010)

The 96-hour acute toxicity of MON 65533 to rainbow trout (*Oncorhynchus mykiss*) was determined in a static test system. Ten fish per treatment level were exposed in moderately-hard water obtained from a well for four days to MON 65533 at nominal concentrations of 0 (control), 7.5, 15, 30, 60 and 120 mg MON 65533 Na salt/L. Mortality and signs of toxicity were recorded at 2.5, 24, 48, 72 and 96 hours after test initiation.

Findings

Mortality and signs of toxicity in control and treated groups are summarised in the table below. No mortalities or effects due to treatment were observed in rainbow trout exposed to MON 65533 at concentrations of 7.0, 14.0, 27.9, 56.8 and 113.6 mg MON 65533/L. The study was considered valid as mortality in the control group did not exceed 10% by the end of the test and dissolved oxygen concentration was at least 60% of the air saturation value throughout the test.

Table 79: Toxicity of MON 65533 to rainbow trout (*Oncorhynchus mykiss*) under static conditions

Nominal Concentration (mg MON 65533 Na salt /L)	**Mean Measured Concentration (mg MON 65533/L)[1]**	**Number of dead fish[2]**			
		24 hours	**48 hours**	**72 hours**	**96 hours**
0 (control)	---	0/10	0/10	0/10	0/10
7.5	7.0	0/10	0/10	0/10	0/10
15	14.0	0/10	0/10	0/10	0/10
30	27.9	0/10	0/10	0/10	0/10

60	56.8	0/10	0/10	0/10	0/10
120	113.6	0/10	0/10	0/10	0/10

[1] Mean of concentrations measured at test initiation, at 48 hours, and at test termination, corrected for purity of test substance and molecular weight of salt.
[2] Number of dead individuals/total number of fish in the group.

Conclusion
The 96-hour LC_{50} for rainbow trout, *Oncorhynchus mykiss*, exposed to MON 65533 under static test conditions was >113 mg MON 65533/L, the highest concentration tested. The no-mortality concentration and the no-observed-effect concentration (NOEC) were both 113 mg MON 65533/L.

MON 65500 Amide Acid (MON 65534): A 96-Hour Static Acute Toxicity Test with the Rainbow Trout (Oncorhynchus mykiss); Schneider, S.Z., Kendall, T.Z., Krueger, H.O. (2010)

The 96-hour acute toxicity of MON 65534 to rainbow trout (*Oncorhynchus mykiss*) was determined in a static test system. Ten fish per treatment level were exposed in moderately-hard water obtained from a well for four days to MON 65534 at nominal concentrations of 0 (control), 3.8, 7.5, 15, 30, 60 and 120 mg MON 65534 Na salt/L. Mortality and signs of toxicity were recorded at 2, 24, 48, 72 and 96 hours after test initiation.

Findings
Mortality and signs of toxicity in control and treated groups are summarized in the table below. No mortalities or effects due to treatment were observed in rainbow trout exposed to MON 65534 at concentrations of 3.5, 6.8, 12.9, 27.6, 56.2 and 110.5 mg MON 65534/L. The study was considered valid as mortality in the control group did not exceed 10% by the end of the test and dissolved oxygen concentration was at least 60% of the air saturation value throughout the test.

Table 80: Toxicity of MON 65534 to rainbow trout (*Oncorhynchus mykiss*) under static conditions

Nominal Concentration (mg MON 65534 Na salt/L)	Mean Measured Concentration (mg MON 65534/L)[1]	Number of dead fish[2]			
		24 hours	48 hours	72 hours	96 hours
0 (control)	---	0/10	0/10	0/10	0/10
3.8	3.5	0/10	0/10	0/10	0/10
7.5	6.8	0/10	0/10	0/10	0/10
15	12.9	0/10	0/10	0/10	0/10
30	27.6	0/10	0/10	0/10	0/10
60	56.2	0/10	0/10	0/10	0/10
120	110.5	0/10	0/10	0/10	0/10

[1] Mean of concentrations measured at test initiation, at 48 hours and at test termination, corrected for purity of test substance and molecular weight of salt.
[2] Number of dead individuals/total numbers of fish in the group.

Conclusion
The 96-hour LC_{50} for rainbow trout, *Oncorhynchus mykiss*, exposed to MON 65534 under static test conditions was >110 mg MON 65534/L, the highest concentration tested. The no-mortality concentration and the no-observed-effect concentration (NOEC) were both 110 mg MON 65534/L.

MON 65561: A 96-Hour Static Acute Toxicity Test with the Rainbow Trout (Oncorhynchus mykiss); Minderhout T, Kendall TZ, Krueger HO. (2008)

The 96-hour acute toxicity of MON 65561 to rainbow trout (*Oncorhynchus mykiss*) was determined in a static test system. Two replicate test chambers, with 10 rainbow trout in each test chamber, were exposed in moderately-hard water obtained from a well for four days to MON 65561 at nominal concentrations of 0 (control), 1.7, 3.3, 6.6, 13.2 and 26.4 mg MON 65561/L. Mortality and signs of toxicity were recorded at 5, 24, 48, 72 and 96 hours after test initiation.

Findings

Mortality and signs of toxicity in control and treated groups are summarized in the table below. No mortalities or effects due to treatment were observed in rainbow trout exposed to MON 65561 at concentrations of 1.6, 2.9, and 4.7 mg/L. The study was considered valid as mortality in the control group did not exceed 10% by the end of the test and dissolved oxygen concentration was at least 60% of the air saturation value throughout the test.

Table 81: Toxicity of MON 65561 to rainbow trout (*Oncorhynchus mykiss*) under static conditions

Nominal Concentration (mg/L)	Mean Measured Concentration (mg/L)[1]	Replicate	Number of dead fish [2]			
			24 hours	48 hours	72 hours	96 hours
0 (control)	---	A B	0/10 AN 0/10 AN	0/10 AN 0/10 AN	0/10 AN 0/10 AN	0/10 AN 0/10 AN
Solvent control	---	A B	0/10 AN 0/10 AN	0/10 AN 0/10 AN	0/10 AN 0/10 AN	0/10 AN 0/10 AN
1.7	1.6	A B	0/10 AN 0/10 AN	0/10 AN 0/10 AN	0/10 AN 0/10 AN	0/10 AN 0/10 AN
3.3	2.9	A B	0/10 AN 0/10 AN	0/10 AN 0/10 AN	0/10 AN 0/10 AN	0/10 AN 0/10 AN
6.6	4.7	A B	0/10 AN 0/10 AN	0/10 AN 0/10 AN	0/10 AN 0/10 AN	0/10 AN 0/10 AN
13.2	10.1	A B	0/7AN; 3N 0/2AN; 8N	0/9AN; 1N 0/4AN;6N	0/9AN;1N 0/8AN;2N	0/9AN;1N 0/8AN;2N
26.4	24.7	A B	0/10R 0/10R	0/10R 0/10R	0/10R 0/10R	1/9R 0/10R

[1] Mean of concentrations measured at test initiation, at 48 hours and at test termination.
[2] Dead = cumulative number of dead fish; Effects = observed effects (AN = appear normal; N = loss of equilibrium; R = lying on bottom of chamber.)

Conclusion

The 96-hour LC_{50} for rainbow trout, *Oncorhynchus mykiss*, exposed to MON 65561 under static test conditions was greater than 24.7 mg/L, the highest concentration tested. The no-mortality concentration was 10.1 mg/L and the no-observed-effect concentration (NOEC) was 4.7 mg/L.

5.4.1.2 Long-term toxicity to fish

The long-term toxicity to fish is summarized below. A chronic toxicity study was conducted with early life stage embryos and larvae of juvenile fathead minnows (*Pimephales promelas*).

Table 82: Summary of long term toxicity studies in fish.

Test organism	Exposure period	Endpoint	Result[1] (mg/L)	Reference Author, year Study number
Silthiofam (MON 65500)				
Pimephales promelas	up to 28 days post-hatch	ELS NOEC	0.89	CA 8.2.2.1/01 Minderhout, 2014 WL-2013-0381

Silthiofam: An Early Life-Stage Toxicity Test with the Fathead Minnow (Pimephales promelas); Minderhout T, VanEvera SM, Gallagher SP, Martin KH. (2014)

The effects of silthiofam (MON 65500) on the early life-stages (time to hatch, hatching success, survival and growth) of fathead minnow was determined in an unaerated, flow-through test system for 33 days (5-day hatching and 28-day post-hatch period). Treatments consisted of a dilution water control, a solvent control (0.1 mL/L HPLC-grade dimethylformamide) and five nominal silthiofam concentrations of 0.13, 0.25, 0.50, 1.0 and 2.0 mg/L.

Findings

Analytical verification of silthiofam concentrations was made on test solutions sampled on Day 0 and at regular intervals during the study. Mean, measured concentrations of silthiofam were 0.12, 0.23, 0.45, 0.89 and 1.8 mg/L representing 92, 92, 90, 89 and 90% of nominal concentrations. All chemical and physical parameters for the 33 day study were within acceptable ranges. All validity criteria were met for the study:

1.) the percentages of embryos in the negative and in the solvent controls that hatched successfully were both 100%, and the post-hatch survival in the negative and solvent controls were 85 and 84%, respectively. The criteria for validity as outlined by the guidelines was >66% control hatchability and >70% control larval survival;

2.) the dissolved oxygen concentration was ≥ 60 percent of the air saturation value (≥ 4.9 mg/L) throughout the test;

3.) the water temperature measurements did not differ by more than ± 1.5ºC between test chambers or between successive days at any time during the test, and were within 25 ± 1°C;

4.) the concentrations of the test substance in solution were satisfactorily maintained within ± 20% of the mean measured values;

5.) no significant effect on survival or any other adverse effects on the early-life stages were revealed by the solvent-only control.

The majority of fathead minnow embryos in the control and treatment replicates hatched on Days 3, 4 and 5 of the test. Hatching reached >90% in the controls and silthiofam treatment groups on Day 5 of the test, at which time the larvae were released to their respective test chambers. No apparent delay in hatching was noted in any of the silthiofam treatment groups in comparison to the control

groups. Test endpoints analyzed statistically were hatching success, larval survival and growth (total length, wet weight and dry weight) for the juvenile fish. Since no differences were detected between the two control groups ($p > 0.05$) for hatching success, survival or growth, the control data for these parameters were pooled for comparison among the treatment groups. In general, the majority of the fish in the control groups and in the silthiofam treatment groups appeared normal throughout the test, with infrequent observations of fish that appeared smaller, weak or noted with a morphological deformity (e.g. crooked spine, curved spine, enlarged abdominal or missing a caudal fin) in comparison to the fish in the control replicates.

A summary of percent hatching success, survival, and growth of surviving fathead minnow is shown in the following table.

Table 83: Summary of effects following exposure of fathead minnow to Silthiofam (MON 65500) for 33 days.

Mean, measured silthiofam concentration (mg/L)	Mean % hatching success	Mean % Survival	Mean term length (mm)	Mean term wet weight (mg)	Mean term dry weight (mg)
Negative Control (0.0)	100	85	24.6	96.7	18.6
Solvent Control (0.0)	100	84	24.5	93.8	19.0
Pooled Controls	100	84	24.6	95.3	18.8
0.12	100	71*[2]	24.4	95.8	18.4
0.23	96*[1]	75	24.3	101.1	18.8
0.45	100	84	24.1	94.4	18.7
0.89	100	80	23.9	91.1	18.3
1.8	96*[1]	83	22.3*	74.0*	15.1*

* Indicates a significant difference in mean percent hatching success, percent survival (Fisher's Exact test, $p \leq 0.05$) and in growth (measured as total length, wet and dry weight) from the pooled controls (Dunnett's one-tailed test, $p \leq 0.05$).

[1] Since the significant difference in percent hatching success found at the 0.23 mg/L treatment level did not follow a dose response pattern and the hatching success in both the 0.23 and 1.8 mg/L treatment levels were well above the validity control criterion for hatching success (70%), the statistically significant differences found were not considered to be biologically meaningful.

[2] Since the significant difference in percent survival found at the 0.12 mg/L treatment level did not follow a dose response pattern, the statistically significant difference found was not considered to be treatment related.

The ECx values (i.e. EC_{10} and EC_{20}) and the 95% confidence intervals based on growth endpoints, measured as total length, wet weight and dry weight are shown in the table below.

Table 84: EC_{10} and EC_{20} Values

Endpoints	EC_{10} (95% Confidence Interval) (mg/L)[1]	EC_{20} (95% Confidence Interval) (mg/L)[1]
Total Length	1.96 (1.63 – 2.36)	3.35 (2.15 – 5.23)
Wet Weight	1.12 (0.77 – 1.63)	1.63 (1.36 – 1.97)
Dry Weight	1.38 (0.95 – 2.00)	1.83 (1.55 – 2.16)

[1] ECx values were calculated using Bruce-Versteeg regression model.

Conclusion

Fathead minnows (*Pimephales promelas*) were exposed to silthiofam at mean measured concentrations ranging from 0.12 to 1.8 mg/L under flow-through conditions for 33 days (a 5-day hatching period plus a 28-day post-hatch growth period). There were no significant treatment-related effects on hatching success or survival at concentrations ≤1.8 mg/L. Growth, measured as total

length, wet and dry weight, was the most sensitive biological endpoint measured in this study. Fathead minnows exposed to silthiofam at concentrations ≥1.8 mg/L had statistically significant reductions in total length, wet weight and dry weight in comparison to the pooled controls. Consequently, the NOEC, based on growth, was 0.89 mg/L. The LOEC was 1.8 mg/L and the Maximum Acceptable Toxicant Concentration (MATC) was calculated to be 1.3 mg/L.

Both NOEC and $EC_{10/20}$ values were present in this study. A preference for the NOEC values was based on this measure being the most sensitive endpoint (lowest value). All estimated EC_x values for growth were greater than the valid and acceptable NOEC for growth.

5.4.2 Aquatic invertebrates

Table 85: Summary of overall summary of the acute aquatic invertebrate toxicity for silthiofam and the metabolites.

Aquatic invertebrates					
Water flea *Daphnia magna*	SILTHIOFAM	48 hours	EC_{50} NOEC	14 4.9	Endpoints List: SANCO/1424/1001 – final 3 July 2003
	MON 65513	48 hours	EC_{50} NOEC	78 30	Endpoints List: SANCO/1424/1001 – final 3 July 2003
	MON 65533	48 hours	EC_{50} NOEC	>110 110	CA 8.2.4.1/01 Schneider, 2010
	MON 65534	48 hours	EC_{50} NOEC	>109 109	CA 8.2.4.1/02 Schneider, 2010
	MON 65561	48 hours	EC_{50} NOEC	>26.8 6.4	CA 8.2.4.1/03 Minderhout *et al.*, 2008

5.4.2.1 Short-term toxicity to aquatic invertebrates

SILTHIOFAM: A 48-hour static acute toxicity test with the cladoceran (Daphnia magna); Graves W.C. and Swigert J. P. (1996)

Daphnids (*Daphnia magna*) were exposed to Silthiofam (MON 65500) at nominal concentrations of 2.6, 4.3, 7.2, 12 and 20 mg a.s./l (measured concentrations: 2.9, 4.9, 7.8, 13 and 20 mg a.s./l), with one negative control (well water) and one solvent control. Test system was static, test duration was 48 h. Twenty neonate daphnia (< 24 h old) per group were divided into two replicates per dose level. Animals were fasted during the test period. Mortality, immobilisation, signs of toxicity and abnormal behaviour were monitored at 20, 24 and 48 h. Water temperature, pH, dissolved oxygen, hardness, alkalinity and specific conductance were monitored as required during the test. Water samples for determination of silthiofam concentrations were collected from each replicate test chamber at the beginning and end of testing.

Findings

The following table summarises the results obtained for mortality, immobilisation, signs of toxicity and abnormal behaviour. In the 7.8 mg a.i./l treatment group, one daphnid appeared normal and the remaining daphnids appeared lethargic. All surviving daphnids in the 13 mg a.s./l appeared lethargic at test termination. By test termination, 95% of the daphnids in the 20 mg a.s./l treatment group were dead and / or immobile and one surviving daphnid appeared lethargic.

Table 86: Toxicity of Silthiofam (MON 65500) to *Daphnia magna*

Test Substance	TG a.s.
Test Object	*Daphnia magna*
Exposure	48h Static
EC_{50} mg a.s./l	14.0 (95% CI: 12 – 16)
Lowest concentration with effect (LOEC) mg a.s./l	7.8
Highest concentration tested without toxic effect (NOEC) mg a.s./l	4.9

Conclusions
Up to the limit of water solubility, Silthiofam is only slightly toxic to the waterflea, *Daphnia magna*.

MON 65513: A 48-hour static acute toxicity test with the cladoceran (Daphnia magna); Palmer S.J. and Krueger H.O. (1998)

Daphnids (*Daphnia magna*) were exposed to MON 65513 at nominal concentrations of 7.5, 15, 30, 60 and 120 mg a.s./l (measured concentrations: 7.5, 15, 30, 59 and 119 mg a.s./l), with one negative control (well water). The test system was static, test duration was 48 h. Twenty neonate daphnia (< 24 h old) per group were divided into two replicates per dose level. Animals were fasted during the test period. Mortality, immobilisation, signs of toxicity and abnormal behaviour were monitored at 5.5, 24 and 48 h. Water temperature, pH, dissolved oxygen, hardness, alkalinity and specific conductance were monitored as required during the test. Water samples for determination of MON 65513 concentrations were collected from each replicate test chamber at 0, 24 and 48 h.

Findings
Results obtained for mortality, immobilisation, signs of toxicity and abnormal behaviour are summarised in the following table. At test termination the percent mortality / immobility for daphnids in the 59 and 119 mg a.s./l treatment groups were 10 and 100% respectively. Lethargy was observed in 16 of the 20 daphnids in the 119 mg a.s./l group at 24 hours and in one daphnid in the 59 mg a.s./l group at 48 hours.

Table 87: Toxicity of MON 65513 to *Daphnia magna*

Test Substance	TG pm*
Test Object	*Daphnia magna*
Exposure	48h Static
EC_{50} mg a.s./l	78.0 (95% CI: 59 – 119)
Lowest concentration with effect (LOEC) mg a.s./l	59.0
Highest concentration tested without toxic effect (NOEC) mg a.s./l	30.0

pm* = pure metabolite

Conclusions
Up to the limit of water solubility, MON 65513 is only slightly toxic to the waterflea *Daphnia magna*.

MON 65500 Allyl Acid (MON 65533): A 48-Hour Static Acute Toxicity Test with the Cladoceran (Daphnia magna); Schneider, S.Z., Kendall, T.Z., Krueger, H.O. (2010)

The 48-hour acute toxicity of MON 65533 to *Daphnia magna* was determined in a static test system. Exposures were conducted in moderately-hard water obtained from a well for two days to MON 65533 at nominal concentrations of 0 (control), 7.5, 15, 30, 60 and 120 mg MON 65533 Na salt/L. Immobility and signs of toxicity were recorded at 3, 24, and 48 hours after test initiation. The results of the study are presented as mean measured concentrations corrected for purity of test substance and molecular weight of salt.

Findings
Immobility and signs of toxicity in control and treated groups are summarised in the following table. After 48-hours of exposure, all surviving daphnids in all treatment groups appeared normal, with no overt signs of toxicity throughout the test. One incidence of immobility occurred at 24-hours in the 14 mg/L treatment group, however it was not considered to be treatment related as no other immobile organisms were observed in any higher concentration treatment groups. The study was considered valid as immobility in the control group did not exceed 10% by the end of the test and dissolved oxygen concentration was at least 60% of the air saturation value throughout the test.

Table 88: Toxicity of MON 65533 to *Daphnia magna* under static conditions

Mean Measured Concentration [1] (mg MON 65533/L)	Replicate	No. of Daphnids	No. Immobilized / Effects [2]			Cumulative Percent Immobility
			3 Hours	24 Hours	48 Hours	
Negative Control	A B	10 10	0 / 1 Q,AN; 9 AN 0 / 2 Q,AN; 8	0 / 10 AN 0 / 10 AN	0 / 10 AN 0 / 10 AN	0
6.7	A B	10 10	0 / 10 AN 0 / 1 Q,AN; 9 AN	0 / 1 Q,AN; 9 AN 0 / 10 AN	0 / 10 AN 0 / 10 AN	0
14.0	A B	10 10	0 / 10 AN 0 / 1 Q,AN; 9 AN	1 / 9 AN 0 / 10 AN	1 / 9 AN 0 / 10 AN	5
27.0	A B	10 10	0 / 10 AN 0 / 10 AN	0 / 3 Q,AN; 7 AN 0 / 1 Q,AN; 9 AN	0 / 10 AN 0 / 10 AN	0
54.9	A B	10 10	0 / 10 AN 0 / 2 Q,AN; 8 AN	0 / 10 AN 0 / 10 AN	0 / 10 AN 0 / 10 AN	0
110.8	A B	10 10	0 / 4 Q,AN; 6 AN 0 / 10 AN	0 / 10 AN 0 / 10 AN	0 / 10 AN 0 / 10 AN	0

[1] Mean of concentrations measured at test initiation and at test termination, corrected for purity of test substance and molecular weight of salt.
[2] Effects = observed effects (AN = appear normal; Q,AN = trapped at water surface but appear normal after gentle submersion).

Conclusions
The 48-hour EC_{50} for *Daphnia magna* exposed to MON 65533 under static test conditions was >110 mg MON 65533/L, the highest concentration tested. The no-immobility and no-observed-effect concentrations (NOEC) were both 110 mg MON 65533/L.

MON 65500 Amide Acid (MON 65534): A 48-Hour Static Acute Toxicity Test with the Cladoceran (Daphnia magna); Schneider, S.Z., Kendall, T.Z., Krueger, H.O. (2010)

The 48-hour acute toxicity of MON 65534 to *Daphnia magna* was determined in a static test system. Exposures were conducted in moderately-hard water obtained from a well for two days to MON 65534 at nominal concentrations of 0 (control), 7.5, 15, 30, 60 and 120 mg MON 65534 Na salt/L. Immobility and signs of toxicity were recorded at approximately 3, 24, and 48 hours after test initiation. The results of the study are presented as mean measured concentrations corrected for purity of test substance and molecular weight of salt.

Findings

Immobility and signs of toxicity in control and treated groups are summarised in the following table. After 48-hours of exposure, all daphnids in all treatment groups appeared normal, with no overt signs of toxicity throughout the test. The study was considered valid as immobility in the control group did not exceed 10% by the end of the test and dissolved oxygen concentration was at least 60% of the air saturation value throughout the test.

Table 89: Toxicity of MON 65534 to *Daphnia magna* under static conditions

Mean Measured Concentration [1] (mg MON 65534/L)	Replicate	No. of Daphnids	No. Immobilized / Effects [2]			Cumulative Percent Immobility
			3 Hours	24 Hours	48 Hours	
Negative Control	A B	10 10	0 / 10 AN 0 / 10 AN	0 / 10 AN 0 / 10 AN	0 / 10 AN 0 / 10 AN	0
6.5	A B	10 10	0 / 8Q,AN; 2AN 0 / 7Q,AN; 3AN	0 / 10 AN 0 / 10 AN	0 / 10 AN 0 / 10 AN	0
12.9	A B	10 10	0 / 4Q,AN; 6AN 0 / 6Q,AN; 4AN	0 / 10 AN 0 / 10 AN	0 / 10 AN 0 / 10 AN	0
26.7	A B	10 10	0 / 3Q,AN; 7AN 0 / 10 AN	0 / 10 AN 0 / 10 AN	0 / 10 AN 0 / 10 AN	0
53.4	A B	10 10	0 / 2Q,AN; 8AN 0 / 8Q,AN; 2AN	0 / 1Q,AN; 9 AN 0 / 1Q,AN; 9 AN	0 / 10 AN 0 / 10 AN	0
109.6	A B	10 10	0 / 1Q,AN; 9AN 0 / 8Q,AN; 2AN	0 / 10 AN 0 / 10 AN	0 / 10 AN 0 / 10 AN	0

[1] Mean of concentrations measured at test initiation and at test termination and corrected for purity of test substance and molecular weight of salt.

[2] Effects = observed effects (AN = appear normal; Q, AN = trapped at water surface but appear normal after gentle submersion).

Conclusions

The 48-hour EC_{50} for *Daphnia magna* exposed to MON 65534 under static test conditions was >109 mg MON 65534/L, the highest concentration tested. The no-immobility and no-observed-effect concentrations (NOEC) were both 109 mg MON 65534/L.

MON 65561: A 48-Hour Static Acute Toxicity Test with the Cladoceran (Daphnia magna); Minderhout T, Kendall TZ, Krueger HO. (2008)

The 48-hour acute toxicity of MON 65561 to *Daphnia magna* was determined in a static test system. Daphnids were exposed to a geometric series of five test concentrations, a negative (UV sterilized well water) control and a solvent control (0.11 mL dimethyl formamide/L). Exposures were conducted in moderately-hard water obtained from a well for 48 hours to MON 65561 at nominal

concentrations of 0 (control), 1.7, 3.3, 6.6, 13.2 and 26.4 mg/L. Immobility and signs of toxicity were recorded at 6, 24, and 48 hours after test initiation.

Findings

Immobility and signs of toxicity in control and treated groups are summarised in the following table. Mean measured concentrations were 1.7, 3.2, 6.4, 12.3 and 26.8 mg/L, representing 93 to 102% of nominal values. The concentrations of MON 65561 remained stable under the conditions of administration. Temperature Range: 19.5 – 20.7°C measured manually daily during the test, and 19.8 – 20.5°C measured continuously in an adjacent container of water; Dissolved Oxygen: 7.8 – 8.5 mg/L; pH: 8.2 – 8.5. Daphnids in the negative control and solvent control appeared normal throughout the test. Percent mortality/immobility in the 1.7, 3.2, 6.4, 12.3 and 26.8 mg/L treatment groups was 0, 0, 0, 0 and 35%, respectively. The study was considered valid as immobility in the control group did not exceed 10% by the end of the test and dissolved oxygen concentration was at least 60% of the air saturation value throughout the test.

Table 90: Toxicity of MON 65561 to *Daphnia magna* under static conditions

Mean Measured Concentration [1] (mg/L)	Replicate	No. of Daphnids	No. Immobilized / Effects [2]			Cumulative Percent Immobility
			3 Hours	24 Hours	48 Hours	
Negative Control	A B	10 10	0 / 10 AN 0 / 10 AN	0 / 10 AN 0 / 10 AN	0 / 10 AN 0 / 10 AN	0
Solvent Control	A B	10 10	0 / 10 AN 0 / 10 AN	0 / 10 AN 0 / 10 AN	0 / 10 AN 0 / 10 AN	0
1.7	A B	10 10	0 / 10 AN 0 / 10 AN	0 / 10 AN 0 / 10 AN	0 / 7AN;3C 0 / 6AN;4C	0
3.2	A B	10 10	0 / 10 AN 0 / 10 AN	0 / 10 AN 0 / 10 AN	0 / 10 AN 0 / 10 AN	0
6.4	A B	10 10	0 / 10 AN 0 / 10 AN	0 / 10 AN 0 / 10 AN	0 / 10 AN 0 / 10 AN	0
12.3	A B	10 10	0 / 10 AN 0 / 10 AN	0 / 8AN;2C 0 / 8AN;2C	0 / 7AN;3C 0 / 6AN;4C	0
26.8	A B	10 10	0 / 6AN; 4C 0 / 6AN; 4C	0 / 6AN; 4C 0 / 5AN; 5C	5 / 4AN;1C 2 / 8C	35

[1] Mean of concentrations measured at test initiation and at test termination.
[2] Effects = observed effects (AN = appear normal; Q,AN = trapped at water surface but appear normal after gentle submersion; C = lethargy).

Conclusions

The 48-hour EC_{50} for *Daphnia magna* exposed to MON 65561 under static test conditions was greater than 26.8 mg/L, the highest concentration tested and the apparent solubility limit of the test substance in the test system. The no-mortality or immobility concentration was 12.3 mg/L and the no-observed effect concentration (NOEC) was 6.4 mg/L.

5.4.2.2 Long-term toxicity to aquatic invertebrates

SILTHIOFAM: A semi-static life-cycle toxicity test with the cladoceran (Daphnia magna); Drottar, K.R., Kendall, T.Z. and Kreuger, H.O. (2000)

Young *Daphnia magna* (< 24 hours old) were exposed under semi-static conditions for 21 days to concentrations (mean measured) of 0.23, 0.47,0.96, 1.8, 3.7 mg a.i./l, a negative (dilution water) control and a solvent (0.1 ml DMF/L) control. Each treatment and control group consisted of 10 replicate test chambers containing 1 Daphnid. Test solutions were renewed three times/week (except

weekends) and water samples were collected at test initiation, at the beginning and end of the longest renewal cycle each week and at test termination.

Daphnids were observed daily for mortality, the onset of reproduction and other sub-lethal signs of toxicity. With the onset of reproduction, the number of second generation daphnids were counted three times/week. At the end of the test daphnid length was determined to the nearest 0.05mm using venire calipers. Daphnids were fed once daily during the test. Stability checks on the test medium were also performed.

Findings

Analytical results showed that mean measured concentrations were in the range 84% to 107% of nominal concentrations. The test substance was stable for the duration of the test. Study results are based on mean measured concentrations.

Table 91: Effects observed following exposure of *Daphnia magna* to MON65500

Parameter	Mean Measure Concentration (mg a.i./L)						
	Control	**Solvent Control**	**0.23**	**0.47**	**0.96**	**1.8**	**3.7**
% Mortality	10	20	0	0	10	40	90[1]
% Immobile	0	0	0	0	0	0	0
No. surviving Adults	9	8	10	10	9	6	1[2]
1st day of Reproduction	9	12	9	9	9	9	9[2]
Mean Live young/adult daphnid	165	176	190	153	154	137	47[2]
Mean length (mm) of surviving 1st generation adults	5.21	5.17	5.18	5.05	4.93[3]	4.40[3]	3.15[3]

[1]Indicates a significant difference from the control on day 21 using a 2 x 2 contigency table ($p \leq 0.05$)
[2]This treatment group was not included in the statistical analyses of reproduction data due to a significant ($p \leq 0.05$) effect on survival.
[3]Inidicates a significant difference from the pooled controls using the Bonferroni test ($p \leq 0.05$).

Observations**:** Survival of adult daphnids in the 3.7 mg a.i./L treatment was significantly different from the pooled controls. No other effects on survival were observed therefore the LOEC was 3.7 mg a.i./l and the NOEC was 1.8 mg a.i./l. The first day of reproduction was day 9 in all treatments with the exception of the solvent control indicating no treatment related effects on the time to first brood. Analysis of variance indicated that there were no statistically significant differences between any of the controls or treatment groups when the 3.7 mg a.i./l data were not included in the analysis. The one surviving adult daphnid in the 3.7 mg a.i./l treatment group produced only 47 young and reproduction appeared to be decreased in a concentration dependent manner. Therefore the NOEC for reproduction was 1.8 mg a.i./l and the LOEC was considered to be 3.7 mg a.i./l. The mean length of daphnids exposed to the 0.96 and 1.8mg a.i./l was significantly reduced when compared to the pooled controls. Therefore the lowest NOEC is based on growth retardation and was 0.47 mg a.i./l.

Table 92: Toxicity of Silthiofam to *Daphnia magna*

Test Substance	TG a.i.		
Test Object	*Daphnia magna*		
Exposure	21d semi-static renewal		
Parameters	Survival	Reproduction	Growth

Lowest test conc. with effect (mg a.i./l)	3.7	3.7	0.96
Highest tested conc. w/out toxic effect (mg a.i./l)	1.8	1.8	**0.47**

The long-term NOEC for *Daphnia magna* exposed to the active substance is 0.47 mg a.i./l.

Conclusion
Silthiofam has a moderate chronic toxicity to *Daphnia magna* with a NOEC of 0.47 mg a.i./l.

Table 93: Summary of the endpoints for the long-term and chronic toxicity to aquatic invertebrates as measured for silthiofam in *Daphnia magna.*

Test organism	Exposure period	Endpoint	Result[1] (mg/L)	Reference
Silthiofam (MON 65500)				
Daphnia magna	21 days	NOEC (surv.) NOEC (growth)	1.8 0.47	Endpoints List: SANCO/1424/1001 – final 3 July 2003

[1]Results based on mean measured concentrations.

5.4.3 Algae and aquatic plants

Silthiofam and its metabolites MON 65513, MON 65533, MON 65534, and MON 65561 were tested in algae. *Pseudokirchneriella subcapitata* (formerly known as *Selenastrum capricornutum*) was the algae species tested with silthiofam and its metabolites.

SILTHIOFAM: A five-day toxicity test with the freshwater alga (Selenastrum capricornutum); Drottar K.R. and Krueger H.O. (1998).

Freshwater algae (*Selenastrum capricornutum*, approximately 3000 cells/ml at test start) were exposed for 5 days to Silthiofam (MON 65500) at nominal concentrations of 1.3, 2.5, 5.0, 10 and 20 mg a.s./l (measured concentrations: 1.1, 2.3, 4.6, 9.0 and 19 mg a.s./l), with one negative control (culture medium) and one solvent control. In addition, an abiotic culture (culture medium with silthiofam at 20 mg a.s./l but no algae) was included to monitor stability of test compound. There were three replicates for control and test series, two replicates for the stability check. Algae samples were collected from each replicate test chamber at approximately 24 h intervals and cell densities were determined for area under the growth curve and growth rate calculations. Samples for the determination of silthiofam concentrations were collected from each dose group at study start, on Day 3 and at the end of the test. Abiotic replicates were sampled on Days 3 and 5.

Findings

Table 94: Effects of Silthiofam (MON 65500) on cell densities of *Selenastrum capricornutum*

Concentration (mg a.s./l)	Mean cell density [1](cells/ml)				
	Day 1	Day 2	Day 3	Day 4	Day 5
Negative control	0 [2]	40'110	199'297	1'202'808	3'492'477
Solvent control	0 [2]	32'910	199'089	1'156'236	3'002'044
1.1	0 [2]	31'400	173'299	1'006'162	2'992'678

2.3	0 [2]	29'377	149'755	916'360	3'039'167
4.6	0 [2]	20'962	124'450	646'257	3'445'618
9.0	0 [2]	20'451	98'656	399'314	963'045
19	0 [2]	0	2'222	18'383	32'339

[1] Mean cell density at initiation was 3000 cells/ml, [2] A zero was substituted for a negative cell density value obtained upon correcting the measured cell density based on the least squares regression analysis performed as part of the electron particle counter linearity check

Table 95: Effects of Silthiofam (MON 65500) on biomass and growth rate of *Selenastrum capricornutum*

Concentration (mg a.s./l)	0 – 72 h inhibition values [1] (%)		0 – 120 h inhibition values [1] (%)	
	AUC [2]	Growth rate	AUC [2]	Growth rate
Negative control	-	-	-	-
Solvent control	-	-	-	-
1.1	14	2.9	11	1.1
2.3	24	6.3	14	0.88
4.6	41	11	34	4.1
9.0	51	16	67	18
19	100	100	99	66
EC_{50} (mg a.s./l) [3]	8.6 (2.9 and 11)	13 (13 and 13)	6.7 (5.2 and 7.7)	16 (15 and 16)

[1] Percent inhibition as compared to pooled controls, [2] Area under the growth curve, [3] Parentheses indicate 95% confidence limits.

Conclusion

There were no statistically significant reductions in cell density, area under the growth curve or growth rate of *Selenastrum capricornutum* exposed to silthiofam at concentrations of 2.3 mg a.s./l. Day 3 EC_{50} values for biomass and growth rate were: E_bC_{50} (0-72 h) = 8.6 (confidence limits: 2.9 and 11) and E_rC_{50} (0-72 h) = 13 (confidence limits: 13 and 13), respectively. Therefore, Silthiofam is slightly to moderately toxic to the green algae, *Selenastrum capricornutum*.

MON 65513: A 72 h toxicity test with the freshwater alga (Selenastrum capricornutum); Palmer S.J. and Krueger H.O. (1998)

Freshwater algae (*Selenastrum capricornutum*, approximately 10,000 cells/ml at test start) were exposed for 72 h to MON 65513 at nominal concentrations of 1.9, 3.8, 7.5, 15 and 30 mg a.s./l (mean measured concentrations: 1.9, 3.7, 7.4, 15 and 30 mg a.s./l), with one negative control (culture medium). In addition, an abiotic culture (culture medium with silthiofam at 30 mg a.s./l but no algae) was included to monitor stability of test compound. There were three replicates for control and test series, one replicate for the stability check. Algae samples were collected from each replicate test chamber at approximately 24 h intervals and cell densities were determined for area under the growth curve and growth rate calculations. Cells were counted using an electronic particle counter. Temperature, pH of the cultures and light intensity was monitored as required during the test. Samples for the determination of MON 65513 concentrations were collected from each dose group at study start and end. The abiotic replicate was sampled at 72 h.

Findings

Table 96: Effects on MON 65513 on cell densities of *Selenastrum capricornutum*

Concentration (mg a.s./l)	Mean cell density[1](cells/ml)		
	24 h	48 h	72 h
Negative control	60,163	352,597	1,826,562
1.9	60,490	398,597	1,896,368
3.7	57,968	431,589	2,011,261
7.4	51,982	350,305	1,772,779
15	54,663	299,349	1,558,546
30	40,724	48,494	93,547

[1] Mean cell density at initiation was 1×10^4 cells/ml

Table 97: Effects on MON 65513 on biomass and growth rate of *Selenastrum capricornutum*

Concentration (mg a.s./l)	0 – 72 h inhibition values [1] (%)	
	AUC [2]	Growth rate
Negative control	-	-
1.9	-6.2	-0.81
3.7	-13	-1.9
7.4	2.9	0.52
15	15	3.0
30	91	58
EC_{50} (mg a.s./l) [3]	21 (18 and 23)	28 (24 and 31)

[1] Percent inhibition as compared to pooled controls, [2] Area under the growth curve, [3] Parenthesis indicate 95% confidence limits

Conclusions:
There were no statistically significant reductions in cell density, area under the growth curve or growth rate of *Selenastrum capricornutum* exposed to silthiofam at concentrations of 15 mg a.s./l. Day 3 EC_{50} values for biomass and growth rate were: E_bC_{50} (0-72 h) = 21 (confidence limits: 18 and 23) and E_rC_{50}(0-72 h) = 28 (confidence limits: 24 and 31), respectively. MON 65513 is slightly toxic to the green algae *Selenastrum capricornutum*.

MON 65500 Allyl acid (MON 65533): A 72-hour toxicity test with the freshwater alga (Pseudokirchneriella subcapitata); Porch, J.R.; Kendall, T.Z.; Krueger, H.O. (2010)

The effects of silthiofam allyl acid (MON 65533) on the growth of the green alga *Pseudokirchneriella subcapitata* (formerly called *Selenastrum capricornutum*) were determined during a 72-hour static laboratory test with orbital shaking. Six control replicate cultures and three replicate cultures per test concentration were exposed to nominal concentrations of 0 (control), 7.5, 15, 30, 60, and 120 mg MON 65533 Na salt/L. The concentration of MON 65533 was determined at the beginning and end of the test for all test concentrations.

Findings:
Mean measured concentrations were 7.0, 14.0, 27.9, 54.9, and 112.7 mg MON 65533/L. Measured concentrations of test solutions at test initiation ranged from 98.9 to 101% of nominal and at test termination ranged from 97.6 to 99.6% of nominal. Since the concentrations were stable, the results of this study are reported as mean measured concentrations corrected for purity of test substance and molecular weight of salt. Algal cells appeared normal at test initiation and test termination. Test results are summarized in the table below. There were no treatment-related differences for any endpoints.

Table 98: Toxicity of MON 65533 to *Pseudokirchneriella subcapitata* under static conditions

Mean measured MON 65533 (mg/L) [1]	Mean cell density [2] (10^4 cells/mL)			Yield [3]	Mean specific growth rate 0-72h (μ)	% Inhibition (72 h)	
	24 h	48 h	72 h			Yield	Growth Rate
Negative Control	4.34	30.4	156	155	0.0701	-	-
7.0	4.46	30.9	119*	118*	0.0663*	24*	5.5*
14.0	4.44	33.2	139	138	0.0684	11	2.5
27.9	4.16	30.4	145	144	0.0690	7.3	1.6
54.9	4.17	32.2	138	137	0.0684	12	2.5
112.7	4.15	29.5	130	129	0.0675	17	3.8

[1] Mean measured concentrations corrected for purity of test substance and molecular weight of salt. LOQ (limit of quantitation) was 3.0 mg MON 65533 Na salt/L.
[2] Nominal cell density at test initiation: 1 x 10^4 cells/mL; mean of 3 replicates.
[3] × 10^4.
* Statistically significant difference from the negative control replicates. Based on evaluation of the dose-response, this reduction was not treatment related.

The test was considered to be valid as the biomass in the control cultures increased by a factor of >16 within three days (actual value was 156x), the mean percent coefficient of variation for the section-by-section specific growth rates in the control replicates did not exceed 35% (actual value was 15.2%), and the coefficient of variation of average specific growth rate in the control replicates for the first 72-hours did not exceed 7% (actual value was 2.04%).

Conclusions
Based on mean measured concentrations, the 72-hour EC_{50} values for MON 65533 toxicity to *Pseudokirchneriella subcapitata* under static test conditions were determined to be:

- EC_{50} (cell density): >112 mg MON 65533/L (95% confidence interval: n/a)
- E_rC_{50} (growth rate): >112 mg MON 65533/L (95% confidence interval: n/a)

The 72-hour NOAEC for both cell density and growth rate was 112 mg MON 65533/L.

MON 65500 amide acid (MON 65534): A 72-hour toxicity test with the freshwater alga (Pseudokirchneriella subcapitata); Porch, J.R.; Kendall, T.Z.; Krueger, H.O. (2010)

The effects of silthiofam amide acid (MON 65534) on the growth of the green alga *Pseudokirchneriella subcapitata* (formerly called *Selenastrum capricornutum*) were determined during a 72-hour static laboratory test with orbital shaking. Six control replicate cultures and three replicate cultures per test concentration were exposed to nominal concentrations of 0 (control), 7.5, 15, 30, 60, and 120 mg MON 65534 Na salt/L. The concentration of MON 65534 was determined at the beginning and end of the test for all test concentrations. The results of the study are presented as mean measured concentrations corrected for purity of test substance and molecular weight of salt.

Findings:
Mean measured concentrations were 6.8, 13.8, 27.6, 54.4, and 110.5 mg MON 65534/L. Measured concentrations of test solutions at test initiation ranged from 98.8 to 101% of nominal and at test termination ranged from 97.3 to 99.1% of nominal. Since the concentrations were stable, the results of this study are reported as mean measured concentrations corrected for purity of test substance and

molecular weight of salt. Test results are summarized in the table below. Cell density was significantly reduced at the highest treatment level. Cell density was the most sensitive parameter.

Table 99: Toxicity of MON 65534 to *Pseudokirchneriella subcapitata* under static conditions

Mean measured MON 65534 (mg/L) [1]	Mean cell density [2] (10^4 cells/mL)			Yield [3]	Mean specific growth rate 0-72h (µ)	% Inhibition (72 h)	
	24 h	48 h	72 h			Yield	Growth Rate
Negative Control	4.33	30.4	146	145	0.0692	-	-
6.8	4.72	29.0	142	141	0.0687	2.7	0.7
13.8	4.53	24.3	125	124	0.0671	14	3.0
27.6	4.20	26.7	135	134	0.0681	7.6	1.5
54.4	4.30	25.5	118	117	0.0662*	19	4.3*,[4]
110.5	3.96	24.0	115*	114*	0.0659*	21*	4.7*,[4]

[1] Mean measured concentrations corrected for purity of test substance and molecular weight of salt. LOQ (limit of quantitation) was 4.0 mg MON 65534 Na salt/L.
[2] Nominal cell density at test initiation: 1 x 10^4 cells/mL; mean of 3 replicates.
[3] × 10^4.
[4] Effects on growth rate of less than 5% may not be considered adverse.
* Statistically significant difference from the negative control replicates.

The test was considered to be valid as the biomass in the control cultures increased by a factor of >16 within three days (actual value was 146x), the mean percent coefficient of variation for the section-by-section specific growth rates in the control replicates did not exceed 35% (actual value was 15.9%), and the coefficient of variation of average specific growth rate in the control replicates for the first 72-hours did not exceed 7% (actual value was 2.44%).

Conclusions

Based on mean measured concentrations, the 72-hour EC_{50} values for MON 65534 toxicity to *Pseudokirchneriella subcapitata* under static test conditions were determined to be:

- EC_{50} (cell density): >110 mg MON 65534/L (95% confidence interval: n/a)
- E_rC_{50} (growth rate): >110 mg MON 65534/L (95% confidence interval: n/a)

The 72-hour NOAEC for cell density and growth rate were 54 and 110 mg MON 65534/L, respectively.

MON 65561: A 72-hour Toxicity Test with the Freshwater Alga (Pseudokirchneriella subcapitata); Minderhout, T.; Kendall, T.Z.; Krueger, H.O. (2008)

The effects of MON 65561 on the growth of the green alga Pseudokirchneriella subcapitata (formerly called Selenastrum capricornutum) were determined during a 72-hour static laboratory test with orbital shaking. Six control replicate cultures and three replicate cultures per test concentration were exposed to nominal concentrations of 0 (control), 1.5, 3.0, 6.0, 12 and 24 mg MON 65561/L.

Findings

Measured concentrations of the test solutions at test initiation ranged from 96.0 to 107% of nominal and at test termination ranged from 94.1 to 104% of nominal. The measured concentration of the abiotic replicate at 72 hours was 96.3% of nominal. Since the test concentrations were stable in the absence of algae, the results of this study were based upon mean measured concentrations. Mean measured concentrations were 1.5, 3.2, 5.8, 11 and 23 mg/L. Test results are summarized in the table below. Algal cells appeared normal at test initiation and test termination. The effects of MON 65561 on growth of *Pseudokirchneriella subcapitata* are summarized in the tables below.

Table 100: Toxicity of MON 65561 to *Pseudokirchneriella subcapitata* under static conditions

Mean measured MON 65561 (mg/L)	Mean cell density [1] (10^4 cells/mL)			Mean specific growth rate 0-72h (μ)	% Inhibition (72 h)	
	24 h	48 h	72 h		Cell Density	Growth Rate
Negative Control	3.01	25.3	169	0.0712	--	--
Solvent Control	3.05	26.6	178	0.0719	--	--
Pooled Control	3.03	25.9	173	0.0715	--	--
1.5	3.24	25.2	188	0.0727	-5.9	-1.6
3.2	2.52	22.3	161	0.0705	9.1	1.5
5.8	2.93	22.4	140 *	0.0686 *	18*	4.0*
11	2.65	19.7	115 *	0.0659 *	32*	7.9*
23	2.33	12.6	77 *	0.0604 *	55*	16*

[1] Nominal cell density at test initiation: 1 x 10^4 cells/mL.
* There were statistically significant differences (p<0.05) in comparison to the pooled control replicates.

The test was considered to be valid as the biomass in the control cultures increased by a factor of >16 within three days (actual value was 173x), the mean percent coefficient of variation for the section-by-section specific growth rates in the control replicates did not exceed 35% (actual value was 33%), and the coefficient of variation of average specific growth rate in the control replicates for the first 72-hours did not exceed 7% (actual value was 1.9%).

Conclusions

The 72-hour EC_{50}, based on cell density, was 19 mg/L with the 95% confidence intervals of 15 to 23 mg/L. The E_bC_{50}, based on area under the growth curve, was 19 mg/L, with the 95% confidence intervals of 16 to 23 mg/L. The E_rC_{50}, based on growth rate, was the least sensitive and was determined to be greater than 23 mg/L, the highest concentration tested. Dunnett's test indicated that cell density, area under the growth curve and growth rate were significantly reduced (p<0.05) in the 5.8, 11 and 23 mg/L treatment levels. Consequently, the 72-hour NOEC in this study was determined to be 3.2 mg/L.

5.4.4 Other aquatic organisms (including sediment)

A Tier 2 summary is summarized below for a non-guideline/non-GLP 48-hour acute toxicity of silthiofam to the African clawed frog (*Xenopus laevis*) in a static renewal test system. While the study is highly relevant it is of informative nature since test concentrations were not measured, and the report contains only limited information on the validation of such a test system.

Test Report on the Acute Toxicity of 97.7% Silthiopham TC to Amphibians (2013). Monsanto Report no. MSL0025641.

The 48-hour acute toxicity of silthiofam to the African clawed frog (*Xenopus laevis*) was determined in a static renewal test system. Thirty tadpoles per treatment level were exposed in dechlorinated water for two days to silthiofam at nominal concentrations of 0 (control), 22.4, 23.8, 25.2, 26.7, 28.3, and 30.0 mg silthiofam/L. Mortality and signs of toxicity were recorded at 6, 24, and 48 hours after test initiation.

Findings:
Mortality and signs of toxicity in control and treated groups are summarised in Table 101. The study was considered valid as mortality in the control group did not exceed 10% by the end of the test.

Table 101: Toxicity of silthiofam to African clawed frog (*Xenopus laevis*) under static renewal conditions.

Nominal Concentration (mg silthiofam /L)	Number of dead tadpoles[1]			48 hour Mortality (%)
	6 hours	24 hours	48 hours	
0 (control)	0/30	0/30	0/30	0
0 (solvent control)	0/30	0/30	0/30	0
22.4	3/30	4/30	6/30	20.0
23.8	7/30	10/30	11/30	36.7
25.2	3/30	10/30	13/30	43.3
26.7	12/30	14/30	15/30	50.0
28.3	9/30	21/30	22/30	73.3
30.0	15/30	24/30	27/30	90.0

[1] Number of dead individuals/total number of tadpoles in the group.

Conclusions
The 48-hour LC_{50} for the African clawed frog (*Xenopus laevis*) exposed to silthiofam under static renewal test conditions was 25.7 mg silthiofam/L (95% confidence interval: 24.8-26.5 mg silthiofam/L).

Sediment-dwelling Organisms
To date no data has been presented by the notifier with respect to the acute or chronic toxicity of Silthiofam to sediment-dwelling organisms. The mode of application and the environmental fate and behaviour data for Silthiofam indicate that there will be only low acute exposure and no significant long-term exposure of the aquatic environment (Section 5.5.1.2) as Silthiofam will be applied only once a year as a seed treatment. This method of application means that there will be no potential for losses to adjacent surface waters via direct overspray or drift and only limited losses via drain flow. Accordingly, specific toxicity data for sediment dwelling organisms is not necessary.

5.5 Comparison with criteria for environmental hazards

Silthiofam is relatively stable hydrolytically at alkaline pH but is unstable in acidic conditions. The hydrolysis half-lives for silthiofam at 25°C ranged from 45 hours (pH 4) to 448 days (pH 7). The hydrolysis half-life at 25°C for pH 9 was 314 days. Using Arrhenius plots, the half-lives at 20°C were

estimated to be 73 hours for pH 4 and 77 years for pH 9. Therefore, silthiofam is rapidly hydrolysed at pH 4 and 20°C but can be considered to be stable at pH 9 and 20°C. The principal hydrolysis product of silthiofam was CP 240659, resulting from acid-catalysed desilylation of the parent molecule. This metabolite was also shown to form hydrolytically in acidic soils. There was no evidence of any significant degradation of CP 240659 during the hydrolysis study with silthiofam.

Photolysis was a significant route of degradation for silthiofam with a DT_{50} value of 16 days. The degradation products each individually account for less than 10% of applied radioactivity. CP 240659 was identified as accounting for up to 2% of applied radioactivity. Considering the relatively short DT_{50} value of 16 days for the irradiated samples, it can be concluded that if silthiofam was exposed to sunlight under field conditions then photochemical degradation could be expected to be an important factor in its degradation.

Silthiofam is not considered to be "rapidly degradable".

In water/sediment systems: FOCUS surface water modelling, for Silthiofam, bias-corrected geometric mean DT_{50} values of 152.3 days for total system, 78.2 days for water column, and 193.6 days for sediment phase are recommended. For the metabolites M1 and M2, no decline phase was established so a worst case default value of 1000 days is recommended by the RMS evaluator.

Silthiofam was shown to have a low potential for bioaccumulation in fish, BCF 98 mg/L.

The acute toxicity studies indicate that silthiofam is slightly toxic to fish and *daphnia* and moderately toxic to green algae. The most sensitive species tested was *Selenastrum capricornutum*, with a 72h EC_{50} of 8.6 mg a.s/l. This represents the lowest available toxicity value for consideration of aquatic acute hazard classification. However, the 72h EC_{50} value of 8.6 mg a.s/l does not satisfy the criteria for Aq. Acute 1 and silthiofam is therefore not classified for aquatic acute toxicity. All aquatic metabolites tested (MON 65513, MON 65533, MON 65534 and MON 65561) were of lower toxicity relative to parent silthiofam at all aquatic trophic levels (fish, daphnia, green algae).

The chronic toxicity studies indicate silthiofam is moderately toxic to fish (*Pimephales promelas*) and aquatic invertebrates (*Daphnia magna*). Aquatic Chronic 2 is considered for silthiofam as explained below.

Classification criteria for Category Chronic 2

Long-term aquatic hazard
Non-rapidly degradable substances for which there are adequate toxicity data available

Category Chronic 2:

Chronic NOEC or ECx (for fish)	*≤ 1 mg/L and/or*
Chronic NOEC or ECx (for crustacea)	*≤ 1 mg/L and/or*
Chronic NOEC or ECx (for algae or other aquatic plants)	*≤ 1 mg/L*

Silthiofam classifies for ecotoxicological effects in accordance with the classification criteria stipulated in Table 4.1.0, (b)(i) in Annex I of CLP Regulation (EC) No. 1272/2007, based upon the results of (1) the 28-day Early Life-Stage Toxicity Test with the Fathead Minnow (*Pimephales promelas*) and (2) the 21 day semi-static life-cycle toxicity test with the cladoceran (*Daphnia magna*). A chronic NOEC of 0.89 mg a.s./L and 0.47 mg a.s./L was established for the Fathead minnow and

Waterflea, respectively. Silthiofam classifies as Aquatic Chronic 2; H411 'Toxic to aquatic organisms with long lasting effects' in accordance with the classification criteria of CLP Regulation 1272/2008.

5.6 Conclusions on classification and labelling for environmental hazards

In accordance with the CLP Regulation, Silthiofam classifies for Aquatic Chronic 2 - H411 'Toxic to aquatic organisms with long lasting effects'.

RAC evaluation of aquatic hazards (acute and chronic)

Summary of the Dossier Submitter's proposal

Degradation

The dossier submitter proposed to consider silthiofam as not rapidly degradable for classification purposes. The basis for this proposal is that silthiofam is only rapidly hydrolysed at pH 4 (half-life at 20 °C > 73 hours) but must be considered to be stable at the more environmentally relevant pH 7 and pH 9 (half-life at 20 °C > 77 years).

A study on aqueous photolysis (Lewis, 1997) found that silthiofam was photodegraded in pH 7 aqueous solution under artificial sunlight with a DT_{50} value of 16 days but it was not shown that under relevant environmental conditions the photochemical degradation could be expected to be above the criteria for classification.

In an OECD TG 301B test system, silthiofam degraded less than 2 % after 28 days and therefore is not readily biodegradable.

In a study (Lewis, 1997) with two different water/sediment systems, silthiofam dissipation resulted mainly in the formation of bound residues. It disappeared from the water phase with $DisT_{50}$ of 5 and 52 days and dissipated extremely slowly in whole systems with $DegT_{50}$s of 269 and 147 days (for the pond and run-off systems respectively). By the end of the study (100 days) the levels of silthiofam detected in the total system accounted for 74 % and 58 % of applied radioactivity for the pond and run-off systems, respectively. The amount of ^{14}C-carbon dioxide was not reported by the dossier submitter.

In a second study (Irmer, 2013), two aquatic systems (river and pond) under aerobic conditions were investigated at 20 °C in the dark. Silthiofam dissipation from water was mainly due to the adsorption to the sediment layers. By the end of the study (118 days), silthiofam in the total system still accounted for 52.2 % and 50.2 % of applied radioactivity for the river and pond systems, respectively. The formation of ^{14}C-carbon dioxide and other volatile products was insignificant.

The findings of two aerobic metabolism studies in soil under laboratory conditions (Lewis, 1996a and Goodyear, 1999a) indicate that silthiofam forms bound residues and may degrade to a certain degree in soil.

Aquatic Bioaccumulation

The dossier submitter proposed to not consider silthiofam as being bioaccumulative in the aquatic environment for classification purposes. The basis for this proposal is a log P_{ow} of 3.72 and a measured steady-state bioconcentration factor (BCF) (total wet weight/normalised to 5 % lipid content) for fish of 98 L/kg.

Acute Aquatic Toxicity

The dossier submitter proposed to not classify silthiofam as acute toxic for the aquatic environment, as all acute toxicity values are above the threshold value of 1 mg/L. Thus, the basis for this proposal is that the available acute toxicity studies indicate that silthiofam is slightly toxic to fish and daphnia and moderately toxic to green algae. The most sensitive species tested was *Selenastrum capricornutum*, with a 72 h EC_{50} (biomass) of 8.6 mg a.s/L and a 72 h E_rC_{50} (growth rate) of 13 mg/L. All aquatic metabolites tested (MON 65513, MON 65533, MON 65534 and MON 65561) were of lower toxicity relative to silthiofam in all aquatic trophic levels (fish, daphnia, green algae).

***Table**: Summary of relevant information on aquatic toxicity*

Method	Results	Remarks	Reference
Fish			
Silthiofam: A 96-hour static acute toxicity test with the rainbow trout (*Oncorhynchus mykiss*) *OECD TG 203 and FIFRA Chapter 72-1 Subdivision E.*	LC_{50} (mg a.s./L) = 14 (95 % CI: 13 - 16) LOEC(mg a.s./L) = 5.3 NOEC(mg a.s./L) = 3.2 Silthiofam is only slightly toxic to rainbow trout up to the water solubility limit.	Performed according to GLP criteria.	Anonymous (1996). MON 65500: A 96-hour static acute toxicity test with the rainbow trout (*Oncorhynchus mykiss*)
Silthiofam: A 96-hour static acute toxicity test with the Bluegill (*Lepomis macrochirus*) *OECD TG 203 and FIFRA Chapter 72-1 Subdivision E.*	LC_{50} (mg a.s./L) = 11 (95 % CI: > 8.4) LOEC(mg a.s./L) = 6.0 NOEC(mg a.s./L) = 3.7 Silthiofam is slightly toxic to bluegill sunfish up to the water solubility limit.	Performed according to GLP criteria.	Anonymous (1996). MON 65500: A 96-hour static acute toxicity test with the Bluegill (*Lepomis macrochirus*)
Silthiofam: An Early Life-Stage Toxicity Test with the Fathead Minnow (*Pimephales promelas*). *ASTM Standard E-1241-05; FIFRA Subdivision E, Section 72-4a; OECD TG 210; OPPTS 850.1400*	Fathead minnows (*Pimephales promelas*) were exposed to silthiofam at mean measured concentrations ranging from 0.12 to 1.8 mg/L under flow-through conditions for 33 days (a 5-day hatching period plus a 28-day post-hatch growth period). There were no significant treatment-related effects on hatching success or survival at concentrations ≤ 1.8 mg/L. Growth, measured as total length, wet and dry weight, was the most sensitive biological endpoint measured in this study. Fathead minnows exposed to silthiofam at concentrations ≥ 1.8 mg/L had statistically significant reductions in total length, wet weight and dry weight in comparison to the pooled controls.	Performed according to GLP criteria.	Anonymous (2014). MON 65500: An Early Life-Stage Toxicity Test with the Fathead Minnow (*Pimephales promelas*)

	Consequently, the NOEC, based on growth, was 0.89 mg/L. The LOEC was 1.8 mg/L and the MATC was calculated to be 1.3 mg/L.		
Aquatic Invertebrates			
Silthiofam: A 48-hour static toxicity test with the cladoceran (*Daphnia magna*). *OECD TG 202, FIFRA Chapter 72-2 Subdivision E.*	EC_{50} (mg a.s./L) = 14.0 (95% CI: 12 – 16) LOEC (mg a.s./L) = 7.8 NOEC (mg a.s./L) = 4.9 Up to the limit of water solubility, silthiofam is only slightly toxic to the waterflea, *Daphnia magna*.	Performed according to GLP criteria.	Graves W.C. and Swigert J. P. (1996). MON 65500: A 48-hour static toxicity test with the cladoceran (*Daphnia magna*)
Silthiofam: A semi-static life-cycle toxicity test with the cladoceran (*Daphnia magna*). *OECD TG 211; ASTM Standard E1193-87*	LOEC (mg a.s./L) survival = 3.7 LOEC (mg a.s./L) reproduction = 3.7 LOEC (mg a.s./L) growth = 0.96 NOEC (mg a.s./L) survival= 1.8 NOEC (mg a.s./L) reproduction = 1.8 NOEC (mg a.s./L) growth = 0.47 Silthiofam has a moderate chronic toxicity to *Daphnia magna* with a NOEC of 0.47 mg a.i./L.	Performed according to GLP criteria.	Drottar, K.R., Kendall, T.Z. and Kreuger, H.O. (2000). MON 65500: A semi-static life-cycle toxicity test with the cladoceran (*Daphnia magna*)
Algae			
Silthiofam: A five-day toxicity test with the freshwater alga (*Selenastrum capricornutum*). *OECD TG 201, EEC method C3 and FIFRA Chapter 123-2.*	There were no statistically significant reductions in cell density, area under the growth curve or growth rate of *Selenastrum capricornutum* exposed to silthiofam (MON 65500) at concentrations of 2.3 mg a.s./L. Day 3 EC_{50} values for biomass and growth rate were: E_bC_{50} (0-72 h) = 8.6 (confidence limits: 2.9 and 11) and E_rC_{50} (0-72 h) = 13 (confidence limits: 13 and 13), respectively. NOE_bC = 4.6 mg a.s./L NOE_rC = 2.3 mg a.s./L Therefore, silthiofam is slightly to moderately toxic to the green algae, *Selenastrum capricornutum.*	Performed according to GLP criteria.	Drottar K.R. and Krueger H.O. (1998). MON 65500: A five-day toxicity test with the freshwater alga (*Selenastrum capricornutum*)

Chronic Aquatic Toxicity

The basis for the dossier submitter's proposal for chronic aquatic toxicity is that silthiofam is considered to be not rapidly degradable and to have a low bioaccumulation potential. Furthermore, the chronic aquatic toxicity studies indicate silthiofam is moderately toxic to fish (*Pimephales promelas*) and aquatic invertebrates (*Daphnia magna*). The 28-day Early Life-

Stage Toxicity Test with the Fathead Minnow (*Pimephales promelas*) resulted in a NOEC (growth) of 0.89 mg a.s./L and the 21 day semi-static life-cycle toxicity test with the Waterflea (Daphnia magna) in a NOEC (growth) of 0.47 mg a.i./L. Thus, the dossier submitter proposed to classify silthiofam as toxic in Category Chronic 2; H411, based on the criteria set in Table 4.1.0 (b)(i).

Comments received during public consultation

Four MSCAs commented on the proposals for environmental classification, all agreeing with the proposed classification as Category Chronic 2; H411.

Industry in their comments from June 2018 disagreed with the proposed classification. They argued that in the early life-stage toxicity test with the Fathead Minnow (*Pimephales promelas*) the EC_{10} of 1.12 mg/L (mean measured) is more appropriate than the NOEC (growth) of 0.89 mg a.s./L for long-term environmental classification. In addition, they claim that for the OECD TG 211 semi-static life-cycle toxicity test with *Daphnia magna* the NOEC (reproduction) of 1.8 mg/L (mean measured) should be used instead of the NOEC (growth, length) of 0.47 mg a.i./L. They stressed that the results in the study report are only given as NOEC and LOEC information; no corresponding EC_{10} values based on a dose-response curve are reported.

The DS noted the comments but did not provide any additional response.

Assessment and comparison with the classification criteria

Degradation

RAC agrees with the proposal of the dossier submitter to consider silthiofam as not rapidly degradable for classification purposes, based on the overall evidence from the hydrolysis, ready biodegradability and simulation studies.

Aquatic Bioaccumulation

RAC agrees with the proposal of the dossier submitter to not consider silthiofam as being bioaccumulative in the aquatic environment for classification purposes, based on an experimentally derived BCF value for fish of 98 L/kg and a measured log P_{ow} of 3.

Acute Aquatic Toxicity

RAC agrees with the proposal of the dossier submitter to not classify silthiofam as acute toxic for the aquatic environment, based on no acute toxicity below the CLP threshold value of 1 mg/L.

Chronic Aquatic Toxicity

RAC agrees with the comment by Industry that for long-term environmental classification in general the EC_{10} value is more appropriate than the NOEC. The reason for this is that the EC_{10} as a regression-based estimate is less influenced by dose selection and makes full use of the dose response curve. In general, the value of the EC_{10} is smaller than the value of the NOEC and leads to a more stringent classification. In the case of the early life-stage toxicity test with the Fathead Minnow (*Pimephales promelas*) the EC_{10} value is larger than the NOEC, which can be explained by the chosen test concentration intervals and by concentration-response modelling. The original study report does not provide an EC_{10} value. In their comments from

June 2018, the Applicant provided an EC_{10} value of 1.12 mg/L (mean measured) while RAC has recalculated the EC_{10} value using the Software ToxRat Professional Version 3.2.1 and found an EC_{10} of 1.059 mg/L. Both values are close to the upper limit of the classification criteria for a not rapidly degradable substance. RAC concludes to take the uncertainty expressed by a lower NOEC than the EC_{10} values into account and not to base the classification on the results from the early life-stage toxicity test with the Fathead Minnow (*Pimephales promelas*).

RAC notes, that the fish species Fathead Minnow (*Pimephales promelas*) used for the chronic endpoint is not represented in the acute fish data set. However, the surrogate approach using the acute data from the other two fish species (Rainbow Trout (*Oncorhynchus mykiss*) and Bluegill Sunfish (*Lepomis macrochirus*)) would not result in a more stringent classification.

In response to the comment by industry, RAC notes that OECD TG 211 states that growth measurements are highly desirable since they provide information on possible sub-lethal effects, which may be useful in addition to reproduction measures alone; the measurement of the length of the parent animals (i.e. body length excluding the anal spine) at the end of the test is recommended. The reporting may include any appropriate justification. Moreover, following the same guideline, a justification is not obligatory, which means that a missing justification does not invalidate the result as such. The endpoint growth based on length *per se* is a relevant endpoint for the purpose of classification. RAC concludes that for the OECD TG 211 semi-static life-cycle toxicity test with *Daphnia magna* the endpoint growth based on length is relevant for the purpose of classification.

RAC agrees with the proposal of the dossier submitter to classify silthiofam as **Aquatic Chronic 2; H411** based on the NOEC (growth) of 0.47 mg a.i./L from the 21 day test with the Waterflea (*Daphnia magna*) and silthiofam being not rapidly degradable.

6 OTHER INFORMATION

7 REFERENCES

8 ANNEXES